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Effect of low-temperature treatment on bacterial cultivation in bacterial induced mineralization

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In this paper, the effect of low-temperature treatment on bacterial activity was investigated and its performance in preparing the consolidated sand was accessed. Four factors, including absorbance (OD600), pH, electric conductivity (EC) and urease activity (UA) were monitored to evaluate the bacterial activity. Moreover, the calcium carbonate yields under different bacterial solution and cementation solution volume ratios were determined. Finally, the compressive strength and microstructure of consolidated sand were investigated and the mechanism was explored by characterising the products among sand particles. The results showed that for both continuous and interval cultivation of bacteria, stable bacterial solution was harvested within the cultivation period of 30 h. Low-temperature treatment showed limited inhibition at early cultivation stage, while it had strong inhibition at middle cultivation stage. The interval cultivation improved the cultivation efficiency of bacteria. It was observed that the low-temperature preservation for a stable bacterial solution has little influence on UA before 4 days, while UA decreased seriously after its preservation time was extended to over 8 days. The optimal volume ratio of bacterial solution and substrate solution was 1:1, resulting in the highest calcium carbonate yield. The improved compressive strength of consolidated sand after 3 injections was attributed to the irregular-shaped calcite products, which coated and bonded the sand grains together.

microbial mineralization, cultivation methods, low-temperature treatment, bacterial activity, microstructure

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1 Introduction

As one of the eco-friendly and sustainable development approaches to enhance the property of materials, microbial mineralization has been extensively investigated from either the natural environment or laboratory observation [1]. Generally, two types of process, in terms of biologically induced mineralization and biologically controlled mineralization, have been identified as the two main approaches of microbial mineralization [2]. For biologically induced mineralization, the minerals are formed extracellularly as a result of the metabolic activity of the organism [3]. The microbial induced mineralization has been applied in many fields, such as soil reinforcement or modification [4–6], sand consolidation or cementation [7,8], performance-modifier of cementitious materials [9,10], crack remediation [11–13], solidification or removal of heavy metals [14], and ancient buildings' restoration [15]. Meanwhile, biologically controlled mineralization commonly occurs due to the cellular activities of the microorganisms [2] and the composition of the biominerals is mainly governed by the environment inside the vesicles or vacuoles [16]. Thus, the application of biologically controlled mineralization is rare.

The main bacterium applied in microbial induced mineralization is a kind of urease-producing bacteria [17], which can generate urease to hydrolyze urea and produce calcium carbonate in the presence of calcium ions. Therefore, the efficiency of microbial mineralization depends on the se-

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cretion of the bacteria and execution of the urease enzyme which can degrade the urea [2]. Generally, the urease-producing bacteria for microbial mineralization is obtained in two ways, either purchasing from a commercial supplier in the USA [18,19], Germany [9,20,21] and China [22], or extracting from the raw materials such as soil [22], solid waste or cement specimen [1,23–25], which requires the involvement of cultivation.

Obviously, the cultivation and preservation of bacteria are very important, which depends on the wide application of microbial induced mineralization. It has been reported that mature bacteria were harvested after culturing between 30 to 40 h, and the prepared bacterial solution was preserved at 4 °C for a few days [19]. Moreover, Mortensen et al. [26] suggested that the maximum period for the preservation of harvested bacteria were reached to 14 days. In addition, Omoregie et al. [25] reported that optimal urease activities were achieved at 25–30°C. The reason could be that the urease was extremely impaired at a low temperature, which may lead to insufficient activity [7]. Therefore, Don et al. [27] reported that the suitable temperature range for the growth of *B*. *megaterium* was between 3 and 45°C. Sun et al. [28] reported that the precipitation of urease-producing bacteria *B*. *megaterium* at the temperature of 10°C was calcite, which was more stable than vaterite precipitated by *Sporosarcina pasteurii* at the same condition. Krajewska et al. [29] reported the lifetime of urease was 5–8 days when the temperature was 25°C. However, it is generally agreed that its half-life could be extended when it is preserved at a lower temperature [29,30]. The reason could be attributed to that lower temperature inhibits bacterial reproduction, which, therefore, provides less intracellular urease to involve the reaction solution [31]. Considering the factor of decreased enzymatic activity at lower temperatures, it was not surprising that the precipitation efficiency of the bacteria was decreased, which has been investigated and confirmed in the literatures [32,33]. Therefore, although it is generally accepted that the urease producing bacterial solutions could be stored at 4°C and retrieved immediately before using [34], the influence of low-temperature (refers to 4°C hereinafter) treatment on both OD600 and urease activity of the stable bacterial solution has not been clear. As reported, the low-temperature treatment could inhibit the activity of bacteria at the stable phase [7]. However, the different treatment on different growth stages of bacteria is also important. Generally, the growth of bacteria can be divided into four stages: a lag phase, an exponential phase, a stationary phase and a decline phase [35]. The effects of low-temperature treatment on different growth stages, which might either inhibit or improve the activity, are still unknown. Furthermore, the influence of treatment temperature during different bacterial growth stages and the preservation time of bacterial solution at low-temperature treatment, the optimal bacterial solution and cementation solution volume ratio for generation of calcium carbonate, in microbial reduced mineralization have not been explored.

Therefore, this paper investigated the influence of low-temperature treatment at different cultivation stages of bacterial solution. Cultivation methods include both continuous and interval cultivation on bacterial-induced mineralization. For interval cultivation, low-temperature treatment was set at exponential phase and stationary phase i.e., at 6, 12, 18 and 24 h, respectively. To evaluate the bacterial activity, four parameters, including optical density at a wavelength of 600 nm (OD600), pH, electric conductivity (EC) and urease activity (UA) were monitored during the cultivation process and the calcium carbonate yields of bacterial-induced mineralization under different bacterial solution and cementation solution volume ratios were determined. Finally, the compressive strength and microstructure of the bacterial-incorporated consolidated sand were determined and their relationship was attempted to be discussed.

2 Materials and methods

2.1 Bacteria and cultivation conditions

The bacteria used in this paper was *Sporosarcina pasteurii* (CGMCC No. 1.3687) strain, which is a type of urea hydrolyzing bacteria. The microbial lyophilized powder of the bacteria was isolated from soil and was purchased from China General Microbiological Culture Collection Centre, which is poisonless and harmless. The ingredients of the recommended culture medium 0907 are shown in Table 1, and the pH of the culture medium was adjusted to 7.0 with 1 mol/L NaOH. All culture mediums used for the subsequent experiments were stored within 30 days [36].

After preparing the culture medium, which includes solid culture medium in a test tube and liquid culture medium in both test tube and Erlenmeyer flask, the inoculation process of the bacteria was conducted, which is displayed in Figure 1. The inoculation process of the bacteria contained at least 3 generations of incubation, and then the stable bacteria were obtained, which was then used in the next experiment. After being inoculated in a liquid culture medium, the bacteria were then aerobically inoculated at 30°C in a shaker with a rotation rate of 150 r/min.

2.2 Cultivation regimes of bacteria

Two cultivation methods, including continuous cultivation and interval cultivation, were applied in this study. The continuous cultivation (Regime 1) indicated the bacteria were cultured for 60 h after inoculation without any interruption. The interval cultivation (Regimes 2–5) mean that the bacteria were first cultured for a certain period of time before

Table 1 Ingredients of the culture medium 0907

Bacterial type	Ingredients of the culture medium 0907 (L^{-1})				
	Peptone	Beef extract	Urea	$MnSO_4 \cdot H_2O$	Agar
Sporosarcina pasteurii	5 g	3 g	20 g	0.01 g	15 g

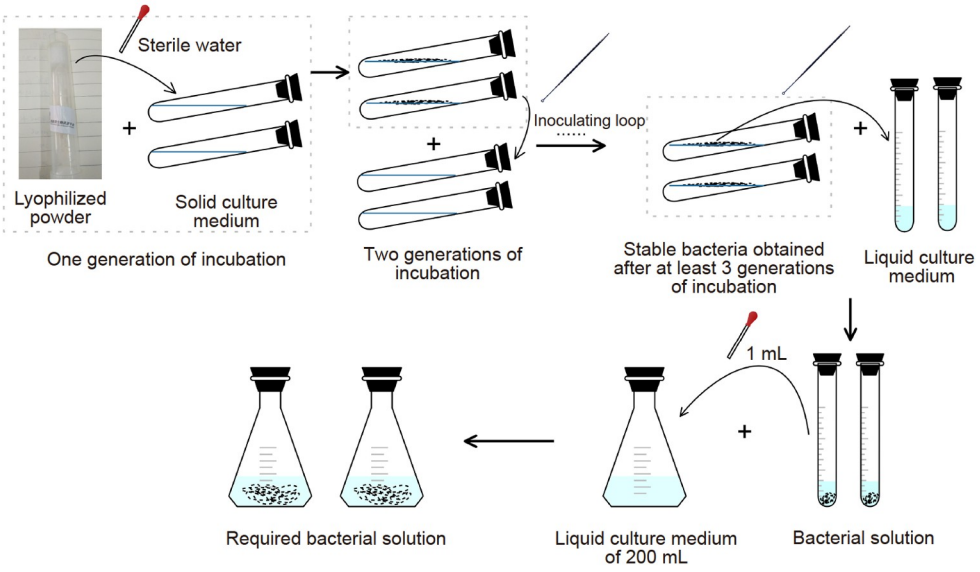

Figure 1 (Color online) The inoculation process of the bacteria.

storing in a refrigerator at 4°C for another 12 h (low-temperature preservation), which were then continuous to be cultured for another period of time till to 60 h. All regimes for bacterial cultivation were recorded to 60 h. And the cultivation regimes of bacteria are shown in Figure 2. As shown in the figure, the total time of cultivation was 60 h. For interval cultivation (Regimes 2–5), the time for the first cultivation stage were selected as 6, 12, 18, and 24 h, respectively. Therefore, the corresponding second cultivation time was 42, 36, 30 and 24 h.

2.3 Tests on the performance of the bacterial solution

During the process of the experiments, the performance of the bacteria in the culture medium was monitored by optical density, pH, electric conductivity and urease activity.

2.3.1 Optical density (OD600)

The biomass concentration was determined by measuring the optical density at a wavelength of 600 nm using a spectrophotometer (Shanghai Jingke 721N, China), which was usually applied to compare different growth characteristics of the bacteria [32]. The spectrophotometer was calibrated by using un-inoculated growth media as blank before the optical density of bacterial cultures grown was measured [37]. Then, 3 mL bacterial solution was placed into a clean cuvette for test [38].

2.3.2 pH value (pH)

After the test of OD600, the bacterial solution in the cuvette was placed into a 7 mL centrifuge tube to measure its pH by using a pH meter (Shanghai Leici PHS-3C, China). The pH electrode was calibrated with pH 6.86 and 9.18 buffer solutions before each test.

2.3.3 Electric conductivity (EC)

After the test of pH, the bacterial solution in a centrifuge tube was used to determine its EC by using an electric conductivity meter (Shanghai Leici DDSJ-308F, China).

2.3.4 Urease activity (UA)

The urease activity of the bacterial solution was determined by using the conductivity measurement method [39]. The principle of the measurement is based on that the urea substrate in the solution is hydrolyzed by urease to produce ammonium and carbonate ions, which can increase the conductivity of the solution. UA was determined by measuring the relative change in conductivity when it was exposed to urea under conditions of 1.5 mol/L urea at 25°C [39]. The change in conductivity per minute (mS/min) was converted to the amount of urease hydrolysis per unit time by eq. (1), and eventually, the rate of hydrolysis of urea per minute (mM urea hydrolyzed/min, refers to "mM/min" hereinafter) was obtained via multiplying by the dilution factor of 10, which represents urease activity [39].

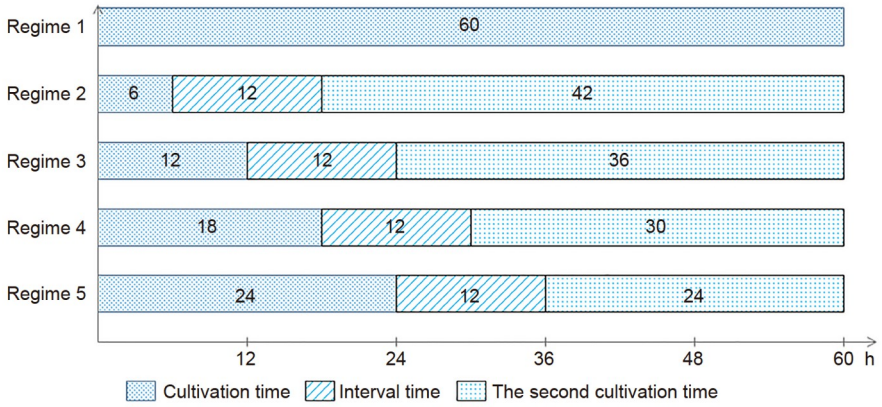

Figure 2 (Color online) The cultivation regimes of bacteria.

Urea hydrolysed (mM)

$$=\text{Conductivity (mS)} \times 11.11 \quad (R^2 = 0.9988). \tag{1}$$

2.4 The calculation of calcium carbonate yield

First, a clean filter paper was put into the oven with 105°C for 1 h, and the quality was weighted and recorded as M1 (g); then the different volume ratios of bacterial solution and cementation solution were mixed together for full reaction and transferred through the filter paper by rinsing thoroughly products three times with distilled water and the weight of the product was recorded as M2 (g); recording the whole solution of bacterial solution and cementation solution as V (mL), so the calcium carbonate yield (mg/mL) was calculated by

$$\text{Calcium carbonate yield}=(M2-M1)\times 1000 / V. \tag{2}$$

2.5 Preparation of sand column specimens

A syringe (50 mL) with the inner direction of 30 mm was used as the mould of sand column. The sand column in the syringe was prepared by the following procedure according to the preliminary experiment: (1) a layer of approximately 1 cm of gauze was placed at the bottom of the syringe, which was applied to prevent the flow of sand particles from the bottom of the syringe; (2) 30 g fine sand with a poorly graded (0.08–0.16 mm) was poured into the syringe, and then the syringe containing fine sand was shaken for one minute; (3) another layer of approximately 1 cm of gauze was placed at the top of the sand column, which was used to prevent the scour of bacterial solution and cementation solution when pouring into the sand column. Three sand column specimens were prepared as a set. All sand columns were positioned vertically. The peristaltic pump was connected to injection points at the top of each column. The distilled water was passed through the sand column to remove the air among sand particles before the test.

From the cultivation experiments of bacteria, in both continuous cultivation and interval cultivation approaches, the stable bacterial solution was obtained at the final. One bacterial solution with OD600 of 1.0, pH of 9.47, EC of 31.5 mS/cm and UA of 29.77 mM/min was selected to prepare the consolidated sand, since the quality between batches was stable based on the previous experiment, which can ensure the reproductivity of the experiment. The cementation solution was prepared by mixing 1 mol/L calcium chloride ($CaCl_2$) solution and 1 mol/L the urea ($CO(NH_2)_2$) solution.

One cycle injection process of bacterial solution and cementation solution for the sand column was described as follows. Firstly, 20 mL bacterial solution was injected and passed through the sand columns. The leaked liquid from the outlet of the syringe was then collected and re-used to be circulated into the same sand column specimen for 1 h. Secondly, the sand column stood for 6 h to make full absorption for bacterium on the surface of sand particles. Thirdly, 20 mL cementation solution was passed through the sand column and stand for 10 h for the full reaction between bacteria solution and cementation solution. Therefore, one cycle injection process for the sand column was completed within a day. Moreover, for each sand column specimen, three cycles of injection process were applied.

2.6 Tests on the performance of the bacterial solution in consolidated sand

After 3 cycles of injection processes for each sand column, every sand column with syringe mould was kept in a drying oven at the temperature of 30°C for 72 h. Then the sand columns were demoulded and both ends of the sand columns were polished for compressive strength. After compressive strength, the debris of the broken sand columns was collected for microscopic observation. The white substance on the surface of the injection point, which could be the product of

the bacteria, was collected for microscopic observation and X-ray diffraction (XRD). The collected debris and white substance were stored at a vacuum drying oven with 30°C till to test.

XRD analysis was used to analyze the crystal type of the precipitated substance and it was tested by X-ray diffractometry (X'Pert Pro produced by RIGAKU, Japan) with copper target and the continuous scan was from 10° to 80° with a step size of 0.02°.

Scanning electron microscope-energy dispersive spectrometer (SEM-EDS) analysis was used to analyze the surface topography, morphology and mineralogical compositions of the precipitated substance. SEM micrographs were obtained using field emission scanning electron microscope (Zeiss SIGMA, Germany) with energy dispersive X-ray unit (Oxford UltimMax 40, UK), with accelerating voltage of 5 kV, magnification 5× up to 100000× and resolution of 1.0–2.0 nm.

3 Results and discussion

3.1 The influence of bacterial activity on cultivation methods

The effect of growth time on OD600, pH, EC and UA of the bacterial solution under continuous cultivation and interval cultivation are shown in Figures 3 and 4. As shown in Figure 3(a) and (c), for continuous cultivation (regime 1), OD600 and EC increased slowly during the early cultivation (up to 5 h), then increased quickly between 5 to 12 h, and finally reached a stable state after 30 h, which were 1.2 and 32 mS/cm, respectively. Since OD600 and EC are related to the number of bacterial cells [40], the results suggested that the number of bacterial cells slowly increased within 5 h. After 5 h, the number of bacterial cells increased quickly, indicating that the growth speed became faster. Finally, the increase in the number of bacterial cells slowed down and gradually approached zero. However, for interval cultivation, when the low-temperature preservation was treated after 6 h's cultivation (regime 2), both OD600 and EC increased during low-temperature treatment. And after then, they increased quickly and finally reached a stable state. When the low-temperature preservation was treated after 12 h's cultivation (regime 3), both OD600 and EC were reduced during the low-temperature treatment, which is different from that after 6 h's cultivation (regime 2). After the treatment, OD600 and EC increased again and reached a stable state. Moreover, as shown in Figure 3(b), for regime 1 pH value increased along with the growth time, which reached a stable value of 9.5 after 10 h. The increased pH value could be attributed to the metabolism of bacteria in the culture medium. During the cultivation of bacteria, expecting the consumption of peptone and beef extract for metabolism, the number of bacterial cells increased, therefore, the quantity of urease increased as well. Meanwhile, in the presence of urea, some urea was hydrolyzed to form carbamate and ammonia, an additional one mole of ammonia and carbonic acid are formed due to the

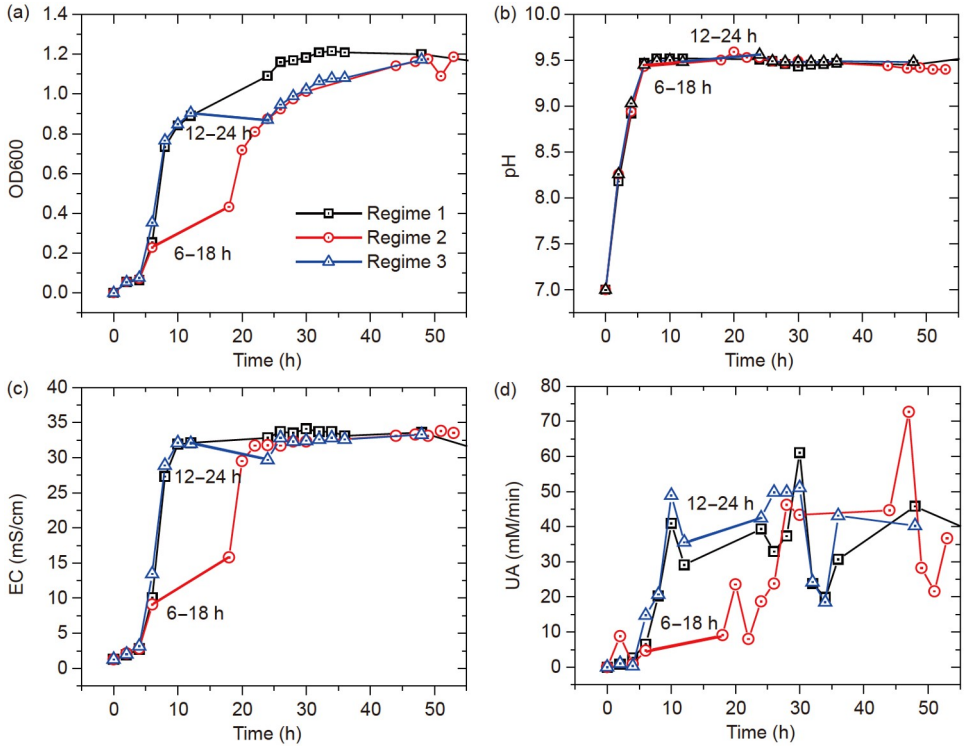

Figure 3 (Color online) Effect of growth time on OD600 (a), pH (b), EC (c) and UA (d) of the bacterial solution under continuous cultivation (regime 1) and interval cultivations (regime 2 and regime 3).

hydrolyzes of the carbamate. These products subsequently form 1 mole of bicarbonate and 2 moles of ammonium and hydroxide ions, respectively, which, therefore, lead to the increased pH of the culture medium [40,41]. For regime 2 and regime 3, pH value only increased a bit during the treatment and then stabilized at around 9.5 after low-temperature treatment. Furthermore, it can be seen from Figure 3(d) that with increasing growth time, UA increased slowly during early cultivation, then increased fast with the vigorous growth of bacteria, and stabilized at about 35 mM/min for regime 1. Meanwhile, for regimes 2 and 3, UA increased during the low-temperature treatment and the final value increased to about 44 mM/min, which was higher than that of continuous cultivation.

As shown in Figure 4, when the low-temperature preservation was treated after 18 h's cultivation (regime 4), similar trends were observed to those from continuous cultivation before the low-temperature treatment. However, apparently, the low-temperature treatment resulted in a decrease of OD600 and EC. Following that, they increased slightly and reached to a stable state. Moreover, the treatment exhibited limited influence on pH value, which was stabilized at 9.5. Furthermore, UA increased during the treatment and was finally stabilized at 33 mM/min. All the results showed a similar trend with the previous regime 3. When the low-temperature preservation was treated after 24 h's cultivation (regime 5), different from the effect under previous regimes, low-temperature preservation showed almost no effect on OD600, pH, EC and UA. The reason could be that after cultivating for 24 h, the growth of bacteria reached a stable state. Therefore, the inhibition of low-temperature treatment on the stable bacterial solution was weak, which, therefore, exhibited little influence on bacterial activity.

As illustrated previously, the influence of low-temperature treatment at different growth stages of bacteria was different. Under continuous cultivation, OD600, EC and UA were very low at early growth time, which indicated that the growth of microorganisms was slow. With the increase of growth time, OD600 and EC increased quickly and UA was further increased, which indicated that the growth of bacteria came into a logarithmic phase with the growth of microorganisms. At last, OD600 and EC reached a plateau, which showed that the growth of bacteria reached a stable stage. During the bacterial growth process, some ions were generated due to the consumption of nutrition for bacterial metabolism, which increased the EC. Under interval cultivation, the low-temperature treatment showed weak inhibition of bacterial growth at the early cultivation stage (after 6 h's cultivation). At the early cultivation stage, a liquid culture medium with rich nutrients contained a small number of microbes, thus the concentration of living bacterium was low. Although low-temperature treatment is unconducive to the growth of microorganisms [32], the increasing mean growth space of the bacterium was a benefit to the bacterial growth. Moreover, the slight increase of UA indicated that low-temperature treatment did not decrease the activity of urease. Instead, it was beneficial to the growth of UA. Under this condition, the promotion effect of rich nutrients was stronger than the in-

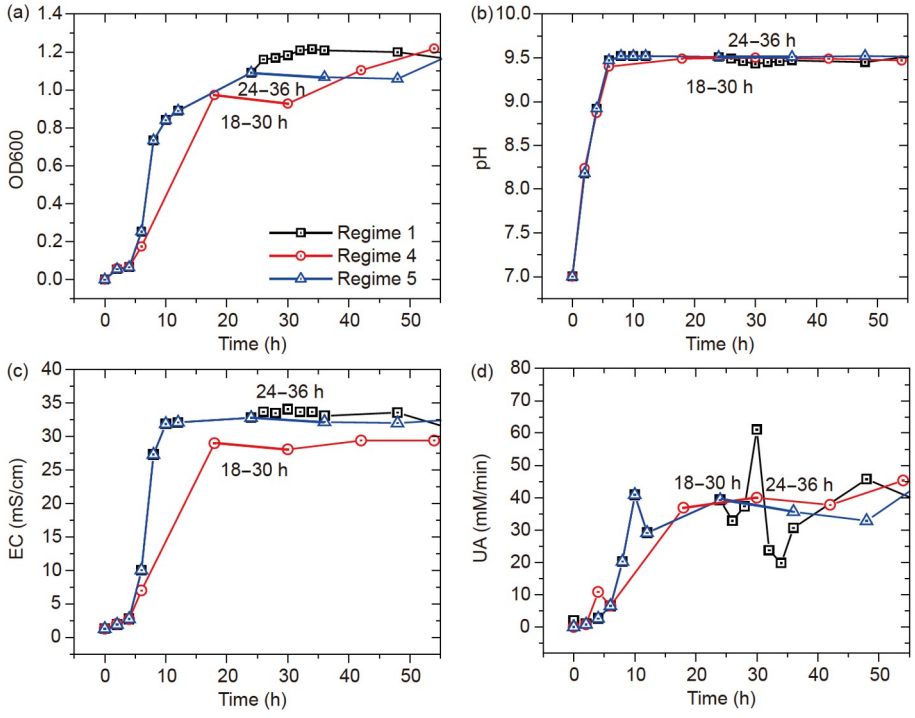

Figure 4 (Color online) Effect of growth time on OD600 (a), pH (b), EC (c) and UA (d) of bacterial solution under continuous cultivation (regime 1) and interval cultivations (regime 4 and regime 5).

hibition effect of low-temperature. Strong inhibition effect of low-temperature treatment was exhibited at the middle cultivation stage (after 12 h's cultivation). It might be caused by the high concentration of the bacterial solution and the mean living space of each bacterium was limited, which slowed down the growth of bacteria. Meantime, the low-temperature treatment was unconducive to the growth of bacteria. Considering the combined effect of the two reasons, the low-temperature treatment not only led to a strong inhibition of further growth of bacteria but also caused some death of bacteria, leading to a significant reduction in OD600 and EC. The inhibition effect of bacterial solutions at the late cultivation stage (after 18 h's cultivation and after 24 h's cultivation) was weaker than that after 12 h's cultivation. After 18 and 24 h's cultivations of the bacterial solution, the growth of bacteria reached a stable state. Therefore, the weak effect of low-temperature treatment on the bacterial solution was confined.

From above, during the process of bacterial growth, low-temperature treatment had weak inhibition of bacterial growth at early cultivation stage, while exhibiting a strong inhibition effect at the middle cultivation stage. Although the low-temperature treatment could inhibit the growth of bacteria to some extent, 12 h's low-temperature intervention during the culture process of bacteria could not prolong the cultivation time of bacterial solution. Therefore, the OD600 and UA could reach the same levels of bacterial solution with continuous cultivation. The low-temperature intervention reduced the cultivation time of bacterial solution in the shaker without any loss of UA. From this point, low-temperature intervention may improve the cultivation efficiency of the bacterial solution.

3.2 The reproducibility of continuous cultivation of bacterial solution

To evaluate the reproducibility of bacterial solution with continuous cultivation and test the stability of the bacterial solution, OD600, pH, EC and UA were tested for another bacterial solution under the exact same procedure, and the results are shown in Figure 5.

As shown in Figure 5, the change trends of OD600, pH, EC and UA with increasing growth time were similar to that of the bacterial solution presented in sect. 3.1. For example, both OD600 and EC increased slowly at early cultivation time and then increased quickly and reached stable values after 30 h's cultivation. pH changed from 7 to 9.5, which rapidly grew at early cultivation time and then the increment speed was slowed down. UA increased slowly at early cultivation time and increased to about 33 mM/min at last. To sum up, all the results were similar to the results described in sect. 3.1. Therefore, the comparison results suggested that a stable bacterial solution can be obtained through this method.

3.3 Effect of low-temperature preservation time on urease activity of the stable bacterial solution

To explore the effect of low-temperature treatment on a

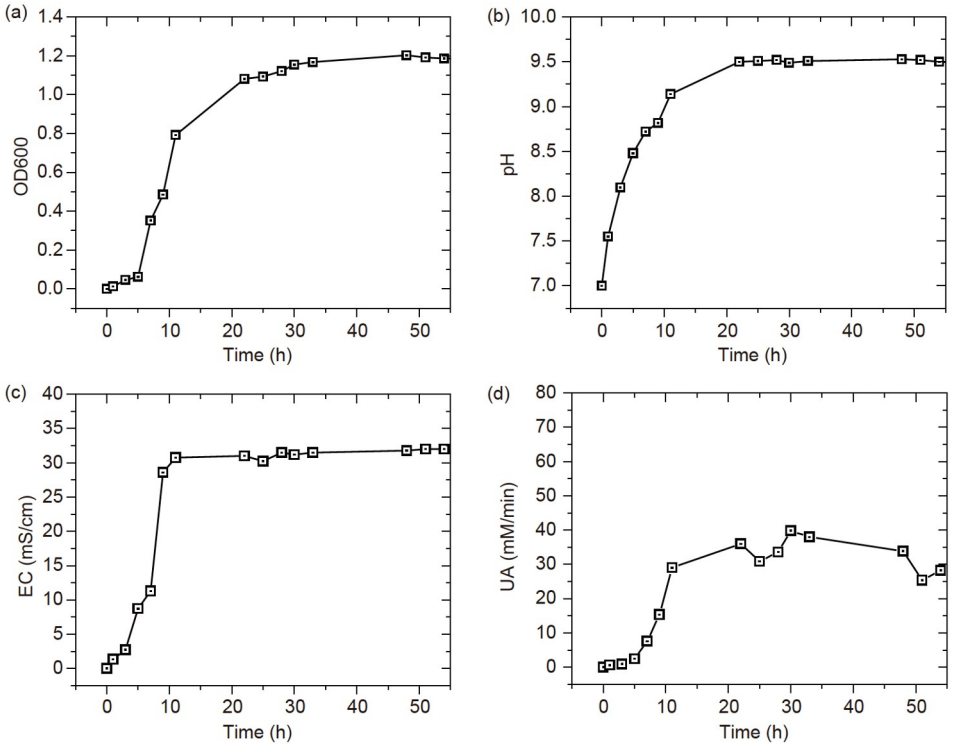

Figure 5 Effect of growth time on OD600 (a), pH (b), EC (c) and UA (d) of the bacterial solution under continuous cultivation (the test of reproducibility).

stable bacterial solution, OD600 and UA of two stable bacterial solutions stored at 4°C were monitored with increasing storage time, and the results are shown in Figure 6.

It can be observed from Figure 6 that OD600 and UA did not change after the first 4 days' preservation, which indicated that 4 days' low-temperature preservation for the stable bacterial solution could not decrease its activity. After 4 days' storage, both OD600 and UA were decreased slightly with the increase of storage time. For example, at 8 days, OD600 and UA of bacterial solution were about 1.12 and 32 mM/min, respectively. However, at 12 days' storage, OD600 was about 1.08 and UA was about 23 mM/min. Although OD600 of the bacterial solution made a slight decrease from 8 to 12 days' storage, UA decreased significantly. Such decreasing UA could lead to the dysfunction of the bacteria in its application. Therefore, based on the previous result, it is best to select the stable bacterial solution, where it was stored at low-temperature treatment within 4 days in its application for preparing consolidated sand. Meanwhile, although the level of OD600 and UA of the stable bacterial solution under less than 8 days' storage at the low temperature was still high, it can also be used in the application for preparing consolidated sand. The exact storage time for the stable bacterial solution can provide enough and qualify bacterial solutions for engineering applications.

3.4 The influence of bacterial solution and cementation solution volume ratios on calcium carbonate yield

The calcium carbonate yield with the increase of bacterial solution and cementation solution volume ratios are shown in Figure 7. As shown in the figure, the calcium carbonate yield increased with the increase of volume ratio of bacterial solution and cementation solution. When the volume ratio was 1, the calcium carbonate yield reached the highest value. When the volume ratio was higher than 1, the calcium carbonate yield decreased with the increase of the volume ratio. From the above, the calcium carbonate yield was different with the increase of bacterial solution and cementation solution volume ratio. The influence factors of calcium carbonate yield here were UA of bacterial solution and the concentration of calcium and urea in cementation solution. The optimal volume ratio of 1:1 indicated that UA at this time was enough to hydrolyze urea and generate carbonate ions. And with the same content of calcium ions, all carbonate ions are combined with calcium ions to generate calcium carbonate. At the same volume of the reaction solution, if the UA could not hydrolyze urea completely, the generated calcium carbonate yield could not reach the highest.

3.5 Stable bacterial solution used in consolidated sand

3.5.1 Compressive strength of the consolidated sand columns

The average compressive strength of the consolidated sand with the selected bacterial solution was 1.38 MPa. Although the value of the strength was low, compared with the pure sand (no compressive strength), the developed compressive strength could still indicate that the bacterial solution could consolidate the sand. Similarly, Yu et al. [42] investigated that the average compressive strength of bio-sandstone with sand particles range from 212 to 425 μm was 0.37, 0.63 and 1.33 MPa when the number of injections was 2, 4 and 6, respectively. Chu et al. [43] had reported that when the content of precipitate calcite was 1.24%, the compressive strength of the treated sand is 454 kPa, to achieve a compressive strength of 1000 kPa, more content of precipitated calcite required. Zhao et al. [44] had reported that the average compressive strength of cemented sand without fiber-reinforced was 1.08 MPa. As reported by other researchers above, although the particle size of the sand was different, the compressive strengths of the cemented sand were generally below 1.3 MPa, which was lower than that investigated in this research. Therefore, the compressive strength of our result can achieve or exceed the results in current literatures. Proper bacterial solution and cementation

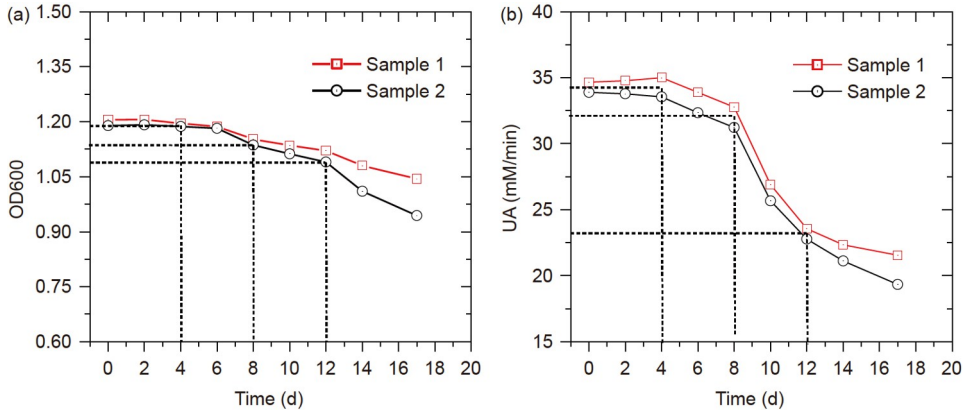

Figure 6 (Color online) Effect of low-temperature preservation time on OD600 (a) and UA (b) of two bacterial solutions.

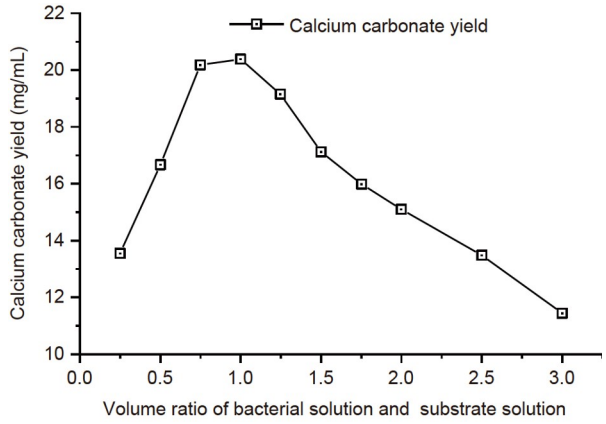

Figure 7 Calcium carbonate yield with the volume ratios of bacterial solution and substrate solution.

solution volume ratio is a benefit to the generation and precipitation of calcium carbonate, which could improve the compressive strength of the consolidated sand.

3.5.2 SEM analysis of the consolidated sand columns

The microstructure and morphology of the consolidated sand specimens are shown in Figure 8. As shown in Figure 8(a), there existed a large number of fine grains, which are filling in the intersections among the sand particles. The fine grains were about 2–5 μm with an irregular shape, which showed a fish scale shape in Figure 8(b). Generally, the large amounts of fine grains were deposited on the surface of sand particles, wrapped around and linked to the adjacent sand particles. During the consolidation experiment, the bacterial solution was first passed through the sand column to ensure that more bacteria could be adsorbed on the surface of sand particles. The biofilm and the extracellular polymers formed by microorganisms' secretion were effective to bind ions from the surrounding environment, which were capable to act as a heterogeneous nucleation site for the mineral deposition. On the other hand, the alkaline conditions were provided by microbial metabolism, which was confirmed by the pH change with the cultivation time of bacterial solution as presented previously. The crystal nucleation could begin at the active site by bonding a wide variety of cations, while the functional groups like carboxyl, hydroxyl, and phosphate from the bacterial cell wall de-protonated to in an alkaline condition [2]. Therefore, a strong electrostatic attraction occurred with the cations and the accumulation of calcium ions on the surface of the cell wall happened. Finally, a sufficient supersaturation state of calcium ions was achieved, which could bond with the carbonate ions to form calcium carbonate [2]. Therefore, the fine grains of calcium carbonate were observed on the surface of sand particles. Although there were many fine particles between the sand particles, there existed some voids among the sand particles, indicating that not all voids were filled by the generated fine grains and more fine grains would be produced to enhance the density of sand columns.

To analyze the chemical compositions of the generated fine grains, the EDS results of the irregular shape grains were tested and the results are shown in Figure 8(b). EDS results showed that the elements of the irregular shape grains were calcium (Ca), carbon (C), oxygen (O), silicon (Si) and chlorine (Cl). Due to the angle problem during the experiment of EDS, the electron beam could hit the surface of the surrounding sand. Therefore, as expected, Si came from sand particle and Cl came from calcium chloride in cementation solution, the fine grains was confirmed as calcium carbonate.

Moreover, it should be noted during the experiments that some white substances were observed at the top surface of the injection point, which was then characterized by SEM. SEM image of the white substance at the injection point is shown in Figure 9. The white substances were sphere-shaped with 2–10 μm. Moreover, some rod-shaped dents on the spherical surface were also observed, indicating that some bacteria have existed. To analyze the chemical compositions of the spherical grains, EDS of the substance was also texted. From the EDS result, the main elements of the substance were calcium (Ca), carbon (C) and oxygen (O), indicating that the spherical substance could be calcium carbonate.

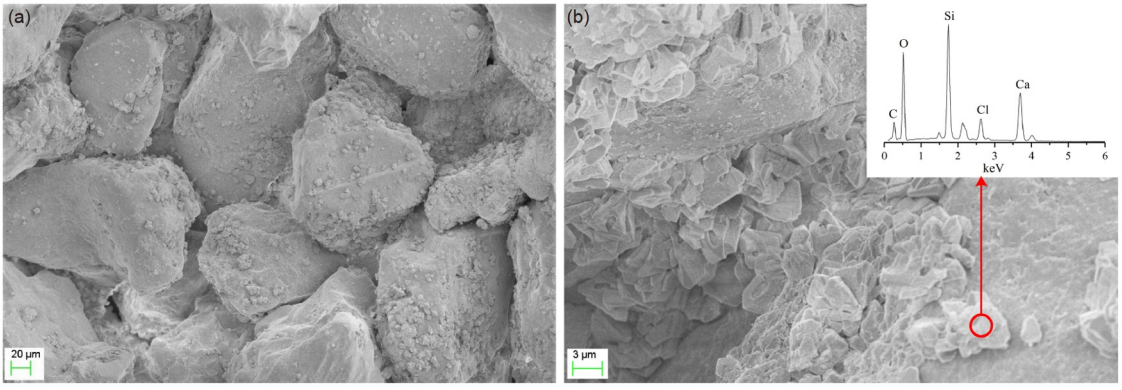

Figure 8 (Color online) The microstructure (a) and morphology (b) of sand particles.

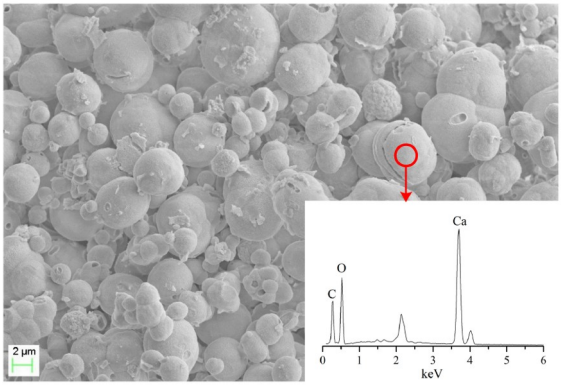

Figure 9 (Color online) The morphology of the white substance at the injection point.

3.5.3 XRD analysis of the consolidated sand columns

The XRD patterns of the consolidated sand columns containing the irregular shape fine grains and spherical substance were determined and the results are shown in Figure 10. As shown in XRD pattern of fine grains, the peaks of XRD pattern were well consistent with silica (PDF No 00-033-1161) and calcite (PDF No 01-086-0174). The peaks of silica were much higher than those of calcite because calcite was generated around the surface of sand particles, and the main substance of sand column was still sand, silica. Therefore, it can be deduced that the mineral of the irregular shape grain was calcite, which was in line with the results in sect. 3.5.2. Moreover, as shown in XRD pattern of the white substance, the peaks of XRD pattern were well consistent with calcite (PDF No 01-086-0174) and vaterite (PDF No 01-072-0506). Therefore, it can be indicated that the minerals of the spherical substance were a mixture of calcite and vaterite. Combining the observation of the morphology in Figure 9, it can be speculated that the white substance on the surface of the gauze was vaterite.

Combined with the results of products among sand particles, it is indicated that calcium carbonate was generated at both injection points and around sand particles. However, the crystal shapes of calcium carbonate generated in different places were different. It may be related to the surroundings where calcium carbonate precipitated. As shown in Figure 8, the space among sand particles is limited, the generated calcium carbonate mainly grows on the surface of sand particles. The space on the gauze was roomy, and calcium carbonate was generated on the surface of the gauze without insufficient space, resulting in relatively intact spherical particles. It can be deduced that the space size of calcium carbonate generated may influence its crystal shape. For example, the space around the sand particles was limited, and the product calcium carbonate was irregular shape calcite. Although sufficient space existed at the injection port on gauze, the product calcium carbonate at the surface was mainly spherical shape vaterite. At the bottom of the white substance, there might be some calcite exist, which could be found in the results of its XRD pattern in Figure 10.

4 Conclusions

Based on the investigation of cultivation methods of bacterial growth and the stable bacterial solution applied in consolidated sand, the following conclusions can be drawn.

(1) Under both continuous and interval cultivation of bacteria, the stable bacterial solution was harvested within 30 h's cultivation. Low-temperature treatment applied at the early cultivation stage exhibited weak inhibition of bacterial

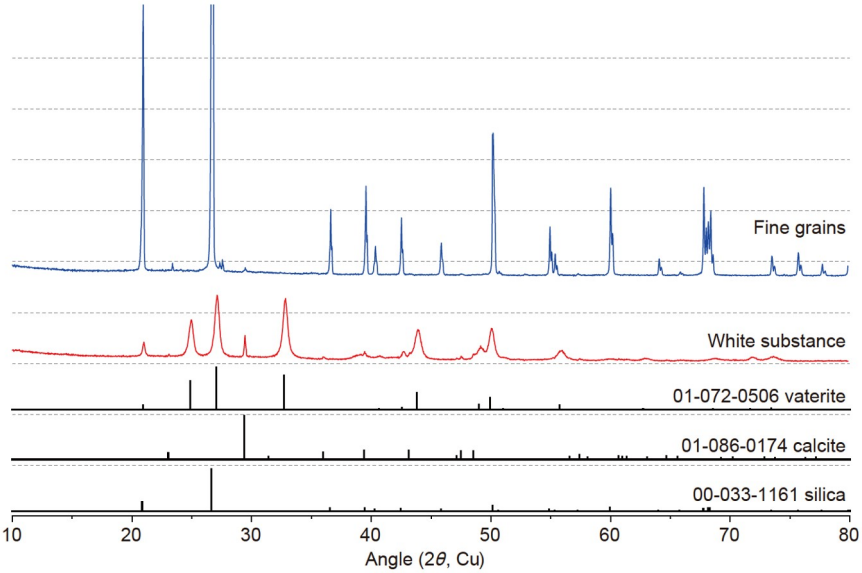

Figure 10 (Color online) The XRD results of fine grains among sands and white substances at the injection point in consolidated sand columns.

growth, while it showed strong inhibition at the middle cultivation stage. Interval cultivation could improve the cultivation efficiency of bacteria.

(2) Low-temperature preservation for stable bacterial solution showed little influence on urease activity before 4 days, while urease activity decreased seriously after it was stored for more than 8 days.

(3) The optimal bacterial solution and cementation solution volume ratio was 1:1, which was beneficial to the generation of calcium carbonate.

(4) The average compressive strength of consolidated sand columns after 3 injections was 1.38 MPa, the improved compressive strength was attributed to the product's irregular shape calcite coated and bonded sand particles together.

(5) Among sand particles, irregular shape grain calcite was observed, while on the surface of the injection point, sphere shape vaterite was investigated.

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1 Joshi S, Goyal S, Reddy M S. Influence of nutrient components of media on structural properties of concrete during biocementation. Construct Building Mater, 2018, 158: 601–613
2 Sarayu K, Iyer N R, Murthy A R. Exploration on the biotechnological aspect of the ureolytic bacteria for the production of the cementitious materials—A review. Appl Biochem Biotechnol, 2014, 172: 2308–2323
3 Dhami N K, Reddy M S, Mukherjee A. Biomineralization of calcium carbonates and their engineered applications: A review. Front Microbiol, 2013, 4
4 Nemati M. Modification of porous media permeability, using calcium carbonate produced enzymatically *in situ*. Enzyme Microbial Tech, 2003, 33: 635–642
5 Nemati M, Greene E A, Voordouw G. Permeability profile modification using bacterially formed calcium carbonate: Comparison with enzymic option. Process Biochem, 2005, 40: 925–933
6 De Muynck W, Cox K, Belie N D, et al. Bacterial carbonate precipitation as an alternative surface treatment for concrete. Construct Build Mater, 2008, 22: 875–885
7 DeJong J T, Fritzges M B, Nüsslein K. Microbially induced cementation to control sand response to undrained shear. J Geotech Geoenviron Eng, 2006, 132: 1381–1392
8 Whiffin V S, van Paassen L A, Harkes M P. Microbial carbonate precipitation as a soil improvement technique. Geomicrobiol J, 2007, 24: 417–423
9 Qiu J, Tng D Q S, Yang E H. Surface treatment of recycled concrete aggregates through microbial carbonate precipitation. Construct Build Mater, 2014, 57: 144–150
10 Achal V, Mukherjee A, Reddy M S. Effect of calcifying bacteria on permeation properties of concrete structures. J Ind Microbiol Biotechnol, 2011, 38: 1229–1234
11 Luo M, Qian C. Influences of bacteria-based self-healing agents on cementitious materials hydration kinetics and compressive strength. Construct Build Mater, 2016, 121: 659–663
12 Chen H, Qian C, Huang H. Self-healing cementitious materials based on bacteria and nutrients immobilized respectively. Construct Build Mater, 2016, 126: 297–303
13 Wang J, Vandevyvere B, Vanhessche S, et al. Microbial carbonate precipitation for the improvement of quality of recycled aggregates. J Cleaner Prod, 2017, 156: 355–366
14 Lesley A W, Patricia A M. Microbially mediated calcium carbonate precipitation: Implications for interpreting calcite precipitation and for solid-phase capture of inorganic contaminants. Geomicrobiol J, 2001, 18: 93–115
15 Dick J, De Windt W, De Graef B, et al. Bio-deposition of a calcium carbonate layer on degraded limestone by Bacillus species. Biodegradation, 2006, 17: 357–367
16 Young J R. Biomineralization within vesicles: The calcite of coccoliths. Rev Mineral Geochem, 2003, 54: 189–215
17 Soon N W, Lee L M, Khun T C, et al. Improvements in engineering properties of soils through microbial-induced calcite precipitation. KSCE J Civ Eng, 2013, 17: 718–728
18 Xu P, Fan H, Leng L, et al. Feasibility of microbially induced carbonate precipitation through a *Chlorella-Sporosaricina* co-culture system. Algal Res, 2020, 47: 101831
19 Zhang J, Su P, Li Y, et al. Environmental investigation of bio-modification of steel slag through microbially induced carbonate precipitation. J Environ Sci, 2021, 101: 282–292
20 Zeng W, Zhao Y, Poon C S, et al. Using microbial carbonate precipitation to improve the properties of recycled aggregate. Construct Build Mater, 2019, 228: 116743
21 Feng Z, Zhao Y, Zeng W, et al. Using microbial carbonate precipitation to improve the properties of recycled fine aggregate and mortar. Construct Build Mater, 2020, 230: 116949
22 Zhan Q, Qian C. Stabilization of sand particles by bio-cement based on CO_2 capture and utilization: Process, mechanical properties and microstructure. Construct Build Mater, 2017, 133: 73–80
23 Siddique R, Nanda V, Kunal V, et al. Influence of bacteria on compressive strength and permeation properties of concrete made with cement baghouse filter dust. Construct Build Mater, 2016, 106: 461–469
24 Ivanov V, Stabnikov V, Stabnikova O, et al. Biocementation technology for construction of artificial oasis in sandy desert. J King Saud Univ-Eng Sci, 2020, 32: 491–494
25 Omoregie A I, Khoshdelnezamiha G, Senian N, et al. Experimental optimisation of various cultural conditions on urease activity for isolated *Sporosarcina pasteurii* strains and evaluation of their biocement potentials. Ecol Eng, 2017, 109: 65–75
26 Mortensen B M, Haber M J, DeJong J T, et al. Effects of environmental factors on microbial induced calcium carbonate precipitation. J Appl Microbiol, 2011, 111: 338–349
27 Don J B, Noel R K, James T S. Bergey's Manual of Systematic Bacteriology—vol 3: The Firmicutes. New York: Springer, 2009
28 Sun X H, Miao L C, Wu L Y, et al. Comparative study of microbially induced carbonate precipitation under low-temperature conditions. Rock Soil Mech, 2018, 39: 224–230
29 Krajewska B, Leszko M, Zaborska W. Urease immobilized on chitosan membrane: Preparation and properties. J Chem Technol Biotechnol, 1990, 48: 337–350
30 Jiang N J, Yoshioka H, Yamamoto K, et al. Ureolytic activities of a urease-producing bacterium and purified urease enzyme in the anoxic condition: Implication for subseafloor sand production control by microbially induced carbonate precipitation (MICP). Ecol Eng, 2016, 90: 96–104
31 Keykha H A, Huat B B K, Asadi A, et al. Electro-biogrouting and its challenges. Int J Electrochem Sci, 2012, 7: 1196–1204
32 Sun X, Miao L, Tong T, et al. Study of the effect of temperature on microbially induced carbonate precipitation. Acta Geotech, 2019, 14: 627–638
33 Sun X, Miao L, Wu L, et al. Improvement of bio-cementation at low-temperature based on *Bacillus megaterium*. Appl Microbiol Biotechnol, 2019, 103: 7191–7202
34 Osinubi K J, Eberemu A O, Gadzama E W, et al. Plasticity characteristics of lateritic soil treated with *Sporosarcina pasteurii* in microbial-induced calcite precipitation application. SN Appl Sci, 2019,

1: 829
35 Siddique R, Chahal N K. Effect of ureolytic bacteria on concrete properties. Construct Build Mater, 2011, 25: 3791–3801
36 Cuzman O A, Richter K, Wittig L, et al. Alternative nutrient sources for biotechnological use of *Sporosarcina pasteurii*. World J Microbiol Biotechnol, 2015, 31: 897–906
37 Omoregie A I, Palombo E A, Ong D E L, et al. Biocementation of sand by *Sporosarcina pasteurii* strain and technical-grade cementation reagents through surface percolation treatment method. Construct Build Mater, 2019, 228: 116828
38 Omoregie A I, Ngu L H, Ong D E L, et al. Low-cost cultivation of *Sporosarcina pasteurii* strain in food-grade yeast extract medium for microbially induced carbonate precipitation (MICP) application. Biocatal Agric Biotechnol, 2019, 17: 247–255
39 Whiffin V. Microbial $CaCO_3$ Precipitation for the production of Biocement. Dissertation for Doctoral Degree. Perth: Murdoch University, 2004
40 Rivadeneyra M Ã A, PÃ¡rraga J Ã, Delgado R, et al. Biomineralization of carbonates by Halobacillus trueperi in solid and liquid media with different salinities. FEMS Microbiol Ecol, 2004, 48: 39–46
41 Bhutange S P, Latkar M V, Chakrabarti T. Role of biocementation to improve mechanical properties of mortar. Sādhanā, 2019, 44: 50
42 Yu X, Qian C, Sun L. The influence of the number of injections of biocomposite cement on the properties of bio-sandstone cemented by biocomposite cement. Construct Build Mater, 2018, 164: 682–687
43 Chu J, Ivanov V, Naeimi M, et al. Optimization of calcium-based bioclogging and biocementation of sand. Acta Geotech, 2014, 9: 277–285
44 Zhao Y, Xiao Z, Fan C, et al. Comparative mechanical behaviors of four fiber-reinforced sand cemented by microbially induced carbonate precipitation. Bull Eng Geol Environ, 2020, 79: 3075–3086