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Running title: urease-producing bacteria from different climate zones

Halotolerant, alkaliphilic urease-producing bacteria from different climate zones and their application for biocementation of sand

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Abstract

Microbially induced calcium carbonate precipitation (MICP) is a phenomenon based on urease activity of halotolerant and alkaliphilic microorganisms that can be used for the soil bioclogging and biocementation in geotechnical engineering. However, enrichment cultures produced from indigenous soil bacteria cannot be used for large-scale MICP because their urease activity decreased with the rate about 5% per one generation. To ensure stability of urease activity in biocement, halotolerant and alkaliphilic strains of urease-producing bacteria for soil biocementation were isolated from either sandy soil or high salinity water of different climate zones. The strain VUK5, isolated from soil in Ukraine (continental climate), was phylogenetically closed with identity 99% of 16S rRNA gene sequence to the strain of *Bacillus* sp. VS1 isolated from sand in Singapore (tropical rainforest climate), as well as to the strains of B*acillus sp*. isolated by other researchers in Ghent, Belgium (maritime temperate climate) and Yogyakarta, Indonesia (tropical rainforest climate). Both strains *Bacillus* sp. VS1 and VUK5 had maximum specific growth rate of 0.09 h^{-1} and maximum urease activities of 6.2 and 8.8 mM of hydrolised urea/min, respectively. The halotolerant and alkaliphilic strain of urease-producing bacteria isolated from water of saline lake Dead Sea in Jordan was presented by Gram-positive cocci close to the species of *Staphylococcus succinus*. However, the strains of this species could be hemolytic and toxigenic ones, therefore only representatives of alkaliphilic *Bacillus sp*. were used for the biocementation studies.

Unconfined compressive strengths for dry biocemented sand samples after six batch treatments with strains VS1and VUK5 were 765 kPa and 845 kPa, respectively. The content of precipitated calcium and the strength of dry biocemented sand at permeability equals to 1% of initial value were 1.24% and 454 kPa, respectively, in case of biocementation by the strain VS1. So, indigenous halotolerant, alkaliphilic urease-producing bacteria isolated from different climate zones have similar properties and can be used for biocementation of soil.

Keywords: Biocementation; Climate zones; Urease-producing bacteria

Introduction

Cement has been the most commonly used as a construction material for the strengthening of soils in civil and environmental engineering applications. However, the production of cement is energy-consuming and environmentally unfriendly process because world production of cement requires about 5% of the global industrial energy consumption and contributes about 5% to global anthropogenic CO_2 emissions (Worrell et al. 2001). Also, in many cases cement cannot be used for the treatment of the fine soils because of high viscosity of its suspension. The chemical grouts that can replace cement for soil improvement are usually expensive and toxic for human and environment (DeJong et al. 2006; Ivanov and Chu 2008).

A new type of construction material, biocement, is developing extensively as an alternative to cement and chemical grouts (Bachmeier et al. 2002; Burbank et al. 2011; Chu et al. 2012a; DeJong 2010; De Muynck et al. 2010a; Ferris and Stehmeier 2002; Stabnikov et al. 2011; Whiffin et al. 2007). Application of most common type of biocementation, microbially inducing carbonate precipitation (MICP), was proposed for the strengthening of soil and for the treatment of the cracks in concrete (De Muynck et al. 2008a, b; Fernandes 2006; Jonkers et al. 2010; Ramachandran et al. 2001; Tittelboom et al. 2010). The application of microbial biotechnology to construction may also simplify some of the existing construction processes and revolutionize the ways that soils and wastes are treated.

MICP is the crystallization of calcite onto solid particle surface initiated by urease-producing bacteria (UPB) in the presence of urea and calcium ions:

$$(NH_2)_2CO + 2H_2O + Ca^{2+} + UPB \rightarrow 2NH_4^+ + CaCO_3 + UPB \qquad (1).$$
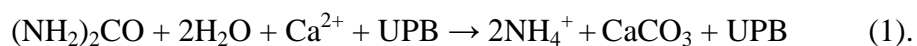
One of the major tasks for the development of biocement is selection of urease-producing bacteria. The most important criterion for selection of urease-producing strain is its ability to

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synthesize active urease. However, there are many pathogens among urease-producing bacteria. For example, active urease producers are *Helicobacter pylori* infecting the human stomach, and the opportunistic pathogens of human such as *Proteus vulgaris, Staphylococcus aureus*, and *Pseudomonas aeruginosa.* Meanwhile, it is known that active non-pathogenic producers of urease can be found among halotolerant and alkaliphilic Gram-positive, spore-forming bacteria such as *Sporosarcina pasteurii* (former *Bacillus pasteurii*), *Sporosarcina urea,* and some species of *Bacillus*. It was shown that *Sporosarcina pasteurii*, a common soil bacterium, can produce intracellular constitutive urease in quantity up to 1% of the cell dry weight (Bachmeier et al. 2002).

Urease-producing bacteria used for biocementation should be active in geotechnical environment with high salt concentration. Thus, halophilic or halotolerant, and alkalihilic urease-producing bacteria should be the preferable choice for the manufacturing of biocement. A microbial urease for biocementation must be active at high concentration of inorganic salt (e.g. $CaCl_2$) and pH above 8.5. The most popular strain of halotolerant and alkaliphilic urease-producing bacteria is *Sporosarcina pasteurii*, especially *S. pasteurii* ATCC 11859, which was used in the numerous studies of MICP (Bachmeier et al. 2002; Bang et al. 2001; DeJong et al. 2006; Ferris et al. 1996; Li et al. 2012; Mitchell and Ferris 2005; Mortensen and DeJong 2011; Whiffin et al. 2007). Urease activity of *S. pasteurii* ATCC 11859 after 24 hours of growth was 13.7 мM hydrolysed urea/min (Whiffin 2007).

Meanwhile, urease-producing bacteria are the common inhabitants of soil in places with regular supply of urea, which is the final product of nitrogen metabolism of mammals. Therefore, enrichment cultures of indigenous urease-producing soil bacteria were used for MICP (Burbank et al. 2012; Chu et al. 2011; Hammes et al. 2003).

The aim of the present study was comparative study of urease-producing enrichment cultures and strains isolated in different climate zones and their applications for biocementation of sand.

Materials and methods

Enrichment cultures of urease-producing bacteria

Liquid medium for preparation of enrichment culture of ureolytic, alkalophilic and halotolerant bacteria had the following composition: Tryptic Soya Broth DIFCO™, 30 g; urea, 20 g; NaCl, 100 g, $MnSO_4 \cdot H_2O$, 12 mg; $NiCl_2 \cdot 6H_2O$, 24 mg, phenol red, 10 mg/l, distilled water 1 l. Phenol red was used as a pH indicator: its color is yellow at the pH 6.8, but gradually changes to red/bright pink color at the pH above pH 8.2. All components of this medium, for exemption of urea, were sterilized at 121^oC for 15 minutes. Stock solution of urea, 100 g/l, was filter sterilized by filtration by using Whatman™ nitrocellulose membrane with 0.2 μm pores to avoid decay of urea during autoclaving. Two ml of the trace elements stock solution was added to 1 l of sterile medium. The trace elements stock solution consists of the following components: $ZnSO_4 \cdot 7H_2O$, 0.1 g; $MnSO_4 \cdot H_2O$, 0.085 g; H_3BO_3, 0.06 g; $CoCl_2 \cdot 6H_2O$, 0.02 g; $CuCl_2$, 0.004 g; $Na_2MoO_4 \cdot 2H_2O$, 0.04 g; $FeCl_2$, 0.3 g, deionized water, 1 l. The pH was adjusted to 2.0 using 1 N HCl.

This medium was inoculated with soil sample in dosage 10 g of soil per 0.5 L. Cultivation was on the shaker with 150 rpm at 30^oC for six days. For enrichment culture of aquatic halotolerant and alkaliphilic ureolytic microorganisms an aliquot of 200 ml of water from

Dead Sea was filtered through the Whatman™ nitrocellulose membrane with 0.2 μm pores and this filter was used as inoculum for 0.5 L of medium. The inoculated flask was placed on the shaker with 150 rpm at 30°C for 6 days.

Three geographic points were selected for production of enrichment cultures and isolation of pure cultures of halotolerant and alkaliphilic ureolytic microorganisms. The sample N1 was from tropical beach sand under coconut tree in Singapore; the sample N2 was taken from the garden sandy soil in Kiev, Ukraine; and the sample N3 was from water of Dead Sea in Jordan. Ten-fold dilutions of enrichment cultures were used to grow colonies at 30°C for 6 days in Petri dishes with liquid medium shown above and solidified by addition of 20 g Bacto Agar (Difco)/l. The strains were isolated from individual colonies grown in Petri dish.

Measurement of urease activity

Urease activity was defined as the amount of ammonium produced from 1 M solution of urea per minute. To measure it, 1 or 10 ml of bacterial suspension were added to 100 ml of 1 M urea solution. Concentration of ammonium produced from urea was determined using electric conductometer showing linear correlation ($R^2 = 0.9986$) between molar concentration of NH_4^+ (Y) and electric conductivity of solution (X) in mS/cm.

Identification of isolated urease-producing bacteria

The morphology of the bacterial cells was observed by scanning electron microscopy (SEM, Zeiss EV050, UK). Gram-staining was performed using Gram-stain kit (Difco Laboratories,

Detroit, MI, USA). The nearly full-length 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) with forward primer 27F and reverse primer Universal 1492R (Lane 1991). Purified PCR products were sequenced using the ABI PRISM 3730xlDNAsequencer and the ABI PRISM BigDye Terminator Cycle Sequencing ready-reaction kit. Primers 27F, 530F, 926F, 519R, 907R and 1492R were adopted to sequence both strands of the 16S rRNA gene. The sequences were finally assembled to produce the full-length sequence and the full-length sequence was compared with all other sequences available in the NCBI Genbank database using BLAST (http://blast.ncbi.nlm.nih.gov).

Physiological properties of selected UPB strains

Batch cultivation of selected strains was conducted in sterile medium in the B. Braun Biostat Fermentor (International Equipment Trading Ltd., Vernon Hills, Illinois, USA) with working volume 4.5 l at $30^{o}C$ under aeration rate 3 l/min for 72 hours. Palm oil was used as an antifoaming agent in quantity 0.2% (v/v). Optical density (OD), pH and urease activity were monitored. Optical density (OD) was measured using UV spectrophotometer at 600 nm and concentration of biomass was determined using calibration curve. The content of dry matter (total solids, TS) was determined by the standard method (American Public Health Association, APHA 2005). Specific growth rate was determined by equation:

$$\mu = (\ln X_1 - \ln X_0)/(t_1 - t_0) \qquad (2),$$

where X_1 and X_0 were concentrations of biomass at time t_1 and t_0, respectively.

Testing calcium precipitation

Composition of a reagent mixture for this experiment was as follows: 90 ml of 1 M urea; 90 ml of 0.5 M $CaCl_2$; 20 ml of cultural liquid of strain VS1 or strain VUK5. Calcium carbonate formation using cultural liquid of bacterial strains was measured by filtration and drying at 60^oC.

Measurement of calcium concentrations

Calcium concentration was determined using a standard method APHA 2340C with ethylene diaminetetraacetate (EDTA) titration (APHA1999). Liquid sample, 50 ml, was placed in a dry conical flask and 1 ml of buffer solution was added to maintain pH of 10.0 and few drops of Eriochrome Black T indicator were added as indicator. The sample was titrated with 0.01 M solution of EDTA until the color was changed from purple to blue.

Testing of urease activity in solution of $CaCl_2$

Final concentrations of $CaCl_2$ were 0.75 M (Stabnikov et al. 2011), 1.5 M and 2.5 M at final concentration of urea 1.5 M and addition of bacterial suspension of 100 ml/l. Initial pH was adjusted to 7 with 1 N HCl and change of pH in controls was monitored for 52 hours.

Biocementation of sand

A standard sand ASTM C778 was used for the experiments. This rounded grain silica sand had a mean grain size of 0.42 mm. The specific gravity was 2.65. Biocementation was performed using (a) suspension of biomass of *Bacillus* sp. VS1 or *Bacillus* sp. UA5 and (b)

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solution containing 82.5 g/l (0.75 M) calcium chloride and 90 g/l (1.5 M) of urea. These solutions were used for several treatments of 200 g of pure sand in a plastic column for 12 h with the drainage of the mixture from the column at the end of each treatment.

For sand biocementation 25 ml of bacterial suspension was injected from the bottom to the top of the 50 ml syringes filled with 50 ml of sand. The inner diameter of the syringe was 27 mm. Sand was incubated for 2 h with bacterial suspension for adsorption of cells and then the suspension was drained off by gravity. The sand was washed with 25 ml of distilled water to remove the remaining cultural liquid. After the water was drained out, 25 ml of solution, which contained 82.5 g/l (0.75 M) calcium chloride and 90 g/l (1.5 M) of urea, was injected from the bottom to the top of the syringes. After incubation for 24 h for crystallisation of calcite, the remaining solution was drained off by gravity. To increase strength of the treated sand, the biocementation solutions were injected repeatedly for five additional rounds. The bottoms of the plastic syringes were cut out at the end of treatment and the specimens were pushed out of the syringe carefully using the plunger. The wet sample was removed from the syringe for an unconfined compression test. The experiments were made in triplicate to check the repeatability and quantify the changes to the sand properties statistically.

Results

Urease activity in enrichment cultures

Growth of halotolerant and alkaliphilic urease-producing bacteria (UPB) in enrichment cultures from the soil samples was detected by color change of the medium from light orange

to crimson after 6 days of cultivation. Not all soil samples showed growth of enrichment cultures.

However, all enrichment cultures, even having high initial urease activity, showed its decrease with each culture transfer to fresh medium after every six days of cultivation. Urease activity of enrichment culture of indigenous bacteria could decrease 20 times after 5 transfers to fresh medium (Fig.1). Considering that one cycle of batch cultivation (Fig. 2) included about four generations, average calculated rate of elimination of urease activity was about 5% per one generation, so urease activity is unstable property of enrichment culture of urease-producing bacteria.

Therefore, even halotolerant and alkaliphilic enrichment cultures of bacteria are also not too suitable for industrial-scale MICP because of instability of urease activity. So, pure cultures of these bacteria were isolated, identified, produced, and biomass of these bacteria with known and stable urease activity was used for biocementation studies and applications.

Isolation and identification of strains of urease-producing bacteria

One biggest colony from each sample was selected as pure culture. The cells from one colony were grown in liquid medium in the tubes for two days and then urease activity for each sample was determined. Conductivities of 1 M urea solution for 30 min increased by 2.47 mS/cm, 3.11 mS/cm, and 3.62 mS/cm using strains of VS1, VUK5, and VDS3, respectively.

Urease-producing strain VS1 was presented by spore-forming, Gram-positive, rod-shaped cells. The determination of the nearest phylogenetic neighbour sequences for 16S rRNA gene sequences of the strain VS1by BLAST search program against known species in NCBI Genbank Database showed that strain VS1 was the representative of genus *Bacillus*

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close to strain *Bacillus* sp. CPB 2 (identity was 99%) isolated to be used for ureolytic microbial calcium carbonate precipitation in Ghent University, Belgium (Hammes et al. 2003). The partial nucleotide sequences were assembled to produce the full-length nucleotide sequence of 16S rRNA gene deposited in NCBI GenBank under accession number JF896459.

Cells of urease-producing strain VUK5 were spore-forming, Gram-positive, and rod-shaped. The determination of the nearest phylogenetic neighbor sequences for 16S rRNA gene sequences of the strain VUK5 showed that this strain was the representative of genus *Bacillus* close to strain *Bacillus* sp. VS1 (identity was 99%) (Chu et al. 2012b) isolated from Singapore sand and *Bacillus* sp. WB7 (identity was 99%) isolated from natural samples from Yogyakarta for bacterial carbonate precipitation (Lisdiyanti et al. 2011). The partial nucleotide sequences were assembled to produce the full-length nucleotide sequence of 16S rRNA gene deposited in NCBI GenBank under accession number KC464455.

Cells of urease-producing strain VDS3, isolated from water of Dead Sea in Jordan were Gram-positive cocci close to species *Staphylococcus succinus* The partial nucleotide sequences were assembled to produce the full-length nucleotide sequence of 16S rRNA gene deposited in NCBI GenBank under accession number KC464456. It is well known that some halotolerant species of genus *Staphylococcus* exhibited high urease activity (Jin et al. 2004; 1994; Christians et al. 1991). However, because the strains of *S. succinis* could be hemolytic and toxigenic ones (Zell et al. 2008) and were associated with some infectious diseases (Novakova et al. 2006; Taponen et al. 2008), only representatives of *Bacillus sp*. were used for biocementation studies.

Physiological properties of selected UPB strains

Strains VS1 and VUK5 were cultivated in medium in the Biostat Fermentor at 30°C under aeration for 72 hours to determine such physiological characteristics of strains as growth rate and specific urease activity. Their physiological characteristics were very similar (Fig.1). Final concentration of biomass and maximum urease activity in cultural liquid after 72 hours of cultivation was 8.4 g dry biomass/l and 6.2 mM hydrolised urea/min for strain VS1 and 10.4 dry biomass/l and 8.8 for mM hydrolised urea/min for strain VUK5. Maximum specific growth rate was 0.10 h^{-1} for period from 12 to 24 hours for strain VS1 and 0.09 h^{-1} for period from 24 to 36 hours for strain VUK5.

Testing of urease activity in the solutions with different concentration of CaCl$_2$

Changes of pH in the solutions with different concentration CaCl$_2$ are shown in Figure 2. Increase of pH indicated on urea hydrolysis due to activity of urease-producing bacteria. It was almost no differences in pH increase in solutions with concentration of CaCl$_2$ 0.75 M and 1.5 M for both strains. However, concentration of CaCl$_2$ 2.5 M inhibited activity of urease.

Changes of urease activity during storage

Urease activity of cultural liquids during storage at 4°C was stable for 8 days and then constantly decreased (Fig. 3). The loss of urease activity was 31% of initial values for both strains after 24 days of storage at 4°C. The similar results were shown for *S. pasteurii*: urease activity of bacterial cells remained stable over 25 days of storage at 4°C (Whiffin 2004). However, urease activity of cultural liquid of strains VS1 and VUK5 dropped very fast and

decrease of activity was around 50% if it was stored at room temperature without aeration for 3 hours.

Strength of biocemented sand

The maximum of unconfined compressive strength (UCS) of wet samples of biocemented sand after six batch treatments was 765 kPa and 845 kPa using strains VS1 or VUK5, respectively. For air dried samples, UCS was 1540 kPa and 1670 kPa for strains VS1 and VUK5, respectively. However, the strength of biocemented sand (Y) depends mainly on the content of precipitated calcium (C) (van der Ruyt and van der Zon 2009; Whiffin et al. 2007). So, the strength must be shown for some defined conditions.

The rate of biocementation will negatively correlate with sand permeability because clogging of the sand pores will diminish flow rate through sand (F) and the supply rate of the reagents for biocementation. These processes could be described as follows:

$$(dY/dt) = k_1 F = k_2 F_0 /C \quad (4),$$

so the rate of its biocementation became low at some content of precipitated calcium in sand. Conventionally, this content of calcium and related strength of biocemented sand could be determined for permeability equals to 1% of initial value. Real experimental data for the strain VS1 (Fig. 4) showed that F and Y can be described by the linear functions:

$$F = 507 - 403 \, C \text{ ms}^{-1} \quad (R^2 = 098) \quad (5), \text{ and}$$

$$Y = 366 \, C \text{ kPa} \quad (R^2 = 0.98) \quad (6).$$

So, the content of precipitated calcium ($C_{0.01}$) and the strength of biocemented sand ($Y_{0.01}$) at permeability equals to 1% of initial value were as follows: $C_{0.01} = (507 - 5.1)/403 = 1.25\%$ and $Y_{0.01} = 366 \times 1.24 = 454$ kPa. Therefore, the strength at defined content of precipitated

calcium or at known value of permeability should be used for the characterization of biocemented samples or bacterial strains used for biocementation.

Discussion

Constitutive production of urease is a common feature of many species of soil bacteria (Burbank et al., 2012). However, the use of indigenous urease-producing bacteria (UPB) for MICP (Burbank et al., 2011) could be limited by the following factors: (1) low concentration of urease-producing bacteria in deep layers of soil and there absence in the rocks; (2) not sufficient urease activity at high concentration of salt or pH above 8.5; (3) potential pathogenicity; (4) not predictable and not stable bioactivity during MICP. Therefore, introduction of enrichment or pure cultures of UPB could be more reliable and effective way for MICP than use of indigenous urease-producing bacteria.

Therefore, pure cultures of UPB were isolated from different climate zones. The halophilic and alkalophilic strains isolated from soil in Ukraine and Singapore were representatives of *Bacillus sp*. showing 99% phylogenetic similarity by 16S rRNA gene sequence between themselves and between the strains isolated in Belgium (Hammes et al. 2003) and Indonesia (Lisdiyanti et al. 2011) to be used for ureolytic microbial calcium carbonate precipitation. So, the source of origin does not affect the results of isolation and efficiency of MICP.

Urease activities of isolated strains were in the range from 6.2 to 8.8 mM hydrolised urea/min. Urease activities for other bacterial strains used for biocementation were also in the similar ranges from 5 to 20 mM of hydrolised urea/min for *Sporosarcina pasteurii* DSMZ 33 (Harkes et al. 2010); 2.2 to 13.3 mM hydrolised urea/min for *Sporosarcina pasteurii* ATCC 11859 (Whiffin 2004); and more than 3.3 mM hydrolised urea/min for three *Bacillus* strains

isolated from Australian soil and sludge (Al - Thawadi and Cord-Ruwisch 2011). It is known that urease activity should not be very high for successful biocementation. For instance, it was shown that stronger aggregates of calcium carbonate were formed at low rate of urea hydrolysis (Qian et al. 2009). Urease activity in the range from 4.4 to 9.5 mM hydrolised urea/min ensured strength of biocemented sand 32 – 35 MPa, however, it became lower at urease activity higher than 9.5 mM hydrolised urea/min (Whiffin 2004).

The maximum of unconfined compressive strength (UCS) of wet samples of sand biocemented with the isolated strains was in the range from 765 kP to 845 kPa. In comparison, the strength of the wet biocemented sand column was 570 kPa (Whiffin et al. 2007). However, the strength of biocemented sand depends on the content of precipitated calcium (van der Ruyt and van der Zon 2009; Whiffin et al. 2007), therefore it was proposed in this paper to compare strength of different samples at some defined content of calcium, for example 1.25% (w/w), or for some defined permeability value, for example for the samples which permeability equal to 1% of initial value. The comparisons of the sample properties at defined conditions will permit compare the strains and conditions for MICP.

Conclusions

Comparative study of urease-producing strains of bacteria for biocementation isolated from sandy soil in different climate zones showed their phylogenetic, morphological and physiological similarity. The strain *Bacillius* sp. VS1, isolated from sand of Singapore, had maximum specific growth rate 0.10 h^{-1} and urease activity 6.2 mM hydrolised urea/min and the strain *Bacillius* sp. VUK5, isolated from sandy soil in Ukraine, had maximum specific growth rate 0.09 h^{-1} and urease activity 8.8 mM hydrolised urea/min. Unconfined

compressive strengths for the sand samples biocemented using strains VS1or VUK5 were 765 kPa and 845 kPa, respectively. The content of precipitated calcium and the strength of dry biocemented sand at permeability equals to 1% of initial value were 1.24% and 454 kPa, respectively, in case of biocementation by the strain VS1.

Acknowledgement

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Figure legends

Fig. 1. Change of urease activity after transfers to fresh medium.

Fig. 2. Dynamics of parameters in batch culture of strains VS1 (A) and VUK5 (B). Data on X axis show biomass concentration, g of biomass/l (curve 1), total urease activity, mM/min (curve 2) and specific urease activity, mM/g of biomass/min (curve 3).

Fig. 3. Change of pH in media with concentrations of $CaCl_2$ 0.5 M, 1.5 M, and 2.5 M for strains *Bacillus* sp. VS1 (A) and *Bacillus* sp. VUK5 (B).

Fig. 4. Urease activity in cultural liquid of strains VS1 and VUK5 during their storage at 4^oC.

Fig. 5. Effect of precipitated calcium content on permeability of wet samples and unconfined compressed strength of dry samples of biocemented sand.