Biomineralization Analysis and Hydration Acceleration Effect in Self-healing Concrete using Bacillus subtilis natto

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Abstract
Since concrete with bacteria incorporated in the matrix shows promising results in initial studies, more research has focused on using bacteria for the strength and durability enhancement of concrete. Bacterial concentrations varying from different positions on the crack surface were recorded and evaluated in conjunction with the amount of self-healing product formed. The change in bacteria concentration with the survival time in concrete was investigated and lasted for two years. The degree of mineralization of calcium carbonate from the microbial activity is also closely related to the level of survival and reduction of bacterial concentrations in concrete compared to the initial amount. These processes were tracked and analyzed through phase composition analysis and microstructure analysis. The role of nucleation sites of bacteria for accelerating mineral deposition was also investigated. The change in the content of hydrated cementitious minerals can be seen in groups of samples with different bacteria regarding cracking age (7-90 days). The increase in C-S-H content in the bacterial samples at early cracking age was significant compared with the control group. The effect on healed crack parameters through microscopic observation contributed to supporting and demonstrating the hypothesis of the combination of the formation of the calcium carbonate crystals around the bacterial cell as crystallization nuclei and the promotion of hydration for C-S-H formation.

1. Introduction
In recent years, microbially calcium carbonate precipitation (MICP) by microorganisms has been studied for application in concrete crack repair to create a sustainable and cost-effective alternative method. Some bacteria strains can convert carbonate ions (CO32-) through urea hydrolysis to bind with calcium ions (Ca2+) to precipitate calcium carbonate (CaCO3) (Naveed et al. 2020; Seifan and Berenjian 2019). However, there are challenges when adding bacteria to concrete directly due to this harsh environment. Firstly, the typical concrete porosity typically ranges from 9% to 10%, and the average pore size is smaller than 1 μm for micropores, 1-10 mm for mesopores, and greater than 10 mm for macropores (Chen et al. 2016; Vicente et al. 2019). However, the pores of cement-based materials will decrease with age, reducing the survival and activities of bacterial-based self-healing agents when added directly into concrete. The average bacterial size is around 0.5-2.0 μm (for spherical bacteria) and 1-10 μm (for rod-like or filamentous bacteria) (Holley 2017; Khalifa 2016; Mitchell and Santamarina 2005). Hence, the bacteria could be easily ‘squeezed’ by the decreasing volume of capillary pores during the cement hydration process. Secondly, the cement matrix in concrete structure is a high-alkaline environment, with a pH of 12-13 and limited moisture and oxygen created by the mineral setting and hardening process. These conditions can challenge bacteria to survive, grow, multiply, and activate. Furthermore, at such a high pH value, the bio-mineralization of bacteria could decrease significantly (Whiffin 2004) due to the decrease in urease activity shown by the low rate of urea decomposition. As reported in a previous study, the compressive strength would only increase at 28 days and later, while measurements at earlier time points showed lower strength than the reference without bacteria (Jonkers 2011).

Additionally, the mechanical forces during concrete mixing and gradual concrete shrinkage can destroy bacteria. The high alkaline pH of concrete, the intense abrasion during the mixing process, thermal and mechanical stresses caused during the setting-hardening of the cement phase, and the lower ingress of oxygen are the main reasons to diminish the possibility of bacterial survival in concrete. In 2011, a study using B. megaterium showed that the bacterial concentration decreased sharply from 107 to 102 CFU/ml after three days in the mortar specimens. After 28 days, the survival rate dropped to 0.1%. Similar results were observed when using S. Pasteurii in cement paste (Basaran 2013), the remaining viable cell concentration decreased by 80% after one day, and only 0.4% survived after 28 days. After 28 days from when B. megaterium was added to the concrete mixture, only 0.06% of the bacte-
ria could survive, a decrease in cell concentration from $5 \times 10^7$ to $3.2 \times 10^6$ CFU/ml (Achal et al. 2011). Another study showed that only 2% of the initial *S. Pasteurii* cells survived (Bundur et al. 2015). As an inevitable consequence, protection was focused on being studied as one of the critical factors for prolonging the bacterial lifetime in concrete. When using diatomaceous earth with a highly porous structure to immobilize *Bacillus subtilis* HU58 (Huynh et al. 2017), a slight reduction of $2.7 \times 10^8$ CFU/g from the initial bacteria concentration was observed after five months in concrete. However, the low-strength property of diatomaceous earth makes this material an unideal environment for long-time storage of the bacteria. As described in Fig. 1, incorporating immobilized bacteria in lightweight aggregate (LWA) in concrete can enhance crack healing ability with the CaCO$_3$ generated by bacterial metabolic activity and subsequent chemical reactions with other hydrated cement minerals and metabolic byproducts. Injections, as surface treatments, could be preferred to add to the concrete mixture, as repairing liquid-based systems with bio-grout can easily transport to the cracks (Putri et al. 2019; Ujike et al. 2014). However, the bonding between the healing agent and the concrete substrate may decrease, resulting in a re-crack or even more severe damage.

In contrast, adding bacteria directly into the concrete mixture can create a “smart-living” material with the ability to self-repair without human intervention. Not only the way of protection or immobilization, but the source of nutrient is still an unresolved issue when setting the goals of a long-time use and repeatable self-healing effect. In the case of using complete nutrients, although adding bacteria directly to concrete may prevent the micro-cracks from appearing, there were adverse effects in the concrete mix reported in a study on using *Bacillus subtilis* JC3 (Rao et al. 2017). The hydrated cement phase integrity disruption, resulting from organic matter exceeding the permissible limit according to Indian Standard IS 456 : 2000, may cause compressive strength reduction in mortar specimens with bacterial concentrations of more than 105 CFU/ml (Rao et al. 2017). Generally, bacterial spores are protected by proteins, and the solubility of these proteins increases when the pH increases, causing foam formation (Cano-Medina et al. 2011; Silva et al. 2014), especially in the high alkali environment as concrete. Besides, the degradation of the protein layers in the bacterial spore can cause localized air voids. Therefore, a high concentration of bacteria and organic compounds could create new voids inside the concrete structure, resulting in adverse effects on the hydration, thus retarding or even hindering the strength of concrete.

Among various nutrients such as lactose, glucose, corn starch, tapioca, and soybean meal, which could be used for bacterial biomineralization, yeast extract has the highest impact on decreasing compressive strength. The cement hydration could be delayed when culture addition is more than 0.85% of cement weight (Wang 2013; Wang et al. 2014). On the other hand, urea has a moderate effect on cement hydration, and calcium sources such as CaCl$_2$ can increase cement strength by accelerating the hydration process.

As mentioned above, in this study, low-nutrient medium was used to minimize the number of organic nutrients while maintaining the functionality and repeatability of the self-healing process over multiple cracking-healing cycles. Unlike previous experiments using protective encapsulation with the nutrient-rich medium, a technique using limited controlled nutrients in relevant environmental factors was investigated. Although *Bacillus subtilis natto* uses glucose as the most preferred carbon and energy source (Stülke and Hillen 2000), a large amount of glucose can impact concrete properties. The low-nutrient medium, which includes only lactose, urea, and calcium chloride (CaCl$_2$), could germinate the bacterial spores to grow with rapid multiplication in the early stage of concrete hardening. Theoretically, after the first cycle of cracking and healing, lactose and bacteria were converted to precipitated products and biofilms (Lindsay et al. 2006). Then, the remaining spores could survive in the unoccupied pores of LWA. For the next cracking cycle, the bacteria must use the remaining urea as a priority nutrient source to get the required carbon and nitrogen and convert it to CO$_3^{2-}$. The secondary CaCO$_3$ formed by urea needs to be more

![Fig. 1 (a) Self-healing mechanism activating *Bacillus subtilis natto* immobilized in LWA (Huynh et al. 2020); (b) Mechanism of bacterial biofilms formation.](image-url)
prolonged than the rapidly forming of primary CaCO$_3$ with lactose in the first cycle. Biominalization was possible with the precipitated products covering the dead bacteria and new spores despite the lack of enrichment medium. The self-healing process could be restarted several times, independently of cracking cycles. This technique is expected to reduce the required bacterial inoculum volumes and avoid potential damage to concrete properties caused by excess nutrients.

Figure 2 shows the strategy of experiment design and the hydration acceleration effect mechanism based on the growth of C-S-H layers on the bacterial calcite surface. Generally, when analyzing the crystalline structure, it could be found that Ca and O atoms in the surface layer of calcite show high similarities and match the CaO layer usually found in the structure of C-S-H (Bentz et al. 2015). Hence, that suggested that bacterial-based calcite could accelerate the cement hydration process to produce C-S-H, the main component of cement phase strength. Furthermore, immobilizing bacteria and the nutrient medium in LWA were expected to extend the bacterial lifespan and the crack self-healing repeatability. Also, reducing initial bacterial concentration and expensive organic components can reduce costs. This study reported the biomineralization process related to the hydration of cement, the self-healing capacity evaluated through compressive strength restoration, and the water capillary absorption through the cracks. A critical need is still a better understanding of the impacts that affect biomineralization rates and bacterial growth. Nevertheless, experimental results can improve the knowledge of self-healing concrete and provide the foundation for future research toward real-scale applications.

2. Materials and Experiments

2.1 Materials

This study used the gram-positive Bacillus subtilis natto bacteria of concentration $6 \times 10^8$ CFU/g obtained from Yuzo Takahashi Laboratory Co. (Yamagata, Japan). Powdered bacteria are bacterial starter spores and sugar (with lactose as the main composition). Microscopic observations show that the average size of the active cell is about 1-10 μm (rod-shaped), and the diameter of the spore is around 1-5 μm (sphere-shaped). The colonies have a grey-white round shape with wrinkled surfaces. The dosage of nutrient sources, including urea, CO(NH$_2$)$_2$, and calcium chloride were controlled with a safety limit according to ACI 318 for maximum chloride ion content in concrete ($m_{\text{urea}}$ and $m_{\text{calcium chloride}}$$\leq$0.45% by mass of cement and $m_{\text{bacteria}}$$\leq$0.5% by mass of cement) to prevent the corrosion of reinforcement.

In this study, the bacterial concentration was calculated to be $3 \times 10^7$ CFU for each concrete specimen (Table 1). As an essential preparation step, Bacillus subtilis natto was dispersed in water by stirring at 120 rpm for 24 hours to transform them into spore-form. Then these spores were mixed with urea solution and CaCl$_2$ to perform the bacterial solution. The pH of the lightweight aggregate is around 7. After immersing in the bacterial solution for 24 hours, the aggregate was taken out and cured in the air at room temperature. Also, LWA without bacteria was used as the reference.

Fig. 2 (a) General strategy of the study and experiment design; (b) Schematic diagram showing how bacterial calcite on C-S-H crystallization creates multi-layers and forms the crack healing materials.
2.2 Experiments

2.2.1 Bacterial activities and its bio-mineralization in concrete

The bacterial survival in concrete with LWA was tested for up to 2 years. Figure 3(a) describes the experimental flow chart for bacterial survival in concrete. After curing in water, the concrete specimens were ground into powder, diluted with nutrient solution, shaken, and cultured in the Petry dishes for colony counting. The results were then calculated and transposed to a Colony-forming unit (CFU) per 1 gram of concrete. Finally, survival rates were compared between the bacteria in LWA without nutrients, with low-nutrient medium (lactose) and nutrient-rich (yeast extract, peptone, nutrient broth). The bacterial concentration was also studied in three cases: non-crack specimen, one cycle, two cycles, and three cycles of cracking-healing. Detailed information about the cracking-healing cycle will be described in the next part about multi-cycle strength restoration and repetitive self-healing.

Figure 3(a) summarizes the steps of bacterial counting and material collecting to demonstrate the survival of bacteria in concrete specimens. The bacterial concentration was determined and calculated through the CFUs counting method. This method also can be used to confirm the survival of bacteria. Cell viability can be determined simply by the ability of the bacteria to reproduce by growing bacteria on agar plates, then placing them under a microscope. While looking under the microscope, the counting process was processed to determine how many colony-forming units were present.

Bacterial concentrations over different positions on the crack surface were performed to study the distribution of bacteria and their activities for biominalization after release from the broken LWA by the crack. Bacterial spores and nutrients (lactose, urea, and CaCl₂)
were immobilized in LWA. First, concrete specimens (40×40×40 mm) with one LWA particle placed in the center were prepared. After 7-day curing in water, specimens were applied load in a 3-point bending test for crack creation. Then, the materials on the crack surface were collected and used for the bacterial concentration measurements via colony counting. Other cracked specimens were cured in 1, 3, 7, and 14 days for the healing process before being taken out for the following materials collection. Then, the second cracking was carried out, and the materials in the re-crack surface were collected. **Figure 3(b)** shows the different positions for bacterial concentration determination on the crack surface of the concrete specimen, which was cast with only one LWA pellet, and the positions where materials on the surface were collected. Actual photos of the crack surface with marked positions are shown in the bottom part of **Fig. 3(b)**.

### 2.2.2 Multi-cycle strength restoration and the repetitive self-healing

The multi-cycle compressive strength restoration test followed the steps in **Fig. 4**. Long-term tests (28, 60, and 90 days) and short-term tests (7, 14 days) were conducted to clarify the effect of late hydration. The cracking-healing cycle with cylinder specimens (φ=50 mm; H=100 mm) was tested for the compressive strength recovery experiment. Each cycle includes the compression test and 7-day curing in water. The first visible cracks usually appear and develop at around 50% of compressive strength. For favorable observation, the load to introduce cracks was defined by 90% of the compressive strength of the "trial specimen". All specimens at the same cracking age were then compressed under the same load (loading rate was kept at 1.17 kN/s). Repeat these processes with the corresponding value of load for the other cases of cracking-day (14, 28, and 60 days). Also, standard procedures are being developed to determine self-healing efficiency. Hence, self-healing capacity was estimated by calculating the strength restoration rate and the crack area reduction ratio. Crack-healing was performed with the cracking ages of 7-90 days to evaluate the self-healing repeatability. For phase composition, crack closure was visualized using optical microscopy with microstructure analysis by SEM and x-ray diffraction (XRD).

### 2.2.3 Microstructure analysis

The microstructure of LWA was observed under a scanning electron microscope (SEM, Hitachi S-4800 FE-SEM) with energy-dispersive x-ray spectroscopy (EDS). The visualization of crack closure was taken with microstructure analysis by SEM/EDS and x-ray diffraction (XRD - Bruker D8 ADVANCE) for phase composition. Pieces of broken LWA and pieces of concrete on the crack surface were collected for SEM/EDS analysis. Small pieces (around 1 cm) of the concrete specimen were taken out and then observed under SEM with EDS mapping function for chemical elements detecting. After drying, the specimen
surface was covered by a thin gold film, and a voltage of 3 kV was applied. Other concrete pieces in the same concrete specimen were collected and ground into a fine powder (passing the 45 μm sieve) for XRD analysis to determine the phase composition and crystal structure of unknown healing materials found within the cracks.

2.2.4 Water permeability and visual evaluation of crack healing
Water can penetrate easily into the concrete structure through the crack. Therefore, the capillary water absorption test (Fig. 6) was carried out to evaluate the self-healing effect on preventing the water. Seven days after casting, concrete specimens in two groups with and without bacteria were taken for crack creation. First, the crack was created under the 3-point bending test, ranging from 0.4-0.7 mm. Then, cracked specimens were measured for water absorption before immersing them to cure in water for 3 and 7 days. Capillary water absorption test used modified total experimental times and measuring intervals from ASTM C 1585 (ASTM 2013) The bottom surface exposed to water was sealed with aluminum tapes except for 10 mm on both sides of the notch to ensure uniaxial water uptake through the crack. The specimens were immersed in distilled water at 3±1 mm during the test.

Two groups of concrete specimens (40×40×40 mm) with bacteria without bacteria as reference were prepared. After casting, specimens were cured continuously in water. The 28-day age specimens were taken for the 3-point bending test for crack creation to remove the late hydration of the complete bacterial self-healing process, including the natural healing phenomenon. Cracks were fixed to 0.5 mm wide by metal wire inside and covered by a rubber band before immersing back into the water for 14-day healing.

A digital microscope was used to quickly check and track the self-healing process in the curing room or the experimental workshop. Then, a multi-function microscope was used with measurement software for detailed observation. Crack width and crack length were then measured, while the crack area was calculated by the image-pixel-counting method (Stefanidou et al. 2021). Next, values calculated before and after self-healing were compared to evaluate the healing capacity. Finally, stereo optical microscope observation of crack healing and the binary-image analysis were compared to get as much accuracy as possible when estimating the crack healing rate. In this part, an essential advantage of using microscopy is assessing the degree of self-healing by combining photomicrography with quantitative image analysis with substantial help from the computer.

3. Results and discussion

3.1 Bacterial calcium carbonate formation and stimulating ability on the formation and development of hydrated cement minerals
From 1 to 14 days, bacteria gradually migrated from the core (LWA) to the outside of the specimen. This process resulted in a gradual decrease in the bacterial concentration in the center of the crack, while the outer part gradually increased as seen in Fig. 7(a). Finally, the bacteria concentration after 14 days was almost uniform on the entire crack area, corresponding to CaCO₃ formed covered this surface. However, there was still a significant amount of bacteria left; they turned into spores and hid under this layer of CaCO₃. When subsequent cracks appeared and exposed spores to the environment, these spores could be reactivated, divided, and triggered growth. As a result, the bacterial concentration increased at the core of the crack in the LWA pellet. SEM/EDS analysis studied the massively precipitates formed inside the LWA. The result revealed calcium, oxygen, and carbon [Fig. 7(b)], while reference specimens did not show them. The concrete specimens containing bacteria were tested using x-ray diffraction to confirm the mineral composition. Besides, due to the formation of not well-crystallized CaCO₃ in the condition of poor nutrient source, different morphology of precipitated products could be obtained, which differ from the typical rhombohedral shape of calcite.
From SEM images, the forming of healing products did not occur uniformly in the entire volume of the LWA. Figure 8 with EDS and EDS-mapping shows a continuous and homogenous layer of materials covering the bacterial cells on the broken LWA. This layer filled the porous zones of the LWA and overflowed across the aggregate interface and the hydrated cement matrix. On the other hand, particular zones (“Empty” zone) deep inside LWA retained their high porosity with almost no bacterial healing products, which should be available for bacteria to take place and maintain their survival under spore-form. These unfilled porous positions were expected to be ready to release the bacteria for the other cracking-healing cycles.

The SEM micrographs from healed-crack surfaces (Fig. 9) showed the unclear border of the interaction zone between precipitated crystals and the surrounding zones due to a layer of cement hydration products (C-S-H, ettringite, portlandite), which connected the original concrete substrate and the healing products. Note that the white arrow in Fig. 9 indicates the bacterial footprints, and the white dot-line rectangular shows the rod-shaped healing product. The dash-curve means the hydrated-cement minerals formed in combination with bacterial CaCO$_3$ in the healing precipitations. The extracellular growth produced by the microorganism was expected to contribute more to the strength of concrete. Even dead cells or live cells without any nutrient source can remain in the matrix as organic fibers. These fibers were demonstrated to improve the strength of early-age specimens (Ramachandran et al. 2001). When bacterial cells were incorporated into the cemented material, they grew after taking up the nutrients from the pores and the surrounding area. Calcium carbonate was precipitated both on the cells and within the cement matrix. Amelioration of compressive strength might be
explained by precipitated calcite around bacteria incorporated as fiber-like aggregates in the cement matrix (Nain et al. 2019; Reddy et al. 2012; Rao et al. 2013) to enhance the cement matrix strength as a complex fiber-reinforced compound or fill crack volumes. Also, the mutuality of CaCO₃ and hydrated cement minerals such as C-S-H and ettringite could make the material robust into cracks with solid bonding with the crack border. This result also provides information on the high efficiency of self-healing ability in concrete whose crack age is less than 28 days. The late hydration of cement particles can still support healing products. These effects could be explained by the phase composition of the healing products on the XRD analysis of the healed-crack surface.

Figure 10(a) shows the XRD analysis of materials collected from concrete specimens with different situations (uncracked, cracked) to clarify CaCO₃ precipitation conditions. The almost absence of CaCO₃ in non-crack reference specimens was predicted. However, a weak signal of CaCO₃ could be obtained in cracked reference specimens, mainly caused by the natural carbonation process when water ingresses the crack. In this case, the average crystalline domain size of calcite estimated by Rietveld refinement is about 42.4 nm. Then, a small peak of CaCO₃ can also be seen in the non-crack specimen with bacteria. Although this specimen was not cracked, a small amount of bacterial healing agents could be released in the early stage of concrete to heal the micro-cracks, which many reasons could cause. The calcite peak intensity resulting from the biomineralization is higher than the specimen without bacteria and much sharper, which is reflected by a more considerable amount and a large average crystalline domain size (around 179 nm). The materials within the healed crack revealed that phase composition included portlandite, ettringite, C-S-H, aragonite and calcite [Fig. 10(b)]. Note that small kaolinite peaks and magnesite peaks can be found come from the composition of the LWA. These components were subtracted when calculating the phase composition of minerals.

As shown in Fig. 10(b), C-S-H could be the main mineral at the early ages of crack (from 1 to 7 days) compared to the other components. Primary CaCO₃ was formed in the favorite polymorph calcite with a small size (baseline at position 2θ =29°), and portlandite still shows a high concentration. Then, phase composition changes as calcite increases and portlandite decrease over the increase of the cracking age. Aragonite could be found in a small amount in the precipitated products, indicating the difference in chemical reactions and bacterial activity [Figs. 10(b) and 11(a)]. With bacteria, the additional formation of C-S-H can be assumed by the role of nucleation sites. Bacteria can affect the hydration...
products, promoting a more uniform distribution of the silicate phases in the hydrated cement matrix. As a result, a denser C-S-H gel can be observed in Fig. 9, which leads to a more compact microstructure that can reasonably explain strength improvement and restoration. Also, reactions between Ca\(^{2+}\) bound in the bacterial cell wall with Al\(^{3+}\) and other anions could lead to the formation of more ettringite. These results are comparable with research findings (Torres et al. 2013; De Muynck et al. 2008; Huang et al. 2020; Xu et al. 2014; Nguyen et al. 2016; Huynh et al. 2017), where calcite, aragonite, and vaterite could be precipitated in different proportions by microorganisms include Bacillus bacteria. Besides, a similar self-healing reaction as the hydration acceleration effect can occur when crystalline admixture helps to increase calcium silicate hydrate (C-S-H) density, then forming water-insoluble pore/crack-blocking deposits (Xue et al. 2020). For 14-day or later-age cracks, although there is still C-S-H inside the healed crack, this C-S-H was formed at the late stage of concrete, in which the amount of unhydrated cement particles is not enough for the high-crystalline structure of C-S-H. Also, the primary healing product, in this case, is calcium carbonate. Therefore, it can be seen that the late-age C-S-H was formed on the surface of calcite crystals to create a mix of healing products. Then, at 14-days, C-S-H can reach the highest content [maximum value in Fig 11(b)], and Portlandite lowered down to the minimum content [minimum value in Fig 11(b)].

Moreover, the presence of aragonite and the absence of such precipitates in the reference specimens clarified the precipitated CaCO\(_3\) caused by bacterial biomineralization. Also, the biological mineral CaCO\(_3\) is generally more resistant to solubility (Abo-El-Enein et al. 2012) than the one formed by inorganic precipitation. The bacterial footprints confirmed the possible bacterial activity of mineralization. Note that the ettringite formation was significantly higher from the age 7-day of crack. At the later age of the crack, the healing materials can be found with calcite and aragonite as the main components. That means late hydration almost stopped its effect on the healing process. This phenomenon could lower concrete strength restoration when the crack appears 28 days or later. One of the critical factors of self-healing is that CaCO\(_3\) precipitation on the crack interface may create a robust interfacial bonding with the original concrete surface. Unhydrated cement particles and the carbonation of calcium hydroxide can only cause tiny cracks, and the healing effect decreases with the age of specimens (Dong et al. 2021). Although the bonding between healing products in late-age cracks can not reach the value of early-age cracks, biomineralization, which occurs layer by layer from the concrete substrate to the empty crack volume, may considerably reduce the crack width and prevent water ingress. Ghosh et al. (2009) and Biswas et al. (2010) found that a silica-leaching bacterial protein formed additional silicate hydrates in the cementitious matrix that fills the micropores, increasing the strength. For the current extrinsic methods, the ability to repeat and maintain the self-healing effect is an issue that needs to be solved. For example, when using hollow-tube systems in concrete, it is possible to inject repair materials from outside when needed. However, it will be easy to clog and have difficulty in the second crack (or more) onwards (if any). In the case of using short-tubes, tablets/pellets containing a chemical or mineral solid, the effect may be high and immediate, but the repeatability is limited.

When the chemical reactions form the healing products, the concrete structure will change from the original, resulting in the difficulty of the subsequent healing process. That is not considered the adequate time for these chemicals or minerals in concrete; how long can it maintain when cracks appear late or very late in the life cycle of the structure. As for the intrinsic method, the most significant advantage is that self-healing is possible without external intervention. However, it is impossible to have a high self-healing effect for all specific conditions or structures. Actual conditions will lead to cracks appearing in mass with different scales, patterns, positions, and morphology throughout the concrete structures, which can easily lead to uneven results or

![Fig. 11](image_url)

**Fig. 11** (a) The phase composition of minerals in the healing product obtained by XRD analysis software; (b) Mineral composition and the relationship between mineral components.
even delay the “feedback” of healing agents to the cracks. In the group, polymers were used as the carriers in the immobilization technique, resulting in a not-fully or low-level breakage rate of the capsules. As a result, the release of healing agents was not high. Other groups of bacteria are immobilized with whole nutrient sources, including many organic compounds; organic matter can be suitable for the activities of bacteria but can cause adverse effects on the hardening and strength development of concrete. Also, the high cost of these nutrients makes the possibility of the bacterial-based self-healing technique for real applications more challenging.

3.2 Bacterial survival rate and the protecting effectiveness of LWA

Without protection, almost no bacterial cell could be found after two months in concrete as seen in Fig. 12(a). According to the previous study (Jang et al. 2020) about *Lysinibacillus boronitolerans* YS11 (non-ureolytic bacteria) and *Bacillus alkaliphilus* AK13, the bacterial survival when adding directly to concrete mix decreased rapidly through the concrete curing process. Also, in this study, with the nutrient source including calcium lactate, and yeast extract, after 28 days, only 0.002-0.006% of live bacteria could be found.

As shown in Fig. 12(b), the more cracking times, the more cracks form the decrease in bacterial concentration. After three cycles of cracking by compression and curing for healing in water, the remaining number of *Bacillus subtilis natto* cells in 1 g of the concrete specimen was 10⁵ CFU/g from the initial concentration of 4×10⁶ CFU/g. In one cracking-healing cycle, the bacterial survival was 10⁴ CFU/g; for non-crack specimens, the survival bacteria was 10⁵ CFU/g after 180 days. For the later concrete age, seven months, specimens showed the result of 6×10⁴ CFU/g and kept in around 10⁵ CFU/g for two years, which suggests that the bacteria could maintain their life for years. The bacterial survival rate was significantly higher than other treatment methods with polymer-based micro-capsules. For the protection performance of LWA, over 70% of the initial bacteria could survive and keep CaCO₃ precipitation. Alkaline bacteria were expected to lie dormant in the concrete for up to 200 years (Akın 2022; Jones et al. 2022; Ylmén et al. 2009) before activating for crack healing. Using just 20% volume of LWA for bacteria that activated when the crack appeared, the bacteria that did not join the biomineralization could take place in these porous zones and wait for the subsequent cracking-healing cycles.

Although *Bacillus subtilis natto* uses glucose as the most preferred carbon and energy source (Stülke and Hillen 2000), a massive amount of glucose can impact concrete properties. When using low-nutrient medium, lactose can be the effective enrichment medium for bacteria growth, while urea and CaCl₂ are the biomineralization media. Suitable lactose can germinate the bacterial spores to grow in the early stages of concrete hardening with rapid multiplication. After the first cycle of cracking-healing, bacteria were converted to precipitated products and biofilms (Lindsay et al. 2006). Then, the remaining spores could save their life in the unoccupied pores of LWA. For the next cycle, the bacteria must use the remaining urea as the priority nutrient and convert it to CO₃⁻. The secondary CaCO₃ formed by urea needs a longer time than the rapidly forming of primary CaCO₃. Biomineralization was possible despite the lack of the enrichment medium, with the precipitated products covering dead bacteria and new spores. The self-healing process could restart several times, independently of cracking cycles.

3.3 Multi-cycle strength restoration and the repetitive self-healing

Figure 13 summarizes the compressive strength restoration rate of the concrete specimens with LWA immobilized bacteria, and the reference specimens range from 7-60 days for cracking. The data tend to lean much below the mean, reflecting that regular concrete without bacteria almost cannot restore the compressive strength over multi-loading as seen in Fig. 13(a). In contrast, all the values over 50% as seen in Fig. 13(b) and the data tend to lean much above the mean (except the third cycle). The gap between the highest and lowest values is less than the reference, which indicates the steady bacterial biomineralization in repairing the damaged structure, compared to the severe damage of the regular concrete after multi-cracking cycles.
Figures 13(c) and (d) show that although the late age of cracks could lead to a low strength restoration rate, CaCO₃ and ettringite, as demonstrated by XRD, can partially recover the strength. However, late cracks could not be healed by combining CaCO₃ and other cement minerals such as C-S-H at the early crack with the substantial help of cement continuous hydration. In addition, a significant difference can be obtained when comparing the reference and specimens with bacteria in LWA. At each cracking age, especially after 28 days, the bacterial concrete specimens got a significantly higher restoration rate after the 3 cycles of cracking than the reference specimens.

The best self-healing performance in strength restoration could be achieved under underwater curing conditions for one week when bacteria with low-nutrient medium were immobilized in LWA. This curing time is not longer than using the nutrient-rich organic calcium source (Jonkers 2007; Jonkers et al. 2010; Jonkers and Schlangen 2008), in which the early strength of concrete was slightly decreased (Singh et al. 2020) and inadequate data on repeatability for multiple cracking cycles (Souradeep and Kua 2016). However, low-nutrient medium was enough for CaCO₃ precipitation when cracks appeared without decreasing early compressive strength. Furthermore, the water absorption of LWA under wet conditions can provide a portion of water to the internal cracks for bio-mineralization under dry conditions.

The multi-cycle of cracking could cause new cracks initiation or re-create the original cracks propagation (Fig. 14). Discrete cracks and pores might join to form healing products transport networks for self-healing. When the crack is almost starting from the weakest point of the concrete specimens, complete healing reinforces this region, and it is regularly that the new crack begins at a position corresponding to the next weakest region in the specimens. Again, new cracks were formed during the multi-cycle of cracking. However, this time, at least after the third cracking cycle, they did not merge completely with the previous cracks, suggesting that the damage was healed with considerable compressive strength after three cracking-cycle.

3.4 Water permeability and visual evaluation of crack healing

After 7-day curing, the reference specimen shows the water penetrated through the unhealed crack up to the top of the specimen. After 30 minutes of testing [Fig. 15(a)], during the specimen with bacteria, the water level penetration did not exceed more than 0.5 cm through the crack, which was partially reduced in width and length [Fig. 15(b)]. The crack became filled with
water 1 minute after the reference specimen contact with water [Fig. 15(b)]. The zone around the crack at the height became filled with water soon. In contrast, precipitated products from bacteria released from the LWA acted as a water barrier. Previous studies (Hong and Choi 2017, 2018; Shim et al. 2018) indicated that the number of healing products was minimal compared to the testing specimen size. However, these materials prevent the water from continuing to penetrate the specimen. A similar result can be obtained in this study. Outlooking of the crack shows that it was not healed completely; however, the internal structure of the crack might be changed, resulting in a small value of water absorption. The self-healing process can reduce water absorption, while the crack volume may not be filled. With the development of crystalline healing products by bacterial metabolism in include CaCO3 and hydrated minerals, as discussed above, these materials could be massive and thicker over time.

The initial sorptivity of concrete specimens can be affected by various crack factors, including the width, length, and surface roughness (Snoeck et al. 2016; Shim et al. 2018). These factors were almost not controllable in the testing specimens. Therefore, it was logical to evaluate self-healing performance by comparing the reduction ratio of the initial absorptivity at each curing period (3 and 7 days) rather than comparing the absolute values of the progressive absorptivity measurements. With the precipitation of CaCO3 inside the cracks, the water absorption significantly decreased after 3 and 7 days of curing. It almost returned the initial value as the original specimen before cracking in both the case of 3 day curing and 7 day curing. This result indicates that the amount of the healing product became stable after rapidly increasing the formation of crystalline materials. At the same time, there is a weak reduction in two curing periods of reference specimens (Fig. 16), although the late-hydration and natural carbonation can help to heal the small cracks (around 0.1 mm). An enormous gap in the water absorption curves between the cracked specimen with LWA immobilized bacteria and the reference specimens help demonstrate the self-healing effect. The curing period in water gave the conditions for bacteria to be activated for biomineralization. As analyzed about microstructure, healing products precipitated from the internal of the specimen to connect two concrete crack surfaces and joined them. Although 7 day curing could not lead to a complete healing crack in the surface of concrete specimens, it still prevented the water from being absorbed inside the crack. It suggests that a longer curing time may be necessary for a higher healing effect through a more significant amount of pre-
cipitated products. Also, a nutrient supplement can be an accelerating factor, promoting the healing process for large-size cracks.

4. Conclusions

*Bacillus subtilis natto* can form CaCO₃ via the urea hydrolysis pathway under low-nutrient conditions. Additionally, calcite, aragonite, C-S-H, and ettringite with more robust mechanical properties were formed in the healing materials, indicating the features characteristic of this type of bacteria. Therefore, the following conclusions can be drawn from this study:

1. The possibility of the bacterial surviving (for at least two years) and maintaining the bio-mineralization for a long time (for at least one year at the concentration of 10⁴ CFU/g of concrete) was demonstrated using a non-significant reduction in concentration compared to other protection methods.

2. *Bacillus subtilis natto* in spore-form with low nutrients to study the repeatable bio-mineralization under harsh conditions demonstrated that the bacteria could adapt almost immediately when cracks appear.

3. The massive amount of self-healing products that could be observed using SEM/EDS, and optical microscopic images were essential in closing the cracks and densifying the concrete structure. Besides, XRD analysis confirmed the formation of CaCO₃ as the main component in the healing products together with high amounts of hydrated minerals such as C-S-H and ettringite.

4. Complex composite minerals were formed within the cracks, resulting in a high level of strength restoration. Also, surface cracks around 1.5 mm could be healed after 21 days, while the internal cracks were closed little by little over the curing time, while it is needed just two weeks to heal cracks around 0.5 mm.

5. The capillary water absorption test shows the high effect of bacterial healing products on preventing water from penetrating the cracks. Furthermore, after 7-day curing, absorption could almost return to the value of the original concrete without cracks. These experimental results add to understanding the bio-mineralization of *Bacillus subtilis natto* in self-healing concrete.

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