Materials Transactions, Vol. 59, No. 9 (2018) pp. 1520 to 1527

Effects of Various Factors on Carbonate Particle Growth Using Ureolytic Bacteria

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In Microbially Induced Carbonate Precipitation (MICP), bacteria can perform metabolic activities that promote the deposition of carbonate particles in the form of calcite. Previously, purified urease and $CaCl_2$ have been used for hydrolysis of urea to deposit carbonate particles. In our present study, Mg^{2+} ions were added to investigate the effect on the deposition of carbonate particles, because Mg^{2+} ions can delay the reaction rate and enhance the crystal deposition rate. Additionally, other parameters (temperature, solvent, bacterial population, and $CaCl_2$ concentration) were taken into consideration to enhance the amount of carbonate deposition by ureolytic bacteria. The aim of this study was to investigate the mechanism of carbonate particle generation using urease producing bacteria (*Pararhodobacter* sp.) in laboratory test conditions using a translucent cell. In this study, marine ureolytic (*Pararhodobacter* sp.) bacteria were used and their urease activity was estimated considering bacterial concentration, temperature, and the effect of Ca^{2+} and Mg^{2+} ions. Digital microscopy analysis revealed the direct involvement of these parameters on the deposition of carbonate particles. The results of this study also showed that the type of deposited crystals, their shapes, and bacterial growth rate change depending on the medium used, the type of carbonate (metal ion used), $CaCl_2$ concentration, and temperature. In addition, when Mg^{2+} and Ca^{2+} ions were used, the amount of particle deposition increased, which enhanced the possibility of becoming a superior binder for sand particles. This study is useful for the various sand solidification experiments and to regulate the most suitable conditions for engineering applications in future studies. [doi:10.2320/matertrans.M-M2018830]

(Received April 17, 2018; Accepted June 16, 2018; Published July 23, 2018)

Keywords: *MICP, carbonate deposition, ureolytic bacteria, urease activity, magnesium ion*

1. Introduction

Microbially Induced Carbonate Precipitation (MICP) using urease-producing bacteria is a promising technique in the field of geotechnology and civil engineering. The mechanism of this technique is elucidated by the following reactions where urea is hydrolyzed by urease (ureolysis) to form ammonium ions and carbamate (eqs. (1)–(3)), which spontaneously hydrolyzes to form a second ammonium ion and bicarbonate. The reactions of urea hydrolysis and calcite formation are shown in eqs. (1)–(3).

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

$$CaCl_2 \rightarrow Ca^{2+} + 2Cl^- \quad (2)$$

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3 \downarrow \text{(deposited)} \quad (3)$$

Research has shown that four factors are mainly responsible for the deposition of calcium carbonate: the concentration of dissolved inorganic carbon, the pH, the concentration of calcium ions, and the presence of nucleation sites.[1)] The first three factors are provided by the metabolism of the bacteria while the cell wall of the bacteria acts as a nucleation site.[2)] In addition, in our study, we considered several parameters like temperature, pH, bacterial concentration, and solvent (distilled water or artificial seawater). In our study, marine bacteria *Pararhodobacter* sp. were used because marine bacteria can survive in extreme environmental conditions such as high salinity/alkalinity and pressure, low/high temperature, and low nutrient content, and their physiological traits are perhaps uniquely suited for many geotechnical engineering application processes by the MICP method.[3,6)] Marine bacteria *Pararhodobacter* sp. isolated (identified by 16S rRNA gene analysis) from the beachrock in Okinawa, Japan[4)] have been used for their urease-producing enzymes that produce carbonate and ammonia, which then combines with natural calcium to precipitate as calcium carbonate[4–6)] and in the presence of Ca^{2+} ions, calcium carbonate is then formed and deposited, which contributes to the binding of sand particles. Additionally, several studies have investigated the calcite formation mechanism and the subsequent effect on carbonate deposition through the MICP method.[4–7)] In our study, magnesium chloride was added to investigate the effect on carbonate particle deposition because magnesium chloride can delay the reaction rate and enhance the crystal deposition rate.[8)] It was previously found that adding magnesium to the inorganic carbonation process affects the carbonate deposition rate and modifies the structure and the size of the deposited crystals.[9)] The concentration of Mg^{2+} ions influences the morphology of the carbonate polymorphs, and deposited crystals have been observed to possibly progress from angular to spherical as the Mg^{2+} ions increased.[10)] The $CaCO_3$ deposition rate is generally affected by the concentration of the Ca^{2+} ion products, and in addition, the presence of Mg^{2+} ions changes the shape and the size of the deposited crystals.[1,11)] It has been widely described that bacteria can serve as nuclei of carbonate deposition upon adsorbing Ca^{2+} and Mg^{2+} cations onto their cell surface due to their negative surface charge[12)] and mineralogical analysis revealed that the presence of magnesium ions can effectively promote the formation of deposited carbonate particles with a significant improvement in cementation strength.[11,13)] Bio-cementation is achieved when calcite crystals are deposited on the surface or form bridges between the existing soil grains. These calcite crystals bond the soil particles and prevent movement of the grains, thereby enhancing the strength and improving the stiffness properties of the soil.[14)] Therefore, to improve the cementation strength of sand or soil particles, different sizes of carbonate crystals may be needed for different engineering applications depending on the distribution of fine or coarse sand.[15)] Further investigation

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is needed of what factors controls the size of these crystals for successful biocementation process.

Therefore, improving the cementation strength of deposited crystal particles' structures is very important. Thus, this study aimed to determine the effect of various parameters (temperature, bacterial population, Ca^{2+} and Mg^{2+} ion concentration, and $CaCl_2$ concentration) on crystal particle deposition to determine appropriate conditions for crystal particle deposition considering their shape, size, and diameter using digital microscopy image analysis, which can be effective for the practical applications from lab to engineering field scale.

2. Materials and Methods

2.1 Screening and cultivation of bacteria

In our study, *Pararhodobacter* sp. (identified by 16S rRNA gene analysis) isolated from the soil near beachrock in Okinawa, Japan were used for calcium carbonate deposition. ZoBell 2216 medium (5.0 g/L polypeptone, 1.0 g/L yeast extract, and 0.1 g/L $FePO_4$ prepared in artificial seawater (Table 1), adjusted to pH 7.6–7.8) was used as the culture medium for cultivating the bacteria. The isolated bacteria were pre-cultured in 5 mL medium at 30°C with shaking at 160 rpm for 24 h. One millilitre of the pre-culture was inoculated into 100 mL of fresh ZoBell2216 medium incubated at 30°C with shaking at 160 rpm. During cultivation, cell concentration was determined by measuring the OD_{600} using a UV-vis spectrophotometer (V-730, JASCO Corporation, Tokyo, Japan) and deposited particle growth was investigated by digital microscopy (VHX-1000, Keyence Corporation, Tokyo, Japan).

2.2 Observation of particle deposition

Pararhodobacter sp. bacteria were cultivated in ZoBell2216E medium (100 mL of culture in a 300-mL Erlenmeyer flask) and cultivated for 3 days at 30°C in a column oven incubator. Then, an Erlenmeyer flask was filled with 1.0 M NaOH solution, pH adjusted to 7.6–7.8, and the top of the flask was covered with aluminum foil before autoclaving (KTS-2346 by ALP Corporation, Japan) at 121°C for 15 minutes and stored on a clean bench (CT-1200N-UV by Tanaka Seiki, Japan) for 24 hours to stabilize the precipitation (at room temperature: 25°C). Then, 1 mL of the prepared bacterial solution was transferred to a translucent cell (Fig. 1) designed for image analysis by a VHX-1000 digital microscope (Fig. 2). Ten deposited particles were randomly selected and the cell area was subjected to image analysis (0.5 cm × 0.5 cm). Image analysis was performed by manipulating the still image analysis software "Win-roof" to investigate the diameter (circle equivalent diameter) of deposited crystal grains and the area ratio (Fig. 3).

Table 1 Composition of artificial seawater (Yashima Chemical Co., Japan) [g/L] (solvent: distilled water).

Elements	[g / L]
$MgCl_2 \cdot 6H_2O$	222.23
$CaCl_2 \cdot 2H_2O$	30.70
$SrCl_2 \cdot 6H_2O$	0.85
KCl	13.89
$NaHCO_3$	4.02
KBr	2.01
H_3BO_3	0.54
NaF	0.06
NaCl	490.68
Na_2SO_4	81.88

2.3 Calculation of deposited particles

"Win-roof 2015" image analysis software was utilized to calculate the circle equivalent diameter of the deposited crystal particles. Figure 4 shows a schematic diagram of a

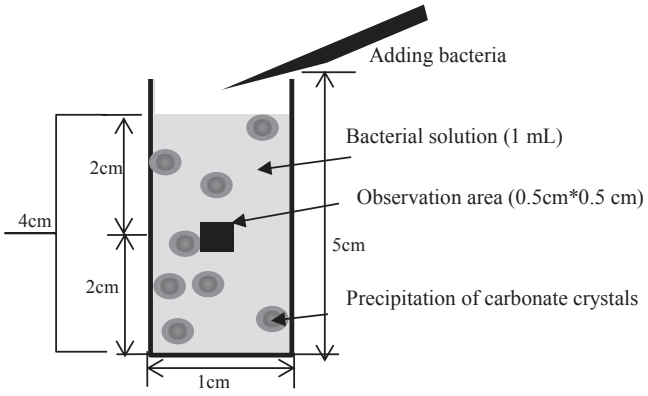

Fig. 1 Translucent cell observation for carbonate precipitation.

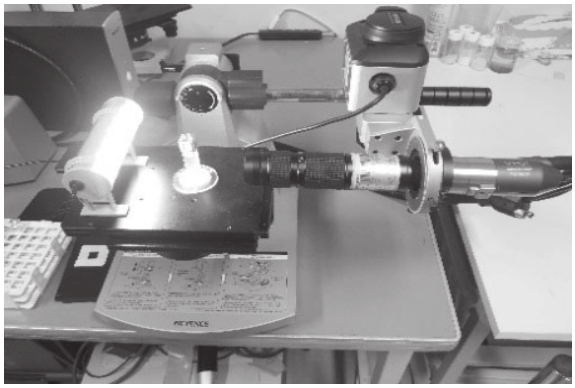

Fig. 2 Digital microscope VHX-1000.

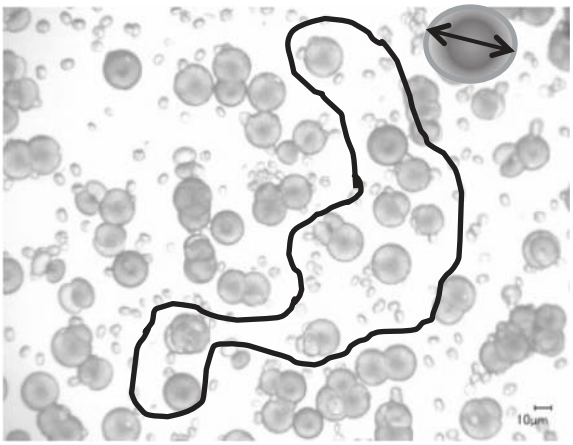

Fig. 3 Particle binarization for measuring diameter and inset picture showing diameter of a particle.

Table 2 Testing conditions.

Elements	Conditions		
Bacterial concentration	Bacterial concentration (high)	Bacterial concentration (less)	
$CaCl_2$ concentration	0.1 M $CaCl_2$	$CaCl_2$ 0.3 M	$CaCl_2$ 0.5 M
Temperature	25 °C	30 °C	35 °C
Culture condition	Adding only $CaCl_2$	Adding both ($CaCl_2$ + $MgCl_2$)	

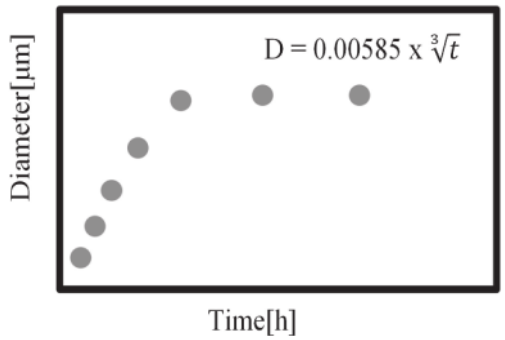

Fig. 4 Schematic diagram of particle diameter over time (standard curve).

graph of particle diameter over time. The vertical axis of the graph is the equivalent circle diameter and the horizontal axis is time. The deposition rate of crystal particles was calculated based on data in this graph. For the data analysis, the graphing software "Origin Pro 2015" was used. The average grain size (D) of deposited crystal grains was expressed by the following formula (4) and the growth rate of carbonate particles was calculated using the eqs. (4)–(8) which is illustrated in Fig. 4.

$$D = A\sqrt[3]{t} \tag{4}$$

Where, D = Diameter; A = Constant; t = Time.

In this equation, the particle diameter (D) will continue to increase exponentially indefinitely over time. As a result, the growth reaction becomes steady and cannot advance. Therefore, according to chemical reaction formulas (1)–(3), it can be said that since the reaction of carbonate is considered to be almost the same as the decomposition rate of urea to be a constant rate, the following relationship expression can be obtained.

$$V = \alpha t \tag{5}$$

Where, V = Average volume per crystal particles; α = Constant; t = Time.

(if the precipitated crystal particles are spherical in shape)

$$\text{Then, } V = \frac{\pi}{6} D^3 = \alpha t \tag{6}$$

$$D^3 = \frac{6\alpha}{\pi} t = A^3 t \tag{7}$$

$$\alpha = \frac{\pi}{6} A^3 \ [\text{mm}^3/\text{h}] \tag{8}$$

From eq. (8), the value of the constant α can be obtained. This α was taken as the deposition rate. Since the unit is [mm^3/h], the unit of the average particle diameter of deposited particles was changed from D [μm] to D [mm] during the calculation process. The deposition rate α [mm^3/h] was calculated for each tested condition. The testing conditions used in the experiment are shown in Table 2.

3. Results

3.1 Effects of bacterial concentration

High concentrations of bacterial cells (from 10^6 to 10^8 cells) increased the amount of calcite deposition by MICP by enhancing the urease concentration for urea hydrolysis.[16,17] Therefore, urea hydrolysis has a direct relationship with bacterial cell concentration for crystal particle deposition. Results of our study (Fig. 5) showed the deposition rate [%] of crystal particles to the number of bacteria (120 hours after the start of the test). The crystal deposition rate is the ratio of the total area of the particles on the photographed image. As shown in Figs. 5 and 12, crystal grain deposition was extremely affected by the number of bacteria and time. With increased time, crystal deposition and size also increased; the deposition of crystal grains was higher when the Bacteria/Solvent (distilled water) ratio was 1:9 compared to deposition when the Bacteria/Solvent (artificial sea water) ratio was 1:1 or Bacteria/Solvent (distilled water) ratio was 1:1. The increased bacterial concentration was measured by a UV-vis spectrophotometer at 600 nm and these results showed that the deposition rate of crystal particles was higher when the Bacteria/Solvent (both distilled water or artificial sea water) ratio was 1:9, compared to when the Bacteria/Solvent (both distilled water or artificial sea water) ratio was 1:1. Therefore, results from this experiment clearly showed that crystal

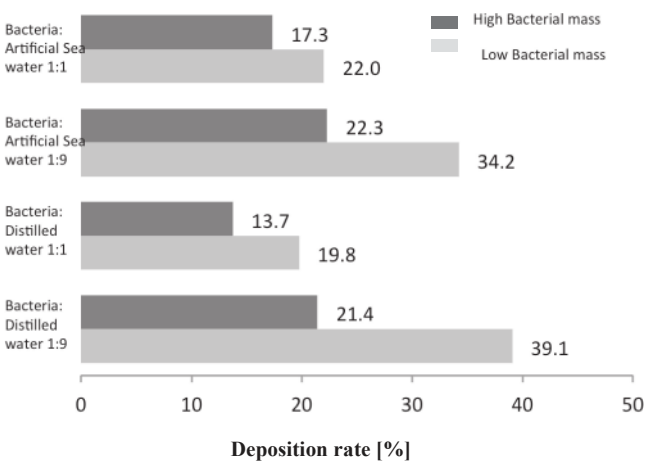

Fig. 5 The deposition rate (α) of particles by the bacterial concentration.

deposition was greatly affected by the bacterial concentration and type of solvent. In this case, the largest deposited particle diameter was observed when the Bacteria/Solvent (distilled water) ratio was 1:9.

3.2 Effects of $CaCl_2$ concentration

Previous studies showed that calcium concentration can greatly accelerate $CaCO_3$ deposition by microorganisms.[5,12] It was revealed that $CaCO_3$ deposition and $CaCO_3$ precipitation consist of three distinct stages, are dependent on the calcium source, and that bacterial cells act as nucleation sites for crystal formation and growth.[5] These results indicated that the calcium concentration influences crystal deposition. Therefore, it is necessary to investigate which calcium concentration is optimal for crystal deposition. Data shown in Figs. 6 and 7 shows that the crystal deposition rate is greatly affected by the concentration of bacteria and the $CaCl_2$ concentrations of 0.1 M, 0.2 M, and 0.5 M. Moreover, the Bacteria/Solvent ratio also affects crystal deposition (Fig. 6). Data in Fig. 6 show that crystal deposition rate increased when bacteria and solvent concentration (artificial sea water) also increased. The crystal deposition rate also correlated with bacteria, solvent, and $CaCl_2$ concentration (Fig. 7). Data in Fig. 7 show that crystal particles were uniform at concentrations of 0.1 M and 0.3 M and the average particle diameter was the largest at 0.5 M. This tendency was not seen at concentrations of 0.1 M and 0.3 M. Therefore, from this experiment, it can be stated that the number of crystal particles on the photographed image increased as the solvent, bacteria, and Ca^{2+} concentration increased, which are significant findings for the application of sand solidification.

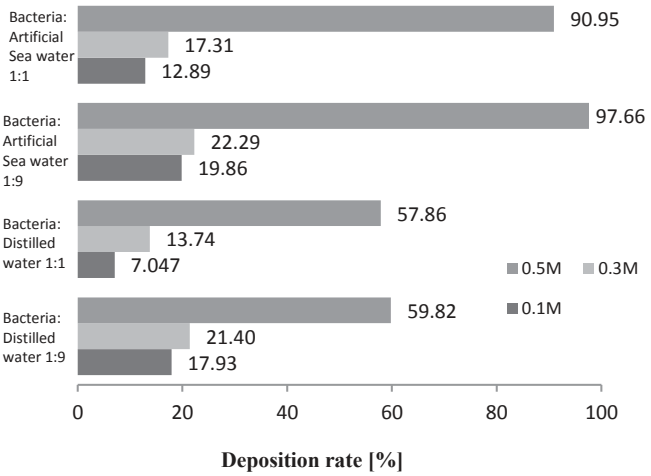

Fig. 6 The deposition rate (α) of precipitated crystals by $CaCl_2$ concentration correspond with solvent and bacteria.

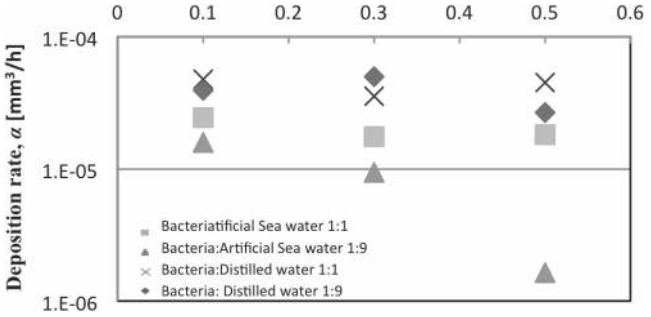

Fig. 7 Effects of $CaCl_2$ concentration on precipitated crystals correspond with solvent and bacteria.

3.3 Effects of temperature

Corresponding with the other enzymatic reactions, the catalysis of urea by urease depends on the temperature. The optimum temperature for activity of most ureases ranges from 20 to 37°C[18,19] and the optimum range of the enzymatic reaction depends on environmental conditions and concentration of reactants in the system.[20] In our study, data in Figs. 8 and 9 reveal the temperature dependence of the reaction. In each condition, three samples were placed at 25°C, 30°C, and 35°C, respectively. The average particle diameter at these temperatures is shown in Figs. 8 and 9. Figure 8 shows the deposition rate [%] of crystal particles at different temperatures after 48 h from the start of the test. The deposition rate is computed from the average particle diameter following eqs. (4)–(8). High urease activity was

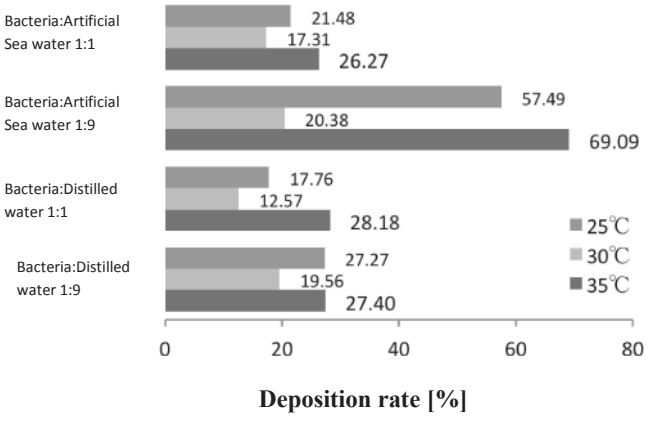

Fig. 8 The deposition rate (α) of crystal particles by the temperature.

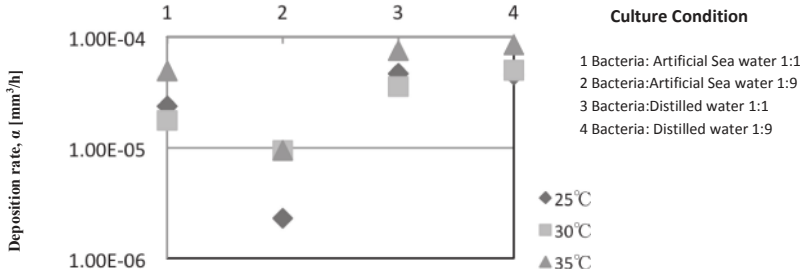

Fig. 9 Effects of temperature on precipitated crystal particles correspond with solvent and bacteria.

obtained for *Pararhodobacter* sp. at 20–60°C and a significant decrease in activity was observed above 70°C, ascribed to thermal denaturation of urease[21] which is directly related to crystal deposition.[5] The maximum urease activity of *Pararhodobacter* sp. was obtained at 60°C. In comparison with the previous studies, the number of deposited crystal particles on the photographed image was almost the same with little change depending on the bacterial concentration. As disclosed in Figs. 8 and 9, the crystal deposition rate was 27% at 25°C and 35°C, but in at 30°C, crystal deposition was considerably reduced by 19% and the average particle diameter was the largest at 25°C, followed by 35°C and 30°C with the lowest deposition rate (Fig. 9). At 30°C and 35°C, crystal growth stopped at about 12 h and reached a steady state, whereas growth at 25°C was stable for about 36 hours.

3.4 Effects of Mg^{2+}

It has been previously found that the maximum deposition ratio in the presence of Mg^{2+} was roughly 70% and the deposition ratio increased rapidly and approached the maximum, i.e., 90% when 10 and 20% of magnesium were used.[22,23] Subsequently, Figs. 10 and 11 depict the influence of Mg^{2+} on crystal deposition. The testing conditions of "$CaCl_2$+$MgCl_2$" and $CaCl_2$ were influenced by the basic solidification promoting solution (Table 1). Data in Figs. 10 and 11 shows that the average particle diameter was influenced by adding both Ca^{2+} and Mg^{2+}. Furthermore, Fig. 10 shows the deposition rate [%] of crystal particles upon adding Mg^{2+} 48 h after the start of the test. The deposition rate was enumerated from the average particle diameter. Finally, the number of crystal particles on the photographed image was larger when $MgCl_2$ was present in the solidification-promoting solution. In addition, as shown in Fig. 11, the average particle size was smaller and particle diameter growth stopped at about 12 h and become stationary, and the $MgCl_2$ deposition rate of crystal particles was influenced by "Ca" and "Ca+Mg" solution, solvent, and bacterial concentration.

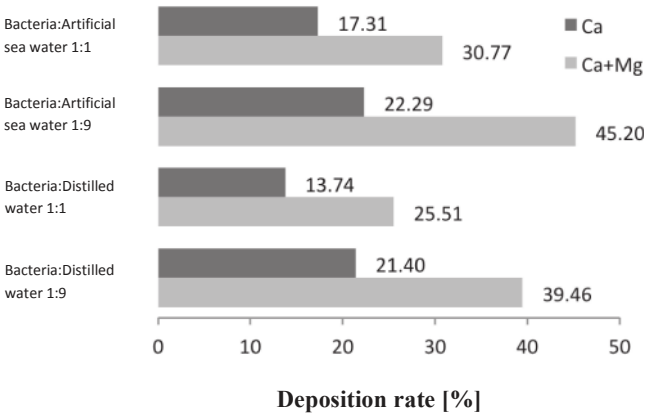

Fig. 10 The deposition rate (α) of crystal particles by the "Ca" and "Ca+Mg" solidification solution.

4. Discussions

4.1 Effects of the bacterial concentration

For $CaCO_3$ deposition, bacterial concentration plays a vital role because the bacterial cells serve as nucleation sites for $CaCO_3$ deposition and the availability of these nucleation sites is very significant for calcite depositions.[24] Additionally, it was previously shown that 98% of the initial Ca^{2+} concentrations were deposited due to bacterial hydrolysis (microbially), but only 35% and 54% was deposited chemically in the water and subsequent medium, respectively.[21,25] Therefore, for $CaCO_3$ deposition and the diameter of the deposited crystal particles, bacterial concentration is very important because the bacterial cell concentration provides the nucleation sites for $CaCO_3$ deposition and creates an alkaline environment for the induction of further calcite growth. Our experimental results indicate that the number and diameter of crystal particles increase with higher bacterial concentration, added promotion solution, and solvent, as shown in Fig. 5. The average particle diameter growth became steady earlier when bacterial concentration was lower compared to that when bacterial concentration was high. Finally, this result indicates how the deposited crystals are influenced by the bacterial concentration that is associated with the sand solidification test. In this experiment, the deposition rate was slightly lower, the number of deposited particles was larger, and the average particle diameter became smaller when bacterial concentration was high. This study also revealed that bacterial cell concentration greatly affects the bio-cementation of sand particles.

4.2 Effects of $CaCl_2$ concentration

$CaCl_2$ concentration is very important for $CaCO_3$ deposition because it has been reported that the ideal calcium source and concentration affects $CaCO_3$ deposition. However, high concentrations of $CaCl_2$ (above 0.5 M)

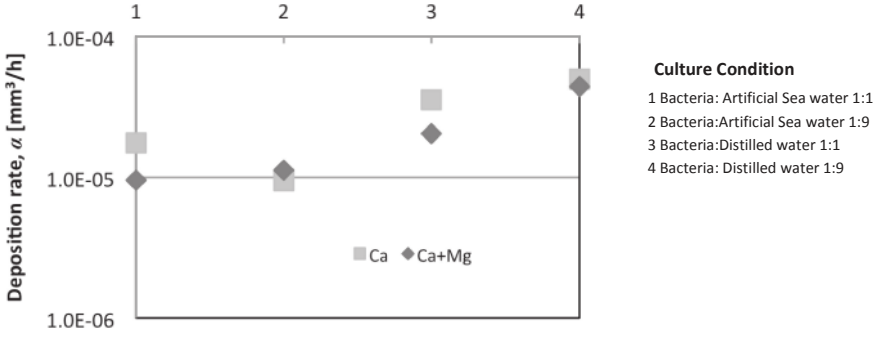

Fig. 11 Influence of "Ca" and "Ca+Mg" solidification solution on crystal deposition rate.

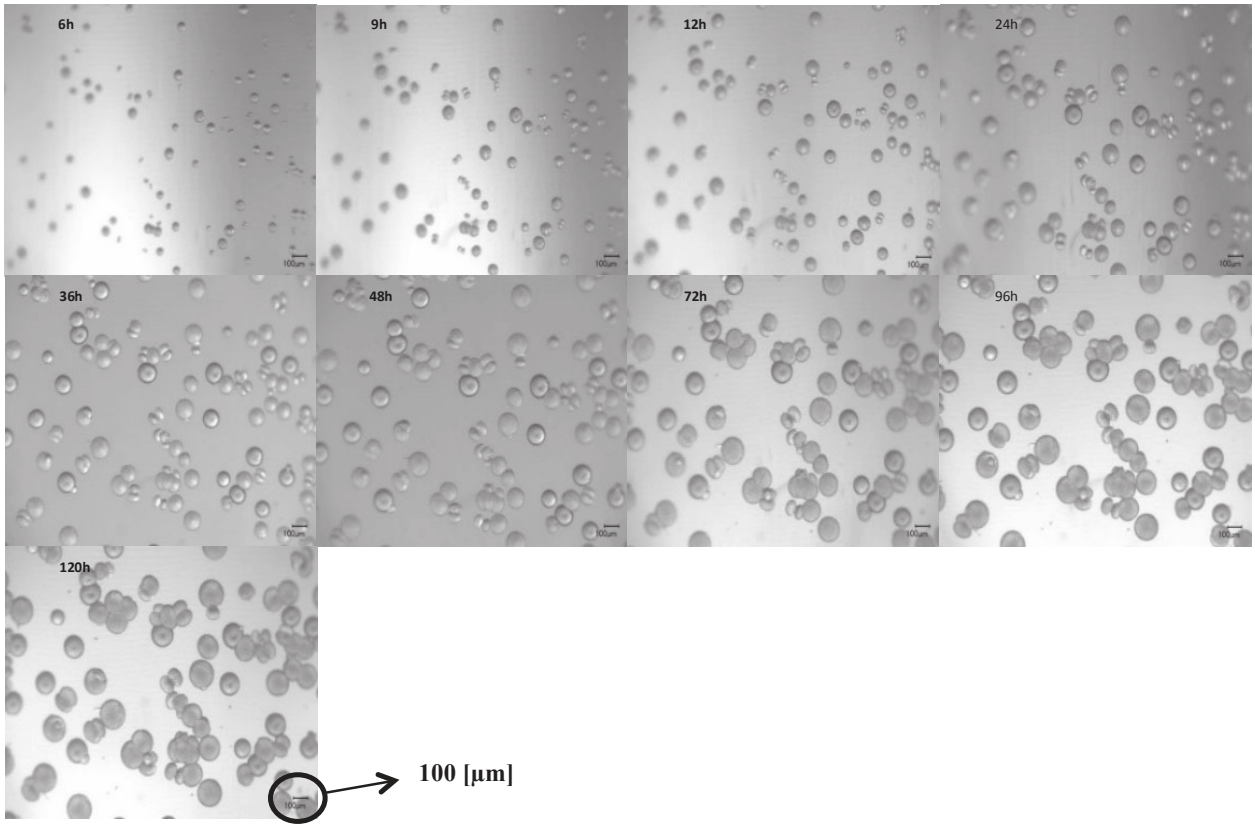

Fig. 12 Crystal deposition at certain times of interval with bacterial concentration.

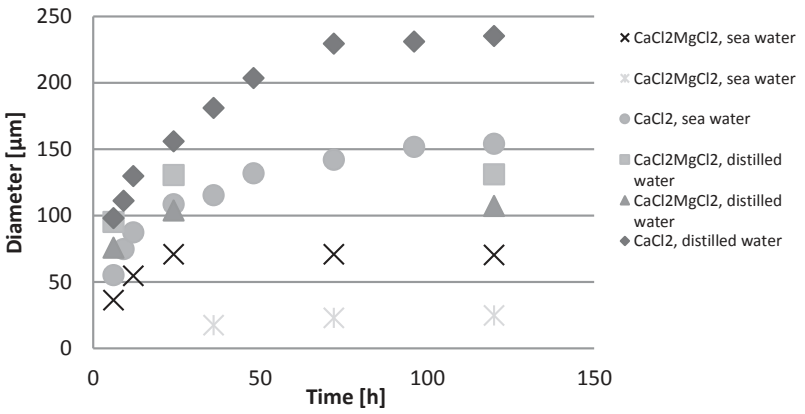

Fig. 13 Deposited crystals diameter change with time.

decreased the capability of calcite deposition and low concentrations (0.05–0.25 M) increased the capability of calcite deposition;[20] this study also found that the optimum $CaCl_2$ concentration for calcite deposition was 0.5 M and 0.25 M, respectively. Correspondingly, the amount of calcite deposition depends more on Ca^{2+} concentration than on urea concentration.[5,11] As shown in Fig. 6, the crystal deposition rate was about 30% lower at a $CaCl_2$ concentration of 0.5 M when distilled water was used as a solvent. In our study, the average particle diameter was smaller with artificial seawater and when Ca^{2+} concentration was high. However, a higher concentration of $CaCl_2$ promoted the deposition rate diameter (Fig. 12) of deposited carbonate when distilled water was used as a solvent (Fig. 13). Additionally, when the concentration of "urea+$CaCl_2$" was higher, the growth of microorganisms was inhibited. Therefore, from our study, it is seen that conditions with an extremely high $CaCl_2$ concentration do not have potential for the deposition of crystal carbonates.

4.3 Effects of temperature

In general, urease activity is increased by about 5 to 10 times when the temperature increased from 15°C to 20°C and 10°C to 20°C, respectively[22,24] and the kinetic rate of urease hydrolysis[26] for $CaCO_3$ deposition was completely stable at 35°C, but when the temperature increased to 55°C, the enzymatic activity declined by almost 47%. Therefore, the temperature is a very significant parameter for urea hydrolysis and $CaCO_3$ deposition, which is a major concern in this study because as the temperature is higher, the

Table 3 The degree of influence on deposited particles of carbonate.

	Bacterial mass	Concentration	Temperature	Mg^{2+}	Solvent	Ratio
Particle size	◎	◎	△	◎	○	△
No. of particles	◎	◎	△	◎	○	△
Particle shape	△	△	△	◎	◎	△
Particle growth	△	△	△	○	○	◎
Deposition rate	△	◎	○	◎	◎	◎
Deposition speed	○	△	◎	◎	◎	○
◎-Strong influence; ○-Moderate influence; △-Small influence						

deposition rate of carbonate also increases.[27] In this study, data in Fig. 8 show that the deposition rate was about 70% at 35°C where Bacteria/Artificial sea water ratio was 1:9. On the other hand, the deposition rate was similar between the artificial seawater and distilled water with changing temperatures, whereas the use of artificial seawater was observed to slow deposition rate at 25°C (Fig. 9). This is because the hydrolysis by microorganisms had decelerated due to the changes in cell growth at a low temperature of 25°C compared to growth at 35°C.

4.4 Effects of Mg^{2+}

It was previously reported that low concentrations of magnesium ion (i.e., 0.02 and 0.04 mol/L) significantly promoted the formation of aragonite and the substitution of a small amount of magnesium ion conveys a significant improvement in the deposition ratio.[23] Furthermore, the acceleration of calcite deposition may be because the Mg^{2+} ions engaged in the reaction and the addition of magnesium also affects the shape of the deposited materials.[14,27] In our experiment, we added $MgCl_2$, and in agreement with the above results, the number of particles and deposition rate and diameter of crystals were decreased in the basic solidification-promoting solution containing $MgCl_2$ (Fig. 10 and Fig. 11). In addition, the average particle diameter was larger when $MgCl_2$ was present in the solidification-promoting solution. This is likely because the existence of multivalent cations suppresses its deposition rate and the crystal gradually grows, consequently, the crystal seems to be rounded. In addition, in this study, we found that the deposition speed was slower when Mg^{2+} was scarce in the solidification-promoting solution. Therefore, Mg^{2+} enhances the crystal deposition speed.

4.5 Summary of the discussions

In brief, this study found that carbonate deposition and crystal formation are greatly affected by temperature and coexisting ions. Based on the findings obtained in this study, the results summarizing the degree of influence on carbonate particle deposition are shown in Table 3. For each item, a ◎ mark in the table represents a strong influence of the parameter, a ○ mark signifies the moderate influence, and a △ indicates a parameter with low influence. From the results of this study, we found that bacterial concentration, temperature, Mg–Ca ion, and solvent played a significant role in $CaCO_3$ crystal deposition (spherical type) and the optimum conditions of these parameters (Table 3 and Table 4) can be applied for engineering applications in sand solidification tests towards soil improvement techniques.

Table 4 Suggested optimum conditions for engineering application for *Pararhodobacter* sp.

Bacterial mass	Abundant
Concentration	0.5M
Temperature	35°C
Mg^{2+}	Include
Solvent	Artificial seawater
Precipitated crystal type	Spherical

5. Concluding Remarks

Our results effectively demonstrate that the feasibility of using “Ca” and “Mg–Ca” ions was considerably influenced by bacterial concentration, temperature, and solvent for crystal carbonate deposition using marine bacteria species. In this work, Mg^{2+} ions were added and other parameters (temperature, solvent, bacterial population, and $CaCl_2$ concentration) were taken into consideration to investigate the rate and the amount of carbonate deposition. The effects of Mg^{2+} on the deposited materials were analyzed to

investigate the microstructure of the deposited materials by a digital microscope. Adding Mg^{2+} ions increased the deposition ratio of carbonate up to 80% with the $CaCl_2$ concentration. The existence of "Ca" and "Mg–Ca" changed the shape and the size of the deposited crystal carbonate materials. This study can be useful for determining the suitable conditions for sand solidification by MICP method that can be applied to various ground improvement and engineering sectors.

Acknowledgement

This work was partially supported by JSPS KAKENHI, Grant number JP16H04404, Japan.

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