AL-KAUNIYAH: Jurnal Biologi, 19(1), 2026, 113-127
Website: https://journal.uinjkt.ac.id/kauniyah/index
P-ISSN: 1978-3736, E-ISSN: 2502-6720

ISOLATION, SCREENING, AND CHARACTERIZATION OF CARBONATOGENIC BACTERIA FROM BUKIT BULAN LIME SOIL AS ALTERNATIVE BIOCEMENT AGENTS FOR SELF-HEALING CONCRETE CRACKS

ISOLASI, SKRINING DAN KARAKTERISASI BAKTERI KARBONATOGENIK ASAL TANAH KAPUR BUKIT BULAN SEBAGAI AGEN BIOSEMEN ALTERNATIF *SELF-HEALING* KERETAKAN BETON

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Submitted: 25 December 2024; Revised: 20 January 2025; Accepted: 12 September 2025

Abstract

Eight carbonatogenic bacterial isolates have been successfully isolated from the limestone soils of Bukit Bulan, Jambi Province, Indonesia. This research aims to obtain superior bacteria as biocement agents that can help the self-healing process of concrete cracks. The eight carbonatogenic bacteria are isolates coded BB1, BB2, BB3, BB4, BB5, BB6, BB7, and BB8. Qualitative screening revealed that all isolates were capable of forming calcium carbonate ($CaCO_3$) precipitation in the test medium. Quantitative tests revealed that all isolates were capable of producing precipitation of calcium carbonate ($CaCO_3$). $CaCO_3$ crystals produced by carbonatogenic bacteria exhibit properties of both calcite and vaterite phases, which can potentially enhance the strength of concrete structures. Seventy-five % of the isolates are gram-positive, and 25% are gram-negative. All isolates can produce the enzyme cytochrome oxidase C, which enhances the natural self-healing ability of bacteria in repairing concrete. All isolates can decompose urea, suggesting that they may contribute to the formation of $CaCO_3$ minerals. The motility test revealed that 50% of carbonatogenic bacteria are motile, which can enhance the efficiency in self-healing concrete. When applied to concrete mixtures, it is known that the carbonatogenic bacteria BB1, BB2, BB3, and BB4 can aid in repairing concrete cracks. The BB3 isolate can close concrete cracks faster than other isolates, indicating that it has the potential to be further developed as a biocement agent.

Keywords: Biocement; Carbonatogenic bacteria; Concrete; isolate; self-healing

Abstrak

Delapan isolat bakteri karbonatogenik telah berhasil diisolasi dari tanah batu kapur Bukit Bulan, Provinsi Jambi, Indonesia. Penelitian ini bertujuan untuk mendapatkan bakteri unggul sebagai agen biosemen yang dapat membantu proses penyembuhan diri retakan beton. Delapan bakteri karbonatogenik adalah isolat berkode BB1, BB2, BB3, BB4, BB5, BB6, BB7, dan BB8. Skrining kualitatif mengungkapkan bahwa semua isolat mampu membentuk presipitasi kalsium karbonat ($CaCO_3$) dalam media uji. Tes kuantitatif mengungkapkan bahwa semua isolat mampu menghasilkan pengendapan kalsium karbonat ($CaCO_3$). Kristal $CaCO_3$ yang dihasilkan oleh bakteri karbonatogenik menunjukkan sifat fase kalsit dan vaterit, yang berpotensi meningkatkan kekuatan struktur beton. Tujuh puluh lima persen isolat adalah gram positif, dan 25 persen adalah gram negatif. Semua isolat dapat menghasilkan enzim sitokrom oksidase C, yang meningkatkan kemampuan penyembuhan diri alami bakteri dalam memperbaiki beton. Semua isolat dapat menguraikan urea, menunjukkan bahwa mereka dapat berkontribusi pada pembentukan mineral $CaCO_3$. Uji motilitas mengungkapkan bahwa 50% bakteri karbonatogenik bersifat motil, yang dapat meningkatkan efisiensi dalam beton penyembuhan sendiri. Ketika diterapkan pada campuran beton, diketahui bahwa bakteri karbonatogenik BB1, BB2, BB3, dan BB4 dapat membantu memperbaiki retakan beton. Isolat BB3 dapat menutup retakan beton lebih cepat daripada isolat lainnya, menunjukkan bahwa ia memiliki potensi untuk dikembangkan lebih lanjut sebagai agen biosemen.

***Kata Kunci**: Bakteri karbonatogenik; Beton; Biosemen; Isolat; Self-healing*

Permalink/DOI: https://doi.org/10.15408/kauniyah.v19i1.43636

INTRODUCTION

Cement is an important building material used worldwide, but its production process results in severe environmental impacts. Cement production accounts for 5–8% of anthropogenic CO_2 emissions and 10–15% of total industrial energy consumption (Benedetti et al., 2023). Concrete is one of the most widely used construction materials due to its superior properties. However, in its application, concrete has problems with the appearance of cracks that can reduce its strength and durability. Liquid infiltration and corrosion in steel are the causes of cracks in concrete. So, it is necessary to find alternative solutions to overcome this problem. One of the solutions that has garnered attention is the automatic crack-healing mechanism using carbonatogenic bacteria. (Gao et al., 2020). To date, microorganism utilization techniques for crack healing in concrete have been explored by engineering the optimal external environment for microbial growth, thereby forming calcium carbonate ($CaCO_3$) particles that adhere tightly to the concrete surface, protecting and strengthening the substrate (Ivaškė et al., 2023).

Carbonatogenic bacteria are known to form calcium carbonate ($CaCO_3$) precipitation under optimal conditions. Thus, they are predicted to protect and strengthen concrete and can be used as candidates for alternative solutions in self-healing concrete (Benedetti et al., 2023). Recent research has been conducted on biotechnology in the field of building materials, utilizing the biomineralization mechanism, which has led to the development of so-called bioconcrete (González-Kunz et al., 2017). Bioconcrete is more environmentally friendly than conventional cement because it does not produce CO_2 in manufacturing. When formed through the metabolic conversion of calcium salts, CO2 is consumed during the process in calcium carbonate mineralization. Biocement can also encourage improvement in cement materials, mechanical properties and durability (Aranda-Usón et al., 2013a). The genus *Bacillus* has been widely studied in its ability to precipitate calcite (Ivaškė et al., 2023). Research on the formation of $CaCO_3$ by microbes through urea hydrolysis has been conducted to support the production of new building materials and the repair of concrete cracks. This method is considered superior to various conventional technologies due to its environmental friendliness. (Yuan et al., 2008). Applying microbial ureolytic mineral bioproduction allows filling pores and crack gaps in concrete, which can be utilized as a surface treatment technique or as a curative component that fuses with the structure (Van Tittelboom et al., 2010).

Indonesia is known as a country with high megabiodiversity, including microbial diversity. However, exploration of biocementation involving local bacteria is still limited. One of the studies that has been developed utilizes *Bacillus galactosidilyticus* JB3, derived from limestone soils in East Java, which can precipitate calcium carbonate ($CaCO_3$). This bacterium has the potential to improve and strengthen concrete structures. (Zulaika et al., 2021). Previous research indicates that the addition of *Bacillus cereus* bacteria improves the mechanical properties of concrete, especially in terms of increased compressive strength and decreased water absorption capacity (Hidayattullah., 2019). Studies show that the addition of a consortium of bacteria from Indonesia, consisting of *Solibacillus*, *Bacillus*, and *Staphylococcus*, in high-quality concrete has a significant effect on improving its performance, especially in the self-healing mechanism of microcracks through calcium carbonate deposition ($CaCO_3$) (Alepu et al., 2024). Therefore, this study aims to explore the diversity of local bacteria in Jambi in search of superior bacteria that have the potential to be used as biocement agents in repairing cracks in concrete.

MATERIALS AND METHODS

This study employs exploratory and experimental methods, comprising the following stages: isolating carbonatogenic bacteria, characterizing and screening potential bacteria for self-healing biocementation of cracks in concrete, and testing bacteria as biofillers in concrete in the final stage.

Sampling Procedure

A sterilized concrete pestle mash was made with 1 g of soil samples from Bukit Bulan, Sarolangun Regency, Jambi Province. It is then dissolved in 9 mL of physiological saline (0.8%) and heated to a temperature of 80 °C for 20 minutes. The sample is then diluted to 10^{-3}. A 100 µL was

inoculated onto the surface of the Calcium Carbonate Precipitation Agar (CCPA) medium, then incubated at 37 °C for 1 week. A $CaCO_3$ precipitation zone surrounds the colony, characterizing the growing isolate of carbonatogenic bacteria. The isolate of carbonatogenic bacteria was grown and then purified using the new CCPA medium until a single colony was obtained. Single colonies of carbonatogenic bacteria will be used for phenotypic characterization and biochemical properties tests (Stabnikov et al., 2013). The phenotypic characters and biochemical tests observed were cell shape, endospore staining, Gram staining, oxidase test, motility test, oxygen requirement test, and catalase test. Confirmation test of urea hydrolysis ability by the scratch conductivity method on Christensen-urea agar (Krishnapriya et al., 2015).

Characterization of Carbonatogenic Bacterial Isolates

Endospore Staining

The process of staining the endospores of carbonatogenic bacteria is carried out by the Schaeffer-Fulton method. The clove preparation is placed on a dye tray, covered with filter paper, and dripped with Malachite Green, then heated for 5 minutes and cooled. After the filter paper is removed and the preparation is dried, contrast staining is performed with safranin, followed by a 1-minute rinse with running water and subsequent drying. The finished preparation is covered with a glass cover, immersion oil is added, and it is observed under a light microscope with 1,000× magnification. Green endospores and pink vegetative cells characterize positive results (Krishnapriya et al., 2015).

Gram Staining

The Gram staining procedure begins with smearing bacteria on a slide and then fixing it using heat from a Bunsen burner. The slides were stained with violet crystals for 1–2 minutes, rinsed with aquades, then given an iodine solution for 1–2 minutes and rinsed again. Decolorization is performed by dripping acetone for 2–3 seconds until the main dye is removed, followed by rinsing with water. The preparation is then stained with safranin for 2 minutes, rinsed, dried, and observed using a light microscope (Siddique et al., 2017).

Motility Test

Bacterial motility is tested based on their ability to produce disulfide acids and gases. The growth pattern that spreads out of the inoculation line indicates the ability of cells to move (Utomo, 2018).

Catalase Test

A drop of 3% H_2O_2 is dripped on the glass of a sterile object, then one ose-bacterial isolate is aseptically transferred. Positive results are characterized by the formation of air bubbles around the inoculum, which indicates that the bacteria belong to the group of aerobes or facultative aerobes (Pourfallahi et al., 2020).

Oxidase Test

One 24-hour-old bacterial isolate is applied aseptically to the surface of the oxidase paper (Oxoid, UK). Positive results are indicated by changing the color of the paper to purple within 5 seconds (Utomo, 2018).

Urease Test

One ose bacterial isolate was inoculated into Urea Base Agar and cultured at 37 °C for 24 hours. A change in the medium's color from yellow to deep pink indicates a positive result, indicating the bacteria's activity of the urease enzyme (Ambarsari et al., 2020).

Production and Calculation of $CaCO_3$ Weight

The selected isolate was tested for its ability to precipitate $CaCO_3$ in Nutrient Broth (NB) medium supplemented with 2% urea and $CaCl_2$ (NB-U/Ca). A total of 2% inoculum was added to 30

mL of NB-U/Ca, and then the mixture was incubated in a shaker at 130 rpm and 30 °C for 7 days. The test was conducted in three replicates. The $CaCO_3$ precipitates are filtered using Whatman filter paper, dried in the oven at 60 °C for 3 hours, and then weighed. The weight of the $CaCO_3$ (Wc) precipitation is calculated based on certain equations, Wc= Wfc - Wf. Where Wfc is the weight of the filter paper containing the sediment, and Wf is the weight of blank filter paper (Zoheir et al., 2013).

$CaCO_3$ Crystal Characterization

The characterization of $CaCO_3$ crystals was carried out using X-ray diffraction (XRD) analysis. The crystalline precipitation from the results of quantitative screening is mashed with a mortar and pestle, then suspended in ethanol for homogenization. XRD analysis was performed with a current of 35 mA and a voltage of 40 kV, in the range of 2θ 20–60° with a scan rate of 0.01°/s. Diffraction peaks in the field (112) are used for calcite mineral identification (Wei et al., 2015).

Application of Carbonatogenic Bacteria as Biocement Agents

The application of bacteria as biocement agents is carried out to evaluate the ability of bacteria to prevent cracks in concrete. Concrete is made by mixing 0.051563 kg of cement, 0.025308 kg of water, and 0.25388 kg of filler material, which is then molded in sizes of 50 × 50 × 50 mm. It is subsequently given a mica plastic coating 0.20 mm thick and 40 mm long in the middle. After drying for 48 hours, the concrete is soaked in water for 28 days. A 500 µL of carbonatogenic bacterial suspension was added to the microcrack and incubated in a humid chamber (100% RH) at 30 °C for 8 days, with macroscopic visual observation of the microcrack (Nimafar et al., 2021).

RESULTS

Isolation of Carbonatogenic Bacteria from the Limestone Soil of Bukit Bulan

From the seven limestone soil samples collected at the selected location, a total of 8 bacterial colonies were obtained. The isolates of BB1, BB2, BB7, and BB8 were isolated from the V/A, V/B, VI/B, and VII/A samples collected from the cave walls, while the BB3, BB4, BB5, and BB6 strains were isolated from the I/D, I/C, I/A, and II/D samples collected from the limestone soil. This can be seen in Table 1 and Figure 1 shows isolated isolates after 7 days of growth at 37 °C in CCPA media.

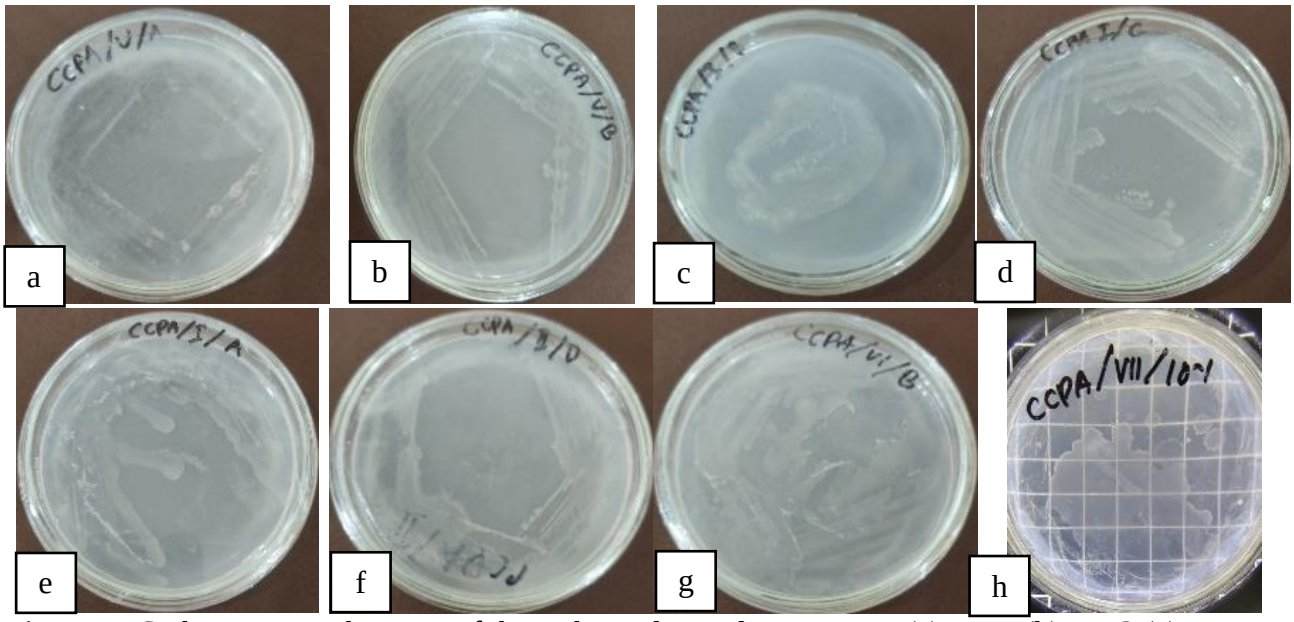

Figure 1. Carbonatogenic bacteria of the Bukit Bulan isolate are BB1 (a), BB2 (b), BB3 (c), BB4 (d), BB5 (e), BB6 (f), BB7 (g), and BB8 (h)

Qualitative Screening

The production of $CaCO_3$ precipitation qualitatively uses the CCP medium. $CaCO_3$ crystal production begins to appear after 24 hours of incubation. The $CaCO_3$ crystals that form are visible

near the bacterial colony. The formation of $CaCO_3$ crystals in the CCP medium takes place faster than in the liquid CCP medium. In this study, the temperature of 37 °C favors faster bacterial growth than that of 30 °C. The results of the qualitative screening of $CaCO_3$ crystals observed under a microscope are presented in Figure 2.

Table 1. Description of the Bukit Bulan carbonatogenic bacteria isolate

Isolate	Sample	Location	Colony color	Margin	elevation	Diameter (mm)	Texture
BB1	V/A	Cave	Beige white	Entire	Flat	±2	Granular
BB2	V/B	Cave	Beige white	Entire	Flat	±2.5	Granular
BB3	I/D	Soil	Beige white	Entire	Flat	±2	Granular
BB4	I/C	Soil	Putih emas	Entire	Convex	±2	Granular
BB5	I/A	Soil	Beige white	Entire	Flat	±1	Granular
BB6	II/D	Soil	Putih krem	Entire	Flat	±1.5	Granular
BB7	VI/B	Cave	Beige white	Entire	Convex	±2	Granular
BB8	VII/A	Cave	Beige white	Entire	Convex	±2	Granular

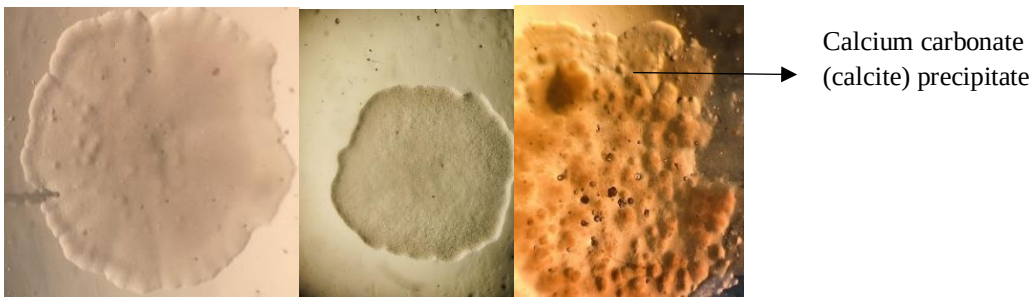

Figure 2. Microscopic $CaCO_3$ crystals (40x) on Calcium Carbonate Precipitation Agar (CCPA) medium 7 days after incubation

Quantitative Screening

One hundred seven cells/mL of carbonatogenic bacteria were inoculated in 30 mL of medium NB-U/Ca, shaken at 130 rpm at 30 °C. After 48 hours, the precipitation in the form of yellowish-white powder (Figure 4) began at the base of the medium and continued to increase until the 7th day of incubation. All bacterial isolates can produce as much as 96 mg of $CaCO_3$ per 30 mL. The isolates that make the most $CaCO_3$ are BB3 bacterial isolates, weighing 135 mg/30 mL, while the isolates that cause the least $CaCO_3$ are BB8 isolates, weighing 102 mg/30 mL. The results of weighing the weight of $CaCO_3$ precipitation of carbonatogenic bacterial isolates are presented in Table 2.

Figure 4. Calcium carbonate precipitation test using NB-U/Ca medium at 30 °C at 130 rpm speed for 7 days

Visually, BB3 isolate produces calcium carbonate precipitate first compared to other isolates. At the 48th hour, this isolate can precipitate calcium carbonate, and the incubation time of calcium carbonate precipitate increases as the thickness of the coating increases. The most significant precipitation of calcium carbonate occurred at the 72nd hour after incubation, compared to the 24th hour and 12th hour after incubation. This is because the bacteria were still adapting to the medium

nutrients at the beginning of inoculation. The formation process of calcium carbonate precipitation in carbonatogenic bacterial cells is illustrated in Figure 3.

Table 2. Weight of the $CaCO_3$ precipitate of the carbonatogenic bacterial isolate

Isolate	$CaCO_3$ precipitation weight (mg/30 mL)	Timing of precipitation formed (h)
BB1	125.29	72
BB2	130.25	72
BB3	135.34	48
BB4	116.53	96
BB5	115.20	72
BB6	125	72
BB7	132	72
BB8	102	72

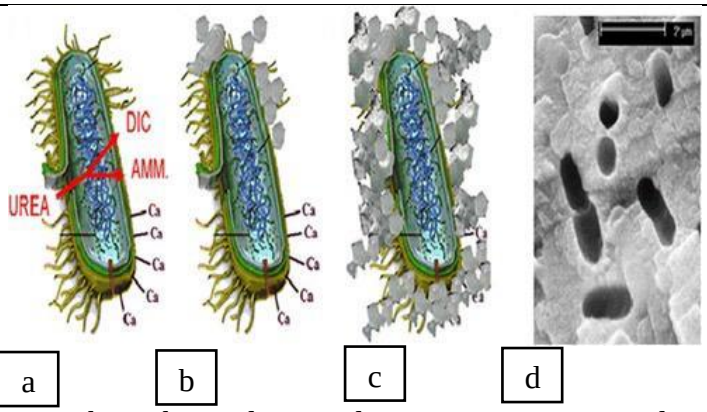

Figure 3. Calcite precipitation through ureolysis, calcium ions are attracted to the surface of the bacterial cell, and when urea is added, dissolved carbon (DIC) and ammonia (AMM) are released into the environment (a), the precipitate encapsulates the cell (b), calcium carbonate settles around bacterial cells (c), traces of bacterial cells are seen as the location of calcium carbonate precipitation (d) (De Muynck et al., 2010)

Partial Characterization of Carbonatogenic Bacteria

The eight selected bacterial isolates were then characterized to determine their properties and characteristics. The following are the results of the characterization of carbonatogenic bacteria from the Bukit Bulan isolate presented in Table 3.

Table 3. Results of characterization of the Bukit Bulan carbonatogenic bacterial isolate

Isolate	Gram	Urease	Oxidase	Catalase	Motility	Endospore
BB1	+	+++	+	+	+	+
BB2	+	++	+	+	+	+
BB3	+	++++++	+	+	+	+
BB4	+	+++	+	+	+	+
BB5	+	++	+	+	-	-
BB6	-	++	+	+	-	-
BB7	+	++	+	+	-	-
BB8	-	+	+	+	-	-

From the above results, it is evident that the carbonatogenic bacteria from Bukit Bulan isolates are Gram-positive. Specifically, six isolates, namely BB1, BB2, BB3, BB4, BB5, and BB7, are Gram-positive, while BB7 and BB8 are Gram-negative. All gram-positive isolates were tested for their ability to produce the enzyme urease. Growing bacteria tested this ability in a urease-based agar medium. The presence of urease is indicated by the change in color of the medium from yellow to deep pink (Figure 5). This color change occurs because the medium contains *phenol red*, which serves as an indicator of pH changes.

According to Table 3 above, the BB3 isolate exhibits a significantly stronger urease production ability than other isolates. Within 24 hours, it was able to produce urease, as indicated by a change in color in the *Urease base Agar medium.*

The results of the oxidase test show that all positive isolates can produce the oxidase enzyme cytochrome c. This is characterized by a change in color to blue on the oxidase disc. The results of the oxidase test are presented in Figure 6.

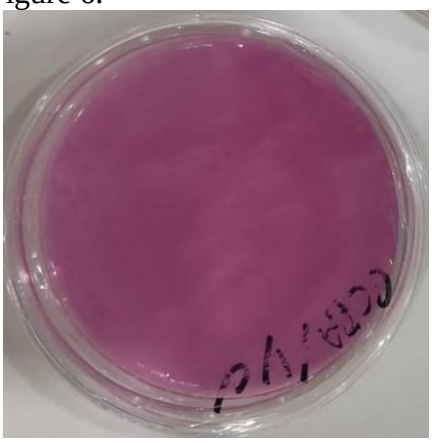

Figure 5. Urease test results of the BB3 isolate carbonatogenic bacteria

Figure 6. Oxidase test results of carbonatogenic bacteria

The catalase test results showed positive results, characterized by the appearance of gas bubbles in the isolate of carbonatogenic bacteria after being given 3% H_2O_2 droplets. The results of the catalase test are presented in Figure 7.

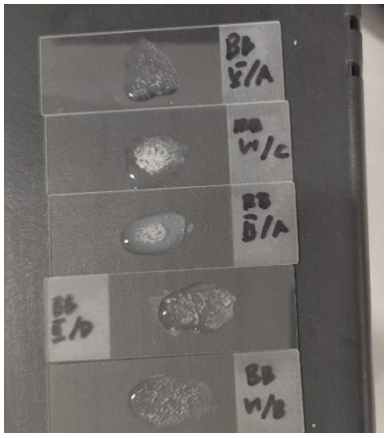

Figure 7. Carbonatogenic bacterial catalase test results

The motility test results showed that the carbonatogenic bacterial isolates with codes BB1, BB2, BB3, and BB4 were motile. Bacteria that produce $CaCO_3$ for *self-healing* concrete must be motile (able to move) because this property increases their ability to spread throughout the cracks in the concrete and cover them with their metabolic product, calcium carbonate. The results of the endospore test showed that carbonatogenic bacterial isolates with codes BB1, BB2, BB3, and BB4 were capable of producing endospores (Table 7). Concrete has tough properties, high pH, mechanical stress, and low water availability.

X-Ray Difraction (XRD) Analysis

X-Ray Diffraction (XRD) analysis aims to determine the phase of $CaCO_3$ crystals produced by carbonatogenic bacteria and identify the crystal phase of calcium carbonate. The data was then analyzed using the Joint Committee for Powder Diffraction Standards (JCPDS) database available in the Match!3 application (Figure 8).

Based on the JCPDS database analysis, three types of $CaCO_3$ crystal morphology formed by bacterial isolates were identified: calcite, vaterite, and other crystals whose phases could not be determined by the chemical formula $CaCO_3$.

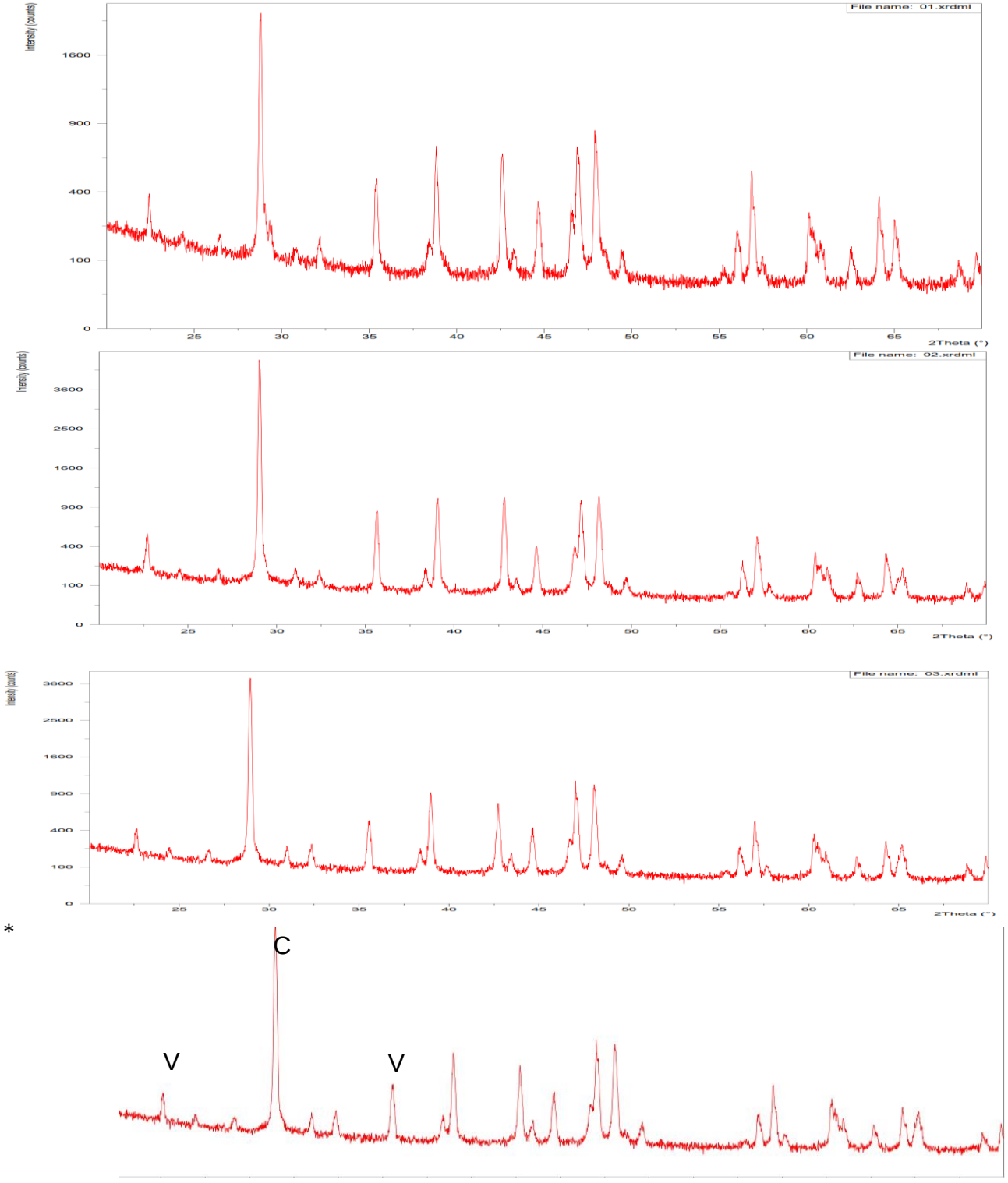

Figure 9. XRD analysis results to determine the crystal shape of $CaCO_3$. C=calcite, V=Vaterite

Application of Carbonatogenic Bacteria as Biofillers in Concrete

The test was carried out by inoculating the isolates of carbonatogenic bacteria BB1, BB2, BB3, and BB4 into the CCP medium. It was carried out on concrete specimens with artificial micro-cracks for eight consecutive days—the results of bioconcrete after the curing process are presented in Figure 10.

Based on the observation results, it can be seen that bacteria's ability to seal concrete cracks varies. Test bacterial isolates are generally known to begin growing on day 10 after mixing. Isolates with the code BB3 close cracks the fastest, at 16 days after mixing, while other isolates close 20 days after mixing.

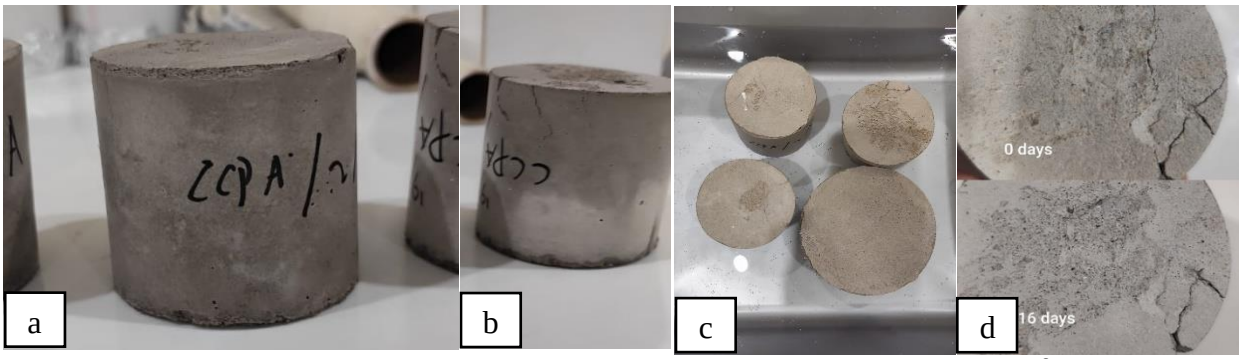

Figure 10. Concrete morphology, control concrete (without the application of carbonatogenic bacteria) (a); bioconcrete with carbonatogenic bacterial application (b); the process of curing concrete specimens (c); morphology of bio-concrete surfaces with the application of carbonatogenic bacteria of the BB3 isolate (d)

DISCUSSION

Isolation of Carbonatogenic Bacteria from the Limestone Soil of Bukit Bulan

The isolation results showed that the Bukit Bulan limestone soil contained carbonatogenic bacteria that could grow on CCPA medium. This condition is in line with the environment where bacteria come from, which has a high pH and temperature, so the diversity of microorganisms, both bacteria and fungi, is relatively high and has the potential to be used as biocement (Pangestu, 2019). The physical properties of the soil in Bukit Bulan are dominated by sand with a clumping structure, and the soil's color ranges from creamy white to deep beige, indicating a high lime content. Previous research has successfully isolated several carbonatogenic bacteria from three strains: *Lysinibacillus fusiformis* 3.20, *Psychrobacillus psychrodurans* 7Mo, and *Lederbergia lenta* Vetro1 obtained from the statue of Il Giovane di Mozia, the Etruria frescoes at Tomba degli Scudi, and the statue of Sant'Eustachio in Matera (Benedetti et al., 2023). Isolation of carbonatogenic bacteria from various sources is essential for application in biomineralization and conservation in cultural heritage restoration (Stabnikov et al., 2013). Research at Bukit Jaddih, Bangkalan, succeeded in isolating six carbonatogenic bacteria with codes JA1, JB2, JB3, JA4 (from limestone soil samples), AK4 (from young stalactites of Goa Akbar), and SU1 (from Bukit Kapur Suci, Gresik), which were used for self-healing applications on concrete cracks (Utomo, 2018).

Qualitative and Quantitative Screening of $CaCO_3$ Crystal Production

Qualitative and quantitative screening processes are conducted to isolate bacteria that can biomineralize by producing calcium carbonate. $CaCO_3$ crystals form after 7 days of incubation in solid medium faster than in liquid medium, because solid medium is incubated at 37 °C, while liquid medium is at 30 °C. Incubation temperature plays a vital role in bacterial growth. Generally, bacteria that live in a moderate environment have an optimal temperature of 30–37 °C, depending on the type. Temperature and incubation duration affect growth phase, generation time, cell concentration, nutrient requirements, enzyme activity, and cell composition (Ivaškė et al., 2023). The biomineralization process of calcium carbonate by bacteria is influenced by four main factors: calcium ion concentration, dissolved inorganic carbon concentration, medium pH, and nucleation site availability (Hammes & Verstraete., 2002). Bacteria can create an alkaline environment and increase concentrations through various autotrophic and heterotrophic metabolic pathways (Joshi et al., 2017). Calcium carbonate mineralization occurs if calcium ions and nucleation sites are available in the environment.

Bacterial surfaces, including cell walls and exopolysaccharides (EPS), serve as nucleation sites because they bind metals. They also act as a template for heterogeneous nucleation and $CaCO_3$ crystal

growth. EPS affects the morphology, polymorphism, spatial location, and growth rate of crystals (Yuan et al., 2008). The EPS is a template matrix that defines the shape, polymorphism, spatial distribution, and growth of $CaCO_3$ crystals (Van Tittelboom et al., 2010). $CaCO_3$ crystals usually grow on the surface of bacterial cells (Utomo, 2018). The resulting polymorphs (calcite, aragonite, and vaterite) depend on environmental conditions and bacterial strains (Krishnapriya et al., 2015).

The process of forming calcium carbonate crystals is affected by the saturation state of the solution, which is the concentration of carbonate ions (CO_3^{2-}) available to form $CaCO_3$ precipitation. High pH values increase the stability of carbonate ions, while the high availability of calcium ions (Ca^{2+}) increases the chances of calcite formation. In addition, nucleation sites are essential to ensure the stable and sustainable formation of calcium carbonate (Siddique et al., 2017). Bacteria act as nucleation sites in biomineralization, where calcium carbonate precipitates in the vicinity of bacterial cells. Various environmental parameters greatly influence ureolytic activity and $CaCO_3$ crystal formation. The surface of bacterial cells has negatively charged groups capable of attracting and binding divalent cations such as Ca^{2+} or Mg^{2+} at a neutral pH, thus providing an ideal nucleation location for calcite precipitation (Pourfallahi et al., 2020). However, Ca^{2+} ions tend to bind to the surface of negatively charged bacterial cells more often than Mg^{2+} because they have higher ionic selectivity. These bonded cations then react with carbonate anions to form calcium carbonate in an insoluble form (Figure 3). Bacterial cells play an essential role in $CaCO_3$ precipitation, since in addition to providing heterogeneous nucleation sites, cells also influence the specific types of minerals that are formed (Ambarsari et al., 2020).

Microbe-induced calcite precipitation refers to the formation of calcium carbonate from saturated solutions caused by the presence of microbial cells and their biochemical activity (Anbu et al., 2016). During microbial-induced calcite formation, microbes can release one or more metabolic products, such as carbonate ions (CO_3^{2-}), which then react with calcium ions (Ca^{2+}) in the environment, thus triggering calcium carbonate ($CaCO_3$) precipitation (Wei et al., 2015). Previously, it was known that calcium carbonate precipitation can occur through various biochemical mechanisms, including photosynthesis, urea hydrolysis, sulfate reduction, anaerobic sulfide oxidation, biofilm formation, and the activity of extracellular polymeric substances (EPS). Each of these mechanisms plays a role in providing carbonate ions (CO_3^{2-}) or facilitating the nucleation of calcium carbonate in the microbial environment (Krishnapriya et al., 2015). However, among these mechanisms, the precipitation of calcium carbonate mediated by bacteria through urea hydrolysis (ureolysis) is the most widely used method, due to the high efficiency of $CaCO_3$ crystal formation and its ease of regulation under laboratory conditions and field applications (Aranda-Usón et al., 2013b).

The mechanism of calcite precipitation occurs when the urease enzyme catalyzes the hydrolysis of urea into ammonium and bicarbonate. In this reaction, one mole of urea is hydrolyzed into one mole of ammonia and one mole of carbamic acid (Equation 1), which is then spontaneously hydrolyzed into one mole of ammonia and carbonic acid (Equation 2) (3)

$$CO(NH_2)_2 + H_2O \longrightarrow NH_2COOH + NH_3 \quad (1)$$

$$NH_2COOH + H_2O \longrightarrow NH_3 + H_2CO_3 \quad (2)$$

The two products, namely NH_3 and H_2CO_3, then react with water to form bicarbonate (Equation 3) and produce two moles of ammonium and hydroxide ions (Equation 4). The hydroxide ions formed increase the pH of the solution, thus shifting the bicarbonate equilibrium towards the formation of carbonate ions (Equation 5). This shift allows metal ions to settle, while the local increase in pH due to NH_4^+ triggers a reaction that results in calcium carbonate precipitation (Pourfallahi et al., 2020).

$$H_2CO_3 \longleftrightarrow HCO_3^- + H^+ \quad (3)$$

$$2\ NH_3 + 2\ H_2O \longleftrightarrow 2\ NH_4^+ + 2\ OH^- \quad (4)$$

$$HCO_3^- + H^+ + 2\ OH^- \longleftrightarrow CO_3^{2-} + 2\ H_2O \quad (5)$$

CaCO precipitation occurs on the surface of bacterial cells if there is a sufficient concentration of Ca^{2+} and CO_3^{2-} in the solution (Fig. 4) (Equations 6 & 7)

$$Ca^{2+} + \text{Sel bakteri} \longrightarrow \text{Sel-}Ca^{2+} \quad (6)$$

$$\text{Sel-}Ca^{2+} + CO_3^{2-} \longrightarrow \text{Sel-}CaCO_3 \quad (7)$$

An increase in temperature will increase the amount of calcium carbonate precipitation; the longer the incubation time, the more calcium carbonate precipitation will form (Pourfallahi et al., 2020).

Partial Characterization of Carbonatogenic Bacteria

The characterization of isolates aims to determine the physiological properties of each isolate. Most isolates are Gram-positive bacteria of the genus *Bacillus*, which are known to produce the enzyme urease (Gao et al., 2020). Gram-positive bacteria are stained purple after Gram staining. This is due to the cell wall having a thick layer of peptidoglycan. When given alcohol treatment, the cell walls are dehydrated, causing the pores to close, which leaves the violet-iodine crystal complex insoluble and the purple color visible until the end of staining. In contrast, Gram-negative bacteria have a thin lipid layer dissolved by alcohol, so the violet-iodine crystal complex is lost from the cell wall, the pores are widened, and the bacteria end up pink due to safranin (Zulaika et al., 2021).

When bacteria hydrolyze urea in the medium with the help of the urease enzyme, the medium's pH increases, and its color changes from yellow to deep red. Urease decomposes urea into ammonia and CO_2, which increases the pH and the concentration of carbonate ions around the bacterial cell (Fitri et al., 2023). In bacteria that can hydrolyze urea, the urease enzyme catalyzes the urea reaction to form carbonate ions. If calcium ions are available, these carbonate ions will settle as calcium carbonate ($CaCO_3$), which can be used for various applications, including as a material for sealing cracks in concrete (Abdelgalil, 2022).

Urea is a decarboxylated product of amino acids. When hydrolyzed by bacteria, urea is broken down to produce ammonia and CO_2. The formation of ammonia increases the pH of the medium, which can be detected through the discoloration of the red phenol indicator, from light orange at pH 6.8 to magenta (pink) at pH 8.1. Urease-positive bacteria will quickly turn the entire medium pink within 24 hours. Weak positive bacteria may need a few days to show discoloration, while harmful bacteria do not cause any changes, or the medium remains yellow due to acid production (Ningsih et al., 2018).

$$\underset{\text{Urea}}{(NH_2)_2CO} + 2H_2O \xrightarrow{\text{Urease}} CO_2 + H_2O + 2\,NH_3 \quad (8)$$

Several researchers have discussed the ability of urease (urea amidohydrolase; EC 3.5.1.5) in microorganisms to induce carbonate precipitation (Hammes & Verstraete, 2002).

Urease activity is found in various microorganisms, but some strains can produce very high amounts of urease. One example is *Sporosarcina pasteurii* (formerly *Bacillus pasteurii*), a non-pathogenic soil bacterium that forms endospores, has an optimal growth pH of 9.0, and is tolerant to extreme conditions. Due to these characteristics, the strain is widely studied for the induction of calcium carbonate precipitation through microbial activity (Zoheir et al., 2013). In addition, the BP-M-3 mutant strain of *Sporosarcina pasteurii* MTCC 1761 has been successfully developed. It shows higher urease activity and calcite precipitation ability than wild-type strains (Dhami et al., 2014). Some pathogenic bacteria, such as *Helicobacter pylori*, *Proteus vulgaris*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, also produce urease, especially during urinary tract infections. Urease plays a role in the formation of intracellular urinary tract stones. The precipitation of calcium carbonate through urease is an easy-to-control induction mechanism that can produce high $CaCO_3$ concentrations in a short time. The urease activity influences biomineral formation through four main parameters (Fitri et al., 2023).

Carbonatogenic bacteria used in biocement need oxidase enzymes, as these enzymes play an essential role in bacterial growth and metabolism. They thus support the formation of calcium carbonate ($CaCO_3$) minerals, a process known as microbially induced calcium carbonate precipitation

(MICP). Isolates of carbonatogenic bacteria, such as *Bacillus* spp., can produce $CaCO_3$ through the MICP mechanism. The oxidase enzyme also helps bacteria survive harsh environmental conditions by regulating energy metabolism and pH (Zhang et al., 2024).

Calcium carbonate-producing bacteria require catalase enzymes to maintain cell health and ensure the mineralization process runs effectively. These enzymes counter oxidative stress during metabolism, especially when bacteria are in extreme environments. During metabolism, bacteria produce hydrogen peroxide (H_2O_2) as a byproduct; if not broken down, H_2O_2 can damage cells. Catalase converts H_2O_2 into water and oxygen, thus protecting cells from damage. By neutralizing H_2O_2, catalase maintains optimal environmental conditions, including pH and ion concentration, to form calcium carbonate ($CaCO_3$). The presence of this enzyme enables bacteria to survive longer, making the process of closing cracks in concrete through the production of $CaCO_3$ more effective. In addition, catalase provides metabolic flexibility, as bacteria can cope with varying oxidative conditions without being hampered by the harmful effects of H_2O_2, supporting survival and mineralization activity (Hayta et al., 2021)

Endospores are dormant structures that allow bacteria to survive in extreme or unfavorable environmental conditions. As conditions improve, such as increased humidity or nutrient availability, endospores can germinate into active vegetative cells, ready to resume their growth and metabolic activity (Van Tittelboom et al., 2010). Bacteria that can biocement calcium carbonate generally belong to the genera Bacillus and Sporosarcina, which are known for their ability to produce endospores. The ability to form endospores is closely related to the effectiveness of biocementation, especially in applications such as self-healing concrete and soil stabilization. Endospores enable bacteria to survive in extreme conditions, including high pH levels, dehydration, and high temperatures, and facilitate reactivation and regrowth when environmental conditions become optimal. In addition, the presence of endospores also increases the long-term stability of the concrete self-healing system (Abdelgalil, 2022).

X-Ray Difraction (XRD) Analysis of $CaCO_3$ Crystals

Based on the characterization of calcium carbonate crystals produced by the isolate of Bukit Bulan's carbonatogenic bacteria, it is known that the crystal phase consists of calcite and vaterite. Calcite has a solid rhombohedral structure and is most effective in strengthening concrete and soil. In contrast, vaterites have a less dense structure and are metastable (can turn into calcite), generally formed in the early stages of the biocementation process. Meanwhile, aragonite is shaped like a rod or needle, stable at high pressure, and is rarely found in concrete (Algaifi et al., 2021). Carbonatogenic bacteria can precipitate calcium carbonate ($CaCO_3$) in various crystalline phases, namely calcite, vaterite, and aragonite. The effectiveness of this biocementation process is influenced by the type of bacteria used, environmental conditions such as temperature, pH, and humidity, as well as the chemical and physical factors that regulate the biomineralization mechanism (Chen et al., 2021).

Application of Carbonatogenic Bacteria as Biofillers in Concrete

The BB03 isolate demonstrates the ability to close cracks more quickly than other isolates. The bacteria used in self-healing concrete can produce enzymes such as urease or carbonate anhydrase to accelerate the production of $CaCO_3$, which plays a vital role in sealing microcracks. The BB03 isolate is possibly more adaptable to high alkaline conditions, so it shows higher activity than other isolates (Dinarvand & Rashno., 2022). The production of exopolysaccharides (EPS) can increase the adhesion ability of bacterial cells to the surface of micro-cracks while retaining moisture that supports bacterial growth. The ability of bacteria to precipitate calcium carbonate ($CaCO_3$) through the activity of the urease enzyme has been widely studied. Research shows that *Bacillus subtilis* can increase the compressive strength of concrete while closing micro-cracks (Haedar et al., 2025). The addition of *Bacillus subtilis* to the mortar mixture has been shown to increase the compressive strength of concrete by up to 25.38% at 3 days of age and can close cracks with a maximum width of 0.22 mm (Elgendy et al., 2024).

CONCLUSION

Based on the results of the study, it can be concluded that eight isolates of carbonatogenic bacteria were successfully isolated from the limestone soil and cave walls, with the isolate codes BB1, BB2, BB3, BB4, BB5, BB6, BB7, and BB8, which can produce $CaCO_3$ crystal precipitation in CCPA medium. The results of quantitative screening indicate that the BB3 isolate produces the highest CaCO3 precipitation, with a total of 135.34 g/30 mL NB-U/Ca. Based on the results of biochemical characterization, it is known that the BB3 isolate is a bacterium with the desired physiological properties in application as biocement. This isolate is a gram-positive bacterium, motile, capable of producing urease, catalase, and oxidase, and forms endospores. The result of XRD analysis was that calcite was the dominant form of $CaCO_3$ crystals from the BB3 isolate. The results of testing as a concrete biofiller indicate that the BB3 isolate can seal cracks in concrete more quickly than other isolates. Hence, this isolate is a promising candidate for application as a biocement agent for concrete cracks.

Further research is necessary to optimize the ability of BB03 bacteria in the Bukit Bulan isolate by engineering the growth conditions and molecular gene manipulation. Molecular identification is necessary to determine the type of BB03 bacteria and to test the compressive strength and tensile strength of concrete with and without a mixture of BB03 bacteria.

ACKNOWLEDGMENTS

The author would like to express his infinite gratitude to the Directorate of Research, Technology, and Community Service (DRTPM) of the Ministry of Education and Culture-Research and Technology for funding this research in the Beginner Lecturer Research (PDP) scheme for the 2024 fiscal year.

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