Biotechnology and Bioprocess Engineering 18: 903-908 (2013)
DOI 10.1007/s12257-013-0030-0

RESEARCH PAPER

Effects of Different Calcium Salts on Calcium Carbonate Crystal Formation by *Sporosarcina pasteurii* KCTC 3558

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Received: 14 January 2013 / Revised: 17 March 2013 / Accepted: 26 March 2013

Abstract We examined the effectiveness of using different calcium salts for bioconsolidation. Four calcium salts were chosen based on their applicability and solubility. Initial experiments demonstrated that the addition of any calcium salt had a negative effect on the urease activity of *S. pasteurii*. Microscopic examinations elucidated the morphological and structural differences of the calcium carbonate ($CaCO_3$) crystals induced. Calcite and vaterite are the prominent forms of $CaCO_3$ detected according to X-ray diffraction (XRD) analysis. Bioconsolidated sand samples were able to significantly resist water flow through a column compared to the non-treated samples. Also, in a tightness test, the differences in the ability to retain water within columns were observed among the samples tested. Moreover, despite the differences, the calcium salts tested still bound the sand together to form blocks. Our results further explain the influence of multiple factors in crystal formation and sand bioconsolidation effectiveness.

Keywords: bioconsolidation, calcium salts, calcium carbonate crystals, *Sporosarcina pasteurii*

1. Introduction

Microbially induced sand bioconsolidation has previously been considered to improve the engineering properties of sand [1,2]. Bioconsolidation as a means of increasing the cohesive properties of sand particles is not sufficient for a construction application. However, it can be very useful in geotechnical engineering to prevent or stabilize erosion and increase slope stability. The application of bioconsolidation can lead to a minimum 10-fold change in the primary properties of the sand such as permeability, stiffness, compressibility, shear strength, and volumetric behavior [1].

Microbially induced calcium carbonate precipitation (MICP) occurs in many bacteria under certain conditions [3,4]. *Sporosarcina pasteurii*, a ureolytic bacteria, is one of the many well-studied organisms which induce calcium carbonate precipitation. It uses the ureolytic pathway as a mechanism for degrading urea to produce ammonium ions (i) as an energy source which consequently causes the alkalinization of the surrounding environment [5,6]. Together with the production of two moles of NH_4^+, a mole of carbonate ion (i) forms which, in the presence of Ca^{2+} (ii), results in the precipitation of $CaCO_3$ (iii) [7]. Moreover, these reactions occurring in the presence of sand will also result in the development of crystals between sand particles that then hold the sand particles together.

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

$$Ca^{2+} + \text{Cell} \rightarrow \text{Cell-}Ca^{2+} \quad \text{(ii)}$$

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

Four parameters are important for the precipitation of calcium carbonate to occur; (1) the calcium concentration, (2) carbonate concentration, (3) the pH of the environment, and (4) the presence of nucleation sites [7]. Each of these parameters greatly affects either the ureolytic bacteria or the formation of crystals. According to de Muynck *et al*. [8], specific urea and calcium chloride concentrations greatly

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influence the effectiveness of biodeposition. In addition, the pH of the environment significantly affects the urease activity of bacteria. In a study conducted by Stocks-Fischer *et al.* [6], while the activity of the enzyme increased at a relatively fast rate as the pH of the reaction mixture increased from 6.0 to 10.0, the activity decreased at a higher pH. Lastly, nucleation sites for calcium attachment have also been considered. Theoretically, as the electro-negative characteristic of the bacteria increases, the calcium attaches more readily to the bacterial cell surface, subsequently inducing more calcium carbonate precipitation.

Many studies have confirmed that differences in the formation of crystals are attributed to several factors. Most of the studies regarding the identification of the possible cause of the differences of crystal formation mainly focus on the strain of ureolytic bacteria and the components that might be present during cultivation and crystal formation. Calcium is an integral parameter of MICP. Since calcium is in a salt form when added, attached anions may also affect crystal formation and thus the effectiveness of bioconsolidation. In this study, we examined the effect of the use of different calcium salts on the urease activity of *S. pasteurii*, crystal formation, and bioconsolidation effectiveness.

2. Materials and Methods

2.1. Microorganism

The strain of *S. pasteurii* KCTC 3558 used in this study was procured from Korean Collection for Type Culture (KCTC, Daejon, Korea). Cultivation was conducted under an aerobic condition in Yeast Extract (YE) medium containing 20 g/L yeast extract and 75 mM NH_4Cl, at pH 9. *S. pasteurii* was first inoculated in YE media and incubated overnight in a shaking incubator at 28°C. One tenth of the culture was transferred to new media and incubated for 8 h to obtain fresh culture.

2.2. Urease activity

Urease activity was determined using the phenol-hypochlorite assay method [9]. A bacterial suspension (250 μL) was added to a previously mixed 250 μL Sodium Phosphate Buffer (0.1 M) and 500 μL urea solution (3 M). The mixture was incubated at 37°C for 5 min, after which 2 mL of phenol nitroprusside solution was added followed by 2 mL of alkaline hypochlorite solution. The mixture was then incubated at 50°C for 10 min. Absorbance was measured at 626 nm. Ammonium Chloride (0 ~ 10 μM) was used as a standard. One unit of urease activity is defined as the amount of enzyme that hydrolyzes 1 μmoL of urea per minute. Specific urease activity can be calculated by dividing the standard urease activity by the total protein concentration measured using a Bradford assay [10].

2.3. Calcium carbonate crystal harvest

A fresh culture of *S. pasteurii* was washed twice with ddH_2O. The washed culture was inoculated in a ddH_2O-urea-calcium salt solution (urea 2%, calcium salt 50 mM) to generate an absorbance of 0.1 at 600 nm. The calcium salts used in this study were calcium chloride, calcium acetate, calcium lactate, and calcium gluconate. The cells were incubated for 3 h in a shaking incubator until precipitation occurred. Precipitates were collected using ash-less filter papers and then dried overnight in an oven at 50°C.

2.4. Scanning electron microscopy (SEM)

A modified SEM method was applied to observe the calcium carbonate crystals produced. Calcium carbonate crystals previously harvested were placed into a suspension by adding water, plated on a glass cover, and then dried in an oven at 50°C. After drying completely, the samples were coated with platinum in an ion sputter. A field emission scanning electron microscope (Hitach S-4300, Japan) was used for visualization of the crystal morphology.

2.5. X-ray diffraction analysis (XRD)

X-ray diffraction analysis was used to identify the crystal structure of the calcium carbonate. The samples of calcium carbonate previously produced using different calcium salts were suspended with 100 μL of sterilized water. The suspension was placed on a cover glass then dried in an oven at a temperature of 50°C. The samples were then analyzed using X-ray diffraction analysis (DMAX-2500, Rigaku, Japan).

2.6. Tightness test

The ability of the bioconsolidated sand to retard water flow through the column was determined using a tightness test. The experimental procedure for the tightness test was formulated from our lab. Three calcium salts were used in this study: calcium chloride, calcium acetate, and calcium lactate. An overnight culture of *S. pasteurii* was harvested (6,000 × *g*, 6 min), washed twice, and re-suspended with saline solution. A set of bacteria was autoclaved at 121°C for 20 min to kill any bacteria and endospores. Cell death was confirmed by plating in YE plates. The cells were suspended in a 20 mL saline solution (0.9% Sodium Chloride Solution) and mixed with 100 g sterile silica sand (0.3 ~ 0.45 mm, Joomoonjin Sand Co., Ltd., Korea). The pH of the sand suspended in distilled water is around 8.5. Sand slurry containing killed or live cells was packed into a 50 mL plastic syringe column (Kovax-Syringe®, Korea). A 30 min waiting time is necessary to at least establish the

binding of the bacteria to the sand. The sand slurry treated with killed bacteria served as the control. Columns were fed once by gravity with 20 mL of the urea and calcium salt solution (30 g/L urea and 20 g/L as calcium ion). The columns were set aside for 24 h to allow maximum crystal growth and to let any excess water to drip off. The columns were then dried at 45ºC for 24 h to facilitate the evaporation of excess water. Before measurement, the columns were allowed to cool. After cooling, the column samples were placed on a fixed rack. A tightness test was carried out by measuring the time required for 20 mL of water to pass through the column. Retention time refers to the time it takes before the first droplet is observed at the tip after the addition of water. The column treated without bacteria and the column treated with autoclaved cells served as the blank and control, respectively.

2.7. Sand blocks

Sand blocks were also produced to determine whether bioconsolidation with the use of different calcium salts could stabilize sand. The sand blocks were prepared similarly to those for the tightness test but were packed in a Petri dish instead of a syringe column. Three calcium salts were used: calcium chloride, calcium acetate, and calcium lactate. Sand treated with autoclaved cells served as the control.

3. Results and Discussion

3.1. $CaCO_3$ crystal morphology studies

Images of the calcium carbonate crystals examined under SEM are presented in Figs. 1A and 1B. Rod-shaped bacteria were prominent in all sediments and appeared fossilized as intact bacilli in the middle of the calcite crystals. The presence of crystalline calcium carbonate associated with the bacteria suggests that bacteria served as nucleation sites during the mineralization process.

3.2. Effect of calcium salts on the urease activity of *S. pasteurii*

Like many other microbial enzymes, the *S. pasteurii* urease is also vulnerable to environmental stresses. However, certain conditions allow urease to perform to its optimum when it is under control. Anything that exceeds the limit creates a drawback for the enzyme. Calcium ion plays an integral role in MICP. To determine the effects of calcium salts on the urease activity of the enzyme, 200 μL of the salts (50 mM) was added to the 1 mL enzyme reaction mixture. The presence of 30 mM of Ca^{2+} in crude enzyme increased by a 10 fold maximum when compared to the activity in the absence of Ca^{2+} [11]. However, in our study, the addition of 50 mM of each calcium compound did not cause an increase in the urease activity; nonetheless, it generated a decrease in the enzymatic activity (Fig. 2). Some reports concluded that Ca^{2+} has little or no effect on urease enzyme, probably due to the dissimilarities in terms of atomic radius or charge densities between nickel, the urease metalloenzyme, and calcium ion [12,13]. All of the calcium compounds tested except calcium silicate have specific urease activity of around 36 Unit/μg. In the case of calcium silicate, the specific urease activity is 59.51 ± 3.25 Unit/μg, which is a value of only about 5 lower than the control. A significant decrease in the enzyme activity can be observed in almost all calcium salts tested. A possible explanation could be the change in pH of the reaction mixture due to the addition of the salts. The activity of urease is optimum at a pH of 8.0 but activity decreases with the increase or decrease of pH [6,12]. Another explanation for the decrease in the urease activity could be due to the salt effect, wherein a decrease of enzymatic activity occurs with the addition of electrolytes [14]. The small decrease of the urease activity caused by calcium silicate is apparently attributed to its low solubility.

3.3. Differences in crystal morphology

The use of calcium chloride as a source of Ca^{2+} caused the

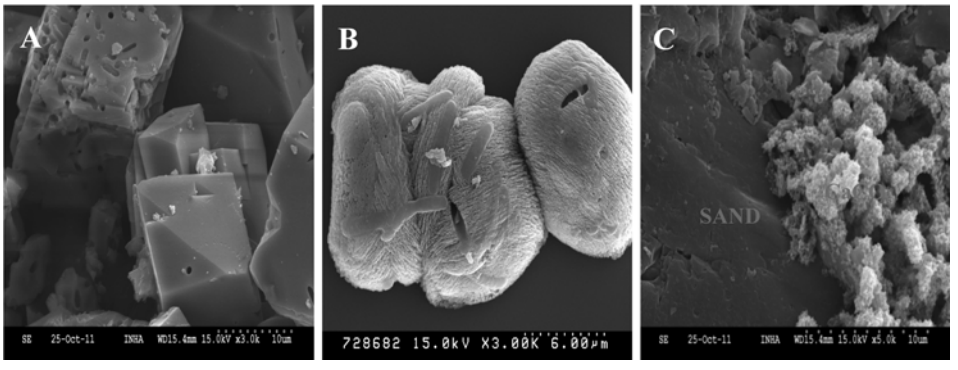

Fig. 1. SEM images of the induced by calcium carbonate by *S. pasteurii*. Bacterial imprints can be observed embedded on the crystal surface (A, B). Bioconsolidated sand showing the presence of $CaCO_3$ attached to the sand particle (C).

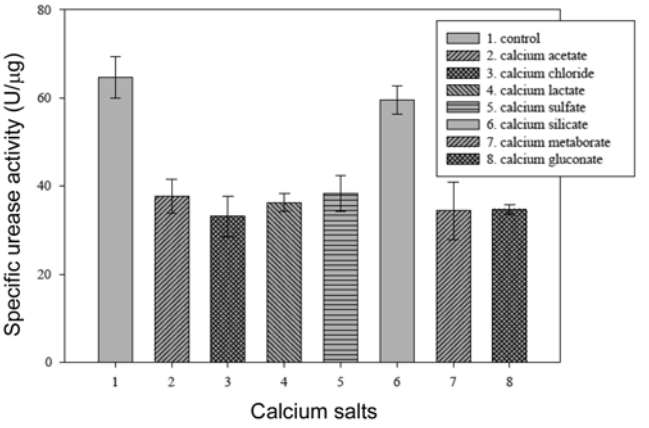

Fig. 2. Effects of different calcium salts on the urease activity of *S. pasteurii*. All of the calcium salts tested decreased the activity of urease when compared to the control. Addition of calcium silicate did not significantly decreased urease activity which might be attributed to its low solubility in water.

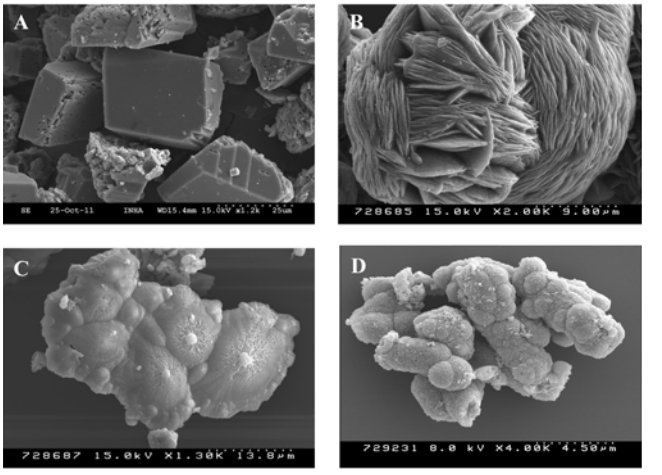

Fig. 3. SEM image showing the effects of the use of different calcium salts on the shape of crystal. The results show that the difference in shape of the calcium carbonate precipitated can be also be attributed to the calcium salt used. Calcium chloride (A), calcium acetate (B), calcium lactate (C), and calcium gluconate (D).

precipitation of a rhombohedral calcium carbonate (Fig. 3A). A rhombohedral shape is a characteristic of the most stable form of calcium carbonate, that of calcite [15]. Conversely, using calcium acetate induced a different shape, a lettuce-like or lamellar shape, which is a type of vaterite, a metastable form of calcium carbonate (Fig. 3B) [16]. Meanwhile, the use of calcium lactate and calcium gluconate (Figs. 3C and 3D, respectively), as a source of calcium ions, gave a spherical shape, which is another form of vaterite [16]. Optical microscopy further revealed the differences in the sizes of the crystals formed (data not shown). The use of calcium lactate resulted in the largest crystals followed by calcium acetate, calcium chloride, then calcium gluconate as the smallest crystals. The morphological differences of the crystals formed may be strain-specific, having different levels of urease activity [11,17]. Also, the composition of the bacterial media or culture may affect the crystal morphology [18]. Moreover, crystal growth can be inhibited or altered by the adsorption of proteins and organic and inorganic components to the specific crystallographic planes of the growing crystal [11,18]. Our study shows that different calcium salts can induce different crystal sizes, and hence can affect the applicability of calcium carbonate for bioconsolidation.

3.4. Differences in the crystal structure

X-Ray diffraction (XRD) analysis is a scientific technique in which X-ray, neutron, or electron diffraction is used on powder or microcrystalline samples for the characterization of the composition, structure, microstructure, and phase conversion of the material [19]. Calcium carbonate crystals were produced using different calcium salts. Calcite and vaterite were produced in all samples (Fig. 4). The results, especially from the use of calcium chloride, do not concur with previous reports in which only calcite was produced [20,21]. The peaks and intensities of calcite and vaterite among samples vary. The differences might account for the differences in morphology. Calcification studies were carried out to provide us with new data on the properties of the calcium carbonate studies. To our knowledge, this is the first report in which calcium salts also caused differences in the crystal morphology. This is a very important finding because the differences in the crystal morphology may affect the effectiveness of calcium salts in bioconsolidation. In the crystal harvest procedure, the only substances present within the solution were deionized water, *S. pasteurii*, urea, and calcium salts in order to prevent other chemicals inducing changes in the calcification.

3.5. Tightness test

The tightness and ability of the bioconsolidated sand column to resist water flow were measured by passing 20 mL volume of water into the column. The first drop falling from the syringe column was recorded. The time gap between the addition of water and the first drop is marked as the retention time. Bioconsolidated sand columns using calcium chloride, calcium lactate, and calcium acetate had longer retention times, with averages of 96, 42, and 78 sec, respectively, when compared to the two control samples of no cells and dead cells with average times of 2.12 and 2.03 sec, respectively (Fig. 5). The increase in the retention time could have been caused by the formation of calcium carbonate crystals causing the addition of plugging materials on the sand column. According to Whiffin *et al.* [2], the bioconsolidation of sandy materials increases strength and

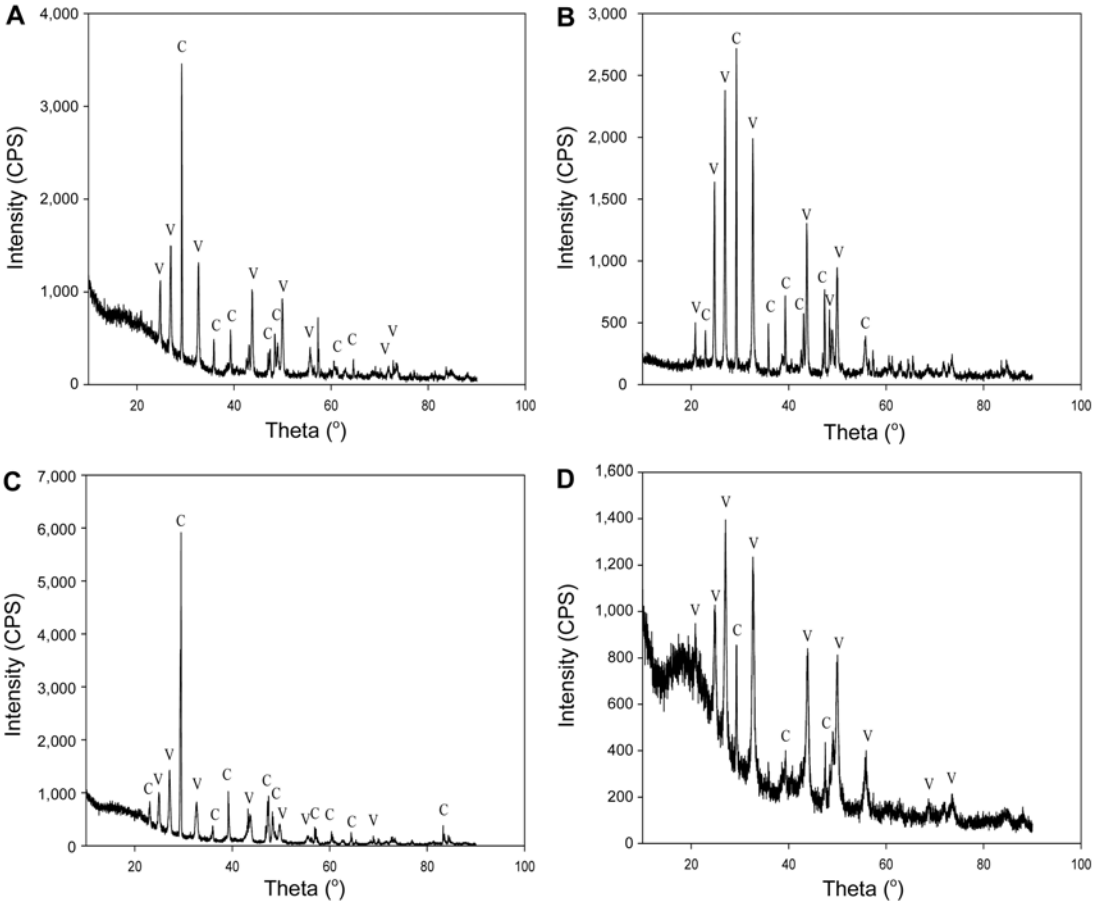

Fig. 4. X-ray diffraction analysis of the induced calcium carbonate crystals showing the differences in the crystal structure. The different calcium salts used: calcium chloride (A), calcium acetate (B), calcium lactate (C), and calcium gluconate (D). In all samples tested, calcite and vaterite were detected. The intensity of the peaks among samples varied.

alters its flow. The differences in the retention time may be attributed to the crystal sizes of the calcium carbonate produced by using different calcium salts, wherein as the crystal size decreases, the retention time concurrently increases. We did not include calcium gluconate because of the problems with its solubility at high concentrations. We also studied the ability of different calcium salts to stabilize sand particles by bioconsolidation. Stable blocks were able to be formed from all of the samples treated by bioconsolidation using different calcium salts (Figs. 6A, 6B, and 6C). However, in the case of the control sample in which dead cells were used (Fig. 6D), the sand blocks collapsed after the Petri dish was de-molded.

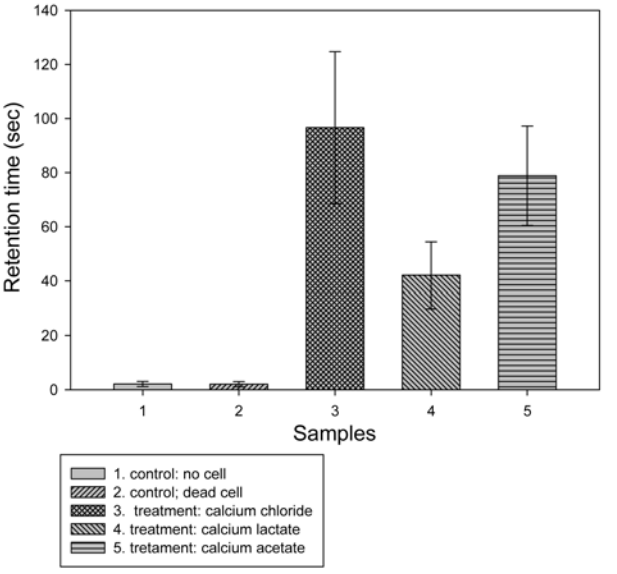

Fig. 5. Tightness test between non-treated samples and the bioconsolidated samples using different calcium salts. Bioconsolidated samples have relatively higher water retention time when compared to the control.

4. Conclusion

Bioconsolidation techniques in improving soil stability are rapidly emerging, and a range of opportunities continues to expand whilst challenges continue [1]. The research for this study was divided into three areas which represent

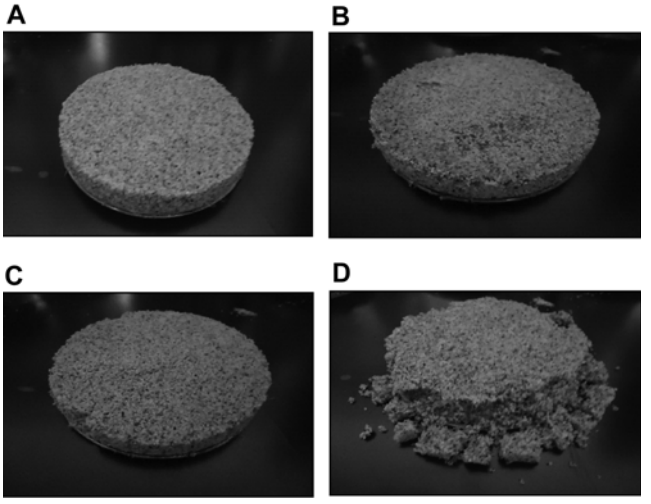

Fig. 6. Bioconsolidated sand blocks made from the use of different calcium salts. All of the calcium salts used were able to fold a solid block. Calcium chloride (A), calcium acetate (B), calcium lactate (C), and the control (D).

three of the most integral parts of the bioconsolidation process. The activity of the urease enzyme should always be kept high; however, stressful conditions cannot be avoided *in situ*. Conversely, many conclusions can be made on the occurrences of different calcium carbonate crystal morphologies with the same calcification conditions. However, our results further explain that the formation of crystals can be influenced by multiple factors. The activity of the enzyme and crystal formation greatly affects bionconsolidation effectiveness. With such results, future research will further elucidate the effectiveness of bioconsolidation. Multiple treatments might be very beneficial in order to achieve optimum sand bioconsolidation. Lastly, a hardness test to measure the effectiveness of bioconsolidation in stabilizing or binding sand particles together should also be made.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2011-0025229) and in part by an Inha University Research Fund.

References

1. DeJong, J. T., B. M. Mortensen, B. C. Martinez, and D. C. Nelson (2010) Bio-mediated soil improvement. *Ecol. Eng.* 36: 197-210.
2. Whiffin, V. S., L. A. van Paassen, and M. P. Harkes (2007) Microbial carbonate precipitation as a soil improvement technique. *Geomicrobiol. J.* 24: 417-423.
3. Boquet, E., A. Boronat, and A. Ramos Cormenzana (1973) Production of calcite (calcium carbonate) crystals by soil bacteria is a general phenomenon. *Nature* 246: 527-529.
4. Rivadeneyra, M. A., R. Delgado, A. Del Moral, M.R. Ferrer, and A. Ramos-Cormenzana (1993) Precipitation of calcium carbonate by *Vibrio* spp. from an inland saltern. *FEMS Microbiol. Ecol.* 13: 197-204.
5. Kroll, R. G. (1990) Alkaliphiles. pp. 55-92. In: Edwards, C. (ed.). *Microbiology of extreme environments*. McGraw-Hill, NY, USA.
6. Stocks-Fischer, S., J. K. Galinat, and S. S. Bang (1999) Microbiological precipitation of $CaCO_3$. *Soil Biol. Biochem.* 31: 1563-1571.
7. Hammes, F. and W. Verstraete (2002) Key roles of pH and calcium metabolism in microbial carbonate precipitation. *Rev. Environ. Sci. Biotechnol.* 1: 3-7.
8. De Muynck, W., K. Verbeken, N. De Belie, and W. Verstraete (2010) Influence of urea and calcium dosage on the effectiveness of bacterially induced carbonate precipitation on limestone. *Ecol. Eng.* 36: 99-111.
9. Natarajan, K. R. (1995) Kinetic study of the enzyme urease from Dolichos biflorus. *J. Chem. Educ.* 73: 556-557.
10. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72: 248-254.
11. Hammes, F., N. Boon, J. De Villiers, W. Verstraete, and S. D. Siciliano (2003) Strain-specific ureolytic microbial calcium carbonate precipitation. *Appl. Environ. Microbiol.* 69: 4901-4909.
12. Mahadevan, S., R. D. Sauer, and J. D. Erfle (1977) Purification and properties of urease from bovine rumen. *Biochem. J.* 163: 495-501.
13. Perez-Perez, G. I., C. B. Gower, and M. J. Blaser (1994) Effects of cations on *Helicobacter pylori* urease activity, release, and stability. *Infect. Immun.* 62: 299-302.
14. Kistiakowsky, G. B., P. C. Mangelsdori, A. J. Rosenberg, and W. H. R. Shaw (1952) The effects of electrolytes on urease activity. *J. Am. Chem. Soc.* 74: 5015-5020.
15. De Yoreo, J. J. and P. G. Vekilov (2003) Principles of crystal nucleation and growth. *Rev. Mineral. Geochem.* 54: 57-93.
16. Tai, C. Y. and F. Chen (1998) Polymorphism of CaCO3 precipitated in a constant-composition environment. *AIChE J.* 44: 1790-1798.
17. Park, S., Y. Park, W. Chun, W. Kim, and S. Ghim (2010) Calcite-forming bacteria for compressive strength improvement in mortar. *J. Microbiol. Biotechnol.* 20: 782-788.
18. De Muynck, W., K. Cox, N. D. Belie, and W. Verstraete (2008) Bacterial carbonate precipitation as an alternative surface treatment for concrete. *Constr. Build. Mater.* 22: 875-885.
19. Cullity, B. D. (1978) *Elements of X-Ray Diffraction*. 2nd ed., Addison Wesley Publishing Compnay, MA, USA.
20. Shirakawa, M. A., K. K. Kaminishikawahara, V. M. John, H. Kahn, and M. M. Futai (2011) Sand bioconsolidation through the precipitation of calcium carbonate by two ureolytic bacteria. *Mater. Lett.* 65: 1730-1733.
21. Achal, V., A. Mukherjee, P. C. Basu, and M. S. Reddy (2009) Strain improvement of *Sporosarcina pasteurii* for enhanced urease and calcite production. *J. Ind. Microbiol. Biotechnol.* 36: 981-988.