Vol. 21, No. 2(2022) Bio2723
Revista Mexicana de Ingeniería Química

Bioleaching of As from mine tailings using an autochthonous *Bacillus cereus* strain

Biolixiviación de As de jales mineros utilizando una cepa Nativa de *Bacillus cereus*

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Received: January 27, 2022; Accepted: April 11, 2022

Abstract

Contamination of heavy metals has been a serious environmental problem due to increasing anthropogenic activities such as mining, smelting, livestock, landfills, waste dumps, and agriculture. Bioleaching experiments were conducted using native *Bacillus cereus* MAMPE19 BCG, isolated and characterized from previous research, to test removal of arsenic (As) from actual mining waste. Mineralogical characterization by XRD was performed to identify mineral associations with As. The experimental design included a bioleaching system with agitated flasks (triplicate) and the effects of pH (5, 7, and 9) and pulp density (10, 15, and 20%) were evaluated. Finally, based on the results of the ANOVA, the system that achieved the highest percentage of As dissolution was selected and scaled to a stirred tank bioreactor. The composition of the mineral was mainly calcite ($CaCO_3$), gismondine ($CaAl_2Si_2O_8{\cdot}4(H_2O)$), akermanite ($Ca_2MgSi_2O_7$), and silicon oxide ($SiO_2$). Native *Bacillus cereus* dissolved 40.6 ± 4.9 and 37.4 ± 2.7 % of As in 10 d, in agitated flasks at a pH of 5 and a pulp density of 10 and 15%, respectively; and a 27.5 ± 2.9% dissolution of As was achieved in the stirred tank bioreactor at pH 5 and pulp density of 15%, supported by significant statistical differences.

Keywords: Arsenic, *Bacillus cereus*, bioleaching, heavy metals, mine tailings.

Resumen

La contaminación por metales pesados es un grave problema ambiental debido al aumento de actividades antropogénicas como la minería, fundición, ganadería, rellenos sanitarios y agricultura. Se realizaron experimentos de biolixiviación utilizando *Bacillus cereus* MAMPE19 BCG nativo, aislado y caracterizado en una investigación previa, para evaluar la remoción de arsénico (As) de muestras de jal minero. Se efectuó una caracterización mineralógica por DRX para identificar las asociaciones mineralógicas con el As. El diseño experimental incluyó un sistema de biolixiviación con matraces agitados (triplicado) evaluando el efecto del pH (5, 7 y 9) y densidad de pulpa (10, 15 y 20%). Finalmente, basado en el ANOVA, se seleccionó el sistema con mayor porcentaje de disolución de As y se escaló a un biorreactor de tanque agitado. La composición del mineral fue principalmente calcita ($CaCO_3$), gismondina ($CaAl_2Si_2O_8{\cdot}4(H_2O)$), akermanita ($Ca_2MgSi_2O_7$) y óxido de silicio ($SiO_2$). La cepa nativa de *Bacillus cereus* disolvió 40.6 ± 4.9 y 37.4 ± 2.7% de As en 10 d, en matraces agitados a pH de 5 y densidad de pulpa del 10 y 15%, respectivamente; y se logró una disolución del 27.5 ± 2.9% de As en el biorreactor agitado, a pH 5 y 15% de densidad de pulpa, respaldado por diferencias estadísticas significativas

Palabras clave: Arsénico, *Bacillus cereus*, biolixiviación, jales mineros, metales pesados.

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https://doi.org/10.24275/rmiq/Bio2723
ISSN:1665-2738, *issn-e*: 2395-8472

1 Introduction

Nowadays, the mining industry is an important economic pillar worldwide. Global markets have grown in the levels of mineral raw materials extracted, with countries such as China, United States, Russia, Australia, Turkey, Canada, India, Japan, South Korea, Indonesia, Peru, Bolivia, Chile, Brazil, and Mexico accounting for more than 70% of global mineral extraction (Concha, 2017). Mining represents one of the main causes of air, water, and soil pollution due to the generation of waste known as *mine tailings* which are resultants of the extraction of metals of major interest, such as gold and silver (Gavilán-García *et al*., 2020). These mine tailings contain many heavy metals, such as Cr, As, Hg, Zn, and Ni, just to name a few (Covarrubias and Peña-Cabriales, 2017; Buendía-González *et al*., 2010). Heavy metals are defined as those elements with a density ≥ 5 g/cm^3 that possess metallic properties such as ductility and conductivity. Their danger lies in their capacity to be easily adsorbed by living organisms since they tend to accumulate even in the environment, causing serious diseases and even death (Meléndez-Sánchez *et al*., 2021; Alcázar-Medina *et al*., 2020; Beltrán-Pineda and Gómez-Rodríguez, 2016). In Mexico, As is found in greater proportion in the states of Durango and Coahuila, where concentration from 0.007 to 0.740 mg/L have been reported in agricultural wells; 0.009 to 0.149 ppm and 0.08 to 0.15 ppm in groundwater, urban and rural zones, respectively; and up to 30 μg/g in soil (Rangel-Montoya and Balagurusamy, 2015; Martínez-Prado *et al*., 2013). However, arsenic also has other important uses in industry, for example the removal of bismuth by chemical co-precipitation processes (Vargas-Rubio *et al*., 2021). Physicochemical, thermal, and biological treatments are the most known for the removal of heavy metals, where biological is friendly with the environment and least expensive. There are many examples, such as biovolatilization, phytoremediation, bioprecipitation, and bioleaching, which consists of the extraction of metals in aqueous phases using microorganisms (Cui *et al*., 2021; Rahman and Singh, 2020). This represents one of the best alternatives at present, since its cost and maintenance are minimal, and it represents a sustainable alternative for the remediation of contaminated soils and include a wide variety of direct and indirect reactions of microorganisms, such as oxidation, reduction, chelation, adsorption, and dissolution processes, which can dissolve insoluble substances from solids (Gómez-Ramírez *et al*., 2021). However, the degree of remediation of the contaminated site will be determined by different factors such as the degradation capacity of microorganisms, available macro- and micronutrients, initial conditions of the contaminated site, among others (Martínez-Prado and Soto-Álvarez, 2017). Microorganisms typically used for heavy metal bioleaching include a wide variety of fungi, yeasts, and bacteria, being acidophilic sulfur-oxidizing and iron-oxidizing bacteria the most used. Among the best known chemolithotrophic organisms are *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*. However, it is also common the use of fungi, like *Aspergillus niger* and *Penicillium simplicissimum*, and *Chromobacterium violaceum* and *Bacillus megaterium* as very promising cyanogenic bacteria; being the *Bacillus* species one of the most efficient (Baniasadi *et al*., 2019). As previously mentioned, the most representative microorganisms used in bioleaching are bacteria, which are the most abundant prokaryotic organisms on earth, having the ability and capacity to live in a vast range of environmental conditions. Bacteria have been used in the removal of contaminants because of their large number of advantages, including their small size, growth rate, and easy reproducibility. An example of this is the ability of marine bacteria to resist concentrations of heavy metals in extreme conditions found in the aquatic environments of Antarctica, as it is the case of the AQ5-AO1 strain, which can degrade diesel and withstand exposure to heavy metals such as Al, Cd, Co, Ni, Zn, etc. (Zakaria *et al*., 2020). Additionally, heavy metal ions are usually adsorbed on the polysaccharide layers of bacteria through functional groups such as carboxyl, amino, and phosphate, allowing bacteria to have a capacity of adsorption ranging from 1 to 500 mg/g and even more, according to the type of bacteria used (Yin *et al*., 2019). It is important to mention that bacteria can bind toxic heavy metal cations to negatively charged bacterial structures and in turn to components of cellular biomass, living or dead, thanks to the surface/volume ratio of bacterial biomass, which allows it to act effectively as a biosorbent (Priyadarshanee and Das, 2021). An example of this is *Bacillus cereus* (*B. cereus*) which spreads in a wide variety of environments including soil, water, vegetation, and the intestinal tract of some invertebrates. It has an elongated stick shape of 1.0 to 1.2 μm, is gram-positive, spore-forming (when stressed), facultatively anaerobic, and has a growth

temperature range from 4 to 48ºC where its spores can tolerate up to 95ºC and grows at pH range of 4.3 to 9.3 (Cortés-Sánchez *et al*., 2018).

The main objective of this research was to determine by XRD the mineralogical associations of the mine tailing with As, as well as to implement a microbial leaching system at flask level using *B. cereus* at different pH (5, 7, and 9) and pulp density (10, 15, and 20%). Finally, to replicate the microcosm test with the highest As removal, a stirred tank leaching system was scaled up 35 times to evaluate these conditions at a larger scale to implement this system at industrial level in the future as an interesting and promising alternative to this major problem of heavy metal contamination in the mining industry.

2 Methodology

2.1 Reactants

The following reagents were purchased from Solbiosa Company® (Guadalajara, Mexico): Peptone casein (batch 2001181), yeast extract (batch 1907228) and bacteriologic agar (batch 3232890). In addition, hydrochloric acid (CAS 7647-01-0), sodium hydroxide (CAS 1310-73-2), and sodium chloride (CAS 7646-14-5) were acquired from Fermont Company® (Monterrey, Mexico). Finally, sodium hydroxide (CAS 1310-73-2) was obtained from J.T. Baker®. These reactants were used both for the preparation of Luria-Bertani medium and for the respective pH adjustment that was made daily to the treatments at the flask and stirred tank level.

2.2 Chemical analysis and characterization of mine tailings

The mine tailing sample used (MAMPE19-C) came from a silver extraction mine located in Coahuila de Zaragoza, Mexico (28°21'32.76", 102°34'30.36"). The sample had a particle size of 200 mesh (0.074 mm), so it did not receive additional particle reduction treatment. Subsequently, the X-ray diffraction (XRD) powder method was used to identify the mineralogical associations of the mine tailing with As. X-ray diffraction patterns of the dry mineral sample were obtained with a D8 Advance diffractometer (Drucker, Germany) applying a Cu - K Kα radiation (wavelength = 15.41 nm) at 50 kV and 150 mA in a 2θ range of 5º - 90º. The diffractograms were created using a step size of 0.02º and a measurement speed of 1º/min as suggested by Akhgar and Pourghahramani (2015). In addition, inductively coupled plasma (ICP) analysis was carried out by ICP - OES (iCAP 7000, Thermo Scientific, USA®) by the SGS company to find the initial and final concentration in the soil sample submitted to evaluation. The technique consisted of an acid digestion with aqua regia (nitric and hydrochloric acid in a 3:1 ratio, respectively) to have As and other metal elements in solution and then quantify their concentrations by using the optical emission spectrum with inductively coupled plasma. It is worth mentioning that this technique applies to the determination of As, Al, Sb, Ba, Be, Bi, Cd, Ca, Cr, Co, Cu, Ni, P, K, Sc, Ag, Na, Sr, S, Sn, Ti, W, V, Y, Zn, and Zr in geological sample matrices.

2.3 Reactivation and morphological characterization of Bacillus cereus

2.3.1 Strain reactivation and culture medium

B. cereus strain used in the current work was previously isolated from a mine tailing sample, identified, and preserved as part of a research project (Meléndez-Sánchez, 2021). This strain was preserved in cryogenic vials containing 800 μL of concentrated bacterial culture and 70 μL of dimethyl sulfoxide (DMSO) as a cryoprotectant. When needed, the strain was reactivated as follows: cryogenic samples were thawed at room temperature, 0.8 mL of the total volume was distributed in two Eppendorf tubes with a capacity of 1.5 mL each, and 0.4 mL of Luria-Bertani medium was added to each tube. Then, the samples were homogenized in a Thermo/IEC Microlite Microcentrifuge 3580 with Rotor® at 13,500 rpm for 5 minutes, the supernatant was discarded and the process was repeated three times, to finally culture on a Luria-Bertani agar plate for 24 h at 30ºC.

2.4 Flask level bacterial bioleaching process

2.4.1 Experimental design

A flask level system was conducted with a 3^2 factorial experimental design, allowing to determine the number of experiments to carry out to analyze the combinations of each of the chosen factors with their respective levels. pH and pulp density were selected as the two factors, setting 3 levels per factor: pH (5, 7, and 9) and pulp density (10, 15, and 20%).

Table 1. Mass of nutrients required for the preparation of Luria-Bertani medium.

Nutrients	Nutrient required (g)	
	To prepare 540 mL of inoculum	To prepare 5,460 mL of medium for the experimental runs
Casein peptone	5.4	54.6
Yeast	2.7	27.3
Sodium chloride	5.4	54.6

A multilevel factorial design was obtained using Minitab 18® software considering 2 factors, 3 levels and 3 replicates each, resulting in 27 experimental runs.

2.4.2 Preparation of experimental phase

The bacterial leaching process was implemented in agitated flasks and 27 experimental runs were performed. A total of 180 mL of Luria-Bertani medium and 20 mL of bacterial inoculum (10% v/v) were added to each flask, medium was autoclaved for 30 minutes at a pressure of 30 psi (lbf/in^2) before use. Table 1 lists the composition of Luria-Bertani medium, both for the bacterial inoculum and for the 27 experimental runs as described by Garboza *et al*. (2011). Subsequently, the reactivated bacteria were inoculated in the medium and incubated in a shaker incubator model CVP-100B® at 130 rpm, for 24 h at 30ºC. Additionally, 3 negative controls (without bacterial inoculum) were included, resulting in a total of 30 experimental runs.

2.4.3 Inoculum: Initial cell count

The cell count was performed to determine the initial concentration (cells per milliliter, Cell/mL) of the bacterial inoculum added in the different tests. A 10^{-1} dilution of the inoculum was prepared, 900 μL of LB medium and 100 μL of bacterial inoculum, dilutions were mixed into a 1.5 mL-Eppendorf tube. Afterward, a 15 μL aliquot of this dilution was added to another Eppendorf tube containing 5 mL of lactophenol blue dye and homogenized. Then, the Neubauer chamber was loaded with 5 μL of the previous dilution and the count cell was completed under the microscope with the 100X objective. Cells per milliliter (Cell/mL) of bacterial inoculum after 24 h were calculated using equation 1, according to the modified equation reported by Kikuti *et al*. (2013).

$$\frac{\text{Cell}}{\text{mL}} = \frac{(\text{Cells counted})(10,000)}{(\text{Number of quadrants counted})(\text{dilution factor})} \quad (1)$$

2.4.4 Mine tailings sample preparation

Tests were performed at 10, 15, and 20% pulp density and mass (g) of the mine tailing sample required was calculated considering the total volume of each flask (200 mL). According to the experimental design, a total of 9 tests were carried out for each pulp density, therefore 180 g of mine tailing sample were required for 10% pulp density tests, 270 g for 15% pulp density tests, and 360 g for 20% pulp density tests. Mine tailing sample was completely mixed and weighed in metal containers previously autoclaved and ultraviolet light sterilized once more under a laminar flow hood for 2 h.

2.4.5 Flask-scale arsenic dissolution experimental test

Each experimental run was prepared as mentioned above (triplicate) in 200-mL flasks containing 180 mL of LB media, 20 mL of inoculum, and mine tailings sample (180, 270, and 360 g, for 10, 15, and 20% pulp density, respectively) in a shaking incubator at 30ºC and 130 rpm, and carried out for a period of 10 d; pH was adjusted daily with 10 M NaOH and 2N H_2SO_4 according to the modified technique reported by Núñez-Ramírez *et al*. (2011). After 10 d, the samples were filtered in a Whatman® grade 2 qualitative filter paper, to collect as much mineral sample as possible. Right after filtration, the filtrate was washed with distilled water to eliminate impurities of some minerals that could remain adhered. Subsequently, the residue was dried in an oven at 60ºC for 24 h to eliminate water and avoiding modification of the properties of the sample. The solid sample obtained was then carefully crushed in a mortar and maintained in labeled plastic bags at room temperature, in a site with little variation in light and humidity ready for ICP analysis to determine dissolution of As and other metals present in the mine tailing sample evaluated.

2.5 Stirred tank-level bacterial leaching process

From the results obtained in the flask level experimentation, the sample with the best As dissolution under the conditions already established was chosen. The process was performed in a stirred tank with a capacity of 9.6 L and a working volume of 7 L, corresponding to a 35-fold scale-up of the flask level experiment to simulate a bioreactor with a constant temperature of 30°C, aeration provided by mixing at 700 rpm propeller with a Rushton turbine in an open tank to ensure transfer of oxygen from the atmosphere to the tank, for a period of 10 d.

2.5.1 Bioreactor experimental phase preparation

Working volume in the bioreactor was 7 L to ensure complete mixing was achieved in the tank. Two different experiments were carried out under the best conditions achieved in experiments at the microcosm level (pH 5 for 10 and 15% pulp density); volume/mass in the tank were as follows: a) Luria-Bertani medium volume of 4,725 mL, 525 mL (10% v/v) of bacterial inoculum, for 525 and 787.5 g of mine tailing sample (10 and 15% pulp density) considering estimates in the previous studies (Núñez-Ramírez *et al*., 2018). A 50 mL sample was taken by triplicate every 24 h until the end of the experiment. Afterwards, samples were filtered and dried in an oven at 60ºC and crushed as described in the experiment at the microcosm level; the percentage of As dissolution was determined for each sample during the experimental time. At the end of experimentation (day 10), the tanks were emptied, and the entire sample was filtered and allowed to dry, a representative sample of all the remaining content was analyzed to determine dissolution of As and the rest of the metals present by ICP-OES. It is important to note that in contrast to the experimentation carried out at the flask level, during the tank level test, conditions of total sterility were not preserved, to demonstrate the capacity of bacterial culture adaptation to circumstances like those used at the industrial actual conditions.

2.5.2 Arsenic dissolution curve

To obtain the As dissolution curve, the values of the initial concentration and the concentrations obtained each day by ICP-OES were used to determine the daily dissolution percentage (DDP) using equation 2, and values were plotted using SigmaPlot 12.0® software.

$$DDP = \left(1 - \frac{\text{Final metal concentration}}{\text{Initial metal concentration}}\right) \times 100 \quad (2)$$

2.6 Statistical analysis

A full factorial design was performed in Minitab18® to find all possible combinations between each factor and level. This allowed the treatment conditions to be identified, in order to determine which factor configuration could best represent the results. To interpret results obtained through the analysis by ICP-OES, an analysis of variance (ANOVA) was first performed with Minitab 18® software, to evaluate the existence of significant differences in the means of the factors analyzed. After that, to obtain more specific information on the results gotten and to compare their means individually and thus finding significant differences, a multiple comparison with Tukey test was conducted at a confidence interval of 95%, to determine which test represented the best performance concerning As/other metals dissolution.

3 Results and discussion

3.1 Concentration and mineralogical characterization of mine tailings

Characteristic crystallographic planes of this sample (MAMPE19-C) corresponded to calcite ($CaCO_3$) whose crystalline system is rhombohedral. Likewise, crystallographic planes corresponding to gismondine (tectosilicate) $CaAl_2Si_2O_8{\cdot}4H_2O$ were observed. Probably the reddish-brown color of this mine tailings sample is due to the presence of gismondine. In addition, the presence of akermanite ($Ca_2MgSi_2O_7$) was detected which has a tetragonal crystalline system. Finally, a crystalline system found in smaller proportion was silicon dioxide (SiO_2), whose unit cell is trigonal trapezohedron. It is important to note that the mine tailings used in the tests were characterized and an As concentration of 2,214 mg/L was found. The detection method was digestion in nitric and hydrochloric acid (aqua regia) with an ICP finish, analysis was performed with duplicate samples. The initial concentrations of some of the other heavy metals found in the sample were much lower than those for As, among them: Ni (9 mg/L), Zr (5.3 mg/L), and Ba (72 mg/L).

3.2 Morphological characteristics of Bacillus cereus

Opaque beige or milky white colonies were observed, with an irregular shape and rough surface which are characteristics that differentiate *B. cereus* when it grows on Luria-Bertani medium plates as reported by Wang *et al*., (2018). The morphological characterization analysis showed that the bacterium used is a Gram-positive *Bacillus*, which agrees with the characteristics described in the literature for *B. cereus* and corresponds to the characteristics of the native bacterium isolated from the mine tailing in the investigation that preceded this research (Meléndez-Sánchez *et al*., 2022). One of the main characteristics of the Luria-Bertani medium is that it contains yeast extract, which is obtained by chemical degradation and subsequent extraction of yeast cells, and casein tryptone, which arises as a product of the hydrolyzation of casein digested by trypsin. Therefore, having relatively small nutrients, it is easily assimilated by bacterial microorganisms (Yamamoto *et al*., 2021). Also, one of the mechanisms that allow *B. cereus* to resist high concentrations of As is in the arsC gene as it encodes the enzyme arsenate reductase, which comprises a detoxification system. Arsenate reductase is made up of several families of enzymes which adopt different protein folds. Its detoxification mechanism consists of a reaction where the enzyme catalyzes the reduction of arsenate in the cytoplasm to subsequently extrude it from the cell through transporters. Once this is done, the enzyme is oxidized and becomes inactive (Zhou *et al*., 2020; Hu *et al*., 2015; Jain *et al*., 2011). Gram staining resulted in blue/purple-colored bacilli distinctive of Gram-positive bacteria (Tallent *et al*., 2012). These tests were performed to confirm that the strain corresponded to the one under evaluation and that there was no presence of other species. Therefore, it was observed that both the colonial morphology and the differential staining correspond to the distinctive and specific characteristics of *B. cereus*. Figure 1 (left) shows the streak obtained on Luria Bertani medium plate, while Figure 1 (right) shows the result of Gram staining as seen under a microscope.

3.3 Bioleaching experiments at flask level

3.3.1 Initial cell count in Neubauer Chamber

The initial concentration of bacterial inoculum after 24 h was estimated using equation 1. The initial cell concentration is important to determine since it gives a clear perspective of the inoculum used at time zero for the experiments conducted.

3.3.2 Effect of pH on flask-level experiments

The batch bioleaching system at the flask level was evaluated for a period of 10 d and daily control of pH was carried out according to the conditions of the tests based on factorial design obtained through Minitab 18®. The pH is a very important indicator because it is a parameter that favors or does not cell growth, which is reflected in aspects such as the color of the medium when compared to controls (without bacterial inoculum). The mean of each triplicate and its standard deviation (error bars) were determined for each day and the results were plotted for each pulp density and pH.

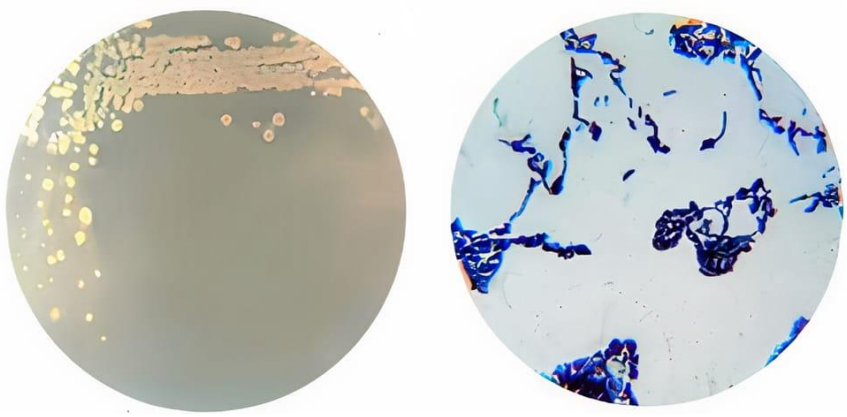

Fig. 1. Morphological characterization to identify *Bacillus cereus*. Left: In Luria-Bertani medium plate, the colonies showed irregular edges characteristic of this bacterium, solid medium was incubated at 30ºC for 24 h. Right: Gram stain, blue/purple-colored colonies of bacilli can be observed as a characteristic of Gram-positive bacteria.

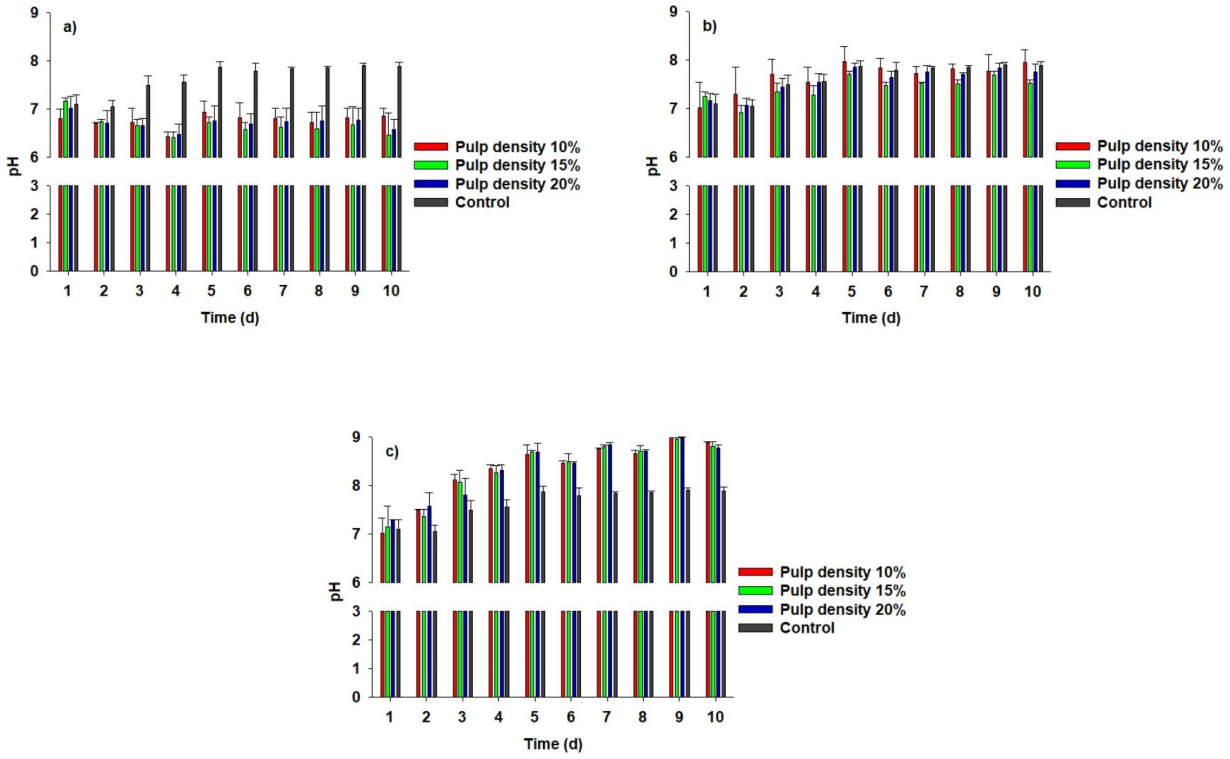

Fig. 2. Variation of pH vs time at different pulp densities compared to a negative control (no inoculum): a) pH 5. b) pH 7. c) pH 9. pH was adjusted daily with 10 M NaOH and 2N H_2SO_4 for 10 d in all tests.

Figure 2 shows pH variations that existed in the 5, 7, and 9 pH tests and those presented by the controls for pulp densities of 10, 15, and 20%. The behavior of the different pulp densities at pH 5 and 9 showed the same variation throughout testing, being the latter where there was the least variation from day to day (pH 9). At pH 5, a higher increase over time was observed, with changes up to one pH unit per day; while at pH 7, there were less significant variations. Another important aspect to emphasize is the rise in pH by almost one unit in the control and the way it stabilized from day 6. As previously mentioned, mine tailings have a high quantity of calcite, and because of constant agitation (even without the presence of microorganisms) for 10 d and at a temperature of 30ºC this led to the release of carbonates that finally ended up alkalinizing the solution. Considering these aspects and in comparison with the control, it was observed that there was a clear bacterial proliferation; likewise, the 10% pulp density tests, at pH 5, 7, and 9, were the ones that indicated the greatest increase in pH and even on day 10 they showed an increasing trend compared to the pulp density tests of 15 and 20%. It is noteworthy that there was a noticeable difference in properties of Luria-Bertani medium containing bacterial inoculum concerning the controls in which no microorganisms were added. The flasks containing inoculum showed a less intense color than the controls, and their density was slightly lower. The most relevant result in the leaching process corresponded to the pH 5 test at different pulp densities, but especially at 10 and 15%. The results obtained are complemented with the information collected for As dissolution as well as the effect of carbonic anhydrase and the influence of calcite in these tests, as discussed in the following sections.

3.3.3 Effect of carbonic anhydrase on pH levels

Carbonic anhydrase (CA) is a very common bacterial enzyme, and it is present in *B. cereus*, which promotes the hydration of carbon dioxide to release HCO_3^- y $CO_3^=$ in the process of calcite precipitation. Moreover, together with the membrane potential generating system (mpsAB), it provides a supply of dissolved inorganic carbon. It is important to note that both systems rarely exist at the same time and that the suppression of either of them inhibits cell growth,

which could only be reversible in the case of adding a CO_2 source. Furthermore, it has been shown that the distribution of both mpsAB and CA is not at all random, with mpsAB representing an advantage in species where the CO_2 supply is hindered, for example, in bacteria that form mucus or biofilms (Fan *et al*., 2021). Due to the joint effect of released ammonia, it has been shown to significantly increase the pH of a solution due to the combined effect of carbonic anhydrase and ammonia that might be released by *B. cereus* (Zhuang *et al*., 2018). It should be noted that all cells express multiple CA isoforms and thus can catalyze the reversible hydration of CO_2 into bicarbonate and protons. Likewise, it has been reported that CA promotes the maintenance of an alkaline intracellular pH (Yan *et al*., 2021). The bacterial process that normally promotes calcite precipitation is ureolysis, which acts as the fastest form of precipitation through a rapid increase in pH due to the release of ammonia. In addition, the extracellular substances produced by the bacteria contribute to the formation of a favorable microenvironment for this event to occur (Hoffmann *et al*., 2021). However, it has also been demonstrated that bacteria possess several enzyme encoding and regulating genes such as urease, which is also involved in calcite precipitation (Ali *et al*., 2022). The above gave the first indication that in those tests where the pH was higher, there was greater cell growth and therefore greater As dissolution, which is a relevant finding in this work. Furthermore, it has been reported that calcite precipitation is absolutely assured because of the ureolytic activity of urease-producing bacteria such as *B. cereus*, whose ureolytic activity has been reported, besides CA. Urea is hydrolyzed into ammonia and carbonate, which are randomly hydrolyzed to produce more ammonia and carbonic acid. All these compounds equilibrate in water to generate bicarbonate, ammonium, and hydroxide ions causing an increase in pH (Oualha *et al*., 2020). Finally, it was observed that the controls did not show a significant change in color or density, while there was a greater variation of these parameters in the tests corresponding to pH 9. However, the change in color and density was evident in the tests in general. It is important to conduct additional experimentation to measure the levels of ammonia produced as well as to verify the presence of carbon anhydrase and urease in *B. cereus*. However, the literature review allows us to assume that the increase in pH is due to these combined factors, since no such substantial increases in pH were observed in pH 7 and 9 tests, where dissolution levels were also lower.

3.3.4 Indicative of cell growth in filtered Luria-Bertani medium

Another indication of cell activity corresponds to the liquid medium which was filtered and kept refrigerated. In treatments that were carried out at pH 5, filtered Luria-Bertani medium exhibited a yellowish color, while in tests at pH 9, turbidity was evident, indicating at first sight that there was not a correct filtration; however, the medium was simply pigmented because it did not contain more mineral material to be filtered. This variation in the color change of the filtered medium as a function of its pH can be explained by the protonation of the surface (becomes positively charged) of *B. cereus* at low pH, as it attracts negative anions of heavy metals (including As) to promote sorption. However, at higher pH, the surface of this bacterium becomes negatively charged by the presence of hydroxyl ions, thereby repelling heavy metal cations and promoting them to remain in solution (Giri *et al*., 2013). That is, at higher pH, heavy metal ions will tend to remain in solution, which explains the dark color that the medium acquires when the pH increases. It should be noted that the Luria-Bertani medium recovered after the flask level experimentation showed that heavy metal ions tend to remain in solution, as it was the case for As, Al, Ba, Bi, Cd, Cu, Mg, Ni, Sn, Sr, and Zr ions.

3.3.5 Final Arsenic concentrations for flask experiments

As already mentioned, the initial concentration of As in mine tailings sample subjected to evaluation corresponded to 2,214 mg/L. It was observed that the tests with the highest dissolution percentages were those corresponding to pH 5 for pulp density of 10 and 15%, reaching up to 40.6 ± 4.9 and 37.4 ± 2.7%, respectively, which is related to the daily constant pH increase, indicating there was a greater bacterial growth and therefore greater As sorption. Table 2 shows the dissolution percentages that existed for the various treatments at different pH and pulp densities.

3.3.6 Behavior of the concentration for other heavy metals for flask experiments

Given the interesting and promising results obtained in the dissolution of As, an analysis of the ICP-OES results on the dissolution of the other heavy metals

Table 2. Arsenic dissolution (Mean ± Standard Deviation) for all treatments carried out at flask level according to experimental design.

Initial Concentration = 2,214 mg/L			
ID Combination	**pH**	**Pulp density (%)**	**% Dissolution ± SD**
1,10,19	5	10	40.6 ± 4.9
2,11,20	5	15	37.4 ± 2.7
3,12,21	5	20	25.4 ± 6.1
4,13,22	7	10	28.2 ± 4.2
5,14,23	7	15	20.8 ± 5.3
6,15,24	7	20	9.8 ± 2.0
7,16,25	9	10	24.6 ± 8.4
8,17,26	9	15	11.5 ± 6.2
9,18,27	9	20	13.8 ± 5.6

Table 3. Other heavy metals dissolution (Mean ± Standard Deviation) for a 15% pulp density and pH 5 at flask level.

Heavy metal	**Pulp density (%)**	**Initial concentration (mg/L)**	**Dissolution (%)**
Barium (Ba)	10	72	69.9 ± 1.6
	15		69.9 ± 0.8
Bismuth (Bi)	10	67	63.7 ± 3.7
	15		59.2 ± 3.4
Cadmium (Cd)	10	104	52.7 ± 4.0
	15		49.7 ± 2.5
Copper (Cu)	10	395	49.9 ± 4.5
	15		48.4 ± 0.5
Nickel (Ni)	10	9	81.5 ± 16.9
	15		88.9 ± 11.1
Tin (Sn)	10	105	54.3 ± 14.3
	15		52.7 ± 14.3
Strontium (Sr)	10	281	40.9 ± 3.0
	15		41.5 ± 2.6
Zirconium (Zr)	10	5.3	71.0 ± 2.9
	15		68.6 ± 4.7

present in the mine tailing sample was carried out, finding a high dissolution of several metals of great industrial and environmental relevance. In the case of As, the most favorable results were obtained at pH 5 and 10 and 15% pulp density as well as for the following metals: Ba, Bi, Cd, Cu, Ni, Sn, Sr, and Zr. Table 3 shows the dissolution for all the heavy metals that were analyzed, finding notable removal levels for Zr, Ni, and Ba, although initial concentrations were not as high.

3.4 Bioleaching experiments at stirred tank level

3.4.1 Arsenic dissolution curve

As dissolution kinetics vs time was conducted for pH 5 at 15% pulp density. The results obtained in the 10-day experiment for this test are shown in Table 4. It is important to consider that the initial concentration value differs from the day 0 concentration, this is due to common variations that exist in the different tests over time, which should not be relevant if the values do not drastically exceed the initial concentration of the mine tailings sample.

Table 4. Arsenic dissolution (Mean ± Standard Deviation) at stirred tank level for 15% pulp density and pH 5.

Initial concentration = 2,214 mg/L		
Day	**Pulp density 15%**	
	Arsenic (mg/L)	**Dissolution (%)**
1	2, 354	0 ± 0.0
2	2, 152	2.8 ± 0.0
3	2, 078	6.1 ± 2.9
4	1, 999	9.7 ± 4.3
5	1, 980	10.5 ± 3.2
6	1, 867	15.7 ± 0.4
7	1, 845	16.7 ± 1.1
8	1, 839	16.9 ± 1.9
9	1, 762	20.4 ± 1.9
10	1, 605	**27.5 ± 2.9**

Table 5. Dissolution of other heavy metals (Mean ± Standard Deviation) at a pulp density of 15% and pH 5 at stirred tank level.

Heavy metal	**Initial concentration (mg/L)**	**Dissolution (%)**
Barium (Ba)	72	56.9 ± 2.4
Bismuth (Bi)	67	44.8 ± 1.5
Cadmium (Cd)	104	49.7 ± 2.5
Copper (Cu)	395	19.9 ± 3.2
Nickel (Ni)	9	66.7 ± 0.0
Tin (Sn)	105	48.6 ± 4.8
Strontium (Sr)	281	39.9 ± 2.0
Zirconium (Zr)	5.3	76.1 ± 3.9

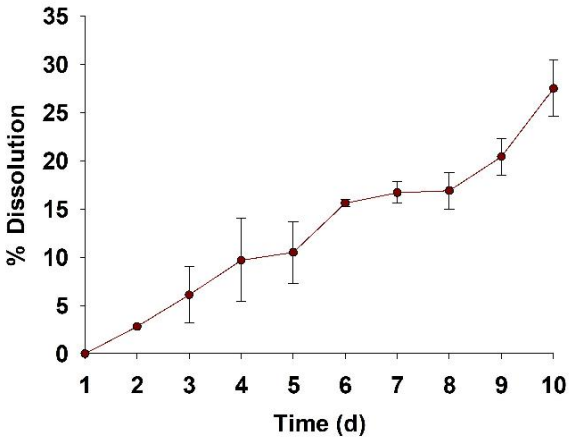

Fig. 3. Arsenic dissolution curve vs time. Bioreactor experiment carried out at pH 5 and 15% pulp density (Mean ± Standard Deviation).

The dissolution behavior, at pH 5 and 15% pulp density, indicated that As dissolved up to 27.5 ± 2.9% in 10 d of treatment. No significant increase in the dissolution was observed at days 5, 6, and 7, achieving only 16.9 ± 1.9% removal up to that time; however, from day 8 to the end of the experiment there was a favorable increase with a tendency to continue over time, as shown in Figure 3.

3.4.2 Dissolution behavior of other heavy metals at stirred tank-level

The dissolution of several heavy metals in the stirred tank (7 L) test was significant. The highest percentage of dissolution was achieved at pH 5 with 15% pulp density as follows: Ni (66.7 ± 0.0), Zr (76.1 ± 3.9), and Ba (56.9 ± 2.4). Like it was observed in the flask level (200 mL) experimentation, the dissolution achieved for the rest of the heavy metals was substantial; as shown in Table 5.

Likewise, Wu *et al.* (2016) concluded that it was possible to achieve >80% Cd removal using active *B. cereus* biomass, reaffirming the ability of

this bacterium for heavy metal capture at this phase. Similarly, the high resistance of this bacterium to heavy metals, especially As, is well known as reported by Aguilar *et al*. (2020) where *B. cereus* achieved removal of 69.38 - 71.88% of arsenite and 82.39 - 85.72% of arsenate, and a resistance up to 3,000 mg/L of As, which is a value close to the initial concentration of As reported in the current investigation; although, the treatment periods only reached 72 h and were not carried out under the same conditions as in this work.

Muñoz-Silva *et al*. (2019) proved in their work that *B. cereus* had a tolerance of up to 84 ± 7% for Cu^{+2}, 80 ± 5% for Zn^{+2}, and 78 ± 7% for Ni^{+2} for a 1 mM concentration of these heavy metals, and 103 ± 10% for Ag^{+1}, 115 ± 9% for Cr^{+6}, and 98 ± 1% for Cd^{+2} for a 0.01 mM concentration; the percentage was calculated with respect to the bacterial culture without the presence of the metal (control) and when the tolerance value was greater than 100%, it indicated a greater growth of the culture in the presence of the heavy metal. Ayangbenro and Babalola (2020) isolated a strain of *B. cereus* from mining soil and determined its resistance to heavy metals on Luria-Bertani agar. The strain showed the ability to resist 200 mg/L of Cd and Cr and up to 1,000 mg/L of Pb. On the other hand, Miyatake and Hayashi (2005) reported As removals of up to 83% in a medium containing 0.2 mg/L of As (III) and As (V), under completely controlled conditions. They also demonstrated that the use of dried *B. cereus* cells could improve removal up to 97% in solutions containing up to 1 mg of As/L, the latter having important advantages since they are not affected by pH or pulp density. This same issue was also discussed by Mohd-Bahari *et al*. (2013) where dead biomass of *B. cereus* was used and additionally demonstrated that once the biomass was used as a sorbent, it can be reused again achieving up to 94% desorption at a pH of 1, which indicates a high potential of this bacterium to be used in various applications during the leaching process. In recent studies, Cheah *et al*. (2022) compared the efficiency of exopolymeric substances (EPS), using live and dead cells of *B. cereus* to remove heavy metals. It was shown that there was a higher removal efficiency with dead cells reaching removals for Cu (63.26%), Pb (70.16%), Zn (58.86%), Cd (57.68%), and Cr (56.48%). The use of dead cells could be a great alternative, although the use of live biomass showed significant removal as shown in this work. Furthermore, the production of EPS means a better biosorption capacity of the bacteria, related to the decrease in the concentration of heavy metals present in the mine tailings, as reported by Kashyap *et al*. (2021) where *B. cereus* resisted concentrations above 1,000 mg/L with a biosorption of 57.2 ± 0.62%. The yields of EPS generation and dissolution of heavy metals by this pathway depends on several factors. It has been shown that the addition of Zn improved the quality of the EPS produced by several species of the *Bacillus* genus and, although its yield decreased, a greater removal of heavy metals was achieved. However, it was concluded that the best strategy to improve the yield and quality of EPS is the addition of molasses. This certainly shows precedents on how to improve the performance of *B. cereus* (Alotaibi *et al*., 2021). It is important to define all the mechanisms that could be involved in the removal of heavy metals, as well as their efficiency, since this will give a broader picture, helping to define strategies that allow better results to be achieved in future experiments.

There are numerous studies about the dissolution of different heavy metals besides As using *B. cereus*. Pérez-Bou *et al*. (2021) demonstrated the efficiency of this bacterium to remove significant amounts of Cd in pure culture and consortia including *Acinetobacter* sp., *B. cereus*, and *Micrococcus* sp., reaching dissolution levels of up to 92.0 ± 0.5% for Cd and 95.0 ± 2.9 for Zn in mixed cultures at initial concentrations of 40 mg/L. Huang *et al*. (2018) showed that at pH 7 *B. cereus* achieved dissolution of heavy metals such as Cu and Cd with removals of 16.7 and 81.4%, respectively, at concentrations up to 10 mg/L which contrasts with the values reported in the present investigation for these heavy metals: Cu dissolutions of 49.5 ± 4.5% and 48.4 ± 0.5% for pH 5 at 10 and 15% pulp densities, respectively, at flask level; 52.7 ± 4.0% and 49.7 ± 2.5% for Cd at the same conditions; while at tank level a 40.2 ± 2.6% and 19.9 ± 3.2% were reached for Cu, whereas 34.6 ± 1.9% and ± 45.2 ± 2.5% for Cd were achieved. It is important to note that promising results were obtained for the dissolution of different heavy metals such as Cd, Ni, and Zr, among others. It is worth highlighting the excellent performance obtained with As, since its initial concentration was higher than 2,000 mg/L, far exceeding the initial concentrations of the other metals present in the mine tailings evaluated. Similarly, Meléndez-Sánchez *et al*. (2022) reported that the heterotrophic *B. cereus* strain, used in the current investigation, withstood amounts up to 909.37 mg/L in the adaptation phase of up to 9 h for sodium arsenite which is considered more toxic than elemental As; furthermore, they described the characteristic curve of *B. cereus* which consisted of the adaptation phase between 0-1 h, the exponential phase at 1-5 h,

and stationary growth until 24 h showing asymptotic behavior, which it was attributed to the ability of this bacterial strain to produce spores after the main carbon source of the medium in which it reproduces has been depleted; that could explain the behavior observed in the arsenic dissolution curve.

Nowadays, heavy metal removal studies using *B. cereus* are very innovative, Sharma and Shukla (2021) reported on the ability of this bacterium to remediate a soil contaminated with Pb obtaining up to 79.26% accumulation of this heavy metal and biosorption capacity of 193.93 mg/g, achieving a minimum inhibitory concentration (MIC) of up to 2,400 ppm for their *B. cereus* BPS-9 strain. Similarly, Rangasamy *et al*. (2021) isolated *B. cereus* and *Paenibacillus pabuli* from alkaline soils with the main intention of remediating Cr (VI) and subjected them to concentrations between 50 and 400 mg/L, finding better removal the lower the Cr concentration, eliminating 74 and 98% in 72 h at a pH of 9.5; pH values higher than those analyzed in the present work, which would suggest analyzing in subsequent studies the influence of the type of soil and how the place where the bacteria were isolated influences; however, it is another proof of the adaptability of *B. cereus* to different types of environments. It is important to emphasize the uses that can be given to the *Bacillus* species in general, Abba *et al*. (2020) used chicken feathers as bioadsorbent for Cd and Pb. Then, they isolated a bacterium tolerant to these heavy metals, which was identified as *B. sp*., to degrade the feathers. Adsorption levels from 15 to 30 ppm in 1 h were achieved, while *B. sp*. was able to degrade between 30 and 40% of these feathers in approximately 7 d. In conclusion, for the present investigation, although the levels of removal did not reach those commonly reported in other studies, the conditions to which *B. cereus* was subjected are the first semi-pilot scale study and have not been previously reported.

In addition, the capacity of this bacterial gene is not only limited to heavy metals. Cisneros-de La Cueva *et al*. (2014) isolated and characterized a strain of *B. sp*. KJ629314 showing that it had a great potential to degrade total petroleum hydrocarbons at concentrations from 10,000 to 50,000 ppm. However, as the concentration increased, hydrocarbon biodegradation decreased. In summary, *B. cereus* and the entire *Bacillus* genus could represent a very viable alternative in the future, thanks to its rapid growth, great potential for heavy metals uptake, and its resistance to adverse situations, as well as their adaptability in the use of living and dead cells.

3.5 Statistical Analysis for flask-level tests

3.5.1 Analysis of variance (ANOVA)

Analysis of variance was carried out to determine whether the means of two or more tests were significantly different. The null hypothesis assumes that there is no difference in the means of As dissolution levels in the test, while the alternate hypothesis indicates that there is a difference in at least one test. ANOVA yielded a p-value of 0.000; if $p < 0.05$ the null hypothesis is false, therefore, an alternative hypothesis is accepted, and it is concluded that there are statistically significant differences in the means for As dissolution.

3.5.2 Tukey test

The Tukey statistical method was used to select the best treatment at microcosm level to carry out the experiment at tank level. Results showed that there was no difference between the means of pH 5, 10% pulp density (95% CI: 34.12 - 47.18) and pH 5, 15% pulp density (95%: CI 30.90 - 43.96), and that As and other heavy metals were dissolved. However, it was decided to use the latter due to its industrial application, which is a great alternative for semi pilot and pilot applications, since the greater the amount of mineral used, the more practical its real application would be.

Conclusions

The crystallographic planes obtained from XRD analysis showed that the mine tailing is mainly composed of calcite ($CaCO_3$), gismondine ($CaAl_2Si_2O_8{\cdot}4(H_2O)$), akermanite ($Ca_2MgSi_2O$), and to a lesser proportion silicon dioxide (SiO_2). Concerning the capacities of the As removal process from *B. cereus* for a period of 10 d at a pH of 5, removal of 40.6 ± 4.9% and 37.4 ± 2.7% can be achieved, at a pulp density of 10 and 15%, respectively. Likewise, the ability of this system to remove other metals of interest between 50 and 80% was also observed. In addition, *B. cereus* demonstrated the ability to grow without problem in concentrations of up to 2,000 mg/L of As and other heavy metals present in the mine tailings, such as Cd (104 mg/L), Cu (395 mg/L), Sn (105 mg/L), and Sr (281 mg/L). It was shown that this native strain can dissolve As and other heavy metals over time. Finally, through this study it

was demonstrated that *B. cereus* can remove relevant concentrations of As, Al, Ba, Bi, Cd, Cu, Mg, Ni, Sn, Sr, and Zr. The results are promising for applications in the mining industry and environmental protection, with the aim of promoting green and sustainable technologies.

Acknowledgment

This research was conducted in the Industrial Biotechnology and Microbial Biotechnology Laboratories of the Postgraduate, Research, and Technological Development Unit (UPIDET) of the TecNM-Instituto Tecnológico de Durango (TecNM-ITD) and the Laboratory of Biotechnology of Materials of the Chemical Sciences Faculty at the Universidad Juárez del Estado de Durango (FCQ-UJED). Financial support was provided by Tecnológico Nacional de México (TecNM), project number 10467.21-P. The authors would like to thank the Biotechnological Processes Group of TecNM-ITD, FCQ-UJED, FQ-UNAM, and Tec de Monterrey. The listed authors are highly obliged to their respective departments, institutes, and universities for providing literature services.

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