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BIOCEMENTATION OF SRI LANKAN BEACH SAND USING LOCALLY ISOLATED BACTERIA: A BASELINE STUDY ON THE EFFECT OF SEGREGATED CULTURE MEDIA

*Pahala Ge Nishadi Nayanthara[1], Anjula Buddhika Nayomi Dassanayake[2], Kazunori Nakashima[3] and Satoru Kawasaki[3]

[1]Graduate School of Engineering, Hokkaido University, Japan; [2]Faculty of Engineering, University of Moratuwa, Sri Lanka; [3]Faculty of Engineering, Hokkaido University, Japan

*Corresponding Author, Received 10 March 2019, Revised 08 April 2019, Accepted: 25 April 2019

ABSTRACT: Bioengineering has paved the way into multidiscipline as a more sustainable and eco-friendly solution to numerous problems. Among them, microbial induced carbonate precipitation (MICP) is a newly emerging microbiological soil/sand stabilization method, where microbial urease is employed for calcium carbonate precipitation throughout the soil matrix by urea hydrolysis which leads to soil strength and stiffness enhancement. This paper investigates avenues for strengthening loose beach sand from Sri Lanka by MICP through a series of laboratory experiments. A bacterial strain belonging to *Sporosarcina* sp., a native ureolytic bacterium was isolated from Sri Lankan soil, and urease activity and induction of calcium carbonate precipitation were quantified. The isolated bacteria showed a stable growth over the test period of 7 days and a maximum urease activity of 2.1 µmol of hydrolyzed urea/min.mL after 48 hours of cultivation. A major part of the study was focused on determining the effect of centrifuged cell pellets resuspended in distilled water and fresh culture medium (NH_4-YE medium) on the effectiveness of MICP. The small-scale column solidification tests revealed that although urease activity is considerably higher for cell pellets suspended in distilled water, the strength improvement on column top by intact cell culture injection is about 5 times higher than that from cell pellets. Further, different size and types of calcium carbonate crystals were examined in MICP tests done for intact culture liquid and centrifuged cell suspensions. However, detailed analysis of biocementation by the selected isolate is highly recommended as future work.

Keywords: Biocementation, Centrifuged cell pellets, Microbial induced carbonate precipitation, Native ureolytic bacterium, Urease activity

1. INTRODUCTION

Bio-mediated soil improvement is one of the research areas that have drawn much attention in recent years in the field of geotechnical engineering for its sustainability. Among them, biocementation by microbial induced carbonate precipitation (MICP) is considered the most common approach which has proved its viability for reducing the liquefaction potential of soil, enhancing soil stability, improving the soil strength and stiffness and high potential for self-healing [1-5].

Biocementation is achieved by hydrolysis of urea by microbial urease which causes calcium carbonate to precipitate in the presence of a calcium source. Loose sand grains are then bound together by the resulting calcium carbonate crystals thereby improving mechanical properties of the soil. The secretion and execution of urease enzyme that degrades urea control the whole mechanism. The overall process can be explained by the following equations [6].

$$\mathbf{CO(NH_2)_2 + H_2O \rightarrow 2NH_3 + CO_2} \quad (1)$$

$$\mathbf{2NH_3 + 2H_2O \rightarrow 2NH_4^+ + 2OH^-} \quad (2)$$

$$\mathbf{CO_2 + H_2O \rightarrow HCO_3^- + H^+} \quad (3)$$

$$\mathbf{HCO_3^- + 2OH^- \rightarrow CO_3^{2-} + 2H_2O} \quad (4)$$

$$\mathbf{Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3\downarrow} \quad (5)$$

There are extensive literatures on various microorganisms capable of producing urease enzyme and showing the ability to be utilized for MICP applications [7]. However, bacteria must produce urease constitutively or inducibly and should be non-pathogenic to be used for MICP successfully [8].

Sporosarcina sp. (formerly known as *Bacillus)* is identified as an ideal strain to catalyze the hydrolysis of urea because of its high biosafety level and high urease activity [9,10]. As per literature, members of *Sporosarcina* genus have been isolated from a wide variety of sources ranging from different types of soils, water, and ocean to human blood samples and soy sauce [11].

The high dependency of MICP on the microbial growth makes it necessary to optimize the process to

provide most suitable conditions for the bacteria in action because each bacterium has unique conditions at which urease activity will be maximum; physical parameters such as temperature and pH greatly influence the bacterial growth [8]. On the other hand, using local bacteria for cementation applications in the real field can provide a more suitable environment for the process while minimizing adverse effects associated with introducing foreign bacteria to the soil in concern.

During a study focused on finding urease producing bacteria capable of MICP process, a strain belonging to *Sporosarcina* genus was isolated from a soil sample collected from the tropical climate in Sri Lanka. The main aim of the research study presented by this paper was to evaluate the feasibility for biomediated sand cementation using the strain isolated to be applied in loose beach sand immobilization applications in Sri Lanka.

However, special concern was taken to determine the effect of segregated cell pellets on microbial induced carbonate precipitation process since it is an area untouched in many types of research related to MICP. As a preliminary investigation, the aforementioned objectives were achieved by conducting a series of laboratory experiments. Therefore, main aims of this research study can be stated as (i) isolation and identification of a native ureolytic bacterium (ii) segregation of cell pellets and comparatively quantifying the microbial growth and urease activity and (iii) evaluating their role in MICP using calcium carbonate precipitation tests and solidification tests.

2. MATERIALS AND METHODS

2.1 Soil Sampling and Isolation of Ureolytic Bacteria

About 50 g of soil was collected from a public park in Sri Lanka (06° 54' 43.2" N, 79° 51' 36.3" E) to a sterile test tube, imported to Japan and stored at 4°C. The soil sample was then serially diluted (10^1 - 10^6 times) and plated on NH_4-YE agar medium (15.75 g/L Tris buffer, 10.0 g/L NH_4SO_4, 20.0 g/L agar, 20.0 g/L yeast extract). After incubating at 30°C for 2 days, colonies were identified from a plate with 30-200 colonies. Different types of colonies were selected and separately cultivated on the plates prepared in the same manner described above.

A simple urease activity test was then conducted for qualitative assessment of urease producing bacteria. Each colony was mixed with 20 mL of cresol red solution containing urea and incubated at 45°C for two hours. Colonies that changed the initial yellow color to pink (Cresol red changes from yellow to pink when pH changes to 7.2-8.8 which is accomplished during urea hydrolysis) were identified as urease producing bacteria. Among the four ureolytic bacteria identified in this manner, the one showing the highest pH increase was selected for further analysis.

2.2 Identification of Bacteria

16S rDNA Gene Amplification and sequencing was carried out for the isolate. The analysis of the DNA sequences was performed by using the DB-BA 12.0 (TechnoSuruga Laboratory) and International Nucleotide Sequence Database (DDBJ / ENA (EMBL) / GenBank) by TechnoSuruga Laboratory, Japan.

2.3 Segregation of Cell Pellets

NH_4-YE medium (15.75 g/L Tris buffer, 10.0 g/L NH_4SO_4, 20.0 g/L yeast extract prepared with distilled water) was used for culturing the bacteria under sterile aerobic conditions. The cells were precultured in 5 mL medium at 25°C and 160 rpm for 24 hours. One mL of the preculture was inoculated with 100 mL of the fresh medium and incubated under the same conditions.

One of the main aims of this study is to compare the urea-hydrolyzing capability of the selected bacterium when intact culture is used against when concentrated cell pellets are used. In order to achieve this, cell pellets were segregated by centrifugation of the bacterial culture at 10°C and 8000 rpm for 5 mins. The supernatant was removed along with dissolved culture constituents and residues, cell pellets were washed and once again dispersed to original OD_{600} value using distilled water for one test case and using NH_4-YE medium for another test case. The schematic diagram for the process of separating cell pellets and supernatant is given below (Fig.1).

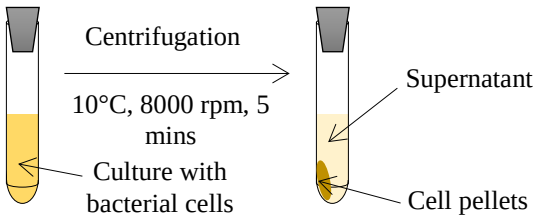

Fig.1 The schematic diagram for the process of segregating cell pellets.

2.4 Measurement of Microbial Cell Growth and Urease Activity

Microbial cell concentration was determined in terms of optical density at a wave length of 600 nm (OD_{600}) using a UV-VIS spectrophotometer (V-730, JASCO Corporation, Tokyo, Japan) at regular

intervals of 24 hours for representative samples collected from the culture medium.

Further, urease activity of the bacterial cells was evaluated by cells suspended in a solution containing urea in terms of the amount of enzyme required to hydrolyze one micromole of urea per minute (U) per milliliter following the method explained by Fujita et al. [12]. The test was repeated at regular intervals of 24 hours to determine the temporal stability of the urease enzyme.

When the bacterial growth was at optimum, cell pellets were separated, resuspended in distilled water and NH_4-YE medium as aforementioned and similar urease activity tests were conducted separately. Activity in the supernatant was also detected as it is a measure of how much enzyme is secreted into the substrate.

2.5 The Effect of Segregated Cell Pellets on Calcium Carbonate Precipitation

In order to distinguish the precipitation capacity of the bacterial strain when intact cell culture is directly used against when cell pellets are used separately, 3 different cases (Case 1, Case 2 and Case 3) were implemented as shown in Table 1.

Table 1 Experimental conditions for the MICP tests.

Case No	Bacteria culture	OD_{600}
1	Intact cell culture with whole cells	0.2
2	Cell pellets resuspended in NH_4-YE medium	0.2
3	Cell pellets resuspended in distilled water	0.2

Microbial induced calcium carbonate precipitation tests were conducted in 10 mL tubes for the above 3 cases keeping all other conditions similar. Calcium chloride reagent was used as the calcium source and the concentrations of $CaCl_2$ and urea in the reaction mixtures were maintained at 0.5 M. Three replicates were tested for each case.

The total volume of all the samples was adjusted to 10 mL with distilled water and kept in a shaking incubator at 25°C and 160 rpm for 48 hours. The supernatant was then filtered through a filter paper and filter papers and tubes with the precipitates were ovens dried at 105°C for 24 hours to measure the dry weights of the precipitates. In order to quantitatively assess the precipitation efficiency, the precipitation ratio (the ratio between the actual mass of $CaCO_3$ precipitated and theoretical mass of maximum $CaCO_3$ to be precipitated) was calculated for each test case. Precipitated specimens were also analyzed using the SEM to evaluate the morphology of the resulting calcium carbonate crystals.

2.6 The Effect of Segregated Cell Pellets on Sand Solidification

Small scale sand solidification columns were prepared in syringes of 30 mL in volume and 25 mm in diameter. Fifty grams of Sri Lankan beach sand was compacted into the syringe in 3 layers after oven drying at 105°C for 48 hours. The grain size distribution curve of the beach sand used (specific gravity 2.8) in this study is shown in Fig.2 below.

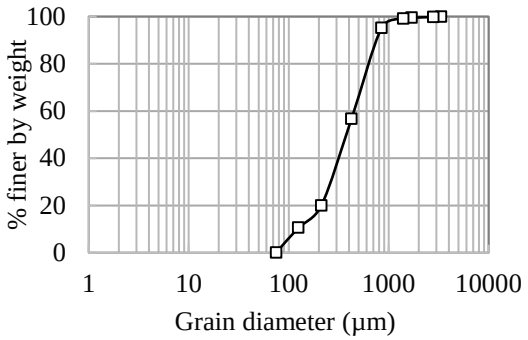

Fig.2 Grain size distribution curve of Sri Lankan beach sand.

Although many sand treatment processes for MICP applications have been introduced so far, cycles of batch treatment were used in this study. Sixteen milliliters of bacteria culture medium was injected from the top, excess solutions were drained out at a controlled rate and allowed for bacteria fixation. 20 mL of 1.0 M Ca^{2+} cementation solution (110.98 g/L $CaCl_2$, 60.06 g/L urea, 3 g/L nutrient broth, 10 g/L NH_4Cl and 2.12 g/L $NaHCO_3$) was then followed maintaining a fully saturated condition over the entire length of the sand column. The culture solution was injected twice on day 1 and day 7 whereas cementation solution was injected every 24 hours for the total curing period of 14 days. The two different test conditions maintained are briefly summarized in Table 2.

Table 2 Experimental conditions for sand solidification tests.

Case No	Bacteria culture	OD_{600}
1	Intact cell culture with whole cells	2.5
2	Cell pellets resuspended in distilled water	2.5

The Unconfined Compressive Strength (UCS) of the treated samples was tested to quantify the effect of segregated cell pellets on sand solidification. A needle penetration device (SH-70, Maruto Testing

Machine Company, Tokyo, Japan) was used to measure the penetration gradient in N/mm and calculate UCS in MPa using regression analysis. As a final step, oven dried solidified samples were observed under SEM to study the soil matrix biocemented with calcium carbonate.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Bacteria

The isolate was identified as a strain of *Sporosarcina* sp. with 99.7% sequence similarity.

3.2 Microbial Growth and Urease Activity

The microbial cell concentration of the bacterium and corresponding urease activity for a period of 7 days are shown in Fig.3. According to the results, cell growth increases when transferred to the fresh medium up to a maximum within 72 hours and remains almost constant for next 72 hours followed by a gradual fall due to cell lysis and depletion of nutrients.

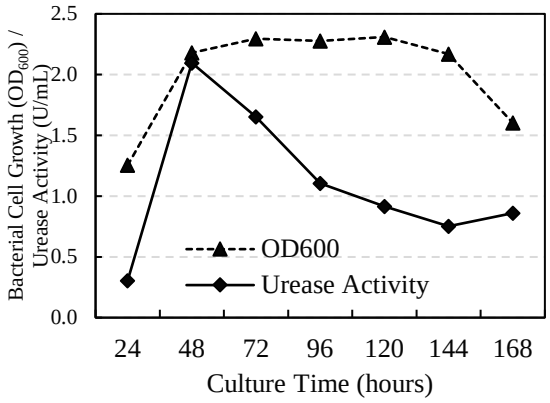

Fig.3 Bacterial cell growth and urease activity of *Sporosarcina* sp. isolated from Sri Lankan soil at 25°C.

The urease activity measurements for the same test period indicate that the highest activity is achieved within the first 48-72 hours after cultivation. However, as cell lysis and denaturation of the loose enzyme take place as described by van Passen [13], the activity of the enzyme substantially decays over time. Overall, the selected bacterium seems to maintain relatively stable growth and satisfactory enzyme stability over a period of 7 days at a temperature of 25°C.

3.3 Urease Activity of the Segregated Cell Pellets

As a major part of this work, segregation analysis was completed by separating cell pellets and extracting them with distilled water and NH_4-YE medium separately and determining their urease activities (Fig.4). The supernatant with negligible OD_{600} value compared to other cases showed no urease activity indicating that no or very lower amount of urease enzyme is released into the substrate. Similar kind of urease activity has been recorded in the literature for other ureolytic bacteria whose urease enzyme is found to be accumulated in/on the cell membrane [12,14].

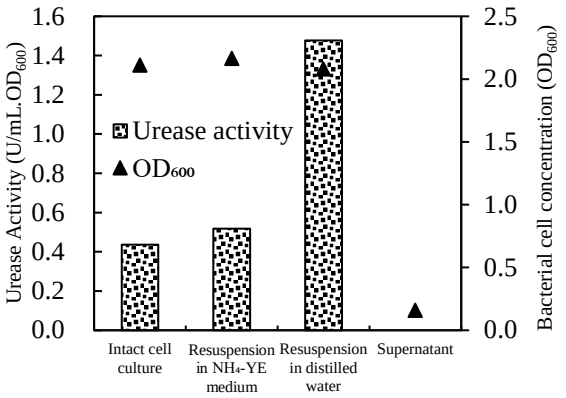

Fig.4 Comparison of urease activity

For the same OD_{600} value, the cell pellets resuspended in distilled water showed 3 fold increment in activity than that from intact cell culture. In contrast, the cell pellets resuspended in fresh NH_4-YE medium indicated only a slight increase in activity. Although the reason for this behavior is not yet fully understood, there are certain hypotheses that can be put forwarded.

Firstly, in a similar kind of research, it is stated that the presence of protease activity in the non-centrifuged culture solution and the absence of the same in the cell pellet resuspensions cause the difference in measured activity as urease is decayed by proteolysis in the presence of protease [15]. Although this hypothesis can be questioned on the fact that cell itself contains protease in whatever suspension, it is acceptable that differences in protease activity in different bacteria suspensions can have effects at varying degrees. Secondly, another report documents that it is unlikely to detect all the urease activity associated with a microorganism from intact cell culture with whole cells, especially when urease is localized as a cytoplasmic enzyme which is the common case for microbial ureases [16]. The hypothesis is that the centrifugation, washing and resuspension process may allow higher passage of intracellularly generated urease across the cytoplasmic membrane resulting in higher levels of urease activity. Although not studied extensively, osmotic shock due to centrifugation and resuspension may cause the release of the enzyme to the solution from cells and have an effect on the activity of urease in intact cell

culture and pellets suspended in distilled water at varying levels [17]. However, none of these reasons can be used to fully explain the considerable difference in urease activities between cell pellets resuspended in distilled water and fresh NH_4-YE medium. Therefore, in addition to the aforementioned possibilities, there are some other unidentified factors that cause urease instability in non-centrifuged culture solution and cell pellets resuspended in NH_4-YE medium.

3.4 Calcium Carbonate Precipitation by *Sporosarcina* sp.

Precipitation tests were done for 3 different cases (Refer to Table 1 for the 3 cases studied). The weight of the precipitate formed for each case is shown in Fig.5 below.

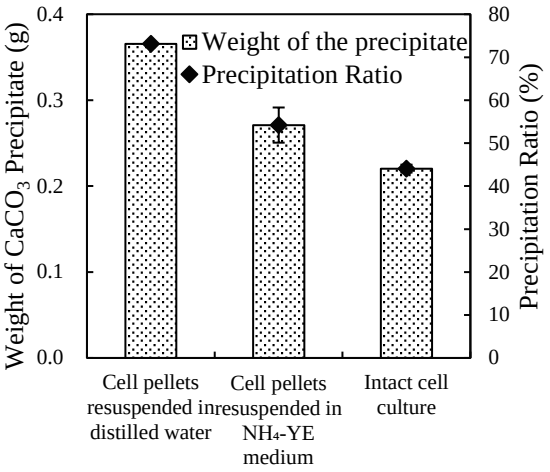

Fig.5 Comparison of $CaCO_3$ precipitation under varying experimental conditions.

The amount of $CaCO_3$ precipitate formed when cell pellets with distilled water resuspension were significantly higher than the other two cases where intact cell culture or the pellets with NH_4-YE resuspension were used for the same OD_{600} value. The resuspension of centrifuged pellets in distilled water gave a precipitation ratio as high as 73% whereas it is only 44% for the intact cell culture. This observation can be directly attributed to the fact that higher urea hydrolyzing capability leads to better precipitation efficiency.

3.5 Morphology of $CaCO_3$ Crystals

When morphology of the $CaCO_3$ crystals precipitated by *Sporosarcina* sp. isolated from Sri Lankan soil was imaged using the SEM, few variations were identified. For the case where total cell culture was used, irregular agglomerations were dominant with the presence of randomly distributed hemispherical crystals (possibly vaterite) (Fig.6). The presence of agglomerations is mostly the effect of nutrient constituents and residues present in the culture medium.

The $CaCO_3$ crystals observed when cell pellets resuspended with NH_4-YE medium was used primarily consisted of larger spherical crystals (possibly vaterite), some randomly covered with irregular agglomerations (Fig.7).

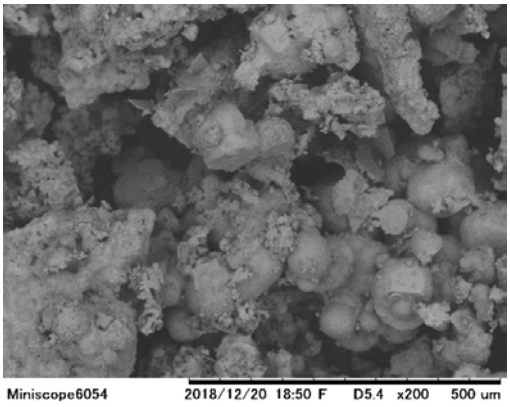

Fig.6 SEM image of $CaCO_3$ crystals formed when intact cell culture was used.

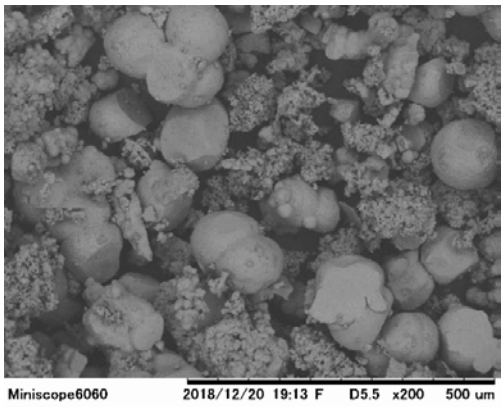

Fig.7 SEM image of $CaCO_3$ crystals formed when cell pellets resuspended with NH_4-YE medium was used.

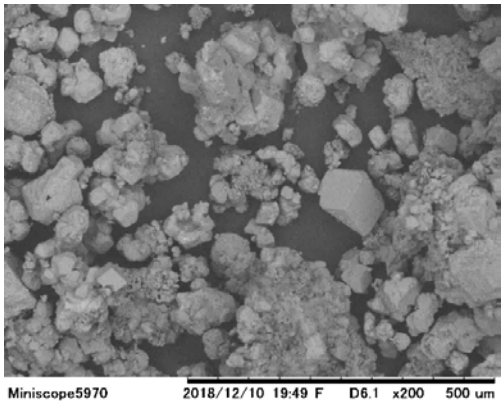

Fig.8 SEM image of $CaCO_3$ crystals formed when

cell pellets resuspended in distilled water was used.

However, well developed rhombohedral crystals (possibly calcite) were observed for the test case in which cell pellets resuspended in distilled water were utilized; as a result of minimum effect from other dissolved nutrient constituents in the mixture (Fig.8).

It is well known that bio-precipitation of carbonates is a complex process giving rise to unusual morphological properties [18]. There are many factors that affect the variations in type, size and morphology of the calcium carbonate crystals precipitated [19]. The phase formation, growth and shape of the crystals are affected by factors like rate of urea hydrolysis [20], effect from other enzymes such as carbonic anhydrase [21], effect of amino acids such as glutamic and aspartic acids [18] and [21], urea and calcium concentrations [22], differences in saturation condition of the system [23] and more importantly differences in the functional attributes of the responsible microorganism [24]. Apart from those, the composition and dosage of extracellular polymeric substance (EPS) in the reaction mixture can have a significant impact on crystal properties as described in previous studies [25]. Some of these factors independently or in combination may have resulted in various calcium carbonate crystal morphologies observed in this study.

3.6 Sand Solidification Tests

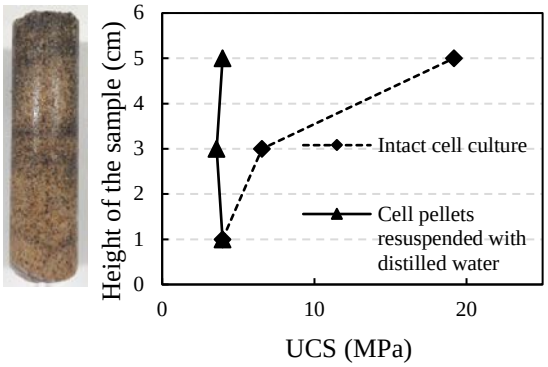

Fig.9 UCS of the solidified sand columns

Small scale tests were carried out under two test conditions to evaluate the effect of cell pellets for loose beach sand solidification by MICP. One sand column was treated with intact cell culture and another with cell pellets resuspended in distilled water keeping all other factors constant. The UCS values as derived from needle penetration test indicate a significant enhancement of strength in both cases over the entire length of the sample (Fig.9). Irrespective of the lower urease activity observed, the strength enhancement in the top and middle parts of the sample treated with total cell culture showed considerable improvement from that of the sample treated with cell pellets (19.2 and 4.0 MPa for top and 6.6 and 3.6 MPa for middle respectively).

One major factor that influences the efficiency of MICP process is how well urease enzyme or the cells of urease producing bacteria is adsorbed on to the sand grains [15]. The adsorption of bacterial cells is primarily controlled by the size, surface properties of the cells and sand grains (surface charge and hydrophobicity) along with the concentration of ions in the extracellular environment [15]. It is possible that the adsorption properties of the cells might have changed after centrifugation, washing and resuspension steps and had an effect on the sand solidification. From the results, it is revealed that bacterial cells have been well adsorbed onto the sand grains and filtered out along the sand column when the intact culture was injected thereby facilitating better calcium carbonate bridge formation between the grain contacts. However, the column strength improvement shows a gradual decline from top to bottom obviously due to higher accumulation of bacterial cells on top and depletion of reactant concentration as they are been readily consumed for biocementation [13].

For the column treated with cell pellets, it seems adsorption of cells on sand grains has not been efficient thus allowing cells to be flushed out along with the cementation solution. However, that has eventually caused lesser pore filling and clogging of voids by calcium carbonate cement on top resulting in a more homogeneous vertical strength enhancement.

The imaging of solidified samples by SEM further helped to confirm this phenomenon. The SEM images of the sample treated with intact culture liquid clearly showed calcium carbonate bridge formation at grain contacts responsible for strength gain (Fig.10) whereas sample treated with cell pellets only caused the surface coating of grains by cement (Fig.11). However, in both cases, well developed rhombohedral calcium carbonate crystals (possibly calcite) were observed.

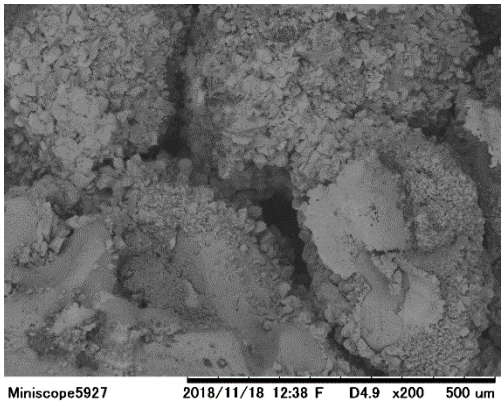

Fig.10 SEM image of the sample treated with intact

cell culture.

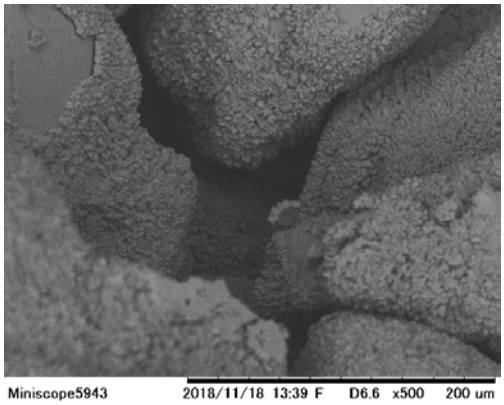

Fig.11 SEM image of the sample treated with cell pellets resuspended in distilled water.

Although this study was aimed at evaluating the effect of employing centrifuged bacterial cells on sand solidification by MICP and satisfactory strength improvement was achieved, it is still debatable how certain associated microbial processes are taking place. Hence, extensive research work on this matter is encouraged for future practical applications.

4. CONCLUSIONS

The main objective of the research presented in this paper was to study the effect of utilizing bacterial cell pellets obtained after centrifugation against intact cell culture containing whole cells for solidification of loose Sri Lankan beach sand by MICP. For this, an indigenous ureolytic bacterium belonging to *Sporosarcina* sp. was isolated and cultured in NH_4-YE medium. The initial laboratory tests revealed that the isolated bacterium has stable growth and urease activity for a period of 7 days.

The urease activity tests conducted for intact cell culture, cell pellets resuspended in NH_4-YE medium and distilled water showed that activity is significantly higher when cell pellets are resuspended in distilled water, although the biological and/or physicochemical mechanisms responsible for the processes are not yet fully understood. MICP tests carried out for same test conditions mentioned above resulted in different types of calcium carbonate morphologies when observed using SEM images which is a result of various factors that decide the size, shape and type of the crystals. Two numbers of small-scale sand solidification tests were done following a methodology based on two-phase injection procedure. Irrespective of the lower urease activity measured, the compressive strength of the top of the sand column injected with intact cell culture was approximately 5 times higher than that treated with cell pellets suspended in distilled water. However, latter resulted in a more homogeneous strength improvement over the entire height of the treated sample. Overall, it is confirmed that the sand solidification by the isolated bacterium results in satisfactory strength enhancement irrespective of whether intact cell culture or cell pellets suspended in distilled water are used. However, further research work is highly recommended to fully understand the bio-precipitation and subsequent strength improvement processes in each scenario.

5. ACKNOWLEDGMENTS

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