Isolation and Characterization of Bacterial Carbonate Precipitation for Biogrouting

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Abstract

Biogrouting is a process that transforms soil or sand into calcarenite or sandstone by bacterial calcium carbonate precipitation . The mechanisms of this process are that the bacteria hydrolyze urea catalyzed by urease, and with the existence of dissolved Ca^{2+}, a solid crystalline calcite or calcium carbonate ($CaCO_3$) will be produced. The main advantage of biogrout is that soil or sand can be strengthened without interfering with the hydraulics of the treated soil or sand and without excavation or replacement. In this study, the isolation and identification of bacterial calcium carbonate precipitation , and characterization of urease produced by bacteria were conducted. In the isolation method, the enrichment method using urea as abacterial carbon source was carried out. The formation of crystalline calcite was observed by a light microscope. The urease enzyme activity was determined by the 3, 5-Dinitrosalicylic acid (DNS) method. The molecular identification of isolates was analyzed by the determination of 16S rRNA gene sequencing. As a result, 19 calcium carbonate precipitation have been isolated from soils, sands, water, and rocks collected from several areas in Indonesia. They showed calcite formation in a medium with urea. Molecular identification of isolates with high urease enzyme activity revealed that the isolates belong to the group Bacilli. The highest enzyme activity produced by ID10-U004 is 374.94 U/mL. The preliminary biogrouting experiment was also conducted using isolated strain, and the reaction was able to make the sand solid and stiff.

Keywords: calcium carbonate precipitation, bacteria, urease, biogrouting, Bacilli

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Introduction

Precipitation of calcite or calcium carbonate ($CaCO_3$) by bacteria is a common natural phenomenon occurring in different environments, from hot springs, marine environments, caves, calcite rocks, soils, geological formations, freshwater biofilms, oceans, and saline lakes (Boquet *et al*., 1973; Dardau *et al*., 2021; Hammes *et al*., 2003a; Lange-Enyedi *et al*., 2022). Various types of polymorphic crystalline carbonate (calcite, aragonite, dolomite, and others) with the number, size, and type, depending on the type of bacteria and growth, could form from this natural phenomenon.

Carbonate precipitation by bacteria has paid the attention of scientists in recent years mainly due to its important role in marine systems as carbon sinks to boost the global production of CO_2 and mineralization of stone. Calcite resulting from carbonate precipitation, is a mineral widely distributed on earth and found in rocks as marble and sand stone in the waters or on land (Hammes, 2002).

In geology, biogrouting is a process that transforms soil or sand into calcarenite or sandstone by bacterial calcium carbonate precipitation developed with mechanisms based on the mediation of carbonate precipitation. Carbonate precipitation can theoretically occur in natural environments by

increasing the concentration of calcium and carbonate in solution or by lowering the solubility of calcium and/or carbonate. The carbonate precipitation bacteria can be used to accelerate the process *in situ*. Precipitation (deposition) of calcite is at least determined by 3 parameters: (1) the concentration of calcium, 2) the concentration of carbonate, and (3) the pH environment and the availability of nucleation sites (Hammes, 2002; Hammes *et al*., 2003b; Kumari *et al*., 2019). The mechanism of calcite precipitation by bacteria depends on urease enzyme activity. Enzymatic hydrolysis of urea presents a straightforward model for studying bacterial carbonate precipitation. In this reaction, urease (EC 3.5.1.5, urea amidohydrolase) will hydrolyze urea to become ammonia. One mol of urea is hydrolyzed intracellularly to 1 mol of ammonia and 1 mol of carbamate, which spontaneously hydrolyzes to form an additional 1 mol of ammonia and carbonic acid. These products subsequently equilibrate in water to form bicarbonate and 2 mol of ammonium and hydroxide ions. The latter gives rise to pH increase, which in turn can shift the bicarbonate equilibrium, resulting in the formation of carbonate ions, which in the presence of soluble calcium ions precipitate as $CaCO_3$ (Stocks-Fischer *et al*., 1999; Hammes *et al*., 2003b; Rodrigues-Navarro *et al*., 2003; Kumari *et al*., 2019).

Biogrouting is also called as biocementation. Ideally the source of microorganisms for biogrouting should be resistant or tolerant to high concentrations of urea and calcium. Urease-producing microorganisms can be classified into 2 groups based on the response to ammonium: (1) urease enzyme activity is suppressed by the presence of ammonium, for example, *Pseudomonas aeruginosa*, *Alcaligenes autrophus*, and *Bacillus megaterium* (Kaltwasser *et al*., 1972) and *Klebsiella aerogenes* (Friedrich & Magasanik, 1997) and (2) urease enzyme activity is not affected by ammonium, for example, *Sporosarcina pasteurii* (*Bacillus pasteurii*), *Proteus vulgaris*, *Helicobacter pylori*. In the carbonate precipitation reaction, the high concentration of urea will hydrolyzed by the group of bacteria that their enzyme activity is not suppressed by ammonium, which is suitable for use. At this time, genus *Sporosarcina* (*Bacillus*) has been applied to biogrouting process because it has a high urease activity and are not pathogenic (Mobley *et al*., 1995; Fujita *et al*., 2000).

Carbonate precipitation has value in technical and industrial applications for preserving and restoring sands or calcareous stone. In this study, the isolation and identification of bacterial calcium carbonate precipitation from Indonesian environments and characterization of urease produced by bacteria were conducted for further uses for the application of biogrouting.

Materials and Methods

Isolation and Purification. Sample were taken from soils, sands, marine water, and rocks in 3 areas in Indonesia. Soil and rock samples were ground before use. The sample was directly plated by serial dilution onto B4 medium with the composition 3 g/L nutrient broth, 20 g/L urea, 2.12 g/L $NaHCO_3$, 10 g/L NH_4Cl, 4.41 g/L $CaCl_2.2H_2O$, and 15 g/L agar if needed. The plates were incubated at room temperature for 5 days. Colonies of bacteria were picked up and purified using four-way streak method to obtain pure bacterial isolates (Cappuccino & Sherman, 2005).

Observation Crystal Using a Light Microscope. Bacterial colonies with crystalline-forming in the medium were observed after 5 and 10 days of cultivation with a light microscope, Olympus CX41. Photographs were made at the time of observation.

Screening of Urease-producing Bacteria. The screening was carried out by growing the isolates in the urea broth medium added with phenol red as pH indicator for qualitative urease test using the method of Hammes *et al.* (2003b). The reaction was incubated at 30ºC for 3 days. Bacterial isolates which urease activity will raise the pH of the medium to alkaline environment and changes the color of the medium from yellow (negative) to fuchsia (positive).

Urease Activity. The quantitative test of urease activity conducted in the following method: isolates were grown in a production medium with the composition of 20 g/L yeast extract, 10 g/L NH_4Cl, and 10 µM of $NiCl_2$ in

1 L distilled water, incubated on shaker incubator at 30ºC for 120 hours. Urease activity was measured using the method of Weatherburn (1967) with modification as follows, Natrium hypochlorite was used in an alkaline solution instead of NaOH. The time of color formation was changed from 20 minutes to 30 minutes. Reactions were carried out in test tubes containing 100 µL of sample, 500 µL of 50 mM urea, and 500 µL of 100 mM KH_2PO_4 buffer (pH 8.0) so that the total volume was 1.1 mL. The reaction mixture was incubated in a water bath with at 37ºC for 30 minutes. This reaction was stopped by transferring 50 µL of the reaction mixture into tubes containing 500 µL solution of phenol-sodium nitroprusside. An alkaline hypochlorite solution 500 µL was added to the tube and incubated at room temperature for 30 minutes. Then the solution was measured the optical density (OD) with a spectrophotometer (Shimadzu UV 160) at a wavelength of 630 nm and compared with a standard curve $(NH_4)_2SO_4$. One unit of enzyme activity is the amount of enzyme required to liberate 1 µmol NH_3 from urea per minute under standard assay.

DNA Extraction. Two-day-old suspension of bacterial colonies was added to a microcentrifuge tube then centrifuged at 13.000 rpm for 1 min. The supernatant were decanted and the pellets were resuspended. The total DNA of the bacteria was extracted using a Genomic DNA mini kit (Geneaid Biotech Ltd.). The DNA was then stored at -20ºC.

Amplification of 16S rRNA Gene and DNA Sequencing. One µL of DNA template was mixed with 49 µL PCR solution containing 20 µL of ddH_2O, 25 µL of buffer ready (PCR) mix (KAPA), 2 µL of 20 pmol primer 9F, 2 µL of 20 pmol primer 1541R in total final reaction volume of 50 µL. Amplification of 16S rRNA genes was carried out using the universal primer 9F (5'-GAGTTTGATCCTGGCTCAG-3') and primer 1541R (5'-AAGGAGGTGATCCAGCC-3'). The PCR (Applied Biosystems, USA) reaction conditions were the initial denaturation step at 96ºC for 5 min, followed by 30 cycles of 96ºC for 30 s, 55ºC for 30 s, 72ºC for 1 min, and the final extension step at 72ºC for 7 min (Lisdiyanti *et al*., 2012). The PCR product was purified using a Gel/PCR or DNA fragments extraction kit (Geneaid Biotech Ltd.). Sequencing of 16S rRNA gene was analyzed by sending the PCR product to Geneaid Biotech Ltd. services in Singapore. DNA sequence information from the sequence database was compared to track homology with known strains in GeneBank/DDBJ/EMBL based on BLAST (Altschul *et al.,* 1997).

Preliminary Test of Biogrouting in Sand. To test the feasibility of bacterial-induced carbonate precipitation, a small-scale tray experiment (12×8×12.5 cm^3) was conducted. A bacterial suspension was grown to late exponential phase to a final optical density of 2,880 (OD_{600}). A 270 mL of diluted bacterial suspension was injected into the sand core and then immediately followed by injection of 270 mL of fixation fluid contains 50 mM $CaCl_2$. The cementation fluid contains 1 M $CaCl_2$ and urea was flushed through the tray. After 2 h of reaction time, the cementation fluid was flushed again through the tray. The fluid in the tray was then allowed to react for 24 h. The next day, the tray was flushed with cementation fluid again (Harkes *et al.,* 2009). The experiment was observed visually.

Results

Isolation and Screening of Bacterial Calcium Carbonate Precipitation.

Various natural habitats samples, including soil, sand, water, and rock were used to isolate bacterial calcium carbonate precipitation. As described in research the samples have been crushed, serially diluted, and directly grown in isolation medium. Then, the bacterial colonies was observed under the microscope. If the observed colony produced crystal, a single colony was picked up, grown in isolation medium, and purified to obtain a pure culture. As a result, a total of 146 isolates are capable crystalline-forming in the medium (Figure 1).

Screening of bacterial biogrouting was conducted to determine the ability of bacterial isolates to produce urease enzyme. This screening was conducted by growing the 146 isolates in a urease test liquid medium. Screening results for urease qualitative with urea broth showed that 19 isolates showed positive reaction on the urease test. The urease test was used for the selection of the bacteria

producing urease enzyme. The hydrolysis of urea by the enzyme urease produces ammonia and carbon dioxide, which increases the pH of the medium and causes the phenol red indicator in medium become fuchsia. This result showed that only 13% of total isolates that could grow on urea medium (B4) expressed urease activity. The bacteria positive of urease test isolated from Papua are ID10-U001, ID10-U002, ID10-U003, and ID10-U004; from Yogyakarta are ID10-U005 until ID10-U014, and from Sulawesi are ID10-U015 until ID10-U019 (Tables 1, 2, 3). Most of isolates have bacilli shape and Gram-positive, and ID10-U001 has coccus shape.

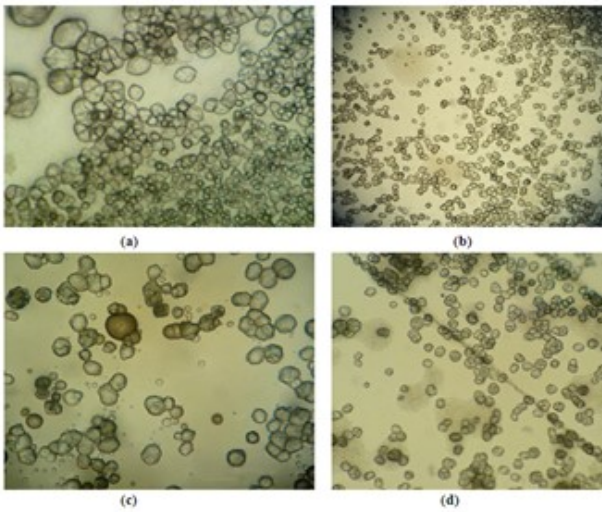

Figure 1. Morphological differences in calcite crystal within bacterial colonies of bacterial carbonate precipitation grown on semisolid medium. The types of crystal, a) spherulite type with fibrous surface texture (ID110-U005), b) rhombohedral type (ID10-U004), c) spherical vaterite type (ID10-U017), and d) trianguler type (ID10-U013) (magnitude, 20×).

Table 1. The number of bacteria with the ability crystalline-forming isolated from Papua

Year	Location	Sample	∑ bacteria
2010	Grasberg	Soil	0
	Grasberg	Soil	0
	Grasberg	Soil	46
	Grasberg	Sands	0
	Grasberg	Sands	19
	Grasberg	Soil	14
	Grasberg	Soil	0
	Total		**79**

Urease Enzyme Activity.

After that, the 19 positive urease test were measured their urease enzyme activity using the method of Weatherburn (1967) with modification. Determination of urease enzyme activity of positive urease test indicated that the isolates had the ability of urease activity (Figure 2). Isolate ID10-U004 has the highest urease activity (374.94 U/mL) (Figure 2). As information, reference strain for biogrouting *Sporosarcina pasteurii* DSMZ 33^T has the urease activity 294.77 U/mL.

Table 2. The number of bacteria with the ability crystalline-forming isolated from Yogyakarta.

Year	Location	Sample	∑ bacteria
	Selarong cave	Rock	0
	Selarong cave	Rock	2
	Parangtritis coast	Sand	1
2010	Parangtritis coast	Sand	10
	Parangtritis coast	Water	9
	Parangtritis coast	Rock	9
	Parangtritis coast	Rock	8
	Total		**39**

Table 3. The number of bacteria with ability crystalline-forming isolated from Southeast Sulawesi.

Year	Location	Sample	∑ bacteria
2010	Batu cave, BNP	Water	0
	Batu cave, BNP	Rock	0
	Batu cave, BNP	Soil	0
	Mimpi cave, BNP	Water	0
	Mimpi cave, BNP	Rock	0
	Mimpi cave, BNP	Soil	0
	Mimpi cave, BNP	Water	0
	Mimpi cave, BNP	Rock	0
	Mimpi cave, BNP	Soil	8
	BNP	Water	0
	Pangkap	Water	0
	Rotterdam castle	Rock	0
	Rotterdam castle	Soil	0
	Lae-Lae island coast	Water	0
	Lae-Lae island coast	Soil	0
	Samalona island coast	Water	14
	Samalona island coast	Soil	0
	Samalona island coast	Rock	0
	Samalona island coast	Soil	0
	Samalona island coast	Rock	6
	Total		**28**

Ammonium was produced variously for each of isolates depending on isolates (Figure

3). Generally, there is a correlation between the enzyme activity and ammonium concentration (Figure 3). In addition, four isolates from Papua area have higher urease enzyme activity than isolates from another areas. The data of urease activity in terms of U/mg protein have not shown.

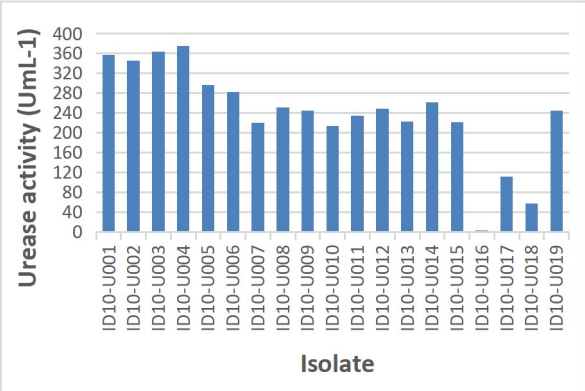

Figure 2. Urease enzyme activity of isolates

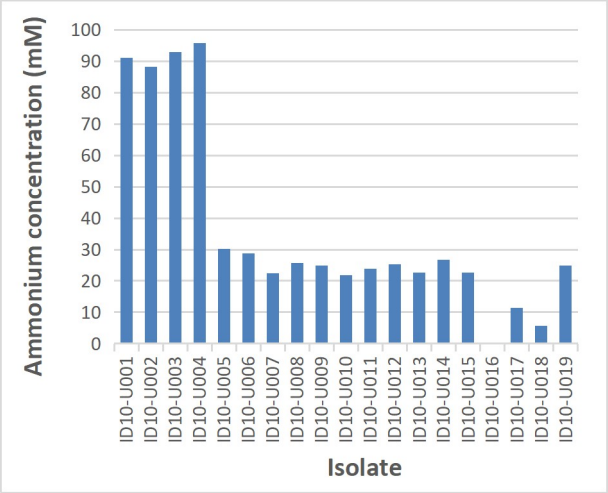

Figure 3. Ammonium concentration of isolates

Molecular Identification of Bacteria.

Almost 1,500 base pairs long of 16S rRNA gene was amplified, purified, and sequenced. All of the 19 bacterial carbonate precipitation were dominated by *Bacillus* genera (8 isolates = 42.1%). Generally, the bacterial carbonate precipitatioon is characterized as an alkalophile bacteria (pH 7-9), bacilli shape, Gram-positive (Lee, 2003). One isolate was identified into the genus *Staphylococcus*, 4 isolates into the genus *Oceanobacillus*, 5 isolates into the genus *Sporosarcina*, and 1 isolate into the genus *Schineria* (Table 4).

Preliminary Test of Biogrouting in Sand.

A small-scale biogrouting experiment for feasibility the capability of the isolate for sand reinforcement have been done. ID10-U004 isolate was used in the experiment through biocementation process. After incubation times for 1 week, the sand particles became compact, strong, and stiff (Figure 4). During reaction, as shown in Figure 5 that the pH was increased for 14-day incubation to near pH 9.

Table 4. Identification of bacterial carbonate precipitation based on 16S rRNA sequencing

Isolat ID	BLAST Similarity with Bacterial strain	Homology (%)
ID10-U001	*Staphylococcus haemolyticus* strain LEH2_2A	98%
ID10-U002	*Oceanobacillus profundus* strain CL-MP28	98%
ID10-U003	*Oceanobacillus* sp. BSi20641	92%
ID10-U004	*Oceanobacillus* sp. R-27401	95%
ID10-U005	*Bacillus pichinotyi* strain RS2	97%
ID10-U006	*Bacillus* sp. strain WCC 4585	96%
ID10-U007	*Bacillus* sp. WB7	98%
ID10-U008	*Sporosarcina* sp. 106	96%
ID10-U009	*Sporosarcina pasteurii* NCCB 48021	97%
ID10-U010	*Schineria* sp. CHNDP40	97%
ID10-U011	*Sporosarcina luteola*	99%
ID10-U012	*Bacillus* sp. VS1	90%
ID10-U013	*Bacillus sp. WB7*	92%
ID10-U014	*Sporosarcina pasteurii* NCCB 4802	98%
ID10-U015	*Sporosarcina soli* strain I80	95%
ID10-U016	*Bacillus* sp. KSM-P358	94%
ID10-U017	*Bacillus lentus* strain UR41	96%
ID10-U018	*Oceanobacillus chironomi* strain T3944D	89%
ID10-U019	*Bacillus lentus* strain NCIMB8773	98%

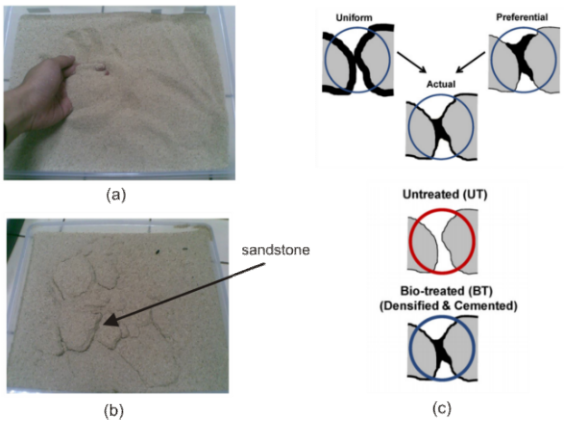

Figure 4. The small scale of biogrouting experiment using ID10-U004 isolate in sands from Pari Island, Indonesia; (a) control negative without adding bacteria and cementation fluid, (b) sand was injected by bacteria and cementation fluid and calcarenite stone resulted by bacteria induce carbonate precipitation activity; and (c) illustration of calcite distribution in biocementation (pick up from DeJong *et al*., 2010).

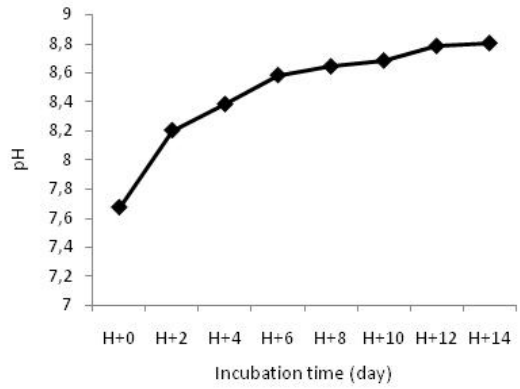

Figure 5. Profile of pH in biogrouting experiment for 14 days.

Discussion

Bacterial-induced carbonate precipitation can be used for several industrial applications, such as restoration of calcareous stone materials (Tiano *et al*., 1999; Castanier *et al*., 2000; Rodriguez-Navarro *et al*., 2003; Joshi *et al*., 2021), bioremediation (Fujita *et al*., 2000; Warren *et al*., 2001; Ivanov *et al*., 2019), wastewater treatment (Hammes *et al*., 2003b), and strengthening of concrete (Ramachandran *et al*., 2001). The advantage of isolation bacterial-induced carbonate precipitation from several areas in Indonesia was to collect isolates producing novel urease activity. Urease produced from these isolates can be used for many purposes.

This study has shown that bacterial-induced carbonate precipitation could be isolated not only from soils, but also from sands, waters, and rocks. We applied urea in isolation medium as a carbon source. When bacteria utilize urea, ammonia is formed during incubation that makes the reaction of these medium alkaline, producing a fuchsia color due to the presence of phenol red, a pH indicator. Aono *et al*. (1999) reported that certain structural component of the cell wall of some alkalophiles, such as teichuronopeptide, may contribute to pH homeostatis at alkaline pH and aid bacteria to survive in alkaline environment. Further, in B4 medium agar, isolates were able to form crystals that is produced by urease enzyme activity (Figure 1).

The urease activity can be measured by phenol hypochlorite. The blue color is a positive reaction of indophenol obtained at high pH of ammonia, phenol and hypocholorite. All 19 isolates produced a significant amount of urease. ID10-U016 isolate has lower urease activity (2.45 U/mL) than others. Bacteria hydrolyze urea by urease for (1) increasing the ambient pH (Burne & Marquis, 2000), (2) utilizing it as a nitrogen source (Burne & Chen, 2001), and (3) using it as a source of energy. Kantzas *et al.* (1992) reported that urease from *Bacillus pasteurii* (*S. pasteurii*) detected in the culture medium was extracelluler and offered an option of using the urease rather than the whole cell to consolidate sand with $CaCO_3$. The subsequent increase of pH in the surrounding medium due to the presence of ammonia ions and the additional release of CO_2 from the enzymatic urea hydrolysis further accelerate the rate of the urease-induced carbonate precipitation.

According to the results, ID10-U004 isolate have high urease activity that is 374.94 U/mL. We have not measured the activity every day, but we harvest the urease enzyme on maximum activity (120 hours) then it measured. Achal and Pan (2011) reported that the maximum urease enzyme activity was shown during the initial phase of bacterial growth and decreased when cells showed maximum growth, followed by slight increase in its activity when growth further declined. They used three isolates that are *Bacillus* sp. AP4, *B. megaterium* AP6 and *B. simplex* AP9, which showed maximum urease productivity at 120 hours for 534 U/mL, 553 U/mL, and 493 U/mL, respectively. The urease activity of ID10-U004 isolate was 294.77 U/mL at 120 h.

The present study showed a possibility that bacteria-induced carbonate precipitation isolated in this study can be exploited as a biologically induced mineralization. Bacteria-induced precipitation of calcium carbonate have been established tool for the *in situ* restoration of building and monuments (Castanier *et al.,* 1999; Stocks-Fisher *et al.,* 1999). Techniques to change soil properties by stimulating natural biochemical processes *in situ* are developed by many researchers (Whiffin *et al*., 2005; van Meurs *et al*., 2006; Ivanov & Chu, 2008). In this preliminary study, we used one isolate that is ID10-U004, which showed high urease enzyme activity as a model of biogrouting (biocementation) in the small scale level using a tray for the biogrouting experiment (Figure 4).

Visually, the results indicated that the surface of sand injected by bacteria and cementation fluid (1 M $CaCl_2$ and urea)

became more compact, strong, and stiff compared with the control negative (Figure 4). Calcite material from precipitate of $CaCO_3$ showed in white colour. However, the mechanical soil analysis of the study is needed. Putri *et al.* (2019) reported that by applying urease enzyme in sand, there is a rise in the cohesion value of the sand due to the addition of the clay mixture. A'la *et al*. (2020) also reported that the increase in shear strength of soil due to the addition of clay and the biocementation process of the urease enzyme.

Bacteria-induced carbonate precipitation are able to improve the mechanical properties of porous materials as reported by Nemati and Voordouw (2003) and DeJong *et al* (2006). In most studies, calcium carbonate precipitation was induced by the hydrolysis of urea in a solution with calcium chloride. Purified urease enzymes or whole bacterial cells containing the enzyme in high concentrations, were used to catalyze the hydrolysis of urea and produce ammonium and carbonate ions as stated in Reaction 1.

$$CO(NH_2)_2 + 2H_2O \rightarrow 2\ NH_4^+ + CO_3^{2-} \quad (1)$$

In the presence of dissolved calcium ions, the produced carbonate ions will precipitate and form calcium carbonate ions (Reaction 2).

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3\downarrow \quad (2)$$

When these crystals form bridges between the existing sand grains, they prevent movement of the grains and improve the materials's strength and stiffness of the properties (Ramakrishnan *et al.*, 2000; Lee, 2003; Whiffin, 2004; van Paassen, 2009).

In this study, the biocementation reaction showed that the pH was increased for 14-day incubation to near pH 9. The pH had a significant biochemical effect on urease activity, which exhibited an optimum activity on pH between 7 and 9. One of the roles of urease plays for *Sporosarcina pasteurii* (reference isolat) is to increase the external pH to 9.25 thus creating an environment conductive to growth (Wiley & Stokes, 1963).

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