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Strengthening Water Retention Capacity of Marine Soft Clay Using Bio-cement

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Abstract 'Bio-cement' is biologically produced salt largely calcium carbonate along with the cell mass of microorganisms. It is mainly generated by urease producing organisms. The present study focuses on the feasibility of using bio-cement to strengthen the marine soft clay in order to increase its water retention capacity. Eight urease producing organisms were initially isolated from sewage and four of them were selected based on their high urea (10%) tolerance. Growth parameters (pH and temperature) of the selected strains were also determined which ideally correlates with the bio-cement production. Further, one strain was selected for bio-cement production based on its maximum urease production studied on Christensen's Urea plate by the well diffusion method. The selected strain was identified to be *Klebsiella pneumoniae* using 16S rDNA sequencing and biochemical tests which produced 0.46 g of bio-cement per liter of seawater after seven days of incubation at room temperature. The dried bio-cement was mixed with marine soft clay in a 1:1 ratio. The water retention capacity was checked intermittently for 72 hours and compared with untreated marine soft clay. It was evident from the results that water retention capacity of marine soft clay was strengthened using bio-cement. Thus, this combination can be utilized for construction of aquaculture ponds in sand, agriculture in marine soil and conservation of concrete structures, amongst several other ecofriendly applications.

Keywords: *bio-cement, Urease, Klebsiella pneumoniae, water retention capacity*

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1. Introduction

Urea is an important nitrogenous end product of the metabolic breakdown of proteins in mammals. It is commonly present in agricultural soils, agricultural runoffs and effluent containing sewage and therefore, such places are ideal sample collection sites for isolating ureolytic microorganisms. Urease-producing organisms instigate calcium carbonate precipitation which is influenced by four main factors such as Ca^{2+} concentration, dissolved inorganic carbon amount, availability of nucleation sites and pH [1]. Microorganisms exert precipitation of calcium carbonate by changing any of these parameters, either individually or in different combinations [2,3]. When urease-producing microorganisms are introduced in a system containing calcium ions, Ca^{+2} gets attracted to the microorganism's cell wall due to its negative charge and hydrolyzes urea to form carbamate and ammonia. Carbamate disintegrates at physiological pH to form carbonic acid and another molecule of ammonia [4]. In water, these products undergo equilibrium to form bicarbonate and a molecule of ammonium which increases the pH of the environment, thus causing precipitation of $CaCO_3$ on the surface of microorganisms. After sometime, $CaCO_3$ precipitate encapsulates the whole cell, transfer of nutrient to the organisms gets restricted and as a result, the microorganisms die and only the bio-cement remains [5].

Water-holding capacity is the measure of the quantity of water that can be effectively accessed by crops. Thus, plants can't grow in sandy or loose soil without soil improvement. There are three soil improvement methods popularly known other than microbial methods. These include treatment of soil with either composted leaf litter, bentonite or termite mound material [6].

Bio-cement is highly desirable because of its natural and environment-friendly approach. There are many other applications for bio-cement. Some of these include contaminant removal of radio-active pollutants; removal of calcium ions from wastewaters and groundwater; restoration and protection of limestone monuments and statuary; pore plugging of the oil-recovery reservoir, rock and stone formation [4,6,7]. The purpose of the present study is to isolate bio-cement producing organisms and use them for strengthening water retention capacity of the loose soil for various purposes.

2. Material and Method

Sample collection: Sewage, sea water, and soil samples were collected for isolation of bio-cement producing organisms.

Initial enrichment, screening, isolation: 150 ml of Christensen's urea broth was inoculated with 3 ml of the sewage sample and incubated at room temperature for 24 hours. Post incubation, loopful of the enriched samples were isolated onto Christensen's urea agar plates and incubated at room temperature for 24 hours to obtain well-isolated colonies.

Urea tolerance: To check the ability of the isolates to tolerate higher concentrations of urea, urea tolerance test was performed. Colonies were individually picked, inoculated in Christensen's urea broth and incubated at room temperature. After 24 hours of incubation, the cultures were adjusted to O.D =0.1 at 580nm and inoculated into Christensen's urea broth containing 3%, 5%, and 10% urea respectively. These were then incubated at room temperature for 24 hours and the growth was measured colorimetrically at 580nm.

Growth optimization of bacterial isolates: Optimization of pH: To determine the optimum pH of the isolates, colonies were individually picked, inoculated in Christensen's urea broth and incubated at room temperature. After 24 hours of incubation, the cultures were adjusted to O.D=0.1 at 580nm and inoculated into Christensen's urea broth having different pH i.e. pH 6, pH 6.5, pH 7.0, pH 7.5 and pH 8 and incubated at room temperature for 24 hours. Absorbances of the broths were measured post incubation at 580 nm to determine the growth at different pH conditions. The experiment was done in triplicate.

Optimization of Temperature: To determine the optimum temperature of the isolates, 0.1 O.D 580nm cultures of individual colonies were obtained by the similar method mentioned in pH optimization and inoculated in three sets of Christensen's urea broth. The tubes were incubated at different temperature conditions i.e. 37°C, Room temperature and 4°C. Absorbances of the tubes were measured at 580 nm post incubation to determine the growth at varying temperature conditions. The experiment was done in triplicate.

Urease activity: To determine the strain that produced maximum urease in a limited amount of time, the urease hydrolysis test was performed using the well diffusion method. Wells were bored using sterile borers on Christensen's urea agar plate. Colonies were individually picked, inoculated in Christensen's urea broth and incubated at room temperature. After 24 hours of incubation, the cultures were adjusted to O.D=0.1 at 580nm and 100µl of cultures were added to the wells and the plates were incubated at room temperature for 24 hours.

Characterization of bacterial isolates: The pure culture of the selected bacterial strain was isolated on Nutrient Agar plates and the colony characteristics and Gram staining were studied after 24 hours of incubation at room temperature. Biochemical tests such as sugar fermentation, salt tolerance, nitrate reduction, motility, indole production, methyl red, Voges Proskauer, citrate utilization, triple sugar iron, oxidase, catalase, and Hugh Leifsons (aerobic and anaerobic) tests were performed. The culture was identified by 16S rDNA sequencing method [9]. Briefly, DNA extraction of the bacterial strain was done and PCR amplification of the extracted DNA was conducted using 1492 R and 27 F primers. The amplified PCR products were run on Agarose gel followed by gel purification and sequencing. Finally, the obtained sequence was compared with existing database sequences.

Analysis of water hardness: The hardness of sea water and sewage samples were determined by titrating them against 0.01 M Sodium EDTA using Eriochrome Black T as an indicator at pH 10. The indicator turns pink in the presence of calcium ions. EDTA chelates with calcium ions. On addition of adequate EDTA to the sample, when all the calcium ions in the system form complexes with EDTA and no free calcium ions are present, the solution turns blue in color [10].

The concentration of $CaCO_3$ is calculated by applying the following equation:

$CaCO_3$ (mg/l) = [volume of EDTA (ml)/ Sample volume (L)]*[$CaCO_3$ (1mol) / EDTA (1mol)].

Bulk production of bio-cement: For the bulk production of bio-cement, 1000ml of seawater was used. To the sea water, 200ml of 24 hour old inoculums of O.D=0.2 at 580nm and 3M urea was added. The flasks were incubated at room temperature for seven days. After incubation, the white precipitates obtained were filtered using Whatman filter paper Grade 1 and dried at 48 °C for eight hours. The Ca^{2+} content of the original sea water and the filtrate was analyzed using the above-mentioned protocol.

Determination of water retention capacity: Three columns were prepared using 2 ml syringes in order to determine the water retention ability of the bio-cement that was obtained. The test column was established by mixing bio-cement and marine soft clay in a 1:1 ratio. Similarly, a positive control column was established by mixing commercially available $CaCO_3$ and marine soft clay in a 1:1 ratio. A negative control column consisted of only marine soft clay. To all the three tubes 1.5 ml of water was added slowly and the leakage of water was observed and recorded intermittently for 72 hours (Figure 5).

XRD Analysis: The XRD analysis was done to determine the crystalline form and the chemical composition of the bio-cement. XRD-spectrum was obtained using Philips X'pert MRD with a Cu anode (40 kV and 30mA).

Statistics: Sigmastat 3.5 Statistical software was used for performing statistical analysis. 'One Way Analysis of Variance' was performed to determine differences between various groups and $P<0.05$ was considered significant.

3. Results

Sewage samples were enriched in Christensen's urea broth and the enriched samples were isolated on Christensen's urea agar plates where phenol red was used as a pH indicator. Pink colonies of urease producing strains were observed after 24 hrs of incubation at room temperature as indicated in supplementary Figure 1. Initially, eight bacterial strains producing urease from sewage sources were isolated and pure cultures were obtained after repeated isolations.

Urea tolerance of the isolates was checked by inoculating the bacterial isolates in Christensen's urea broth having three different concentration of urea i.e. 3%, 5% and 10%.

Four isolates were selected based on their high urea tolerance at 10% concentration. Growth parameters (pH and temperature) of these bacterial isolates that could tolerate high urea were optimized. (shown in Figure 1 and Figure 2 respectively). Urea hydrolysis test was performed by the agar well diffusion method to qualitatively determine urease production. Isolates 1 and 2 produced no distinct pink zone while isolates 3 and 4 produced 13mm and 22mm zone diameter respectively after 24 hrs of incubation at room temperature as shown in Figure 3. Based on biochemical analysis (data not shown) and 16S rDNA sequencing, strain number 4, the selected urease- producing organism was identified as *Klebsiella pneumoniae*. The optimum pH and temperature of the selected strain was found to be pH 8 and 24°C respectively (Figure 1 & Figure 2).

For the production of bio-cement, hard water systems i.e. seawater and sewage water were analyzed for the presence of Ca2+ ions in it by titrimetric analysis. The hardness of seawater and sewage water was 2677.34 mg/l and 1135.06 mg/l respectively. Thus, for the bulk production of bio-cement, sea water was chosen. After seven days of incubation at RT, a white powdery substance was found to precipitate at the bottom of the flask as shown in Figure 4. 0.46 g of white mass was obtained per 1000ml of seawater. After filtration, the filtrate was analyzed for hardness, mainly for Ca^{2+} content. The filtrate showed a significantly low amount of Ca^{2+} (1242.48 mg /L) compared to control. The bio-cement obtained was analyzed for its water retention ability as per the arrangement of the experiment shown in Figure 5. On checking the water leakage capacity intermittently, the column containing untreated soft clay started leaking in less than 5 minutes on the addition of water. The column containing soft clay mixed with commercially available $CaCO_3$ in a 1:1 ratio started leaking after 3 hrs. However, the column containing soft clay treated with bio-cement in a 1:1 ratio was able to retain the water even beyond 72hrs. The XRD analysis of the synthesized bio-cement further confirmed the presence of calcium carbonate which perfectly corroborates previous literature [11]. The signature peaks at 2θ = 29.8°(104) and43.7 (202)clearly show the presence of $CaCO_3$. However, it is also evident from the pattern that the calcite is not produced in its pure form; peaks at 2θ =16.7, 21.7, 32.4 represent some other crystal structure of aragonite and dolomite which are also present in the bio-cement produced by *Kelbsiella pneumoniae*.

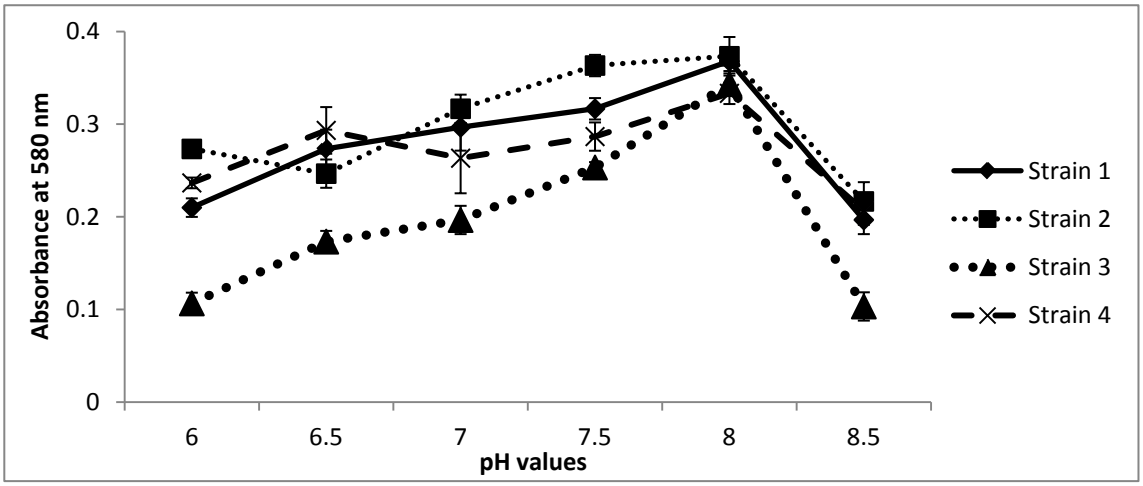

Figure 1. Effect of pH on growth of urea tolerant bacterial strains: Four selected strains which can tolerate 10% urea were checked for their growth at six different pH. The point indicates mean absorbance ± SD of a representative experiment (n=3).

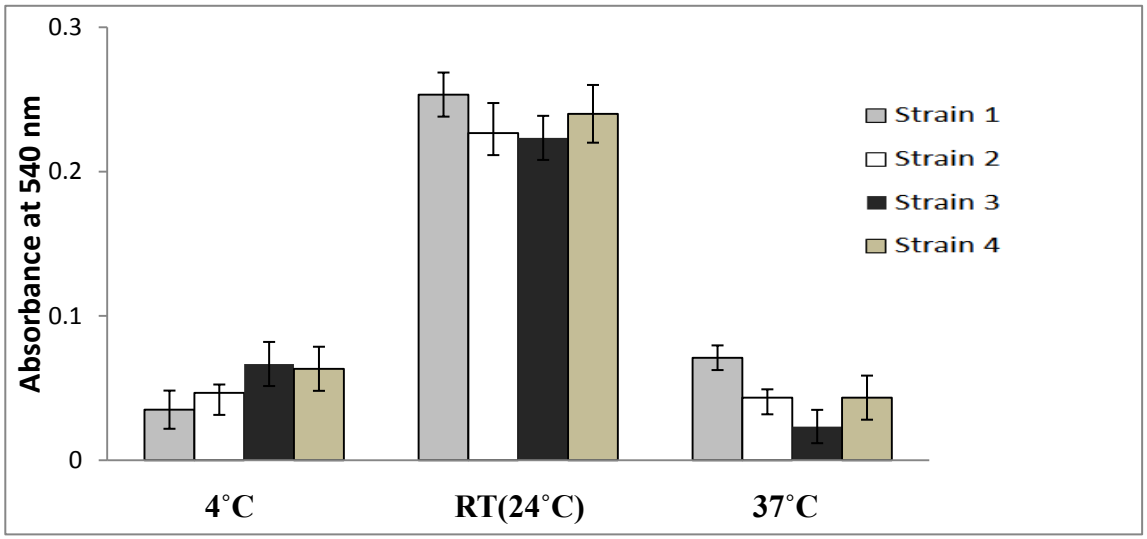

Figure 2. Effect of temperature on growth of urea tolerant bacterial strains: Four bacterial strains were checked for their growth at three different temperatures. The result is represented as mean ± SD (n=3).

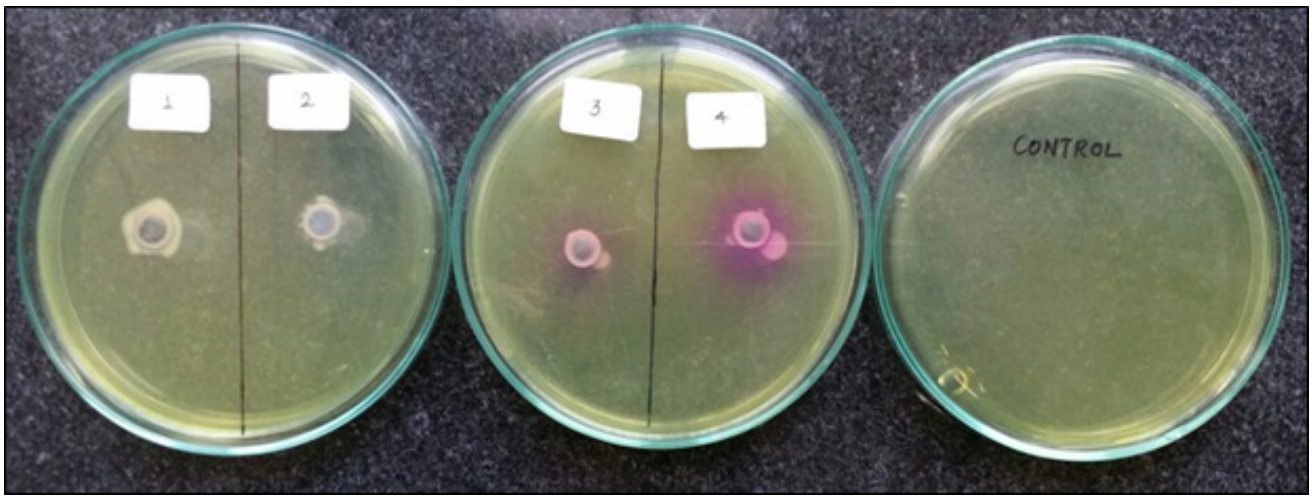

Figure 3. Urea hydrolysis test by agar cup method: All selected strains were grown in Christensen's Urea broth for overnight and 100ul of bacterial suspension (OD=0 .1) were added to the wells of Christensen's Urea agar plate. Isolate 1 and 2 produced no distinct pink zone while isolate 3 and 4 produced 13mm and 22mm zone diameter respectively after 24 hrs of incubation

Figure 4. Mass production of Bio-cement: 200ml of 24hour old inoculum (O.D $_{580nm}$=0.2) and 3M urea were added to 1000ml of seawater. After 7 days incubation at room temperature, white powdery substance was precipitated which was filtered using Grade 1 Whatman filter paper(Left); Filtered powder dried at 48°C for 8 hours (Right)

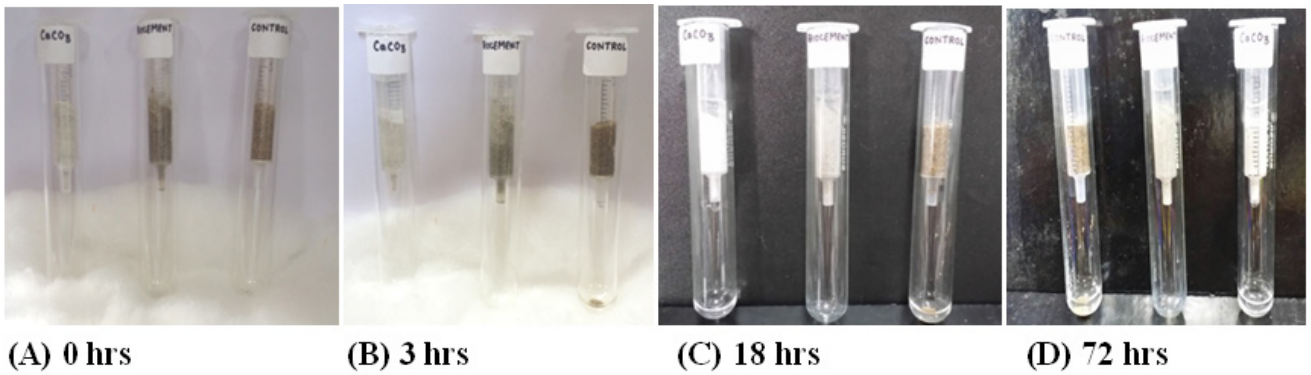

Figure 5. Determination of water retention capacity: Three columns were prepared using 2 ml syringes placed in test tubes to check the water retention ability of the bio-cement. Positive control column was established by mixing commercially available $CaCO_3$ and marine soft clay in 1:1 ratio; Test column was established by mixing bio-cement and marine soft clay in 1:1 ratio. Negative Control column consisted of only marine soft clay. To all the three tubes 1.5 ml of water was added slowly and water leakage was recorded intermittently for a period of 2 days. The representative pictures were taken after 0 hours (A), 3 hours (B), 18 hours (C) and 72 hours(D) respectively

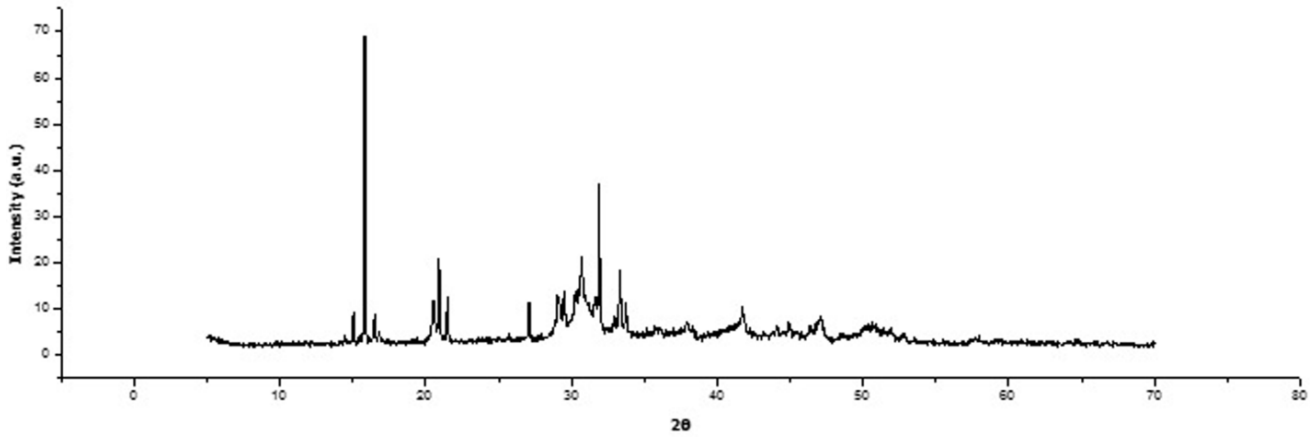

Figure 6. XRD analysis of white precipitate, obtained from isolate *Klebsiella pneumoniae*

4. Discussion

Four groups of microorganisms which are mainly alkaliphiles are involved in the bio-cement precipitation process. They include photosynthetic organisms (cyanobacteria and algae), organisms utilizing organic acids, sulfate-reducing bacteria responsible for dissimilatory reduction of sulfates and organisms that are involved in the nitrogen cycle either by ammonification of amino acids, nitrate reduction or hydrolysis of urea [12,13,14]. In the current study, a single isolate was chosen based on its ability to tolerate urea and the amount of urease production which was later identified as *Klebsiella pneumoniae*. The optimum temperature for growth of the isolate was ambient temperature and its optimum pH was 8. Previously, in other studies, seawater, sewage water and effluent contaminated sources of water were used for the purpose of bio-cement production [15]. In this study, seawater was used since it is an abundant source of calcium compared to sewage water. The bio-cement produced by the test organism was found to be 0.46g/ 1000ml in seawater. The lower amount of Ca^{2+} in filtrate confirmed the utilization of Ca^{2+} for $CaCO_3$ precipitation. XRD analysis of the bio-cement further confirmed the presence of $CaCO^3$.

The water retention ability of bio-cement produced was compared with commercially available calcium carbonate and untreated marine soft clay. The bio-cement was found to tightly pack the marine clay samples while the control column containing untreated marine soft clay and the column containing calcium carbonate mixed with marine soft clay started leaking much earlier. It indicated that the porosity of the soft clay was reduced by adding the bio-cement increasing its water retention ability considerably.

5. Conclusion

Bio-cement was obtained successfully using the isolated organism in a seawater system with 3M urea concentration. It was later utilized to increase the water retention capacity of marine soft clay which could further be carried out on a large scale. The retention capacity was found to be higher as compared to untreated soft clay. Therefore this study was able to provide a microbiologically induced cementing substance which could be used as the alternative to the commercially available concrete for the construction of aquaculture ponds in the sand, agriculture in marine soil and conservation of heritage structures.

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