

## Application of Antifungal CFB to Increase the Durability of Cement Mortar

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**Antifungal cement mortar or microbiological calcium carbonate precipitation on cement surface has been investigated as functional concrete research. However, these research concepts have never been fused with each other. In this study, we introduced the antifungal calcite-forming bacteria (CFB) *Bacillus aryabhatai* KNUC205, isolated from an urban tunnel (Daegu, South Korea). The major fungal detriogens in urban tunnel, *Cladosporium sphaerospermum* KNUC253, was used as a sensitive fungal strain. *B. aryabhatai* KNUC205 showed CaCO<sub>3</sub> precipitation on B4 medium. Cracked cement mortar pastes were made and neutralized by modified methods. Subsequently, the mixture of *B. aryabhatai* KNUC205, conidiospore of *C. sphaerospermum* KNUC253, and B4 agar was applied to cement cracks and incubated at 18°C for 16 days. *B. aryabhatai* KNUC205 showed fungal growth inhibition against *C. sphaerospermum*. Furthermore, *B. aryabhatai* KNUC205 showed crack remediation ability and water permeability reduction of cement mortar pastes. Taken together, these results suggest that the CaCO<sub>3</sub> precipitation and antifungal properties of *B. aryabhatai* KNUC205 could be used as an effective sealing or coating material that can also prevent deteriorative fungal growth. This study is the first application and evaluation research that incorporates calcite formation with antifungal capabilities of microorganisms for an environment-friendly and more effective protection of cement materials. In this research, the conception of microbial construction materials was expanded.**

**Keywords:** Antifungal, calcite-forming bacteria, *Cladosporium sphaerospermum*, *Bacillus aryabhatai*, crack remediation, water permeability

colonization on the building surface [4, 23]. In particular, destruction of the rock or cement structure is processed by the acidic metabolite from the growing tip of fungal hyphae and physical distortion due to penetration of hyphae into the cement structure [5, 13]. The potland cement that was commonly used as construction material, Ca(OH)<sub>2</sub>, gives unique compressive strength to cement mortar. However, the chemical reaction between Ca(OH)<sub>2</sub> with environmental carbon dioxide (CO<sub>2</sub>) or vapor can lead to neutralization of the cement surface (lower than pH 10.0) [19]. This phenomenon can cause surface cracking or reduction of compressive strength and can lead to microbial colonization on the surface of cement [19, 24]. Fungal growth is a major cause of deteriorative reaction (biodegradation), so fungal growth on cement cracks have to be prevented [4, 23]. To prevent these microbially induced biodegradation, application of an antimicrobial agent like azole, carbamate, silver, copper, zeolite, and zeolite carbon has been researched [3, 5, 17]. However, the antifungal properties of antifungal-activated cement mortar (AACM) have been tested against not purely isolated/identified milk-derived fungi [17].

Independent of this trend, promising microbial construction materials like calcite-forming bacteria (CFB) have been suggested as environment-friendly biomaterials, and application of CaCO<sub>3</sub> precipitation in cement structure as a promising field for further research [2, 7, 19, 20]. In this context, application of microbiologically induced calcite precipitation (MICP) to the cement structure or monumental stones for the purpose of crack remediation have been attempted and then evaluated with diverse architectural assays or for the purpose of strength improvement [16, 21, 22, 24]. Furthermore, biodeposition can decrease the permeation properties of mortar or cement surface [2]. Specifically, calcium carbonate deposition on the surface of the mortar can lead to reduction of water permeability. Cement, which has low permeability, lasts longer without exhibiting signs of distress and deterioration [10]. Crack remediation using MICP or antifungal cement mortar has been researched separately

Discoloration of a building structure is caused by natural oxidation of itself or by specific pigments from microbial

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even though antifungal activity derived from bacteria is well known in diverse microbial research [6], and isolation and application of antifungal calcite-forming bacteria on cement mortar has never been attempted yet.

In this research, an antifungal calcite-forming isolated from an urban tunnel [14] was used for crack remediation and fungal growth inhibition on mortar cracks and then evaluated. This antifungal calcite-forming showed antifungal activity against the species *C. sphaerospermum* KNUC253, revealed as a major fungal deteriogen in the representative building structure [14]. Moreover, *B. aryabhatai* KNUC205 showed water permeability reduction ability on cement mortar pastes.

## MATERIALS AND METHODS

### Microorganisms and Culture Media

We used an urban tunnel-derived bacterial strain *Bacillus aryabhatai* KNUC205, which was reported to precipitate the  $\text{CaCO}_3$  crystals in urea- $\text{CaCl}_2$  agar medium and also have antifungal activity [14]. *Rhodococcus erythropolis* KNUC201, which shows no antifungal activity [14] was used as a negative control for fungal growth inhibition on cement mortar surface. These bacterial strains were subcultured in TSB (tryptic soy broth, Difco, USA) agar medium and incubated at 30°C for further assays. To assay the  $\text{CaCO}_3$  precipitation or antifungal activity, B4 agar medium was used. The sensitive fungal strain was *C. sphaerospermum* KNUC253, which is reported as a major fungal deteriogen [14]. *C. sphaerospermum* was subcultured on PDB (potato dextrose broth) agar medium and incubated at 18°C, the optimal growth temperature of each fungal strain.

### Selection of B4 Medium to Verify Fungal Growth Inhibition

Up to now, urea- $\text{CaCl}_2$ , B4, M-3, M3-P, CC, and SF<sup>b,c</sup> media have been used for assaying MICP [3].  $\text{CaCl}_2$ ,  $\text{Ca}(\text{NO}_3)_2$ , and  $\text{Ca}(\text{CH}_3\text{COO})_2$  have been used as the calcium source for microbial crack remediation along with treatment of urea solution [24]. However, fungal deteriogen cannot be cultured in urea- $\text{CaCl}_2$  media or urea-containing calcium media (data not shown), so we needed to select a new medium to verify the growth inhibition on mortar surface when treated with *B. aryabhatai* KNUC205. In this research, B4 medium was selected via calcite formation assay of *B. aryabhatai* KNUC205, and hyphal growth or spore germination of *C. sphaerospermum* KNUC253 on B4 agar medium. It was reported that B4 media never show lower efficiency than urea- $\text{CaCl}_2$  media [1]. B4 agar plates (yeast extract 4 g, glucose 5 g, calcium acetate 15 g, distilled water 1,000 ml) were prepared with 1% (w/v) agar concentration for spore germination. Calcium acetate and the other constituent of B4 agar were autoclaved separately to prevent agglutination and precipitation between calcium acetate and after media components. Hyphal growth and spore germination on B4 medium were tested. Prepared spores of *C. sphaerospermum* KNUC253 in  $10^5$ – $10^6$  spore/ml was added to molten B4 agar plates, and then incubated at 18°C in dark condition.

### $\text{CaCO}_3$ Precipitation on B4 Agar Medium

To identify the  $\text{CaCO}_3$  precipitation on B4 agar, *B. aryabhatai* KNUC205 was streaked on B4 agar medium and incubated at 30°C

for 5 days. Crystals of  $\text{CaCO}_3$  were visualized by the optical microscope (Sw 804425, Samwon, Seoul, Korea) and illumination system (light solution LS-100W). Digital images were captured by a Zentech digital camera [15].

### Preparation of Cement Mortar Pastes

For assaying the crack remediation, fungal growth inhibition, and water permeability reduction, cement mortar paste were made by mixing aliquots of distilled water with cement in a 0.4 water/potland cement mortar weight ratio and poured into a 35 × 10 mm style petri-dish (Falcon, Becton Dickinson Labware Co., USA), and then cured for 72 h [15].

### Neutralization of Cement Mortar

A modified method was designed for neutralization of cracked cement mortar pastes. Cracked regions and surface of cement mortar pastes were brushed with sterile cotton and clearly washed off with sterile distilled water (SDW). The cleaned pastes were loaded in a glass flask and cooled at –80°C. The flask was filled with saturated  $\text{CO}_2$  gas (Korea Standard Gas Co., Korea), sealed with a rubber stopper, and autoclaved at 121°C, 15 lb condition to accelerate the rate of reaction between the  $\text{CO}_2$  gas and cement disk surface. All steps were repeated 10 times. Neutralization of the cracks was confirmed with pH paper (Panoehaolus, MicoScience Co., Germany). Growth of fungi on the surface and cracks of cement pastes was tested using spores of *C. sphaerospermum* KNUC253 in 500 µl of PDB, concentration adjusted to  $1 \times 10^5$ – $10^6$  spore/ml, by spraying on the surface of the cement disc.

### Crack Remediation on Mortar Crack

*B. aryabhatai* KNUC205 was assayed for its ability to remediate cement mortar cracks. Cement mortar pastes were made as described above. Cracks were made by artificial forces. Crack width was measured by optical microscopy (×10), and cement pastes with cracks of 0.3 mm width were selected for further experiments [15]. A single colony of *B. aryabhatai* KNUC205 was inoculated on 5 ml of B4 liquid medium and precultured at 30°C for 18 h. Then 250 µl of culture suspension of *B. aryabhatai* KNUC205 was mixed with the same volume of B4 liquid medium and treated into mortar cracks. Thereafter, B4 liquid media were treated into mortar cracks every 12 h intervals. A mixture of *Escherichia coli* K12 and B4 liquid medium alone was used as negative controls. All samples were incubated at 30°C for 24 h. This procedure was repeated four times over 5 days at 24 h intervals. After 5 days, the images were taken by a Zentech digital camera [15].

### Water Permeability

The treatment of *B. aryabhatai* KNUC205 on cement mortar pastes was completed as described below. *B. aryabhatai* KNUC205 was inoculated on 50 ml of B4 liquid medium and incubated at 30°C for 24 h at 180 rpm. Cultural suspension was poured on a 1,000 ml volume sterilized pot containing the six cement mortar pastes and 400 ml of sterilized B4 liquid medium. These samples were incubated at 30°C for 9 days at 50 rpm. Treated cement mortar pastes were dried at 55°C in a ventilated dry-oven for 24 h, until a constant weight was obtained ( $W_1$ ). After that, these pastes were immersed onto the SDW and its weight measured ( $W_2$ ). Pastes were removed from SDW and weighed after eliminating the surface water using sterile moisture tissue [10]. The cement mortar pastes treated

with *E. coli* K12 and B4 liquid medium were used as negative controls. The water permeabilities were then measured using the formula for absorption and bulk specific gravity of stone, KS F 2518, as described by the Korea Standard Association [10].

$$N (\text{water permeability}) = W_2 - W_1 / W_1 \times 100$$

### Fungal Growth Inhibition on Mortar Crack

A mixture containing spores of *C. sphaerospermum* KNUC253, *B. aryabhatai* KNUC205, and B4 agar medium was inoculated into cracks. To make the mixture, cell culture of *B. aryabhatai* KNUC205 and spores were prepared as described below,

1) *B. aryabhatai* KNUC205 was precultured in 10 ml of TSB at 30°C for 18 h using a rotary shaker, and then 2 ml of culture was centrifuged at 4°C, 1,000 rpm. Supernatant was discarded and cell pellets were washed 3 times with sterilized distilled water. 2) Concentrations of spore were adjusted to  $10^5$ – $10^6$  spore/ml. The control and experimental groups of this assay were divided into three groups. For fungi growth inhibition assay, (i) B4 agar, (ii) fungi spore with B4 agar, (iii) mixture of fungi spore/*B. aryabhatai* KNUC205 with B4 agar, and (iv) mixture of fungi spore/*R. erythropolis* KNUC201, which has no antifungal activity, with B4 agar controls were established. To assay the fungi growth inhibition, cement mortar pastes were made as described above, and then cured for 72 h. Cracks 2 mm deep and 0.5 mm wide on the cement mortar pastes were made using a compass saw. Neutralization of cement mortar was done by a newly designed method. All mixtures were inoculated and incubated at 18°C for 16 days. All fungal growth inhibition tests were triplicated. The images were captured by a digital camera (Lxus 70; Canon inc, Tokyo, Japan).

### Preparation of Spore Suspension

For harvesting the conidiospore of newly isolated fungal deteriorogen *C. sphaerospermum* KNUC253, V8 juice agar medium [300 ml of V8 juice (Campbell Soup Company, Camden, NJ, USA), 4.5 g of calcium carbonate (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA), and 15 g of Bacto agar (Becton, Dickson and Co., Sparks, MD, USA)] was used [12]. The strain was incubated at 18°C under dark condition for 3 weeks. Spore formation was identified using an optical microscope (Sw 804425; Samwon, Seoul, Korea). Subsequently, 5–10 ml of sterilized 0.1% (w/w) Tween 80 (Junsei Chemical Co., Ltd, Japan) was inoculated onto the fungal colony, and then scrubbed by a sterile plastic inoculating loop. Obtained spore suspension was vortexed vigorously, and then centrifuged at 12,000 ×g, 4°C for 15 min. Subsequently, pellets were washed with SDW two or three

times, and then its concentration was adjusted to  $1 \times 10^5$ – $10^6$  spore/ml using a hemacytometer (Marienfeld Co, Germany) [18]. For the storage of spores, the obtained spore suspension was centrifuged at 12,000 ×g, 4°C for 15 min, and then the supernatant was eliminated. The pellet was resuspended in 20% (v/v) sterile glycerol solution and refrigerated at –80°C. Contamination of the spore suspension was examined by incubation using NB (nutrient broth, Difco, USA) or TSB agar media in a 25°C chamber.

## RESULTS AND DISCUSSION

### Selection of B4 Medium

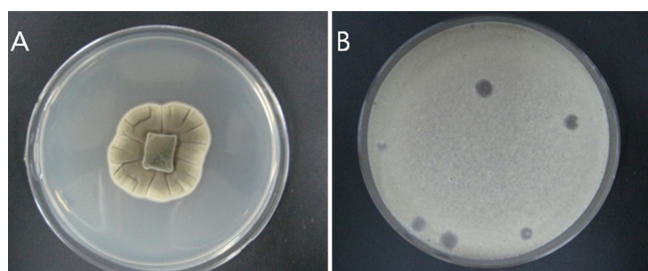
Up to now, crack remediation with MICP and development of antifungal-activated cement mortar have been researched separately so that calcium carbonate precipitation and antifungal activity were not verified simultaneously. Spore germination of fungal strain was identified within 96 h. Hyphal growth of the sensitive fungal strain was identified after 2 weeks of incubation on B4 agar medium under the same conditions (Fig. 1).

### CaCO<sub>3</sub> Precipitation on B4 Agar

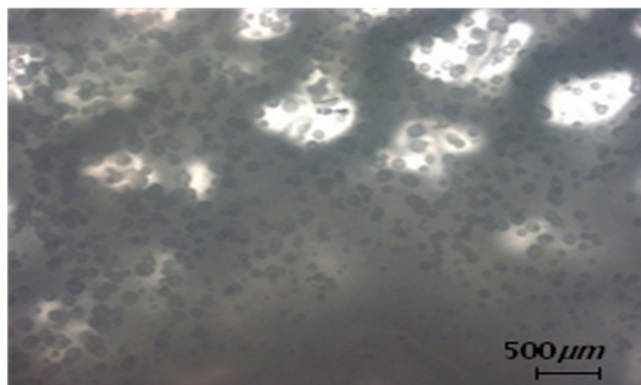
*B. aryabhatai* KNUC205 showed CaCO<sub>3</sub> precipitation (Fig. 2). Crystallizations were observed only inside the bacterial colony.

### Remediation of the Cracks on Cement Mortar

Cracks of concrete building structure can lead to acceleration of the neutralization process or enlargement of cracks by water and gas penetration. These deterioration processes can lead to a collapse of the building structure or corrosion of the rebar, inner structure of a construction building [11], thus remediation using MICP has been researched vigorously [2, 9, 15, 19, 24]. This method is regarded as environmental-friendly or shows long-lasting effects [21]. To remediate the cracks, MICP has been applied using diverse bacterial strains or bacteriological media, and then



**Fig. 1.** Hyphal growth and germination of fungal strains in B4 agar. (A) Hyphal growth of *C. sphaerospermum* KNUC253 and their morphology in B4 medium and (B) spore germination of *C. sphaerospermum* KNUC253.



**Fig. 2.** Crystal images of *Bacillus aryabhatai* KNUC205. The images were captured by a biology stereomicroscope (×40) equipped with a Zentech Digicam (Sentech Co., Ltd., Japan).



verified by thermogravimetric analysis, water permeability test, and ultrasonic measurements [24].

To remediate the artificially induced crack in cement mortar, a culture suspension of *B. aryabhatai* KNUC205 was used. *B. aryabhatai* KNUC205 showed the ability for crack remediation (Fig. 5).

### Water Permeability Reduction

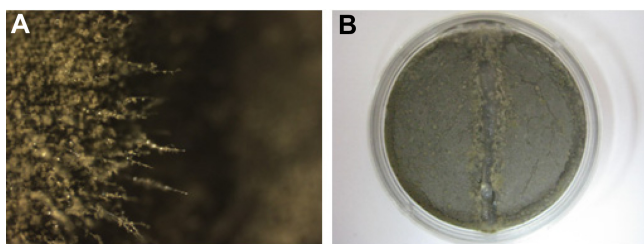
*B. aryabhatai* KNUC205-treated mortar paste showed significant reduction of water permeability (Fig. 6) compared with that of negative controls. After 24 h immersion in SDW, *B. aryabhatai* KNUC205-treated mortar paste showed 19.86% water permeability, that of *E. coli* K12 showed 24.13%, and that of B4 liquid medium showed 24.19%. *B. aryabhatai* KNUC205-treated mortar paste showed water permeability reduction compared with that of *E. coli* K12. This result means *B. aryabhatai* KNUC205 successfully remediated the mortar cracks with its biodeposition. In this test, *B. aryabhatai* KNUC205 showed the possibility of applicable microbial resources for water penetration reduction, which can reduce the risk of cracks in buildings by penetrated water or related serial reactions.

### Fungal Growth Inhibition on Mortar Crack

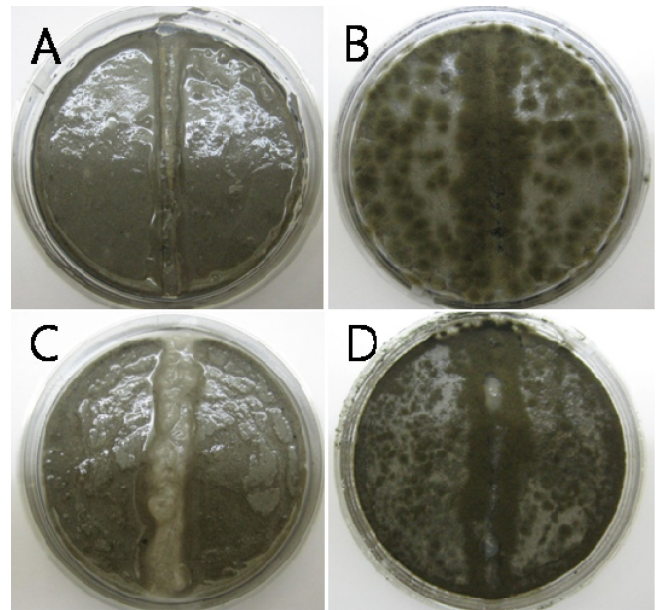
**B4 agar only.** In the case of B4 agar inoculants, no changes were observed compared with *B. aryabhatai* KNUC205 with B4 agar or fungal spore with B4 agar.

**Fungi spore with B4 agar.** To assay the fungal growth inhibition in mortar crack, fungal spore with B4 agar was established. The mixture was inoculated, and then incubated at 18°C. After 5 days of incubation, germination of spores were verified with the optical microscope, and after 8 days of incubation, strain-specific morphologies were identified with the naked eye (Fig. 3).

**Mixture of fungi spore/*B. aryabhatai* KNUC205 with B4 agar.** The mixture of fungi spore/*B. aryabhatai* KNUC205 with B4 agar was inoculated, and incubated at 18°C for 16 days. *B. aryabhatai* KNUC205 showed fungi growth inhibition at all three samples. This result means that *B. aryabhatai* KNUC205 successfully inhibits the fungal growth on mortar cracks (Fig. 4). *R. erythropolis*



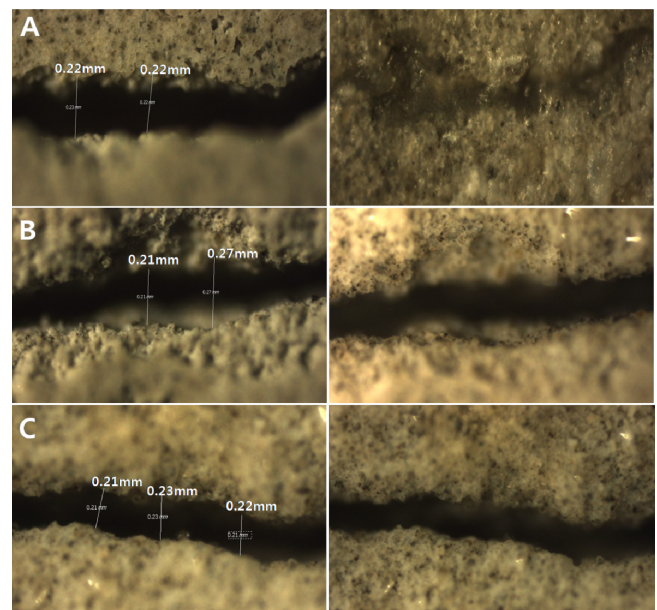
**Fig. 3.** Inoculant of fungi spore with B4 agar. (A) The magnified microscopic image of fungi growth in the cement mortar crack after 5 days of incubation, and (B) fungi growth, identified with the naked eye after 8 days of incubation.



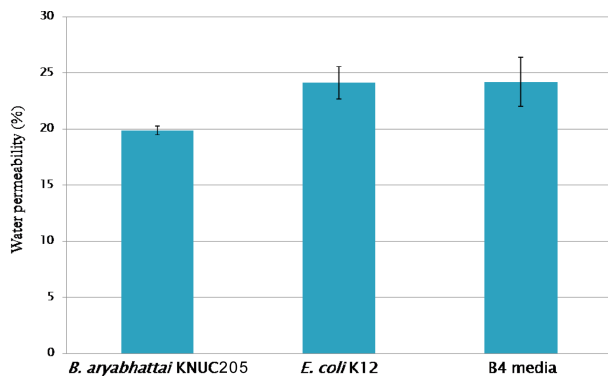
**Fig. 4.** Fungi growth inhibition on neutralized cement mortar crack after inoculation and 16 days incubation of CFB, fungi spore with B4 agar.

(A) Inoculant of B4 agar; (B) inoculant of fungi spore with B4 agar; (C) inoculant of *B. aryabhatai* KNUC205/fungi spore with B4 agar; and (D) inoculant of *R. erythropolis* KNUC201/fungi spore with B4 agar.

*KNUC201*, which showed no antifungal activity on B4 agar, cannot inhibit fungal growth on cement crack. This result suggested that *B. aryabhatai* KNUC205 can prevent



**Fig. 5.** Crack remediation using antifungal CFB strains. Initiative images of crack on cement mortar (left) and crack remediation after 5 days of incubation (right). (A) *B. aryabhatai* KNUC205; (B) *E. coli* K12; (C) inoculant of B4 liquid medium alone as control.



**Fig. 6.** Water permeability of *B. aryabhatai* KNUC205-treated cement mortar compared with that of *E. coli* K12 or B4 medium.

fungal infections in cracks or on the surface of cement mortar.

In a previous study, construction sites were dispersed with diverse fungal species and many construction building-habiting fungal strains have been reported for biodegradative or biodeteriorative abilities [4, 13, 14, 23]. Furthermore, calcite-forming bacteria can be found in construction building or related environments [8]. Therefore, there are enormous possibilities of isolating the CFB that have antifungal properties against co-habiting deteriorative fungal species. In this study, urban-tunnel-derived antifungal CFB successfully inhibited the fungal growth isolated in tunnel surfaces so that there are possibilities of control of the fungal growth on the same sites. Therefore, to prevent biodeterioration of the construction site or monuments, further investigation or application study about construction site-florating antifungal CFB is needed acutely. In this context, urban-tunnel-derived *B. aryabhatai* KNUC205 showed fungi growth inhibition ability against deteriorative fungal strain *C. sphaerospermum* KNUC253, which was derived from the same site, so that treatment of B4 medium to the surface of the tunnel can lead to effective prevention of tunnel deteriorations.

In conclusion, we clearly showed the  $\text{CaCO}_3$  precipitation of *B. aryabhatai* KNUC205 on B4 agar media. The mortar crack remediation and fungi growth inhibition abilities of *B. aryabhatai* KNUC205 were verified. Furthermore, water permeability reduction on cement surface was evaluated. In this research, introduction of a multifunctional (antifungal/ $\text{CaCO}_3$  precipitation) biomaterial to cement mortar and further application study are required to obtain diverse positive effects on cement concrete structures.

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