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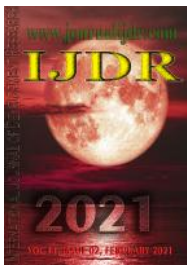

RESEARCH ARTICLE **OPEN ACCESS**

LYSINIBACILLUS SPHAERICUS AS A SELF-HEALING AGENT FOR CEMENT-BASED MATERIALS: A PRELIMINARY INVESTIGATION

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ABSTRACT

Self-healing in cement-based materials occurs in order to plug microcracks that can cause harmful agents to enter the material. In this context, the objective of this work is to study the microorganism Lysinibacillus sphaericus and submit it to tests that prove its application in concrete structures to act as a healing agent. For this, it was exposed to growth in a culture medium with different pH alkalis. Then, the concentration of microorganisms in the culture medium was verified by the Petri dish counting method. After this step, the microorganism underwent a 14-day growth in a liquid culture medium rich in calcium to verify its capacity to precipitate calcium carbonate minerals. Investigations of precipitated minerals were conducted by XRD and SEM. The bacterium Lysinibacillus sphaericus precipitated the calcium calcium carbonate and showed growth in the culture medium with alkaline pH. This result is a strong indication that the microorganism is suitable to be applied as a healing agent in cement-based materials.

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INTRODUCTION

Nature has long served as a source of inspiration for humans in the development of technology. In the last decades, there has been an increase in studies on biomaterials that improve various properties of materials (Aizenberg and Fratz, 2009). According to Teeri *et al*. (2007), biomimicry is a field of science that investigates biological structures and processes of materials that have considerable potential in the development of new high-performance materials with low environmental impact. In the context of biomimetics, self-healing materials can be defined as those that have the ability to repair themselves without external intervention (Seifan *et al*. 2016) (Ghosh, 2009). According to Zwaag (2007), a recurring goal in the development of self-healing materials is to prevent damage caused by their use. In concrete, the presence of cracks is inevitable due to the shrinkage resulting from the reduction in the volume of hydration products, leading to low tensile strength and material degradation over time (Rauf *et al*. 2020). The internal cracks act as pathways for chemicals that are aggressive to the structure, affecting durability and reducing its service life (Wang *et al*. 2012) (Xu *et al*. 2013). The consequences of these cracks can lead to a reduction in the service life of these structures (Jonkers, 2011) (Tittelboom *et al*. 2010) (Chahal *et al*. 2012). Damage to concrete structures can impact their cost over time, given that interventions will be necessary to treat pathological manifestations, a fact that will possibly generate costs for maintenance and losses if the building has to be isolated to perform some repair (Medeiros *et al*. 2011). In this respect, techniques called self-healing of cracks have been studied as a way to increase the service life of buildings. Among them, there is the use of alkali-resistant bacteria that deposit carbonates as a result of their metabolic activities. However, the efficiency of this self-healing depends on the use of special bacteria with characteristics that include spore formation, high tolerance to basic pH and microbial induction with mineral precipitation (De Muynck *et al*. 2010). Thus, it is important to confirm bacterial resistance to adverse conditions such as the high pH and low oxygen level characteristic of concrete (Han *et al*. 2020). In this context, research on microorganisms and different enrichment mediums has been carried out, such as: Bacillus alkalinitrilicus with calcium lactate (Wiktor and Jonkers. 2011), Bacillus sphaericus with chloride calcium (De Muynck *et al*. 2010) (Tittelboom *et al*. 2010) and Bacillus megaterium with calcium lactate and urea (Andalib *et al*. 2016). In order to incorporate knowledge to the theme that has been investigated, this work aims to verify whether the microorganism B. Sphaericus presents growth when in contact with nutrient mediums that are pH neutral (pH 7) and alkaline (pH 9, 10, 11, and 12) through the addition of calcium hydroxide as a pH-modifying agent in the medium. It will also be verified if this

microorganism precipitates the chemical compound calcium carbonate when enriched with Calcium Nitrate. Thus, its applicability as a microorganism capable of being used as a self-healing agent in cement-based structures can be suggested. B. Sphaericus was recently reclassified and is now part of the genus Lysinibacillus. As can be seen in Table 1, this microorganism has already been studied by Wang *et al*. (2014) with the same enrichment method proposed here. The researchers studied absorption, permeability, porosity and resistance to compression and flexion. They reported that absorption was reduced by up to 30%, permeability was 10 times lower, and pore size decreased. However, the compressive strength was reduced and the tensile strength in flexion was not changed. The researchers reported that the healing rate in samples with biocapsules was about 2 times higher than in those without bacteria, and the thickness of the cured cracks reached the order of 4 times that of the non-bacterial series.

Although the researchers carried out a study with the same microorganism and enrichment medium, the in vitro method performed here was not followed. In this context, this research proposes the indication of a preliminary path for the identification of microorganisms with the potential to be successful in self-healing in Portland cement composites. The importance of an in vitro method is to generate preliminary information about a specific microorganism based on rapid tests, even before a study on concretes and mortars, in which the verification of the effectiveness in healing cracks can take a few months.

MATERIALS AND METHODS

Concrete is a highly alkaline medium with a pH around 12.5. Therefore, knowing whether the microorganism remains alive and active in this environment is a key issue for the viability of its use in the development of self-healing concretes. In addition, a question to be answered is whether the microorganism disposed in a calcium-rich medium tends to generate calcium carbonate. This material can be precipitated in the cracks, generating self-healing, as reported in studies by Tittelboom (2010), Wang *et al*. (2014) and Jonkers (2011).

In this context, the experiments were developed in order to follow the line of reasoning as shown in the flowchart of Figure 1, which indicates that two experiments were carried out in parallel.

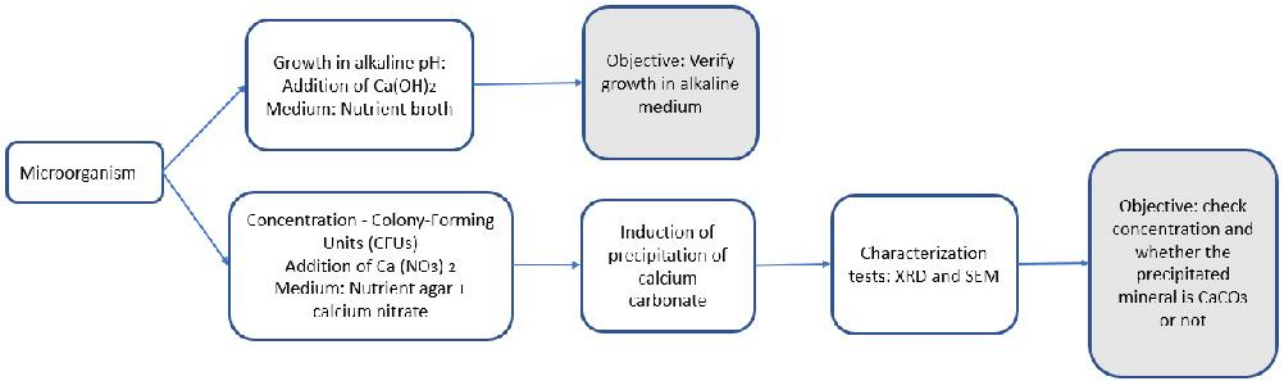


Figure 1. Flowchart of the experiment

First: the growth of the microorganism in an alkaline pH was induced in order to verify its growth in this medium. Second: the chemical compound Calcium Nitrate was added in order to make the medium rich in calcium. In this line of study, a serial dilution experiment was carried out to verify the concentration of the culture medium. Finally, the precipitation of the microorganism L. sphaericus was induced to verify the precipitation of the mineral calcium carbonate. The response variables (dependent) of this experiment are the growth of the microorganism at pH 7, 9, 10, 11 and 12, the concentration of the culture medium in CFU/ml (CFU – colony-forming unit), and the identification of calcium carbonate crystals ($CaCO_3$) using characterization by XRD and SEM. With this, it is intended to investigate the applicability of Lysinibacillus sphaericus to act as a healing agent in cementitious matrices. The parameters set in this experiment, or independent variables, were: the base nutritional medium for the growth of the microorganism (Agar/Nutrient Broth), the incubation temperature (30°C) and addition of the calcium-rich reagent in the concentration of 5 g/l of distilled water (Calcium Nitrate).

MATERIALS

Lysinibacillus sphaericus: The microorganism used was Lysinibacillus sphaericus (L.S.) INCQS-422 (ATCC 14577). This was provided by the Oswaldo Cruz Foundation (Fiocruz), a Brazilian public entity linked to the Ministry of Health, which is a research and development institution in biological sciences. The use of this microorganism was due to the ease in its acquisition and its use by researchers who obtained satisfactory results related to the porosity of the cement matrix (Titelboom *et al*. 2010), (Wang *et al*. 2014).

Calcium-based reagent: The reagent used to potentiate the growth of the microorganism and induce the formation of carbonate minerals was Calcium Nitrate ($Ca(NO_3)_2$). The researchers Wang *et al*. (2014) used L.S. in the same nutrient medium, with melamine microcapsules. They indicated a 20% to 30% reduction in absorption. Calcium nitrate was diluted in the proportion of 5 g to 1 liter of distilled water.

Base nutrient: The base nutrient for the growth of the microorganisms in a liquid medium was Nutrient Broth. The nutrient acquired was the Kasvi brand. The composition of the nutrient is given by the proportion of 1.0 g of meat extract, 2.0 g of yeast extract (protein base rich in carbohydrates), 5.0 g of peptone (semi-digested protein that serves as a source of nitrogen and carbon) and 5.0 g of sodium chloride. The dilution adopted was 13 g to 1 liter of distilled water, as indicated by the manufacturer. For the growth of the microorganisms in a solid medium, nutrient Agar was used. Agar is a polysaccharide extracted from red seaweed and has a gelatinous texture. It is used in microbiology to prepare solid cultures, such as those produced in Petri dishes. For this work, Nutrient Agar was used, which has the same composition as Nutrient Broth, the only difference being the addition of the compound Agar in the fraction of 15.0 g to 13.0 g of the base nutrient (broth). The difference between the two culture mediums in solid and liquid mediums can be seen in Figure 2.

METHODS

Growth in alkaline pH: The microorganisms were grown with a change in the pH of the culture medium. In addition to the neutral pH (pH 7), four alkaline pHs were adopted: pH = 9, pH = 10, pH = 11 and pH = 12. The objective of this experiment is to simulate the medium of a concrete structure, which has an alkaline characteristic.

The pH change of the culture medium was carried out by the chemical compound calcium hydroxide ($Ca(OH)_2$) to keep the medium rich with the calcium element and similar to the concrete pore solution. The control was done with the aid of a benchtop pH meter and the $Ca(OH)_2$ additions were made until reaching the pH measurements cited. After changing the pH of the culture medium, the microorganism was added to induce growth. For that, the culture medium was subjected to inoculation of the microorganism and placed in a heating chamber at 30°C for 24 hours to induce growth.

After this period, the liquid medium was subjected to reading by spectrophotometry to assess growth according to the concentration of the medium.

Concentration of medium - Petri dish count: This method consists of determining the size of a bacterial population. The method considers that each colony formation in the Petri dish comes from a single microbe, so the Petri dish count is often called Colony-Forming Units (CFUs) (Tortora *et al*. 2005). In the counting procedure, a volume of 1 ml is removed from the sample to be quantified, which is diluted in 0.9% saline medium with a volume of 9 ml. Thus, each test tube containing 9 ml of saline solution receives the volume of 1 ml of the previous tube. After the dilutions are completed, the mediums are transferred in the amount of 0.1 ml to the Petri dishes of Nutrient Agar and Nutrient Agar + Calcium Nitrate (5 g/l), which are subjected to a heating chamber at a temperature of 30°C for 24 hours to start the growth. After the elapsed time, the Petri dishes are counted by the colonies that showed growth and can be seen with the naked eye. The addition of Calcium Nitrate aims to increase the concentration of calcium in the medium and also to increase the nitrogen that serves as a nutritional source for microorganisms. Precipitation induction of the mineral calcium carbonate. This part of the experiment was divided into two phases.

Phase 1: Induction of precipitation - For this experiment, two samples were prepared, one without the addition of Calcium Nitrate and the other with the addition. The nutritional culture medium used for this procedure was the liquid culture medium with a Nutrient Broth base. 50 ml of nutrient medium was prepared with Calcium Nitrate (5 g/l), as well as 50 ml of nutrient medium without Calcium Nitrate. The microorganisms were transferred to these mediums and submitted to a heated shaker at 100 rpm with controlled temperature at 30°C for 14 days, in order to stimulate the precipitation of the chemical compound calcium carbonate. Phase 2: Collection of the precipitate - After the precipitation induction process, the samples were transferred from the culture mediums to 15 ml Falcon type reagent tubes, which were centrifuged at 1000 rpm for a period of 20 min. This procedure was applied to induce the accumulation of precipitated material at the bottom of the liquid. Figure 3 illustrates the precipitated material after centrifugation. Then, the sediments were transferred to glass Petri dishes and subjected to drying in a heating chamber with a temperature of 40°C for 48 hours. After that, the material was collected, which was in the form of powder.

Characterization of precipitated material: The material collected in the previous phase was submitted to diffraction tests (XRD) and scanning electron microscopy (SEM) with energy-dispersive spectroscopy (EDS). The aim was to identify the products that make up the precipitated material.

XRD: The powdered material resulting from the precipitation of the microorganism was pressed manually in the sample holder and submitted to the Shimadzu MAXima XRD-7000 equipment, operating at 40 kV and 30 mA. The test parameters were: angle from 10° to 70°, step of 0.017° and scan speed of 2°/min. To refine the data, the X'Spert Highscore Plus software with crystallographic database from the International Center for Diffraction Data (ICDD) of 2003 was used.

SEM with EDS: The sample was prepared by placing the precipitate (powder collected from the test tubes) under carbon strips and then carrying out the metallization, which consists of depositing a thin layer of gold on the sample surface. The images were taken with the TESCAN S8000 equipment, which made it possible to visualize the shape of the precipitated minerals. The chemical elements present in the samples were investigated by energy-dispersive spectroscopy analysis (EDS) with the Oxford Instruments equipment (x-Act detector) that is coupled to the SEM.

RESULTS AND DISCUSSIONS

Growth of the microorganism in different pHs: The microorganism showed greater growth when at pH = 7, as can be seen in Figure 4. In this neutral pH culture medium, no calcium hydroxide was added, since it was only used to effect the variation of neutral pH to alkaline. The growth in neutral pH was statistically different from the other series. On the other hand, the series with pH = 9, pH = 10, pH = 11 and pH = 12 were statistically equal. The statistical method used was analysis of variance (ANOVA) with multiple comparisons between means using the Tukey test with a decision limit less than 0.05. Considering that the growth at pH greater than or equal to 9 has no statistical difference, the average growth can be considered 0.336, which represents 1/3 to ¼ of the result at pH = 7.0. It is important to consider that, despite less growth in alkaline pH, Figure 4 shows that there is growth of Lysinibacillus sphaericus at pH 9 to 12. Bacterial growth can be influenced by several factors, especially the nutrient medium, temperature, humidity, oxygen content and pH. It is known that for each microorganism there is an optimal pH value at which its maximum growth occurs (Stocks *et al*. 1999). According to Stocks *et al*. (1999), pH is an important calcification factor for microorganisms, since precipitation is directly related to pH and the molar concentration of Ca2+ in solution. The pH also has an effect on the calcium carbonate morphology. According to Rodriguez-Navarro *et al*. (2012), vaterite may be more favorable than calcite in a high pH environment (pH 8.5-9.0) and the reaction rate may occur more slowly compared to lower pH values.

Petri dish counting: effect of the presence of calcium nitrate: Figure 5 illustrates the count made in the Petri dishes. It is observed that the dilution increases (varying from $1/10^4$ to $1/10^7$) from left to right, and the count is displayed just below each Petri dish. The dilutions occurred up to $1/10^9$, but as there was no growth in the last two dilutions, it was decided not to present their illustration. For the Petri dishes with the addition of Calcium Nitrate, the dilution with $1/10^4$ presented an excessive number of CFUs, which made counting impossible due to the spread of the colonies. Counting was only possible starting at the $1/10^5$ dilution Petri dish, showing 766 CFUs. Table 1 shows the concentrations of microorganisms after Petri dish readings. It should be noted that the microorganism obtained close growths, since for the medium without Calcium Nitrate addition, an average concentration of 1.22×10^8 CFU/ml was obtained, and in the medium with Calcium Nitrate addition, an average concentration of 0.97×10^8 was obtained.

Table 1 .Count and concentration of microorganisms in medium with and without calcium nitrate

Dilution	Agar Nutrient (CFU)	Agar Nutrient + Calcium Nitrate (CFU)
$1/10^4$	excessive nº of CFUs	excessive nº of CFUs
$1/10^5$	958	766
$1/10^6$	101	96
$1/10^7$	9	12
$1/10^8$	2	0
$1/10^9$	0	0
Mean (CFU/ml)	1.22×10^8	0.97×10^8

X-Ray Diffractometry (XRD): For the culture medium without the addition of Calcium Nitrate, there was no precipitation of minerals. This suggests that for the precipitation of the chemical compound calcium carbonate to occur, there must be an addition of some medium rich in calcium. Figure 6 shows the diffractogram of the precipitated material. The diffractogram shows that the precipitated mineral is vaterite, which is a variation of the chemical compound calcium carbonate ($CaCO_3$).

Scanning Electron Microscopy (SEM): Figure 7 shows the scanning electron microscopy images for the microorganism Lysibacillus sphaericus when exposed to the Calcium Nitrate reagent. Figure 7 (a) has a magnification of 400 times and the image of Figure 7 (b) has a magnification of 8,500 times, which has been focused on at the location indicated in the circle of Figure 7 (a). By observing the precipitate, it is suggested that they are calcium carbonate mineral formations. There are six morphologies known as calcium carbonate (Jonkers and Schlangen, 2007), calcite, aragonite, vaterite, monohydrocalcite, ikaite and amorphous calcium carbonate. Calcite and vaterite are the most common phases in applications of microorganisms to cementitious matrices (Addadi *et al*. 2003) (Lian *et al*. 2006). Figure 8 shows the EDS result for the microorganism in the presence of the Calcium Nitrate reagent.

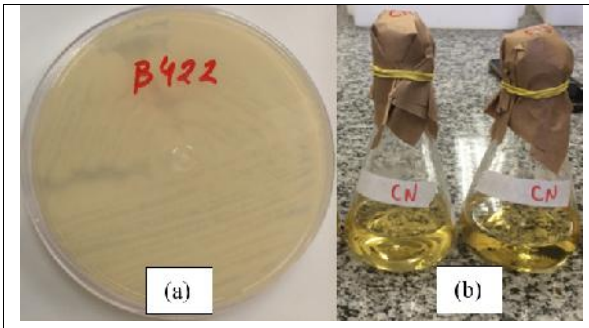


Figure 2. (a) Solid medium - (Agar in Petri dish), (b) Liquid medium (Nutrient broth)

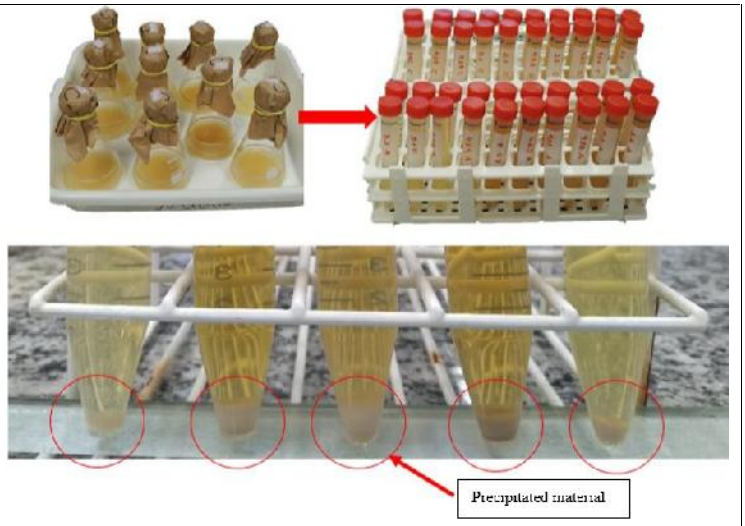


Figure 3. Precipitate of microorganisms

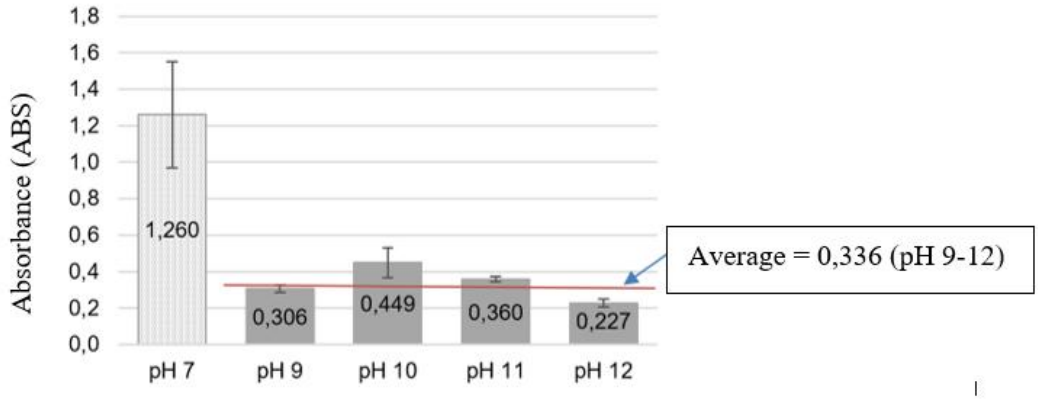


Figure 4. Growth of Lysinibacillus sphaericus under different pHs

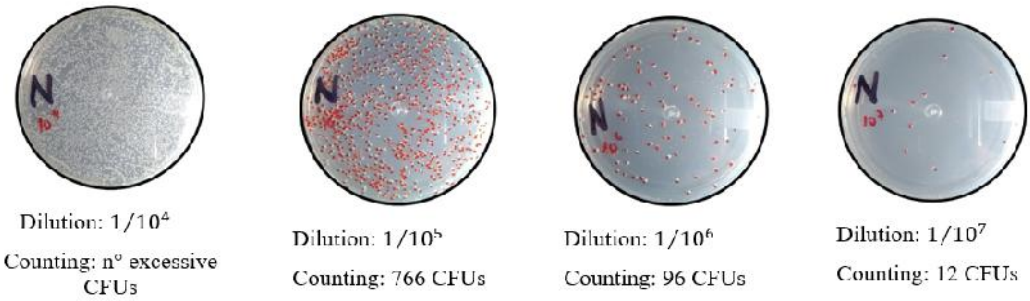


Figure 5. CFU count in Petri dishes with culture medium Nutrient Agar + Calcium nitrate (5 g/l)

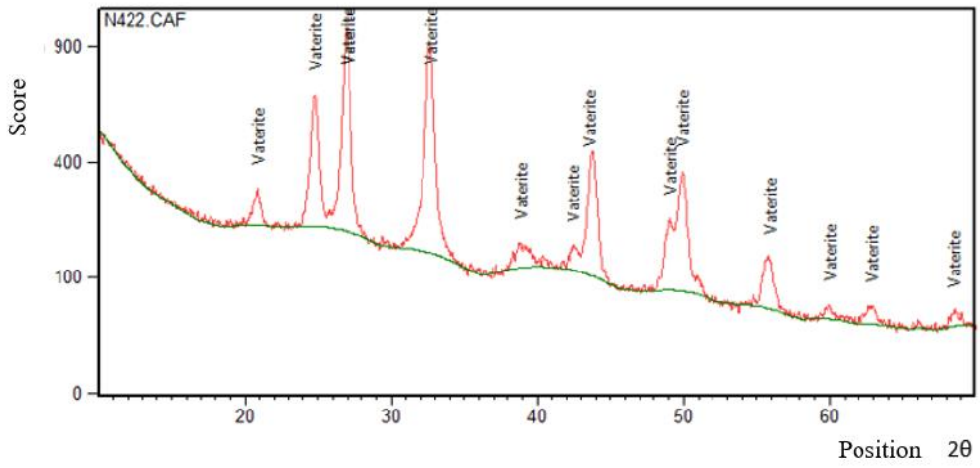
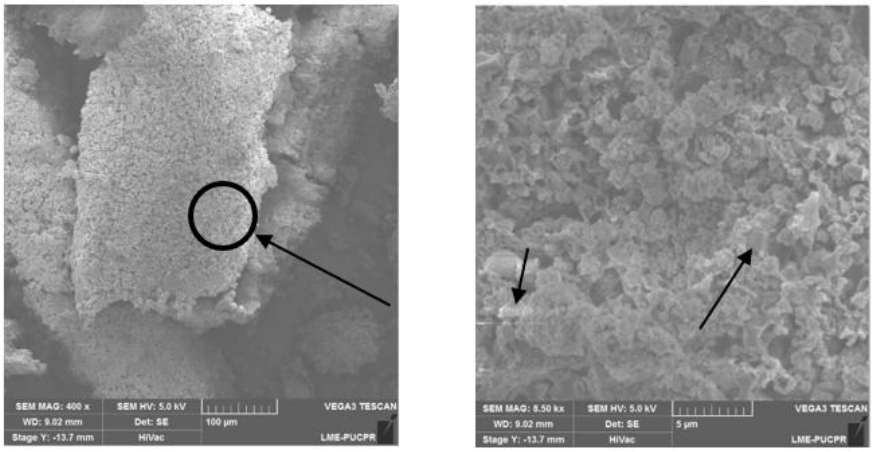
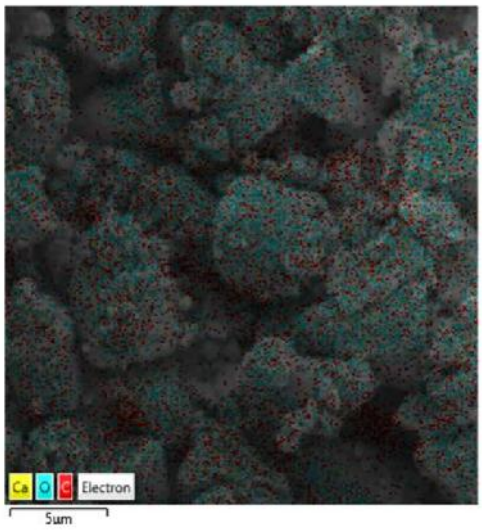


Figure 6. Diffractogram of the precipitated mineral of Lysinibacillus sphaericus in the presence of Calcium Nitrate (pH 7)



(a) Magnification of 400x (b) Magnification of 8.50 kx

Figure 7. SEM of the precipitate of the microorganism Lysinibacillus sphaericus when exposed to the Calcium Nitrate reagent: (a) 400 times magnification; (b) 8,500 times magnification.



Chemical element	Percentage (%)
Ca	38,9
O	38,1
C	20,7
P	1,8
Na	0,5

Figure 8. Energy-Dispersive Spectroscopy (EDS) image of the microorganism in the presence of Calcium Nitrate

Once again, the result supports the hypothesis that the compound precipitated by this microorganism is calcium carbonate, since the percentage of the predominant elements in the sample are calcium, presenting 38.9%, followed by oxygen with 38.1%, and finally carbon with 20.7%. Now it is the time to articulate the research work with ideas gathered in above steps by adopting any of below suitable approaches: A. Bits and Pieces together In this approach combine all your researched information in form of a journal or research paper. In this researcher can take the reference of already accomplished work as a starting building block of its paper.

Jump Start: This approach works the best in guidance of fellow researchers. In this the authors continuously receives or asks inputs from their fellows. It enriches the information pool of your paper with expert comments or up gradations. And the researcher feels confident about their work and takes a jump to start the paper writing. B. Use of Simulation software There are numbers of software available which can mimic the process involved in your research work and can produce the possible result. One of such type of software is Matlab. You can readily find Mfiles related to your research work on internet or in some cases these can require few modifications. Once these Mfiles are uploaded in software, you can get the simulated results of your paper and it easies the process of paper writing. As by adopting the above practices all major constructs of a research paper can be written and together compiled to form a completeresearch ready for Peer review.

CONCLUSION

This work is focused on investigating the possibility of using the microorganism Lysinibacillus sphaericus as a self-healing agent in Portland cement composites. In this context, the following conclusions were drawn from the experiment:

- The microorganism Lysinibacillus sphaericus can precipitate calcium carbonate when exposed to a medium rich in calcium (calcium nitrate).
- In the absence of a calcium-rich medium, the microorganism Lysinibacillus sphaericus did not precipitate calcium carbonate. This proves that it is necessary to have a calcium-rich medium for the effective precipitation of calcium carbonate.
- The growth of the microorganism Lysinibacillus sphaericus was observed in alkaline pH (pH 9, 10, 11 and 12). This fact is evidence that this microorganism can be used as a healing agent in Portland cement-based structures.
- Studying the range of pH variation from 7.0 to 12.0, neutral pH proved to be the most favorable medium for the growth of the microorganism Lysinibacillus sphaericus. However, there was growth of the microorganism Lysinibacillus sphaericus at pH 9, 10, 11 and 12, which may be sufficient to allow self-healing of Portland cement composites.

Other research needs to be developed by testing the application of the microorganism Lysinibacillus sphaericus directly on cracked Portland cement composites to prove the effectiveness of the microorganism. These experiments are of long duration, involving molding and curing of the composite, subsequent induction of cracks and conditioning of the specimens in favorable conditions for the precipitation of calcium carbonate in the cracks. This work acted in the way of proposing preliminary and faster tests in vitro to identify the tendency of a microorganism to succeed in the self-healing of Portland cement concrete.

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