ADAPTATION AND CONTINUOUS CULTIVATION OF MICROORGANISMS USED IN THE BIOLEACHING OF SULPHIDE MINERALS: A BIOTECHNOLOGICAL APPROACH

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ABSTRACT: The processes of Bioleaching of sulphide minerals, aiming at extracting the metallic values from these mineral species, as well as the bio-oxidation of these sulphides so as to release gold particles encapsulated in these mineral matrices for further extracting the remaining gold, require, at first, a stage of adaptation of the involved microorganisms to the growing ionic strengths, due to these bio-oxidative processes, followed by the continuous cultivation of these microorganisms in order to use them in the aforementioned extractive processes at the appropriate production scales. Therefore, this technological contribution aims at defining the conditions, in the first place, for adapting those microorganisms to increasing ionic strengths and for their continuous cultivation, considering the bio-oxidative processes in which such cultures will be used.
KEYWORDS: bioleaching, bio-oxidation, microorganisms, sulphide minerals.

1 | INTRODUCTION

Bioleaching can be defined as a dissolution process of sulphide minerals that results from the action of a group of microorganisms (PRADHAN et al., 2008). Ferric ions (*i.e.*, Fe^{3+}), generated by microbial action, oxidize the sulphide minerals with consequent release of the constituent metals in their ionic forms, generating the leachate from which they will later be recovered according to their physicochemical characteristics. In this case, the main function of microorganisms is to regenerate Fe^{3+} ions. This process has advantages such as low cost, friendly to the environment and adequate in the treatment of ores with low levels of metals of economic

interest. It is a bioprocess widely recognized as an interesting route from an economic and environmental point of view, since it requires little expenditure on consumables (*i.e.*, acids and oxidizing agents), which are produced by the microorganisms themselves, reduced energy expenditure, low capital investment, low operating costs, reduced skilled labour in the operation compared to other metal extraction processes, use of ore tailings with reduced contents of metals of interest, in addition to not issuing SO_2, as in the pyrometallurgical process. Therefore, bioleaching can also be an alternative for small-sized deposits and/or far from centres with the necessary infrastructure.

Bioleaching is a biotechnological process that is based on the use of natural microorganisms capable of solubilizing sulphide minerals resulting in the release of constituent metals (GARCIA, 2007). Generally, these microorganisms involved in the natural processes of bioleaching in mining have well-defined characteristics. They preferentially grow in acidic environments (*i.e.*, they are acidophilic), and can even grow at pH close to zero (OTTOBONI & SATO, 2000), and such processes occur through the action of bacteria such as *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, and *Acidithiobacillus thiooxidans* etc. (BRIERLEY, 2010; RODRIGUES, 2015; TAO & DONGWEI, 2014; WATLING, 2006). According to Oliveira (2009), due to physiological and environmental similarities, these bacteria coexist simultaneously, intensifying the solubilization of the constituent metals of sulphide minerals, and are classified as chemoautotrophic, since they obtain energy from the oxidation of inorganic compounds such as sulphides (ALMEIDA, 2005; SILVAS, 2010), using CO_2 as the only carbon source for biomass production and are classified according to the temperature at which they develop, distinguishing themselves into: mesophiles (up to ~40 °C), moderate thermophiles (~40 - ~55 °C) and extreme thermophiles (~55 -~80 °C) (CHANG et al., 2000; NAGPAL et al., 2000).

The bioleaching of metals has been applied on an industrial scale, especially for copper, through static leaching in heaps. However, for metals with high added value, such as, for example, gold, the operating system used is tanks under agitation. Regardless of the system, the use of bacterial *inocula* is increasingly being understood as an optimization of the process, either in heaps or in reactors.

Thus, the production of large volumes of inoculum presents itself as a new challenge for the process. Generally speaking, this production can be obtained by a batch system or by the continuous cultivation of the microorganisms to be used. The continuous system can produce, in an accelerated way, substantial amounts of the different microbial consortia for an inoculation operation of bioleaching heaps on an industrial scale, for the extraction of the metals of interest from the sulphide minerals bearing these elements.

However, bioleaching is a biological process and, therefore, several factors affect the growth and activity of the microorganisms used in the process, including bacterial strains, pH, Eh, temperature and heavy metals in the leachates (YAMANE, 2012; KIM et al., 2021). During bioleaching processes, bacterial viability can be especially affected by increasing the

ionic strength in solution, resulting in decreased recovery of available elements in sulphide minerals (KIM et al., 2021), since microorganisms are aerobic, *i.e.*, need oxygen for their survival. With increasing salinity, the solubility of a gas is reduced, which makes it difficult for microorganisms to access oxygen. Therefore, bacterial adaptation to heavy metals could play a significant role for increasing bioleaching efficiency.

Thus, this work aimed at investigating the process of continuous cultivation of adapted acidophilic microorganisms, more specifically consortia of mesophilic iron oxidizing microorganisms, which are used in bioleaching of sulphide minerals.

2 | EXPERIMENTAL

2.1 Adaptation of microorganisms

Tests for adapting the microorganisms to increasing ionic strength, involving a consortium of *A. ferrooxidans*, LR strain, and *L. ferrooxidans* (ATCC53992), using an aliquot of 10 mL of the culture of *A. ferrooxidans* and 10 mL of *L. ferrooxidans*. The tests were accomplished in duplicate and included a negative control, in which no microorganisms were added (Control), only 200 mL of deionized H_2O acidified to pH 1.8 with 5M H_2SO_4 and 0.5g/L of ore. Using 500mL Erlenmeyer flasks, initially an aliquot was taken from the culture carried out in fresh medium and transferred to a new medium containing 0.5g/L of gold ore (*i.e.,* bearing chalcopyrite, pyrite and galena) and 32.16g/L of $FeSO_4$ (*i.e.*, ferrous sulphate, source of energy for the microorganisms), together with MKM culture medium (*i.e.*, 0.4 $g.L^{-1}$ of ammonium sulphate; 0.4 $g.L^{-1}$ of heptahydrate magnesium sulphate and 0.04 $g.L^{-1}$ of monopotassium phosphate). The Erlenmeyer flasks were placed in an orbital shaker (Eppendorf Innova S44i) at 30°C and under orbital agitation of 150 rpm, for a period of 3 days. In each new subculture, 10% v/v of inoculum from the previous culture was added to the fresh medium, in a solid/liquid ratio higher than that of the previous culture, being inversely proportional to the $FeSO_4$ concentrations, that is, as the ore concentration increased, the concentration of ferrous sulphate decreased, considering that the added ore contains sulphide minerals that are responsible for supplying such demand during their oxidative biological transformations.

During 28 days of adaptation, cell viability tests were performed to verify the growth of the bacterial population. An aliquot of the subculture, containing ore, was removed and placed in a fresh medium, under ideal growth conditions, being conditioned in the Eppendorf Innova S44i shaker for another 3 to 5 days. Another way of observing growth was through cell counting using an optical microscope coupled to a Thoma's chamber. A new propagation was performed when the count reached at least 10^7 cells per millilitre of culture. The technique of spectrophotometric analysis was used to determine the concentrations of Fe^{2+} and total iron in the samples undergoing the adaptation process. The spectrophotometer (HACH DR 6000) was set to a wavelength of 510 nm, suitable for the analysis of Fe^{2+} and

total iron. The samples were analysed in duplicate, that is, two aliquots of each sample were evaluated for Fe^{2+} and two aliquots for total Iron, in order to guarantee the precision and reproducibility of the results obtained.

The initial pH was recorded daily and, when necessary, adjusted with H_2SO_4 (5M), also recording the final pH. The number of drops of H_2SO_4 (5M) used to adjust the pH in each Erlenmeyer flask was recorded in order to assess acid consumption. The ore concentration was gradually increased at each propagation, starting with 0.1% of the total desired volume and reaching a solid-liquid ratio of 10% w/v.

2.2 Continuous cultivation

From the adaptation of the microorganisms, the microbial cultivation process was carried out through a continuous feeding of "new" MKM culture medium, in which the reaction volume (20L) was kept constant through the continuous removal, by overflow, of the "old" medium used.

The continuous cultivation process (Figure 1) began discontinuously, in which the reactor with MKM culture medium and solids (*i.e.*, 1 $g.L^{-1}$ of the gold ore produced by Euro Metal, the gold mining sponsor of this study), was inoculated with consortia of mesophilic microorganisms *A. ferrooxidans*, LR strain, and *L. ferrooxidans* (ATCC53992) in a 20L reactor useful volume. After a period of discontinuous operation, in which the culture reached a high cell population density, in the order of 10^7 cells/mL, the reactor was fed with culture medium and consequent spill over of the medium as a whole, containing the microorganisms of interest, initiating, effectively, the continuous process.

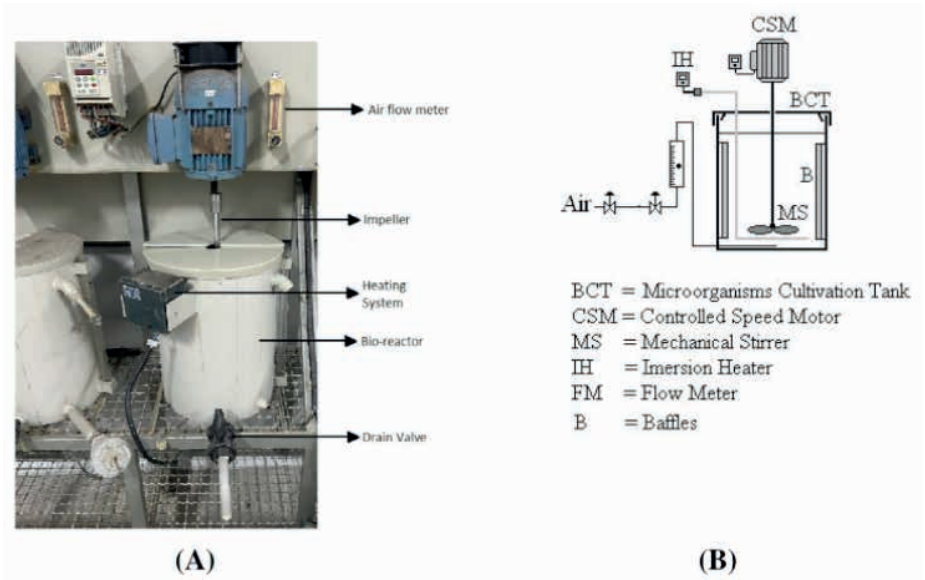

Figure 1- Continuous microorganism cultivation system, where Figure (A) shows the reaction system used and in Figure (B) the details of this reaction system.

The vertical stirrer is equipped with metal pallets, made of 316L stainless steel, and positioned in the centre of the reactor, as shown in Figure 2. This stirrer rotated at speeds ranging from 160 to 180 rpm. Air was injected into the lower part of the reactor and below the pallets fixed to the stirrer shaft at a flow rate of 10 to 15 L/h. Temperature control was carried out through a resistance located inside the tank.

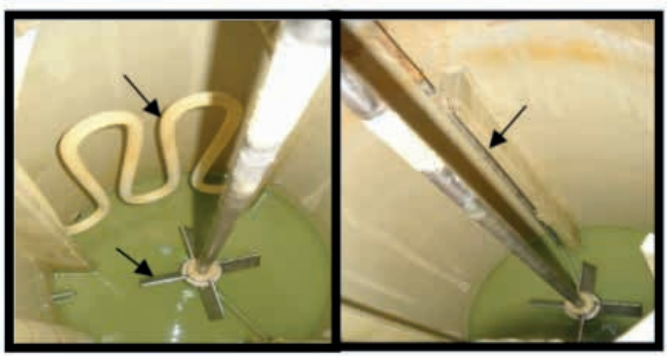

Figure 2 - Interior of the microorganism culture tank.

The reactors were kept with 0.1% w/v of solid (*i.e.*, gold ore) and gases (*i.e.*, O_2 and CO_2) at the aforementioned flow rate. The temperature of the cultures, the inlet flows of MKM (*i.e.*, nutrient solution), the solid addition (*i.e.*, gold ore), and the retention times of the microorganisms in the reactor, that is, the time elapsed for renewing the reactor cultivation volume, are described in Table 1.

Mesophilic Microorganisms			
Variables			
Temperature/ °C	30		
Retention Time/days	5	3	1
MKM Flow Rate/(L.day^{-1})	3.4	5.67	17
Solids Addition/(g.day^{-1})	68	113,3	340

Table 1- Conditions used during continuous cultivation of microorganisms.

Figure 3 shows the daily feed rates of nutrients and solids during the period of continuous cultivation of mesophilic microorganisms. Due to the need for less time for the growth of the microorganisms that constitute the mesophilic consortium, it was possible to carry out the test in two stages with different retention times.

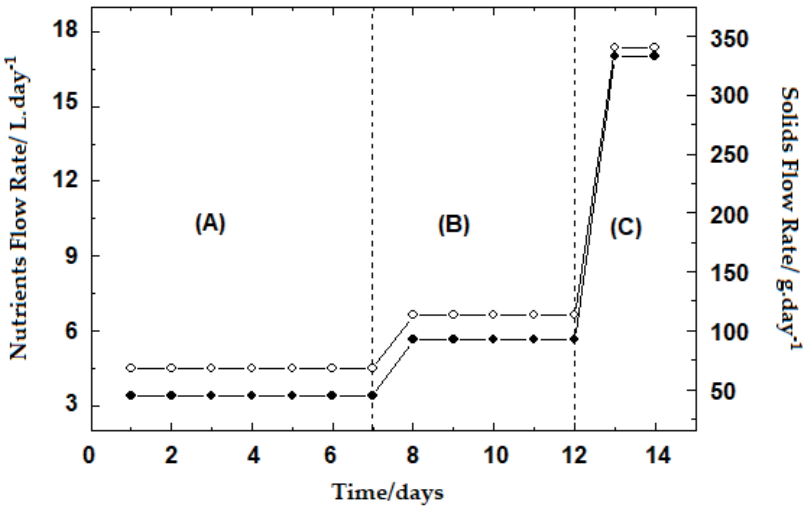

Figure 3 - Profile of the continuous production of Mesophilic Microorganisms, over a period of 14 days. The solid symbols (●) represent the Nutrient Feed and the empty symbols (○) the Solids, obtaining the following retention times (A) 5 days; (B) 3 days and (C) 1 day.

2.3 Concentration of ionic iron species

For determining the concentration of ionic iron species in the microbial inoculum, analyses of Fe^{3+} and Fe^{2+} concentrations were carried out using a spectrophotometric method with the use of orthophenanthroline. To quantify the Fe^{2+} concentration, the diluted sample was mixed with orthophenanthroline and the pH adjusted with acidic water at pH 2.1. After the formation of the Fe^{2+}/orthophenanthroline complex, the absorbance was measured at 510 nm. For total iron determination, Fe^{3+} ions were reduced to Fe^{2+} using hydroxylamine. The Fe^{3+} concentration was obtained by the difference between total iron and Fe^{2+} concentrations.

2.4 pH and Eh Monitoring

The pH and Redox Potential were monitored during the tests with an Analion pH-meter AN2000 Microprocessed device, using a pH combined glass electrode and Pt electrode (*i.e.*, with Ag°/AgCl reference), respectively. The electrodes were previously sterilized (*i.e.*, 20 minutes of immersion in 5% v/v formaldehyde solution (HCOH)) to avoid contamination of the culture.

2.5 Microbial Concentration

Microbial quantification was accomplished using a Thoma chamber with the following dimensions: area of 0.0025 mm^2 and depth of 0.100 mm, equipped with quadrangular divisions. Thus, counting the four quadrants of the extremities was adopted as a methodology. After counting, the equation below was used to determine the value corresponding to the

number of microorganisms per mL of the analysed suspension, remembering to add the dilution factor to the calculation, should this have been done.

$$M^{os}/mL = average\frac{16.10^5}{6.4}$$

3 | RESULTS AND DISCUSSION

3.1 Adaptation

Figure 4 shows the variation of the redox potential throughout the bioprocess. It can be observed that the potential gradually increases, indicating the evolution of the oxidative process, with a consequent increase in the total iron concentration due to the oxidation of sulphide minerals bearing iron in their structures, that is, pyrite (FeS_2), pyrrhotite ($Fe_{(1-x)}S$) and chalcopyrite ($CuFeS_2$). As expected, the fluctuations in the concentrations of the ionic iron species cause the variation of the redox potential throughout the experiment.

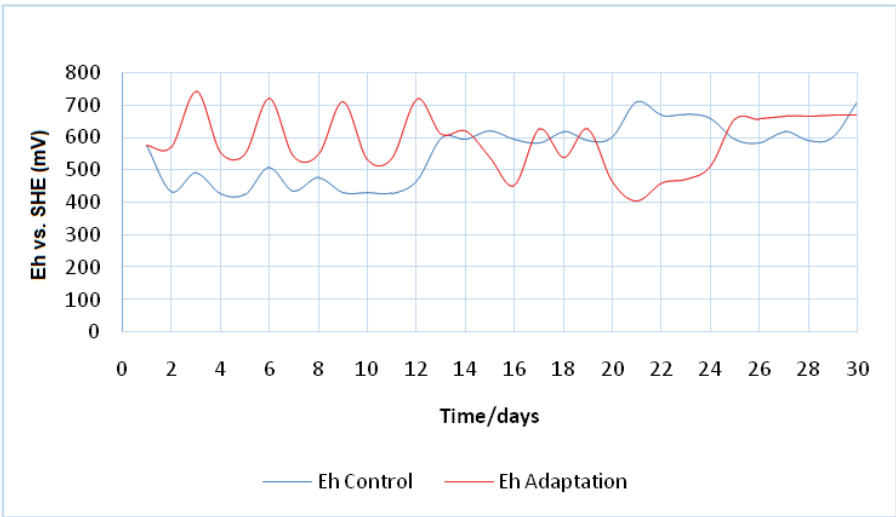

Figure 4 - Eh variation throughout the test period.

An increase in pH was observed in the first two days of the test; however, this parameter did not reach a value higher than 3 (Figure 5). This increase in pH is directly related to the reaction of sulphuric acid with the mineralogical species that make up the ore's gangue, which readily react with the available acid. The addition of sulphuric acid was carried out in the first four days, adding the equivalent of 39 kilograms of acid per ton of ore.

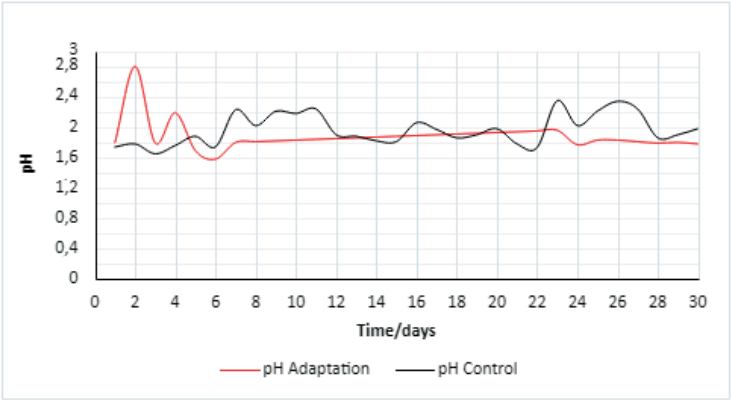

Figure5- pH variation throughout the test period.

3.2 Continuous cultivation

It was observed that in the cultivation of Mesophilic Microorganisms, with longer process time (Figure 6), the values of redox potential decrease, taking into consideration the concentrations of iron species (*i.e.*, Fe^{2+} and Fe^{3+}) in solution, according to the Nernst equation (*i.e.*, Eh a $[Fe^{3+}]/[Fe^{2+}]$) (Figure 7) and microbial population density increases. However, both presented a satisfactory microbial population, that is, values higher than 10^7 cells/mL.

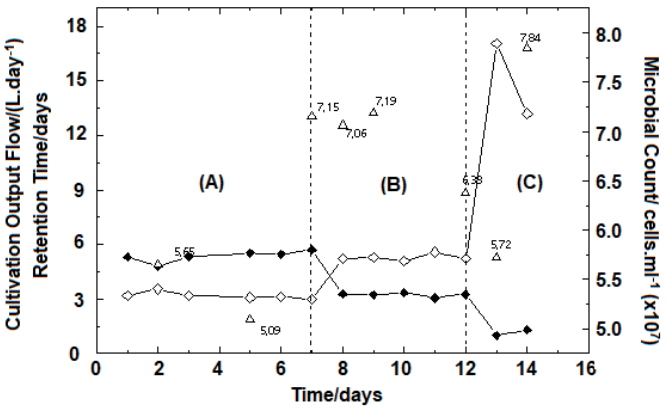

Figure 6- Culture outflow (◊), retention time (◆) and microbial count (Δ) of Mesophilic Microorganisms, with the increase in nutrient flow: (A) 3.4 L/day; (B) 5.67 L/day and (C) 17.0 L/day.

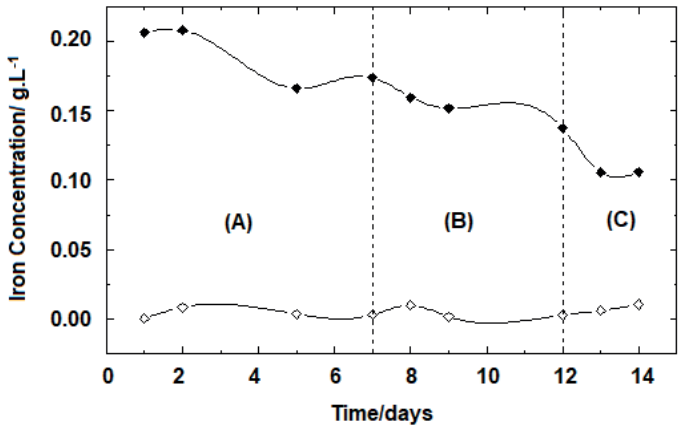

Figure 7- Variations in the Fe^{2+} concentration (◊) and Fe^{3+} (◆), in the continuous growth of Mesophilic Microorganisms, as the flow of nutrients increases: (A) 3.4 L/day; (B) 5.67 L/day and (C) 17.0 L/day.

The low values of redox potential in the test, in a shorter period of time (around 0.600 V *vs.* SHE), Figure 8, may be directly related to the duration of the test, causing an increase in the flow of culture medium solution, in shorter time.

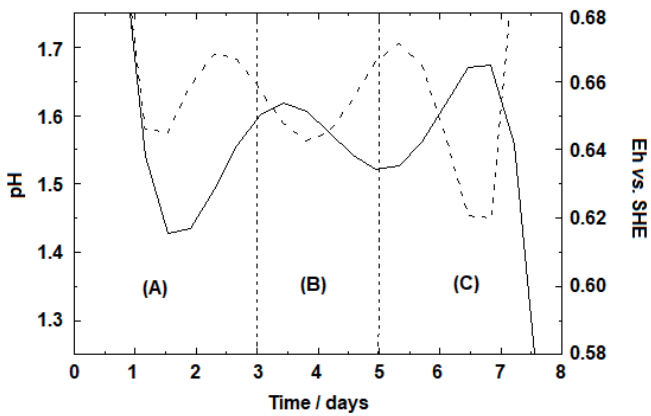

Figure 8- Variations of pH (-) and Redox Potential (--), in the continuous cultivation of Mesophilic Microorganisms, as the nutrient flow increases: (A) 3.4 L/day; (B) 5.67 L/day and (C) 17.0 L/day.

Observing the Figure 9, it is possible to notice that during the first four days of the experiment, there were small variations in the concentrations of Fe^{2+} and Fe^{3+} ions. However, from the 4th day onward, it is evident that the variation of these concentrations was quite expressive. There was a considerable increase in the concentration of Fe^{3+}, while the concentration of Fe^{2+} decreased, that is, the concentration of Fe^{2+} was inversely proportional to the concentrations of Fe^{3+}. It is possible that the demand for ferrous ions by microorganisms has increased, leading to an increase in the concentration of Fe^{3+} in solution. The decrease in Fe^{3+}, from the fifth day onward, may be linked to its reduction to

Fe^{2+} by the indirect mechanism of sulphide minerals oxidation. Additionally, the increase in Fe^{2+} concentration may have caused greater microbial growth, because Fe^{2+} becomes an energy source for the microorganisms metabolic processes; however, the increase in the concentration of ferrous ions and the reduction in the concentration of ferric ions may also be related to the cell concentration, that is, it is possible that there is no enough microorganism to oxidize these ferrous to ferric ions, so that these ions could oxidize the sulphide minerals present and, with that, solubilize the metal of interest, reducing it, consequently, to its respective ferrous ions. On the other hand, the reduction in the concentration of Fe^{3+} may be related to a possible decrease in the availability of the mineral substrate, that is, the sulphide minerals that are responsible for providing energy sources, Fe^{2+} in particular. This means that it is necessary, in order to make continuous cultivation possible, to feed the nutrient solution containing, in a mechanical suspension, some mineral substrate.

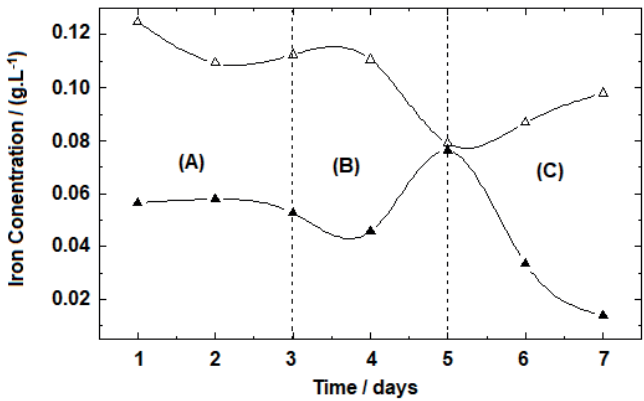

Figure 9- Variations in the concentration of Fe^{2+} (Δ) and Fe^{3+} (▲), in the continuous growth of Mesophilic Microorganisms, as the flow of nutrients increases: (A) 3.4 L/day; (B) 5.67 L/day and (C) 17.0 L/day.

The purpose of using the continuous microbial inoculum production process was to produce, in an optimized way and in less time, a greater volume of inoculum. Thus, the Table below shows, with respect to time, the volume produced in the different processes:

Consortium	Duration of Test (Days)	Time for Optimum Growth (Days)	Continuous Process (Volume/L)	Discontinuous Process (Volume/L)
Mesophilic	6	3	55.54	30

Table 2 - Production of microbial inoculum in continuous and batch systems.

4 I CONCLUSION

The adaptation process occurred as expected, since there was an evolution of the oxidative process;

It is possible to observe that cultivation in a continuous process proved to be effective in terms of inoculum production, considering the microbial population density values presented in this study (*i.e.*, at least 10^7 cells/ml), provided that the adaptation of microorganisms to increasing ionic strength is previously carried out;

The present study proved that it is possible to cultivate acidophilic iron-oxidizing microorganisms in a continuous system, maintaining a microbial density higher than 10^7 cells/mL, which is so necessary for the bioleaching process;

The addition of ore, bearing pyrite, was efficient in controlling the pH, causing it to decrease from 2.2 to 1.9 in 4 days;

Evaluating Table 2, it can be concluded that cultivation in a continuous system proved to be more efficient in the production of microbial inoculum, in particular, the production of consortiums of mesophilic microorganisms.

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