Biocementation effect of high-efficiency urease-producing bacteria mutagenized from indigenous bacteria

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Abstract: A strain of urease-producing bacteria (UPB) was isolated from soil collected from the south of China, and a mutated strain with a high urease-producing efficiency was obtained through ultraviolet mutagenesis technology. The morphological characteristics of the isolated strain were examined under a transmission electron microscope. Numerous controlled trials were conducted to determine the optimal culture conditions of the mutated strain. Biocemented treatments were conducted on tailing sand with the mutated strain, and the uniaxial compressive strength of the biocemented samples was measured. SEM imaging and EDS were used to identify the type and morphological characteristics of calcium carbonate formed during biocementation. Results showed that the urease activity of the mutated strain was the highest at pH 9, and the optimum growth temperature was between 25°C and 35°C, which is similar to climate conditions in southern China. The urease activity of the mutated strain and the production of calcium carbonate were 2.18 and 1.16 times that of commercially used Sporosarcina pasteurii, respectively. The SEM images indicated that crystals with an unusual shape, such as long grains and broccoli shape, formed in the biocemented tailing sand. Raman test revealed that they were calcite. Therefore, using indigenous bacteria is practical and feasible, and ultraviolet mutagenesis technology has great potential for application in the improvement of the performance of isolated strains and their biocementation effect.

Keywords: Microbially induced calcite precipitation; Indigenous bacteria; Ultraviolet mutagenesis; Crystallization

1. Introduction
As a promising and green soil strength technology, microbial induced carbonate precipitation (MICP) technology is an environment-friendly solution for addressing the concerns of environmental and architectural [1]. MICP involves urea hydrolysis, which is a natural process of bio-induced mineralization [2-3]. Urea is hydrolyzed into \( NH_4^+ \) and \( CO_3^{2-} \) by urease enzyme [4], and calcium carbonate can be produced in the presence of \( Ca^{2+} \) in a solution [5]. Calcium salts form during biotreatment and adsorb on soil particles; then, they create bridges between soil particles, thereby
significantly increasing the friction among soil particles [6]. Soil pores become filled with large amounts of calcium carbonate, so the strength and impermeability of materials can be enhanced [7].

Given the effectiveness of MICP, engineers have developed strategies on how to improve biocemented efficiency. Many researchers discussed the factors that affect the uniform distribution and the affective attachment of bacteria to soil particles. Abiotic factors can also significantly affect the urease-producing activity of bacteria. When bacteria are injected into soil, the pH, temperature, and nutrient conditions of soil limit the urease-producing activity of bacteria; these bacteria eventually become inactive if the soil conditions are unsuitable for their survival [8]. Nevertheless, the urease-producing activity can be maintained and efficient mineralization can be achieved by using indigenous bacteria. These bacteria can adapt to the pH, temperature, and nutrient condition of the soil from where they are screened. In a previous study, various indigenous urease-producing bacteria (UPB) are isolated to verify their application potential in bioengineering [9]. Specifically, phenotypic mutants are developed to perform well on alkali resistance [10] and bioremediation of Pb-contaminated soil [11]. Although ultraviolet mutagenesis is a reliable bacterial modification technology, few related studies in the field of MICP have been conducted. Further studies should reveal whether ultraviolet mutagenesis is effective in the improvement of UPB.

In this work, UPB were isolated from soils collected from several locations in a humid hot area in China (Fuzhou, Fujian Province). Isolates with a stable urease-producing ability were obtained, and the morphological characteristics, growth conditions, and 16S rRNA sequence were explored. Many ultraviolet mutagenesis tests were conducted on the isolates to obtain a strain of ultraviolet-mutagenized bacteria, which likely had a high urease-producing efficiency. Tailing sand was subjected to bio-grouting. Scanning electron microscopy (SEM), energy dispersive spectrometry (EDS), and Raman spectroscopy were performed to determine how the characteristics of soil particles influence the morphological characteristics, size, spatial distribution, and crystallization state of calcium carbonate. The crystallization process, including the role of bacteria in nucleation, crystal growth, influencing factors of crystallinity, and crystal defect, was discussed. The uniaxial compressive strength of biocemented samples was measured to verify the MICP feasibility of mutagenic bacteria. Ultraviolet mutagenesis provided new insights into the improvement of biocemented efficiency.

2. Materials and Methods

2.1. Soil Collection
Soil samples were collected at 4:00 p.m. in autumn and separately stored in aseptic plastic bags to minimize the impact of climate change and temperature. The four locations of soil sample collection are shown in Fig. 1. The soil in position 1 was 20 cm below the vegetable garden ground near Fuzhou University and composed of a small number of plant roots. The soil in position 2 was slightly wet and loose topsoil of the vegetable field near the Fujian University of Technology. The soils in positions 3 and 4 were from the campus of Fuzhou University.

![Fig. 1. Location of each soil sample](image)

2.2. Enrichment cultures of indigenous UPB
An enrichment medium was used to promote the growth and reproduction of the target bacteria. The composition of the enrichment medium was 10 g/L *Saccharomyces*, 10 g/L (NH₄)₂SO₄, 8.2 g/L sodium acetate anhydrous, and 3.3 mol/L urea. Each soil sample (1 g) was placed in the enrichment
medium, and the enrichment medium was treated in an Innova 44 rotary shaker at 150 rpm and 34°C for 48 h. After the treatment, the suspension contained numerous target bacteria, and the suspension was collected to isolate and screen isolated bacteria.

2.3. Isolation and screening of indigenous strain
A selective medium was used to select the UPB from all kinds of bacteria in the suspension, and the formulation of the selective medium was as follows: 5 g/L NaCl, 0.012 g/L phenol red, 2 g/L potassium dihydrogen phosphate, 1 g/L glucose, 0.2 g/L peptone, and 20 g/L urea of solution. Phenol red was used in the selective medium as a pH indicator because the surrounding environment changes to alkaline during urea hydrolysis, i.e., it changes from its original color of yellow to red when a solid medium is alkaline. The red region indicated that the bacterial strain produced urease. Urea-hydrolyzing bacteria could be preliminarily identified by distinguishing the change in the color of the medium.

The process of inoculation and culturing of indigenous bacteria is shown in Fig. 2. An inoculating loop was used to isolate a single strain in the red region of the selective medium, and the bacteria were inoculated on the culture medium. In this test, the selective culture medium (NH₄-YE) of UPB was the same as that of Bacillus pasteurii and composed of the following: 20 g/L Saccharomyces, 10 g/L (NH₄)₂SO₄, and 0.13 M Tris buffer (pH = 9.0). After the UPB were inoculated, the culture medium was treated in an Innova 44 rotary shaker at 150 rpm and 34°C. In Fig. 2(c), a fresh bacterial solution was obtained after 24 h of treatment of the inoculated culture medium.

![Fig. 2. Inoculation and culturing of indigenous bacteria](image)

2.4. UV mutagenesis technology of isolated bacteria
In the practical application of bacteria, mutagenesis is usually needed to improve the characteristics of bacteria and meet application and production requirements. Therefore, UV was applied to mutate the naturally selected bacteria and thus obtain high-efficiency UPB adapted to the humid and hot climate in southern China. The specific process of the UV mutagenesis test is summarized in Fig. 3.

![Fig. 3. UV mutagenesis](image)

UV mutagenesis involves three processes:
(1) In UV mutagenesis, the isolated bacteria were placed in a UV mutation box to expose them to UV. The bacterial liquids were separately inoculated on the solid medium. The bacteria that grew on the solid medium were considered the mutant strain.
(2) In secondary screening (Fig. 3), a screening plate was divided into six regions. The isolated strain was inoculated on one of the six regions, and the mutant strains were inoculated on the
remaining regions. After they were treated in an incubator at constant temperature, the strains with a larger or faster flushing area than the isolated strain were selected for further rescreening.

(3) In the extended cultivation, as shown in Figs. 3 (5) and (6), the strains selected in process (2) were inoculated on the culture medium (NH₄-YE) for the extended cultivation. The urease activity of each group was measured with a conductivity meter. The strain whose enzyme activity initially increased but subsequently decreased significantly after several generations of culture was discarded. Afterward, a mutant named YB7 with a high enzyme activity and a stable performance was obtained.

2.5. MICP treatment and soil samples for bio-grouting
The common grouting mold is generally made of a PVC material, so it easily deforms during bio-grouting. As such, a new grouting mold made of 302 stainless steel was used for bio-grouting in this study. The detailed design of the mold is shown in Fig. 4. A peristaltic pump was used to inject liquid into the steel grouting mold, and three specimens were prepared by using tailing sand. A fresh bacterial liquid was treated in an Innova 44 rotary shaker at 150 rpm and 34°C and obtained. Then, 150 mL of fresh bacterial liquid was injected into the grouting mold at a rate of 2 mL/min, and 160 mL of cementing liquid (a mixture of CaCl₂ and urea with a mixing ratio of 1:1 by volume) was injected at a rate of 1 mL/min. This two-step process was considered a bio-grouting cycle. The sample was allowed to stand for 24 h to ensure the complete reaction of MICP. Mine tailings were used in MICP treatment. The grain gradation curve is presented in Fig. 5.

3. Results

3.1. Morphological characteristics and rDNA gene sequences of the isolated bacteria
The bacterial solution was diluted and coated on the culture medium (NH₄-YE) that was then placed in an incubator at 34°C. After 24 h of treatment, several colonies formed on the medium, as shown in Fig. 6 (a). They were round, moist, and smooth. They had the same color at the top and bottom and uniform texture. They were large and protruding, opaque, and easy to pick. One of the strains was selected and observed under a transmission electron microscope at 100× magnification. The results showed that the isolated bacteria were long, rod-shaped, and semicircular at both ends.
The isolated bacteria were subjected to genome extraction, pre-PCR experiment, and formal PCR experiment (completed by Xiamen Jingju Technology Co., Ltd.) to measure their 16S rDNA gene sequences. Then, the 16S rDNA gene sequences of the isolated bacteria were compared with those in all the databases in the Nucleotide BLAST of NCBI. The result showed that the similarity of 16S rDNA between the isolated bacteria and *Bacillus lentus* strain NBRC 16444 reached 99%. The phylogenetic trees of CX21 are shown in Fig. 7.

### 3.2. Urease activity

OD$_{600}$ and conductivity change rate were used to accurately calibrate the optimal cultivation time of the isolated and mutagenized UPB and characterize bacterial growth and urease activity. The conductivity method was used in the test to measure conductivity [2]. The curves with culture time as the abscissa and conductivity change value and OD$_{600}$ as the main axis were plotted separately (Fig. 8). In Fig. 8(a), the mutagenized UPB entered the logarithmic growth period after 6 h of culture, reached the stable phase at 12 h, and obtained the highest urease activity at 18 h. In Fig. 8(b), the highest conductivity change rate of the mutagenized UPB was 0.72 ms (0.144 ms/min) at 18 h, which was 2.18 times that of common *Sporosarcina pasteurii* and 1.33 times that of the isolated UPB. Hence, the cultivation time of the mutagenized UPB was determined to be 18 h in the test. Moreover, the superior performance of the mutagenized UPB confirmed that ultraviolet mutagenesis was practical and could be used to improve the MICP efficiency of indigenous bacteria.

![Fig. 7 Phylogenetic trees of the isolated bacteria](image)

**Fig. 7** Phylogenetic trees of the isolated bacteria

![Fig. 8](image)

**Fig. 8** Growth characteristics and changes in the activity of indigenous and mutant bacteria: (a) growth curve and (b) change in conductivity difference

### 3.3. Morphological characteristics of calcium carbonate crystals in biocemented tailing sand
The samples were observed using a Zeiss Sigma 300 field emission scanning electron microscope (SEM Raman imaging correlation system; WITec) and an EDS detector (Bruker).

Strain-specific precipitation occurs in MICP, and different strains influence the morphological characteristics of calcium carbonate \(^{[12]}\). Calcium carbonate crystallization is a vital process of MICP so that soil particles can accumulate. Hence, the SEM images and EDS of two kinds of biocemented samples were captured and compared to reveal how mutagenic bacteria and soil type would influence the morphological characteristics of calcium carbonate.

In Figs. 9(a) and 9(b), long-grain- and broccoli-shaped calcites were observed in the biocemented tailing sand. They were stacked on top of each other in large numbers and arranged disorderly. They also had different sizes. The crystal of the long-grain-shaped calcite had narrow ends and a wide middle portion. Additionally, some rose-shaped materials were captured. They were adhering to the surface of broccoli-shaped calcite and composed of leafy lamellar structures similar to desert rose stones commonly found in antique markets. Their components were revealed using an EDS.

EDS analysis indicated that the rose-shaped material was mainly composed of S and O and had a small amount of Si and Ca. C was also detected. The element composition and morphological characteristics of the rose-shaped material were similar to sand roses described in a previous study \(^{[13]}\). Khadidja Zouaoud \(^{[13]}\) suggested that S in flotation reagents in mine tailings mixed with calcium chloride in a cementing liquid participate in a metallogenic process. Thus, the symbiotic rose-shaped crystal forms. In our study, Raman spectroscopy was applied to determine the crystal type of long-grain- and broccoli-shaped crystals. The results revealed obvious characteristic peaks of Raman spectroscopy (Fig. 10). Two peaks corresponded to the lattice vibration of calcite and the in-plane bending vibration peak of the \([\text{CO}_3]\) group of calcite (\(\nu_4\) of approximately 712 cm\(^{-1}\)). Therefore, the two kinds of crystals with different shapes were calcite.
The low cementation level at which the tailing sand still had some pore spaces, a large number of bacteria with complete morphology, and small particles on and around their surfaces were detected. The role of bacteria (including their organic substrate) in calcium carbonate crystallization could be further analyzed by observing the state of calcium carbonate around the bacteria and the bacteria themselves. Furthermore, the mineralization mechanism of mutagenic bacteria (YB7) could be initially explored. In Fig. 11, numerous porous materials, which were similar to previously described amorphous calcium carbonate (ACC) \[14\], with adherent bacterial cells could be captured around the YB7. Under the action of these bacteria, pre-nucleated clusters initially formed in the solution and subsequently aggregated to produce ACC nanoparticles. Afterward, these nanoparticles aggregated and grew on the surface of organic matter to crystallize. These nanoscale grains further aggregated and developed into single crystals on a relatively stable template under bacterial regulation. Therefore, ACC formed, grew, and transformed into a single crystal. These processes occurred in the early stage of crystallization.
3.4. Uniaxial compressive strength of biocemented samples

A microcomputer-controlled electronic universal testing machine was used to conduct the uniaxial compression test on the dried and polished samples at a loading speed of 1 mm/min. The uniaxial compressive strength of the three groups was measured. The average UCS of the biocemented tailing sand was 4.70 MPa. The tailing sand had a wide range of particle sizes, mineral integrity, less clastic material, and a rough surface of soil particles. These characteristics improved the biocementation effect of the soil. The solution had a good flowability in the tailing sand. The cementation effect of the soil was uniform, and the surface area of coarse soil particles was larger than that of fine particles; as a result, more calcium carbonate was formed. In a sufficient grouting environment, after the calcium carbonate generated on the surface of each soil particle accumulated, it could effectively contribute to cementation and delay the formation of new cracks. During loading, the pores filled with calcium carbonate could not be easily penetrated, and the bearing capacity strengthened.

4. Conclusions

In this test, indigenous bacteria were isolated from soils, and a mutant strain was obtained from the indigenous strain through UV mutagenesis. The morphological characteristics of the mutant strain were determined, and the MICP feasibility of the mutant strain was discussed by examining the morphological characteristics, size, crystal form, spatial distribution, and crystalline form of calcium carbonate. The ability of the mutant strain to control calcium carbonate phase formation and nucleation mechanism was also described. The conclusions were summarized as follows:

(1) The urease activity of the mutant strain was 2.18 times that of commonly used S. pasteurii. The higher urease activity of the mutant strain confirmed that ultraviolet mutagenesis technology improved the urea hydrolysis ability of UPB. Long-grain- and Broccoli-shaped crystals were observed in the biocemented tailing sand. Raman test revealed that they were calcite.

(2) The environmental change caused by the mutant strain itself indicated the calcium carbonate crystallization induced by the mutant strain. Nucleation occurred on the nanoparticles formed on EPS rather than on the surface of the bacterial cell wall. Therefore, the commonly accepted model of bacterial cell surfaces as nucleation sites for precipitates during MICP could not accurately describe the entire nucleation process of MICP. Further studies should be performed to reveal the accurate nucleation model of MICP technology.

(3) UCS tests showed that the average UCS of the biocemented tailing sand could reach 4.70 MPa. The high urease activity, stable reproduction, and excellent mineralization effect indicated that using indigenous UPB and ultraviolet mutagenic UPB was practical and could improve biocementation efficiency.

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