Improvement of the Sand Quality by Applying Microorganism Induced Calcium Carbonate Precipitation to Reduce Cement Usage

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Abstract: - This research aimed to study the utilization of microorganisms in the soil. These microorganisms were applied to improve the quality of soil and sand to reduce cement consumption. The process of microbial-induced precipitation of calcium carbonate (MICP) was used in this study, a bonding process to create a binding between sand particles from the naturally occurring processes of microorganisms. The study was conducted to determine the maximum growth rate of bacteria (maximum growth curve) of five strains as follows Proteus mirabilis TISTR 100, Bacillus thuringiensis TISTR 126, Staphylococcus aureus TISTR 118, Bacillus sp. TISTR 658 and Bacillus megaterium TISTR 067 in Nutrient Broth (NB) culture medium at 37°C. Christensen's Urea Agar slant (UA) was a preliminary screen for urease-producing bacteria. The color change of indicator color was seen in the slant, comparing the results (+) and control (-). The researchers have selected bacterial strains capable of producing significant amounts of enzymes to hydrolyze urea. It was found that the amount of calcium carbonate sediment produced by five strains of bacteria, namely Proteus mirabilis TISTR 100, Bacillus thuringiensis TISTR 126, Staphylococcus aureus TISTR 118, Bacillus sp. TISTR 658 and Bacillus megaterium TISTR 067 had the amount of sediment produced as follows: 3.430, 3.080, 2.590, 1.985, and 1.615 mg/ml, respectively. It was found that the species Bacillus sp. TISTR 658 does not produce the most calcium carbonate sediment. Nevertheless, it made the sand samples stick together tightly and form the perfect lumps. Therefore, improving the sand's composition has shown the potential to apply the MICP process to reduce the cement used.

Key-Words: - MICP, Cement consumption, Biofilm, Improvement, Ureolytic bacteria, Cemented sand.

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1 Introduction

The current world is facing the issue of global warming, partly caused by environmental degradation resulting from the mining of limestone to produce cement. This process leads to pollution, such as dust and air pollution, and the destruction of natural habitats for wildlife and forests. Therefore, researchers have recognized the importance of biotechnology and industrial construction in developing innovative approaches to reduce the use of cement for environmental sustainability.

Microbially induced carbonate precipitation (MICP) is one environmentally friendly alternative that can improve the properties of soil and sand [1, 2]. The MICP method involves using ureolytic bacteria that produce the enzyme urease, which accelerates the process of urea hydrolysis, resulting in the formation of calcium carbonate precipitates [3, 4].

In this study, suitable bacteria were carefully selected, mainly from soil samples within Thailand. The growth of these bacteria was controlled by adjusting temperature and pH to ensure optimal conditions to produce urease enzymes, which are crucial in inducing calcium carbonate precipitation from various sources of calcium, such as calcium lactate and calcium nitrate [5, 6], and calcium chloride [7, 8]. Among these sources, calcium chloride is commonly used in MICP processes due to its higher calcium carbonate precipitation yield than other calcium sources.

The mechanism of microbial-induced carbonate precipitation begins with the acceleration of urea hydrolysis through the urease enzyme produced by

ureolytic bacteria. This reaction leads to carbonate and ammonium compounds forming, as shown in Equation 1. Subsequently, the presence of ammonium increases the pH of the solution, promoting the precipitation of calcium carbonate, as indicated in Equation 2 [9].

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

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

In general, MICP is mainly focused on inducing calcium carbonate precipitation in soil and sand to enhance their stability and reduce soil erosion [10-12]. It can also be used to create bonds between sand particles, improving their cohesiveness and formability, which is superior to untreated sand [13-16]. Furthermore, forming a bacterial biofilm enhances the adhesion of bacteria to sand surfaces, allowing them to adhere to surfaces effectively. This biofilm formation also contributes to the efficiency of the MICP process, resulting in higher stability of the sand [17-21].

Therefore, this research aims to investigate the quality improvement of sand particles by applying MICP to produce calcium carbonate precipitates as a binding material between sand particles. The study explores the potential of bacterial strains found in Thai soils. Experimental trials are conducted to determine the optimal concentration of calcium chloride solution that induces the highest calcium carbonate precipitation for each bacterial strain. These selected conditions will be further studied to improve the bonding characteristics and strength of the sand, which can be applied for future developments.

2 Materials and Methods

2.1 Materials Preparation

The sand used in this research was obtained from the Chao Phraya River, Nonthaburi Province, Thailand. The particle size of the sand was controlled to be within the range of 75-600 μm using a sieve that complied with ASTM E11 standards [21]. Initially, the sand was washed three times with deionized water (DI) and then dried in a hot air oven at 105°C for 48 hr. Subsequently, the dried sand was sieved through a 30-mesh sieve. The sand that passed through the sieve was then wet sieved to separate dust particles using a 200-mesh sieve. The sand retained on the 200-mesh sieve was then dried in an oven at 105°C for 48 hr. before being used in the subsequent experiments.

The maximum dry density of the sand was conducted according to the ASTM D698 standard [22], and it was found that the sand in this research had an optimum moisture content of 17.5% and a maximum dry density of 1.60 g/cm^3.

2.2 Media Preparation and Cultural Method

2.2.1 Cultural Media Preparation

The preparation of Nutrient Broth containing urea (NBU) began by weighing 13 g of the Nutrient Broth powder (HIMEDIA M002) and dissolving it in 1000 ml of distilled water. Then, 20 g of urea (KEMAUS) were added, and the medium was dissolved completely. Next, 100 ml of the prepared NBU were transferred into a 250-ml Erlenmeyer flask. The flask was then sterilized in an autoclave (HIRAYAMA, HICLAVE HVA-110, Japan) at a temperature of 121°C and a steam pressure of 15 psi for 15 min.

Christensen's Urea Agar (UA) (HIMEDIA M112) was prepared by weighing 24 g of nutrient powder and dissolving it in 950 ml of distilled water. The medium was dissolved completely. Subsequently, the solution was sterilized in the autoclave at a temperature of 115°C and a steam pressure of 10 psi for 20 min. Afterward, the solution was left to cool to 50°C.

A concentrated urea solution of 40% w/v with a volume of 50 ml was aseptically added to the solution. Finally, the UA solution was transferred into sterilized vials with a volume of 5 ml, and the vials were placed at an inclined angle of 30 degrees to allow for proper solidification.

The preparation of Stuarts' Urea Broth (UB) (HIMEDIA M111) begins by weighing 18.71 g of the nutrient powder and dissolving it in 950 ml of distilled water. The medium is dissolved completely. Subsequently, the solution is sterilized in the autoclave at a temperature of 121°C and a steam pressure of 15 psi for 15 min. Afterward, the solution cools to 50°C.

A concentrated urea solution of 40% w/v with a volume of 50 ml was aseptically added to the solution. Finally, the UB solution is divided into sterilized vials with a volume of 5 ml, which have been previously sterilized.

The preparation of Tryptone Soya Broth (TSB) (HIMEDIA M011) begins by weighing 30 g of the nutrient powder and dissolving it in 1000 ml of distilled water. The medium is dissolved completely. Next, 100 ml of the TSB solution is transferred into a 250-ml Erlenmeyer flask. The flask is then sterilized in the autoclave at a temperature of 121°C and a steam pressure of 15 psi for 15 min.

2.2.2 Calcium Source Preparation

The calcium source in the research is a calcium chloride solution (KEMAUS) that has sterile. The concentration is modified to determine the optimal conditions for producing calcium carbonate in each bacterial strain. The concentrations range from 100 to 300 mM.

2.2.3 Bacteria Cultural Method

The selection of pure bacterial strains used in the experiments was conducted at the Thailand Institute of Scientific and Technological Research (TISTR). Five pure strains were chosen, including Proteus mirabilis TISTR 100, Bacillus thuringiensis TISTR 126, Staphylococcus aureus TISTR 118, Bacillus sp. TISTR 658, and Bacillus megaterium TISTR 067. Cell activation was performed by adding freeze-dried cells in an ampoule to vials containing Nutrient Broth (NB) as the culture medium, with a volume of 5 ml (comprising NB at a concentration of 13 g/L). The cells were incubated at 37°C for 24 hr. in an incubator. Subsequently, the bacterial strains were inoculated into the NBU medium to achieve a cell density of 1%. The cultures were further incubated at 37°C for 24 hr.

Following incubation, each bacterial strain was subjected to a growth curve analysis. The growth rate was measured at regular intervals using a spectrophotometer (JENWAY 7200, UK) with a wavelength of 600 nm. This analysis aimed to determine the maximum growth rate for each bacterial strain, which would be utilized in subsequent experimental steps. From the reading $O.D._{600}$, calculate the cell concentration using Equation 3 [23].

$$Y = 8.59 \times 10^7 \cdot Z^{1.3627} \quad (3)$$

Where, Y is the cells concentration (cells/ml)
Z is the reading $O.D._{600}$

2.3 Microbiological analysis

2.3.1 Bacteria Cultural Method

The glass slides were cleaned using 95% ethanol and then passed through the flame of a Bunsen burner using a slide holder. Following this, a loop sterilized by flame was used to transfer a small amount of bacterial-suspended solution onto the glass slide. The slide was allowed to air dry and then held at one end while passing through the flame of the Bunsen burner several times with the smear-side facing up. Afterward, the smear on the slide was stained with crystal violet for 1 min. The slide was then gently rinsed with distilled water. Next, Gram's iodine was applied to the smear for 1 min., followed by elution with 95% ethanol until the eluted solution ran almost clear. The slide was rinsed again with distilled water and then stained with safranin O for 1 min. Subsequently, the slide was rinsed with distilled water and blot dried before being observed under an optical microscope (Nikon, Eclipse E100, Japan) at 100x magnification.

2.3.2 Biofilm Detection and Screening

To investigate biofilm formation by the Microtiter plate assay of different bacterial strains' attachment to calcium carbonate granules [17]. Each strain of bacteria was cultured in TSB at 37°C for 24 hr. Subsequently, the bacterial strains were diluted to a concentration of 1% in TSB and dispensed into a 96-well Microtiter plate. Incubate the plate at 37°C for 24 hr. After incubation, the bacteria were washed twice with phosphate-buffered saline (PBS) and allowed to air dry for 1 hour. The biofilm staining is performed by adding 1% crystal violet solution (200 µl) for 5 min., resulting in a purple coloration. Subsequently, the stain was rinsed off with distilled water thrice. Finally, the absorbance of the wells with 95% ethanol (200 µl) measures at 570 nm using a 96-well plate reader (Metertech, M965+, Taiwan)—this measurement allowed for the quantification of light absorption, indicating the extent of biofilm formation.

2.3.3 Urease Screening

The bacteria were cultured on the UA slant using a sterile loop to screen bacteria capable of producing urease for urea hydrolysis reaction between urea and calcium chloride to form calcium carbonate precipitate. The slants were then incubated at 37°C for 24 hr. to test for any color changes in the culture medium. A pinkish-red color change in the culture medium indicated a positive result (+).

2.3.4 Urease Activity

Using Berthelot's Method, the enzyme samples were collected from the bacterial culture medium in Section 2.2. The bacterial culture was transferred into tubes and then centrifuged at 4000 rpm for 30 min. at 4°C. After centrifugation, the supernatant with 250 µl was collected. Potassium phosphate buffer with a pH of 8 and a concentration of 100 mM (1 ml) was added. Then, a starting solution of urea with a concentration of 100 mM (2.5 ml) was added. The mixture was incubated at 37°C for 5 min. Subsequently, ammonia production was measured by adding Phenol nitroprusside and Alkaline hypochlorite, each with a volume of 1 ml, to generate ammonia. The mixture was further incubated at 37°C for 30 min. Finally, the

absorbance of the sample was measured using a spectrophotometer at a wavelength of 626 nm, and the data were analyzed to determine the urease enzyme activity produced by each bacterial strain.

To prepare the standard ammonium chloride solution, solutions of ammonium chloride were mixed to achieve concentrations of 200, 400, 600, 800, and 1000 μmol. The absorbance of these solutions was measured at a wavelength of 626 nm against a blank solution. The obtained absorbance values were then plotted on a graph representing the standard ammonium chloride solution, as shown in Fig. 1.

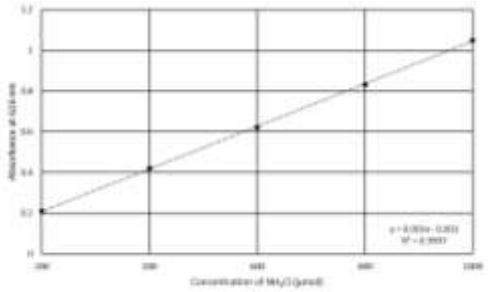


Fig. 1 Standard curve of ammonium chloride

The absorbance values at 626 nm to analyze urease activity for each bacterial strain were compared to the standard solution graph of ammonium chloride. Ammonium chloride concentration (X) in each bacterial strain was calculated using Equations 4 and 5 derived from the standard solution graph of ammonium chloride. Subsequently, the enzymatic activity of urease was determined using Equation 6, utilizing the calculated concentration (X).

$$(y \times \text{dilution}) = ax + b \quad (4)$$

$$x = \frac{(y \times \text{dilution}) - b}{a} \quad (5)$$

Where, y is the reading $O.D._{626}$
X is an NH_4Cl (μmol)

$$\text{Urease activity} = \frac{X \times V_t}{V_{sub.} \times t_i \times V_{smp.}} \quad (6)$$

Where, V_t is the total volume (ml)
$V_{sub.}$ Is the volume of the substrate (ml)
t_i is the incubation time (min.)
$V_{smp.}$ is the volume of enzyme (ml)

2.3.5 Calcium Carbonate Precipitation

Add the calcium chloride solution with a concentration of 100-300 mM [15] to the bacterial-suspended solution to induce the precipitation of calcium carbonate. After adding the calcium chloride solution, incubate the resulting solution in an incubator at 37°C for 1-7 days. [24] Filter the precipitate obtained using a vacuum filtration system through a Büchner funnel with Whatman No.4 filter paper. Then, dry the precipitate by subjecting it to hot air and drying it at 60°C for 24 hr.

2.3.6 Criteria for Microorganism Selection

Select the concentration of calcium chloride solution that produces the highest level of calcium carbonate precipitation among the five bacterial strains by weighing the dried precipitates obtained from each strain and comparing them.

2.4 Sand Sample Preparation

The sand samples obtained from Section 2.1 were molded into cube shapes with 5 cm in width, length, and height dimensions, as shown in Fig. 2. The sample was achieved by taking 200 g of sand per sample, and three samples were prepared for each bacterial strain. The sand was then mixed with the bacterial-suspended solution obtained from Section 2.2.3, with a bacterial volume of 35 ml. The sand was compacted into molds, providing a height of 5 cm. Subsequently, an optimum concentration of calcium chloride solution, selected based on the criteria outlined in Section 2.3.6, was added to the sand. The volume of the calcium chloride solution was also 35 ml, marking the completion of one cycle. Following this, a mixture containing NBU and a calcium chloride solution, each with a volume of 35 ml, was added to the molds every 24 hr. for seven days.

Fig. 2 Casting mold

3 Results

3.1 Growth Curve

Based on the study, growth rates were determined by measuring the light absorbance at a wavelength of 600 nm, and cell concentration was calculated as shown in Table 1.

Table 1. Maximum growth rate

Microorganism	Maximum growth rate (hr.)	Cell concentration (cells/ml)
P. mirabilis TISTR 100	12	1.60×10^8
B. thuringiensis TISTR 126	32	1.98×10^8
S. aureus TISTR 118	22	1.61×10^8
Bacillus sp. TISTR 658	17	1.75×10^8
B. megaterium TISTR 067	30	1.91×10^8

3.2 Gram Strain

Based on the study, Gram staining following the method of Hans Christian Gram was performed, and observations were made using a microscope with a magnification of 100x. The results are shown in Fig. 3, indicating that among the five bacterial strains examined, P. mirabilis TISTR 100 was the only strain that exhibited Gram-negative (-) characteristics, as shown in Table 2.

(a) (b) (c) (d) (e)

Fig. 3 Gram strained bacteria: (a) P. mirabilis TISTR 100, (b) B. thuringiensis TISTR 126, (c) S. aureus TISTR 118, (d) Bacillus sp. TISTR 658, and (e) B. megaterium TISTR 067

Table 2. Gram strain

Microorganism	Gram
P. mirabilis TISTR 100	-
B. thuringiensis TISTR 126	+
S. aureus TISTR 118	+
Bacillus sp. TISTR 658	+
B. megaterium TISTR 067	+

3.3 Biofilm Detection and Screening

From the results of the experiment on biofilm formation, as shown in Fig. 4, it was observed that each bacterial strain could produce biofilms. This phenomenon can be attributed to the binding of crystal violet, which affects the adhesion of bacteria and the resulting production of calcium carbonate. Intense staining indicates higher biological adhesion compared to faded staining [25].

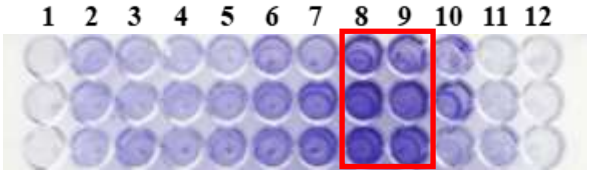


Fig. 4 96-well plate of biofilm detection. Where, 1 and 12 are control (blank) 2-3: biofilm formation from P. mirabilis TISTR 100, 4-5: B. thuringiensis TISTR 126, 6-7: B. megaterium TISTR 067, 8-9: Bacillus sp. TISTR 658, and 10-11: S. aureus TISTR 118

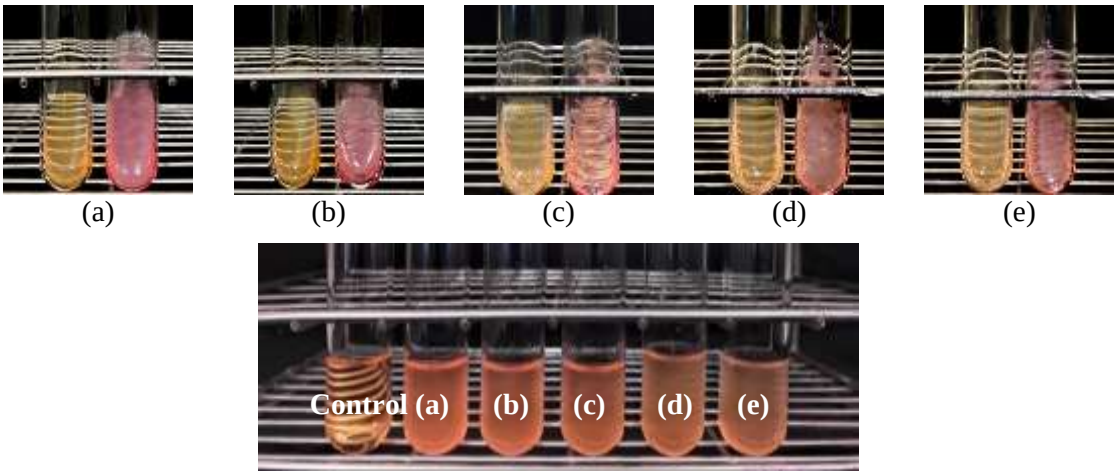
The measurement of $O.D._{570}$, as shown in Table 3, confirms these findings.

Table 3. The reading $O.D._{570}$ (nm) of biofilm

1	2	3	4	5	6	7	8	9	10	11	12
0.073	0.273	0.239	0.218	0.188	0.245	0.302	0.638	0.582	0.316	0.170	0.102
0.103	0.220	0.172	0.201	0.213	0.238	0.332	0.468	0.543	0.387	0.193	0.107
0.101	0.175	0.237	0.199	0.215	0.218	0.267	0.357	0.512	0.177	0.225	0.085

3.4 Urease Screening and Activity

It was observed that all bacterial strains could produce the enzyme urease, which catalyzes the hydrolysis of urea. This reaction occurs with the presence of urea as the substrate in the medium, increasing the pH of UA and UB. Consequently, the color of the medium changes from the control (-) to a positive (+) result, exhibiting a pinkish-red color [26], as depicted in Fig. 5.



(a) (b) (c) (d) (e)

(f)

Fig. 5 Urease screening on UA: (a) P. mirabilis TISTR 100, (b) B. thuringiensis TISTR 126, (c) S. aureus TISTR 118, (d) Bacillus sp. TISTR 658, (e) B. megaterium TISTR 067, and (f) urease screening on UB of all bacteria strains

3.5 Calcium Carbonate Precipitation

The induction of calcium carbonate precipitation through microbial calcification was investigated in the experiment outlined in Section 2.3.5. Interestingly, distinct variations in the characteristics of the calcium carbonate precipitates were observed among the different bacterial strains, as illustrated in Fig. 6.

(a) (b) (c) (d) (e)

Fig. 6 Precipitated calcium carbonate: (a) P. mirabilis TISTR 100, (b) B. thuringiensis TISTR 126, (c) S. aureus TISTR 118, (d) Bacillus sp. TISTR 658, and (e) B. megaterium TISTR 067

Furthermore, it was found that the maximum yielding of calcium carbonate precipitation, represented by the formation of precipitates, differed among the various strains of bacteria in the presence of calcium chloride as the inducing agent. This phenomenon occurred at a pH of 9.1, as depicted in Fig. 7.

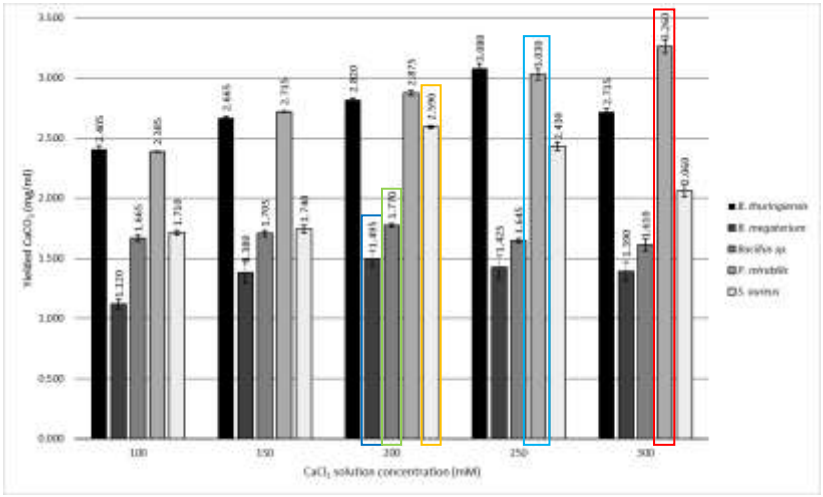


Fig. 7 Calcium carbonate yield of each bacteria strain

3.6 Improvement of Sand Bonding

The improvement of sand quality by selecting calcium chloride concentrations that induce optimal calcium carbonate precipitation was investigated. This process resulted in the formation of the highest level of calcium carbonate precipitates for each bacterial strain, as observed in the experimental results depicted in Fig. 7, as shown in Fig. 8.

It was observed that the sand samples that experienced quality improvement, which allowed them to maintain their structure and stability most effectively, had undergone quality improvement through applying Bacillus sp. TISTR 658, as represented in Figure 8d. This finding aligns with the results obtained from the investigation on biofilm formation, even though P. mirabilis TISTR 100 exhibited the highest capability to produce calcium carbonate precipitates. However, it does not imply that P. mirabilis TISTR 100 exhibited the highest stability in sand quality improvement.

(a) (b) (c) (d) (e)

Fig. 8 Improvement of sand: (a) P. mirabilis TISTR 100, (b) B. thuringiensis TISTR 126, (c) S. aureus TISTR 118, (d) Bacillus sp. TISTR 658, and (e) B. megaterium TISTR 067

4 Conclusion

This study, we proposed and demonstrated a new eco-friendly bio-mineralization process known as MICP for sand improvement. The procedure involved the selection of bacteria capable of producing urease enzymes to facilitate the precipitation of calcium carbonate through urea hydrolysis, which occurred when the pH was shifted from neutral to mildly alkaline conditions. The selected bacteria were found to form biofilms with varying intensities of coloration, which directly correlated with the amount of biofilm formation and inversely correlated with the sand's structural stability.

These findings indicate that by utilizing this approach, it is possible to enhance the structural integrity of sand, offering an alternative method to reduce the reliance on cementitious materials. Stabilizing sand through biofilm mediated MICP provides a promising avenue for sustainable sand improvement practices.

Further research and development are warranted to optimize the MICP process parameters, including the selection of specific microorganisms and their cultivation conditions, and to evaluate the long-term performance and durability of the MICP-treated sand in practical applications. Nevertheless, the results of this study highlight the potential of MICP as a viable and environmentally friendly technique for sand improvement, contributing to the advancement of sustainable construction practices.

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The authors have no conflicts of interest to declare that are relevant to the content of this article.