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IMPROVEMENT OF USING CRUDE EXTRACT UREASE FROM WATERMELON SEEDS FOR BIOCEMENTATION TECHNOLOGY

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ABSTRACT: In recent years, the formation of artificial beachrock and bio-cementation method has gained considerable attention as a sustainable alternative tool in the area of geotechnical and geo-environmental engineering field for soil improvement and construction materials. In general, earlier methods of soil improvement were mostly concentrated on microbes (Bacteria, Fungi, etc.) as a source of urease enzyme widely known as MICP method (Microbial Induced Carbonate Precipitation). To address some of the key limitations of MICP method this study focused on using crude enzyme (low cost, eco-friendly). Crude enzyme was extracted from watermelon seeds (*Citrullus lanatus)* considered as "food waste material" and the carbonate formation process known as EICP "Enzyme Induced Carbonate Precipitation." Crushed and blended watermelon seeds (both dry and germinated) used as a source of urease enzyme. Subsequently, their urease activity was also investigated with various environmental parameters (Temperature, pH, etc.) and investigated the carbonate precipitation trend using calcium chloride ($CaCl_2$) and urea [$(CO(NH_2)_2$]. The form of carbonate (calcite, aragonite, vaterite, etc.) was also confirmed by XRD and SEM-EDX analysis. Finally, syringe (d = 2.3 cm, h = 7.1 cm) sand solidification test was conducted using commercially available "Mikawa sand" (mean diameter, D_{50} = 870 mm) and successfully achieved unconfined compressive strength (UCS) of about 1.2 MPa at neutral pH (~7) and temperature condition (30 °C) considering various curing days and conditions. This study could be useful as an eco-friendly and sustainable method for numerous bio-geotechnical applications (for instance, ground improvement, liquefaction mitigation, artificial beach rock formations, coastal erosion protection, etc.) and the extracted crude urease from watermelon seeds could play as an alternative to replace commercially available urease for carbonate precipitation.

Keywords: EICP, Watermelon seeds, Biocementation, Urease enzyme, Crude extract, Artificial beachrock

1. INTRODUCTION

Recently bio-inspired techniques have been widely used for different geotechnical applications. The maintenance, management of all of these methods were expensive and not eco-friendly [1, 2, 3]. Therefore, a new alternative countermeasure was needed. One of the emerging alternative approach known as "Microbial Induced Carbonate Precipitation-MICP method," learned from "natural beachrock." A beach rock consisted of marine sediments that have been cemented in the intertidal layer mostly by $CaCO_3$, and its deposition time was much shorter than that of other sedimentary rocks. The cemented portion of beachrock primarily made up of both calcium carbonate and silica.

Several researchers have studied the beachrock formation mechanisms [3, 4]. However, some of the difficulties related to the current MICP method, have created requirements for alternative demand of solutions considering economic and ecological sustainability. For instance, the screening, isolation transportation, filtering, physical non-homogeneity, oxygen requirement, urease activity, viscosity, deep penetration, microorganism strength, etc. marked this approach a complicated process. It challenged the use of MICP method in real field applications. Likewise, researchers around the world have tried to overcome some of these leading limitations, but yet, some unresolved issues related to MICP persisted. As an alternative technology, EICP (Enzyme Induced Carbonate Precipitation) has drawn considerable interest to the researcher for investigating the feasibility of using this method from laboratory scale to real field application.

The demand for using the EICP process may be fulfilled with commercially available urease enzyme, which is very costly because it has been manufactured for use in some specific applications [5] in small amounts at high purity levels [6,7].

Therefore, it was essential to find an alternative source of urease enzyme to optimize the benefits through bio-inspired engineering applications and making artificial beachrock considering cost, environmental safety, and sustainability. This study focused on using crude urease extracted from

watermelon (*Citrullus lanatus*) seeds as the source of urease enzyme considering waste utilization, cost-effectiveness, availability of the material, and environmental safety.
During the $CaCO_3$ precipitation, the following reaction occurred in the presence of urea, urease enzyme, and calcium chloride, Eq. (1-5).

$$CO(NH_2)_2 + H_2O \xrightarrow{Urease\ Enzyme} NH_2COOH + NH_3 \quad (1)$$

$$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3 \quad (2)$$

$$H_2CO_3 \leftrightarrow HCO_3^- + H^+$$

$$(3)\ HCO_3^- + H^+ + 2NH_4^+ + 2OH^- \leftrightarrow$$

$$CO_3^{2-} + 2NH_4^+ + 2H_2O \quad (4)$$

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

The primary objectives of this study were to find out investigate an effective extraction procedure of crude urease enzyme from watermelon seeds and to develop cemented samples towards making artificial beachrock. In this study series of test-tube experiments were conducted to identify the variations of precipitated crystals and their morphology considering their reaction rate with time. The precipitated crystals were analyzed with SEM (Scanning electron microscope) and XRD (X-Ray Diffraction) to confirm their morphology. Finally, the extracted crude enzyme was analyzed and compared with a pure commercial enzyme (Urease from Jack bean: Wako pure chemical industries, Ltd., Japan) for measuring urease activity.

Besides, syringe sand solidification tests were conducted using the extracted crude enzyme and successfully obtained the cemented sand specimen in a laboratory scale up to several MPa, which could be a mimic of natural beachrock. The findings of these studies could play a significant role in the development of bio-cemented sand (natural beachrock imitation) using low-cost plant-derived urease enzyme. Moreover, it could also be possible to recycle waste food waste materials like watermelon seeds.

2. METHODS

2.1 Extraction of Crude Urease

Finely crushed and blended watermelon seeds (0.5 g) using 10 mL of distilled water (concentration estimated 50 g / L) stirred at 600 rpm for around 1 h. The crude extract was collected after centrifugation and filtration. After centrifugation, the collected transparent supernatant and pellets were used to investigate the urease activity considering different environmental parameters (temperature, pH, etc.) followed by the indophenol method [8,9]. The extraction procedure presented in Fig. 1. The urease activity of the enzyme (crude extracts) solution was calculated by dividing the slope of the initial linear part of the ammonium corresponds to time curve. Finally, to validate the data, the urease activity was compared to available commercial urease enzymes (jack bean, Wako pure chemical industries, Ltd., Japan).

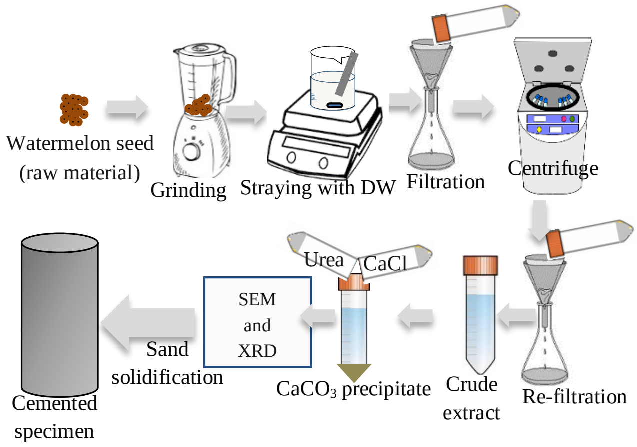

Fig. 1 Extraction procedure of crude enzyme for making artificial beachrock

2.2 $CaCO_3$ Precipitation Test

A series of experiments were conducted to confirm the morphology of $CaCO_3$ precipitation and its precipitation trend, by various combinations of $CaCl_2$-urea and urease. The set of experiments conducted in transparent polypropylene tubes. 10 mL of equimolar solutions of $CaCl_2$-urea was formulated as 0.3 mol/L, 0.5 M, and 0.7 M and adjusted the concentrated distinctly by adding crude extract solution from watermelon seed. The adjusted solution was then added to the test tube and kept in a shaker at 30 °C for 48 h. The obtained precipitation ($CaCO_3$) was separated from the solution using a filter paper (Whatman filter paper, grade 4) and kept in an oven drier for about 24 h at 100 °C. The crystal precipitation ratio (%) was calculated by the mass of the precipitated materials obtained from the test divided by the theoretical mass of $CaCO_3$ (g) crystals. The sand solidification testing conditions were shown in table 1.

Table 1 Testing conditions

	Control			
Urea-$CaCl_2$ (M)	0.3/0.5/0.7 M	0.3	0.5	0.7
Enzyme Solution	No	Yes	Yes	Yes
Initial pH	7.5/7.6/7.6	7.6	7.7	7.6
Final pH	7.5/7.6/7.7	7.9	8.1	7.9

The hydrolysis rate was measured immediately after mixing of solutions with time, and a standard curve was established using the obtained results after complete hydrolysis of several concentrations of urea. Finally, precipitated $CaCO_3$ weighed using a scale to calculate the total carbonate precipitate amount. Scanning Electron Microscope (SEM) and X-ray diffraction (XRD) analysis were also conducted to investigate the morphology of the precipitated carbonate.

2.3 Sand Solidification (Syringe) Test

Commercially available dried "Mikawa" sand (Fig. 2) placed at 110 °C for 24 h and were then transferred (85 g) into a 50 mL syringe (diameter, φ= 2.3 cm, height h=7.1 cm) and compacted gently.

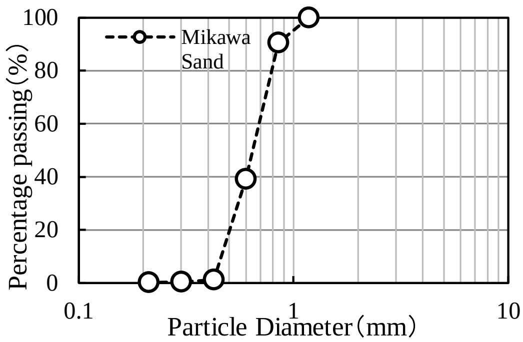

Fig. 2 Particle size distribution of Mikawa sand

The prepared samples were then placed in an incubator (30 °C). Subsequently, 26 mL of prepared urea-$CaCl_2$-urease solution (crude extract) was added to the syringe and maintained the final level of the solution above the top surface of the sand sample. After 24 h, a new cementation solution (urea-$CaCl_2$) was injected into the samples with the same volume and concentration and drained the previously injected solution gradually (Fig. 3). The crude urease injection interval was 48 h. Different curing days were chosen for investigating the effect, variations, and distribution of precipitated calcium carbonate within the sand particles. The condition of the syringe solidification test outlined in Table 2.

Fig. 3 Status of sand solidification test using extracted crude enzyme

Table 2 Testing conditions

Cases	Crude urease	Crude urease Injection interval	Urea-$CaCl_2$ (M) (added everyday)	Temp (˚C)	Curing days
1	No	Control sample			
2	Added	48 h	0.5	30	14
3	Added	48 h	0.5	30	21

3. RESULTS AND DISCUSSION

3.1 Results of Urease Extraction

A summary of urease activity (U/mL) of the extracted crude enzyme from watermelon seeds presented in Fig. 4. The urease activity was also compared with the commercially available urease enzyme made from jack bean (Wako pure chemical industries, Ltd., Japan). According to the results, the urease activity was highest for non-treated crude extract (around10 U/mL) and gradually decreased with time (up to 07 days). For the treated sample, the urease activity was somewhat lower than the non-treated sample (8 U/mL) but almost constant with time (up to 07 days).

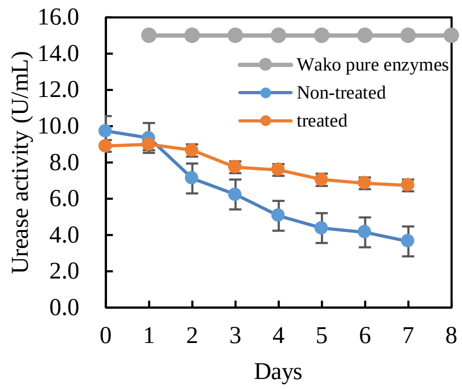

Fig. 4 Urease activity of the extracted solution

For the commercial jack bean urease, the urease activity was observed around 15 U/mL. The purification level of the extracted crude enzyme was around 60-70% (Table 3). The fluctuation of these purification level and urease activity of the crude enzyme to commercial enzyme was influenced by certain metabolic factors [10,11] of seeds, including some biological reactions and presence of specific organic compounds. The maximum urease activity was obtained from jack bean seeds, as reported previously [9,11]. However, from the findings of this study, the urease enzyme could be extracted up to a purity level of 60 percent, which could be sufficient to make bio-cemented for different geotechnical approaches.

Table 3 Crude urease extraction results with their purification percentage

Product	Urease activity (U/mL)	Volume of extraction (mL)	Total Units (U)	Specific activity (U/mL)	Purification fold	Loss of enzyme (%)
Wako pure enzymes	15	100	150	10	0.67	0
Non-treated	10	100	100	10	1	33
Treated	8	100	80	10	1.25	47

* Loss of enzyme = [Sum of crude extract (U) – Total Units after treatment) / Sum of crude extract (U)]

3.2 $CaCO_3$ Precipitation Test

It is reported that, $CaCO_3$ acts as the main binding materials in between the sand particles to stabilize the soil for producing artificial beach rock and bio-cemented sand [12-15]. In EICP process, the crystal precipitation amount closely related with $CaCl_2$-urea concentration, and the $CaCO_3$ crystals started to form immediately because of the accessibility of free urease enzyme (Fig. 5), which was also reported by previous studies [16]. Fig. 5 showed the evidence of forming and enlargement of $CaCO_3$ crystals.

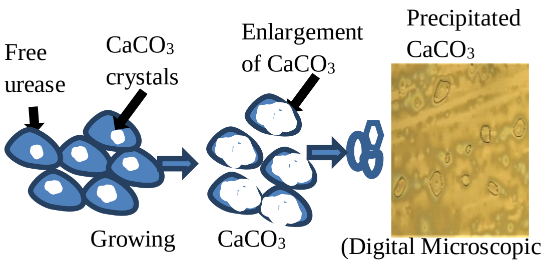

Fig. 5 Growing of $CaCO_3$ crystals

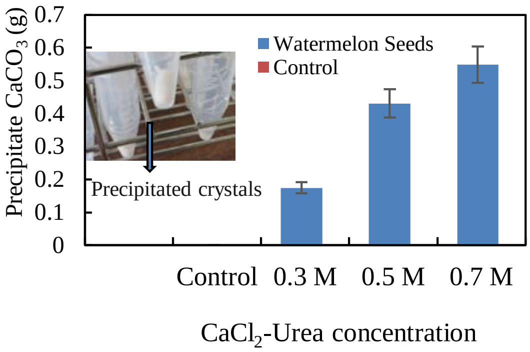

Fig. 6 Trend of $CaCO_3$ precipitation

It was revealed that, the efficiency of bio-cemented samples depends on the type and structure of the precipitated $CaCO_3$ polymorphs (vaterite or calcite). When the crystals formed bridges between the existing sand/soil grains, they prevented the movement of the grains, which identified as a primary reason for improving the strength and stiffness of the material [16]. Fig. 6 indicated the trend of $CaCO_3$ precipitation at different $CaCl_2$-urea concentration. Results showed that, higher concentration of $CaCl_2$-urea leads to precipitate higher amount of $CaCO_3$ crystal.

In addition, from the SEM images it showed that that the precipitated crystals morphology ($CaCO_3$) is highly inconsistent in shape and size (Fig. 7). But the value for crystals precipitation depends on the concentration of urease enzyme and concentration of reactant solutions, which was also reported in previous studies [17-18]. The results of this study showed that, precipitated calcium carbonate crystals from crude extract enzyme may appear as highly disorder and amorphous due to presence or inheritance of different organic compounds confined to the raw materials (crude extract solution from watermelon seeds).

Furthermore, the results also indicated that the reaction rate of EICP process were very fast which leads to precipitated different crystalline phases of $CaCO_3$ crystals. However, the observation of this study could be considered as significant outcomes for various bio-geotechnical applications.

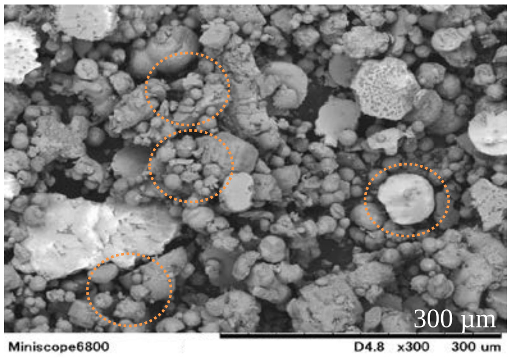

Fig. 7 SEM images of precipitated crystals with their variations in-terms of size and morphology

3.3 Sand Solidification Test (Small Scale)

Mikawa sand was used for sand solidification using the extracted crude solutions from watermelon seeds. From the results, it showed that the higher UCS was obtained for the longest curing sample (case 2) compared with case 1. The variations of the UCS strength was also observed in top, middle and bottom portions of the both specimens (case 1 and case 2). From the results, it was observed that, the bottom portion showed the higher UCS value compared to top and middle portions of the both treated samples (Fig. 8). The possible reason was the fast reaction rate of the urea hydrolysis and distribution variations of precipitated $CaCO_3$ within the specimens (case 1 and case 2). Because of the reaction rate, most of the precipitated $CaCO_3$ tends to deposit at the middle and bottom portion and increased the bonding capacity in between the sand particles at the bottom and middle portion and increased the strength (UCS) (for both case 1 and case 2). But it was also observed that, with increasing the curing time (21 days), the distribution of $CaCO_3$ precipitation was also fluctuate at the top, middle and bottom portion (case 2) and strength (UCS) was also increased. Further investigation is needed for obtaining homogenous strength of the sample by ensuring uniform distribution of $CaCO_3$ precipitation.

In addition, another reason could be higher solubility, lower viscosity, fast penetration and deposition at the bottom portion of the sand, which also supported by previous studies [19,20]. A higher UCS was also observed obtained (around 1.2 MPa) after continuous curing (21 days) at 2.5% of $CaCO_3$ for case 2. In case of 1, maximum UCS was obtained (below 1 MPa) with less than 2% of $CaCO_3$ content and the bottom portion bearing the highest strength Fig. 9. And Fig. 10 also showed the results of the precipitated crystal behaviour in between the cemented sand particles.

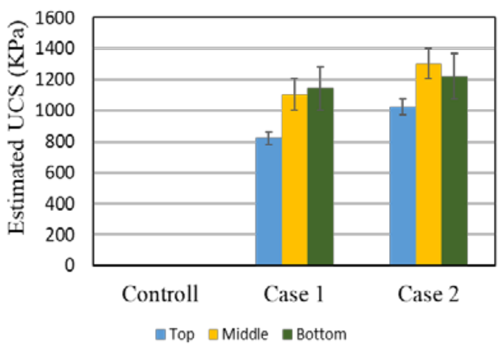

Fig. 8 Estimated UCS of the solidified sand

Form the results, it showed that, $CaCO_3$ is the primary binding materials for improving the strength of sand particles which is an agreement of previous studies [21]. Another important observation was viscosity of the cementation solution could affect the crystal precipitation rate to form $CaCO_3$ and improving the sand strength to a most stable phase of calcium carbonate. However, further investigation is needed to improve this methodology, which could be appropriate for field application.

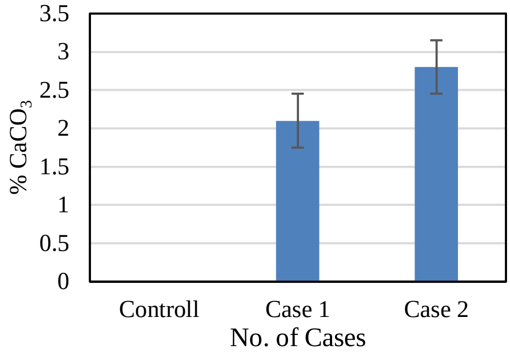

Fig. 9 Percentage (%) of $CaCO_3$ content of the cemented sand specimen

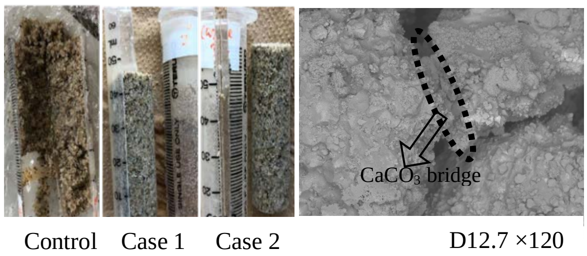

Fig. 10 Status of solidified sand (left images) and SEM images of solidified sand showing the $CaCO_3$ bonding in between the sand particles (right images)

4. CONCLUSIONS

The prospect of using the enzyme induced carbonate precipitation (EICP) method as an effective bio-inspired technique has been evaluated in this study using natural source of urease enzyme (watermelon seeds). The development of alternative sources for urease enzymes which are economical and easy to regulate in the natural world was a major problem in the feasibility of calcite Precipitations Strategies. Through extracting the enzyme from watermelon seeds (upto 60% purity level) in a sustainable, cost-effective, and eco-friendly way, this research successfully addressed these challenges. Furthermore, this study successfully obtained solidified sand sample upto 1.2 MPa using watermelon seeds as a source of urease enzyme and the results from this study clearly indicated that, plant derived urease enzymes could play a vital role for making artificial beachrock. However, still some major challenges for field implementations of this EICP method remained in this novel area. Nevertheless, the findings of these studies could play a substantial role for soil/sand stabilization, coastal erosion protection, ground improvement in deeper portion of the soil, improvement of piles and slope stability, to support foundation and embankment, improvement soils liquefactions under the existing structures and other bio-geotechnical applications.

5. ACKNOWLEDGEMENTS

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