The influence of micro-environment on the depositing rate and morphology of microbially induced carbonate precipitation

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Abstract: Microbially induced carbonate precipitation (MICP) mainly uses urease-producing bacteria to hydrolyze urea to produce carbonate ions and contact the introduced calcium ions. The gelatinous calcium carbonate is deposited on the surface of loose particles and binding unconsolidated materials together. It has a great potential to complete many applications as a technologies of construction. In this work, the rate and ratio of depositing CaCO₃ in the process of MICP at various micro-environments was evaluated. Furthermore, more suitable conditions to promote the process of depositing was presented

1. Introduction
In recent decades, a cost-efficient and environmentally friendly method called microbially induced carbonate precipitation (MICP) has been extensively studied[1-2]. Urease-producing bacteria and cementing solution are injected into the granular material to promote calcite formation that in turn binds unconsolidated materials and reduces the permeability of the material by several orders of magnitude[3-4]. Compared with traditional stabilization methods, it has stronger fluidity and can penetrate deep into the material to strengthen the material performance. It shows bright prospects in the field of geotechnical engineering for several different applications, such as ground improvement and groundwater control[3].

Sporosarcina pasteurii(SP), previously known as Bacillus pasteurii, is the most commonly used bacteria for conducting MICP[5]. It is a non-pathogenic gram-positive bacterium with high urease producing capacity and activity. It is identified Gram-positive by gram staining microscopic examination, as shown in figure 1. The mechanism of MICP have been fully clarified[6]. On the one hand, SP can produce highly active urease enzyme to catalyze the hydrolysis of urea to raise the pH of the system and improve the production of carbonate ion. The reaction in this process is shown as follows (Eqs. [1] - [5]):

\[
\begin{align*}
\text{CO(NH}_2\text{)}_2 + \text{H}_2\text{O} &\rightarrow \text{NH}_2\text{COOH} + \text{NH}_3 \quad [1] \\
\text{NH}_2\text{COOH} + \text{H}_2\text{O} &\rightarrow \text{NH}_3 + \text{H}_2\text{CO}_3 \quad [2] \\
\text{H}_2\text{CO}_3 &\leftrightarrow \text{HCO}_3^- + \text{H}^+ \quad [3] \\
2\text{NH}_3 + 2\text{H}_2\text{O} &\leftrightarrow 2\text{NH}_4^+ + 2\text{OH}^- \quad [4] \\
\text{HCO}_3^- + \text{H}^+ + 2\text{NH}_4^+ + 2\text{OH}^- &\leftrightarrow \text{CO}_3^{2-} + 2\text{NH}_4^+ + 2\text{H}_2\text{O} \quad [5]
\end{align*}
\]

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On the other hand, soluble calcium ions are adsorbed on the surface of bacteria which is negatively charged[6-7]. With the introduced calcium source, the calcium carbonate forms based on the following chemical reaction (Eqs. [6] - [7]):

\[
\text{Ca}^{2+} + \text{cell} \rightarrow \text{cell-Ca}^{2+} \quad [6]
\]

\[
\text{cell-Ca}^{2+} + \text{CO}_3^{2-} \rightarrow \text{cell-CaCO}_3 \quad [7]
\]

The gelatinous calcium carbonate is filled between loose particles to contact them together. It is fact that materials which is of poor mechanical properties are endowed with excellent strength and impermeability.

![Figure 1. Gram staining microscopic examination of *Sporosarcina pasteurii*.](image)

In the field of MICP, calcium carbonate in bio-grouting under multiple treatment factors was studied to achieve the application of MICP on engineering projects. The production of calcium carbonate and the bonding strength of materials was measured by changing a single variable (e.g., bacterial strain, bacterial concentration, calcium source concentration, nutrient concentration, pH, temperature). The mineralization process of carbonate microbial has always been a hotspot in the study of MICP. It said that calcium carbonate deposition depended on four key factors including calcium source concentration, nutrient concentration, pH, as well as available nucleation sites[6].

With the advancement in concrete research, MICP has been widely applied to the filed of civil engineering and has achieved well reinforcement effects. In 1992, MICP was used to reduce the porosity of foundation soil for the application of oil extraction[8]. Urease-producing bacteria and cement were injected into the cracks of historic buildings and artwork to protect relics[9]. Hereafter, bacterial-based self-healing concrete was developed to increase durability of architecture[10]. Based on the fact that MICP can reduce material permeability by several orders of magnitude, it was used to plug leaks and strengthen dams[11]. The successful application in engineering shows the bright prospects of this technology and inspires related researchers. Meanwhile, the reinforcement strength of the material and the amount of formation in the entire process have been often widely reported. However, the formation efficiency of calcium carbonate in a single cementing process has been rarely studied. In this work, the formation rate and situation of calcium carbonate by simulating different growth micro-environments of MICP was evaluated in the laboratory.

2. Materials and Methods

2.1 Bacterial Media and Growth

*Sporosarcina pasteurii* [BeNa Culture Collection(BNCC) 337394] was used in MICP experiments due to its high ability to produce urease. Appropriate culture medium and culture conditions was referred to the literatures. An ammonium-yeast extract medium[20 g/L yeast extract, 10 g/L (NH₄)₂SO₄, 0.13 mol/L Tris buffer (pH = 9.0)] was sterilized at 121 °C for 20 min by steam under high pressure after mixing. Activated *Sporosarcina pasteurii* was cultured on the growth medium in the proportion of 1:150. It was placed in a shaking incubator [180 revolutions per minute (rpm)] at 30 °C. Optical
density of 600 nm (OD\textsubscript{600}) was used as an indicator to measure the growth of microorganisms\cite{5}. Some movement of OD\textsubscript{600} within 36 hours is clearly presented in figure 2.

![Figure 2. Temporal evolution of bacterial growth.](image)

The solution to suspend and preserve the bacteria was prepared in advance. Tris buffer (0.05 mol/L) with different pH (pH = 7, 7.5, 8, 8.5, 9) were configured by changing the ratio of 0.1 mol/L Tris buffer, 0.1 mol/L HCl and ultrapure water. It was also sterilized at 121°C for 20 min by steam under high pressure. Cells in the ammonium-yeast extract medium were centrifuged at 8000 rpm for 10 min for two times to remove the by-products. It was washed with saline solution, harvested, and resuspended in Tris buffer which has been prepared. The OD\textsubscript{600} of all bacteria buffers were designed to 1.5 as an invariant and stored at 4°C prior to use.

Urea-CaCl\textsubscript{2} mixed liquid was chosen to induce ureolytic-driven calcite precipitation in all experiments. Urea was added as an ammonium source and energy for the process of hydrolyzing, and CaCl\textsubscript{2} was introduced as a calcium source to promote the formation of precipitation. It was reported that urea-CaCl\textsubscript{2} mixed liquid at the ratio of 1:1 was efficient and economical. The work solution containing urea and CaCl\textsubscript{2} was adjusted to the given pH before sterilized.

2.2 Microbiologically-induced CaCO\textsubscript{3} precipitation
Microbial CaCO\textsubscript{3} precipitation experiments were carried out with different conditions. The weight of the dry centrifuge tubes (m\textsubscript{0}) was recorded before the work solutions and bacteria buffers were put into the bottom of tubes. Multiple samples with the same configuration were terminated at 2-h intervals form 0 to 12 h afterward. Immediately, tubes with the reaction solution were be centrifuged at 3000 rpm for 5 min. Removing supernatant and washing precipitant with ultrapure water for three times. The weight of tubes with CaCO\textsubscript{3} precipitant (m\textsubscript{1}) were measured after desiccation. The weight of CaCO\textsubscript{3} precipitant (m) was equal to the difference between m\textsubscript{1} and m\textsubscript{0}.

2.3 CaCO\textsubscript{3} precipitation at various pH
Urea-CaCl\textsubscript{2} mixed liquid at various pH was added to the centrifuge tubes as table 1 (N=3). Then, 2 mL bacteria buffers were introduced into corresponding tubes to initiate CaCO\textsubscript{3} precipitation. The process of MICP was carried out at 30°C. CaCO\textsubscript{3} precipitant was measured by the methods proposed above.

| Group | pH | T(°C) | SP suspension | Cementing solution |
|-------|----|-------|---------------|--------------------|
|       | OD\textsubscript{600} | V(mL) | c(mol/L) | V(mL) |

Table 1. Reaction conditions of groups at various pH.
Table 2. Reaction conditions of groups at various temperature.

| Group | T(℃) | pH | SP suspension | Cementing solution |
|-------|------|----|---------------|--------------------|
|       |      |    | OD600 | V(mL) | c(mol/L) | V(mL) |
| 1     | 20   | 7.5 | 1.5   | 2     | 1        | 20    |
| 2     | 25   | 7.5 | 1.5   | 2     | 1        | 20    |
| 3     | 30   | 7.5 | 1.5   | 2     | 1        | 20    |
| 4     | 35   | 7.5 | 1.5   | 2     | 1        | 20    |
| 5     | 40   | 7.5 | 1.5   | 2     | 1        | 20    |

2.4 CaCO$_3$ precipitation at various temperature

Urea-CaCl$_2$ mixed liquid (pH = 7.5) was introduced to the centrifuge tubes as table 2 (N=3). Then, bacteria buffers of 2 mL (pH = 7.5) were added into corresponding tubes to induce CaCO$_3$ precipitation. The tubes with working solution were placed in incubator at different temperatures. CaCO$_3$ precipitant was measured by the methods proposed above.

2.5 CaCO$_3$ precipitation at various calcium concentrations

Urea-CaCl$_2$ mixed liquid (pH = 7.5) which is designed ranging from 0.5mol/L to 2 mol/L was added into tubes as table 3 (N=3). Then, bacteria buffers of 2 mL (pH = 7.5) were put into tubes to promote the process of CaCO$_3$ deposition at 30 ℃. CaCO$_3$ precipitant was measured by the methods proposed above.

Table 3. Reaction conditions of groups at various calcium concentrations.

| Group | pH | T(℃) | SP suspension | Cementing solution |
|-------|----|------|---------------|--------------------|
|       |    |      | OD600 | V(mL) | c(mol/L) | V(mL) |
| 1     | 30 | 7.5  | 1.5   | 2     | 0.5      | 40    |
| 2     | 30 | 7.5  | 1.5   | 2     | 1        | 20    |
| 3     | 30 | 7.5  | 1.5   | 2     | 1.5      | 13.33 |
| 4     | 30 | 7.5  | 1.5   | 2     | 2        | 10    |

3. Results and Discussion

3.1 The depositing rate and ratio of CaCO$_3$ at various conditions

The depositing ratio of CaCO$_3$ is considered as the standard to measure the efficiency of MICP. As reported that the depositing CaCO$_3$ was controlled by micro-environments in the process of MICP. A series of experiments was conducted to present the difference of depositing CaCO$_3$ at various micro-environments (Figure 3). We conducted a series of experiments to present the difference of
depositing CaCO₃ at various micro-environments (as presented in Figure 3). Figure 3a showed the difference of CaCO₃ deposited rate at various pH. The CaCO₃ depositing rate of all samples was increased over time. The samples (pH = 9) have higher depositing efficiency compared with others. The variations of depositing rate at various temperature are presented in Figure 3b. The depositing ratio of CaCO₃ has the same increasing feature in the course of 12 h. The samples (T = 35°C) are provided with efficient depositing CaCO₃ at all measuring nodes. Figure 3c displayed the effects of calcium ion concentration on calcium carbonate deposition. Although the deposition rate of CaCO₃ in different concentration samples keep an increasing trend, the deposition rate of low concentration samples was significantly higher than that of high concentration samples.

Figure 3. The depositing ratio of CaCO₃ at different (a) pH, (b) temperature, and (c) calcium concentrations. Errors bars represent average and standard deviation of three replicates.

3.2 The morphology of depositing CaCO₃ at various conditions
Calcite calcium carbonate is known as the products of MICP. The morphology control of deposited CaCO₃ is related to the factors of micro-environments in the process of MICP. Figure 4 demonstrated the morphology of depositing CaCO₃ at various pH. The CaCO₃ shapes obtained in Figure 4a and 4b are mainly hexahedron in different size. Larger hexahedral particles were wrapped in a layer of unknown material (as presented in Figure 4c). The depositing CaCO₃ in Figure 4b are composed mainly of hexahedron and cuboidal polycrystalline aggregates with a layered structure. Besides, the CaCO₃ shaping of a ball of string congregates together in Figure 4e.

Figure 4. SEM morphology images of depositing CaCO₃ at (a) pH 7, (b) pH 7.5, (c) pH 8, (d) pH 8.5, (e) pH 9.
4. Conclusions

It was found that the depositing rate and ratio of CaCO₃ were influenced by micro-environments (i.e., calcium concentrations, pH, temperature). The cementing solution at lower calcium concentrations is more suitable to promote the process of depositing. The samples (pH = 9) at 35 °C can improve the efficiency of depositing in the process of MICP. SEM analysis showed that morphology of depositing CaCO₃ was affected by pH of working solutions.

Acknowledgements

The work was supported by the Special Project on Development of National Key Scientific Instruments and Equipment of China (2011YQ03013403), National Natural Science Youth Foundation of China (81600407), and the Fundamental Research Funds for the Central Universities.

References

[1] Whiffin, V.S., van Paassen, L.A. and Harkes, M.P. (2007) Microbial carbonate precipitation as a soil improvement technique. Geomicrobiol. J., 24: 417–423.
[2] DeJong, J.T., Mortensen, B.M., Martinez, B.C. and Nelson, D.C. (2010) Bio-mediated soil improvement. Ecol. Eng., 36: 197–210.
[3] Seifan, M., Sarabadani, Z., Berenjian, A. (2020) Microbially induced calcium carbonate precipitation to design a new type of bio self-healing dental composite. Appl. Microbiol. Biotechnol., 104: 2029–2037.
[4] Zhu, X., Wang, J., De Belie, N., Boon, N. (2019) Complementing urea hydrolysis and nitrate reduction for improved microbially induced calcium carbonate precipitation. Appl. Microbiol. Biotechnol., 103: 8825–8838.
[5] Wong, L.S. (2015) Microbial cementation of ureolytic bacteria from the genus Bacillus: A review of the bacterial application on cement-based materials for cleaner production. J. Clean. Prod., 93: 5–17.
[6] Zhu, T., Dittrich, M. (2016) Carbonate precipitation through microbial activities in natural environment, and their potential in biotechnology: A review. Front. Bioeng. Biotechnol., 4: 1–21.
[7] Hammes, F., Verstraete, W. (2002) Key roles of pH and calcium metabolism in microbial carbonate precipitation. Rev. Environ. Sci. Biotechnol., 1: 3–7.
[8] Ferris, F.G., Stehmeier, L.G., Kantzas, A. and Mourits, F.M. (1996) Bacteriogenic mineral plugging. J. Can. Petrol. Technol., 35: 56–61.
[9] Stocks-Fischer, J.K., Galinat, S.S. Bang. (1999) Microbiological precipitation of CaCO₃. Soil Biol. Biochem., 31 (11): 1563-1571.
[10] Gupta, S., Pang, S.D., Kua, H.W. (2007) Autonomous healing in concrete by bio-based healing agents—A review. Constr. Build. Mater., 146: 419–428.
[11] DeJong, M.B., Fritzges, K. (2006) Microbi ally induced cementation to control sand response to undrained shear, J. Geotech. Geoenviron. Eng., 132 (11): 1381–1392.