Article

Biomineralization of calcium carbonate in bioconcrete by the action of *Bacillus subtilis* ATCC 6633

Biomineralização de carbonato de cálcio em bioconcreto pela ação de Bacillus subtilis *ATCC 6633*

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Abstract

In this study, the viability of bacterium *Bacillus subtilis* (ATCC 6633) as a biological agent in the process of precipitation of calcium carbonate ($CaCO_3$) on the surface of concrete was investigated. This evaluation was first carried out in a curing tank for concrete samples, using a nutrient solution enriched with *B. subtilis* for comparison with control samples without the addition of microorganisms. The biomineralized samples with B. subtilis showed a 21.01% reduction in void content and a 25.31% reduction in water absorption by capillarity. Due to the surface protection, the microorganisms reduced the porosity of the material, resulting in an increase in compressive strength of about 6.41%. The mineral morphologies analyzed by SEM included cubic, polygonal and rhombohedral crystals. Chemical evaluation by EDX and characterization by XRD of the bioconcretes indicated the presence of $CaCO_3$ precipitated by the bacteria. The results obtained show that the superficial application of *B. subtilis* (ATCC 6633) on concrete leads to an improvement in mechanical and durability properties.

Keywords: Self-healing. *Bacillus subtilis*. Bioconcrete. Biomineralization. Biological agent.

Resumo

Neste estudo foi investigada a viabilidade da bactéria Bacillus subtilis *(ATCC 6633) como agente biológico no processo de precipitação de carbonato de cálcio ($CaCO_3$) na superfície do concreto. Esta avaliação foi realizada primeiramente em tanque de cura para amostras de concreto, utilizando solução nutritiva enriquecida com* B. subtilis *para comparação com amostras controle sem adição de microrganismos. As amostras biomineralizadas com B. subtilis apresentaram redução de 21,01% no conteúdo de vazios e de 25,31% na absorção de água por capilaridade. Devido à proteção superficial, os microrganismos reduziram a porosidade do material, resultando num aumento da resistência à compressão de cerca de 6,41%. As morfologias minerais analisadas por MEV incluíram cristais cúbicos, poligonais e romboédricos. A avaliação química por EDX e a caracterização por DRX dos bioconcretos indicaram a presença de $CaCO_3$ precipitado pelas bactérias. Os resultados obtidos mostram que a aplicação superficial de* B. subtilis *(ATCC 6633) no concreto leva a uma melhoria nas propriedades mecânicas e de durabilidade.*

Palavras-chave: Self-healing. Bacillus subtilis. *Bioconcreto. Biomineralização. Agente biológico.*

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Recebido em 28/06/24
Aceito em 01/08/24

Introdução

The occurrence of cracks is an unavoidable event during the wear and tear process of concrete structures exposed to climatic changes. If the cracks are not repaired, they can widen and lead to damage caused by the easy penetration of moisture (Jonkers, 2021; Wong, 2015). This allows open access to aggressive physical, chemical and biological agents that can degrade the concrete and lead to premature corrosion, affecting the long-term durability of the structure (Kashif Ur Rehman *et al*., 2022), which is why an effective method of crack healing is interesting (Jonkers; Schlangen, 2008; Soda; Madhavan, 2022; Wong, 2015). The alternative, which aims to harness biological activity by precipitating minerals, is called biomineralization, which is a widespread phenomenon in nature (Chen; Chen; Tang, 2020) and significantly improves concrete properties such as durability (Tittelboom *et al*., 2010). The natural process of calcium carbonate precipitation (Shastri, 2015) is associated with a variety of bacteria from the marine environment such as *Sporosarcina* sp., *Bacillus* sp. and *Brevundimonas* sp. that affect the marine carbonate cycle in the natural environment through the hydrolysis of urea (Wei *et al*., 2015). From sediment samples of mangroves in China, Zhang *et al.* (2016) studied the effect of the incorporation of *Bacillus aerius* into rice husk ash concrete. They observed reduced water absorption and porosity due to calcite precipitation, which increased the durability of concrete structures. In a study with *Bacillus megaterium*, Andalib et al. (2016) reported increased calcite precipitation and a 24% increase in compressive strength when added to the concrete. In addition, *Bacillus subtilis increases* the compressive strength of concrete, resulting in lower maintenance costs for concrete structures (Kalhori; Bagherpour, 2017) and performs well in mineral precipitation and growth in high pH environments (Feng *et al*., 2021). A recent study showed an average reduction of approximately 0.03 g cm^{-2} of water absorption in specimens cured with *B. pumilus* (Santos *et al*., 2023).

The involvement of bacteria and nutrients in the repair process of concrete is essential for the functioning of smart concretes with autogenous healing or self-healing of cracks in concrete (Dhami; Reddy; Mukherjee, 2013). Spore-forming bacteria of the genus *Bacillus* are better suited for concrete. Cracking is one of the main forms of concrete ageing (Kashif Ur Rehman *et al*., 2022). Sealing these cracks in concrete structures with calcium carbonate has proven to be effective (Marín *et al*., 2021). As it is an advantageous alternative for the concrete industry due to its efficiency and sustainability, it has been considered as a biotechnological and environmentally friendly option (Abudoleh *et al*., 2019).

Autonomous self-healing repair mechanisms are beneficial as they reduce manual maintenance and lower costs (Wong, 2015). Self-healing concrete, known as bioconcrete, is very important in today's world. This ability is derived from microorganisms with special properties (Castro-Alonso *et al*., 2019).

Bioconcrete is considered one of the most ecological and cost-effective technologies that is gaining importance due to its self-healing properties and improved mechanical and durability properties of concrete structures (García-González *et al*., 2017). Bacterial spores are cells that can withstand high mechanical and chemical stress and survive in alkaline environments, making them ideal for use in bioconcretes (Kalhori; Bagherpour, 2017).

The genus *Bacillus* is one of the most suitable groups of microorganisms for a biologically induced mechanism of calcium carbonate precipitation. They are abundant in natural environments, can be easily cultivated and have a remarkable potential to produce large amounts of calcite in a relatively short time (Morohashi *et al*., 2007). *B. subtilis* produce heterogeneity during sporulation, which is often used as a cure for crack repair (Feng *et al*., 2021; Laborclin, 2018).

Handling microorganisms in the laboratory requires strict biosafety protocols to protect researchers, the community and the environment. The Centers for Disease Control and Prevention (CDC) and the National Institutes of Health (NIH) are divisions of the Department of Health and Human Services that have established criteria for four levels of safety, called Biosafety Levels (BSLs). These criteria consist of combinations of laboratory practices and techniques, safety equipment, and laboratory facilities. Each combination is specific to the operations performed, the biological materials to be used, and the function or activity of the laboratory (NIH, 2024). The practices and equipment used in a BSL-1 facility are appropriate for working with defined and characterized strains of viable microorganisms that are not known to cause disease in healthy adult humans or to adversely affect the environment. Examples of microorganisms that can be handled with in BSL-1 include *B. subtilis* (NIH, 2024). In the Brazilian scenario, the use of *B. subtilis* has been officially authorized as a source of enzymes and enzyme preparations for use in food (Brazil, 2022) and as an agent for biological control of agricultural pests and diseases (Brazil, 2024).

Therefore, this study investigated the viability of the bacterium *Bacillus subtilis* (ATCC 6633) as a biological agent in the process of biomineralization of calcium carbonate in concrete. *B. subtilis* is a gramme-positive bacterium with a high sporulation capacity that allows it to survive in hostile environments.

Materials and methods

Materials for biodeposition

Bacillus subtilis (ATCC - American Type Culture Collection), provided by the Centre for Higher Education of the West – CEO, at the State University of Santa Catarina in Chapecó, Santa Catarina, Brazil, was used in this study. Standard microbiological practices recommended for BSL-1 laboratories were applied in the present study (NIH, 2024). For the microorganism reactivation a Tryptic Soy Agar (TSA) culture medium (pH 7.3 ± 0.2 at 25 °C) was used. Figure 2b shows TSA media with microbial growth in a Petri dish. Table 1 shows the composition of the culture medium.

The activation medium aims to induce microbial growth through a nutrient broth (Feng *et al*., 2021; Saricicek *et al*., 2019), which was used in the curing of biomineralized test specimens. The nutrient broth used is Brain Heart Infusion Agar (BHI) (pH 7.4 ± 0-2 at 25 °C). The medium is buffered with disodium phosphate. The composition of the BHI liquid medium is described in Table 2.

Materials for concretes

The Portland cement used for the concrete mix was CP II Z-32. The aggregates - fine and coarse were sourced from the state of Santa Catarina/SC, Brazil. The fine aggregate has a fineness modulus of 2.25 and a specific gravity of 2.56 g/cm³. The coarse aggregate was crushed gneiss with a specific gravity of 2.80 g/cm³, a fineness modulus of 5.99 and a maximum diameter of 12.5 mm. The water used was supplied by the local company Águas de Joinville and had a pH of 6.0 to 9.0.

Production of the concretes

Three types of concrete samples were produced: Reference Concrete (RC), Biomineralized Concrete (BMC) and Bioconcrete (BC). The concrete was produced in accordance with NBR 12821 (ABNT, 2009). The concretes were dosed according to the methodology of the Institute of Technological Research of the State of São Paulo (IPT/SP). The characteristic compressive strength assumed for the formulation of the reference concretes and the biomineralized concretes was 25 MPa, and the consistency (slump test) was 10 ± 2 cm. Table 3 shows the dosages of the materials.

Table 1 – **Typical composition of the growth medium TSA**

Formulation	Concentration (g L^{-1})
Pancreatic digestion of casein	15
Soy digestive enzyme	5
Sodium chloride	5
Agar	15
Deionized water	15

Table 2 – **Composition of the broth BHI**

Formulation	Concentration (g L^{-1})
Brain, 200g infusion	7.7
Heart infusion starting from 250g	9.8
Proteose peptone	10
Dextrose	2
Sodium chloride	5
Sodium phosphate	2.5

Table 3 – **Materials used to produce the concretes**

Materials (kg)	Reference (RC)*	Biomineralizated (BMC)*	Bioconcrete (BC)**
Portland cement (CP II Z-32)	16.32	16.32	1.63
Fine sand aggregate	18.50	18.95	1.89
Gnaisse coarse aggregate	30.22	30.22	3.02
Water	6.60	6.00	0.50
Water to cement ratio	0.40	0.36	0.30

Note: *to produce 30 liters of concrete; and **to produce 3 liters of concrete.

Molding of the test specimens

The test specimens for the reference concrete and the biomineralized concrete were produced in accordance with standard NBR 5738 (ABNT, 2015a). A total of 24 cylindrical specimens of 100x200 mm were produced (12 specimens for compressive strength tests and 12 specimens for the determination of water absorption, void index and density). After molding, the reference concrete specimens were immersed in a tank of water saturated with calcium hydroxide at 23°C, where they remained in a hardened state until the tests were carried out. The biomineralized test specimens were cured in a plastic box with a nutrient broth of 3 g L^{-1} and urea of 2.4 g L^{-1}. The liquid media were sterilized in an autoclave for 25 minutes at 120 ºC and 1 atm pressure.

Bioconcrete molds were performed in glass petri dishes, single-walled molds with a thickness of 1.2 mm and 60x15mm in size. For the crack closure test in bioconcrete by $CaCO_3$ biodeposition, a crack was induced visually with the help of a metallic sharp object (scratching the surface of the sample). The bacteria were inoculated into the cracks in the cementitious matrix (bioconcrete) using a pipette (Figure 1). The test solution applied consisted of 10 g L^{-1} nutrient solution, 0.5 g L^{-1} water, and 0.5 g urea. The application was carried out over 6 months, on two bioconcrete samples, at room temperature.

Tests in the hardened state

The physical properties of water absorption, void content and specific gravity of RC and BMC concretes were determined using the immersion and boiling method according to NBR 9778 (ABNT, 2005). The compressive strength was measured after 28 days in accordance with NBR 5739 (ABNT, 2018).

Microstructural analysis

Images of the microstructure of the concrete were taken with a field emission scanning electron microscope (SEM), model JSM-6701F, in conjunction with an energy dispersion spectrometer (EDS).

To characterize BMC concrete, X-ray diffraction (XRD) studies were performed with a Shimadzu diffractometer (model 6000) using the copper Kα line (1.5418 Å). Measurements were made between 10.00° and 80.00° with an increment of 0.02°, using a voltage of 40 kV and a current of 30 mA. The scanning speed was set to 2.0° per minute.

Results and discussions

Biological agent

The growth and multiplication of *B. subtilis* in the culture medium were satisfactory, as shown in Figure 2a after Gram staining. A rod-shaped morphology with a size of almost 4 µm was observed.

Characterization for reference concretes and biomineralized concretes

Water absorption, void index, and density

The results of the physical properties were determined after 28 days for the RC and BMC concretes, as shown in Figure 3. A one-sided hypothesis test with a significance level below the value of $\alpha = 0.05$ was performed.

B. subtilis improved the biomineralized concrete in terms of cell concentration. A reduction of 25.31 % in water adsorption was observed compared to the RC samples. In the same trend, the pore index of the biomineralized concrete showed a reduction of about 21 %. Although higher values for water absorption and void index were found for conventional concrete (CPC) compared to biomineralized concrete (CPB) due to exposure to *B. subtilis*, the significance level shows that the data does not indicate a significant difference. In terms of density, all concretes were classified in the normal category, with values between 2000 and 2800 kg m^{-3}, according to NBR 8953 (ABNT, 2015b).

The water absorption test indirectly quantifies the ability of the microorganisms to close the pores by forming calcium carbonate (Kalhori; Bagherpour, 2017; Sri Durga *et al*., 2021), which is formed by the bacteria on the concrete surface and reduces the porosity and capillary pores. This contributes to increasing the durability of biomineralized concrete by preventing the penetration of water and harmful substances that reduce the service life of the material. The increase in water absorption reduces the compressive strength of the concrete (Kunal; Siddique; Rajor, 2014).

Figure 1 – **The bacteria deposition into the cracks in the bioconcrete using a pipette for $CaCO_3$ biodeposition**

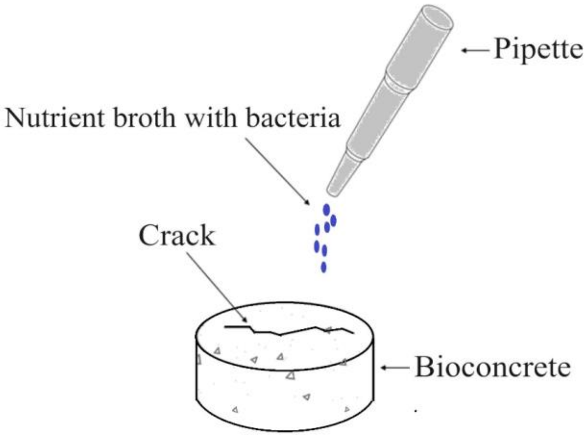


Figure 2 – **(a) Microscopic observation of *Bacillus subtilis*; and (b) observation of colonies of *B. subtilis* after 3 days; and (c) after 7 days of cultivation**

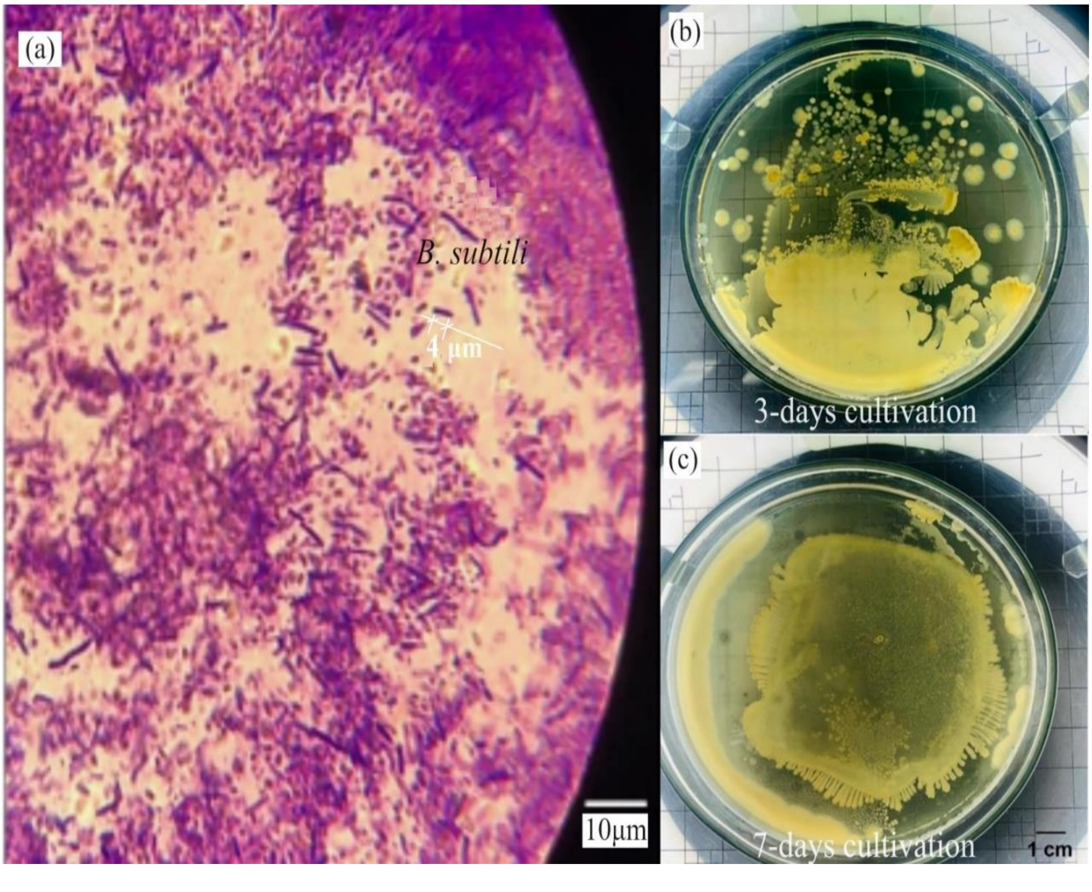
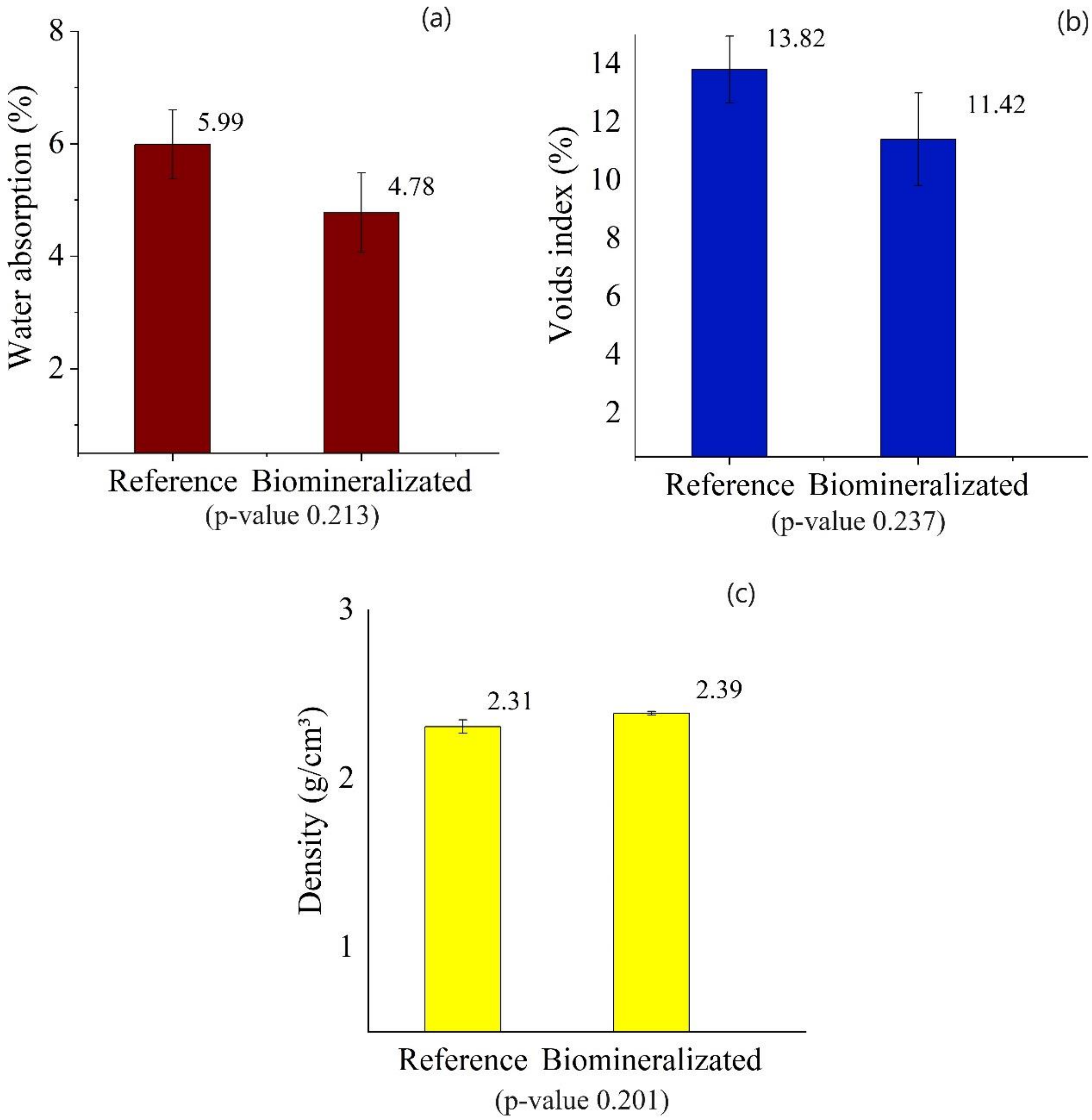


Figure 3 – **Physical properties of the concretes (a) water absorption; (b) void index; (c) density**



Considering these results, the potential of bacteria excreting calcium carbonate to improve strength, durability and therefore overall performance can be recognized. For self-healing concretes, durability and mechanical strength are evaluated by permeability and water absorption tests. Filling the pores with calcium carbonate, which has a relatively low solubility, leads to an absolute reduction in permeability due to the action of bacteria (Andalib *et al*., 2016).

Compressive strength

Figure 4 shows the compressive strength results for the concretes. The compressive strength of the RC concrete was 6.41 % lower than that of the BC concrete. The increase in strength is due to the presence of a sufficient amount of organic matter in the concrete matrix produced by microbial biomass (Muynck; Belie; Verstraete, 2010).

In other studies, the use of bacilli was found to increase compressive strength by 15% to 25%, with the bacilli excreting significant amounts of calcium carbonate, which could increase the strength of the microstructure of the cementitious matrix. The use of the microbial solution, both as part of the mix and as a curing medium, provided benefits for nucleation in the microbial-induced calcium carbonate precipitation (MICP) process (Mondal; Das; Kumar Chakraborty, 2017; Wangui; Karanja Thiong'o; Wachira, 2020). Compared to other studies (Jonkers; Schlangen, 2008; Soda; Madhavan, 2022), the results are similar to the 28-day curing time (Table 4).

The carbonate reacts with cement hydration products such as calcium hydroxide and causes the precipitation of calcium carbonate. This process fills the pores on the surface of the aggregate (Jonkers; Schlangen, 2008). In addition to strengthening the concrete and/or repairing cracks, the bacteria increased the strength of the biomineralized samples after curing, indicating that the curing process carried out by the bacteria increases compressive strength. This is due to the metabolic activity of the bacteria, which leads to increased precipitation of calcite (Abudoleh *et al*., 2019). The formation of calcium silicate hydrate is related to the development of compressive strength, suggesting that the higher the concentration of CSH, the greater the mechanical strength (Lasseuguette *et al*., 2019).

Microstructure analysis using SEM, EDX and XRD

The microstructure image of the concrete surface samples (Figure 5) shows differences in the phases. Figure 5a shows part of an aggregate and the CSH phase attributable to cement composition (Roig-Flores; Formagini; Serna, 2021). The presence of the CSH phase, which contributes most to the strength of the concrete, resulted in more voids being sealed. Figure 5b shows that the bacteria assisted in the formation of the CSH phase and the precipitation of calcium carbonate by the MICP process (Wangui; Karanja Thiong'o; Wachira, 2020).

The calcium carbonate precipitated by the bacterial cells is clearly visible in the pores of the matrix (Figure 5b). During curing, the formation of hydration products such as CSH and ettringite is observed (Lasseuguette *et al*., 2019).

According to Kunal, Siddique and Rajor (2014) the high alkali content of concrete increases the solubility of sulfate ions in the solution, which, when absorbed by CSH, lead to the formation of ettringite, as shown in Figure 5b. Ettringite with a needle-like shape is observed in the cavities of the sample, increasing the density, decreasing the porosity and strengthening the concrete due to the presence of calcite. Figure 5b also shows concentrations of $CaCO_3$ crystals where both polygonal and spherical particles can be seen. The formation of ettringite is responsible for the rapid hardening of the material and compensates for shrinkage in mixtures dominated by Portland cement (Sievert; Wolter; Singh, 2005), resulting in a dense structure and an increase in compressive strength (Kunal; Siddique; Rajor, 2014). The rhombohedral calcite crystals were formed from spherical deposits and occurred after the biocementation reaction stage; however, not all spheres transformed into rhombohedral calcites. The rhombohedral calcite crystals were formed from spheroidal deposits and occurred after the stage of biocementation; however, not all spherules transformed into rhombohedral calcites (Al-Thawadi, 2008).

Figure 5d shows the sites selected for semi-quantitative chemical composition analysis by EDX. Site 1 shows large amounts of calcium (Ca), carbon (C) and oxygen (O) as well as magnesium (Mg), aluminum (Al), silicon (Si) and potassium (K). The chemical composition of site 1 (EDS) showed calcium (22.10 %), oxygen (61.00 %) and carbon (9.20 %), with small amounts of magnesium (0.60 %), aluminum (1.30 %), silicon (3.50 %) and potassium (0.50 %). The spectrum of site 2 showed calcium (25.30%), carbon (7.70%) and oxygen (57.85%), with small amounts of magnesium (1.10%), aluminum (1.60%) and silicon (4.55%).

Figure 4 – **Compressive strength of the concretes**

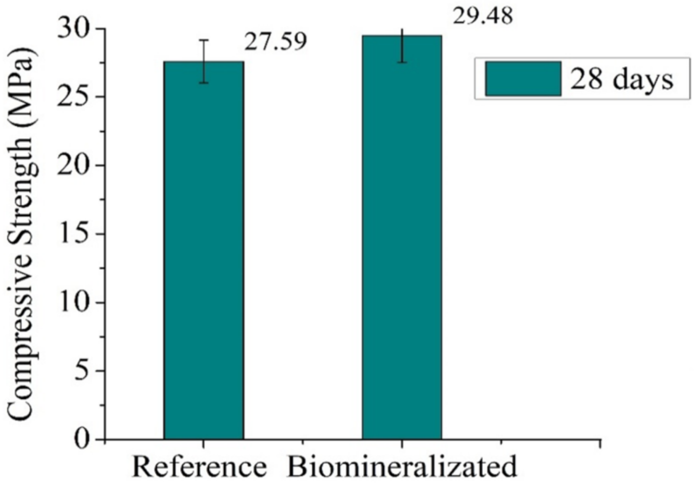
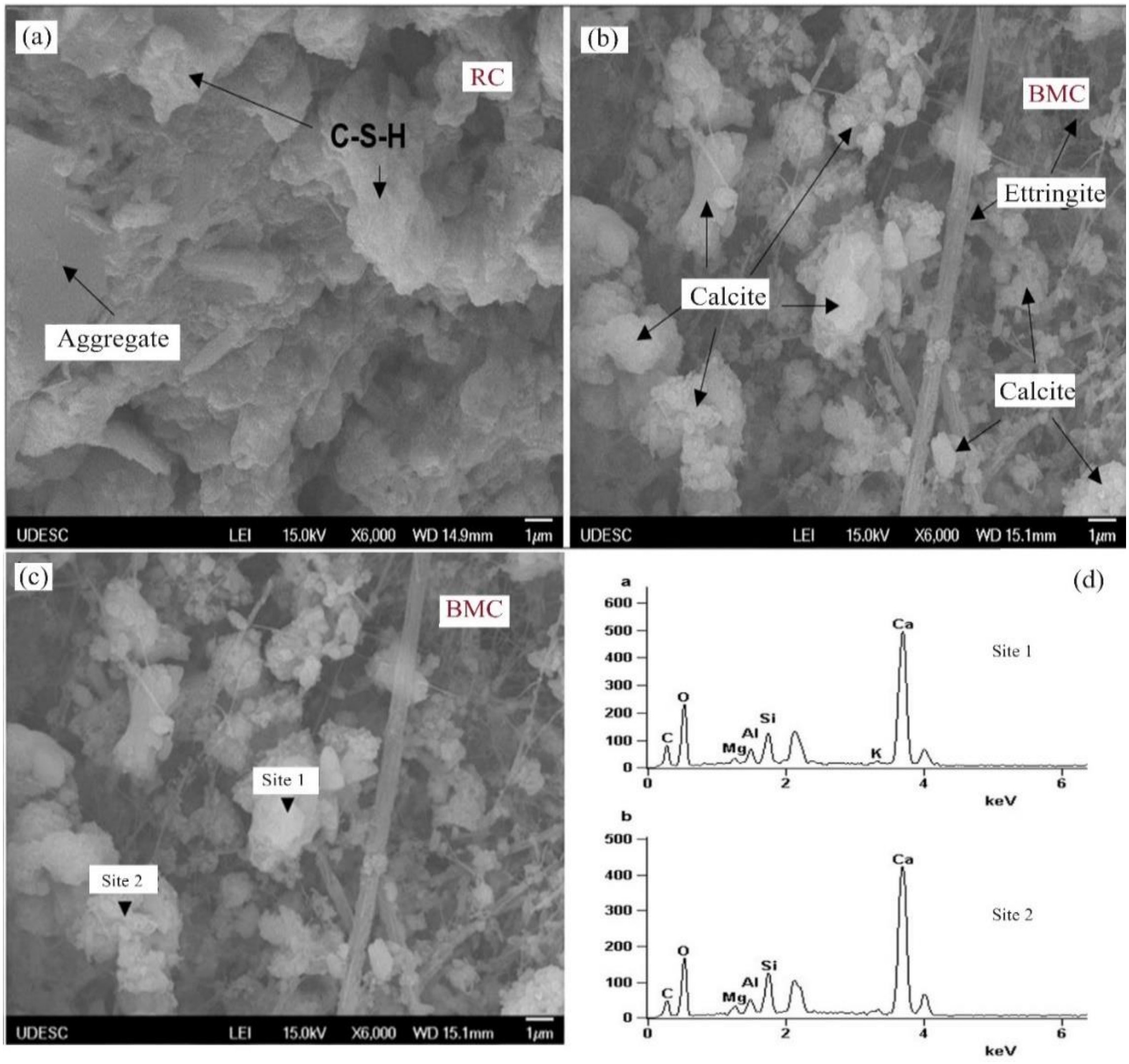


Table 4 – **Comparison of compressive strength results with other studies**

Compressive strength at 28 days (MPa)		
Samples	Reference Concrete	With the addition of bacteria
This study (2023)	27.59 ± 1.56	29.48 ± 1.97
Chen, Chen and Tang (2020)	26.00	29.96 30.96
Soda and Madhavan (2022)	18.05	20.67

Figure 5 – **SEM images at 6000x magnification of (a) reference cocrete, (b and c) biomineralized concrete and (d) EDX of site 1 and 2 from Figure 3c**



According to the semi-quantitative XRD analysis, calcium carbonate was found in both the control sample and the biomineralized concrete. The XRD spectrum in Figure 6 for the biomineralized concrete shows the crystalline structure of two polymorphs, consisting of a combination of vaterite and calcite. Vaterite belongs to the hexagonal crystal system, while calcite is trigonal (and aragonite is orthorhombic). It can be concluded that the presence of vaterite is caused by bacteria, as this phase is not present in samples without the addition of microorganisms (Tittelboom *et al*., 2010).

Small amounts of vaterite were detected in the BC sample. Vaterite, a rare polymorph in nature, is metastable and has a hexagonal structure. It is unstable and quickly transforms into calcite or aragonite at room temperature and/or in aqueous solutions. However, vaterite often forms in synthetic processes and is often reported to develop in the presence of microorganisms (Qian *et al*., 2010; Rodriguez-Navarro *et al*., 2003).

The presence of calcite can be associated with biomineralization as the samples were immersed in the solution containing the bacteria and could not carbonize in air. For the XRD analysis, a sample was taken from the surface of the BC concrete.

Results for the bioconcrete samples

When visually analyzing the cracks in the samples, it is noticeable that the cracks in the samples treated with microorganisms are closing, as can be seen in the images in Figure 7. The deposition of reactive bacteria influenced the closing of the cracks in the samples due to the deposition of $CaCO_3$. The performance of *B. subtilis* in the precipitation of calcium carbonate and in crack healing is satisfactory. To confirm the deposition of $CaCO_3$, a sample was taken from the sedimentation of the cracks to check which chemical compounds were present in the bioconcrete matrix (using EDX). The bacteria acted as a catalyst, feeding on calcium lactate and producing calcite, the crack-sealing material (Abudoleh *et al.*, 2019).

Figure 6 – **X-ray diffractogram of biomineralized concrete**

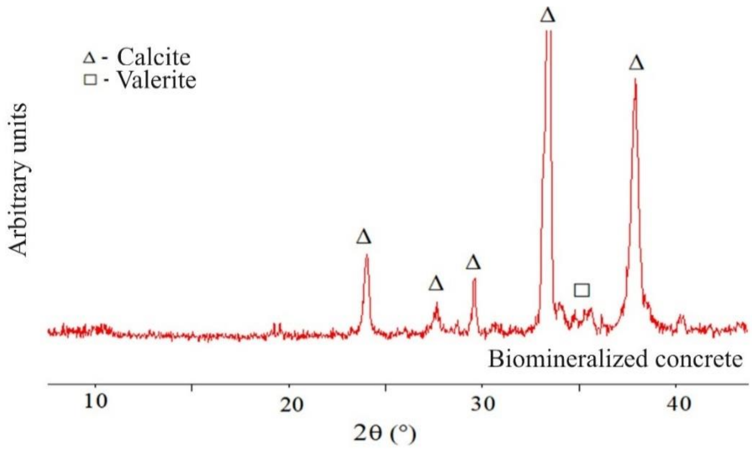
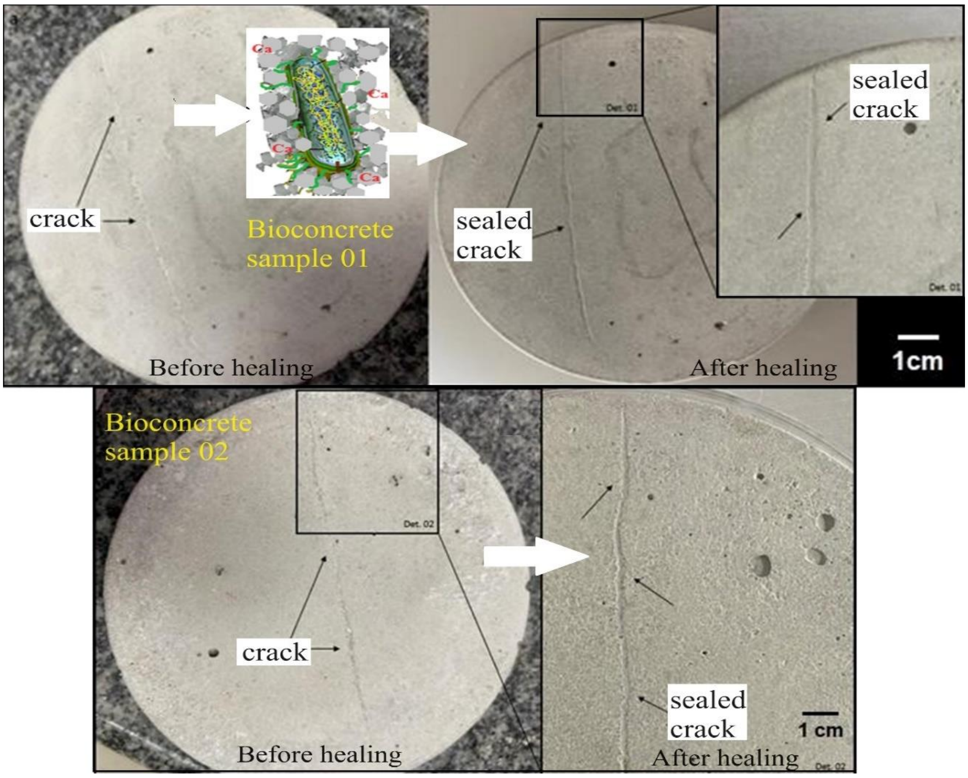


Figure 7 – **Sealed cracks of bioconcrete samples after 6 months of bacterial deposition**



The open pores near the crack were not sealed as the nutrient broth was applied directly to the cracked area. When cracks form in the concrete, the bacteria are enriched with oxygen and water so that they can begin to utilize the nutrients available in the concrete, including calcium lactate, to initiate the calcite precipitation process that is responsible for healing (Abudoleh *et al.*, 2019). The spores must germinate rapidly to form cells that precipitate calcite. Figure 8 shows calcite crystals in the mineral phase.

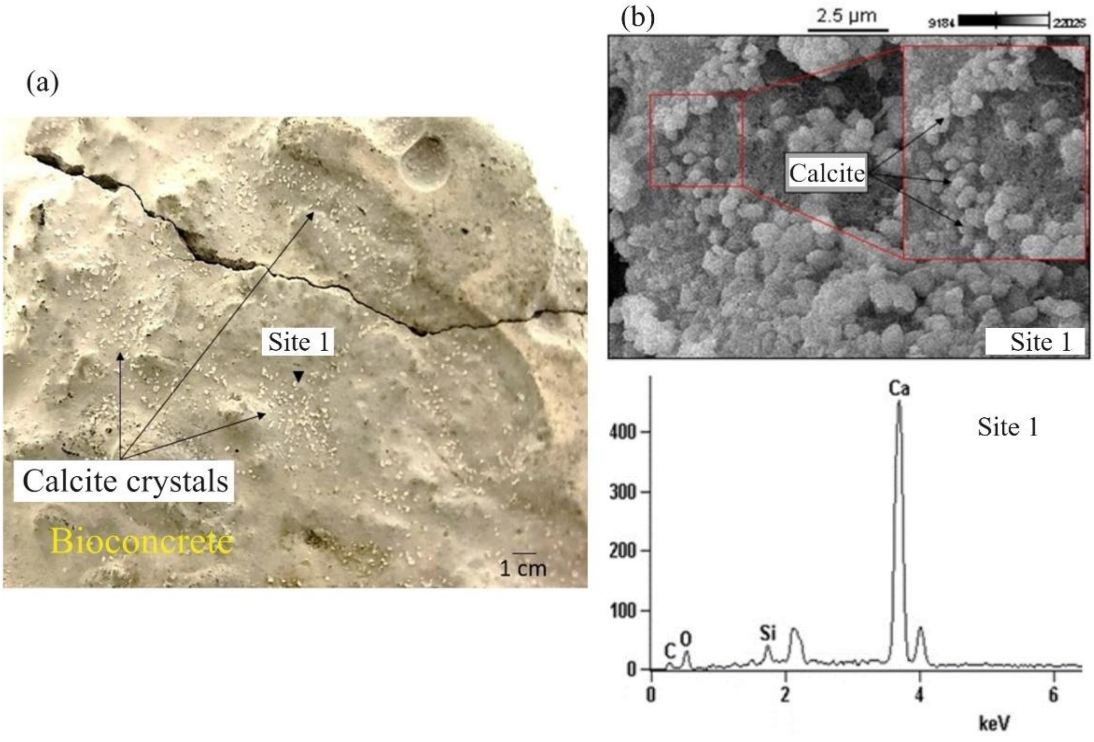
The spectrum (EDX), Figure 8b, of site 1 indicates that the mineral precipitates consist of calcium carbonate ($CaCO_3$) (Wiktor; Jonkers, 2011). The bulk chemical composition (%) revealed 60.10 % calcium, 32.60 % oxygen, 1.95 % silicon and 2.55 % carbon, elements of calcium carbonate. Figure 8b shows the microscopic image of the bioconcrete sample at a scale of 2.5 µm, it is possible to visualize the distribution of calcite in the cementitious matrix with regular sizes. There are spherical and rhombohedral calcite crystals. The rhombohedral calcite crystals have developed from spherical crystals. The spherical crystals initially increase in size, followed by the appearance of the rhombohedral crystals. The bacterial activity improved the physical and mechanical properties of the concrete. By incorporating *B. subtilis* into the concrete matrix, an improved crack repair treatment can be achieved.

The TSA cultivation solution used was satisfactory and proved to be suitable for the growth and sporulation of *B. subtilis* as it met the nutrient requirements of the microorganisms and provided favorable physical conditions. The BHI nutrient solution proved to be effective and provided nutrients for the development of microorganisms.

Bacterial treatment of concrete has been found to significantly reduce water absorption and improve permeability, indicating an improvement in durability. The presence of *B. subtilis* on the concrete surface resulted in a 25.31% reduction in water absorption compared to reference samples and a 21.01% reduction in void index.

The compressive strength of the microorganism-treated concrete was 1% higher compared to the reference, suggesting that biomineralization can repair damaged concrete and improve its strength. The polymorphic calcite crystals seen in the SEM images could explain the improvement in compressive strength.

Figure 8 – **Precipitation of $CaCO_3$ crystals in the bioconcrete; (b) EDX spectrum of the bioconcrete from site 1**



In addition to its many merits, this study has four major research limitations. Although the study produced important results, it is limited by the lack of a urease assay. This shortcoming is a result of resource, financial and time constraints. Larger but similar studies are required to confirm the results, including monitoring pH levels over time. The second limitation is the lack of an assessment of microbial growth at the end of the biomineralization process. In addition, we were unable to assess the risk of contamination from the presence of spores in the bioconcrete. Future work is planned to evaluate the effect of painting the surface already treated with microorganisms with a biocidal paint to prevent humans from being contaminated with the microorganisms used to seal the trenches. Finally, the standardization of methods to evaluate the effects of self-healing is an important task to be addressed in future work, including the production of larger numbers of test specimens.

Conclusion

Crack healing was confirmed by SEM tests in combination with EDS spectra, which gave positive results. This is attributed to the nutrient solution applied to the crack penetrating into the cementitious matrix and precipitating calcite. The surface protection was effective and the crack sealing by the biological treatment led to a reduction in water permeability. It was observed that *Bacillus subtilis* precipitated calcite efficiently by enzymatic reactions, tolerating the alkaline environment of the cementitious matrix, as shown by SEM, EDS and XRD results. Calcite and vaterite crystals adhered strongly to the pore surfaces, providing a protective effect. Thus, the durability of biomineralized concrete structures can be improved by the proper use of bacteria. The standardization of methods to evaluate the self-healing effects of concrete is still a challenge to be overcome.

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Ambiente Construído
Revista da Associação Nacional de Tecnologia do Ambiente Construído
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