A Preliminary Investigation on Isolation and Identification of Marine Bacteria for Biocementation in Nearshore Environments

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Abstract

Microbial Induced Carbonate Precipitation (MICP) is a widely explored technique that involves utilizing bacterially produced carbonate biominerals for improving the engineering properties of soils. When this novel approach is used in cementing sandy soils in nearshore areas, it is necessary to identify suitable bacterial strains which are resistant to high saline dynamic marine environments. Thus, current study was carried out to isolate and identify ureolytic bacteria from Sri Lankan beach sand and to check their suitability for use in MICP. To accomplish this, bacterial strains were isolated from beach sand samples and urease activity was determined. MICP capability was evaluated by cultivating the species on agar plates containing $CaCl_2$ and urea. Based on these results, four isolates having high feasibility to induce bacterially precipitated calcium carbonates were selected and identified by 16S rDNA gene sequencing. Two strains were identified as belonging to *Halomonas* sp. and other two to *Sulfitobacter* sp. and *Oceanobacillus* sp. genera. Further analysis was done to determine the bacterial cell growth of isolates at different temperatures and concluded that all four isolates have a more stable growth at temperature close to 30°C. Isolates were evaluated for their biosafety and found to be non pathogenic. However, detailed analysis on biomineralization by the selected isolates and their biological behaviour is recommended prior to any large scale applications.

Keywords: Biomineralization, Calcium Carbonate, Urease Activity, Ureolytic Bateria

1. Introduction

Coastal erosion is a naturally occurring process which adversely affect the coastal communities all around the world. Global climate change coupled with unfavourable anthropogenic activities have been identified as the main drivers of the erosion in near shore areas. Among the nations tormented by this problem, Sri Lanka is on the top of the list due to its location in the Indian Ocean which causes Sri Lankans to suffer two seasons of monsoons. The Southern, South Western and Western areas of the country are the most prone to

erosion of sandy slopes due to strong waves created by South Western monsoon [1].

The conventional engineering solutions that have been adopted so far towards erosion mitigation includes hard structures such as breakwaters, revetments, sea walls which are economically and ecologically unsustainable [2]. More sustainable approaches such as implementing green belts after tsunami disaster in 2004 also have been failed due to lack of maintenance [3].

On the other hand, beachrocks which are consolidated coastal sediments cemented with calcium carbonate polymorphs have the ability to act as a natural barrier against erosion from wave actions [4]. As a lesson learnt from nature, man–made rocks similar to beachrocks that have the ability to auto repair using sunlight, sea water and bacteria have been recently identified as a more eco friendly solution against coastal erosion [5]. The strengthening and stabilization of the sand is achieved by cementation induced by microbes [6]. The most common approach is Microbial Induced Carbonate Precipitation (MICP) where urea is hydrolysed by microbial urease thereby increasing the pH of the reaction medium which results in calcium carbonate precipitation in the presence of a calcium source. Calcium carbonate crystallization within the soil matrix bound the loose sand grains together improving the strength and stiffness of the treated soil. The overall process can be explained by Equation 1 below [7].

$$CO(NH_2)_2 + 2H_2O + Ca^{2+} \xrightarrow{\text{Urease}} 2NH_4^+ + CaCO_3\downarrow \quad (1)$$

The whole mechanism is therefore dependent on the secretion and execution of urease enzyme that degrades the urea [7]. Number of non-pathogenic bacteria that can produce urease constituively or inducibly and ideal for MICP have been isolated from inland soil samples [8]. However, high saline and dynamic nature of the coastal zones make it difficult to employ foreign microbes for MICP applications in such environments. Therefore, the main aim of this research study was to isolate native bacteria capable of inducing biostimulation to strengthen and stabilize sandy slopes in erosion prone areas in Sri Lanka. This was achieved by conducting field investigations and preliminary laboratory experiments to isolate urease producing bacteria and determining their growth conditions and urease activity.

2. Materials and Methods

2.1 Study Area

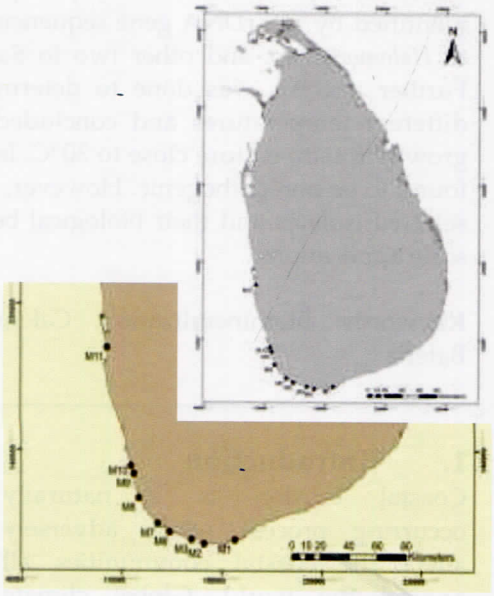

Figure 1 – Sampling locations.

The field investigations and sample collection were done beachrock occurring locations along the coastal strip extending from Dickwella to Uswatekkeiyawa, Sri Lanka. Beach sand samples of approximately 100 g were collected to sterile test tubes from 9 locations as shown in Figure 1. The containers were tightly closed to avoid any contamination and kept at ambient conditions until specimens were exported to Laboratory of Biotechnology for Resources Engineering, Hokkaido University for further analysis. The samples were imported from Sri Lanka to Japan following the Japanese Minister Permission System.

2.2 Isolation of Bacterial Isolates

Each sand sample was then serially diluted (10^{-1} – 10^{-6} times). A definite weight of sample (5.0 g) was homogenised aseptically in 9 volumes of sterilized distilled water (45 mL) to get a homogenous suspension of bacteria. A definite volume (50 µL) of the suspension of bacteria from each dilution was inoculated onto agar plates with Zobell 2216E medium (5.0 g/L polypeptone, 1.0 g/L yeast extract, 0.1 g/L $FePO_4$ and 30g/L agar prepared with artificial seawater and pH adjusted to 7.6-7.8 with 1.0 mol/L NaOH. Liquid medium was sterilized by autoclaving prior to use) and spread properly, so as to space the individual bacteria cells wide apart and isolate them from each other. The inoculated plates were incubated at 30°C for 48 hours.

Once the bacterial cells were well developed to form colonies, different isolates were identified based on the colony size, colour and shape from plates having 30-200 colonies. Different isolates were then streaked on fresh agar plates with a set of parallel strokes. The inoculum was continuously streaked with overlapping sets of similar strokes so that isolated colonies could be observed in the last streaked area after incubating at 30°C for another 24 hours.

2.3 Identification of Urease Producing Bacteria (UPB)

A simple urease activity test was conducted for qualitative assessment of urease producing ability of the isolates. Each separated colony was mixed with 20 mL of cresol red solution containing urea and incubated at 45°C for 24 hours. A control sample without any bacterial species was subjected to similar conditions for comparison purposes. pH of the reaction solutions were measured after 2 hours and 24 hours. Colonies that changed the initial yellow colour 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 (UPB). The schematic of the isolation and identification of UPB is given below in Figure 2.

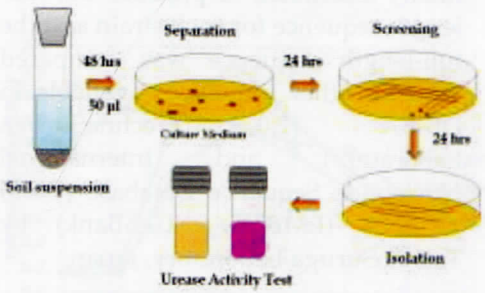

Figure 2 – Schematic of the isolation and identification of UPB

2.4 Microbial Induced Carbonate Precipitation Capacity of the Bacterial Isolates

The ability of biomineralization of calcium carbonate precipitation by the UPB identified from the above step was determined by a specially prepared agar plate containing $CaCl_2$ and urea (5.0 g/L polypeptone, 2.5 g/L yeast extract, 15 g/L agar, 1.0 g/L glucose, 0.05 mol/L $CaCl_2$ and 0.5 mol/L urea). The Microbial Induced Calcite Precipitation (MICP) ability of the isolates were detected qualitatively by the degree of calcium carbonate precipitates formed around the colonies.

2.5 Identification of Bacterial Isolates

16S rDNA gene amplification and sequencing were carried out for the selected isolates. 16S rDNA gene was amplified by Polymerase Chain Reaction (PCR) with forward primer 9F and reverse primer 1541R. Purified PCR products were then sequenced where primers 9F, 515F, 1099F, 1541R, 1115R and 536R were adopted to sequence both strands of the 16S rDNA gene. These sequences were finally assembled to produce the full-length sequence for each strain and the full-length sequence was compared with all other sequences available in DB-BA 12.0 (TechnoSuruga Laboratory) and International Nucleotide Sequence Database (DDBJ / ENA (EMBL) / GenBank) by TechnoSuruga Laboratory, Japan.

2.6 Effect of Temperature on Bacterial Growth

Bacterial isolates were separately precultured in 5 mL aliquots of sterilized and pH adjusted liquid Zobell 2216E medium (5.0 g/L polypeptone, 1.0 g/L yeast extract and 0.1 g/L $FePO_4$) and aerobically incubated at 20°C , 30°C and 40°C at 160 rpm for 24 hours. One mL of the precultures was then inoculated in 100 mL of fresh medium in Erlenmeyer flask and incubated under similar conditions. Bacterial cell growth was monitored every 24 hours for 7 days 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).

3. Results

3.1 Isolation of Urease Producing Bacteria

The collected samples consisted of silica rich sand along with other coastal sediments found in the intertidal zone.

From the 15 sand samples, 74 bacterial isolates were successfully isolated. However, after a visual inspection of the agar plates on which isolates were cultivated, isolates collected from different locations but looked similar to the naked eye were discarded. The urease activity test conducted on the remaining isolates resulted in colur change from yellow to pink for 6 different isolates. The pH change due to realesed NH_4^+ by urea hydrolysis during first 24 hour test period for the 6 isolates are tabulated in Table 1.

Table 1 – pH change due to urea hydrolysis by the bacterial isolates.

Laboratory ID	pH	
	After 2 hrs	After 24 hrs
M1-1-1	8.998	9.080
M2-2-1	8.848	9.085
M7-1-2	8.426	8.812
M11-1-5	9.246	9.395
M11-1-6	9.429	9.270
M11-1-7	8.940	9.130
Control	6.726	7.599

3.2 Microbial Induced Carbonate Precipitation Capacity of the Bacterial Isolates

The MICP tests conducted on agar plates containing $CaCl_2$ (0.05 M) and urea (0.5 M) showed significant positive results for only two bacterial isolates (laboratory ID M1-1-1 and M11-1-7) indicating calcium carbonate crystallization around the colonies while M11-1-6 did not grow on the plates at all. However, considering the pH change during urease activity test and slow calcium carbonate precipitation around the colonies in MICP test, 2 other isolates (laboratory ID M2-2-1 and M11-1-5) were also selected for future work.

3.3 Identification of Urease Producing Bacteria (UPB)

The analysis of 16S rDNA sequences of the 4 isolates yielded following results as shown in Table 2.

Table 2 – Results of 16S rDNA sequence analysis.

Lab. ID	Bacterial Species	Sequence similarity
M1-1-1	*Halomonas* sp.	99.8%
M2-2-1	*Halomonas* sp.	99.9%
M11-1-5	*Sulfitobacter* sp.	99.9%
M11-1-7	*Oceanobacillus* sp.	99.9%

According to the test results, the four species are belonging to three bacterial genera namely, *Halomonas* sp., *Sulfitobacter* sp. and *Oceanobacillus* sp. However, as per the literature none of them have been enagaged in MICP applications up to date.

The reference materials as well as laboratory analysis were used to confirm that none of the isolates are pathogenic. Further, three of the isolates are identified to be gram negative having a thin cell wall whereas M11-1-7 is a gram positive bacterium with a thick mesh type membrane (Table 3).

Table 3 – Gram stain and biosafety level of the bacterial isolates

Lab. ID	Gram Stain	Biosafety level	Ref.
M1-1-1	*Negative*	1 Risk group (German classification)	[9]
M2-2-1	*Negative*		
M11-1-5	*Negative*		
M11-1-7	*Positive*		

3.4 The Effect of Temperature on Bacterial Cell Growth

The bacterial cell growth measured in terms OD_{600} yielded following growth patterns for temperatures 20°C , 30°C and 40°C. (Figure 3 – Figure 6)

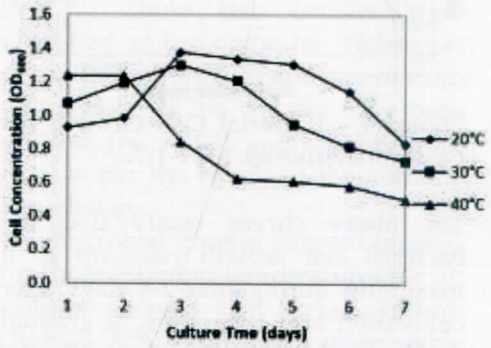

Figure 3 – Bacterial Cell Growth for *Halomonas* sp. M1-1-1.

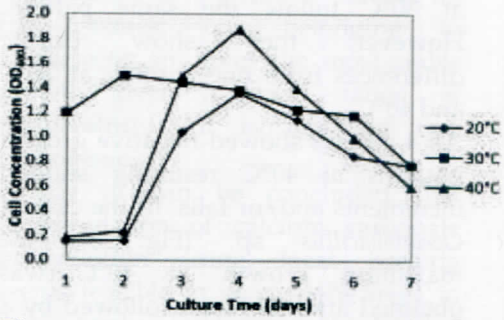

Figure 4 – Bacterial Cell Growth for *Halomonas* sp. M2-2-1.

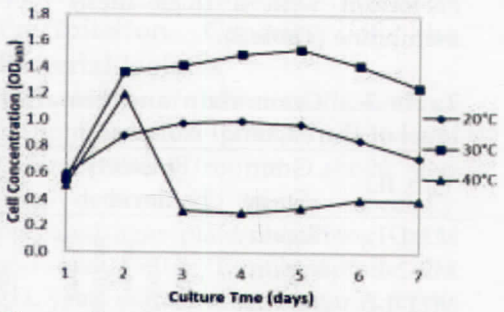

Figure 5 – Bacterial Cell Growth for *Sulfitobacter* sp. M11-1-5.

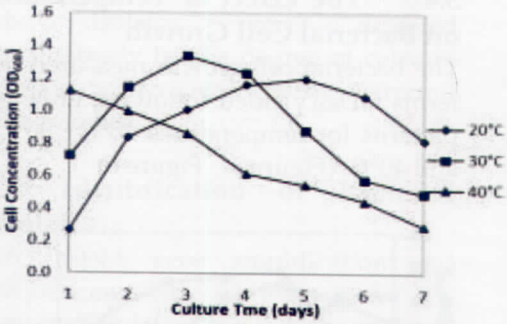

Figure 6 – Bacterial Cell Growth for *Oceanobacillus* sp. M11-1-7.

The above curves testify that the bacterial cell growth rises up to a maximum during first 2-4 days after cultivation and then show a gradual decline in the following days. For the two isolates belonging to *Halomonas* sp. (Figure 3 and Figure 4), the curves at 30°C follow the same pattern. However, they show drastic differences from one another at 20°C and 40°C.

All 4 species showed negative growth patterns at 40°C resulting sudden increments and/or falls. In the case of *Oceanobacillus* sp. (Fig. 6), the maximum growth at 40°C was obtained after 24 hours followed by a gradual decline during the rest of the investigation period.

Overall, it can be stated that the growth conditions for all 4 species at 30°C is more stable than at temperatures as low as 20°C or as high as 40°C.

Further, the isolate belonging to *Sulfitobacter* sp. Showed more stable growth over a wide temperature range of 20°C-40°C making it a suitable candidate for MICP process.

4. Discussion

The current research study was conducted as a basic step towards implementing Microbial Induced Carbonate Precipitation (MICP) to build artificial rocks similar to natural beachrocks as a novel solution against coastal erosion in Sri Lanka. According to extensive number of literature that have been published up to date, there are several physical and biological factors that determines the success of the process [10]. Selection of an appropriate bacterial species which can catalyse urea hydrolysis efficiently and effectively is a key factor in MICP applications [10].

Furthermore, when MICP is applied in a very dynamic natural environments such as near shore areas, it is mandatory that a bacterial species capable of withstanding the changing conditions is utilized for the process. The most appropriate solution to this issue is utilizing indigeneous urease producing bacteria for the process through biostimulation.

Thus, field and laboratory investigations were carried out to isolate and identify indigeneous urease producing bacteria from Sri Lankan soils. The study was carried out focussing the high erosion prone areas in Sri Lanka, that is Southern, South western and Western regions of the island.

A total of 74 bacterial isolates were isolated from the sand samples collected from 9 different locations and only 7 were identified as ureolytic

bacteria based on the conventional urease activity test. This confirms the fact that ureolytic bacteria are not ubiquitous in environment and require certain growth conditions [11]. Our findings suggest that UPB are not very common in the area under concern. This can be attributed to either of the following two cases. First case is that the collected sand samples mainly contained silica rich sand with very lower amount of organic matter thus assuming that the lower nutrient content lead to low levels of bacterial populations. Secondly, there is a lack of laboratory culture media that are capable of isolating all kinds of marine bacteria which may result in isolation of only a smaller proportion of the wide diversity of existing bacteria [12]. The culture medium used in this study was Zobell 2216E and there is a possibility that the growth of certain proportion of bacterial isolates were inhibited in the particular medium.

However, only 2 bacterial isolates showed significant positive results for microbial induced carbonate precipitation on agar plates with $CaCl_2$ and urea. Two other isolates with slow response to the precipitation test were also identified. 16S rDNA gene sequencing and identification showed that 4 isolates were belonging to only 3 bacteria genera and none of them are pathogenic making them suitable for MICP applications.

The evaluation of effect of temperature on microbial growth showed that, all the isolates show astable growth at 30°C which is appropriate for the tropical climate of Sri Lanka. This also confirms the fact that isolates belonging to aforementioned genera show the optimum growth at a temperature range of 25-30°C concluded by numerous previous studies [9]. However, one species belonging to *Halomonas* genus showed its maximum growth at 40°C.

5. Conclusions

This preliminary investigation was carried out to isolate and identify urease producing bacteria from tropical beach sand collected from Sri Lanka. From the data gathered from field and laboratory studies, following conclusions can be made.

Although a large number of bacterial isolates were found from the collected sand samples, only few of them have the capability for urease secretion and execution. Among the isolated urease producing bacteria, two isolates showed high feasibility for Microbial Induced Carbonate Precipitation (MICP). Those two isolates were identified as belonging to *Halomonas* sp. and *Oceanobacillus* sp. genera from which none of the isolates have been utilized for MICP applications upto date as per the extent of the authors' knowledge.

The microbial growth curves obtained for three different temperatures for all isolates showed that a temperature close to 30°C results in a more stable growth with no sudden declines making them appropriate for MICP applications in the future. Among all the isolates, one belonging to *Sulfitobacter* sp. gives the most stable growth over a wide range of temperature. All isolates are non pathogenic.

Finally, it can be concluded that biostimulation of calcium carbonate precipitation using local bacteria seems feasible in near shore areas in Sri Lanka. However, detailed investigations on the bacterial isolates are mandatory prior to up-scaling the process.

Acknowledgements

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