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Factors affecting the urease activity of native ureolytic bacteria isolated from coastal areas

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Abstract. Coastal erosion is becoming a significant problem in Greece, Bangladesh, and globally. For the prevention and minimization of damage from coastal erosion, combinations of various structures have been used conventionally. However, most of these methods are expensive. Therefore, creating artificial beachrock using local ureolytic bacteria and the MICP (Microbially Induced Carbonate Precipitation) method can be an alternative for coastal erosion protection, as it is a sustainable and eco-friendly biological ground improvement technique. Most research on MICP has been confined to land ureolytic bacteria and limited attention has been paid to coastal ureolytic bacteria for the measurement of urease activity. Subsequently, their various environmental effects have not been investigated. Therefore, for the successful application of MICP to coastal erosion protection, the type of bacteria, bacterial cell concentration, reaction temperature, cell culture duration, carbonate precipitation trend, pH of the media that controls the activity of the urease enzyme, etc., are evaluated. In this study, the effects of temperature, pH, and culture duration, as well as the trend in carbonate precipitation of coastal ureolytic bacteria isolated from two coastal regions in Greece and Bangladesh, were evaluated. The results showed that urease activity of coastal ureolytic bacteria species relies on some environmental parameters that are very important for successful sand solidification. In future, we aim to apply these findings towards the creation of artificial beachrock in combination with a geotextile tube for coastal erosion protection in Mediterranean countries, Bangladesh, and globally, for bio-mediated soil improvement.

Keywords: microbially induced carbonate precipitation; ureolytic bacteria; urease activity; coastal erosion protection; artificial beachrock

1. Introduction

The problem of coastal erosion is not new, but over recent years, it seems to have increased rates worldwide, particularly in the Mediterranean countries and Bangladesh. The erosional problem worsens whenever the applied countermeasures (i.e. hard or soft structural options) are inappropriate; improperly designed, built, or maintained; or if the effects on adjacent shores are not carefully evaluated. To prevent, or at least minimize, damage from erosion, a combination of various structures and processes has been traditionally used, including embankments, revetments, jetties, artificial reefs, offshore breakwaters, and sand bypasses. However, considering the costs related to the maintenance and management of concrete structures, the use of inexpensive alternative materials should be considered. Recently, microbially induced carbonate precipitation (MICP) using ureolytic bacteria is a promising technique in the field of geotechnology and civil engineering. To minimize environmental problems, in recent years MICP techniques have been developed as an alternative method for ground improvement (Danjo and Kawasaki 2014a, Sidik *et al.* 2014).

As previously mentioned, MICP using ureolytic bacteria is a promising technique in the field of geotechnology and civil engineering. There are various metabolic pathways leading to MICP and urea hydrolysis is the most prominent for the formation of beachrock (Danjo and Kawasaki 2014b). The reactions occurring in this mechanism are represented by the following equations (Eqs. (1)-(3)); where urea is hydrolyzed by urease (ureolysis) to form ammonium ion and carbamate (Eq. (1)), and subsequently, the carbamate ion is hydrolyzed to form a second ammonium ion and bicarbonate (Eq. (2)).

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{urease}} H_2NCOO^- + NH^+_4 \quad (1)$$

$$H_2NCOO^- + H_2O \rightarrow HCO^-_3 + NH_3 \quad (2)$$

In the presence of Ca^{2+} ions, calcium carbonate is then formed and precipitated, which is effective in the binding of sand particles and plugging of microfractures as follows

$$Ca^{2+} + HCO^-_3 + NH_3 \rightarrow CaCO_3\downarrow + NH^+_4 \quad (3)$$

Danjo and Kawasaki (2014a, b) proposed a new ground improvement technique referred as “artificial beachrock” using the MICP method. To create artificial beachrock, various factors have been considered. Danjo and Kawasaki

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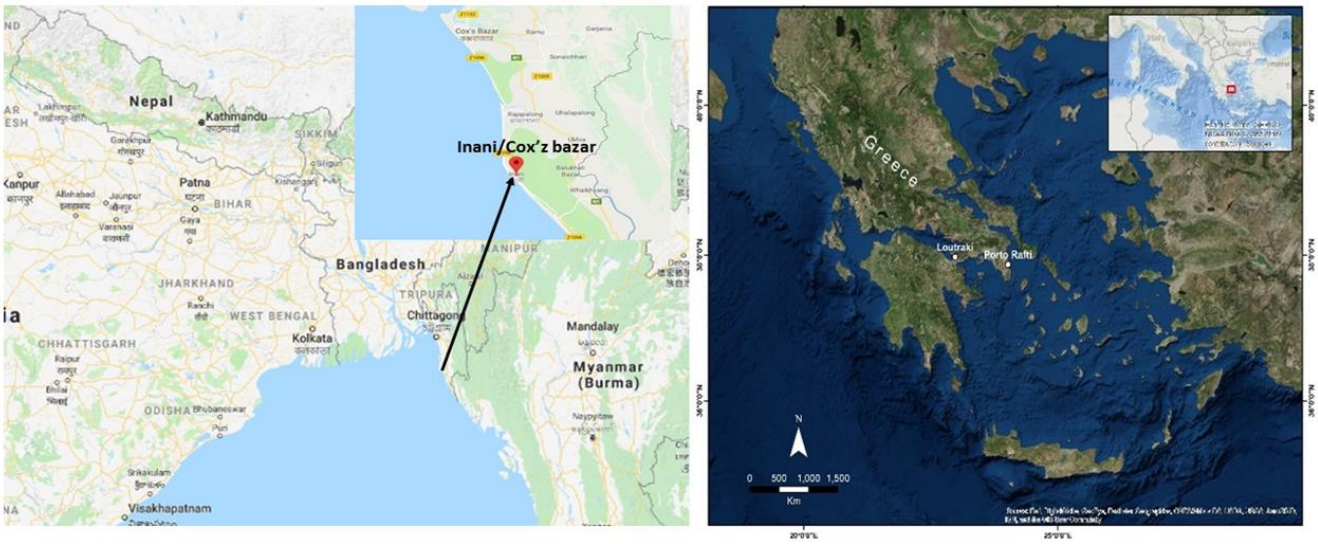

(a) (b)

Fig. 1 Map of Bangladesh showing the sample collection site (Cox'z bazar: N 20° 58′ 19.6″, E 092° 11′ 58.4″) and (b) Map of Greece showing the sample collection site (Porto Rafti: N 37° 52′ 57.1″, E 24° 00′ 51.1″ and Loutraki: N 37° 57′ 02.3″, E 22° 57′ 37.7″)

(2014a) focused on the possibility of promoting solidification using microbial processes, specifically urea decomposition by microorganisms. Previously, most research has been confined to use of the MICP method with land ureolytic bacteria (Whiffin *et al.* 2007); Donovan *et al.* 2016, Keykha *et al.* 2017). However, in the present study, we selected marine ureolytic bacteria isolated from coastal regions in Greece and Bangladesh, which have the most extensive coastlines and coastal erosion in these regions is severe (Orfanidis *et al.* 2005). For the protection of these long coastal zones from erosion, the application of the proposed MICP method was suggested to create artificial beachrock using local ureolytic bacteria, because of its long-term sustainability considering the local coastal climatic environment. For the creation of the artificial beachrock, it was necessary to determine the effectiveness of the urease activity, including the amount of calcite precipitation from the isolated bacterial species. Previously, it has been demonstrated that the formation mechanism of beachrock is greatly influenced by coastal ureolytic bacteria, which shows a great affinity for $CaCO_3$ precipitation and can be sustained for a long time (Danjo and Kawasaki 2014a). We isolated two ureolytic bacterial species from the coastal areas of Greece and Bangladesh. We selected local ureolytic bacteria (from Greece and Bangladesh) because of their local suitability, which is very important for the creation of artificial beachrock using the MICP method.

The present study is very useful for successful sand solidification using the "bio-stimulation" and/or "bio-augmentation" method. The aim of this study was to improve the understanding of the relevant phenomena that affect urease activity by considering different factors using local coastal ureolytic bacteria towards a commercial application in coastal erosion protection in Bangladesh, Mediterranean countries, and worldwide using our proposed method (Imran *et al.* 2017) in the near future.

2. Materials and methods

2.1 Screening and cell culture

Soil samples were (approximately 100 g) collected from the sampling sites (Fig. 1) using conical bottle-shaped plastic tubes and stored in a thermos cool box (at the sampling site) before being transported to the laboratory for further analysis. Under laboratory conditions, 5 g of soil was taken and serially diluted (up to six-fold) with sterile distilled water and 0.1 mL aliquots of serially diluted samples were inoculated in a petri dish containing nutrient agar (5.0 g/L of polypeptone, 1.0 g/L of yeast extract, 0.1 g/L of $FePO_4$, and 150 g/L of agar). The sample plates were then incubated under aerobic conditions at 30°C for 48 h. Upon the growth of bacterial colonies, sub-culturing was performed until single bacterial colonies were obtained. For the identification of the isolated bacterial species, 16s rDNA base sequence analysis (approximately 1500 bp) and BLAST analysis were performed at the Techno Suruga Laboratory, Japan. The identified bacterial species were precultured (5 mL) in a ZoBell2216 medium (5.0 g/L of polypeptone, 1.0 g/L of yeast extract, and 0.1 g/L of $FePO_4$, prepared with artificial seawater (Table 5), pH 7.6–7.8, shaken at 160 rpm for 24 h at 30 °C). After 24 h, 100 mL of the ZoBell2216 medium was used as the culture medium for the cultivation of the species. Subsequently, it was continuously cultivated for 10 days. During cultivation, the cell concentration was measured (OD_{600}) using a UV-vis spectrophotometer (V-730, JASCO Corporation, Tokyo, Japan) and then the urease activity was checked using the indophenol method (Natarajan 1995).

2.2 Urease activity test

For the determination of urease activity the indophenol method (Natarajan 1995) was used. In this method, the

bacterial cells were evaluated by ureolysis that was catalyzed by the cells' suspension in a solution. Considering a certain interval of time, 1 mL of the cell culture was added to 100 mL of the solution composed of 0.3 M urea in a 0.1 M EDTA buffer and phenol-nitroprusside. The desired pH, temperature, and cell culture time were controlled during this stage. A constant water bath was used to keep the reaction mixture at a controlled level. From the reaction mixture 10 mL was sampled at a certain time of interval (5 min for 4 sampling points) and simultaneously 0.1 mL of 10 M NaOCl was added to the sampling solution to stop the enzymatic reactions. After this, the sampling solutions were incubated at 50-60°C for 10 min. The temperature dependency (10-60°C) and pH dependency (6-9) were also investigated using EDTA buffer solutions at 30°C.

2.3 Microbial $CaCO_3$ precipitation test

The selected species was cultured with a ZoBell2216 medium (5.0 g/L of polypeptone, 1.0 g/L of yeast extract, and 0.1 g/L of $FePO_4$) prepared with artificial seawater at a pH of 7.6-7.8, until the maximum cell growth was obtained. The cell growth (OD_{600}) was measured using a UV-vis spectrophotometer (V-730, JASCO Corporation, Tokyo, Japan). 0.5 M urea and 0.5 M $CaCl_2$ were placed in a test tube considering the final volume of the test tube as 10 mL. The bacterial culture was centrifuged at 12,000 rpm for 10 min. The supernatant and cell pellets were separated, and the bacterial cell concentration was adjusted (OD_{600}) using distilled water. The adjusted bacterial cell concentration was then added to the test tube and kept in a shaker at 30°C for 48 h. Then the precipitation (white crystals) was observed and the precipitated calcite (centrifuged at 12,000 rpm for 10 min.) was separated from the culture solution using a filter paper. Precipitated crystal tubes were then kept in an oven drier (100°C, 24 h) and then weighed using a scale to calculate the total calcite precipitate amount.

2.4 Distribution of urease

To investigate the secreted urease distribution, the urease activities of the culture supernatant were also examined by the indophenol method. The cell culture (1.2 mL) was centrifuged (10,000 rpm, 5 min, 10°C) and 1 mL of the supernatant was used to examine the urease activity using the indophenol method. 40 mL of the culture solution was collected from the cell culture and centrifuged (10,000 rpm, 5 min, 10°C) to remove the supernatant. The cell pellet was washed with a 0.1 M Tris/HCl buffer twice and resuspended in the same buffer to obtain a cell suspension. The urease activity test was conducted using cell suspensions with different OD_{600} values. The urease activity of the whole-cell, cell lysate, and soluble and insoluble fraction of the cell lysate were also evaluated. After removal of the culture supernatant, 10 mL of the resuspended solution was collected from the cultured cell and centrifuged using the following method with 500 mL of sonication buffer (20 mM Tris/HCl, 0.1 M NaCl, 1 mM EDTA). This cell suspension was denoted as the "cell pellet." After centrifuging (8000 rpm, 20 min, 4°C), the supernatant and cell debris were separated, and the urease activity tests were conducted to locate the distribution of the urease compared to the supernatant and cell pellet.

3. Results and discussion

3.1 Screening and cell culture

For the molecular identification and confirmation of the species. 16s rDNA analysis was conducted, and the result was confirmed using a third-party company (DB-BA12.0, TechnoSuruga Laboratory) compared to the result using DDBJ/ENA(EMBL)/GenBank, Japan. The identified species are shown in Table 1. As far as is known, this is the first attempt to isolate and characterize the efficiency of ureolytic bacteria that can be applied to protect severe coastal erosion areas such as those of Bangladesh and Greece.

From the previous results, it was found that the *Lysinibacillus* sp. is a gram-positive, rod-shaped, and round-spore-forming bacterial genus in the family Bacillaceae. It can survive in harsh conditions and is able to survive within a pH range of 6-10 (Seifan *et al.* 2017). *Micrococcus yunnanensis* is a gram-positive, non-endospore forming bacteria and its temperature and pH tolerance ranges for growth are 4-45°C and pH 6-8, respectively (Zhao *et al.* 2009). *Pseudoalteromonas* sp. is a gram-negative, rod-shaped bacterium, motile by a polar flagellum which helps it move back and forth between solid surfaces. It requires sodium ions for growth and has an oxidative metabolism (Vera *et al.* 1998). It has also been found that bacterial cell growth is closely related to cell culture duration (Okwadha *et al.* 2010). Moreover, it has been concluded that the rate of urea hydrolysis is directly proportional to the concentration of bacteria, which is an important factor for the success of the MICP application

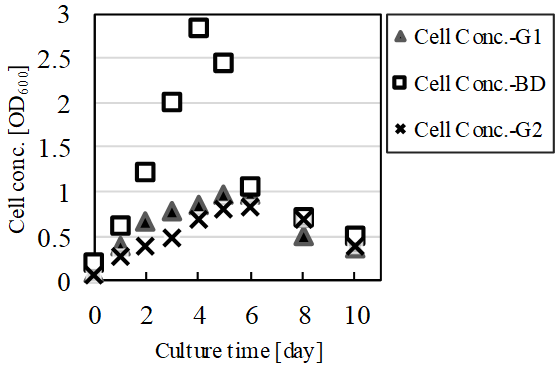

Fig. 2 Relationship of cell growth with culture time of the isolated species

Table 1 Identified species used in this experiment

Sample name/code	Sampling country	Identified species	Biosafety level
BD	Bangladesh	*Lysinibacillus* sp.	1
G1	Greece	*Micrococcus yunnanensis*	1
G2	Greece	*Pseudoalteromonas* sp.	1

(Lauchnor *et al.* 2015). Fig. 2 shows the cell growth with culture duration of the isolated species. As shown in Fig. 2, it can be seen that initially the cell concentration increased and thereafter urease activity decreased with time producing a bell-shaped profile for each species.

However, from this experimental result it has been shown that the behavior of individual bacterial species has a dissimilar tendency for cell growth even under extended cultivation. The findings of this study could support to regulate the tentative duration/time for the sand solidification process using the re-injection or bio-grouting method (Amarakoon and Kawasaki 2017, Kim and Park 2017) during the MICP process.

3.2 Urease activity

Various factors have been proposed that affect urease activity and calcite precipitation (Yasodian *et al.* 2012) but limited attention has been paid to determining the effect of cell culture duration to urease activity. Because bacterial cell culture duration is related to the urease activity of bacterial cells, it has been noted that a high bacterial cell concentration enhances the amount of calcite precipitated using the MICP method (Fukue *et al.*, 2011). Moreover, the rate of urea hydrolysis is directly proportional to the concentration of bacteria, which is an important factor for the success of the MICP application (Ferris *et al.*, 2004), which also supports our research findings in this study. Fig. 3 shows the urease activity of the isolated species. As shown in Fig. 3, it can be seen that initially the urease activity increased with time and, thereafter, the urease activity declined nearly forming a bell shape. However, the maximum urease activity is dissimilar for each individual species.

The findings of this study are correlated with those of previous studies (Fujita *et al.* 2017) and can aid in improving the sand solidification process by determining the time of adding/re-adding bacteria to the sample before the urease activity becomes insignificant.

3.3 Effect of pH on urease activity

It has been reported that urease activities are closely related to pH value (Stocks-Fischer *et al.* 1999), although the endurability and growth of bacteria in a relatively harsh environment has been reported by Kim *et al.* (2015). It has also been found that some of the bacterial isolates, including *Bacillus megaterium* and *B. cereu*, could grow well in a pH range of 6.5 to 11.5, while other isolates (*B. thuringiensis*, *B. subtilis*, and *Lysinibacillus fusiformis*) are capable of surviving within a pH range of 6-10 (Kaur *et al.* 2013). In another investigation, Kim *et al.* (2015) tested different bacterial strains using Difco nutrient broth with a pH of 11 to figure out whether they can grow under an alkaline condition. Among the isolates, only *Sporosarcina* sp. and *Bacillus* sp. can grow in such an alkaline environment. In general, bacterial cells quickly adapt to environmental conditions at a lower pH by showing a shorter lag phase. Regardless of all of these findings, urease activity in such environments has remained a matter of argument. Therefore, it is very important to investigate the

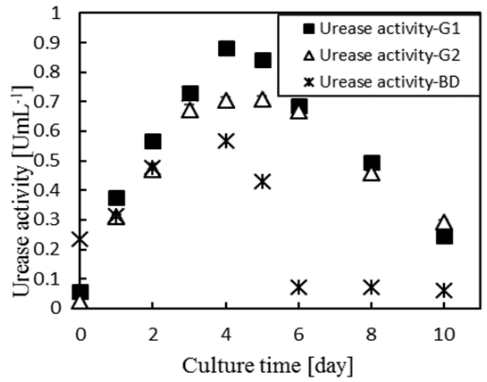

Fig. 3 Relationship of urease activity with culture time of the isolated species

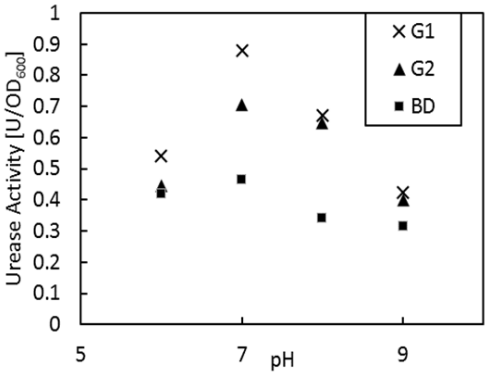

Fig. 4 Effect of the pH of the reaction

urease activity of bacterial species that can lead to the determination of appropriate conditions to induce $CaCO_3$ precipitation within different ranges of pH. Fig. 4 shows urease activity under different pH conditions at a certain temperature (30°C) for the G1, G2, and BD species. The results show a nearly bell-shaped profile with the maximum activity approximately at pH 7 for the G1, G2, and BD species. Under the same conditions, the G1 species showed the highest urease activity compared to that of the G2 and BD species. This result showed that the bacterial cells are highly sensitive to different pH conditions and the selected bacterial species are able to grow under alkaline conditions.

However, under a high alkaline condition their urease activity decreased. From previous research, it was noted that the sand solidification process using the MICP method favored an initial pH environment of 6.5-9.3 (neutral to basic condition) (Amarakoon & Kawasaki, 2017). Additionally, it was also shown that in the case of some bacterial species (*Pararhodobacter* sp.), a significant decrease in urease activity occurred under acidic (lower than pH 6) and alkaline (higher than pH 9) conditions, which was attributed to protein denaturation (Fujita *et al.*, 2017). However, the experimental findings presented in this study can provide very useful information for conducting an efficient sand solidification and biomineralization process.

3.4 Effect of temperature on urease activity

Earlier studies reported that bacterial enzymatic reactions (urea hydrolysis by urease) are dependent on

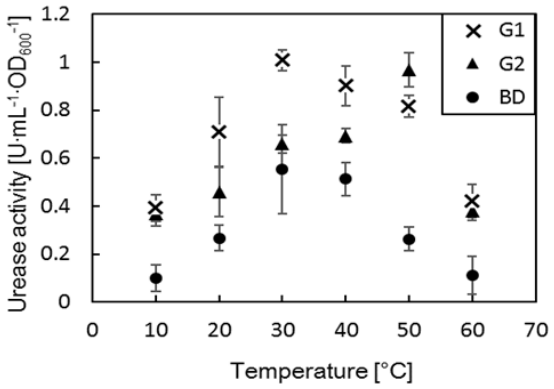

Fig. 5 Effect of temperature on urease activity of the reaction media of the isolated species

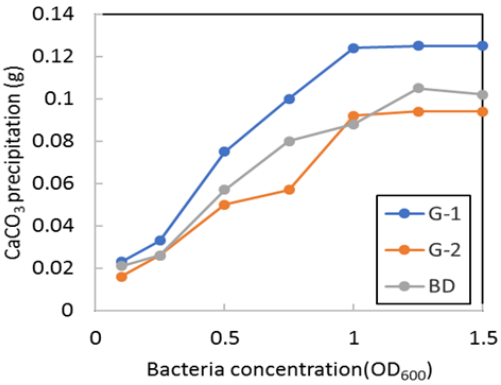

Fig. 6 Effect of bacteria concentration (OD_{600}) on $CaCO_3$ precipitation

temperature and the optimum temperature that favors the urease activity ranges between 20°C and 37°C (Okwadha *et al.* 2010, Mitchell *et al.* 2005). However, bacterial enzymatic reactions for optimal urease activity are greatly influenced by environmental parameters and the concentration of reactants in the system. A study conducted by Mitchell and Ferris (2005) reported that urease activity increased from 5 to 10 times when the temperature increased from 10 to 20°C. The kinetic rate of urease and the temperature dependency on *Sporosarcina pasteurii* was also investigated by Ferris *et al.* (2004) and Dhami *et al.* (2012). Their findings showed that urease activity was very stable at 35°C but it decreased by 47% when the temperature increased by 55°C. Other studies have also reported that physical parameters such as incubation temperature play important roles in affecting urease activity (Harkes, *et al.* 2010). Additionally, the critical temperature parameter depends on individual bacterial species (Helmi *et al.* 2016). Therefore, among all parameters, temperature is considered the most important factor to be studied to determine the optimal temperature that can facilitate selecting an appropriate condition for the maximum urease activity of ureolytic bacteria. Urease activity under different temperature conditions is shown in Fig. 5 for the isolated species. The results presented in Fig. 5 show that high urease activity was obtained at temperatures above 30°C for the G1 and BD species and thereafter gradually decreased, except in the case of the G2 species whose high urease activity was obtained at 40°C. These temperatures correspond to the standard temperatures of Greece and Bangladesh. Therefore, it is possible that the selected bacterial species would yield substantial urease enzymes at a temperature between 25°C to 40°C as shown in our experiment. Therefore, on the basis of these experimental findings and corresponding with previous research, our study found that the urease activity of individual bacterial species strongly relies on different temperatures because the rate of urea hydrolysis changes with changes in temperature, as the kinetic energy induces the collisions between an enzyme and its substrate (which can be evaluated by whole cell evaluation) which is capable of stimulating and altering the cellular membrane of the bacteria for urease activity. These results are very useful in a practical manner for soil treatment using the MICP method with "bio-augmentation" or "bio-stimulation" which could be favorable in the local environment.

3.5 Microbial $CaCO_3$ precipitation test

Earlier studies showed that it is possible to control the strength of the treated sand by adjusting the amount of precipitated minerals (Putra *et al.* 2017). It has also been reported that various environmental factors, including temperature, pH, bacterial size, and cell concentration, control the amount of calcite precipitation during the MICP process (Keykha *et al.* 2017). Therefore, it is very important to investigate the trend in $CaCO_3$ precipitation for individual bacterial species because the $CaCO_3$ acts as the main binder material in between the substrate particles for soil improvement during the MICP process. The $CaCO_3$ precipitation trend for the G1, G2, and BD species is shown in Fig. 6. From the figure, it is clear that $CaCO_3$ precipitation is closely related to the OD_{600} values, which supports previous studies. From these studies, it has also been observed that higher OD_{600} values influence the $CaCO_3$ precipitation amount. Under the same condition, the G1 species has the advanced ability to precipitate a higher amount of $CaCO_3$ compared to the G2 and BD species, leading to a higher possibility of sand solidification. At the same time, the rate of the precipitated $CaCO_3$ decreases and it almost constant with a further increase in the bacterial cell concentration. However, this study suggests that the variation in the $CaCO_3$ precipitation trend is related to the bacterial population of individual species, which supports earlier studies.

3.6 Distribution of urease

Urea hydrolysis is a very significant pathway during the MICP process as the urease enzyme is directly used during the MICP process as a whole-cell during the biomineralization process (Danjo and Kawasaki, 2016, Al-Thawadi and Cord-Ruwisch 2012). The evaluation of urease distribution in the cell (secretion or non-secretion, cytoplasm or cell membrane) is substantial information for an effective solidification process because complicated bi-/tri-phase reactions occur in the mixture of solution, cells. and sand particles during urea hydrolysis (Fujita *et al.* 2017). However, insufficient information is available regarding specific secretion pathways of urease enzymes for ureolytic bacteria. Thus, we investigated the secretion pathway of the urease enzyme using the soluble fraction of

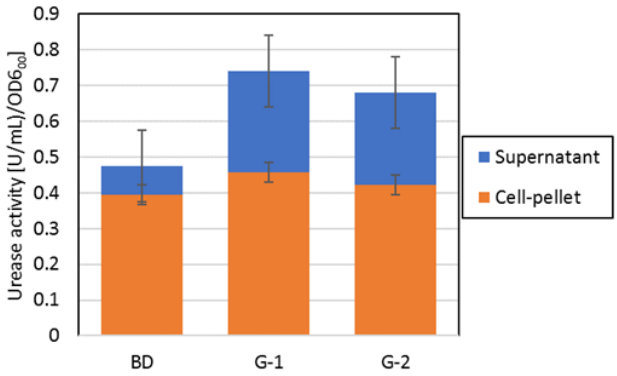

Fig. 7 Effect of bacterial concentration (OD_{600}) on calcite precipitation

the cells of the ureolytic bacteria (using the G1, G2, and BD species).

The total cell culture (supernatant and cell-pellet) and the urease activity is shown in Fig. 7 for the isolated species. Fig. 7 shows that considerably lower activity was observed (BD species) in the culture supernatant compared to the supernatant of the cell culture. However, substantial urease activity was found on the supernatant and cell pellet for both the G1 and G2 species. This indicates that urease would not be secreted to the extracellular medium but would likely have urease enzymes on the cell membrane, similar to urease from *Helicobacter pylori* (Phadnis *et al.* 1996) where it is localized on the cell membrane. Because the enzyme likely accumulated in/on the cell membrane, quite low enzymatic activity was found in the culture supernatant. The urease enzymes (proteins) are released by autolysis linked with the cell surface by reabsorption and secretion of the urease enzyme (protein) relying on selective bacterial species as investigated during the growth phase in this study. However, the findings in this study can be useful information to determine the phase of the reaction/mixture in a culture medium and for treating soil improvements using the MICP method either by enzymatic extraction and/or centrifugation of the cell or modification of the injection pattern (Chang *et al.* 2016).

4 Conclusions

Most studies have investigated urease activity using land ureolytic bacteria using the MICP method with a focus on coastal erosion protection. Therefore, to overcome these research gaps, in this experiment we used ureolytic bacteria isolated from the local coastal area. The results of our experiment provide insight into the factors that affect urease activity of the local ureolytic bacteria species with a prospect for successful sand solidification. This study also presents the effects of various cultural conditions to optimize the urease activity for the isolated bacterial species that could be applied to solve problems relating to environmental biotechnology, civil engineering, and geotechnical engineering. Moreover, the isolated ureolytic bacteria described in this study may hold additional potential in the field of MICP and can serve as an advantageous reference resource for researchers in microbial biotechnology and construction microbial biotechnology. The results of this study can also be applied to the creation of artificial beachrock using the MICP method for coastal erosion protection in Mediterranean countries, Bangladesh, and other coastal areas as well for bio-mediated soil improvement, establishing an economical and environmentally friendly countermeasure for coastal erosion protection and bio-stabilization.

Acknowledgements

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