Experimental Study on the Preparation of a Highly Active Bacterial Suspension for MICP in the South China Sea

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Abstract: Most previous studies investigating the use of microbial-induced calcium carbonate precipitation (MICP) to reinforce foundations have indicated that the final curing effect can be improved by changing the nutrient environment parameters during the MICP reaction. However, using such methods to improve the construction process increases the construction cost and the impact on the surrounding environment. This study was conducted to determine if we could prepare a bacterial suspension with high activity in a short period of time by examining the effects of inoculation time, the concentration of the inoculated bacterial suspension, and shaker speed during expansion to determine whether sporosarcina pasteurii would vigorously grow. Based on the analysis of the pattern of activity variations in the bacterial suspension, the most appropriate growth scheme for preparing high-activity bacterial suspensions when using MICP to strengthen structures in the South China Sea was proposed. In terms of the results, it was found that the trend of changes in activity and the peak time of maximum activity in the bacteriophage cultured under low-speed conditions in the expanded culture tended to be the same. The value of the bacteriophage’s activity was low. During medium speed culture of the bacterial suspension, urease activity peaked much higher than that of the other comparison groups, with the medium speed bacterial broth having the highest peak. As a result of the prolonged shaking incubation time, the effect of prolonged shaking on urease activity in the bacterial suspension was mainly reflected in the fact that the activity decay cycle of the colony itself was slowed.

Keywords: South China Sea environment; microbial-induced carbonate precipitation; training program; urease activity; change rule

1. Introduction

Due to the policy of “One Belt, One Road,” China is paying increasingly more attention to developing and constructing islands and reefs in the South China Sea. Calcareous sand is widely distributed in the South China Sea, and its particles are irregular in shape, rich in micropores, and easily broken, which easily causes uneven settlement of the foundation; therefore, it should be appropriately treated during its development and construction [1,2]. Despite the maturity of traditional foundation treatment methods (such as vibratory punching), they do not apply to calcareous sand foundations, and the construction is difficult, expensive, and not environmentally friendly.

In recent years, MICP technology has emerged as a research hotspot in civil engineering at home and abroad, and is widely used for sand reinforcement [3–6], restoration of ancient sites [7–10], and remediation of contaminated soils [11–14]. The advantages of this type of construction are its ease of construction, low cost, and environmental friendliness. The main mechanism of reinforcement is as follows: in its metabolism, sporosarcina pasteurii produces urease, which hydrolyzes urea in the environment to form carbonate, which...
combines with calcium ions in the environment to create CaCO$_3$ crystals that can function as cement. The main chemical reaction equations can be summarized as follows [15–17].

$$
\text{CO}(\text{NH}_2)_2 + 2\text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3 + 2\text{NH}_3
$$

$$
\text{H}_2\text{CO}_3 + 2\text{NH}_3 \leftrightarrow 2\text{NH}_4^+ + 2\text{OH}^-
$$

$$
\text{H}_2\text{CO}_3 \rightarrow \text{H}^+ + \text{HCO}_3^-
$$

$$
\text{HCO}_3^- + 2\text{OH}^- + \text{H}^+ \leftrightarrow \text{CO}_3^{2-} + 2\text{H}_2\text{O}
$$

$$
\text{O}_3^{2-} + \text{Ca}^{2+} \leftrightarrow \text{CaCO}_3
$$

Researchers Masahiro Fujita et al. [18,19] found that the high pH of the solution affected the growth of bacteria, resulting in a weaker curing effect of MICP. As determined by Lv et al. [20], calcium carbonate precipitates were strongly influenced by temperature, solution pH, bacterial suspension concentration, and colloidal solution concentration, with the size of the resulting calcium carbonate crystals increasing with temperature. Liang et al. [21] investigated the effect of cement content on the physical and mechanical properties of reinforced ISO standard sand specimens. Tan et al. [22] demonstrated that binder concentrations less than 1 mol/L had a good effect on geotechnical properties. At a cement content of 0.25 mol/L, the permeability and/or long-term stability of geotechnical soil was significantly increased, however, it took longer, while at a cement content of 0.5 mol/L, the calcium carbonate content and unconfined compressive strength of the specimens were relatively high. Al Thawadi [23] and Lai et al. [24] found that high cement concentrations delayed or even terminated the MICP process, with the delay effect gradually accounting for the obvious when cement concentrations exceeded 1 mol/L. Cement concentrations up to 2.5 mol/L will lead to the termination of the calcium carbonate production process during MICP. Han et al. [25] investigated the effect of three different calcium salt–urea nutrient salt solutions on the consolidation of liquefiable sand by MICP and found that a Ca(CH$_3$COO)$_2$–urea nutrient salt consolidation was the most effective. Zhao et al. [26] found that Ca(CH$_3$COO)$_2$ was more suitable as a calcium source than CaCl$_2$ or Ca(NO$_3$)$_2$, and its pore size distribution was wider after consolidation by the MICP process. The specimens had a wider range of pore sizes and higher strength. Cheng et al. [27] investigated the effect of four calcium sources and Ca$^{2+}$ concentrations on the induction of calcium carbonate precipitation by autonomously screened UPB1 bacteria, with CaCl$_2$ as the calcium source producing the most stable rhomboid-type calcium carbonate with the highest deposition efficiency at a Ca$^{2+}$ concentration of 0.25 mol/L. In a study by Maleki Kakelar et al. [28], using different injection steps (reaction time, fixative, and number of injections), the sand sample strength increased from 0.25 MPa to 1.55 MPa when the calcium carbonate content increased from 7.7% to 18.9%. Typically, MICP studies are carried out under nutrient-sufficient conditions. Yusuf Atay Ersan et al. [29] used a special elastomeric strain to increase the yield of CaCO$_3$ precipitation in low-nutrient environments, which greatly improved the efficiency of nutrient utilization in the environment.

Even though most of the existing studies alter the nutrient environment parameters of the MICP construction process in order to increase its performance, such alterations may increase construction costs and have adverse effects on the surrounding environment, such as imbalances in the proportion of elements in the environment and eutrophication of the water column. This study investigated the origin of the MICP process (i.e., the ability of bacteria to hydrolyze urea), developed a highly active bacterial suspension in a short time by optimizing the culture protocol of sporosarcina pasteurii, and performed variation pattern analysis to determine the most suitable conditions for preparing an active bacterial suspension for MICP in the South China Sea. The growth, reproduction, and metabolism of microorganisms are closely related to external factors and are influenced by environmental conditions. To reduce the influence of environmental factors on the test results and enhance the project’s practicality, the test site was chosen in Haikou, China.
2. Materials and Methods

Before preserving strains may be used for expanded cultures, they must first be activated and stimulate their bacterial activity. Current preservation methods include liquid paraffin, sand preservation, vacuum freeze drying, preservation in liquid nitrogen, and subculturing [30]. The strains used in this study were maintained primarily through subculturing.

The test procedure is illustrated in Figure 1. The liquid medium needs to be prepared first. After sterilization in an autoclave, the prepared medium is cooled on a sterile bench until it is ready for use. As soon as the preserved culture medium has been taken out from the refrigerator, it is brought to room temperature on the sterile bench. The room-temperature medium is inoculated on the aseptic table and the medium sealed to prevent contamination. After inoculation, the broth is incubated in a constant temperature shaker. It is important to note that the activity of the strain is tested after incubation, and the activation process is repeated until the activity of the bacterial suspension is fully stimulated. After activating the strain, the nutrient solution is added to the liquid medium in a set proportion for incubation and the temperature and speed of the shaker are set according to the experimental protocol.

The strain used in the test was sporosarcina pasteurii, a Gram-positive bacterium purchased from the General Microbial Strain Repository of China. In geotechnical engineering, this bacterium is the most commonly used and is typically ovoid and approximately 1 mm to 3 mm in size.

Based on the study by Chu et al. [31–33], the parameters of the media used in this experiment are listed in Table 1. The solid and liquid media were used in this experiment. The solid medium is used for the long-term preservation of the strains and the liquid medium is used for the activation and expansion of the bacteria. In this case, the solid medium had the same nutrient composition as the liquid medium, differing only in the inclusion of agar for solidification.

Figure 2 shows the experimental apparatus and the experimental operations used during the experiment.

Wiffin et al. [34] found a linear relationship between the amount of urea hydrolyzed in solution and the value of conductivity change so that conductivity values may be used as an indirect index of urease activity. A bacterial suspension of 2 mL was added to 18 mL of
urea solution containing a concentration of 1.5 moles per liter, and the mixture was stirred
thoroughly.

Table 1. Composition and proportion of culture medium.

| Ingredients            | Content/L |
|------------------------|-----------|
| MnSO₄·H₂O              | 10 mg     |
| NiCl₂·6H₂O             | 24 mg     |
| NH₄Cl                  | 10 g      |
| Yeast extract          | 20 g      |
| Agar powder            | 15 g (for solid media) |

Figure 2. Experimental process diagram. (a) Preparation of chemicals to be used; (b) configuration of liquid medium; (c) sterilization of liquid culture media; (d) dispensation of liquid medium and cooling to room temperature; (e) inoculation of bacterial suspension; (f) stationary culture; (g) shaking of culture; (h) detection of urease activity.

The conductivity change values were measured over 5 min using a conductivity meter, and the actual conductivity change (mS/cm/min) was used to characterize the urease activity. Where the following equation can express the relationship between the rate of change in conductivity and urease activity,

\[ U = C \times 11.11 \]

\[ U_{AR} = \frac{C_6 - C_1}{5} \times 10 \times 11.11 \]

\[ U_A = \frac{C_6 - C_1}{5} \times 10 \]
where \( U \) is the amount of urea hydrolyzed (mM); \( C \) is the conductivity change value (mS/cm); \( U_{AR} \) is the actual urea hydrolysis rate (mM urea/min); \( C_6 \) and \( C_1 \) are the conductivity values at minute 6 and minute 1, respectively; and \( U_A \) is the urease activity (mS/cm/min).

Three parallel groups were tested using the gradient method, and the mean value of the urease activity was used as the standard value to characterize the results. The colonies were expanded under different experimental conditions, and the level of urease activity was assessed at the end of the experiment. Experimental conditions included the volume of inoculation (1%, 2%, 3%), rotational speed (160 rpm, 190 rpm, 220 rpm), and time of shaking in cultivation (12 h, 24 h, 36 h, 48 h). The effect of bacteria broth prepared for a period of time (12 h, 24 h, 36 h) was studied at 30 °C. The specific experimental protocols are summarized in Table 2. This study aimed to assess the most appropriate culture protocols and recommendations for using highly active bacterial suspensions in the environment of the South China Sea for future applications.

Table 2. Experimental conditions.

| Experimental Number | Inoculation Volume (%) | Rotational Speed (rpm) | Cultivation Time (h) | Temperature (°C) | Interval of Activity Measurement (h) |
|---------------------|------------------------|------------------------|----------------------|------------------|-------------------------------------|
|                     |                        |                        | 48 h in Total        |                  |                                     |
|                     |                        |                        | Shake Culture (h)    |                  |                                     |
|                     |                        |                        | Stationary Culture (h)|                  |                                     |
| A₁₁₂                | 1                      | 160                    | 12                   | 24               | 30                                 | 12                                 |
| A₁₂₄                |                        |                        | 36                   | 24               |                                     |                                     |
| A₁₃₆                |                        |                        | 48                   | 0                |                                     |                                     |
| A₁₄₈                |                        |                        | 12                   | 36               |                                     |                                     |
| A₂₁₂                | 2                      |                        | 24                   | 24               |                                     |                                     |
| A₂₂₄                |                        | 160                    | 36                   | 12               |                                     |                                     |
| A₂₃₆                |                        |                        | 48                   | 0                |                                     |                                     |
| A₂₄₈                |                        |                        | 12                   | 36               |                                     |                                     |
| A₃₁₂                | 3                      |                        | 24                   | 24               |                                     |                                     |
| A₃₂₄                |                        |                        | 36                   | 12               |                                     |                                     |
| A₃₃₆                |                        |                        | 24                   | 24               |                                     |                                     |
| A₃₄₈                |                        |                        | 36                   | 12               |                                     |                                     |
| B₁₁₂                | 1                      |                        | 12                   | 36               |                                     |                                     |
| B₁₂₄                |                        |                        | 24                   | 24               |                                     |                                     |
| B₁₃₆                |                        |                        | 36                   | 12               |                                     |                                     |
| B₁₄₈                |                        |                        | 48                   | 0                |                                     |                                     |
| B₂₁₂                | 2                      | 190                    | 12                   | 36               |                                     |                                     |
| B₂₂₄                |                        |                        | 24                   | 24               |                                     |                                     |
| B₂₃₆                |                        |                        | 36                   | 12               |                                     |                                     |
| B₂₄₈                |                        |                        | 48                   | 0                |                                     |                                     |
| B₃₁₂                | 3                      |                        | 12                   | 36               |                                     |                                     |
| B₃₂₄                |                        |                        | 24                   | 24               |                                     |                                     |
| B₃₃₆                |                        |                        | 36                   | 12               |                                     |                                     |
| B₃₄₈                |                        |                        | 48                   | 0                |                                     |                                     |
Table 2. Cont.

| Experimental Number | Inoculation Volume (%) | Rotational Speed (rpm) | Cultivation Time (h) 48 h in Total | Temperature (°C) | Interval of Activity Measurement (h) |
|---------------------|------------------------|------------------------|-----------------------------------|-----------------|-------------------------------|
|                     |                        |                        | Shake Culture (h) | Stationary Culture (h) |                |                                |
| A1-12               |                        |                        | 12 | 36 |     |                                |
| A1-24               |                        |                        | 24 | 24 |     |                                |
| A1-36               |                        |                        | 36 | 12 |     |                                |
| A1-48               |                        |                        | 48 | 0  |     |                                |
| A2-12               |                        | 2                      | 220 |                | 30 | 12 |
| A2-24               |                        |                        | 12 | 36 |     |                                |
| A2-36               |                        |                        | 24 | 24 |     |                                |
| A2-48               |                        |                        | 36 | 12 |     |                                |
| A3-12               |                        |                        | 12 | 36 |     |                                |
| A3-24               |                        |                        | 24 | 24 |     |                                |
| A3-36               |                        |                        | 36 | 12 |     |                                |
| A3-48               |                        |                        | 48 | 0  |     |                                |

3. Results

The purpose of this chapter is to discuss the effects of different experimental conditions on the expanded culture of the bacteriophage. The effect of different experimental environments on bacterial culture activity was investigated using single factor analysis. The control experiments in the article were set up with three parallel control groups with the same experimental conditions, and the results of the three experimental groups were plotted in a line graph. The upper end of the error bar represents the maximum value of the measured experimental results in the three groups, and the lower end of the error bar is the minimum value of the experimental results in the three groups, and the average value of the three experimental results is taken as the characteristic value.

3.1. Test Results

3.1.1. Effect of Inoculum Volume on Urease Activity

As shown in Figure 3, the trend of urease activity $U_A$ and the time point of peak activity were both consistent, the effect of inoculum level on peak activity was not significant, and the stability of peak activity was better when a 3% inoculum volume was selected. In both the shaking and static incubations, the peak urease activity was reached after 24 h (up to 0.36 mS/cm/min). In general, the higher the inoculum volume, the faster the decay of urease activity.

As can be seen in Figure 4, the pattern of variation in urease activity between bacterial suspensions varied based on the inoculum used. The urease activity was 2% higher than 1% if the culture was shaken only for 12 h. The urease activity decayed rapidly if shaking was not continued for 12 h. As to this point, the 1% inoculum had slightly less decay, but the activity would remain stable if left to stand. If the shaking was continued for a period of 12 h, the activity of the 1% inoculum tended to decrease, whereas in the other two groups, the opposite is true. With increasing inoculum levels, urease activity changed from increasing to decreasing after 24 h of shaking if the broth was left to stand. When shaking was continued, urease activity increased in all groups, and the higher the inoculum level, the more significant the increase.
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Figure 3. Variation in urease activity with rotational speed of 160 rpm at (a) 12 h; (b) 24 h; (c) 36 h; (d) 48 h.

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Figure 4. Variation in urease activity with rotational speed of 190 rpm at (a) 12 h; (b) 24 h; (c) 36 h; (d) 48 h.

As shown in Figure 5, the activity of different inoculum volumes varies with time during vacuum incubation at a speed of 220 rpm. As can be observed from Figure 5, the inoculum volume did not have much influence on the shaking culture when the speed was 220 rpm. During the shaking culture and the 12 h of resting time for each group, urease activity was always maintained within a certain range and stable. The activity of urease varied with time and inoculum amount, with the highest activity (0.32 mS/cm/min) at 1% inoculum after 36 h.

Figure 5. Variation in urease activity with rotational speed of 220 rpm at (a) 12 h; (b) 24 h; (c) 36 h; (d) 48 h.
As shown in Figure 5, the activity of different inoculum volumes varies with time during vacuum incubation at a speed of 220 rpm. As can be observed from Figure 5, the inoculum volume did not have much influence on the shaking culture when the speed was 220 rpm. During the shaking culture and the 12 h of resting time for each group, urease activity was always maintained within a certain range and stable. The activity of urease varied with time and inoculum amount, with the highest activity (0.32 mS/cm/min) at 1% inoculum after 36 h.

3.1.2. Effect of Rotational Speed on Urease Activity

As shown in Figure 6, the urease activity varies with time when incubated at different rotational speeds. The urease activity increased with the increase in rotation speed in all time periods. The activity of the bacteria cultured at high speed (220 rpm) remained stable after resting, the activity of the bacteria cultured at medium speed (190 rpm) decreased and then stabilized after resting, and the activity of the bacteria cultured at low speed (160 rpm) continued to decay slowly after resting. The urease activity of all groups increased to varying degrees with increasing shaking time after 2 days of incubation. The urease activity of the medium and high-speed cultures was more stable, and the medium-speed culture was slightly better, while the urease activity of the low-speed shaking culture began to decay after reaching its peak at 24 h, and the activity decayed more quickly under rest.

In Figure 7a, the values of urease activity were 190 rpm, 220 rpm, and 160 rpm, respectively, and in the medium and high-speed shaking cultures, values of urease activity were closer. Following 36 h or 48 h of shaking the cultures, the values of urease activity were similar in low and high-speed cultures, but not as good as the medium-speed culture.
Figure 6. Urease activity after inoculation with 1% of the bacterial suspension with different shaking culture times at (a) 12 h; (b) 24 h; (c) 36 h; (d) 48 h.

Figure 7. Urease activity after inoculation with 2% of the bacterial suspension with different shaking culture times at (a) 12 h; (b) 24 h; (c) 36 h; (d) 48 h.

Figure 8. Urease activity after inoculation with 3% of the bacterial suspension with different shaking culture times at (a) 12 h; (b) 24 h; (c) 36 h; (d) 48 h.
Based on Figures 6–8, it can be concluded that, with the increase in inoculum, the pattern of change in urease activity of the bacterial bacterial suspension incubated at low speed was always virtually the same. On the other hand, the activity of the medium to high-speed shaking culture for 12 h gradually changed from stable to decaying after resting, and the activity of the shaking 24 h after resting became larger. After 36 h of shaking, the activity of the medium-speed culture was significantly higher than the activity of the other two groups in both the shaking period and the resting period, and the inoculum level had only a minor effect on the urease activity of the high- and low-speed shaking cultures.

Figure 7. Urease activity after inoculation with 2% of the bacterial suspension with different shaking culture times at (a) 12 h; (b) 24 h; (c) 36 h; (d) 48 h.

Figure 8. Urease activity after inoculation with 3% of the bacterial suspension with different shaking culture times at (a) 12 h; (b) 24 h; (c) 36 h; (d) 48 h.

3.1.3. Effect of Shake Culture Time on Urease Activity

In Figure 9, the urease activity is depicted at different times after inoculation with a range of bacterial suspension concentrations and after expansion of the culture at multiple speeds. The urease activity values of all groups increased significantly from 12 to 24 h in the shaking culture, with a peak value of 0.36 mS/cm/min. After 24 h, the urease activity of all the bacteria began to decay, and the shorter the shaking culture time, the faster the decay rate, and the longer the shaking culture time, the higher the final urease activity value. Even if the culture was shaken for only 12 h and left to rest, the urease activity decayed continuously, and with the increase in the inoculum, the final urease activity value decayed to 0.12 mS/cm/min, which is about 1/3 of the peak value. It is recommended to use it immediately following a peak in urease activity at 24 h.

As shown in Figure 10, urease activity varies with time when incubated at varying rotational speeds. The urease activity increased with an increase in rotation speed across all time periods. In terms of bacterial activity, the bacteria cultured at a high speed (220 rpm) remained active, the bacteria cultured at a medium speed (190 rpm) decreased and then stabilized, and the bacteria cultured at a low speed (160 rpm) continued to decay. After two days of incubation, the urease activity of all groups progressively increased with increasing shaking time. While the urease activity in both medium- and high-speed cultures was more stable and the medium-speed culture was slightly better, the urease activity in the
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Figure 9. Urease activity at different moments of expansion with 160 rpm rotation speed after inoculation with (a) 1%; (b) 2%; (c) 3% bacterial suspension.
After two days of incubation, the urease activity of all groups progressively increased with increasing shaking time. While the urease activity in both medium- and high-speed cultures was more stable and the medium-speed culture was slightly better, the urease activity in the low-speed shaking culture began to decline after reaching its peak at 24 h, and the activity decayed more quickly under rest.

Figure 10. Urease activity at different moments of expansion with 190 rpm rotation speed after inoculation with (a) 1%; (b) 2%; (c) 3% bacterial suspension.

The results in Figure 11 show that when incubation was carried out for only 12 h, the urease activity varied with the inoculum level, with the low inoculum level maintaining the activity for a longer period. In contrast, the medium and high inoculum levels declined after 12 h. When shaking time reached 24 h and above, the urease activity values and the changing pattern were almost similar for all groups at all time periods, and the urease activity values were always maintained within a certain activity range. When the rotation speed is high, using a low inoculum level and a short shaking time is recommended.
3.2. Discussion of Peak Urease Activity

To replenish the bacterial suspension in time and to avoid prolonging the construction process, a bubble map of the peak urease activity and the corresponding program parameters can be used to produce a bacterial suspension with the target urease level. By replacing scattered points with individual bubbles, the bubble map represents three-dimensional information instead of a scattered point map. As shown in Figure 12, the X axis represents the volume of inoculum, the Y axis represents time, and the Z axis represents the size of the bubbles. In addition, the color mapping allows visualization of the peak activity that a culture of bacteria can achieve under specific conditions.

It was observed that the high-activity-value bubbles were mainly distributed in the region of inoculum $\geq 2\%$ and shaking incubation time $\geq 36\, h$. Under the conditions of suitable inoculum level, the peak urease activity could be greatly increased after 24 h after continued incubation, while when the inoculum level was too low; the effect after 48 h of incubation was almost identical to that of 12 h of incubation under optimum conditions. In this experiment, the urease activity values of the bacterial suspension prepared using
Scheme B2-36 were the highest at all time points. Table 3 lists the experimental conditions when the activity of the bacterial solution reached its maximum value.

Table 3. Urease activity peak and scheme.

|       | 12 h | 24 h | 36 h | 48 h |
|-------|------|------|------|------|
| 1%    | 0.38 | 0.36 | 0.36 | 0.42 |
| Number| B1-36| C1-36| B1-24, B1-48, C1-12, C1-24 | B1-48 |
| 2%    | 0.42 | 0.42 | 0.48 | 0.52 |
| Number| B2-36| B2-36| B2-36 | B2-36 |
| 3%    | 0.42 | 0.40 | 0.46 | 0.50 |
| Number| B3-36| B3-36| B3-36 | B3-36, B3-48 |

It was found that incubating the bacterial suspension at low rotational speed had a relatively small effect on the peak of urease activity, and the trend in activity change and the time point of peak activity tended to be similar. Nonetheless, as the speed of shaking incubation was increased and the incubation time was extended, there were significant differences in the peak activity and the pattern of change in the bacterial suspension as a function of the inoculum level. An inoculum level of 2% was found to be most effective.

The pattern of urease activity change between the two protocols differed as the shaker speed was varied. Generally, the urease activity levels were low for the medium and high speeds, whereas the values for the low speed were higher. The peak urease activity of the medium-speed condition was much higher than that of the other two conditions in the optimum environment.

The effect of prolonging the shaking time on the urease activity of the broth was mainly in terms of “slowing down the decay of activities” and “promoting the increase in activities”. The continued shaking of the bacterial suspension will promote the maintenance of urease activity if the urease activity of the broth has reached its peak; if the urease activity of the broth has not reached its peak, extending the shaking time will promote the increase in urease activity.
Following 36 h of shaking at the optimal inoculum and speed, the urease activity values significantly increased both in shaking and standing cultures, and for economic reasons, the standing culture should be used. At all test time points, the highest levels of urease activity were measured for the solution prepared in accordance with protocol B2-36, which involves inoculation of 2% bacterial suspension under 190 rpm rotation speed and shaking for 36 h.

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**References**

1. Yang, S.; Shen, X.; Liu, H.; Ge, H.; Rui, X. Gradation affects basic mechanical characteristics of Chinese calcareous sand as airport subgrade of reefs. Mar. Georesour. Geotechnol. 2020, 38, 706–715. [CrossRef]
2. Wang, X.; Liu, J.Q.; Cui, J.; Wang, X.Z.; Shen, J.H.; Zhu, C.Q. Particle breakage characteristics of a foundation filling material on island-reefs in the South China Sea. Constr. Build. Mater. 2021, 306, 124690. [CrossRef]
3. Cheng, L.; Cord-Ruwisch, R.; Shahin, M.A. Cementation of sand soil by microbially induced calcite precipitation at various degrees of saturation. Can. Geotech. J. 2013, 50, 81–90. [CrossRef]
4. Han, Z.; Cheng, X.; Ma, Q. An experimental study on dynamic response for MICP strengthening liquefiable sands. Earthq. Eng. Eng. Vib. 2016, 15, 673–679. [CrossRef]
5. Lin, H.; Suleiman, M.T.; Brown, D.G. Investigation of pore-scale CaCO₃ distributions and their effects on stiffness and permeability of sands treated by microbiologically induced carbonate precipitation (MICP). Soils Found. 2020, 60, 944–961. [CrossRef]
6. Whitaker, J.M.; Vanapalli, S.; Fortin, D. Improving the strength of sandy soils via ureolytic CaCO₃ solidification by Sporosarcina ureae. Biogeoosciences 2018, 15, 4367–4380. [CrossRef]
7. Liu, S.; Yu, J.; Zeng, W.; Peng, X.; Cai, Y.; Tu, B. Study on the effect of microbiially induced calcium carbonate precipitation on repairing cracks in triangular soils. J. Rock Mech. Eng. 2020, 39, 191–204.
8. Yue, J.W.; Chen, Y.; Zhao, L.M.; Zhang, B.; Kong, Q.M.; Gu, L.H.; Lu, H.F. Strength characteristics and mechanism of action of glutinous rice slurry improved imitation site soil at Chi Cheng site. J. Civ. Environ. Eng. 2022, 44, 195–204. (In Chinese and English)
9. Mu, B.; Gui, Z.; Lu, F.; Petropoulos, E.; Yu, Y. Microbial-Induced Carbonate Precipitation Improves Physical and Structural Properties of Nanjing Ancient City Walls. Materials 2021, 14, 5665. (In Chinese) [CrossRef] [PubMed]
10. Liu, S.; Yu, J.; Peng, X.; Cai, Y.; Tu, B. Preliminary study on repairing tabia cracks by using microbiologically induced carbonate precipitation. Constr. Build. Mater. 2020, 248, 118611. (In Chinese) [CrossRef]
11. Cheng, L.; Shahin, M.A. Stabilisation of oil-contaminated soils using microbi ally induced calcite crystals by bacterial floccs. Géotechnique Lett. 2017, 7, 146–151. [CrossRef]
12. Chen, X.; Zhang, D.; Larson, S.L.; Ballard, J.H.; Knote-Smith, H.M.; Nie, J.; Hu, N.; Ding, D.; Han, F.X. Microbi ally Induced Carbonate Precipitation Techniques for the Remediation of Heavy Metal and Trace Element–Polluted Soils and Water. Water Air Soil Pollut. 2021, 232, 268. [CrossRef]
13. Wei, T.; Yashir, N.; An, F.; Imtiaz, S.A.; Li, X.; Li, H. Study on the performance of carbonate-mineralized bacteria combined with eggshell for immobilizing Pb and Cd in water and soil. Environ. Sci. Pollut. Res. 2022, 29, 2924–2935. [CrossRef] [PubMed]
14. Lyu, C.; Qin, Y.; Chen, T.; Zhao, Z.; Liu, X. Microbial induced carbonate precipitation contributes to the fates of Cd and Se in Cd-contaminated seleniferous soils. J. Hazard. Mater. 2022, 423, 126977. [CrossRef] [PubMed]
15. Rahman, M.M.; Hora, R.N.; Ahenkorah, I.; Beecham, S.; Karim, M.R.; Iqbal, A. State-of-the-Art Review of Microbial-Induced Calcite Precipitation and Its Sustainability in Engineering Applications. *Sustainability* 2020, 12, 6281. [CrossRef]

16. Stocks-Fischer, S.; Galinat, J.K.; Bang, S.S. Microbiological precipitation of CaCO$_3$. *Soil Biol. Biochem.* 1999, 31, 1563–1571. [CrossRef]

17. De Muynck, W.; De Belie, N.; Verstraete, W. Microbial carbonate precipitation in construction materials: A review. *Ecol. Eng.* 2010, 36, 118–136. [CrossRef]

18. Fujita, M.; Nakashima, K.; Achal, V.; Kawasaki, S. Whole-cell evaluation of urease activity of Pararhodobacter sp. isolated from peripheral beachrock. *Biochem. Eng. J.* 2017, 124, 1–5. [CrossRef]

19. Zhao, X. Experimental Study of Microbially Induced Calcium Carbonate Precipitation (MICP) Curing Soil. Ph.D. Thesis, China University of Geosciences, Beijing, China, 2014. Available online: https://cdmd.cnki.com.cn/Article/CDMD-11415-1014249450.htm (accessed on 14 June 2022). (In Chinese)

20. Lv, C.; Tang, C.; Zhu, C.; Li, W.; Chen, T.; Zhao, L.; Pan, X. Environmental Dependence of Microbiially Induced Calcium Carbonate Crystal Precipitations: Experimental Evidence and Insights. *J. Geotech. Geoenviron. Eng.* 2022, 148, 04022050. [CrossRef]

21. Liang, S.; Xiao, X.; Li, Z.; Feng, D. Effect of Nutrient Solution Composition on Bio-Cemented Sand. *Crystals* 2021, 11, 1572. [CrossRef]

22. Tang, C.; Yin, L.; Jiang, N.; Zhu, C.; Zeng, H.; Li, H.; Shi, B. Factors affecting the performance of microbial-induced carbonate precipitation (MICP) treated soil: A review. *Environ. Earth Sci.* 2020, 79, 94. [CrossRef]

23. Al-Thawadi, S. High Strength In-Situ Biocementation of Soil by Calcite Precipitating Locally Isolated Ureolytic Bacteria. Ph.D. Thesis, Murdoch University, Perth, Australia, 2008. Available online: https://researchrepository.murdoch.edu.au/id/eprint/721/ (accessed on 14 June 2022).

24. Lai, H.J.; Cui, M.J.; Wu, S.F.; Yang, Y.; Chu, J. Retarding effect of concentration of cementation solution on biocementation of soil. *Acta Geotech.* 2021, 16, 1457–1472. [CrossRef]

25. Han, Z.G.; Cheng, X.H. Exploration of the effect of nutrient salts on microbial reinforcement of liquefiable sandy soils. *Ind. Constr.* 2015, 45, 19–22. (In Chinese)

26. Zhang, Y.; Guo, H.X.; Cheng, X.H. Influences of calcium sources on microbially induced carbonate precipitation in porous media. *Mater. Res. Innov.* 2014, 18 (Suppl. S2), S2-79—S2-84. [CrossRef]

27. Li, C.; Wei, T.; Ji, B.; Lei, X.; Wang, X. Effects of different calcium sources and Ca$^{2+}$ concentrations on MICP. *Environ. Sci. Technol.* 2018, 41, 30–34. (In Chinese)

28. Maleki Kakelar, M.; Yavari, M.; Yousefi, M.R.; Nimtaj, A. The Influential Factors in the Effectiveness of Microbial Induced Carbonate Precipitation (MICP) for Soil Consolidation. *J. Hum. Environ. Health Promot.* 2020, 6, 40–46. [CrossRef]

29. Ersan, Y.C.; de Belie, N.; Boon, N. Microbially induced CaCO$_3$ precipitation through denitrification: An optimization study in minimal nutrient environment. *Biochem. Eng. J.* 2015, 101, 108–118. [CrossRef]

30. Wang, Y.; Jin, Z.; Wu, L. Preservation and management of microbial strains. *Shanghai Prev. Med.* 2007, 19, 87–88.

31. Chu, J.; Stabnikov, V.; Ivanov, V. Microbially Induced Calcium Carbonate Precipitation on Surface or in the Bulk of Soil. *Geomicrobiol. J.* 2012, 29, 544–549. (In Chinese) [CrossRef]

32. Chen, H. Study on the Influence of Compression and Shear Properties of Grade Paired MICP Reinforced Sandy Soils. Master’s Thesis, Chongqing University, Chongqing, China, 2019. Available online: https://cdmd.cnki.com.cn/Article/CDMD-10611-1019909598.htm (accessed on 14 June 2022).

33. Xu, W.; Zheng, J.; Chu, J.; Zhang, R.; Cui, M.; Lai, H.; Zeng, C. New method for using N-(N-butyl)-thiophosphoric triamide to improve the effect of microbial induced carbonate precipitation. *Constr. Build. Mater.* 2021, 313, 125490. (In Chinese) [CrossRef]

34. Whiffin, V.S. Microbial CaCO$_3$ Precipitation for the Production of Biocement. Ph.D. Thesis, Murdoch University, Perth, Australia, 2004. Available online: https://researchrepository.murdoch.edu.au/id/eprint/399/ (accessed on 14 June 2022).