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Novel carbonatogenic bacterial strain isolated from limestone quarry in East Java, Indonesia to improve concrete performance

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Abstract. *Zulaika E, Utomo MAP, Pangestu AS, Alami NH, Shovitri M, Prasetyo EN, Setiawan E, Luqman A, Kuswytasari ND, Irawan C. 2021. Novel carbonatogenic bacterial strain isolated from limestone quarry in East Java, Indonesia to improve concrete performance. Biodiversitas 22: 3890-3898.* Carbonatogenic bacteria can precipitate $CaCO_3$ in the form of calcite, aragonite, or vaterite. Calcite has the potential to be applied for strengthening concrete structures. This research aims to explore several new bacterial strains that can precipitate calcium carbonate leading to produce calcite and could be useful for strengthening concrete structures. Soil and stalactite samples were taken from a well-known limestone quarry in East Java, Indonesia. The isolated bacteria species were identified using 16S rRNA gene sequences. $CaCO_3$ crystal properties were characterized using X-Ray Diffraction and Scanning Electron Microscopy. Six novels isolated $CaCO_3$ precipitating bacterial strains; *Bacillus huizhouensis* JA1; *B. galactosidilyticus* JB3; *B. niacini* AK4, *B. lentus* SU1, *Lysinibacillus macroides* JB2, and *Sporosarcina soli* JA4 were successfully isolated and have the potential to enhance concrete strength. All isolates were able to produce $CaCO_3$ in calcite form except *B. galactosidilyticus* JB3. The experimental concrete with the addition of bacterial cells showed higher compressive strength and maximum load compared to control concrete and met the requirements for building construction so that it could be applied for building structure materials.

Keywords: Bacteria bio concrete, building materials, $CaCO_3$, carbonatogenic

INTRODUCTION

Concrete is the most widely used construction material made up of cement, sand, gravel, and water (Jonkers et al. 2010; Manasa et al. 2019). It has several advantages such as high compressive strength, resistance to fire, and relatively inexpensive. Within the concrete, one of the most crucial materials is cement which acts as a materials binder. Cement is made from limestone with calcium carbonates ($CaCO_3$) as the main ingredients. However, the process of making artificial cement remains an environmental issue. It contributes around 6% of global anthropogenic CO_2 emissions and depleting the extensive limestone area (Achal and Mukherjee 2015). To maintain the quality of environments, it is needed environmentally friendly and renewable $CaCO_3$ sources. Recently, microbial-induced calcium carbonate precipitation (MICP) for concrete application is of great interest and attracted our attention since $CaCO_3$ can be produced naturally through this process.

Microbial induced calcium carbonate precipitation (MICP) is the geomicrobiological process in which bacteria as inducers play several mechanisms toward calcium carbonates precipitation in the soil matrix, thereby soil strength and stiffness can be increased (Mortensen et al. 2011). The mechanisms of $CaCO_3$ precipitation are various, yet the most frequently used bacteria for concrete application is carbonatogenic bacteria. It is one of the bacteria groups which can perform the MICP process through urea hydrolysis and increase the pH of the environment around bacteria cells. The hydrolysis process generates carbonates (CO_3^{2-}) and ammonium (NH_4^+), thus environmental calcium ions (Ca^{2+}) around bacteria cells will be combined with carbonates to precipitated calcium carbonates (de Muynck et al. 2010). Calcium carbonates in calcite form have promising potential to be applied for concrete reinforcement and self-healing concrete (Ni and Ratner 2008). Besides its ability to produce urease, carbonatogenic bacteria are alkaliphilic and resistant to high urea and calcium content, making them easy to find in limestone areas and calcareous caves (Jimenez-Lopez et al. 2008; Li and Qu 2015).

Carbonatogenic bacteria can be applied as an additive to concrete and mortar for materials binder (Dhami et al. 2014), strengthen concrete structures (Wang et al. 2016; Zulaika et al. 2020), and covers concrete microcracks by self-healing concrete (self-healing concrete) (Siddique and Chahal 2011). Some species can form endospores, so they can form up to 50 years and are resistant in extreme environments (Gavimath et al. 2012). Vegetative cells and endospores can be applied for bio-concrete. Bio-concrete is a concrete technology infused with carbonatogenic bacteria

cells or spores and it has natural self-healing ability (Alves et al. 2019; Ponraj et al. 2015; Zulaika et al. 2020).

There are several methods for making bio concrete ,i.e., introduce directly bacteria cells into the concrete mixture (Krishnapriya et al. 2015), encapsulated bacteria cells in concrete structures (Wang et al. 2014), direct application of bacteria-free cells into the concrete crack surface (Sharma et al. 2017), and immerse concrete specimens in bacterial suspensions (Wiktor and Jonkers 2011). Indonesia has a vast limestone area especially in the northern part of East Java province. Species and novel strains of indigenous bacteria from tropical lime habitats in East Java province are very potential to be applied and developed as concrete reinforcement agents and self-healing concrete, particularly in tropical countries. Here we reported the characterization of novel carbonatogenic bacterial strains isolated from the limestone quarry in East Jawa Indonesia

MATERIALS AND METHODS

Bacteria isolation

Soil and stalactite samples were taken from Jaddih Bangkalan limestone quarry, Suci karst in Gresik, and growing stalactites in the Akbar cave Tuban, East Java, Indonesia. Screening of isolates ability to precipitate $CaCO_3$ was carried out using CCP (Calcium Carbonate Precipitation) agar containing (per liter) 20 g of urea, 2.12 g $NaHCO_3$, 10 g NH_4Cl, 3 g of nutrient broth, 30 mM $CaCl_2$, 20 g agar then the pH of the medium was adjusted to 8.5 (Wei et al. 2015). Bacteria isolates were incubated at room temperature for 7 days. Carbonatogenic bacteria were evaluated for their ability to manifest $CaCO_3$ precipitation zones around the colony.

Urease screening and biochemical test

A qualitative urease assay on Christensen-urea agar was carried out to determine isolates' ability to produce urease (Hammad et al. 2013). Plates were incubated at room temperature for 2-5 days. Shifting of the medium color from yellow to cherish pink indicates urease produced by carbonatogenic bacteria. Urease hydrolyzes urea and releases ammonium that increases pH in the medium until the medium color turns to cherish pink. Morphological and biochemical characterization such as cell shape, Gram staining, endospore staining, catalase test, oxidase test, motility test, and oxygen demand test were performed following standard protocols (Holt et al. 1994).

Species and strain identification

Sequences for strain identification and phylogenetic analysis were obtained from genomic DNA extraction using the DNeasy UltraClean Microbial Kit (Qiagen®, Germany) according to the manufacturer's protocol instructions. The extracted bacterial DNA was amplified by PCR (Polymerase Chain Reaction) in 50 µL PCR mixture which consists of 25 µL OneTaq® Quick-Load 2X Master Mix with Standard Buffer containing 25U / ml OneTaq DNA polymerase, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, and 20 mM Tris-HCl pH 8.9 (New England BioLabs, US), 1 µL forward primer 27F 5'-AGAGTTTGATCCTGGCTCAG-3 'and 1 µL reverse primer 1492R 5'-TACCTTGTTACGACTT-3' (Baker et al. 2003), 2 µL genomic DNA templates bacteria, and nuclease-free water. The PCR program on the BioRad® CFX96 thermocycler was started with an initial denaturation at 94°C for 30 seconds. The process is continued with 35 cycles consisting of denaturation at 94°C (30 seconds), then annealing at 50 °C (50 seconds), extension at 68 °C (1.5 minutes), and final extension at 68 °C (10 minutes) (Wei et al. 2015). The PCR results were then visualized on agarose gels. Amplicons were purified using AccuPrep® PCR Purification Kit (Bioneer, South Korea) and were then sequenced with the ABI 3730XL DNA Analyzer (Bioneer, South Korea).

The obtained sequences were processed and stored in FASTA format using the BioEdit program (Hall 1999). 16S rRNA sequences of carbonatogenic bacteria were compared with related sequences in the Ribosomal Database Project from GeneBank with the BLASTN program (Altschul et al. 1997). The phylogenetic tree was constructed and analyzed using MEGA 7 (Molecular Evolutionary Genetics Analysis) software (Kumar et al. 2016). The distance between nucleotides was calculated using the Neighbor-Joining algorithm (Saitou and Nei 1987). Statistical support data and representative phylogenetic trees according to evolutionary history was performed in 1000 replication of bootstrap (Felsenstein 1985).

CaCO3 precipitation and characterization

Liquid CCP media was used for $CaCO_3$ precipitation. As many as 108 bacteria cells from enrichment media were inoculated into liquid CCP. Liquids were incubated in the rotary shaker (130 rpm) for 10 days at room temperature. Precipitated $CaCO_3$ was separated by membrane filtration and followed by drying in an oven at 60°C for 4 hours. The weight of precipitated $CaCO_3$ powder was weighed using an analytical balance (Hammad et al. 2013).

$CaCO_3$ crystals were ground and washed with 70% ethanol. The crystalline phase was observed using X-Ray Diffraction (XRD). The parameters of the XRD instrument were set in 40-kV voltage and 35-mA current. Scanning was carried out at 20 °-60 ° 2θ at a speed of 0.01o / s. Calcium carbonate was determined at the peak points of d (112) (Wei et al. 2015).

The morphology of calcium carbonate crystal was observed using a Scanning Electron Microscope (SEM-Carl-Zeiss®). Samples were mounted and coated with a gold-palladium structure. The scanning process was carried out at 25 kV voltage acceleration with 3000-4000 times magnification (Hammad et al. 2013).

Bacteria application for bio concrete

Concrete raw materials consist of a mixture of natural sand, coarse aggregate, cement, and water with a modified composition according to 20 MPa concrete calculations (Alkhaly 2016). Control concrete (C0) was made without bacteria cell application, whereas bio concrete was added 200 ml of 12 hours old bacteria culture with a density of 10^6 CFU/ml of the identified bacterial culture. The bacteria

culture was able to produce $CaCO_3$ crystals at pH 9. A compressive strength test was performed after the water curing process. The test of concrete compressive strength was carried out using a Compression Testing (MPa) machine, then compared with concrete compressive strength standard. The concrete compressive strength score was calculated using the following SNI-1726 (SNI 1726:2002, 2002) formula as described below:

$$fc' : \frac{F}{A} \quad (1)$$

Where:
f_c': Concrete compressive strength (MPa)
F: Load (N)
A: Cross-sectional area (mm^2)

RESULTS AND DISCUSSION

Isolates characteristics

Among the carbonatogenic bacteria isolated, only six isolates (JA1, JB2, JB3, JA4, AK4, and SU1) showed both $CaCO_3$ precipitating zone under bacteria colony and urease activity. Isolates JA1, JB2, JB3, and JA4 were isolated from limestone quarries, AK4 was isolated from cave stalactites and SU1 was isolated from karst. The $CaCO_3$ precipitation zone was formed as a result of urease activity which provides free carbon ion (CO_3^{2-}), thereby free calcium ions (Ca^{2+}) in CCP media can be bound and form $CaCO_3$ crystals. Generic assignment test showed that all isolates were rod-shaped, Gram-positive, endospore-forming, and urease positive. Among all isolates, only JA1 was a facultative anaerobe.

Identification of carbonatogenic bacterial strain

Six carbonatogenic isolates were identified using 16S rRNA sequences. All 16S rRNA carbonatogenic bacteria sequences were deposited in GenBank and compared against the 16S rRNA sequence database through the BLAST-N program. BLAST result showed that six carbonatogenic isolates belonged to three different genera, i.e., Bacillus (JA1, JB3, AK4, SU1), Lysinibacillus (JB2), and Sporosarcina (JA4). The phylogenetic tree of six new isolated $CaCO_3$ precipitating bacteria and another $CaCO_3$ precipitating bacteria is shown in Figure 1.

Based on BLAST results, isolate JA1 (MT197306) showed 99% similarity (100% coverage) with *Bacillus huizhouensis*, isolate JB2 (MT197307) showed 99% similarity (100% coverage) with *Lysinibacillus macroides*, isolate JB3 (MT197308) showed 99% similarity (100% coverage) with *B. galactosidilyticus*, isolate JA4 (MT197309) showed 98% similarity (100% coverage) with *Sporosarcina soli*, isolate AK4 (MT197310) showed 99% similarity with *B. niacini* and isolate SU1 (MT197311) showed 99% similarity with *B. lentus*. Genera *Bacillus*, *Lysinibacillus*, and *Sporosarcina* belonged to Firmicutes phylum have the characteristics as Gram-positive, rod shape, heterotroph metabolism, aerobic/ facultative anaerobic, and generate endospore.

$CaCO_3$ production and characterization

This experiment was conducted to measure the ability of carbonatogenic isolates to produce $CaCO_3$ quantitatively. All isolates can produce $CaCO_3$ above 14 mg/ml. Isolate *L. macroides* JB2 has the highest ability to precipitate $CaCO_3$ compared to other bacterial isolates with a crystal weight up to 16.2 mg/ml, while *B. niacini* AK4 is a bacterial isolate with the lowest ability to produce $CaCO_3$ with the weight of crystal of 14.0 mg/ml (Figure 2).

There are 3 types of $CaCO_3$ crystals precipitated through MICP namely calcite, aragonite, and vaterite. Resulted data from XRD were compared to Joint Committee for Powder Diffraction Standards (JCPDS) for the identification of crystal polymorphs. Results in this study showed two types of CaCO3 crystal morphology formed by isolates, namely calcite and vaterite (Figure 3). Calcite-shaped crystals showed the highest intensity at 29 ° 2θ, while vaterite at 27 ° 2θ and 32 ° 2θ.

As shown in Figure 4, *B. huizhouensis* JA1, *L. macroides* JB2, *S. soli* JA4 and *B. lentus* SU1 were able to form calcite, *B. niacini* AK4 was able to form both calcite and vaterite, whereas *B. galactosidilyticus* JB3 was only able to form vaterite. In the present study, *B. galactosidilyticus* JB3 was unable to form calcite as it is only showed the highest intensity on 32,83° and 27,1075° from 2θ degree.

Visual analysis by SEM was performed to confirm the crystal shape and morphology of the calcium carbonate crystals. Micrographs through SEM showed identical results from XRD analysis. All bacterial isolates were able to form calcite, except *B. galactosidilyticus* JB3 (Table 1). The calcite morphology was identified by the rhombohedral or rhombohedral aggregate, while vaterite was hexagonal or spherical form. Figure 4 depicts the morphology of calcite and vaterite produced by carbonatogenic bacteria.

Bacteria application for bio concrete

The concrete tube specimen was designed based on Talinusa et al. (2014) with 10 cm diameter and 20 cm height. Cylindrical concrete of 10 x 20 cm is used more often because it requires fewer materials and is lighter. Carbonatogenic bacteria cultures were mixed directly with other concrete ingredients since it can improve the spatial distribution of carbonatogenic bacteria cells (Xiao et al. 2020). Morphologically, control concrete and bio concrete with the bacterial application were similar. Bio concrete yields after the curing process are provided in Figure 5.

Table 1. The $CaCO_3$ crystal morphology and type produced by bacterial isolates

Isolate	$CaCO_3$ crystal morphology	Crystal type
B. huizhouensis JA1	Rhombohedral	Calcite
L. macroides JB2	Rhombohedral	Calcite
B. galactosidilyticus JB3	Hexagonal	Vaterite
S. soli JA4	Rhombohedral	Calcite
B. niacini AK4	Rhombohedral, hexagonal	Calcite, vaterite
B. lentus SU1	Rhombohedral	Calcite

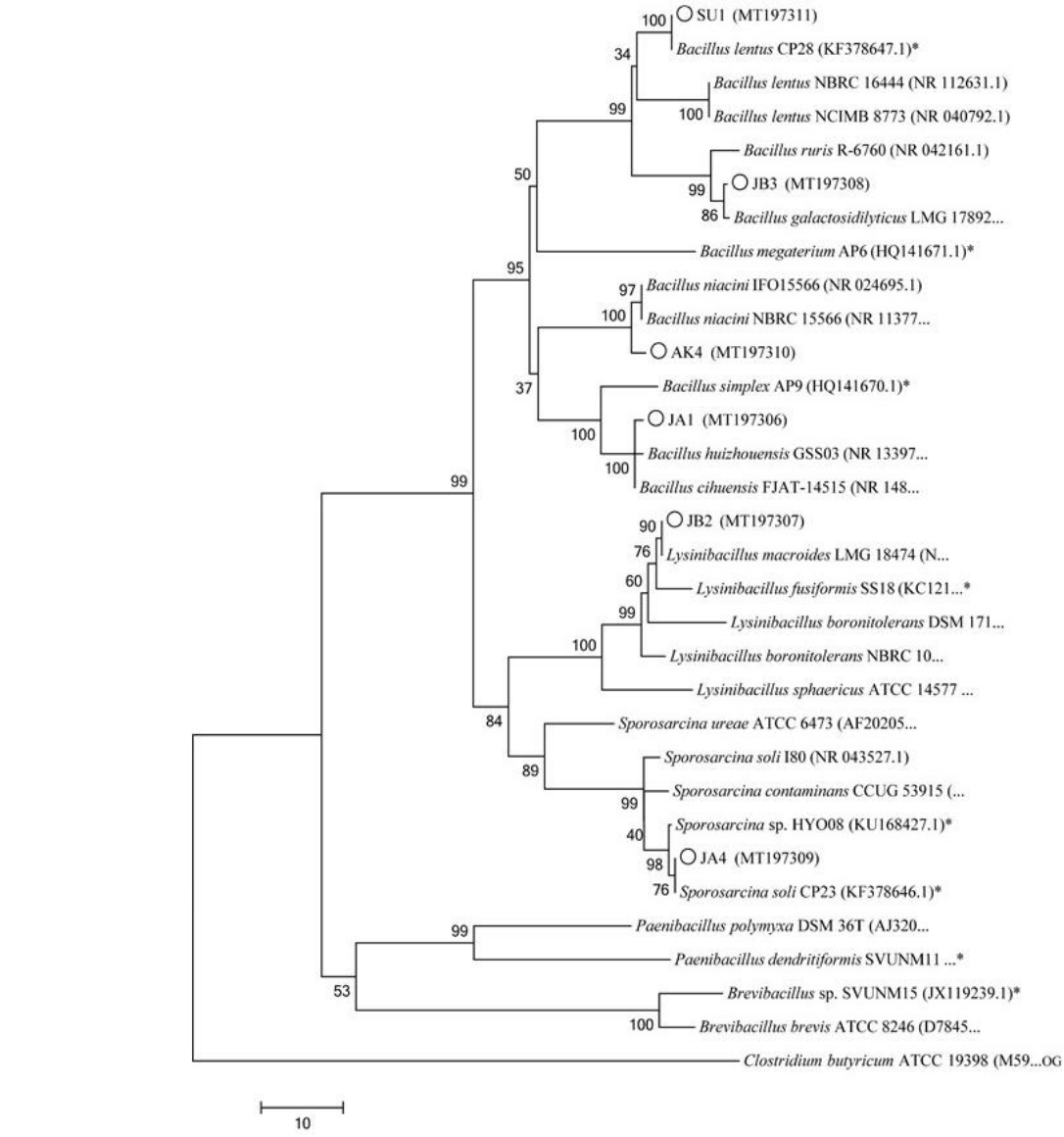


Figure 1. Phylogenetics tree of isolated carbonatogenic bacteria based on 16S rRNA gene sequences. *: Another bio cement/carbonatogenic bacteria from references. OG: Out Group. The scale shows the phylogenetic distance based on the number of DNA substitutions divided by the length of alignment sequences.

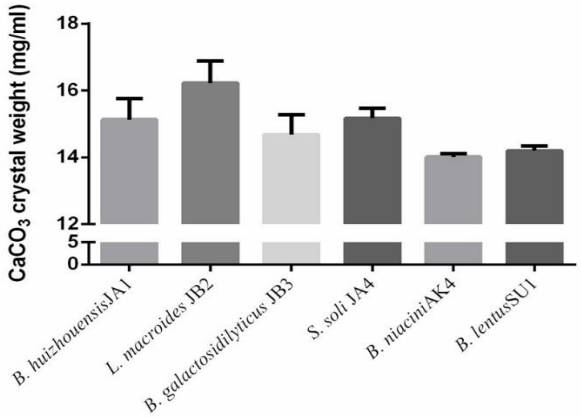


Figure 2. The weight of $CaCO_3$ crystals precipitated by carbonatogenic bacteria

The compressive strength and maximum load of bio concrete specimens vary depending on the bacterial species applied. The higher the compressive strength, the higher the maximum load. All bio concrete with bacteria application had higher compressive strength and maximum load compared to control concrete. The highest compressive strength and maximum load of bio concrete were found in bio concrete with the addition of *L. macroides* JB2 bacterial cells (Figure 6).

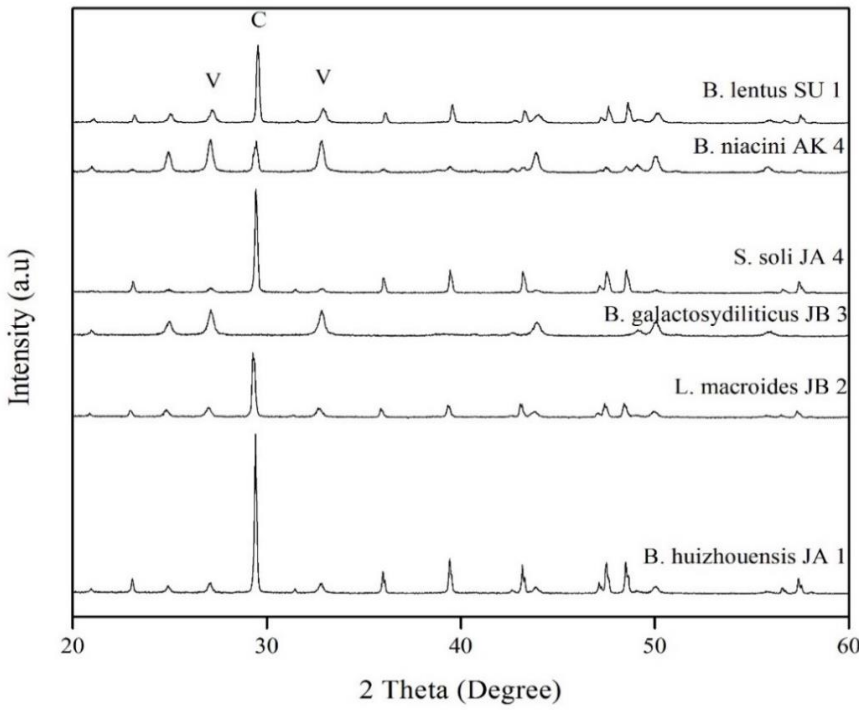


Figure 3. The result of XRD analysis for identified $CaCO_3$ crystal forms. (C stands for calcite peak, the highest peak occurs in 29°2θ, V: stands for vaterite peak, the highest peak occurs in 27°2θ and 32°2θ).

Statistical analysis using One-Way ANOVA showed a significant difference between control concrete and bio concrete (p-value <5%). These results indicate that the addition of carbonatogenic bacteria that produce $CaCO_3$ was able to improve the compressive strength and maximum load of bio concrete specimens. In addition, bio concrete specimens in this study have compressive strength and a maximum load that meets the requirements for building construction with a compressive strength of 17-40 MPa and a maximum load of 2200-2500kg/m3 (Alkhaly 2016). Therefore, bio concrete in this study can be applied for construction materials. The improvement in the compressive strength of the bio concrete is mainly due to the presence of $CaCO_3$ (calcite) crystals that fulfill the micropores in the bio concrete so that the pore size will be shrinkage and maximize the compressive strength of bio concrete.

Discussion

The use of microbes in construction technology is a new field. Many previous studies mentioned the ability of microbes to precipitate CaCO3, especially in the bacteria kingdom (Achal and Pan 2014). Several bacterial groups have particular metabolic pathways to produce carbonate ion (CO_3^{2-}) through photosynthesis (Dupraz et al. 2009), urinalysis (Fujita et al. 2000), denitrification (van Paassen et al. 2010), ammonification (Rodriguez-Navarro et al. 2003), sulfate reduction (Braissant et al. 2007) and methane oxidation (Reeburgh 2007). One of the most well-known metabolic pathways for generating carbonate ions is urea hydrolysis (Dhami et al. 2014; Stocks-Fischer et al. 1999; Wang et al. 2016). This study succeeded in isolating six carbonatogenic bacteria from calcareous areas namely, *B. huizhouensis* JA1, *L. boronitolerans* JB2, *B. galactosidilyticus* JB3, *S. soli* JA4, *B. niacini* AK4, and *B. lentus* SU1. These bacteria isolates can withstand an alkaline environment which is essential for the calcium carbonates precipitation (Zulaika et al. 2019).

In a previous study, *L. macroides* was isolated from soil contaminated with alkaline petroleum in China (Liu et al. 2017). *B. lentus* is a facultative alkaliphile bacterium that produces alkaline protease (Aono et al. 1996; Goddette et al. 1992). *B. niacini* has been isolated from alkaline soil and releases alkaline protease (Badoei-Dalfard et al. 2012). Of these carbonatogenic isolates, only *S. soli* has the potential to fill mortar pores with $CaCO_3$ (Kim et al. 2016). Based on these results, the isolates of alkaliphilic carbonatogenic bacteria have been obtained in previous studies, except *B. huizhouensis* and *B. galactosidilyticus* were isolated from an alkaline environment in this study.

Besides their ability to survive in an alkaline environment, several other properties of carbonatogenic bacteria in this study meet the criteria to be applied in bio concrete such as forming endospores, resistance to minimal oxygen conditions, thick peptidoglycan layer, and produce carbonate ions through urea hydrolysis (Ivanov et al. 2015; Sharma et al. 2019). Previous studies showed that the genera *Bacillus, Lysinibacillus,* and *Sporosarcina* were succeeded in increasing the strength of concrete (Chidara et al. 2014; Dhami et al. 2014).

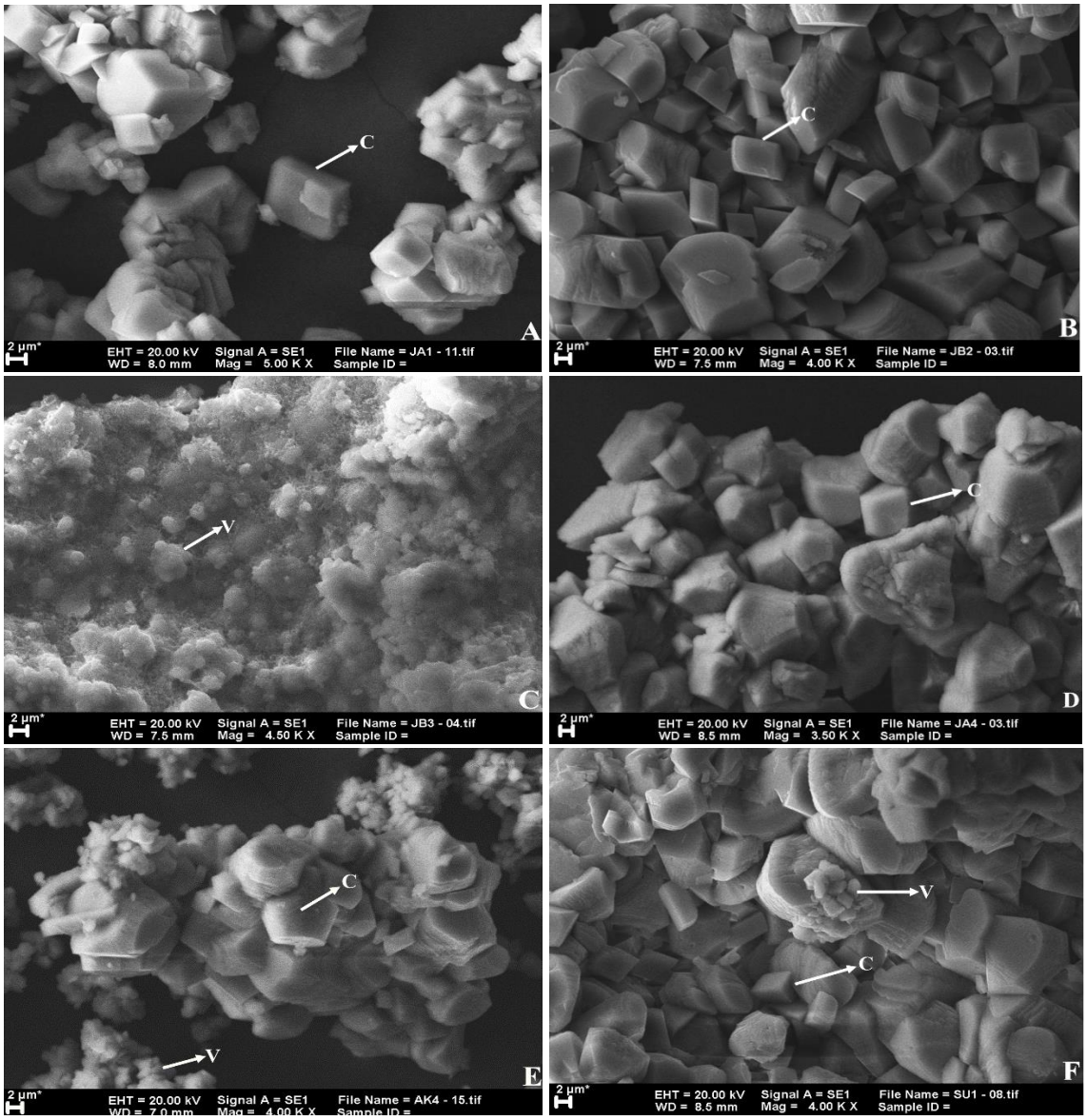
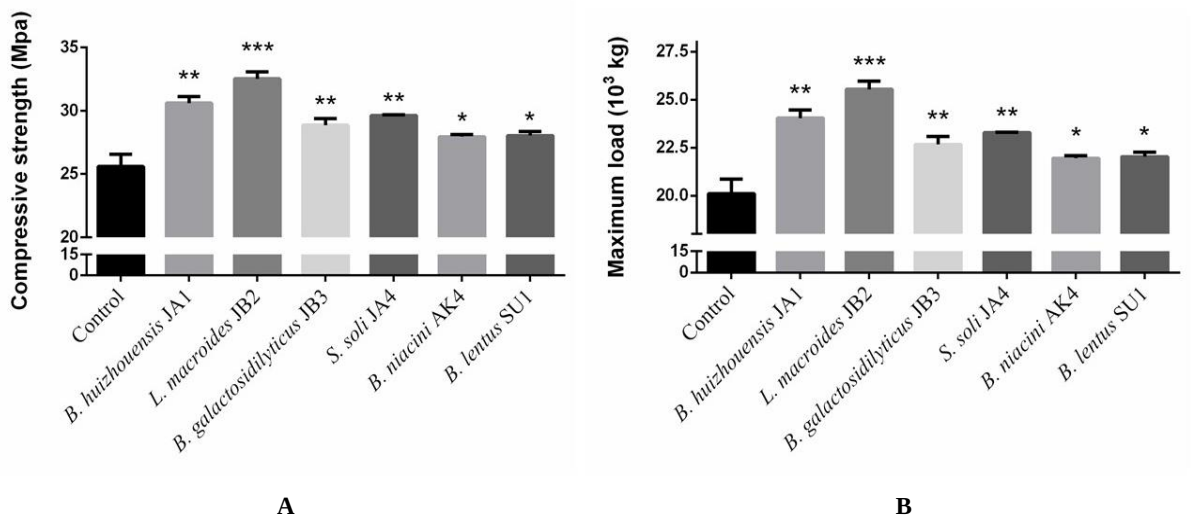


Figure 4. The shape of the CaCO3 crystal was revealed by the SEM micrograph. A. *B. huizhouensis* JA1 formed rhombohedral crystal; B. *L. macroides* JB2 formed rhombohedral crystal; C. *B. galactosidilyticus* JB3 formed hexagonal crystal; D. *S. soli* JA4 formed rhombohedral crystal; E. *B. niacini* AK4 formed rhombohedral and hexagonal crystal and F. *B. lentus* SU1 formed rhombohedral and hexagonal crystal (C stands for calcite, V stands for vaterite)

Figure 5. Morphology of concrete. A. Control concrete (without carbonatogenic bacterial application); B. Bio concrete with carbonatogenic bacterial application; C. Curing process of concrete specimens; D. Surface morphology of control concrete; E. Surface morphology of bio concrete with carbonatogenic bacteria application



A **B**

Figure 6. The compressive strength and maximum load of control concrete and bio concrete with the carbonatogenic bacterial application

Carbonatogenic bacteria use urea in CCP liquid media to produce carbonate and ammonium ions through urease activity. Carbonate ion is the material to produce calcium carbonates $CaCO_3$ while ammonium contributes to increasing the pH of CCP media. At the beginning of the incubation period, no crystal appears and the pH of CCP media being 6.8, however, after incubation crystals and powder-like structures appear at the bottom of CCP media, and the pH reached 11. At first, urease hydrolyses urea to ammonia and carbamate, then carbamate spontaneously transformed into ammonium ion (NH_4^+) and carbonate (CO_3^{2-}) ion (Mobley and Hausinger 1989). Since CCP media contained a high calcium concentration, these cations will strongly bond to the carboxyl, hydroxyl, and phosphate groups on the cell wall of Gram-positive bacteria as a site for crystal nucleation. Then, calcium ions accumulate in the bacterial cell wall and bind to carbonates ions to form calcium carbonates ($CaCO_3$) on the bacterial cell wall (Obst et al. 2009; Sarayu et al. 2014; Tourney and Ngwenya 2009). The ability to precipitate $CaCO_3$ in carbonatogenic bacteria varies which might be affected by the diversity of the ureC gene among bacteria isolates. The diversity of *ureC* affects hydrolysis rate and crystal formation in calcium carbonate precipitation bacteria (Goddette et al. 1992). Zulaika et al. (2019) has been successfully isolated and amplified the *ureC* gene from carbonatogenic bacteria and revealed that the amino acid sequences of the alpha-subunit urease of carbonatogenic bacterial isolates were different from each other.

Two forms of calcium carbonates crystal were found in this study, namely calcite and vaterite. *B. huizhouensis* JA1, *L. boronitolerans* JB2, *S. soli* JA4, *B. niacini* AK4, and *B. lentus* SU1 showed the ability to induce calcite precipitation, only *B. galactosidilyticus* JB3 was unable to precipitate calcite. The calcite and vaterite forms of calcium carbonate are the most common polymorphs induced by carbonatogenic bacteria through urea hydrolysis (Kaur et al. 2013). The results of studies from (Barabesi et al. 2007; Kim et al. 2015; Mitchell and Grant Ferris 2006; Wei et al. 2015), indicated that *Bacillus, Lysinibacillus* and *Sporosarcina* are predominantly precipitate calcite than vaterite. Aragonite was not observed in this study since its precipitation requires temperatures above 60◦C and sufficient magnesium (Mg^{2+}) ions (Trushina et al. 2014; Zhang et al. 2012). Besides, biochemical factors such as the amino acid content of urease play an important role in determining the crystal form of the precipitated calcium carbonate. A study from (Sondi and Salopek-Sondi 2005), suggested that a low concentration of Asp and Glu resulted in calcite predominant, while a high concentration of Asp and Glu will be predominated by vaterite form.

Based on Fig. 6, all concrete specimens with carbonatogenic bacteria application have higher compressive strength compared with control specimens. Concrete specimens with *L. boronitolerans* JB2 application showed the highest increase in compressive strength (27%), while the lowest increase in compressive strength is concrete specimens with the application of *B. niacini* AK4 (9,22 %). The results showed that the amount of precipitated calcium carbonate (Fig. 2) and the compressive strength (Fig. 6) were congruent where the higher the amount of calcium carbonate precipitation the higher the compressive strength. The improvement in the compressive strength of concrete was due to calcite and vaterite precipitated by bacterial cells that clog the concrete pores and reform the concrete microstructure (Balam et al. 2017). The results in this study showed that carbonatogenic bacteria isolated from the calcareous area in East Java have a promising potential to be applied as a healing agent for bio-concrete applications. Sustainable bio concrete application reduces carbon dioxide emission, inspection labour, and additional costs (Jonkers et al. 2010; Wang et al. 2012). To achieve sustainable bio concrete and improve the ability of carbonatogenic bacteria as healing agents, several prospective studies need to be carried out such as the selection of microcapsule/ microencapsulated methods,

binding agents to protect bacteria cells, nutritions, and real-time monitoring of bacterial activity inside the concrete.

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