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Selection of bacteria inducing calcium carbonate precipitation for self-healing concrete application

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ABSTRACT

A modification of bacterial medium using calcium lactate pentahydrate was developed for calcium carbonate precipitation. A total of five strains of bacteria were cultivated on the solution medium containing nutrient broth and calcium lactate pentahydrate. In this study, the variation of 3.2 mM and 16.2 mM of calcium lactate pentahydrate was used to obtain the optimum condition for bacterial growth. The results showed that isolated strains CPB 1, CPB 3, and CPB 5 with medium containing nutrient broth and 3.2 mM calcium lactate pentahydrate gave the optimum growth, pH and Eh, thus being favourable for the process of calcium carbonate precipitation. Hence, this will be useful for self-healing concrete.

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1. Introduction

Bacterially produced calcium carbonate precipitation has been widely observed for many practices, such as heavy metal and radionuclide removal (Fujita et al., 2000; Warren et al., 2001); clogging and grouting of soil in situ (Ivanov and Chu, 2008; van Paasen et al., 2010); self-healing agent and improvement of the material characteristics (Zhang et al., 2016; Achal et al., 2013; Jonkers et al., 2010). Urease activity has emerged to be the most desired mechanism for precipitating calcium carbonate owing to the controllable mechanism for producing a high amount of calcium carbonate in a short time (Dhami et al., 2013). Urea hydrolysis is the onset of this mechanism followed by calcium carbonate precipitation from the Ca2+ source addition (Dhami et al., 2013; Mujah et al., 2016). The urea hydrolysis stage is derived as follows:

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

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

$$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^- \quad (3)$$

$$H_2CO_3 \leftrightarrow 2H^+ + 2CO_3^{2-} \quad (4)$$

Ammonia equilibrates in water to form ammonium and hydroxide ions that contribute to the increase of pH, while carbonic acid is in equilibrium reaction that tends to form carbonate ion. The Ca^{2+} source will generate calcium carbonate precipitation due to its solubility with CO_3^{2-}.

$$Ca^{2+} + CO_3^{2-} \leftrightarrow CaCO_3 \quad (5)$$

Despite high-yield calcium carbonate generated by urease activity, this mechanism has a negative impact on human health and environment due to the excessive ammonia production as a by-product (Yu et al., 2015). Jonkers et al. (2010) reported a novel method of calcium carbonate precipitation, by utilizing alkaliphilic strains bacteria such as *Bacillus cohnii* and *Bacillus pseudofirmus*. These bacterial strains were able to degrade organic compounds into dissolved carbon CO_2, which could be easily converted to CO_3^{2-} at high pH environment to form $CaCO_3$, as long as Ca^{2+} source available (De Belie et al., 2017). Calcium lactate was used as organic carbon and calcium source. This method can be utilized as an alternative mechanism for the urease activity system. The metabolism of aerobic alkaliphilic

bacteria spore can generate calcium carbonate by oxidation process according to the reaction (Jonkers et al., 2010):

$$CaC_6H_{10}O_6 + 6O_2 \rightarrow CaCO_3 + 5CO_2 + 5H_2O \quad (6)$$

This becomes a highly promising method for strengthening and self-healing concrete (Jonkers et al., 2010; Khaliq and Ehsan, 2016). Jonkers et al. (2010) described the mechanism with an alkaline medium containing per litre (diluted with MilliQ ultra-pure water): 0.2 g NH_4Cl, 0.02 g KH_2PO_4, 0.225 g $CaCl_2$, 0.2 g KCl, 0.2 g $MgCl_2$•$6H_2O$, 0.01 g $MnSO_4$•$2H_2O$, 1 ml trace element solution SL12B, 0.1 g yeast extract, 5.16 g citric acid trisodium salt, 4.2 g $NaHCO_3$ and 5.3 g Na_2CO_3, which produced 20–80 μm-sized mineral-like precipitates on crack surfaces of young cement stone specimens cured for 7 days. Khaliq and Ehsan (2016) found that the maximum healing of cracks on cement mortar with graphite nanoplatelets (GNP) as a carrier compound was 0.81 mm after 28 days. Most of the authors utilized complex and expensive media for calcium carbonate production. Therefore, this study investigated affordable materials as a source of the medium, i.e., nutrient broth and calcium lactate pentahydrate. This composition was used for selecting the bacteria of producing calcium carbonate. Elevated concentrations of calcium lactate were tested at 3.2 mM and 16.2 mM. These concentrations were used for investigating the optimum composition of calcium lactate pentahydrate that was suitable for bacterial growth. Parameters observed were the optical density (OD_{600}) of the bacterial growth, pH, and Eh (redox potential). The optimal condition from both medium and bacteria will be a candidate for calcium carbonate precipitation system, especially for biocementation process.

2. Materials and Methods

2.1. Microorganisms

Five bacterial strains were isolated and purified from crude oil pipeline, designated as CPB 1 to 5. Growth medium containing 1.5 g/L nutrient broth (Oxoid) and 1 g/L (3.2 mM) calcium lactate pentahydrate ($CaC_6H_{10}O_6.5H_2O$) was sterilized by autoclave (121°C; 15 min), and subsequently inoculated by 10% v/v bacterial inoculum of each strain separately, which was used as a stock culture. They were incubated on orbital shaker (rotation speed of 180 rpm) at room temperature (28°C) for 3 days.

2.2. Bacterial growth on calcium lactate as a substrate

The medium for bacterial selection was identical to that for cultivation, which was supplemented with elevated concentrations of calcium lactate pentahydrate (3.2 mM and 16.2 mM). Each of the bacterial stock cultures (10% v/v) was inoculated into Erlenmeyer flask containing the sterilized medium. The procedure of this experiment was identical to section 2.1. Samples were withdrawn periodically for analyses, such as optical density for bacterial growth at 600 nm wavelength using UV-Vis spectrophotometer (Genesys 10.5). All experiments were performed in duplicate. The rate of bacterial growth was calculated as follows (Powell, 1956; Maier, 2009):

$$\frac{dx}{dt} = \mu x \quad (7)$$

The reaction above (Equation 7) can be rearranged to:

$$\mu = \frac{\ln x_1 - \ln x_0}{t_1 - t_0} \quad (8)$$

Where μ: the rate of bacterial growth; x_0: optical density of bacterium at initial log phase (t_0); x_1: optical density of bacterium at log phase at a certain time (t_1); t: time.

The pH and redox potential (Eh) of each bacterial solution was measured by using pH meter (Lutron pH-201) and ORP meter (Lutron ORP-203), respectively. Eh was recalculated by using standard hydrogen electrode (SHE) as follows (Inzelt et al., 2013):

$$\text{Eh vs. SHE (mV)} = \text{Eh} + 197 \quad (9)$$

2.3. Identification of isolates based on 16S rRNA

Each bacterial isolate was streaked separately on Luria-Bertani agar plates and incubated for 48 hours at room temperature (28°C). Amplification, cloning, and sequencing of 16S rRNA genes were conducted by 1st BASE Pte. Ltd., Singapore via PT. Genetika Science Indonesia.

2.4. Generation of bioconcrete and self-healing observation

Bioconcrete specimens were prepared to investigate the crack remediation, commonly referred to as self-healing concrete, due to calcium carbonate precipitation from bacterial metabolism. Ordinary Portland cement (Type 1, SNI 15-2049-2004) was mixed with water in a water-cement ratio of 0.5 by weight. The inoculum (10% v/v) of the selected bacteria was poured as water replacement and calcium lactate pentahydrate (0.5% of cement weight) was added. The paste was cast in 45 mm diameter of PVC pipe mould and sealed with a plastic wrap for 24 hours. Then, the moulds were removed and the samples were immersed in water for 7 days. Subsequently, the samples were cut by cutting machine to make approximately 22.5 mm in length and were compressed by a computer-controlled, servo-hydraulic concrete compression test machine (HT-8391, Hung Ta, China) to create the artificial crack. The samples were observed and immersed in the water for 14 days. Finally, the samples were withdrawn from the water, dried up, and observed.

3. Results and Discussion

3.1. Bacterial growth

Bacterial growth based on optical density measurement can be observed in Figure 1. Strain CPB 1, CPB 2, CPB 3, and CPB 5 had the similar growth behaviour for both elevated concentrations of the medium (3.2 mM and 16.2 mM), except for CPB 4 which showed the lowest turbidity and sluggishly increasing turbidity. This suggested that these strains were adapting well with the medium containing calcium carbonate and organic matter mixture, or even utilizing them as a nutrient source for the growth, while

strain CPB 4 was inhibited by the presence of these components.

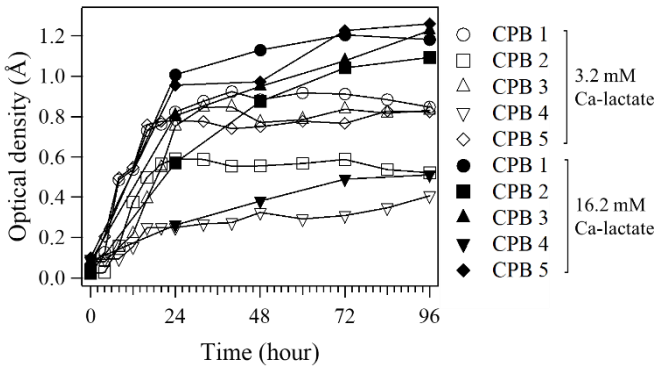

Fig. 1. Bacterial growth in nutrient broth medium supplemented with 3.2 mM and 16.2 mM calcium lactate pentahydrate.

It was shown that turbidity of strains CPB 1, CPB 2, CPB 3, and CPB 5 supplemented with 16.2 mM calcium lactate pentahydrate was higher than with 3.2 mM, indicating that calcium lactate acted as a nutrient source. Jonkers et al. (2010) and Zhang et al. (2017) reported that calcium lactate included as one of the most favourable carbon sources for aerobic bacteria. Despite the higher turbidity given by the higher calcium lactate concentration, the growth rates on the logarithmic phase were lower on average, as shown in Table 1.

Table 1. Growth rate of bacteria with 3.2 mM and 16.2 mM calcium lactate pentahydrate on the medium.

Bacteria	Growth rate; μ (Å/hour)	
	3.2 mM	16.2 mM
CPB 1	0.0602	0.0395
CPB 2	0.141	0.0343
CPB 3	0.0661	0.0273
CPB 4	0.0101	0.0178
CPB 5	0.0921	0.0222

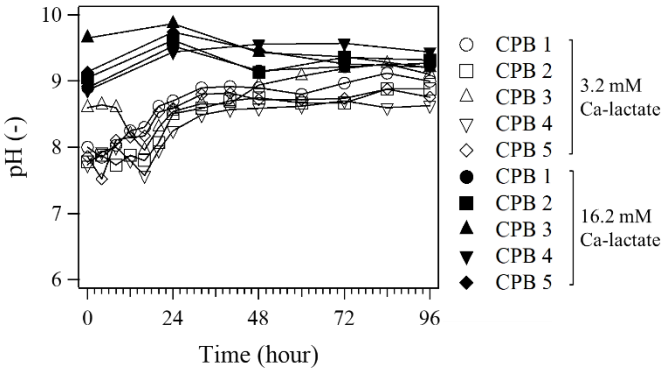

Fig. 2. Changes of pH in nutrient broth medium supplemented with 3.2 mM and 16.2 mM calcium lactate pentahydrate.

Elevating the calcium lactate concentration resulted in the longer lag phase of bacterial growth, which was similar to a previous study reported by Thingstad and Langeland (1974). This phenomenon was also supported by Edwards (1970), describing that high concentration of calcium source might inhibit the bacterial growth. Therefore, calcium lactate was proven to be a limiting factor for bacterial growth (Monod, 1949). As shown in Figure 2, pH values of all strains for both concentrations of calcium lactate pentahydrate concentrations were in the range of 7.8-9.6. The pH value in the first 24 hours by the addition of 3.2 mM was lower than 16.2 mM. This corresponded to the higher generation of calcium carbonate in the addition of 16.2 mM, resulting in higher pH values. Up to 96 hours, the pH values of all strains were similar.

Figure 3 shows the dynamics of redox potential (Eh). The resultant Eh was similarly behaved, approximately 175-320 mV. The positive Eh value implied that the condition was in oxidation state. This condition corresponded to Equation 6, where oxygen supply was derived from the aeration due to the agitation process. According to Figure 3, Eh of all bacteria with 3.2 mM calcium lactate tended to decrease up to 24 hours followed by gradual Eh increase, while in the addition of 16.2 mM calcium lactate, Eh decreased up to 48 hours (except CPB 2, 24 hours). This was due to the bacterial metabolic reaction of calcium lactate pentahydrate decomposed into CO_2 (Equation 6), which subsequently formed CO_3^{2-} according to the equilibrium reaction (Stumm and Morgan, 1981):

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \quad (10)$$

$$H_2CO_3 \leftrightarrow 2H^+ + {CO_3}^{2-} \quad (11)$$

The soluble calcium lactate provided Ca^{2+} ion that would bind CO_3^{2-} to form calcium carbonate. However, Eh decreased due to the calcium carbonate precipitation. This cycle proceeded as long as the calcium source was available while generating proton (H^+) due to the metabolism of bacteria. This mechanism caused a slight increase of Eh values for all strains for both calcium lactate pentahydrate concentrations.

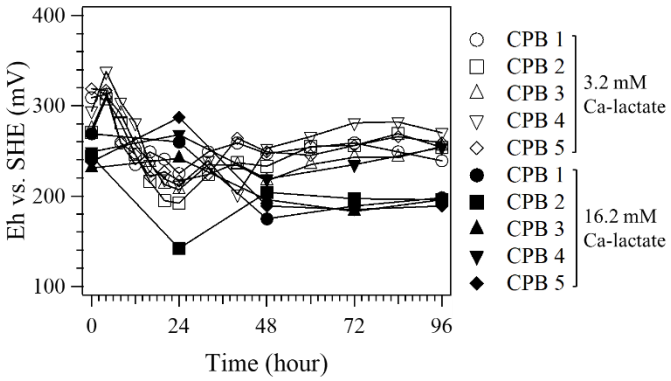

Fig. 3. Changes in Eh in nutrient broth medium supplemented with 3.2 mM and 16.2 mM calcium lactate pentahydrate.

3.2. Selection of bacteria and concentration of calcium lactate

Based on the results, the medium with 3.2 mM calcium lactate pentahydrate addition was more favourable and recommended due to the rapid growth rate of the bacteria, yet more economical. In addition, there was no significant difference in pH and Eh between the additions of two concentrations of calcium lactate pentahydrate.

The CPB 1 had the highest optical density with 3.2 mM calcium lactate pentahydrate followed by CPB 3, CPB 5, and

CPB 2, while CPB 4 was the lowest. However, the strain CPB 4 was eliminated because of the inhibition effect of calcium lactate. The CPB 2 and CPB 5 had the fastest growth rate (24 hours), followed by CPB 1 and CPB 3, both 40 hours. Although CPB 1 and CPB 3 required a longer time to achieve maximum optical density compared to CPB 2, it was assumed that they had slightly higher cell number due to their higher optical density. All bacteria cultivated on this medium showed an insignificant difference in pH and Eh. It can be concluded that strains CPB 1, CPB 3, and CPB 5 were selected to be the most optimum bacteria along with the addition of 3.2 mM calcium lactate pentahydrate to the medium. The three bacteria were further tested on biocementation process (data not shown).

3.3. Identification of isolates based on 16S rRNA

The most optimum bacterial strains were identified using 16S rRNA analysis. According to the results, both strains CPB 1 and CPB 5 were identified as *Bacillus* sp., and strain CPB 3 as *Neisseria* sp. These bacteria were reported to be involved in the generation of calcium carbonate precipitates in biocementation process (Ettenauer et al., 2011).

3.4. Self-healing observation

Based on the visual observations, an uneven calcium carbonate mineral deposit was produced at some points along the crack of the sample as shown in Figure 4. This suggested that bacteria distribution in the sample was uneven. The calcium carbonate phase was indicated as calcite (unpublished data, XRD data was not shown).

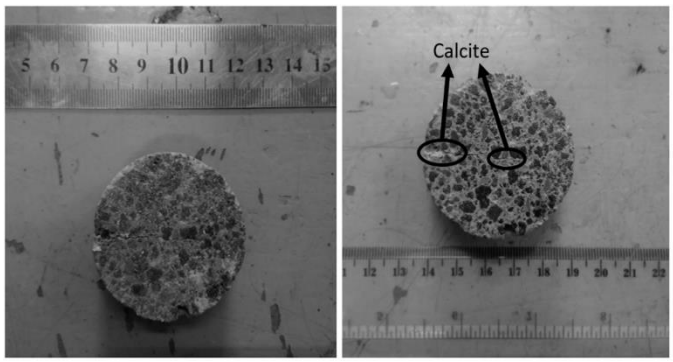

Fig. 4. Visual observation of self-healing bioconcrete using CPB 3.

4. Conclusion

Based on the results of this study, aerobic mixotroph bacterial strains CPB 1, CPB 3, and CPB 5 were selected with the addition of 3.2 mM calcium lactate pentahydrate on the liquid medium of nutrient broth. These results can be recommended for an eco-friendly mechanism of calcium carbonate precipitation.

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Conflict of Interest

The authors declare no conflict of interests in this research.

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