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 CaCO3 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. CaCO3 crystal properties were characterized using X-Ray Diffraction and Scanning Electron Microscopy. Six novel isolated CaCO3 precipitating bacterial strains; Bacillus huizhouensis JA1; B. galactosidilyticus JB3; B. niacinii 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 CaCO3 in calcite form except B. galactosidilyticus JB3. The experimental concrete with the addition of bacterial cell 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, CaCO3, 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 (CaCO3) as the main ingredients. However, the process of making artificial cement remains an environmental issue. It contributes around 6% of global anthropogenic CO2 emissions and depleting the extensive limestone area (Achal and Mukherjee 2015). To maintain the quality of environments, it is needed environmentally friendly and renewable CaCO3 sources. Recently, microbial-induced calcite carbonate precipitation (MIPC) for concrete application is of great interest and attracted our attention since CaCO3 can be produced naturally through this process.

Microbial induced calcium carbonate precipitation (MIPC) 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 CaCO3 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 MIPC process through urea hydrolysis and increase the pH of the environment around bacteria cells. The hydrolysis process generates carbonates (CO32-) and ammonium (NH4+), thus environmental calcium ions (Ca2+) 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 Java 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’-AGAGTTTGATCCTGCTCAG-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).

CaCO_3 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 ° of 2θ at a speed of 0.010 / 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 (Hamad 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 10⁶ CFU/ml of the identified bacterial culture. The bacteria...
culture was able to produce CaCO₃ 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:

\[
f_{c'}^c = \frac{F}{A}
\]

Where:
- \( f_{c'}^c \): Concrete compressive strength (MPa)
- \( F \): Load (N)
- \( A \): Cross-sectional area (mm²)

### RESULTS AND DISCUSSION

#### Isolates characteristics

Among the carbonatogenic bacteria isolated, only six isolates (JA1, JB2, JB3, JA4, AK4, and SU1) showed both CaCO₃ 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 CaCO₃ precipitation zone was formed as a result of urease activity which provides free carbon ion (CO₃²⁻), thereby free calcium ions (Ca²⁺) in CCP media can be bound and form CaCO₃ 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 CaCO₃ precipitating bacteria and another CaCO₃ 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.

#### CaCO₃ production and characterization

This experiment was conducted to measure the ability of carbonatogenic isolates to produce CaCO₃ quantitatively. All isolates can produce CaCO₃ above 14 mg/ml. Isolate L. macroides JB2 has the highest ability to precipitate CaCO₃ 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 CaCO₃ with the weight of crystal of 14.0 mg/ml (Figure 2).

There are 3 types of CaCO₃ 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° 20, while vaterite at 27° 20. 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 20 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 CaCO₃ crystal morphology and type produced by bacterial isolates |
|-----------------------------|-----------------------------|-----------------------------|
| Isolate | CaCO₃ 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.

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 2.** The weight of CaCO₃ crystals precipitated by carbonatogenic bacteria.
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 meet the requirements for building construction with a compressive strength of 17-40 MPa and a maximum load of 2200-2500kg/m$^3$ (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 CaCO$_3$, 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 (Braisant et al. 2007) and methane oxidation (Reebugh 2007). One of the most well-known metabolic pathways for generating carbonate ions is urea hydrolysis (Dhari 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. niacinii 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. macrorides 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. niacinii 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; Dhali 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
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 carbonate 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 were 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, nutrients, and real-time monitoring of bacterial activity inside the concrete.

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