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Enhancement of the Yield and Strength of Microbially-induced Carbonate Precipitation by Optimum Cultivation and Grouting Measures for Civil Engineering Applications

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The application of microbial technology has created new opportunities for the development of civil engineering. Microbial induced carbonate precipitation uses the microbially catalyzed hydrolysis of urea to produce calcium carbonate crystals which can bind sand particles to give similar chemical and physical qualities of sandstone and can increase the strength and stiffness of sand and stone. In this work, we investigated the process of calcium carbonate precipitation induced by *Sporosarcina pasteurii*. Effects of bacterial cultural and cementation conditions on the urease activity and stability are discussed. Add 10 μM $NiCl_2$ in the culture could improve the urease activity. Adding carboxymethyl cellulose (CMC) could improve the amount of calcium carbonate; the strength and the acid tolerance properties of sand columns. Optimal conditions obtained allow for the practical uses of this new technology in civil engineering applications.

Key words: Enzyme Biocatalysis; Microbial Growth; Enzyme Activity; Microbial-induced carbonate precipitation; Bio-grouting.

Recent advances in the study of microbial mineralogy and chemistry reveal that under certain environmental and nutritional conditions, microorganisms in soils and rocks can, through metabolism and degradation, precipitate significant amounts of various mineral crystals such as carbonates, phosphates, oxides, sulfides and silica sinters, although these processes typically occur very slowly in the natural environment [1-3]. Bacterial processes that bind metals and form minerals are widespread and represent a fundamental part of key biogeochemical cycles [4, 5]. Microbially Induced Carbonate Precipitation (MICP) is an important aspect of biomineralization, and has been investigated extensively due to its wide range of technological implications, especially by the urea hydrolysis processes [6, 7]. *Sporosarcina pasteurii* is a commonly used bacterial species in biochemical engineering studies aimed at using mineral crystals for civil and environmental engineering applications [8-11]. Recent scientific findings show that *S. pasteurii* have a marked ability to adhesively solidify loose sand, which can be used to increase the shear resistance of sand and influence the seismic wave propagation of sand [12-13]. Earthquake triggered liquefaction could be therefore mitigated by the MICP technology [14]. Also the effects of various conditions on the enzyme activity of *S. pasteurii* for long-term and large scale foundation improvement have also been studied [15-17]. The ability of the organism to produce calcium carbonate in the pore space of sand columns, and the relationship between the strength acquired from the bonding of sand columns by the microorganism

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and the production of calcium carbonate have been investigated. Some of these studies have included simulation analysis [18-20]. And scaled model tests have shown the effectiveness of MICP at healing or stabilization of cracks in concrete and masonry [21-26], and immobilization heavy metals [28-30].

This study investigated the characteristics of *S. pasteurii* and optimization of culture conditions to increase the urease activity of this species. In order to provide solid basic microbial experimental data for microbial cementing and bio-grouting methods as a large-scale ground liquefaction remedy, the study also includes research on the conditions required to induce the production of calcium carbonate to consolidate loose sand-filled columns.

MATERIALS AND METHODS

Strain and culture medium

The *Sporosarcina pasteurii* strain (ATCC 11859) was obtained from the American Type Culture Collection and was cultured in NH_4-YE medium with: yeast extract 20 g/l, $(NH_4)_2SO_4$ 10 g/l, [13]. $NiCl_2$ was added in the medium with final concentration of 0 µM, 5 µM, 10 µM, 50 µM and 100 µM. All the media were autoclaved at 121°C for 20 min. *S. pasteurii* was cultured at 30°C and 200 rpm for 12h.

Measurement of optical density (OD600)

During the course of the experiments the optical density measured at 600 nm was used as an indication of biomass concentration. The optical density at 600 nm (OD_{600}) was measured using a Unico 2000 absorption spectrophotometer (UNICO Instruments Co., Shanghai, China)

Measurement of urease activity

Urease activity was measured immediately after sampling. In the absence of calcium ions, urease activity was determined by a conductivity method. The urease reaction involves the hydrolysis of non-ionic substrate urea to ionic products thus generating a proportionate increase in conductivity under standard conditions. One ml of bacterial suspension was added to 9 ml of 1.11 M urea and the relative conductivity change in ($mS.cm^{-1}.min^{-1}$) was recorded over 5 min at 20°C. In the measured range of activities $mS.cm^{-1}.min^{-1}$ correlated with a hydrolysis activity of 11.1 mM $urea.min^{-1}$.

Sand column packing and biochemical treatment

Sand columns were packed with Chinese ISO standard silica sand produced by Xiamen ISO Standard Sand Co., Ltd. The sand was manually screened into different particle sizes; the diameter of the screened sand was 0.16-0.315 mm. The sand column mold was a 50 ml syringe case that was 30 mm in diameter and 110 mm long. In the middle of the sand column mold, there was a 100 mm long sand column that needed to be bonded. To avoid congestion of the grout port on the top section, double-layered gauze filters were placed on both ends of the sand column. The top end of the syringe was tightly capped by a rubber stopper with a three-way tube.

Each syringe case was packed with approximately 50 ml of sand. For groups 1-4, deionized water was added before sand packing to pack sand densely and avoid air bubbles; group 5 was packed with sand soaked with bacterial broth.

To saturate the sand prior to the experiment, 150 ml of deionized water was pumped into each sand column at a speed of 1ml/min using a peristaltic pump. Each column was then infused with 40 ml of bacterial broth followed by 40 ml of additives. The columns were then grouted with a Ca/urea mixture solution in batches to generate MICP *in situ* in the sand columns. The Ca/urea mixture solution contained 1 M $CaCl_2$ and 1 M urea. Each time, 40 ml of a Ca/urea mixture solution was injected at a speed of approximately 0.1ml/min 6 times at intervals of 24 h, as shown in Table 1. During the experiment, the volume of outflow fluid was recorded, and 5 ml of each sample was saved for further use.

Measurement of solution calcium concentration

The groups of samples according to grouting procedure were digested with acid for total calcium measurement. The calcium concentration was measured using a calcium ion detector (Shanghai San-Xin Instrument, MP518) following the electrochemical analysis method. To ensure accuracy, the calcium ion detector was calibrated before each use with two concentrations of calcium chloride (two-point calibration); during detection, the calcium concentration was kept between the concentrations of the two calibration solutions and as close to the concentration of one of the calibration solutions as possible. In order to maintain the Ca^{2+} concentration of the diluted

solution close to that of one of the calibration solutions, the dilution of the sample was varied.

Morphology of treated sand columns

An environmental scanning electron microscope (ESEM) FEI Quanta 200 (FEI Company, USA) was operated between 10 and 20 kV. The samples were placed onto an aluminum stub and viewed at a working distance of 2.0mm.

Acid resistance test

A series of solutions with different pH was prepared for testing the acid resistance of the deposited MICP. The pH was as follows from 0.5 to 5.5 at an interval of 0.5 pH unit. A drop of dilute HCl solution with pH 5.5 was first dripped on the deposits and a magnifier was used to observe the reaction with the deposits carefully for 2 min to see whether air bubbles appeared. If no air bubbles appeared, it could be concluded that the deposited $CaCO_3$ could resist the corrosion of HCl solution at this pH. Decreased pH of HCl solution was subsequently dripped on the same deposits until CO_2 bubbles were generated under a certain pH, the acid resistance value of the deposited layer was the previous pH value preceding the current one [24].

RESULTS AND DISCUSSION

The influence of nickel ions on urease activity

Urease has been reported to be a nickel-containing oligomeric enzyme, and nickel ions have been shown to be very important in the process of urea hydrolysis [27]. We therefore tested the effect of nickel ions on the urease activity by including various concentrations of nickel ions in the culture medium. The results of these tests are shown in Figure 1. When the final concentration of $NiCl_2$ in the culture medium was 10 μM, the urease activity reached a maximum of 17.8 $mM.min^{-1}$. Therefore in all subsequent experiments, 10 μM $NiCl_2$ was added to the culture medium.

Optimization of sand column cementation

In this study, the sand column was grouted 3 times, which were designated A, B, C; before each grouting, *S. pasteurii* were cultured. Using a multi-step grouting method in which grouting was performed in two rounds. In each round, the bacterial culture was infused first, followed by infusion of various additives; the reaction solution (calcium chloride and urea mixture) was then infused six times every day.

Table 1. Grouting procedure and solution compositions

Group No.	Filler	Day 1	Day 2	Day 3-8
1	sand	bacterial broth	deionized water	$CaCl_2$+urea
2	sand	bacterial broth	nutrient solution	$CaCl_2$+urea
3	sand	bacterial broth	glutinous rice paste	$CaCl_2$+urea
4	sand	bacterial broth	CMC*	$CaCl_2$+urea
5	sand soaked with bacterial broth	bacterial broth	deionized water	$CaCl_2$+urea

*CMC: sodium carboxymethyl cellulose

This experiment included five groups of samples, which were numbered 1-5. Each group consisted of three columns, which were labeled A, B, and C, respectively. The additives used were as follows: micro-biological culture medium (yeast extract/ammonium sulfate), which provides nutrition for bacteria in the sand column, promotes their growth, and increases calcium carbonate production; glutinous rice paste, which was historically used as mortar in ancient Chinese buildings and shows excellent bonding strength, stiffness, and impermeability [31]; and sodium carboxymethyl cellulose (CMC), which has the functions of bonding, thickening, enhancement, emulsification, water conservation, and suspension. The columns that received these additives were designated groups 2-4, respectively; group 1 was the control.

After grouting, the columns were allowed to stand for 48 h before the mold was removed to observe the condition of bonding. Then, each sand column was removed, and the plastic case of the syringe was discarded. The results demonstrate that the loose sand grains in the 12 sand columns

of groups 1-4 formed into bonded sand columns with characteristic shapes. The sand grains in the sand columns in group 5 did not form into a definite shape, and the strength of these columns was low; as the sand was wet and the porosity was too great.

The concentration of Ca^{2+} in each column was measured so that the amount of MICP generated at each time could be calculated. The results show that group 4 had the highest average calcium carbonate production rate; however, the overall levels, which were approximately 0.022-0.024 M/day, did not differ greatly between the groups. These values indicate that the conversion rate of $CaCl_2$ was above 50%. Unexpectedly, group 2, which was supplemented with nutrient solution, had a lower rate of $CaCO_3$ production than the other groups, while group 1, which was not supplemented with any extra nutrients, had a remarkable production rate.

In this experiment, a hydraulic universal testing machine used in the building materials laboratory, with an accuracy of 40 N per grid, was used. The loading speed was controlled at

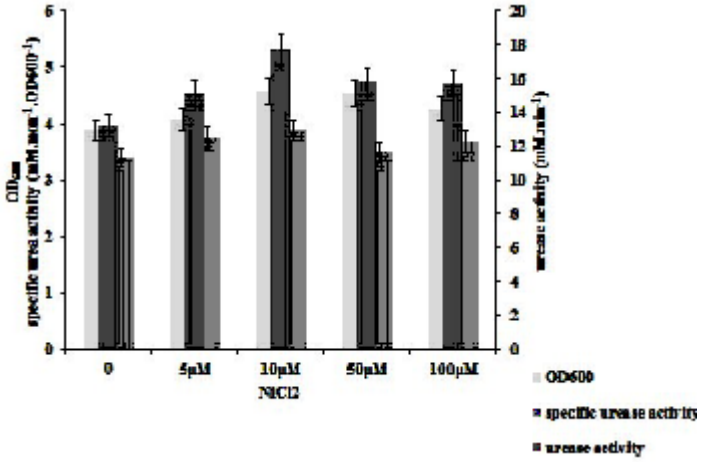

Fig. 1. The influence of the addition of $NiCl_2$ to NH_4-YE medium on urease activity and on specific urease activity

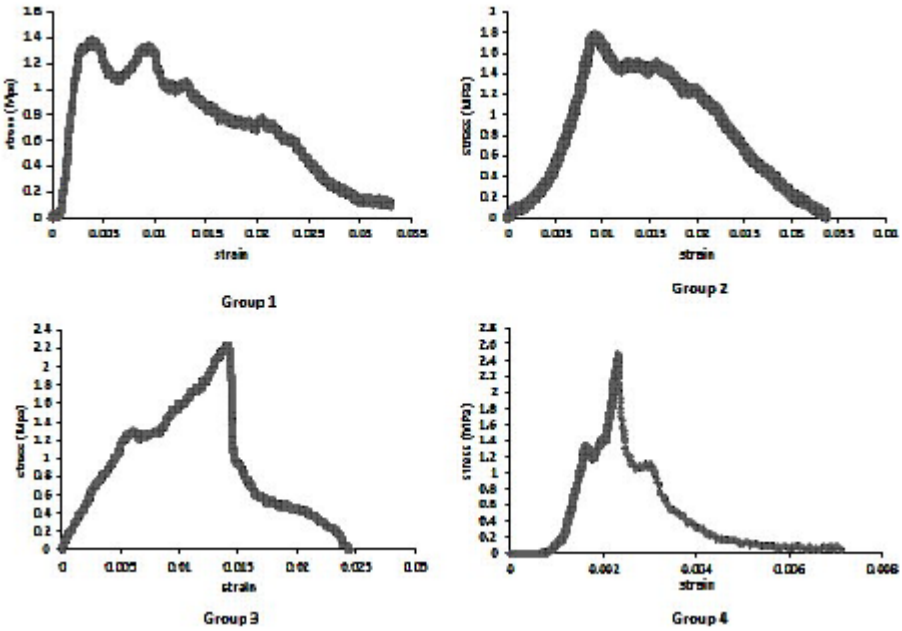

Fig. 2. Uncompressed tensile strength stress-strain curves of the cemented sand columns

approximately 0.2 N/s. The measured uniaxial compressive strengths (UCS) and the stress-strain curves are shown in Figure 2.

The data show that the compressive strength of the columns in group 4 was the highest, reaching 2.490 MPa. The compressive strength of

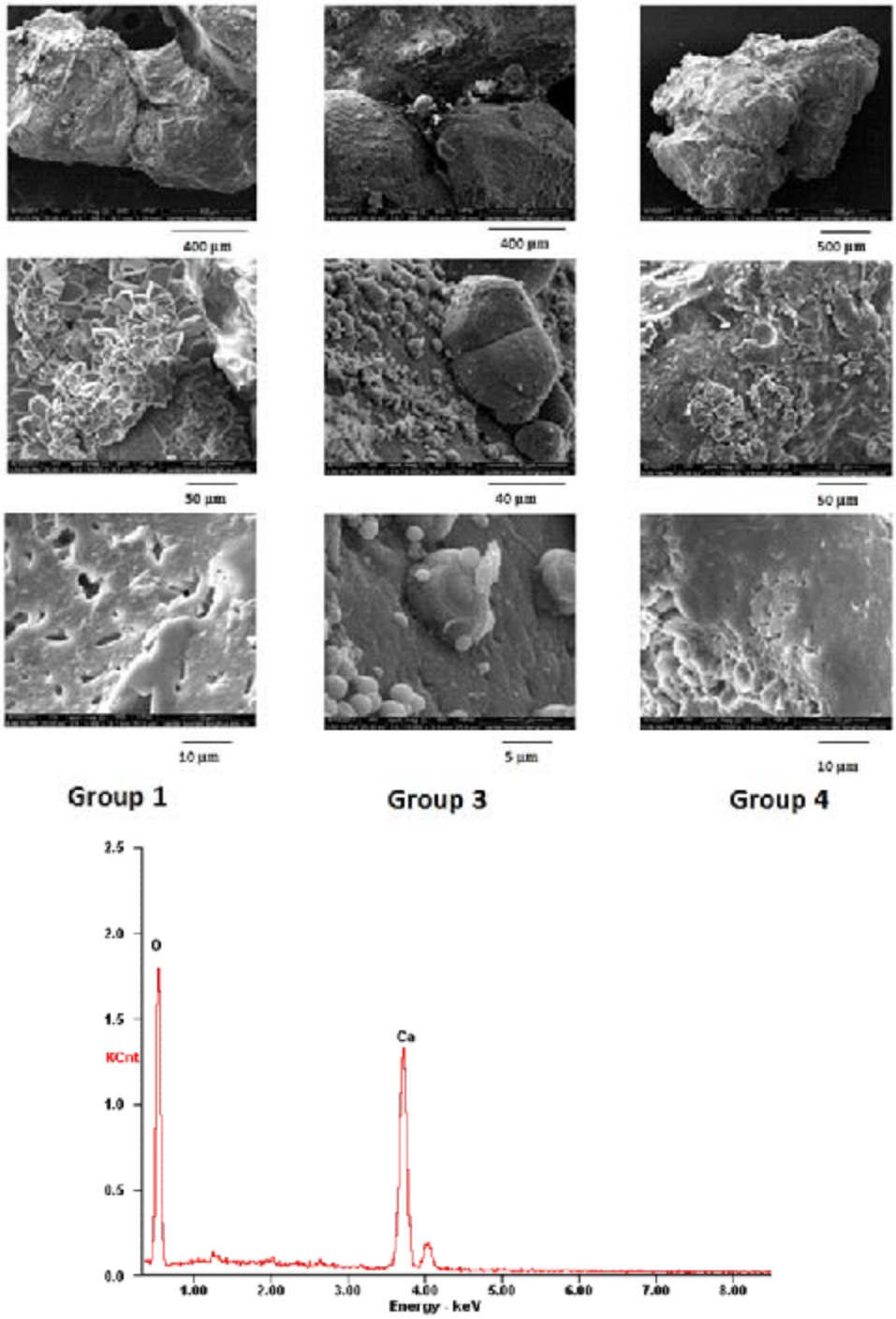

Fig. 3. (A) Scanning electron micrographs of cemented sand columns (B) Chemical element analysis of MICP by EDAX

the columns in group 3, which reached 2.265 MPa, was the second highest. The uniaxial compressive strength of the columns in group 2 was also higher than that of those in the control group 1. These results indicate that the all of the treatments used during the grouting process can increase the uniaxial compressive strength. The effect of CMC was the most prominent, and the addition of glutinous rice paste also played a role. These effects are likely a result of CMC and glutinous rice paste having a certain viscosity; they not only function in sand bonding but also prevent the loss of bacterial broth as a kind of porous material and adsorb calcium carbonate on their surfaces. The addition of culture medium in group 2 can promote bacterial growth in situ, thus increasing urease activity in situ and increasing calcium carbonate production.

Scanning electron microscopy analysis of sand columns

Bonded sand samples from groups 1, 3, and 4 were randomly selected for scanning electron microscopy analysis, as shown in Figure 3A. In group 1, hexahedral calcium carbonate crystals were observed between the sand grains, and there were numerous holes with lengths of 3 μm and diameters of 0.5 μm in the crystals. This result suggests that *S. pasteurii* acts as a crystal nucleus during MICP production. In group 3, spherical and hexahedral calcium carbonate crystals were also observed; in addition, small spherical glutinous rice particles could also be seen adsorbed on the sand, indicating that these particles have an adhesive bonding function. In the group 4 samples, the bonding between sand grains was even tighter, and there was more calcium carbonate, which existed in pieces, possibly as a result of the adhesive bonding effects of CMC. Chemical element analysis of MICP with EDAX was shown in Figure 3B, which confirmed the presence of MICP.

Analysis of acid-tolerance properties

The microbial cement and grouting technology is mainly used outdoors. When exposed to the environment, bio-grouted structures will be threatened by acid rain; therefore, the acid-tolerance of the material needs to be examined. The acid-tolerance capacity was defined as the pH level was the closest to that at which the sand column generated bubbles. The acid-tolerance pH of the sand columns in groups 1, 2, and 5 was 2.5 and that the acid-tolerance pH of those in groups 3 and 4 was 2. Thus, all of the tested sand columns would be expected to be resistant to acid rain. We therefore conclude that they can withstand acid rain and other acid materials in the environment. Furthermore, the sand columns supplemented with the glutinous rice paste and CMC had greater acid tolerance than the other columns.

CONCLUSIONS

This study investigated the culture characteristics of *S. pasteurii* and optimization of its culture medium. As a cofactor of urease, $NiCl_2$ plays an important role in urease activity. The concentration of $NiCl_2$ was optimized as 10 μM. For the efficient production of urease, bacteria that have reached the logarithmic growth phase can be maintained at room temperature for a period of time no longer than 5 days. During the bonding process, sand columns prepared by the grouting method using CMC as an additive produce the greatest amount of calcium carbonate. They have the highest strength and exhibit the best acid tolerance properties.

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REFERENCES

1. Beavon, J., Heatley, N.G.. The occurrence of struvite (magnesium ammonium phosphate hexahydrate) in microbial cultures. *J. Gen. Microbiol.,* 1962; **31**: 167-9.
2. Van Lith, Y., Warthmann, R., Vasconcelos, C., McKenzie, J. Microbial fossilization in carbonate sediments: a result of the bacterial surface involvement in dolomite precipitation. *Sedimentology.*, 2003; **50**: 237-45.
3. Rivadeneyra, M.A., Martín-Algarra, A., Sánchez-Navas, A., Martíin-Ramos, D. Carbonate and phosphate precipitation by chromohalobacter marismortui. *Geomicrob. J.,* 2006; **23**: 89-101.
4. Ferris, F.G., Fyfe, W.S., Beveridge, T. J. Bacteria as nucleation sites for authigenic minerals. Diversity of Environmental Biogeochemistry. *Develop. in Geochem.,* 1991; **6**: 319–26.

5. Ehrlich, H.L.(ed): Geomicrobiology, 4th edn. Revised and Expanded. 2002; New York: Marcel Dekker, Inc
6. De Muynck, W., De Belie, N., Verstraete, W. Microbial carbonate precipitation in construction materials: A review. Ecological Engineering., 2010; **36**: 118-36.
7. Mitchell, J.K., Santamarina, J.C. Biological considerations in geotechnical engineering. J. Geotech. Geoenviron. Eng., 2005; **131**: 1222-33.
8. Ramakrishnan, V., Ramesh, K.P., Bang, S.S. Bacterial Concrete. *Proceeding of SPIE*., 2001; 168-76.
9. Stocks-Fischer, S., Galinat, J.K., Bang, S.S. Microbiological precipitation of $CaCO_3$. *Soil Biology & Biochemistry*., 1999; **31**: 1563-71.
10. Fujita, Y., Taylor, J., Gresham, T., Delwiche, M., Colwell, F., McLing, T., Petzke, L., Smith, R. Stimulation of microbial urea hydrolysis in groundwater to enhance calcite precipitation. *Environ. Sci. Tech*., 2008; **42**: 3025-32.
11. Qabany Al, A., Soga, K., Santamarina, J.C. Factors affecting efficiency of microbially induced calcite precipitation. *J. Geotech. Geoenviron. Eng*., 2012; **138**(8): 992–1001.
12. Burbank, M., Weaver, T., Green, T., Williams, B., Crawford, R. Precipitation of calcite by indigenous microorganisms to strengthen liquefiable *soil. Geomicrob. J.* 2011; **28**: 301-12.
13. Whiffin, V.S., van Paassen, L.A., Harkes, M.P. Microbial carbonate precipitation as a soil improvement technique. *Geomicrob. J*., 2007; **24**: 417-23.
14. DeJong, J.T., Fritzges, M.B., Nusslein, K. Microbially induced cementation to control sand response to undrained shear. *J. Geotech. Geoenviron. Eng*., 2006; **132**: 1381-92.
15. Ivanov, V., Chu, J. Applications of microorganisms to geotechnical engineering for bioclogging and biocementation of soil in situ. Rev. *Environ. Sci. Biotech*., 2008; **7**: 139-53.
16. Harkes, M.P., van Paassen, L.A., Booster, J.L., Whiffin, V.S., van Loosdrecht, M.C.M. Fixation and distribution of bacterial activity in sand to induce carbonate precipitation for ground reinforcement. *Ecolog. Eng.*, 2010; **36**: 112-7.
17. van Paassen, L., Ghose, R., van der Linden, T., van der Star, W., van Loosdrecht, M. Quantifying biomediated ground improvement by ureolysis: large-scale biogrout experiment. *J. Geotech. Geoenviron. Eng*., 2010; **136**: 1721-8.
18. Wijngaarden, W., Vermolen, F., Meurs, G., Vuik, C. Modelling biogrout: A new ground improvement method based on microbial-induced carbonate precipitation. Tran. *Porous.Media*., 2011; **87**: 397-420.
19. Wu, Y., AF, J., Spycher, N., Hubbard, S., Zhang, G., Williams, K., Taylor, J., Fujita, Y., Smith, R. Geophysical monitoring and reactive transport modeling of ureolytically-driven calcium carbonate precipitation. *Geochem. Trans*., 2011; **12(**1): 7.
20. Barkouki, T., Martinez, B., Mortensen, B., Weathers, T., De Jong, J., Ginn. T., Spycher. N., Smith. R., Fujita. Y. Forward and inverse bio-geochemical modeling of microbially induced calcite precipitation in half-meter column experiments. *Tran. Porous. Media*., 2011; **90**: 23-39.
21. Le, M.G., Castanier, S., Orial, G., Loubiere, J.F., Perthuisot, J.P. Applications of bacterial carbonatogenesis to the protection and regenerationof limestones in buildings and historic patrimony. *Sed. Geo*., 1999; **126**: 25-34.
22. Shen, J.Y., Cheng X.H. Laboratory investigation on restoration of Chinese ancient masonry buildings using microbial carbonate precipitation. In Proceedings of 1st international conference BGCE, TU Delft, Netherlands, 2008; 14-18.
23. Bang, S.S., Lippert, J.J., Yerra, U., Mulukutla, S., Ramakrishnan, V. Microbial Calcite, a Bio"Based Smart Nanomaterial in Concrete Remediation. *Int. J. Smart and Nano Mat.*, 2010; **1**: 28-39.
24. Qian, C.X., Wang, J.Y., Wang, R.X., Cheng, L. Corrosion protection of cement-based building materials by surface deposition of $CaCO_3$ by *Bacillus pasteurii. Mat. Sci. Eng*., 2009; **29**: 1273-80.
25. Tittelboom, K.V.. Belie a Nele De., Willem, M. De, Willy, V. Use of bacteria to repair cracks in concrete. Cem. Con. Res., 2010; 40: 157-66.
26. Yang, Z., Cheng, X.H., Li, M. Engineering properties of MICP-bonded sandstones used for historical masonry building restoration. Geo Frontiers 2011, ASCE, Dallas, Texas USA , 2011; **40**: 31-40.
27. Mobley, H. L., Island, M. D., Hausinger. R. P., Molecular Biology of Microbial Ureases. *Microbiol. Mol. Biol. Rev*., 1995; **59**(9): 3451-80.
28. Li, M., Guo, H.X., Cheng, X.H. Application of response surface methodology for carbonate precipitation production induced by a mutant strain of *Sporosarcina pasteurii.* Geo Frontiers 2011, ASCE, Dallas, *Texas USA*., 2011; 4079-88.
29. Li, M., Cheng, X.H., Guo, H. X. Heavy metal

removal by biomineralization of urease producing bacteria isolated from soil. Int. *Biodeter. Biodegr.*, 2013; **76**: 81-5.

30. Fujita, Y., Taylor, J.L., Wendt, L.M., Reed, D.W., Smith, R.W. Evaluating the potential of native Ureolytic microbes to remediate a ^{90}Sr contaminated environment. *Environ. Sci. Tech.*, 2010; **44**: 7652-8.

31. Yang, F.W., Zhang, B.J., Ma, Q.L., Study of sticky rice-lime mortar technology for the restoration of historical masonry construction. Acc. *Chem. Res*., 2010; **43**(6): 936–44.