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Microbiologically induced carbonate precipitation in the consolidation of marble slabs

Trabajo de integración curricular presentado como requisito para la obtención del título de Química

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To the loving memory of my brother

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Abstract

Microbiologically induced carbonate precipitation (MICP) has been used to restore and consolidate heritage sculptures damaged by various environmental and anthropogenic factors. This method has been tested in several countries around the world, where its effectiveness in restoring deteriorated samples was shown under controlled laboratory conditions. However, Ecuador presents no report on using this method, even though it has three cities considered cultural heritage of humanity. This work tests the capacity of a local bacterial strain (RTB017) to produce a consolidating layer of calcium carbonates on carbonate samples (Travertines). The results show that the precipitated carbonate crystals are firmly attached to the surface of the treated samples. Also, it shows that the calcium carbonates formed fill the cracks and holes present in the samples. Therefore, the bacterial strain used can precipitate calcium carbonate crystals that consolidate and restore carbonate samples, improving their mechanical properties and resistance to damage caused by acid rain.

Keywords: Microbiologically induced calcium carbonate precipitation (MICP), consolidation, restoration, calcium carbonates.

Resumen

La precipitación de carbonatos inducidos microbiológicamente (MICP), por sus siglas en inglés) ha sido utilizada como un método de restauración y consolidación de esculturas patrimoniales deterioradas por varios factores ambientales y antropogénicos. Este método ha sido probado en varios países del mundo donde se mostró su eficacia en la restauración de muestras deterioradas bajo condiciones controladas de laboratorio. Sin embargo, en Ecuador no hay ningún reporte sobre la utilización de este método, a pesar de que, cuenta con tres ciudades consideradas patrimonio cultural de la humanidad. En este trabajo se evalúa la capacidad de una cepa bacteriana local (RTB017) de producir una matriz consolidante de carbonatos de calcio en muestras carbonáticas (Travertinos). Los resultados muestran que los cristales de carbonatos precipitados están fuertemente ligados a la superficie de las muestras tratadas. Así también, se muestra que los carbonatos de calcio formados rellenan las fisuras y huecos presentes en las muestras. Concluyendo que, la cepa bacteriana usada es capaz de precipitar carbonatos de calcio que consoliden y restauren muestras carbonáticas, mejorando sus propiedades mecánicas y resistencia a daños ocasionados por la lluvia ácida.

Palabras clave: Precipitación de carbonatos de calcio inducida microbiológicamente (PCIM), consolidación, restauración, carbonatos de calcio.

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1. Introduction

Throughout history, various artists such as sculptors and architects have used carbonate stones (limestone, marble, and dolostone) to represent and capture the history of humanity with sculptures, monuments, or buildings, becoming the heritage of humanity. However, this historical, cultural heritage is affected by the physical-chemical, environmental and anthropogenic changes that it suffers over time and deteriorates [1] The acid attack (acid rain) becomes one of the most remarkable problems. Therefore, many researches focused on investigate methods to restore and preserve this cultural heritage [1]. The conventional methods of conservation and consolidation include inorganic and organic products [2]. These products could be acrylic, epoxy resins, copolymers, or oxalates [3]. However, some of these treatments have many disadvantages[4]. These methods (i) can block the porous system of the stone, (ii) are incompatible with the original substrate, (iii) have poor performance and low adhesion, and (iv) accelerate the deterioration of the treated stone [5][6].

Research has been focused on developing a more effective and compatible method for the conservation and protection of cultural heritage. Looking for an alternative compatible with the original structure [3]. In the last decades, it has been proposed that bacterial biomineralization is a compatible and environmentally friendly alternative for restoring and consolidating heritage buildings [7]. Microbiologically induced carbonate precipitation (MICP) treatment must produce a coherent calcium carbonate layer that protects the deteriorated stone against water uptake and consolidates its inner structure [5]. MICP is present in nature and involves a broad variety of microorganisms [8] and is mainly driven by factors like pH, Ca^{2+} concentration, dissolved inorganic carbon concentration, and availability of nucleation sites [9]. The shape and size of calcium carbonate can vary eg. rhombohedral (calcite), hexagonal (vaterite),or needle-like crystal (aragonite) [10], depending on the cell surface properties of bacteria. Calcite is the most stable molecular structure [11] present in this process. Also, during the bio-precipitation process, particles in suspension, dust particles, and bacteria themselves serve as active sites for calcite nucleation [12][13][14]. The bacterial cell surface is typically negatively charged; hence, it is able to attach divalent cations like Ca^{2+} or Mg^{2+} [15].

This treatment was patented in 1990 (expired in 2010) where a bacterial strain was used to restore carbonate stones [16]. In the next years the method was improved looking for better conditions to produce carbonate crystal and improve the consolidation on carbonate samples. For example, in 1999 Métayer-Levrel, G.Le. et al, showed that bacteria strains were capable to precipitate carbonates on deteriorated limestone samples spraying the entire surface with a suitable bacterial suspension [17]. In the last decade several investigations have been carried out in countries like: Rumania, China, Alemania, España, Francia, Brasil, Colombia and Rusia, with different species of microorganisms, proving the effectiveness of the method in the restoration and consolidation of cultural heritage [18]. The microorganism used in some of research include: *Pseudomonas, Pantoea, Cupriavidus, Myxococcus xantus, Bacillus* and others. Specifically, studies with *B. subtilis* have shown the ability to consolidate and restore deteriorated carbonated samples by improving their mechanical properties [19]. It is reported that *B. subtilis* can form a $CaCO_3$ coating on the surface of materials exposed to corroding environments, increasing their durability [20]. Also, it has been shown that microbiologically treated samples preserve their porous system and shows other advantages compared to conventional methods [20].

It is presumed that MICP occurs via various metabolic pathways such as nitrogen, sulfur, iron reduction or urea degradation [21] being the last one the most common. Also, some theories establish that certain bacteria convert carbon dioxide into carbonate ions CO_3^{2-} and it reacts with calcium Ca^{2+} ions attached to the cell surface, forming crystals of calcium carbonate ($CaCO_3$) [22]. In more detail, bacteria such as *Bacillus subtilis* could promote the precipitation of $CaCO_3$ by converting CO_2 into HCO_3^- through the carbonic anhydrase (CA)[23]. Precipitation begins with the dissolution of gaseous CO_2 in water, to form aqueous CO_2. Aqueous CO_2 reacts with H_2O to form carbonic acid (H_2CO_3), here the CA catalyzed the formation of carbonic acid, increasing the hydration coefficient of CO_2 by 107 times [24]. The ionization of H_2CO_3 generates HCO_3^- and H^+. Under alkaline conditions HCO_3^- ionizes to form CO_3^{2-} and H_2O. The reaction continues towards the precipitation of calcium carbonate by binding Ca^{2+} ions to the bacterial cell surface [25].

Despite this method has been tested in many countries around the world, Ecuador presents no records of the use of this technique until 2020. Two years ago, preliminary research tested the growth of a local bacterial strain (RTB017), identified as *Bacillus*

subtilis, and its ability to precipitate calcium carbonates in two liquid media "Minimal yeast extract (MYM)" and the B4 modified (B4M). It research showed that B4M medium is a more suitable culture media for carbonate precipitation[27] . B4 medium has been used in many researches to study mineral precipitation using bacterial strains since 1973 and this liquid medium has been modified over the time to get better results [26].

1.1.Problem statement:

Ecuador possesses three cities considered Word Cultural Heritage Sites (Quito, Cuenca, and Guayaquil). These cities present a wide variety of statues, facades, fountains, and mausoleums that have been affected by many deteriorating factors. Therefore, a few years ago, the "Instituto Nacional de Patrimonio Cultural" (INPC) proposed a project to restore marble sculptures called "The four seasons" located in Quito, Ecuador. These sculptures are in the installation of INPC and have been deteriorated by pollution and anthropogenic factors. Preliminary research was performed by Ortega-Villamagua [27] to test the ability of a local bacterial strain (RTB017) to precipitate calcium carbonate in two liquid media. In this work is proposed to test the ability of RTB017 to create a protective and consolidating carbonate matrix on carbonate stone samples (travertines) and to assess the efficacy of this method to restore and consolidate culture heritage improving the mechanical properties of these.

1.2. Objectives:

General Objective

- To test microbiologically induced precipitated calcium carbonates in travertine samples, under controlled conditions in the laboratory.

Specific Objectives

- To determine the ability of the bacterial strain RTB017 to create a protective and consolidating carbonate matrix in travertine samples.
- To assess that the Calcium crystals are adhered to the travertine samples by means of sonication and to assess the resistance of samples treated to the acid corrosion by an acid-resistant test.
- To characterize the samples using XRD and SEM-EDS.

2. Methodology

2.1. Reagents

Nutritive Agar was purchased from Difco. Peptone water was purchased from Merck KGaA. Yeast extract was purchased from Bacto. Glucose was purchased from Botica Alemana. Calcium Acetate monohydrate was purchased from In-Qui-Lab with a technical purity grade. Urea Broth was purchased from Difco. Agarose was purchased from Fluka-Garantie. Sulphuric Acid with 98% of purity was purchased from Fisher Chemical. Ethyl alcohol was purchased from La Casa de los Químicos with 96% of purity. Agarose was purchased from Fluka-Garantie.

2.2. Equipment

Plating of bacterial strains was performed on a biosafety cabinet (SterilGARD®) model: SG403A-HE from The Baker Company. Incubation of cultures was performed on Memmert Incubator Oven INB200code: E208.0092. Sterilization was performed on an electric autoclave model 25x-1 from ALL AMERICAN. The reagents and samples were measured in an Adventurer Analytical Balance from OHAUS. A Neubauer Chamber made in China and a ZEISS Axioscope 5 was used for cell counting bacteria. A shaking of culture was performed in a Vortex mixer MRC. The inoculation of culture media was performed with an Eppendorf™ Micropipette. A Digital Ceramic Hot Plate Stirrer from AREC was used for the Erlenmeyer heating. Agitation of incubated samples was performed on 2506 Reciprocating Shaker from MaxQ™. pH measurements were performed in a HANNA brand pH meter model HS5222. Sonication tests were performed in a Brain Bee ultrasonic cleaner model DUC-3110, ultrasound frequency: 40 KHzand ultrasound power: 50 W. A convention electric oven OSK 9500D was used to dry the samples after the treatment. To analyze the surface of samples an Olympus brand stereo microscope model SZHILLD 101005 was used as well as a scanning electron microscope tandem EDS, JEOL IT300 XMAN1 from Oxford Instrument using high vacuum and changing pressure conditions according to samples, detector/SED/LVSED/BED-C. The morphological observation conditions were BED-S detector, 20 KV voltage acceleration, and 11 mm working distance (WD). XRD was performed on a D8-Advance X-Ray Diffractometer from BRUKER with a copper anode (λ= 1.5406 Å).

2.3. Microorganisms

The microorganism used was a bacterial strain (RTB-017) belonging to the genus *Bacillus*, specifically to the specie *Bacillus subtilis* provided by Mgs. Eliana Barba, Laboratory of Zoonosis, Faculty of Chemical Sciences, Universidad Central del Ecuador. For inoculum preparation, the RTB017 strain was precultured in nutrient broth and incubated at 25ºC for 48 hours until it reached a concentration of 2.7325 $x10^8$ cell/ml. Also, another bacterial strain (*Staphylococcus saprophyticus*) provided by Cristina Monserrat Naranjo Lopez, Clinical Biochemist- Universidad Central del Ecuador, was used as positive control to the urease test. *S.saprophyticus* was culture on Trypto-Casein Soy Agar (TSA) and incubated at 35 ºC, its optimal growth temperature, for 24 hours.

2.4. Culture Media

For the preparation of the inoculum, the nutrient broth was prepared using peptone water (5 g) and yeast extract (3 g) in 1000 mL of distilled water. Also, a liquid culture media without pH adjustment called “modified B4” (B4M) was used as calcifying containing yeast extract (1g), glucose (1g), and calcium acetate monohydrate (5g) per 1 liter of deionized water [28]. Both culture media were sterilized in the autoclave at 120ºC for 20 min. Finally, both culture media were sealed with parafilm-covered and restored at an environmental temperature into the biosafety cabinet for their later use.

2.5. Urease Test

The urease test was performed to identify that the strain RTB-017 has the urease enzyme capable of hydrolyzing urea. So, 38.7g of urea broth was dissolved in 1L of distilled water and was sterilized by filtration. The test was performed in the reported procedure [29]. Urea broth was transferred to three test tubes, 5 mL each one, one of them was inoculated with an inoculum of RTB-017 from 24-hour pure culture and shook gently to suspend the bacteria. A second one was inoculated with *Staphylococcus saprophyticus* (urease positive) and shaken gently. Moreover, the last one remained sterile as the negative control. All samples were incubated at 35° C and observed for a color change (from an orange color to a bright pink- fuchsia color) at 8, 12, 24, and 48 hours.

2.6. Carbonate stone samples

The samples used in the consolidation tests were carbonated stones (travertine) from Cuenca-Sector Sinincay and provided by the INPC. Travertines are sedimentary stones formed by calcium carbonate deposits. These are composed of calcite, aragonite, limonite, and iron oxides, giving different colors like yellow, white, and pink. Travertines are frequently used as ornamental stones in heritage monuments of churches and others [30].

Four samples (see Figure 1) were cultivated (submerged) in liquid media B4M to test the consolidation ability of the strain RTB017. The composition of these samples was calcite, anhydrite, siderite, and hematite, according to mineralogical composition in XRD. Three of them were cultivated under shaking conditions to enhance bacterial growth, and the last one was cultivated under stationary conditions. A fifth sample composed of calcite, magnesium calcite, and aragonite was used to simulate a scenario closer to reality (*in situ* application). It was cultivated by poulticing method and under stationary conditions. All pieces were sterilized in the autoclave at 120ºC for 20 min and weighed before bio-mineralization treatment.

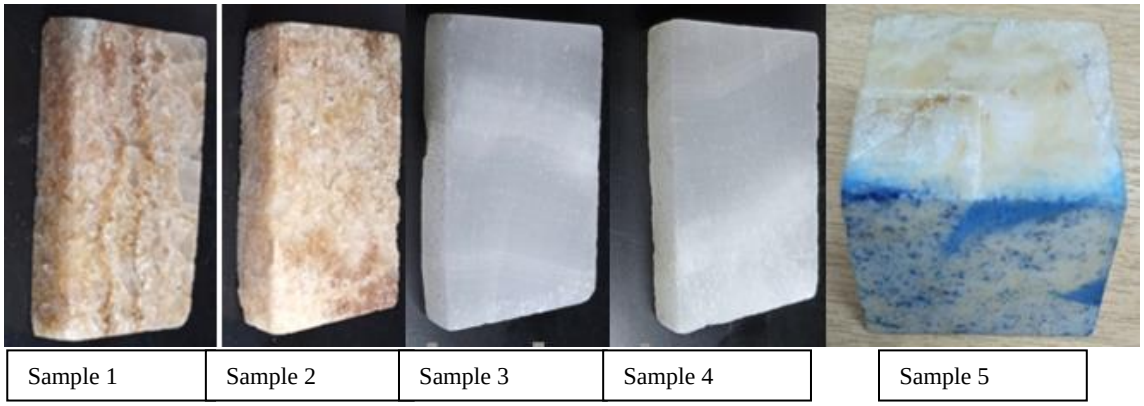

Figure 1. Carbonate stone samples (Travertine) used in the bio-mineralization test.

2.7. Cell counting of inoculum

A Neubauer chamber was used to count bacteria of the inoculum used in the bio-mineralization test. With a micropipette, take 10 µm of inoculums solution (the inoculum was prepared on 5mL of nutrient broth and incubated at 25°C) and carefully fill the chamber, avoiding bubbles or sample overflow. Then, place the Neubauer chamber in the microscopy stage. Cell counting was performed in the central square,

each of the 25 inner squares was focused on the 40x lens. The central square is used for cells with the greatest concentration and cells with a small size, like bacteria. It is split into 25 squares and each one of these has 16 small squares. In the red squares (see Figure 2), was performed the cells counting in a zigzag (see Figure 2). When the counting was finished, the chamber and glass cover was washed first with 70% EtOH and then with distilled water after each count. The procedure was performed three times. Finally, the concentration was calculated with the following formula [31][32].

$$\text{Concentration } \left(\frac{\text{cell}}{mL}\right) = \frac{\text{number of cells}}{\text{volume in mL}}$$

The cell count was performed after 24 and 48 hours. For the second, a dilution must be prepared because the cell density is greater and is difficult to count. The concentration range for the cell count is 250.000 cells / mL and 2.5 million cells / mL [33].

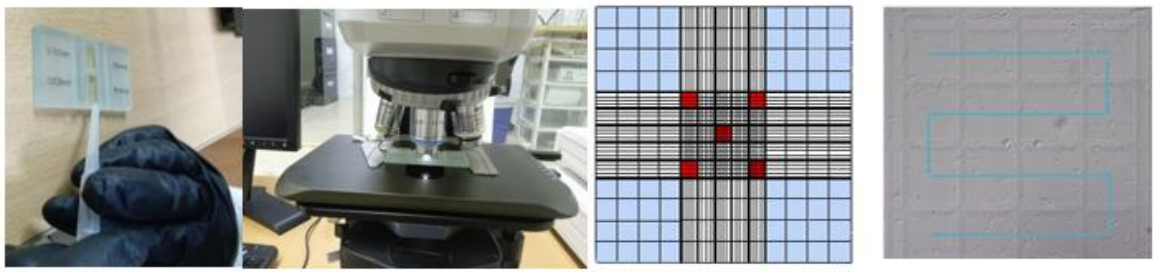

Figure 2. Graphical procedure to cellular counting.

2.8. $CaCO_3$ consolidation on carbonate stone

Once bacterial concentration was calculated, each sample was placed in 50 mL of B4M culture media contained in Erlenmeyer and inoculated with 1ml of the RTB-017 of (2.7325 $x10^8$ cell/ml) each one. The bio-mineralization test was performed as was previously described on [34]. The procedure was performed with three samples in inoculated media, and one remained sterile as the negative control. Two of the samples and negative control were incubated using a system to provide shaking and temperature conditions. The system uses a sand bath that keeps the temperature constant (30°C). Then, the sand bath was placed over a heating plate, and this, in turn, was placed over a shaker to obtain 24/7 shaking conditions. Finally, the system was covered with packing film to avoid external contamination (See figure 3). A third sample was incubated in the incubator oven at 30ºC and under stationary conditions. The pH of the culture medium

was measured before and after the treatment. All samples and control were maintained under the conditions mentioned above for 23 days to test the ability of the bacterial strain to create a consolidated coating. After 23 days all samples were collected and washed with distilled water several times, then, samples were dried at 40°C until weight stabilizes. Finally, the samples were weighed and analyzed by SEM-EDS and XRD.

Figure 3. Adapted mechanism to incubate and agitate bacterial culture.

2.9.Poulticing

To simulate a scenario closer to reality (*in situ* application) bio-consolidation was performed by poulticing method reported on [35]. Poulticing is a process of applying a moist mass of a substance with a soft, absorbent or pasty consistency to a surface for different purposes. This process can be performed with different materials like soft fibers, gelling materials, and clay [36].Also, agarose is considered a poultice material [37]. Then, in this work, we used 3% Agarose gel for the bio-consolidation test on sample 5. The gel was wetted with inoculated liquid media until soaked. This was applied to the up face of the sample on three alternate days, covered with plastic wrap, and incubated at 30°C for 14 days (see figure 4). After this time, the sample was rinsed, dried and weighed for later analysis on SEM.

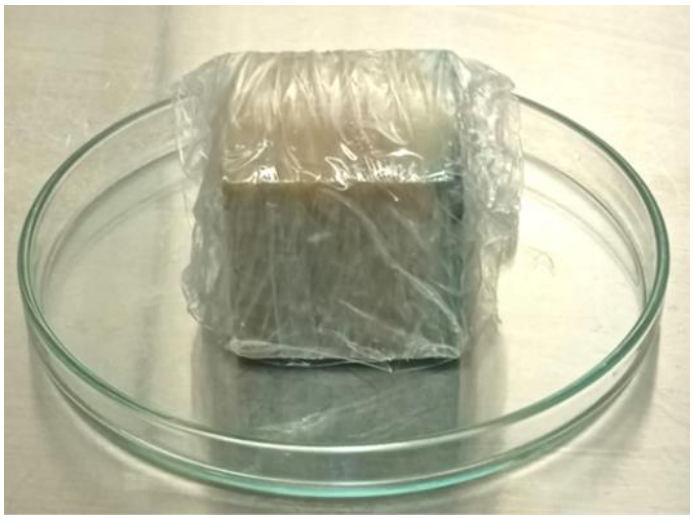

Figure 4. Application of inoculated culture media over sample 5.

2.10.Ultrasonic Test

Sonication has been used to clean or disrupt materials adhered to a surface of different materials[38]. It removes material from the surface of the sample and gives an indirect estimate of the adhesion force between the material-substrate interface [39]. Therefore in the case of the consolidation test, the sonication test gives an estimate of the adhesion force of the newly formed carbonate and the consolidation and/or protection efficacy of the newly formed carbonates [40]. The samples were sonicated in deionized water for 7 min, five times in succession, using a 40kHz ultrasonic bath. Samples were collected, dried for 24 h in an oven at 80°C, and weighed after each 7min sonication cycle. SEM was used to study the final appearance of the stone surfaces.

2.11.Acid-resistant test

Rain erosion is one of the most critical factors causing the deterioration of heritage. Weathering damage generally proceeds from the surface to the interior; thus, protecting sculptures' surfaces is an effective method to resist weathering. Then, an acid-resistant test was performed to test the resistance of the newly formed $CaCO_3$ coating.

On [41] the composition of the rain in the city of Quito, the capital of Ecuador, was analyzed; pH, conductivity, calcium, nitrates, and sulfates were measured in rain water. According to this research, 50% of the city's surface is affected by acid rain. Sulfur and nitrogen oxides are the main compounds in the contamination of the city, which by

oxidation have become sulfuric and nitric acid, respectively. Here, it was reported that the pH of acid rain has values from 4.5 to 5.6 depending on the sector. The acid resistance of the coating $CaCO_3$ layer was tested by the drop acid method reported on [42]. Considering the pH range mentioned on [41] were prepared different concentrations of H_2SO_4 solution (pH = 5.6, 5, 4.7). The test starts with the weakest acid solution (pH = 5.6). A drop of solution was dripped onto the coating $CaCO_3$ layer, and the layer was carefully observed by magnification on a stereo microscope for 2 min to determine any kind of reaction. If no bubbles appeared, it was declared that the layer could resist the corrosion of this acid solution with a specific pH value.

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3. Results and Discussion

3.1. Urease Test

The urease test tested the ability of the strain RTB-07 to hydrolyze urea. After 24 hours, a change of color was observed on the test tube inoculated with *Staphylococcus saprophyticus* (urease positive). While in the test tube inoculated with the strain RTB017, there was no change of color (see Figure 5). It means that RTB017 is negative for the urease test. It matches with the information reported in the literature for a *B. subtilis* [43]. On the negative control, there was no change of color too, which means that there was not any contamination.

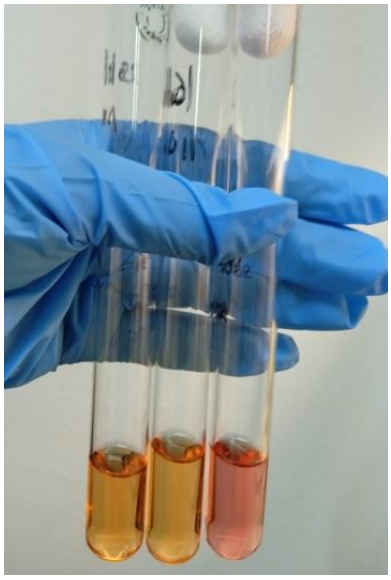


Figure 5 . Urease test left one (negative control), the middle one RTB017, right one (positive control).

Then, as RTB017 cannot hydrolyze urea, it is not necessary to use urea in the culture media to produce calcium carbonate crystals.

3.2. Calculating cell counts

The cell counting of inoculum was performed after 24 and 48 hours (see Tables 1 and 2). For the second, a dilution was prepared for cell counting because there was higher cell concentration and was difficult to count. The bacteria were counted within each red square. Then, the average of each square was calculated for each repetition. Finally, the total number of cells per microliter of sample can be calculated with the formula

described before where the total number of cells bacteria counted is divided by the chamber volume. To the cell count after 48 hours a dilution was prepared, the dilution factor is multiplied by the value previously obtained. Then, we used the inoculum cultured for 48 hours with a cell concentration of $2.735x10^8$ cell/ml.

Table 1: Cellular density calculation after 24 hours

Square/repetition	1	2	3	
1	252	198	182	
2	190	210	190	
3	204	204	197	
4	215	187	166	
5	210	181	190	
Mean [cell]	214.2	196	185	198.4
Chamber Vol. [ml]	0.000004			
Concentration [cell/ml]	$4.96x10^7$			

Table 2: Cellular density calculation after 48 hours

Square/repetition	1	2	3	4	
1	114	80	156	158	
2	101	76	157	117	
3	105	88	123	111	
4	130	89	114	94	
5	93	71	98	111	
Mean [cell]	108.6	80.8	129.6	118.2	109.3
Chamber Vol. [ml]	0.000004				
Concentration [cell/ml]	$2.735x10^8$				

3.1. $CaCO_3$ consolidation on carbonate stone

To test the ability of RTB017 to create a consolidant coating layer over carbonate stone (Travertine), samples were submerged in B4M liquid culture media. Three samples

were cultured under shaking conditions at 30°C, two of them were inoculated, and one remained sterile. A third sample was cultured under stationary conditions at 30°C. After 23 days, it was assumed that maximum carbonate precipitation was reached at this time; samples were collected, rinsed, dried, and weighted. A dense and homogeneous whitish-coating layer of calcium carbonate crystals can be observed on the surface of samples 1 and 2, which were cultured under shaking conditions (see Figure 6 and Figure 7). This coating layer can be seen with the naked eye. These results confirm that the bacterial strain RTB017 is capable of forming a consolidated coating layer of $CaCO_3$ over-carbonated stones, and it matches with the information reported in the literature [35][45]. The third sample that was cultured under stationary conditions does not show visible changes in its surface. However, it is possible to see a little bit of $CaCO_3$ crystal under the surface, marked with red circles (see Figure 8).

Furthermore, it could appreciate an increase in the weight of samples and a change in the pH of the medium (see Table 3). The increase in weight and pH is attributed to the formation of the newly coating layer of $CaCO_3$ [46][47][48][20]. The change of weight for the samples cultured under shaking conditions is greater than the sample cultured under stationary conditions. Also, in the sample 3 it is not possible to see a visible coating layer as in samples 1 and 2. Then, the best results are observed in samples 1 and 2 because the shaking enhances bacterial growth and this, in turn, improves the formation of $CaCO_3$ crystals [49]. Sample 4 was takend as control and it remains without changes and kepts its initial weight, and the initial pH of the medium did not change.

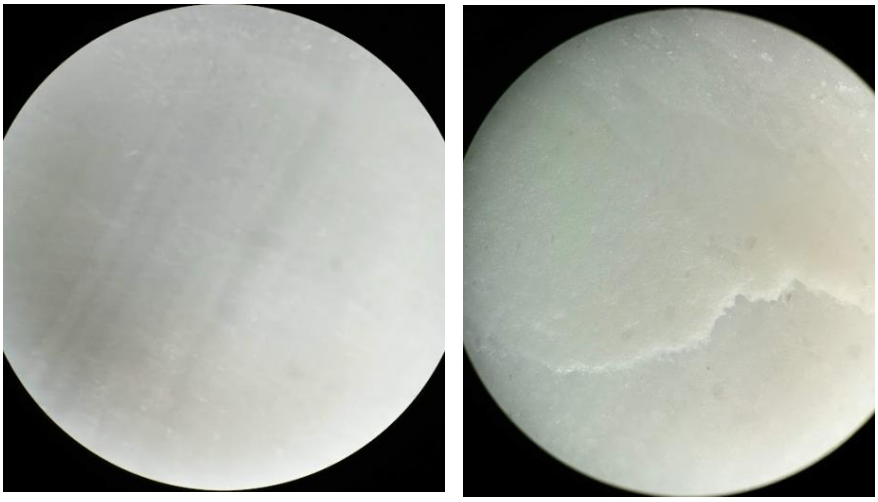


Figure 6. Sample 1, before (left) and after (right) treatment, magnification 20x

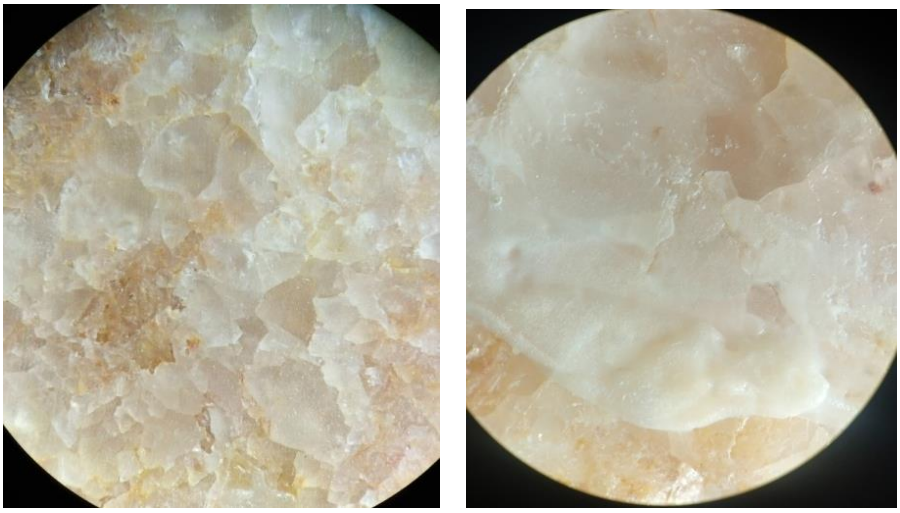


Figure 7. Sample 2, before (left) and after (right) treatment, magnification 20x.

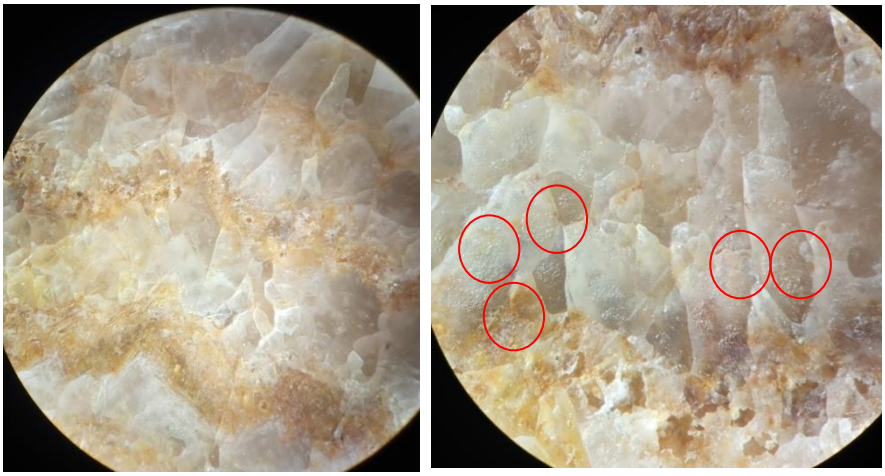


Figure 8. Sample 3, before (left) and after (right) the treatment, magnification 20x.

Table 3. Change of weight and increase of pH after treatment.

Weight	Initial weight (g)	Final weight (g)	Weight increase (g)	Initial pH	Final pH
Sample 1 (Shaking conditions)	13.0464	13.1001	0.0537	6.55	8.24
Sample 2 (Shaking conditions)	14.3354	14.3659	0.0305	6.52	8.25
Sample 3 (Stationary conditions)	7.5662	7.5964	0.0302	6.43	8.47
Sample 4 (Control)	13.3383	13.3383	0	6.60	6.60

3.2.*In Situ* application simulation

To simulate a scenario closer to reality, bio-consolidation was performed by poulticing method. To these, agarose was used to apply inoculated medium over the surface of sample 5 and incubated for 14 days. After 14 days sample was rinsed with distilled water several times and dry at 80 ° C until the weight stabilizes. There was not a visible change in the surface of the sample, however, an increase in the weight of the sample (11, 8 mg) was obtained, which means that there was a deposition of $CaCO_3$ over the sample. Then, analysis in SEM will help to verify if the methods works and if the newly $CaCO_3$ crystal were formed.

3.3.Characterization by XRD analysis

XRD analyses were carried out for samples under shaking and stationary conditions (see Figures 9, 10, 11). For XRD analysis, part of the coating formed on samples 1 and

2 was removed with a scalpel. Analysis of these samples showed that bacterial strain (RTB-017) was able to create a consolidated coating composed of calcite (see Figure 9 and 10), and it matches with the literature [19][50][20][51]. In the case of sample 3, there was not possible to remove a piece of coating formed because there was not a coating formed. Then, for the XRD analysis of sample 3, the precipitated formed on the Erlenmeyer used for this sample, was filtered, and analyzed. Then, the formation of calcite and small quantities of vaterite were detected (see Figure 11).

Calcite and vaterite are the most common precipitated forms of calcium carbonates, with calcite being the dominant and most thermodynamically stable phase [44][52][53], which supports the results. Furthermore, calcite was the unique phase detected in consolidated coating present on samples 1 and 2 and the main phase in sample 3. Finally, as vaterite is a rare metastable calcium carbonate polymorph, the presence in sample 3 is minimal. In nature, vaterite is unstable, and given enough time vaterite crystals tend to transform into the more stable calcite [53].

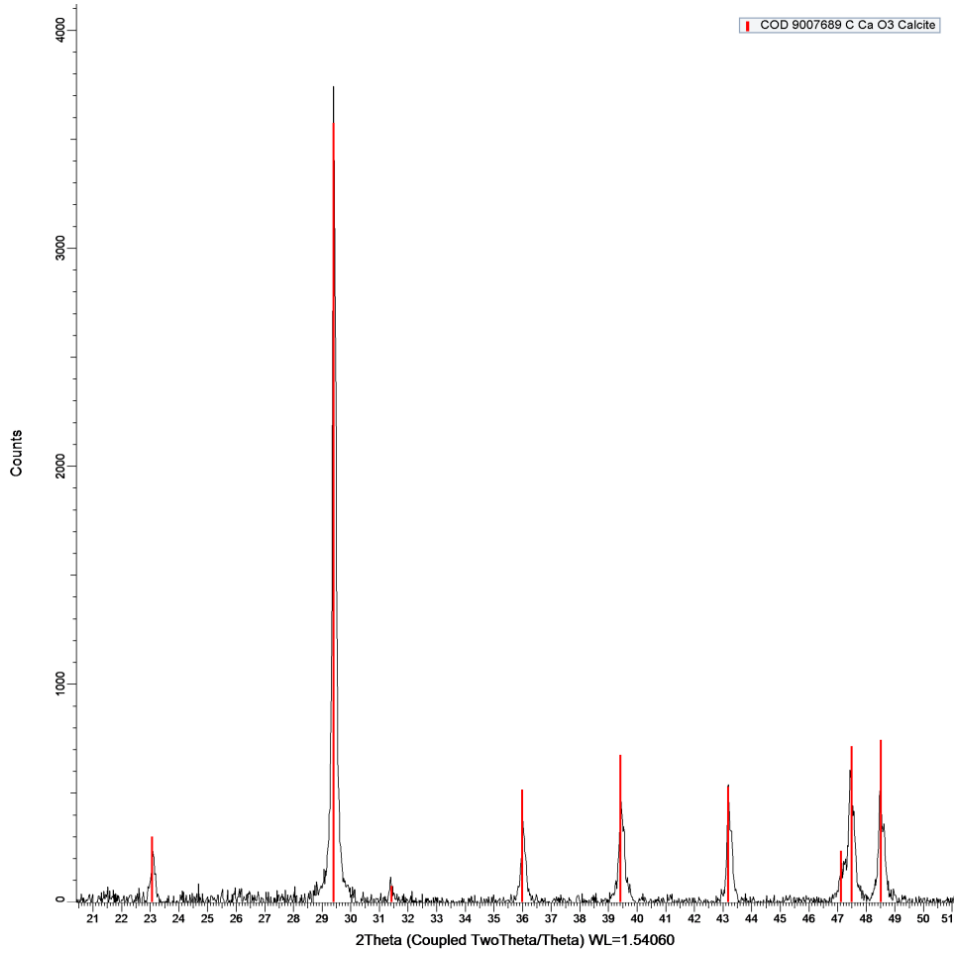


Figure 9. X-Ray diffraction spectra ofprecipitated crystals from Sample 1

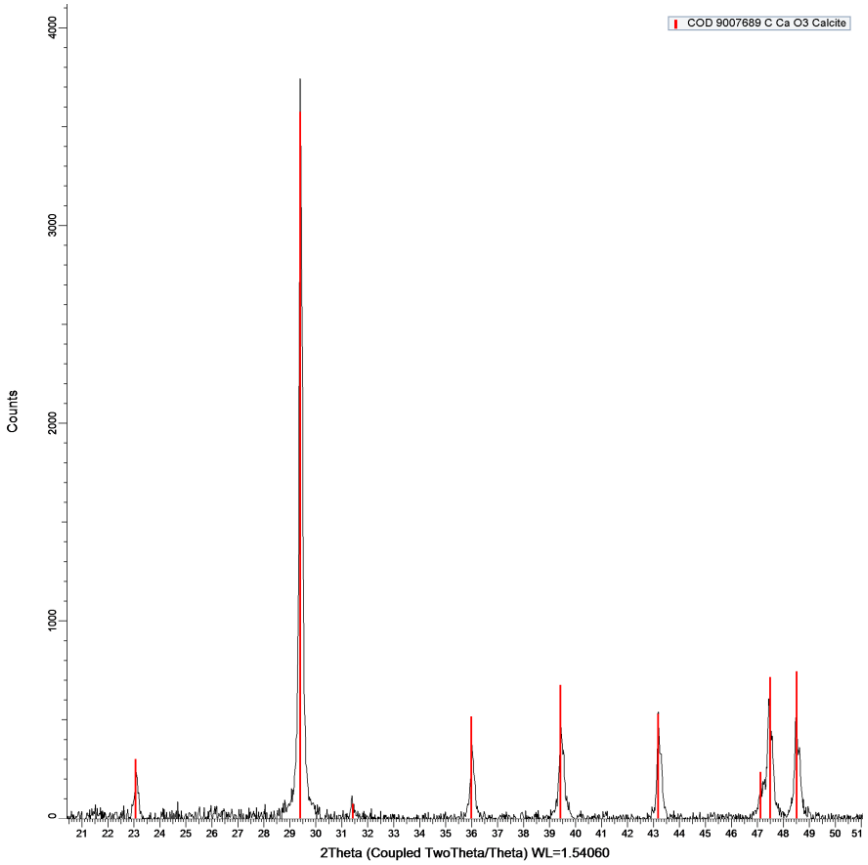


Figure 10: X-Ray diffraction spectra of precipitated crystals from Sample 2.

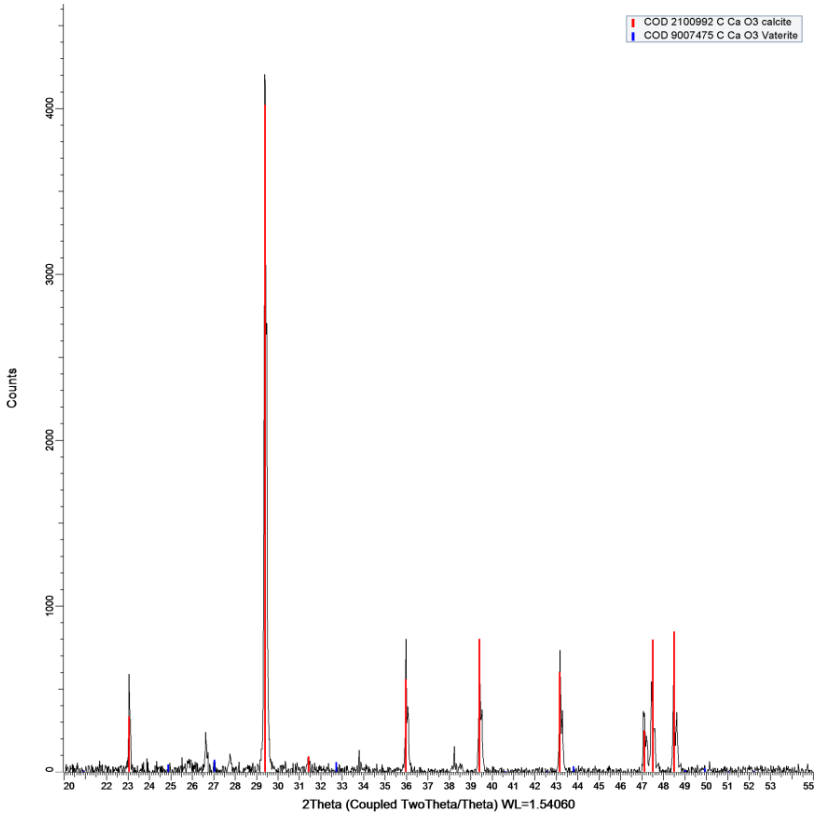


Figure 11: X-Ray diffraction spectra of precipitated crystals from Sample 3.

3.4.SEM-EDX analysis

The homogeneity of the calcite layer precipitated on samples, morphology, and composition after treatment was evaluated by SEM/EDS. Then, sample 1 exhibits a homogeneous and dense calcite deposition (see Figures 12a and 12b) and rhombohedra calcite shapes crystals (see Figure 12c) on the coated edges, which is in accordance with reported in the literature [54][19]. Sample 2 shows a homogeneous calcite deposition and crystals with rhombohedra irregular shapes. Some of these crystals are in scales or curtains distributed heterogeneously over the sample surface as is reported in literature [55][20][48]. These bio-precipitated calcium carbonate crystals fill some of the cracks and holes of sample 2 (see Figure 14b and 14c). Sample 3 shows a less quantity of precipitated carbonates than the two first samples and the shape of these crystals are rhombohedra too (see Figure 15). Also, the calcium carbonate grains are more spread out and there is no presence of a homogeneous coating on the surface. Finally, sample 5 was used as a simulation of *in situ* application of the MICP. This sample has part of its surface damaged, which was expected to improve and restore. In figure 18a is easy to see the damage surface of the sample before the treatment and the results show the new $CaCO_3$ crystals filled the cracks and restored the damaged surface of the sample (see Figures 18b and 18c). These carbonate crystals do not have a defined shape. It could be because of the time it took them to form [30]. It confirms that the bacterial strain RTB017 can be used to restore deteriorated samples. Sample 4 (control) didn't show changes, then, it was not analyzed by SEM/EDS.

All samples showed similar calcium carbonate crystal shapes. However, the size was different for each sample. Sample 1 exhibits crystals between 5 to 20 µm (see Figure 12c). On the other hand, samples 2 and 3 exhibit crystals sizes between 1 to 5 µm. Sample 5 is the sample with a smaller crystal size (1 to 3 µm). Sample 1 has larger carbonate crystals. It could be due to its surface being more homogeneous than other samples. The carbonate crystal deposited over samples 2, 3, and 5 are smaller compared to crystal deposited over sample 1, this could be to the different composition and morphology of the samples. Sample 1 has a crystal structure more compact and samples 2,3 and 5 have a crystal structure less compact, crystals are arranged in such a way that gaps and cracks are present in the surface (see figure 15a). Therefore, the newly calcium carbonates formed fill these gaps and cracks first.

Through the consolidated coating layer formed on samples 1 and 2 is possible to see tiny “voids” present, revealing that bacteria cell surfaces also serve as nucleation sites[27], [56]. Also, the results show that the porosity of the treatment travertine was preserved and matches with the previous studies[35]. Finally, all samples’ results were consistent with calcium carbonate based on EDS analysis (see Figures 13 and 17, and Tables 4 and 5).

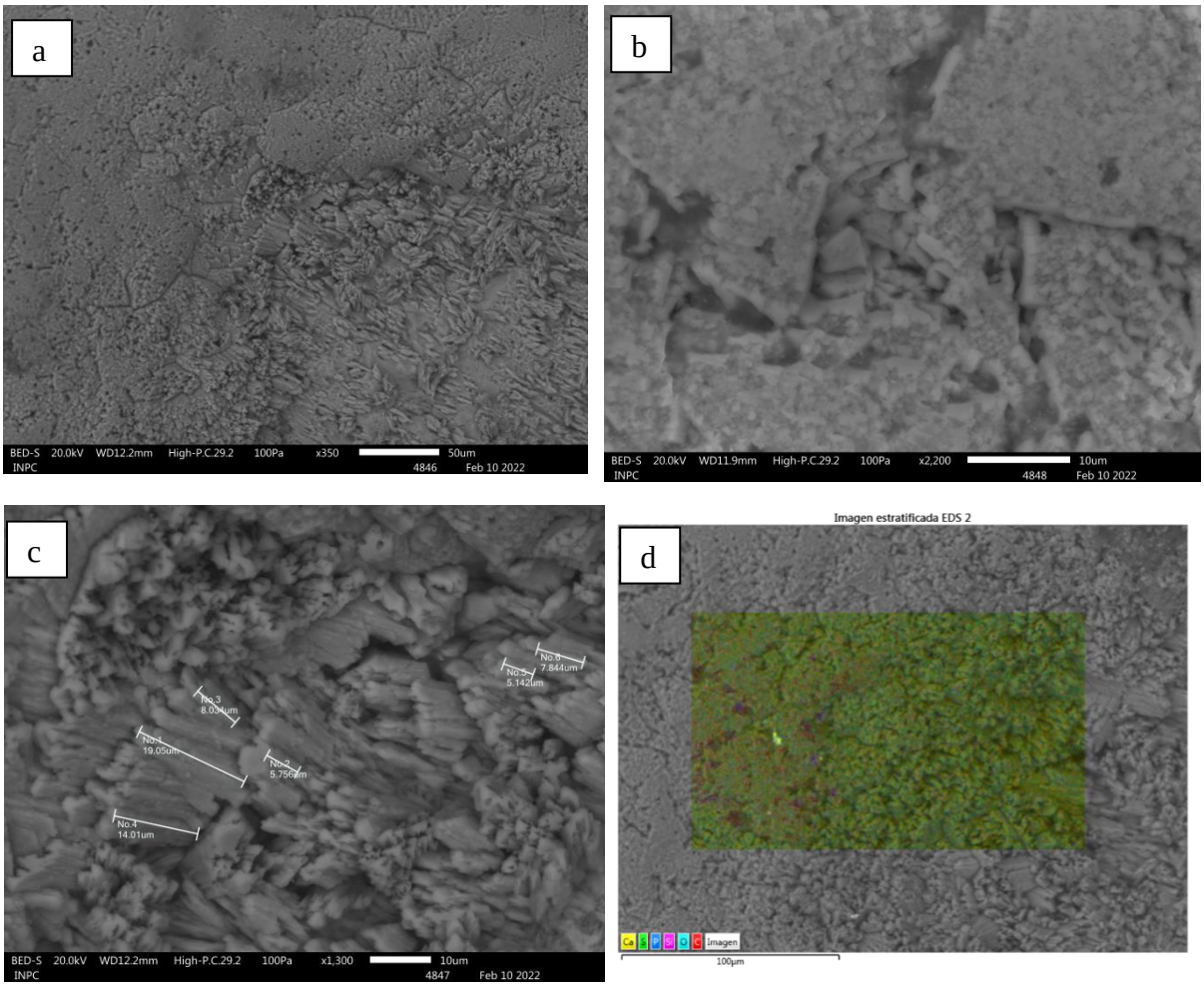


Figure 12. SEM micrographs for sample 1 of a) CaCO3 homogenous layer x350 magnification b) x 2200 magnification preserved porosity c) rhombohedra calcite x 1300 magnification crystal measurement d) selected zone for chemical composition determination via EDS

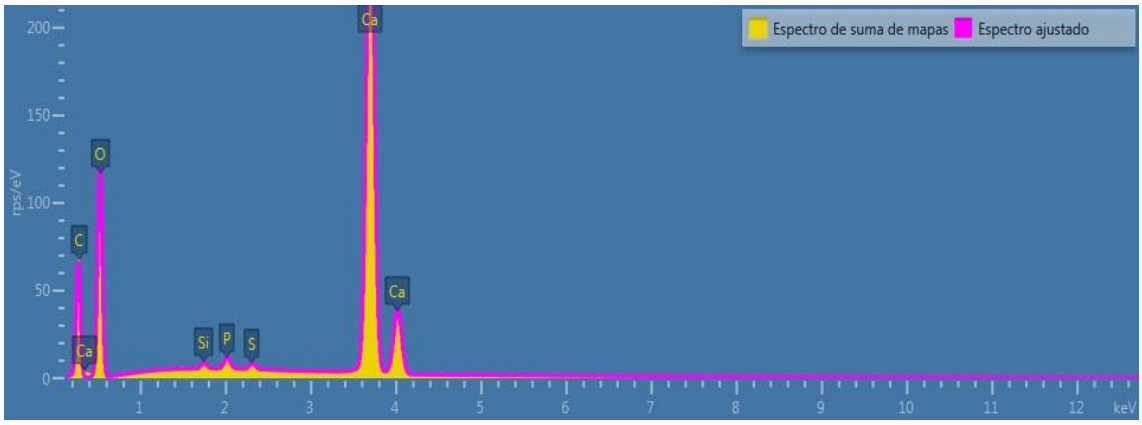


Figure 13: EDS analysis results from Sample 1, shaking conditions.

Table 4. Chemical composition of sample

Result type	Spectra tag	Ca	C	O	Others	Total
%Weight	Map sum spectra	33.29	18.43	47.20	1.08	100

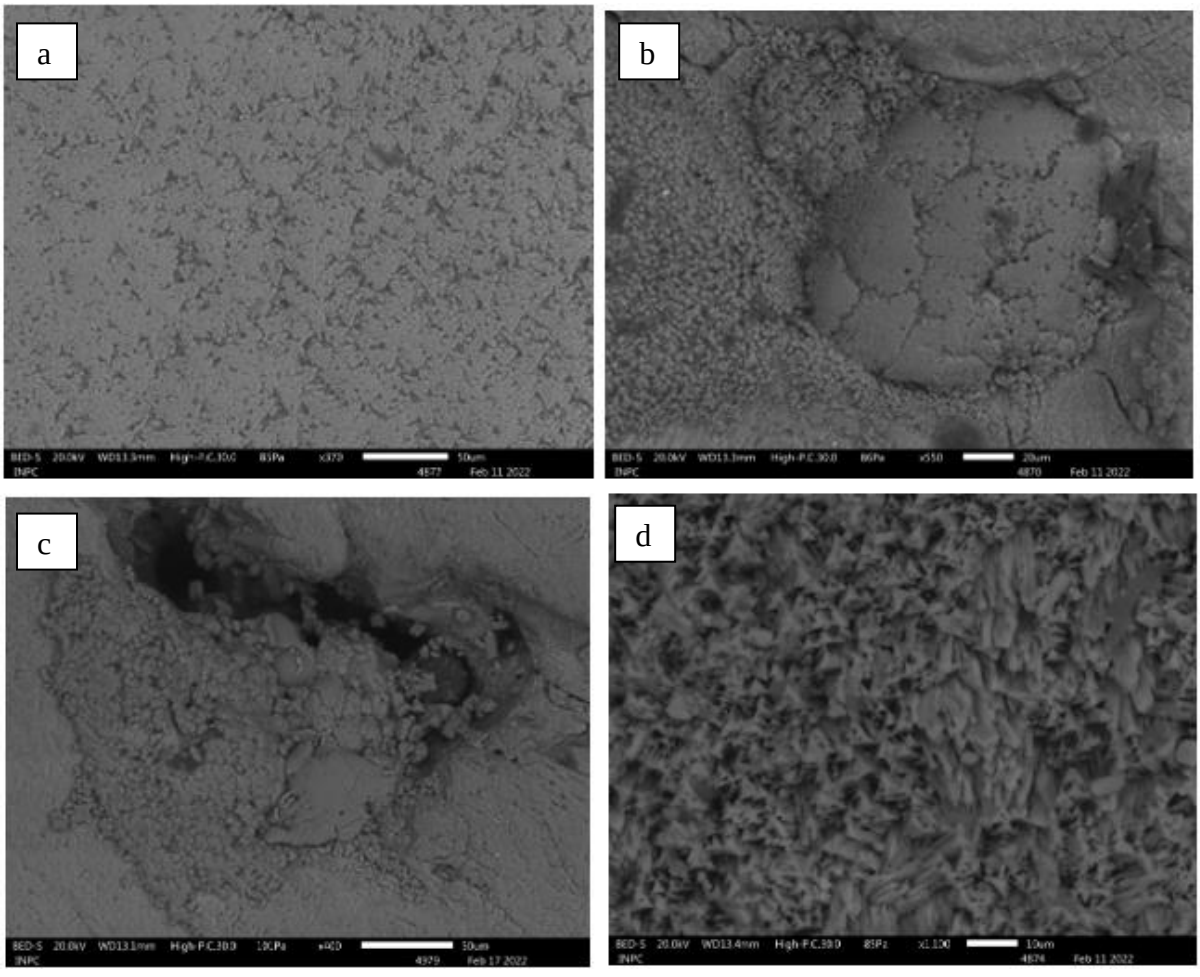
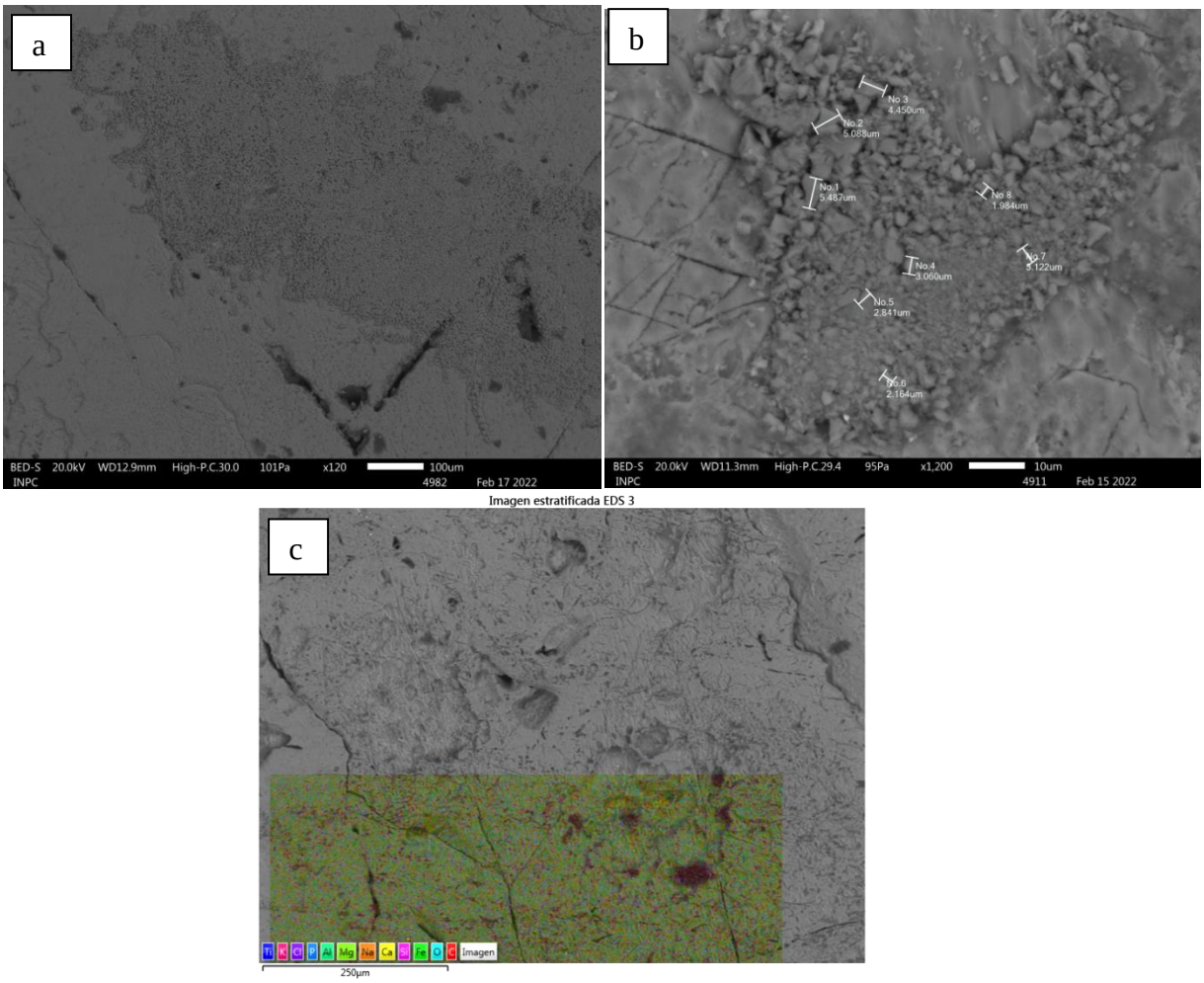


Figure 14. SEM micrographs for sample 2 of a) CaCO3 homogenous layer x370 magnification b) and c) consolidated carbonate filling the cracks d) scales of carbonate precipitated crystals.



F*igure 15. SEM micrographs for sample 3 of a) CaCO3 crystal precipitated heterogeneous over the sample surface b) rhombohedral calcite crystals measurement x 1, 200 magnification c) selected zone for chemical composition determination via EDS.*

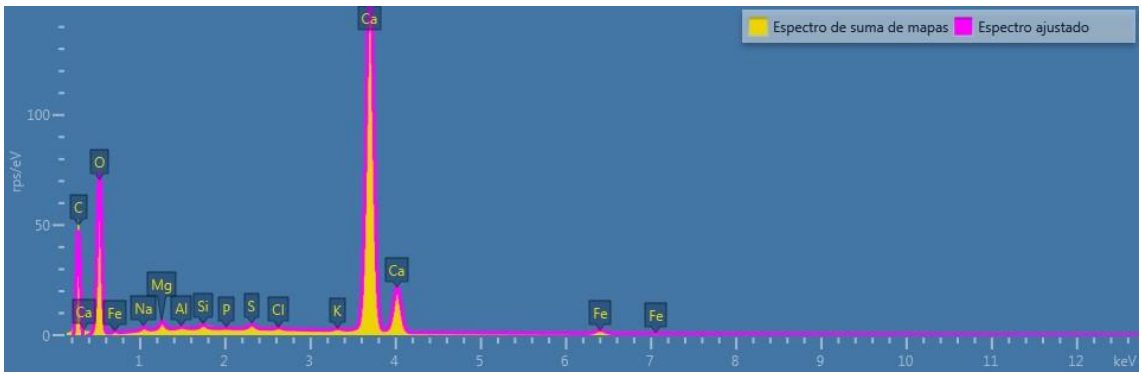


Figure 16. EDS analysis results from sample 3, stationary conditions.

Table 5. Chemical composition of sample 3

Result type	Spectra tag	Ca	C	O	Others	Total
%weight	Map sum spectra	29.63	22.60	45.42	2.35	100

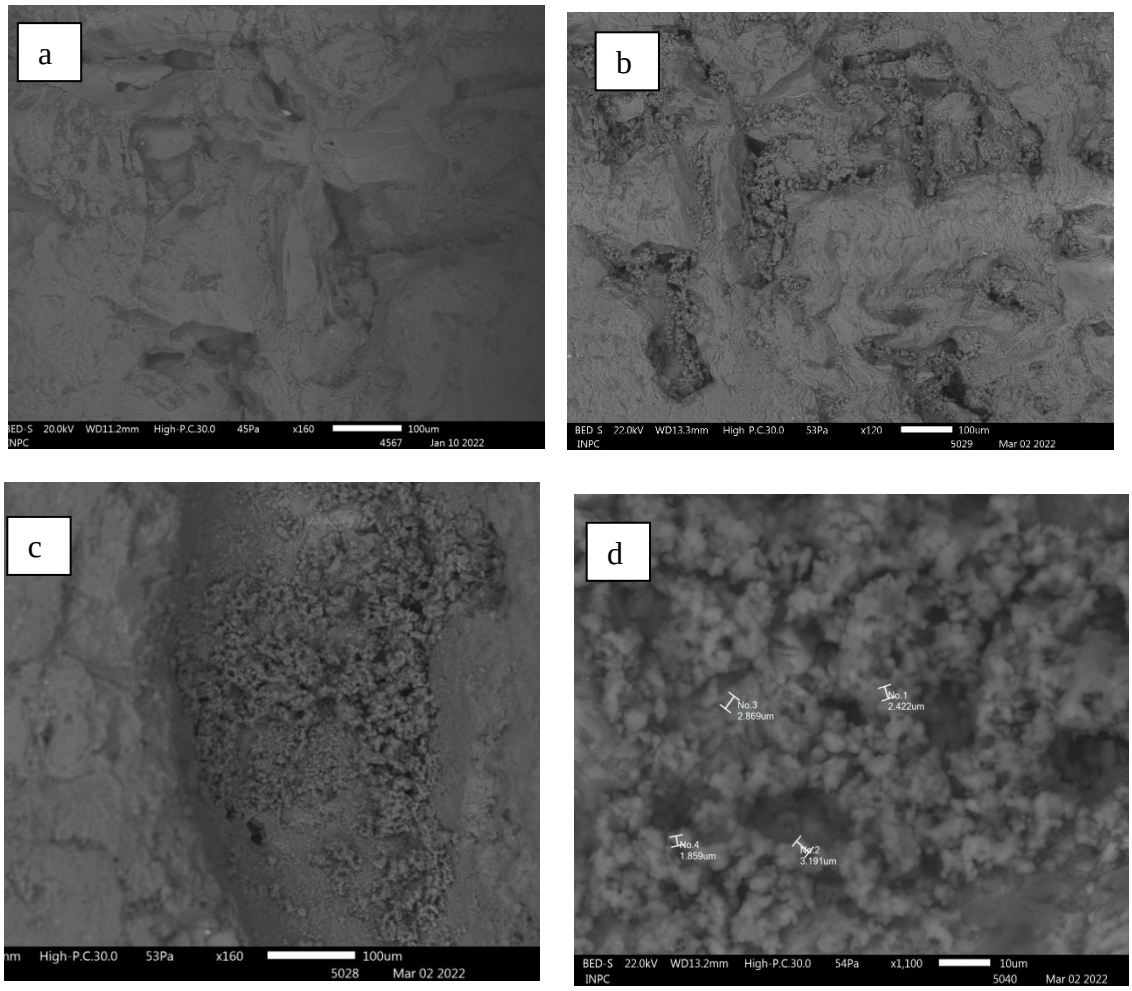


Figure 17. SEM micrographs for sample 5 of a) before biomineralization test b) after treatment, cracks were filled c) $CaCO_3$crystal in a crack at x160 magnification d) magnification of c) and carbonates crystal measurement at 1.100 magnification.

3.5. Ultrasonic test

This test helps to assess the adhesion force and the consolidation efficacy of the newly formed calcium precipitate [47]. After five cycles of ultrasonic treatment the samples that were MICP treated shows less damage than sample that was not treated. As we can see in the previous results all samples treated show a weight increase which is consistent with the production of new biologically induced $CaCO_3$ crystals. Samples 1 and 2 exhibits a consolidated coating strongly attached to sample surface and these samples shows the smaller weight loss after sonication test (see table 6). The Sample 3 lost more weight than samples 1 and 2, it could be, due to the new carbonate precipitated were not formed a consolidated coating over the sample surface, instead, the carbonate grains are poorly attached to sample surface. Sample 4/ control shows the greater weight loss compared to the MICP treated samples .

In the last column of Table 6, the percentage of the weight loss after 5 cycles-sonication was calculated in terms of initial weight after sonication test. Instead of sample 4 shows the biggest weight lost, sample 3 shows the greater percentage of weight loss in terms of its initial weight. However, it is important to mention that the weight loss could be from the original material as well as from the new $CaCO_3$ crystals. Here it should be noted that the initial weight of the samples before sonication test are not the same as the weights obtained after the consolidation treatment because part of the coating formed was removed for XRD analysis. In addition, sample 3 lost a part of its initial material. Thus, the weight loss obtained in sample 3 must be mostly from the original sample material. Taking this into account, the results shows that the more consolidated the stone, the more attached the grains are and, consequently, the less weight is lost by mechanical stress [57]. Finally, the SEM micrographs for sample 1 after five cycles of sonication show that there was not greater damage to the carbonate layer formed on the sample (see Figure 19). Then it was possible to assess a significant increase in the mechanical resistance of all treated samples compared to control sample, and we can to prove that MICP could provide a consolidated structure on carbonate samples, as is reported in the literature [58].

Table 6. weight loss after ultrasonic test

	Weight before sonication (g)	Weight After 5 cycles-Sonication (g)	Weight loss (g)	% Weight loss
Sample 1	13.0661	13.0567	0.0094	0.072
Sample2	14.3597	14.3506	0.0091	0.063
Sample 3	7.5526	7.5424	0.0102	0.135
Sample 4/control (sample without treatment)	13.3383	13.322	0.0163	0.122

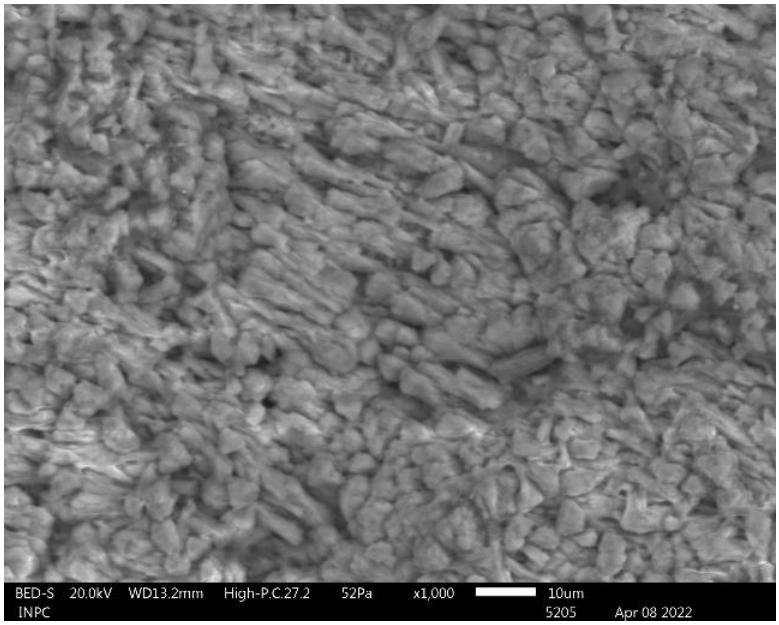


Figure 18. SEM micrograph for sample 1 after five cycles of sonication test.

3.6. Acid resistant test

Three solutions of H_2SO_4 were prepared with different pH (4.7, 5, 5.6), the range of pH reported in the rain of Quito-Ecuador. None of the samples shows a reaction with the acidic solutions, then we can say the coating formed on the samples can resist the corrosion of the rain of the city. These results are consistent with the literature [59].

4. Conclusions

After verifying that RTB017 is not ureolytic bacteria, we can presume that the mechanism by which these bacteria precipitate calcium carbonate crystals ($CaCO_3$) could be by converting carbon dioxide into carbonate ions CO_3^{2-} and it reacts with calcium Ca^{2+} ions attached to the cell surface, forming crystals of calcium carbonate ($CaCO_3$), using carbonic anhydrase as catalyst. Also, the results show that the bacterial strain RTB017 can produce a consolidated coating layer over the sample's surfaces, this coating layer are firmly attached to the treated stone. Furthermore, the new calcium carbonates fill the cracks and holes present in the samples. Then, was assessed the ability of RT017 to consolidate and restore carbonate samples, improving their mechanical properties and resistance to damage caused by acid rain. Finally, SEM micrographs show that the porosity system of the sample was preserved compared to conventional methods.

5. Recommendations and Outlook

Genetic sequencing of the bacterial strain RTB017 gives more information about how to use the strain to get better results.

Carry out an *in situ* application of MICP with different methods like spraying.

To perform research about yield and cost to apply the method *in situ* to restore the heritage of "Instituto Nacional de Patrimonio Cultural".

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