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Biomineralization of Calcium Carbonate by *Bacillus Cereus* for Self-Healing Biocement

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Concrete cracks can induce the disruption of dams, bridges, tunnels, and some structures that assist the human life. The agglomeration component of the concrete and the most applied compound, the Portland Cement, can be utilized in combination with calcium lactate and the *Bacillus* bacteria to produce the crystals of $CaCO_3$ (raw material of the cement production) through adsorption, biomineralization, and crystallization, reducing the risk of ruptures. Thus, a screening of the biotechnological potential of bacterium *Bacillus cereus* (UCP 1615) under a concentration of 10^7 cells/mL was carried out to evaluate the production of $CaCO_3$ crystals with 20 g of the Portland Cement II (PC-II), and 0,58, 1,00, and 1,42 g of calcium lactate. These Petri Dishes were incubated at 28°C and during 168 h. Besides, the crystals produced the *B. cereus* were analysed by an X-Ray Diffractometer (XRD) to estimate the relation (%) between the mainly and possible components. After 17 h, some crystals had already started to appear in the Petri Dishes of 1,00 and 1,42 g of calcium lactate, and after 168 h, the crystals were scraped of all Petri Dishes. The diffractometric analyse of the condition with the highest lactate condition, of 1,42 g, presented percentages of 82% SiO_2 (basic structure of cement) and 18% $CaCO_3$. Therefore, the *B. cereus* strain shows a biotechnological potential to produce crystals of $CaCO_3$ and that this bacterium is able to be tested under different conditions of calcium lactate, microbial concentration, and water according to a factorial design.
Keywords: Concrete cracks. Calcium Lactate. Crystals. XRD.

1. Introduction

In the urban, building and industrial areas, effects of solar rays, winds, rainings, and any kind of weathering are able to wash out the concrete of structures as bridges and roads. The inside and outside cracks of this concrete will reduce the compressive, tensile, and flexural strength limits of the material, increasing the probability to cause any accident. Furthermore, the concrete composition is an essential factor to give a long shelf life for the concrete, requiring a suitable cement for the current application with the ideal proportions of water, and fine and coarse aggregates (Neville, 2016).
For the production of cement, initially, there is the extraction of limestone ($CaCO_3$) from mines with the addition of iron ore and clay. This mixture will enter into a precalcifier (at about 1000 °C) to release CO_2 and CaO and after into a rotational furnace (at about 1500 ºC) to vitrify the clay, constituting a new mixture called clinker (Neville, 2016). These two processes represent the focus of the cement industry. Only this process represents approximately 5-7 % from all CO_2 production of the Earth, mainly because cement is the most utilized mixture of the world (Shanks et al., 2019).

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The clinker can be combined with a diversity of compounds as plaster ($CaSO_4$), slag (SiO_2), pozzolan (silicates), filler ($CaCO_3$), and including the same limestone utilized before the clinker formation in order to reduce the cost of the combustion from the precalcifier and also the release of CO_2 in the atmosphere. These mixtures will characterize the cement with a variety of compositions (Neville, 2016).

In Brazil, the legislation through the NBR 16697 unified the older standards that specified the composition of the different kinds of Portland Cement (PC). This classification can divide the cements in five groups with: I) at least 95 % of clinker with one or more kind of plaster; II) with a maximum mixture of 94 % of clinker with one or more kind of plaster; III) an elevated percentual of slags utilized in heavy constructions; IV) an elevated percentual of pozzolans to reduce the corrosion and the permeability; and V) a lower amount of additives, ensuring a higher initial strength since the first day (ASSOCIAÇÃO BRASILEIRA DE NORMAS TÉCNICAS, 2018).

The combination between cement, water and aggregates will compose the concrete, depending of the application. For common uses, there are pumpable, reinforced and a diversity of concretes, and for new trends, there is the self-healing concrete, for example (Neville, 2016).

A self-healing concrete should present: 1) an efficient sealing; 2) a compatibility from a healing agent with the concrete matrix; 3) a long shelf-life; and 4) an eco-friendly viability. The healing agent can be polymeric, inorganic, or biological. The first two present the disadvantage to react chemically in a wide range of temperature. This is a disadvantage because at any moment the self-healing reaction should stop to avoid the excess of self-healing chemical agent into the concrete matrix (Sidiq et al., 2019).

In this sense, self-healing biological agents as the bacteria *Bacillus* are able to recover cracks inside and outside concrete with a controlled range of temperature by the biomineralization. This phenomenon is usually known as the inorganic precipitation from organisms and microorganisms as: the pearl formed by oysters as a defense mechanism; the production of gold from kinds of bacteria; and the calcium carbonate precipitation by *Bacillus* bacteria in the self-healing concrete (Bastrzyk et al., 2019; Zhang et al., 2019).

There are some biomechanisms that the *Bacillus* species utilize to produce calcium carbonate, depending of the medium. The ureolithic and denitrifying biomechanisms are pathways to convert urea and organics compounds, respectively, into the intended inorganic compound with celerity, however there are two sequential disadvantages: the production of ammonium that can be converted into nitrous or nitric acids according to the nitrogen cycle, raising the corrosion in reinforced concrete for example; and the generation of a suitable environment for *Thiobacillus* species due to the lower pHs, whose presence may causes the concrete degradation (Sidiq et al., 2019; Vijay et al., 2017).

On the other hand, the biochemical mechanism by aerobic oxidation from calcium lactate in Equation 1 will produce the calcium carbonate, water, and carbon dioxide. This dioxide will be combined with some hydrated lime in cement or in concrete to produce more calcium carbonate, according to the Equation 2. This set of reactions is not as faster as the ureolithic and denitrifying biomechanisms, but it will crystallize $CaCO_3$ with lower risks of concrete degradation (Sidiq et al., 2019; Vijay et al., 2017).

$$CaC_6H_{10}O_6+6O_6 \rightarrow CaCO_3+5CO_2+5H_2O \quad (1)$$

$$5CO_2+5Ca(OH)_2 \rightarrow 5CaCO_3+5H_2O \quad (2)$$

Crystallization is a phenomenon that can build geometric inorganic structures with the concentration of a saturated solution. That can be exemplified when steam collides against dust grains under conditions of temperature and pressure, forming the hexagonal snowflakes. In the biomineralization of $CaCO_3$ under the calcium lactate biomechanism, three processes are in evidence: the adsorption of the microorganism into calcium lactate, the biochemical and chemical reactions, and finally the crystallization of $CaCO_3$ (Foust et al., 2013).

Crystals formed by biomineralization can be analysed according to the composition using the X-Ray Diffraction (XRD). This petrographic technique consists on the application of an x-ray beam on a sample. The deviation of the rays will compose a crystallographic profile that can be compared to a diversity of samples profiles around the world (Mors, Jonkers, 2017).

This data infinity can be easier to analyse if a tool that can build a diffractogram, identify the peaks, and distinguish these peaks is used. In this sense, the HighScore Plus® of the PANalytical B.V.© is a software that can reduce the analyse time with an effective comparation of the diffractograms database of inorganic compounds, allowing the user to select which compound will probably be in the crystallographic analysis (Kaur et al., 2017).

Therefore, the aim of this work was to screen the crystal production of $CaCO_3$ under the calcium lactate biomechanism, utilizing the bacterium *Bacillus cereus* (UCP 1615) into a Portland Cement II to investigate the biotechnological potential of *Bacillus cereus* as a biomineralizer microorganism.

2. Material and Methods

2.1 Microorganism and Maintenance and Growing Media

The biomineralizer bacterium was the *Bacillus cereus* (UCP 1615), the maintenance medium of the microorganism to conserve the strain was Agar Nutrient (AN), and the growing media was the Nutrient Broth (NB). Strain of *B. cereus* was pre-inoculated aseptically from AN to NB medium to activate the bacterium in 125 mL Erlenmeyer Flasks, under an agitation of 200 rpm and 28 °C, and during 24 h (Ostendorf et al., 2019). The microorganism utilized was cataloged in the Catholic University of Pernambuco which has a biotechnological potential to produce biosurfactants. The alcoholic fermentation also occurs in the production of crystals of calcium carbonate by the release of CO_2, justifying the choice of the microorganism.

2.2 Production Media

The biomineralization of calcium carbonate had the production medium composed by: 20 g of PC II; 0.58, 1.00, and 1.42 g of calcium lactate; and 10 mL of distilled water. This distribution occurred to follow future conditions of an experimental design called Central Composite Rotatable Design (CCRD) and to maintain the relation water/cement in a range from 40 to 60 % (m/m) (Zheng et al., 2017). This production medium was mixed manually in Petri Dishes, as illustrated in Figure 1, dried in a drying oven under 50 °C under 4 h, and decontaminated by an ultraviolet radiation inside a laminar flow chamber.

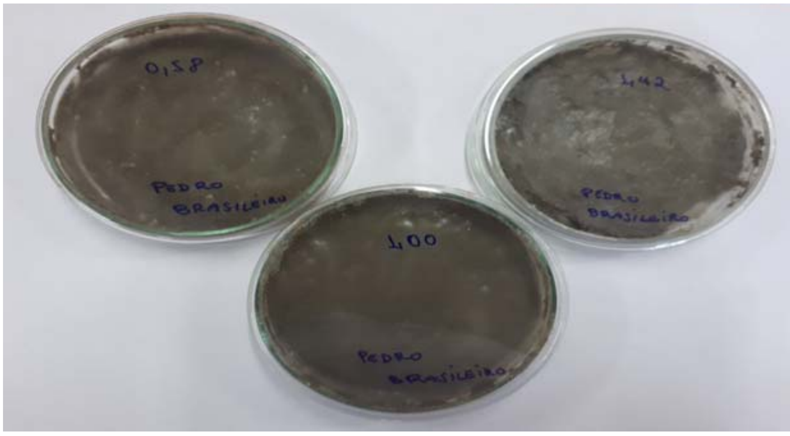

Figure 1: Production medium before the drying oven

2.3 Crystal Production

A 1 mL volume of growing medium containing a concentration of 10^7 cells/mL, analyzed by optical density through an ultraviolet spectrometer, was inoculated aseptically on the Petri Dishes with the dried production medium.

The samples were maintained inside a drying oven, under 28 °C and during 168 h to ensure the adsorption, biochemical and chemical reactions (Equations 1 and 2), and crystallization processes.

2.4 Crystallographic Analysis

The diffractometer Bruker D2 PHASER, under a voltage of 30 kV, a current intensity of 10 mA, and a radiation of Cu- Kα under 1.54 Å. The screening was made between 10 and 80 ° with a step of 1,0 s and angle of 2θ (Lam et al., 2010).

The software HighScore Plus® of the PANalytical B.V.© was utilized to compose the diffractogram with a Rietveld refinement. This tool could also quantify the peak relation (%) between the common components in PC II compared with the software database.

3. Results and Discussion

3.1 Crystal Production

After 17 h of production, in the Figure 2, there was observed that crystals had being formed in the surrounding of Petri Dishes by a higher contact with the air on the three Petri Dishes, according to the Equation 1. Besides, the Petri Dish with 1.42 g of calcium lactate have presented some crystals on the middle of the dish by the elevated concentration of lactate. These features indicated the requirement of an aeration flow inside the dishes with filters on the inlet and outlet flows to avoid the contamination of external microorganisms. As an alternative, it is possible to construct a workable model with a 3D printer and to combine with a microcontroller to produce a prototype with aeration.

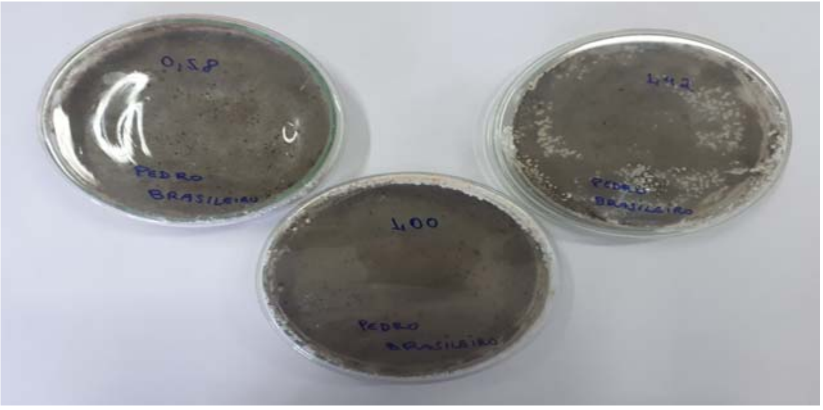

Figure 2: Crystal production after 17 h on the three Petri Dishes

After 168 h of experiment, the crystals were more evident at the end of the process, as be can seen in the Figure 3 on Petri Dishes and Figure 4 under an optical microscopy view, mainly on 1.42 g Petri Dish. These crystals were collected using platinum handles to reduce the number of cement grains. Amounts of crystals were sent to XRD analysis.

Figure 3: Crystal production after 168 h on the 1.42 g calcium lactate Petri Dish

Figure 4: Optical microscopy of crystals of Bacillus cereus

3.2 Crystallographic Analysis

The XRD analysis resulted in 82 % of SiO_2 and 18 % of $CaCO_3$, considering the peak percentual. This distribution might indicate that particles of SiO_2 must have spread from the cement to inoculum volume. Probably, at the moment that the liquid was evaporating, the bioreaction of Equation 1 and the reaction of Equation 2 had also been occurring with the crystallization of these components.

In the Figure 5A, it can be observed that the crystals obtained from *Bacillus cereus* presented an elevated compatibility with the bioconcrete obtained by a *Bacillus* specie, in the Figure 5B (Lucas et al., 2018), according to the comparation between the diffractograms. In this experiment the authors combined bacterium spores, calcium lactate, and the concrete CEM I.

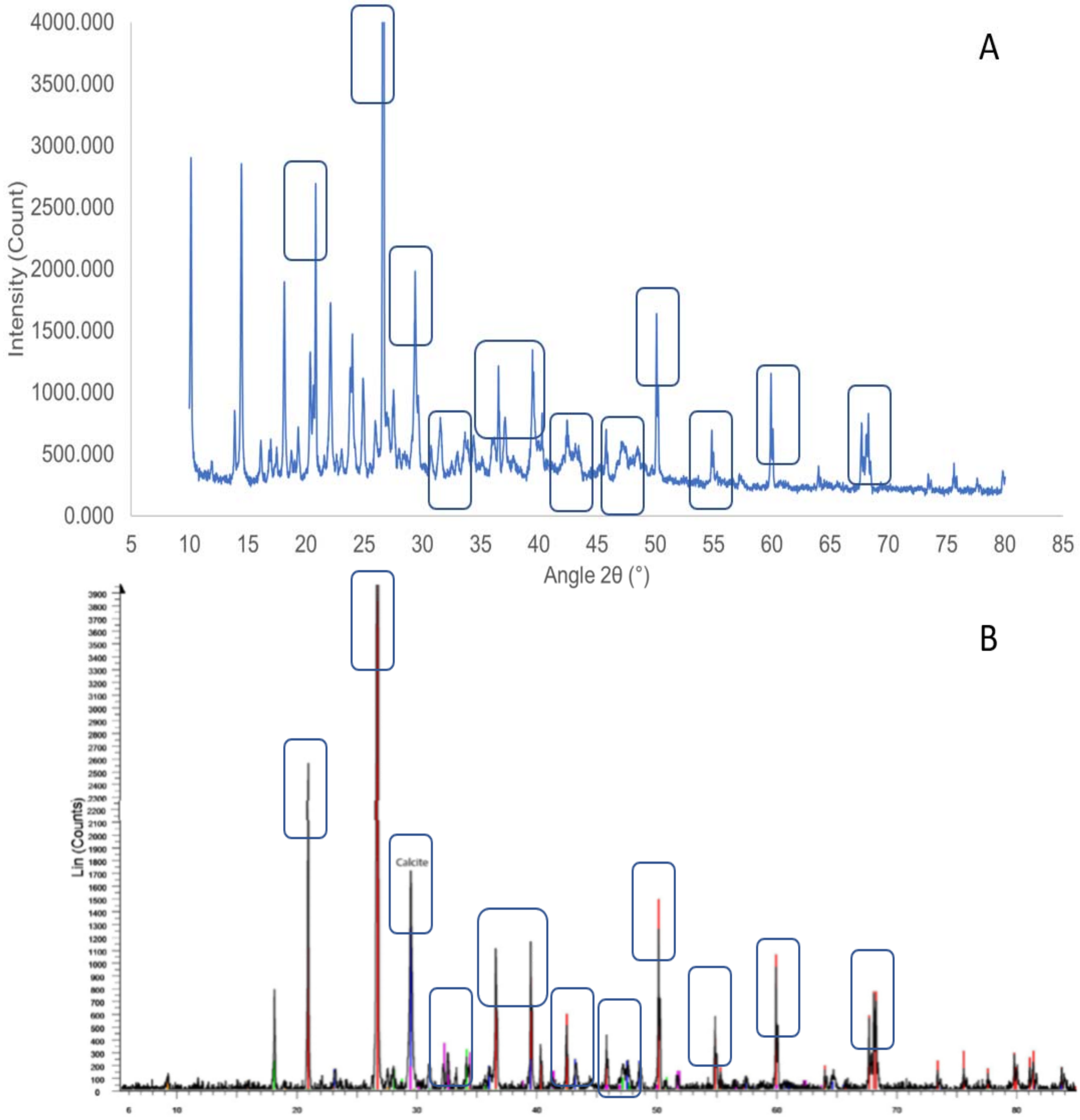

Figure 5: Peaks distribution of crystal formed in: A) biocement produced by B. cereus, and B) bioconcrete (Lucas et al., 2018).

It is clear that the Figure 5A showed more residues than the Figure 5B, and it can be explained by the possible crystallization of other components inside the biocement.

It is important to emphasize that tests with the biocement are only a screening of $CaCO_3$ production. It means that the bacterium *Bacillus cereus* has the biotechnological potential to produce calcium carbonate, however it is necessary to test others factors as the resistance to compression, traction, and bending. Furthermore, the material properties should also be evaluated.

4. Conclusions

The biocement produced by the bacterium *Bacillus cereus* has presented a biotechnological potential to produce crystals of calcium carbonate through the biomineralization under the biomechanism of calcium lactate, mainly in the condition of 1.42 g of lactate that represents a cement ratio of 46.7 %. The crystal production should be investigated by the variation of pH from the distilled water, the calcium lactate concentration, the bacterium concentration, and the volume of distilled water added. These combination will build a Central Composite Rotatable Design that is an useful statistical tool.
Tests with Scanning Electron Microscopy should also be done to know the topography of the crystals. Besides the individual investigation between different kinds of cement and *Bacillus* species are essential to understand which bacterium has the best biotechnological potential.

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