A Study on Biomineralization using Bacillus Subtilis Natto for Repeatability of Self-Healing Concrete and Strength Improvement

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Abstract
Recent studies in the field of concrete materials show that the early cracking criteria in micro-size can occur as soon as the cement matrix becomes hardened. In many ways, these cracks can become macro-size and opened cracks resulting in significant issues for the durability and appearance of concrete structures as water leakage and corrosion. The technique of self-healing using bacteria has recently received attention for its potential applications. However, the effectiveness and the repeatability of this method over a long period have not been clarified. The information on both the survival and the number of bacteria after healing is limited. This paper aims to improve the self-healing ability and repeatability of concrete when using Bacillus subtilis natto. The experimental studies evaluate the effect of biomineralization with lightweight aggregate as the protecting-carrying vehicle, which can control the release of healing fluid through four cracking-healing cycles. The urease activity and the biomineralization of the bacteria with urea as the main carbon source were assessed and the effect of cracking age on the self-healing capacity, associated with the compressive strength improvement was studied. The results obtained from the optical microscope and SEM/EDS analysis indicated the existence of bacteria CaCO₃ forming in concrete after four healing cycles. During long duration, bacterial concentration in concrete was determined by microscopic counting method. Based on experimental results, the restoration of the compressive strength confirmed the high self-healing ability of concrete when using bacteria in lightweight aggregate.

1. Introduction
Concrete is probably the most important and commonly used construction materials. However, cracking at any stage of the service life of concrete structure has been experienced by more clients, designers, researchers, and contractors than any other impact, and overall by an average of 90% of the respondents (Gardner et al. 2018). Even when reinforced with rebars, mineral fibers, or polymer, concrete is sensitive to crack formation - one of several types of damage. Cracking, especially below the groundwater level, can lead to many problems, such as water leakage and reinforcement corrosion. For many years, a full array of crack repair solutions has been developed with deliberate external intervention. However, it is difficult to repair the micro-cracks or crack embedded deep in concrete structures (highways, tunnels, or bridges). A smart and automated method to repair cracks is necessary for sustainable concrete infrastructure.

In general, humans, animals, or plants, can heal their damages themselves if those damages are within a small range. In many cases, cracks lesser than 60 μm in concrete can be repaired autogenously due to the hydration of residual clinker powder or the carbonation of dissolved calcium hydroxide (Wu et al. 2012; Van Tittelboom and De Belie 2013; Reinhardt et al. 2013). Inspired by natural and biological systems, self-healing concrete has been researched and developed. For concrete materials, self-healing can occur as a natural phenomenon (autogenic) or a result of engineering (autonomic) techniques (Gardner et al. 2018; Joseph et al. 2010). The engineering self-healing can be obtained by fiber reinforcement (Gray 1984; Hannant and Keer 1983; Li et al. 1998; Koda 2011; Mihashi et al. 2011; Zhu et al. 2012), using additives with chemical agents (Van Tittelboom and De Belie 2010), mineral geomaterials (Ahn and Kishi 2010), and microbial-induced calcium carbonate precipitation (MICP) (Ramachandran et al. 2001; Jonkers 2007; Jonkers and Schlangen 2008; Van Tittelboom et al. 2010). Therefore, self-healing concrete using biomineralization by bacteria can be a sustainable solution to extend the service life and durability of concrete structures. According to previous studies, limited types of bacteria can be used for MICP, such as Bacillus cohnii (Jonkers 2007), Bacillus pasteurii (Ramachandran et al. 2001), Bacillus pseudofirmus (Jonkers and Schlangen 2008), Bacillus subtilis (Huynh et al. 2017; Matsushita et al. 2010; Rao et al. 2013). Each type of bacteria needs proper nutrients for their growth. Based on the metabolic pathways involved in MICP, the self-healing mechanism includes the ureolytic
and the non-ureolytic process. According to many research findings, the most effective way of producing calcium carbonate (CaCO₃) is urea hydrolysis. Also, non-ureolytic bacteria have been explored (Lee et al. 2017) to prevent the adverse effects on the mechanical properties of concrete from ammonia produced by urea hydrolysis (Dhami et al. 2013; Zhu and Dittrich 2016). However, this mechanism can lead to high costs for organic nutrients and other treatments.

In this study, Bacillus subtilis natto, a native Japanese microorganism, will be used with a suitable proportion of nutrients to form CaCO₃ and prevent any adverse effects on the durability of concrete structure. Bacillus subtilis natto, the main factor for fermented soybean, was reclassified as Bacillus subtilis based on bacteriological characterization in Bergey’s Manual of Determinative Bacteriology (Bergey et al. 1974). Although the scientific name “Bacillus natto” was abolished, this unofficial name is often used for the food industry to distinguish them from common Bacillus subtilis strains that cannot produce natto. As a gram-positive bacteria, Bacillus subtilis natto can survive in the high-alkaline environment of concrete by its ability to form spores (Samanya and Yamauchi 2002; Kawai et al. 2017).

Figure 1 demonstrates that the biomineralization mechanism of Bacillus subtilis natto is relatively similar to Bacillus subtilis HU58 (Huynh et al. 2017) and other members of the subtilis family. Based on the hydrolysis of urea, bacterial cells become negatively charged, leading to the rapid attraction of surrounding calcium ions. Also, bacteria can degrade organic compounds included lactose (sugar), as a carbon source for growth and activation. Therefore, these processes controlled the adverse effects of nutrients on the properties of fresh and hardened concrete. Note that, bacteria can naturally produce CaCO₃ in environments with a high concentration of Ca²⁺ by changing the precipitation factors, separately or in combinations (Krajewska 2018; Dhami et al. 2013; Hammes and Verstraete 2002). Their primary role is often recorded in increasing the pH value. After the first stage with the forming of nucleation sites, the amount of CaCO₃ crystals begins to increase. When CaCO₃ crystals cover all of the cell wall surfaces, new crystals may not form. Instead, the crystals start to grow larger and become compact. Also, Bacillus subtilis natto does not cause disease (Brenner and Miller 2014). This strain is almost safe and easy to work within the laboratory. Bacillus subtilis natto would be an economical solution because of the low cost of bacteria spores, compared to the other microorganisms. As mentioned, urea hydrolysis is one of the most efficient ways of CaCO₃ forming. However, previous researches have studied the biomineralization with adequate organic carbon sources for bacterial growth and activation.

The urease activity and bacterial CaCO₃ precipitation with limited organic nutrients (yeast extract and peptone) to prevent rapid activation during the early stage of concrete hardening were tested in this study. This test condition also simulated the harsh conditions of lacking nutrients after a long time. Note that yeast extract and other organic carbon sources have a considerable impact on the decreasing of compressive strength of concrete due to delays in hydration. Consequently, bacterial spores and sugar were immobilized inside the lightweight aggregate to minimize the negative impact on the hydration and the compressive strength. The optical counting method was used to estimate the survival and the concentration of bacterial cells after self-healing. Another aim of this study was to determine whether or not the self-healing ability would manifest in the case of inadequate carbon source. Besides, to solve one of the significant challenges into the self-healing mechanism (Mahmoody and Sadeghian 2019; Mihashi and Nishiwaki 2012; Li et al. 2018), the repeatability of self-healing concrete specimens with the bacteria in lightweight aggregate was evaluated through the compressive strength recovery of the four cracking and healing cycles test.

2. The originality of the proposed self-healing system

2.1 Paste experiments

The effect of immobilizing bacteria and its essential nutrients in lightweight aggregates was assessed first. Note that a high concentration of bacteria and nutrients is necessary for long duration and high capacity of the self-healing effect. However, using nutrients, included lactose, as mixing water with a large dosage can lead to the adverse impacts on the hardened properties of concrete. Therefore, the lightweight aggregate can be used for not only protecting bacteria but also controlling the release of nutrients. In this work, it is checked whether lactose, a moderate retarder, can slow down or even stop concrete from setting hard in the case of with and without bacteria. Based on this test, the way that bacteria degrade the nutrients to use the carbon source can be explained. Three groups of cement paste were prepared: adding urea (U), adding bacteria (B), adding bacteria...
and lactose (BL), and regular cement paste as reference (R). The microstructure of cement pastes was observed with SEM.

In the case of direct mixing, lactose with high concentration prevented the setting process of cement particles, resulting in the unhardened paste after 14 days [Fig. 2(a)]. After 14 days, hardening can be seen clearly (not allowing the needle to sink into the paste) in the cases of the group “R”, “U”, and “B”. These groups also show the typical color of hydrated cement paste, while group “BL” shows dark gray with a soft surface. In addition, by SEM images, less main hydrated minerals could be found [Fig. 2(c)], as the result of the disruption of the cement matrix. Note that the retarding effect of sugar was highest when it was dissolved in the mixing water. In contrast, it can be seen that the regular formation hydrated minerals as C-S-H and ettringite in the case absence of lactose [Figs. 2(b) and 2(d)].

2.2 Concrete experiment

The hardening properties were tested with two ways of adding lactose immobilized in lightweight aggregate with the mixture proportion shown in Table 1. Figure 3(b) shows that even immobilized in the lightweight aggregate (group “S”); lactose could release and prevent the hydration of cement particles as the description in Fig. 3(a). This effect can be clearly observed by the difference between the unhydrated zone [the areas within yellow dashed lines in Fig. 3(b)] around the aggregate [denoted by the red circles in Fig. 3(b)] and the other position where the hydration could occur usually. Therefore, strong bonding could not be created between the cement matrix and aggregate for all of the volume of the concrete specimen, resulting in the low compressive strength. In contrast, the normal hydration in the case of using lactose with bacteria (group “NS”) indicates that the bacteria could degrade lactose for their growth and activity, resulting in early age hydration. In addition, the 7 day compressive strength (1.5 N/mm²) of the specimen used 100% lactose showed significant retarding. The specimen used 100% bacteria (35 N/mm²) and the controls (34 N/mm²) well hardened. It could be seen that Bacillus subtilis natto itself did not cause any negative effect in setting property and compressive strength of concrete. Besides, immobilization of bacteria and organic components with LWA can not only protect the bacteria from the alkaline environment but also prevent adverse effects of organic matters on the properties of concrete. The bacteria can be active with lactose. Hence, bacteria, together with lactose in LWA, which is market available, would be the best combination and originality of this self-healing system.

![Fig. 2 Hardening test of 4 cement paste groups (a), cement matrix microstructure after 14 days of the controlled cement paste (b), the paste microstructure with bacteria and lactose (c), and the paste microstructure with only bacteria (d).](image-url)
3. Materials and Experiments

3.1 Urease activity of Bacillus subtilis natto

In this study, the gram-positive Bacillus subtilis natto bacteria of concentration $6 \times 10^8$ CFU/g obtained from Yuzo Takahashi Laboratory Co. (Yamagata, Japan) were used. The powdered bacteria composed of bacterial starter spores and sugar (with lactose as the main composition). Microscopic observation [Fig. 4(a)] shows that the average size of the active cell is about 1 to 10 $\mu$m (rod-shaped), and the diameter of the spore is around 1 to 5 $\mu$m (sphere-shaped). The colonies have gray-white round-shaped with the wrinkled surface [Fig. 4(b)].

The urea hydrolysis process not only provides alkaline pH but also generates an available supply of carbonate with the potential to produce high ion concentrations in a short time (Whiffin 2004). Therefore, the urease activity test was studied to determine the bioactive ability of Bacillus subtilis natto. The composition of the test urea solution included urea, NaCl, glucose, and bacterial spores. Four groups of the test solution were prepared with the differences in the number of the bacterial spores as the following proportions of urea: bacteria cell of 1 : 0.075 (UB1), 1 : 0.150 (UB2), and 1 : 0.225 (UB3), and the controlled urea solution (U). Before testing, bacterial spores and glucose were mixed with water and stirred at 120 rpm for 12 hours to turn

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**Table 1 Mixture proportion and materials properties for concrete experiment.**

| Group | w/c | Water (kg) | Cement (kg) | Sand (kg) | LWA (kg) | Flow (mm) | T (°C) |
|-------|-----|------------|-------------|-----------|----------|-----------|-------|
| S     | 0.4 | 0.66       | 1.66        | 2.9       | 2.31*    | 8.89×8.95 (before flow table drops) | 24.1  |
| N     | 0.4 | 0.66       | 1.66        | 2.9       | 2.31**   | 9.57×9.42 (before flow table drops) | 24.3  |
| NS    | 0.4 | 0.66       | 1.66        | 2.9       | 2.31***  | 100.8×97.9 (before flow table drops) | 22.3  |
| R     | 0.4 | 0.66       | 1.66        | 2.9       | 2.31     | 9.59×9.75 (before flow table drops) | 23.8  |

Materials properties of cement, sand, and lightweight aggregate (LWA):

- **Cement**: 3.16 g/cm³
- **Sand**: 2.59 g/mm³
- **LWA**: 1.68 g/mm³

Particle size distribution of lightweight aggregate:

| Particle Size (mm) | Percent passing (%) |
|--------------------|---------------------|
| 20                 | 100                 |
| 15                 | 99                  |
| 10                 | 47                  |
| 5                  | 8                   |

*: 100% lactose in LWA: $m_{lactose} = 0.163$ kg

**: 100% bacteria in LWA: $m_{lactose} = 0.163$ kg

***: (50% bacteria + 50%) lactose in LWA

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Fig. 3 Control release mechanism of bacteria (a), and the unhardened concrete specimen in case of immobilized in lightweight aggregate (b).
into active-form. After that, the other components were
added to the solution and cured at 40°C. For assessing
the effect of bacteria on the pH change, a series of the
reference solutions, including “Urea-CaCl₂”, “100% 
Urea”, and “100% CaCl₂”, was prepared. The pH value
was measured at each period. In addition, phenolphthalein
was used as the pH indicator. Note that phenolphthalein turns pink when exposed to substances above a 
PpH of 8 and becomes purple at higher pH values.

3.2 Biominalization of Bacillus subtilis natto
immobilized in lightweight aggregate

The degradation of urea to carbonate and ammonium,
resulting in an increase of the pH and carbonate ion
concentration in the bacterial environment, combines
with calcium ions to precipitate CaCO₃. The effect of
urea and calcium sources on the activity of Bacillus 
subtilis natto was studied. The bacteria were mixed with
the solution of urea and calcium chloride (UCB). A so-
lution with just urea and calcium chloride (UC) was also
prepared as the reference. Both of these solutions were
measured the change in pH over time to determine the
role in the biominalization. The bacterial solution was
cultured at 40°C for the growth and activation. The
biomineralization of CaCO₃ was observed and recorded
under an optical microscope. For the immobilization test,
LWA was prepared through immersion in the bacterial
solution for 48 hours before drying at 40°C for 24 hours.

Scanning Electron Microscope (SEM) and Energy-
Dispersive X-ray Spectroscopy (EDS) were used to ob-
serve the presence of bacteria and its ability to form 
CaCO₃.

3.3 Compressive strength improvement using 
bacterial repairing solution

The compressive strength of mortar specimens may
increase due to the precipitation of CaCO₃ as an en-
hancement. To imitate the degradation structures after a 
long time using, low-strength mortar specimens with 
w/c = 1.5 were prepared to make the cement matrix less
dense and accelerate the repairing process. Mortar 
specimens (40×40×40 mm) were cast and cured in the
air for one year. The specimens were then split into two
groups to immerse in bacterial solution and water for 7
days. In the bacterial solution, the specimens were
expected to increase the compressive strength when
compared to the group immersed in water.

For concrete case, low-strength concrete specimens
(w/c = 1.5) were used to imitate the existing old con-
crete structures with air-curing for one year before the
test. A group of trial specimens was tested to determine
the 1 year compressive strength (5.5 N/mm²). For
concrete material, the first visible crack is usually
developed at around 50% of compressive strength.
Therefore, the load rate was then maintained at 1.17
kN/s until 90% of the strength of the trial was reached
to create not only tensile cracks but also irreversible 
crack at the aggregate-matrix interface. This value was
recorded as F_damage (F_damage = 5 N/mm²) and was applied
to the bacterial specimens to generate cracks. Cracks
with a maximum width of 1.2 mm on the specimen
surface can be observed. The effect of using the bac-
terial solution was carried out as follows: (1) creating 
cracks and damage by compression; (2) immerse the 
specimens in bacterial solution for 1, 3, 5, and 7 days;
(3) for each stage, measure the compressive strength of 
the test specimen and returned it to the curing environ-
ment. In the case of the controlled specimens, water was
used instead of the bacterial solution for curing.

3.4 Self-healing repeatability and strength im-
provement using bacterial lightweight aggregate

Table 2 shows the mixture proportion for preparing two
groups of cylindrical concrete specimens (diameter = 50
mm, H = 100 mm). The cement, sand, and LWA were
used with the same properties as Table 1. For the group
“Natto”, the LWA (maximum water absorption = 29.5%)
immobilized bacteria was used according to the pro-
goed in Fig. 5(a). The dosage of urea and CaCl₂
was controlled with a safety limit according to ACI 318
for maximum chloride ion content in concrete (m_urea ≤ 
0.45%m_cement, m_bacteria ≤ 0.5%m_cement and m_CaCl₂ ≤
0.45%m_cement) to prevent the corrosion of reinforcement.
In this case, the bacterial concentration was calculated

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Fig. 4 Microscopic image of spore-forming Bacillus subtilis natto (a), and the bacterial colony morphology in Petri dish (b).
to $3 \times 10^7$ CFU for each concrete specimen. For the group “Controls”, the lightweight aggregate without bacteria was used as the reference.

For the strength development after inducing cracking [Fig. 5(b)], the load value was defined based on the 7 day compressive strength of the “trial-specimen” with the mixture as the controlled. After measuring this strength, the load with 90% of value was applied for crack creation ($F_{\text{Damage}}$). Both of group “Natto” and “Controls” were compressed under the same load ($F_{\text{Damage}} = 11 \text{ N/mm}^2$, loading rate = 1.17 kN/s) for all specimens to induce cracks. The continuously self-healing effect was expected to occur through the curing process in water. The compressive strength of the specimens was measured again after curing periods of 7 days, 14 days, and 28 days. The recovery of the compressive strength of concrete specimens can translate into the self-healing efficiency. This test gives information about the relation between self-healing capacity and curing time.

For the repetitive cracking-healing test, the specimens were separated into two series (“Natto” and “Controls”) with different cracking age (Natto 7: 7 days, Natto 14: 14 days, and Natto 28: 28 days from casting). For each cracking-healing cycle, the specimens were compressed and then cured in water for 7 days. Four cycles were studied to clarify the effectiveness and the repeatability of the self-healing process [Fig. 5(c)]. As a useful technology for detecting changes in structure, the pulse velocity was measured using the ultrasonic technique to check and analyze the difference in the concrete structure during the self-healing process. After four cycles of cracking-healing, the concentration of bacteria was determined by microscopic counting method.

### Table 2 Mixture proportion (per m³ of concrete) and materials properties for compressive strength test.

| Group   | w/c | Water (kg) | Cement (kg) | Sand (kg) | LWA (kg) | Bacteria (kg) | Flow (mm) | Air content (%) | T (°C) |
|---------|-----|------------|-------------|-----------|----------|----------------|------------|-----------------|--------|
| Natto   | 0.4 | 147        | 370         | 953       | 618      | 0.163          | 100.8×97.9 | 11              | 22.3   |
|         |     |            |             |           |          |                | 119.2×110.8|                 |        |
| Controls| 0.4 | 147        | 370         | 953       | 618      | -              | 104.5×102.9| 3.9             | 19.3   |
|         |     |            |             |           |          |                | 153.4×144.0|                 |        |

Fig. 5 Flow chart for the preparation of lightweight aggregate immobilized bacteria (a), flow chart for the continuous strength recovery test (b), and flow chart for the four cycles of the repetitive cracking-healing test (c).
4. Results and discussion

4.1 Urease activity of Bacillus subtilis natto

All of the tubes with bacteria showed the color changes of the phenolphthalein indicator [Fig. 6(b)] associated with the difference in the concentration of the bacteria. After 10 days, the color of the solution in the tube “UB_3” began to turn pink [Fig. 6(c)] indicated the alkaline environment. In contrast, the controlled solution (UB) without bacteria did not change the color. For the pH measurement, Fig. 7(a) showed a stable pH value (around 7.5) of the “Urea-CaCl₂”, “100% Urea”, and “100% CaCl₂”. The bacterial solutions, including “UB_1”, “UB_2”, and “UB_3” showed the pH changes with the quickly decreasing from beginning to 3 hours before increasing to become alkali associated with the degradation of urea. There is no significant difference between the pH value of group “UB_1” and group “UB_2”. All of them showed lower results in comparison to the group “UB_3”. Group “UB_3” with the highest initial concentration of bacteria showed the most effective result in the urea degradation test. In the case of the proportion of the bacteria and the carbon source was not suitable, the reaction rate might decrease. This result suggests that biomineralization can be controlled by increasing or decreasing the bacterial concentration to reach the limit with a suitable amount of nutrient.

The pH change has closely related to the CaCO₃ formation corresponding to the biochemical reactions. As a result of urea hydrolysis, ammonia ions accumulated in the medium and made it alkaline. Generally, bacterial urea hydrolysis includes a series of complex reactions. First, one mole of urea was hydrolyzed by bacterial urease to one mole of ammonia (NH₃) and carbamate (NH₂COOH). This carbamate was then spontaneously hydrolyzed to produce one mole of ammonia (NH₃) and carbonic acid (H₂CO₃). After that, H₂CO₃ was converted to bicarbonate (HCO₃⁻) by carbonic anhydrase in bacteria. Next, two moles of ammonium (NH₄⁺) and hydroxide (OH⁻) were formed due to ammonia hydrolysis. Finally, as a consequence, the pH around the bacterial cell was increased. This phenomenon can lead to induce the precipitation of CaCO₃ in the case of the presence of soluble Ca²⁺ in the surrounding environment. Note that the CaCO₃ forming process requires alkaline pH as an indispensable condition to shift the bicarbonate equilibrium carbonate ions. Therefore, the changing of pH indicated the urease activity and induction of Bacillus subtilis natto.

![Fig. 6](image1.jpg)
![Fig. 7](image2.jpg)

Fig. 6 Change in the color of the bacterial solution compared to the controls at the beginning (a), after 7 days (b), and after 10 days (c).

Fig. 7 Change in pH of three series of the bacterial-urea solution compared with the urea controlled solution (a), and the fitting curve for the solution “UB_3” (b).
*Bacillus subtilis* natto to form CaCO₃. This increase in pH also caused the color changing of phenolphthalein indicator from transparent to pink. When compared the trend of pH changes of three groups of the bacterial solution, there were no significant differences (Fig. 7). Although the bacterial groups reached to alkaline pH, the decreasing of the group “UB 1” and the group “UB 2” after 4 days was recorded. According to previous studies (Hammad et al. 2013; Burbank et al. 2012), the pH meter response delay could cause some discrepancies in the measurement data.

Generally, for rapid urease-positive organisms, the degradation process of urea solution follows Equation (1):

\[
\text{CO}(\text{NH}_2)_2 + \text{H}_2\text{O} \xrightarrow{\text{Urease}} \text{CO}_2 + \text{H}_2\text{O} + 2\text{NH}_3
\]  

(1)

\[
\text{CO}(\text{NH}_2)_2 + \text{H}_2\text{O} \xrightarrow{\text{Urease}} \text{NH}_2\text{COOH} + \text{NH}_3
\]  

(2)

\[
\text{NH}_3 + \text{H}_2\text{O} \rightarrow \text{NH}_4\text{OH}^{-}
\]  

(3)

After 7 days, the pH of the bacterial-urea solution was lower than previous studies about urease bacteria as a result of not wholly reactions [Equations (2) and (3)]. Environmental factors could cause a low reaction rate. These processes increased the pH inside the bacterial cell and decreased the pH gradient so that the rate of pH increase in the surrounding environment became slow down. Besides, the carbon source from urea might not be enough for the rapid growth and activation of the bacteria. The pH of the bacterial-urea solution started to increase quickly after 7 days and reached a value of 9.49 after 10 days. This result confirmed the color change of the indicator and positive urease activity of *Bacillus subtilis* natto, which is the base for CaCO₃ forming. In the next paragraph, the self-healing effect of concrete specimens at the period 7 days curing after cracking can demonstrate that the bacteria could renew a biomineralization cycle. In addition, the low-rate activity may be useful for the penetration process of bacteria in the lightweight aggregate. If the reaction rate is too high, the rapid forming of CaCO₃ can become a barricade to prevent the immersion. In the case of full nutrient included yeast, peptone, and glucose, the activity of bacteria was vigorous via the quick degradation of urea within 24 hours. However, the bacterial concentration can decrease quickly. A large amount of CaCO₃ crystals in the early stage of concrete hardening could lead to the volume expanding. On the contract, with urea as the primary carbon source, bacteria can turn into the spore (low-activity stage) to maintain the life cycle in concrete for a long time. Without external stress, the micro-cracks can be controlled and healed within the limit activity of bacteria. Therefore, the biomineralization should be slow down in the early stage of concrete curing. In addition, urea and CaCl₂ were used with a safety limit dosage to prevent corrosion problems. Furthermore, the nutrient needed to be immobilized inside the lightweight aggregate to reduce unexpected problems for concrete hardening.

### 4.2 Biomineralization of *Bacillus subtilis* natto immobilized in lightweight aggregate

Figure 8(a) shows the pH changes in the bacterial solution (UCB) and the controls (UC). According to the reactions of the bacteria, the pH value of the “UCB” solution started to drop suddenly at the exponential phase (from 10 to 20 minutes) and reached the lowest level (pH = 5.25) before increasing and stabilizing. This pH decreasing during the first stage might be caused by the CO₂ release from urea and the merging of Ca²⁺ by the bacteria, leading to OH⁻ consume. Note that a small amount of chemically induced CaCO₃ precipitation might occur in the absence of bacteria. After 7 days, there is a significant difference between the pH of the bacterial solution and the controls. The “UCB” solution changed from transparent to translucent because of the presence of precipitated crystals [Fig. 8(b)]. This phenomenon can be explained by the transparency of materials based on the Mie theory for light scattering. If the

![Fig. 8 Change in pH by the MICP process (a), and the difference in the transparency of the bacterial solution in comparison with the controlled solution (b).](image-url)
crystal size is significantly smaller than the wavelength of visible light, the material will be transparent. In contrast, in the presence of large crystals, the material will be translucent or colored, instead of transparent. After 14 days, the “UCB” solution changed from translucent to opaque (Fig. 9, upper photo). The microscopic image shows the presence of a compacted layer of CaCO₃ crystals [Fig. 9(c), lower photo]. After 45 days, the crystal grains became massive and settled to the bottom of the test tube [Fig. 9(d)]. This result indicates the increased precipitation over time. Furthermore, the ion concentration of the “UCB” solution (3 mV) was also lower than the controls (27 mV) due to the decrease of free Ca⁺ concentration.

Figure 10(a) shows the presence of white-color inside the porous structure of the lightweight aggregate. By SEM/EDS analysis [Fig. 10(b)], these materials were defined as CaCO₃. The SEM image shows that groups of bacterial colonies covered the surface of CaCO₃ crystals as biofilms. The activation of bacteria formed not only CaCO₃ but also the other biomass or biofilms. The combination of CaCO₃ and these bio-products can create a complex repairing material helped to fill the crack volume and densify the concrete matrix. Although CaCO₃ crystals were not homogeneous for all the positions of the aggregate, such as the position “2” [Fig. 10(b), lower photo], lightweight aggregate showed the promising ability for carrying and protecting the bacteria in the concrete mixture. In addition, the remaining space in LWA plays an important role in protecting and curing the remaining bacterial spores for the other cracking cycles. Furthermore, the penetrating technique should be considered to improve the transport and distribution of bacteria into the aggregate. Note that water and oxygen are necessary to activate the biomineralization of bacteria. In this case, these components could penetrate from the curing environment to the concrete structure through the cracks. After that, the transport of bacteria and nutrients with CaCO₃ (healing fluid) through the lightweight aggregate to the cement matrix could densify the structure and repair the cracks.

4.3 Compressive strength improvement using bacterial repairing solution

After 7 days of immersing in the bacterial solution, the compressive strength of the mortar specimens showed higher value (6.9 N/mm²) compared to the controls cured in water (4.3 N/mm²). By using the phenolphthalein indicator [Fig. 11(a)], the structure of the controlled mortar specimens showed a more pink-color

![Fig. 9 Change in transparency and microscopic images of CaCO₃ crystals in the bacterial solution after 7 days (a), 10 days (b), 14 days (c), and 45 days (d).](image)

![Fig. 10 Microscopic images and SEM/EDS result obtained inside the lightweight aggregate immobilized bacteria after 7 days.](image)
area than the bacterial specimens with the presence of biomineral. It could be seen that the increase in compressive strength is related to the biomineralization by bacteria, resulting in a positive effect on improving the physical properties and mechanical resistance of the mortar specimens.

For the concrete test, it can be seen that the deformation of the water-cured specimen increased after the 1st test, while the bacterial specimen was nearly able to maintain its shape until the 4th test [Fig. 11(b)]. The normalized compressive strength (the ratio between the strength after curing and the initial strength of the same specimen) of the specimen cured in water and cured in the bacterial solution is shown in Fig. 11(c). Both of the specimens were almost the same as the initial compressive strength (the 1st test). After cracking, the specimen cured in water decreased the strength rapidly and was broken after 7 days (the 4th test). Note that after 3 days of curing in water, the late hydration helped the compressive strength increase slightly (the 2nd test) before decreased rapidly by the shear and splitting stress. This behavior is similar to the one-cycle cracking-healing test in a study using silica-based microcapsules (Tan et al. 2016). Regarding many pores formed by the high w/c, the late hydration could not densify and repair the structure after three cycles of cracking. In contrast, from the 2nd test to the 3rd test, the specimen cured in bacterial solution shows the lower decreasing rate of compressive strength than the controls. Besides, the compressive strength became more stable after 7 days (three cycles) and started to increase [the angle $\alpha_3$ in Fig. 11(c) is $>90^\circ$]. The broken surface was also checked with phenolphthalein, and similar to the case of mortar, the controls showed a larger pink-color area than the bacterial specimens. This result confirmed the positive effect of biomineralization within the concrete structure.

4.4 Self-healing repeatability and strength improvement using bacterial lightweight aggregate

(1) Self-healing repeatability and compressive strength improvement

For the continuous strength recovery test, Fig. 12 (a) shows that the longer the curing time, the higher the compressive strength. In addition, the compressive strength of the group “Natto” was significantly higher than the “Controls” at every period. This difference could be caused by the healing effect with the capacity of over 40% just after 7 days of curing before the cracking day. This result is higher than the previous study (20%) when using Bacillus subtilis JC3 (Rao et al. 2017) directly with a concentration of $10^5$ CFU/ml as mixing water. Therefore, the immobilization of bacteria and nutrients in lightweight aggregate could not only prevent the disruption of concrete with the presence of organic matter but also fill the cracks and densify the pore structure. Besides, with this technique, the higher initial concentration of bacteria can be used for a longer duration, and higher healing capacity. Moreover, from the 14 day curing period, the gap of compressive strength between the “Natto” and “Controls” groups became higher. This result was relevant in the self-healing process with the increasing of CaCO$_3$ over time.

For the repetitive cracking-healing test, Fig. 12(b) shows the compressive strength of two series of concrete specimens (“Natto” and “Controls”) with three cracking ages (7, 14, and 28 days) after four cracking-healing cycles. Generally, the compressive strength of the series “Natto” was higher than the “Controls” at

![Fig. 11 Immersion process and broken surface of mortar specimens with phenolphthalein indicator (a), surface observation (b), and normalized compressive strength of the concrete specimens cured in water and bacterial solution (c).](image-url)
every testing time. The trend of strength development for both the “Natto” and the “Controls” shows that the younger cracking age, the lower strength at the 1st test, and the 2nd. After the 2nd test, the behavior of specimens became more complex, associated with the different damaged levels. After 7 days of curing, the group “Natto 7” shows the increase of strength, the group “Natto 14” shows a stable value, while the group “Natto 28” shows the decrease. A similar trend with lower values of strength can be seen for the series “Controls”. Even though the absence of bacteria in, the compressive strength of the “Controls 7” increased quietly [Fig. 12(a)] as an expected result from the late hydration of cement particles. Under the optical microscope, the autogenous healing phenomenon can be observed with cracks smaller than 60 μm on the surface of the concrete slice [Fig. 12(c)]. However, after this period, the strength increasing rate became lower and reached a plateau state. In contrast, the compressive strength of the group “Natto 7” increased rapidly by the formation of CaCO₃ inside the cracks. Besides, the initial strength of all specimens in series “Natto” was higher than the series “Controls”. The transportation of water from outside to inside the lightweight aggregate might cause the activation and biomineralization of bacteria. In this case, the gap over 20% compared to the series “Controls” in the early stage is a considerable result. As discussed, the natural healing through late hydration of cement particle could only make a positive effect on the early stage. Therefore, the age of cracking for more than 7 days caused slow recovery and ineffective with massive cracks. Group “Controls 14” and “Control 28” shows the decreasing continuously from the 1st test. The level of this decreasing was significantly higher than the group “Natto 14” and “Natto 28”. Although the healing capacity depends on the quality of immobilizing bacteria into lightweight aggregate and curing conditions, the recovery of compressive strength after four cracking-healing cycles showed a satisfactory result.

(2) Ultrasonic monitoring for self-healing ability
The change of pulse velocity can monitor the deterioration of structure to clarify the repetitive strength recovery behavior. Figures 13(a) and 13(b) show that the

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![Graphs and images related to the text content](image-url)
pulse velocity of both groups “Natto” and “Controls” decreased suddenly after cracking. However, the pulse velocity of the group “Natto” increased quickly through the curing process shown by the slope at the testing time. This significant recovery in the velocity indicated that the voids or cracks were filled with healing material. It can be seen that the longer the curing time, the higher is pulse velocity [Figs. 13(a) and 13(b), upper graphs]. For the repetitive cracking-healing, with the CaCO$_3$ precipitation, the pulse velocity went up to a higher value in comparison with the group “Controls” [Figs. 13(a) and 13(b), lower graphs] after every 7 days curing.

In the case of the velocity through diameter direction, the structure of the group “Controls” became unstable with damages and massive cracks. With the bacterial group, the decreasing rate of velocity got slower than the controls, resulting in the ability to maintain the specimen shape (almost 90%). Besides, after the 2nd compression test, the pulse velocity of the group “Controls” could not increase as the first stage [Figs. 13(a) and 13(b), upper graphs]. This phenomenon can be explained by autogenous healing by the late hydration that reached the limit, and it was not enough for most damages. In contrast, with the CaCO$_3$ forming,

![Fig. 13 Change of pulse velocity through the continuous strength recovery test (a) and through the repetitive cracking-healing test (b). The relationship with compressive strength and pulse velocity is shown in (c).](image)
the autonomic self-healing process led to a positive effect on the compacting of structure and minimized the cracks for a long time.

Furthermore, Fig. 13(c) shows the quite complexity between the compressive strength and the pulse velocity. The aggregate immobilized bacteria might cause a significant difference in the correlation between the “Self-healing” group and the “Controls” group. Although the mix proportion remains constant, a small part of urea and CaCl2 might be released to the cement matrix and changed the formation of the hydrated mineral. Also, cracking under compression many times could lead to shear, and splitting damage prevented the pulse velocity from getting the same value for each position in the length of the concrete specimen. However, the trend of velocity changing has related to the densifying of microstructure caused by the increasing of CaCO3 amount.

(3) Self-healing flow analysis
As the main self-healing factor, a massive amount of CaCO3 from the activation of Bacillus subtilis natto could be observed by naked eyes [Fig. 14(a)]. The shape of these CaCO3 crystals, formed inside the cracks, was similar to the finding of the study using Sporosarcina pasteurrii bacteria (Ameri et al. 2019). Rod-shaped crystals [Fig. 14(b)] could be a reinforced component in cracks. This reinforcement is similar to the strength improvement mechanism of cementitious composite through whisker CaCO3 (Cao et al. 2019). The bacterial activation to form CaCO3 in the pores of concrete structure could also densify the matrix contributes to the high compressive strength. Figure 14(c) shows a thick layer of the precipitated CaCO3 on the surface of the specimen at 60 days of curing, associated with a significant amount of CO2 and O2. This layer also included natural carbonation. The thickness decreased as it entered the core and linked to the lightweight aggregate position. A layer of the precipitated products covered the lightweight aggregate and connected it to the outer CaCO3 layer. This interface reaction could lead to a strong bonding enough to maintain the strength of concrete over four compression test cycles by enhancing the aggregate-matrix interaction. The CaCO3 network within the concrete structure could deliver healing products (healing fluid) to cracked positions and help for the water ingress into LWA as well as the carbonation process. Also, when compared to the controls, natural carbonation was very slow, resulting in the almost alkaline surface [Fig.14(c), right hand side]. Therefore, the thick CaCO3 layer was assumed to be formed primarily from bacterial activity. Furthermore, this phenomenon can demonstrate that the bacteria could

Fig. 14 Surface appearance of the bacterial concrete specimen during the cracking-healing cycles (a), SEM/EDS result of precipitated CaCO3 inside the crack (b), and carbonation through the crack (c).
active and form CaCO\textsubscript{3} below the surface (subsurface) or in the core of the concrete structure, especially in the case of deep cracks.

Moreover, after four cracking-healing cycles, the remaining bacterial concentration in the concrete specimen was 1.5×10\textsuperscript{3} CFU/specimen from the initial concentration of 3×10\textsuperscript{7} CFU/specimen. Note that almost no bacterial cell could be found after one month in unprotected cases. Data obtained after healing cycles indicated many bacterial life cycles, including CaCO\textsubscript{3} forming, died, multiply, and spore-forming with the protection of lightweight aggregate. Furthermore, after 24 hours in the culture medium, the remaining bacteria could become active with a significant increase in concentration. This result indicates that a suitable supply of nutrients can help to maintain bacterial biomineralization for a long time.

5. Conclusions

The experimental results demonstrated that Bacillus subtilis natto could produce urease enzyme to breakdown urea in the hash condition of organic carbon source. The ability of this bacteria type to form CaCO\textsubscript{3} through the MICP process after 7 days inside the lightweight aggregate was also confirmed. The combination of bacteria and nutrients in lightweight aggregate was an effective technique to control the release of healing fluid without adverse effects on setting and hardening properties. Bacillus subtilis natto showed a unique ability to create a massive amount of CaCO\textsubscript{3} for self-healing effect in concrete specimens for a long time.

The use of the bacteria-based repairing solution through immersing showed positive results for both mortar and concrete. In this study, the four cycles cracking-healing test was investigated to analyze the self-healing effect and the repeatability of concrete specimens through compressive strength recovery and pulse velocity. Also, it could be seen that the strength recovery capacity is higher for younger cracking ages. Regarding the age of cracks, the results showed that concretes could achieve the self-healing ability with high performance between 7 days and 28 days of age after casting. The concentration of the bacteria after many healing cycles shows a considerable impact on the immobilizing method. Generally, in this study, the behavior of concrete specimens indicated that Bacillus subtilis natto was forced to work with controlled nutrient sources immobilized in LWA to optimum the self-healing ability for multiple loading cycles without degradation of concrete properties.

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