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To cite this article: Farzin Kalantary, Dariush Abbasi Govanjik & Mahdi Safdari Seh Gonbad (2019): Stimulation of Native Microorganisms for Improving Loose Salty Sand, Geomicrobiology Journal, DOI: [10.1080/01490451.2019.1579876](https://doi.org/10.1080/01490451.2019.1579876)

To link to this article: <https://doi.org/10.1080/01490451.2019.1579876>



Published online: 12 Mar 2019.



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Stimulation of Native Microorganisms for Improving Loose Salty Sand

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ABSTRACT

Prolonged droughts and excessive water harvesting in western Asia has accelerated desertification and caused longer dry seasons of salt lakes. The Aral Sea experience has proven that dust from saline soil is a serious health issue. Various stabilization techniques to reduce wind erosion have been used in the past. However, in recent years, a potentially viable method has been developed; microbial induced calcite precipitation (MICP) has been introduced as a method of soil stabilization, though its effectiveness in saline soils remains to be examined. The effect of salt content in loose sandy soil on calcite precipitation of calcite through stimulation of native bacteria is investigated in this article. Samples with salinity up to 30% salt content were prepared and treated with different culture medium compounds. A number of tests were used to evaluate the effect of the mentioned parameters. The results show that improvement increases with increasing salinity up to 5% salt, and further increase in salinity reduces the effectiveness of improvement. It is also shown that the addition of urea in the culture medium has a significant effect on the urea hydrolysis which resulted in a five-fold increase in compressive strength. Four native strains of halotolerant urease-positive bacteria were also identified.

ARTICLE HISTORY

Received 5 June 2018
Accepted 3 February 2019

KEYWORDS

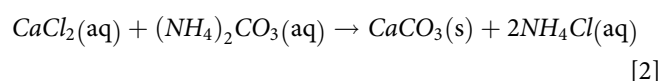
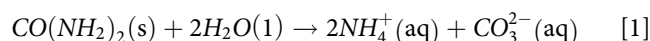
Microbial induced calcite precipitation (MICP); native bacteria; saline soil; culture medium; soil stabilization

Introduction

Wind erosion in saline lands such as saline deserts, dried wetlands, hypersaline lakes, salt marshes etc., causes many problems for the people of adjacent areas (Gerivani et al. 2011; Gholampour et al. 2017; Opp et al. 2017). Salinization of farmlands affecting agricultural products as well as health issues such as respiratory and pulmonary diseases are among the problems of wind erosion of saline soil. Due to the presence of salt, the erosion in the saline lands is more important than the ordinary lands (Abuduwaili et al. 2015; Ataniyazova 2003; Gholampour et al. 2015). In general, the effect level and the sensitivity of surface soil layers to the wind is a key factor in controlling wind erosion. Hence, the formation of a hard shell on the soil can reduce the risk of wind erosion (Gillette et al. 1980, 1982; He et al. 2008). Soil stabilization methods require solutions that guarantee the efficiency and effectiveness of improvement, have high durability and are environmentally friendly (DeJong, Soga, et al. 2010; Gomez et al. 2015).

In recent years, microbial induced calcite precipitation (MICP) has been shown to be effective as well as being environmentally friendly (DeJong et al. 2006; DeJong, Mortensen, et al. 2010). This method is meant for the improvement of the mechanical properties of soil based on a biochemical process. Hydrolysis of urea to ammonia and carbonate is induced by an enzyme secreted by bacteria

(Equation 1). In the presence of calcium chloride, the released carbonate readily reacts to form calcium carbonate (CaCO_3) (Equation 2) (Whiffin et al. 2007).



Various studies have proposed this method for mitigation of soil liquefaction (Montoya et al. 2013), improvement of the load bearing capacity of foundation and slope stability (Van Paassen et al. 2010, Cheshomi et al. 2018), construction of impervious shells with soils (Smith et al. 2017), concrete and masonry repair (Wiktor, & Jonkers, 2011; Amidi and Wang, 2015), wastewater treatment (Hammes et al. 2003), accumulation and stabilization of heavy metals (Fujita et al. 2010; Mwandira et al. 2017), capture and store atmospheric carbon (Washbourne et al. 2012), increasing the consolidation of fine materials (Liang et al. 2015) and improving the shear strength of organic soils (Canakci et al. 2015).

Microbial induced calcite precipitation is thought to be affected by various parameters such as dissolved properties (Mortensen et al. 2011; Tang et al. 2017), type and characteristics of urease-positive bacteria (Mobley et al. 1995), soil type and properties (DeJong et al. 2017; Saricicek et al. 2018; Terzis and Laloui 2018), injection method (Harkes

et al. 2010), effects of air compositions and culture media of urease-positive bacteria (Bundur et al. 2017; Li et al. 2018), effect of pH (Seifan et al. 2017), growth and curing time (Sotoudehfar et al. 2016), and temperature (Keykha et al. 2017).

Other researchers, including Gomez et al. (2014, 2017, 2018) and Amini Kiasari et al (2018) have introduced the simpler and less costly method for biological improvement of poor sandy soils in the term of biostimulation which is the modification of the environment to stimulate existing bacteria capable of bioremediation. In this method, they used indigenous bacteria inside the soil to produce the urease enzyme and sedimentation of calcite. In order to evaluate the efficiency of this method, they have measured the pH, shear and compressive strength, permeability and amount of sediment formed, and sample dimensions. The research results of this group showed that indigenous urease-positive bacteria could be stimulated in order to improve the engineering properties of the soil.

Salt concentration in the environment is one of the factors affecting the growth and activity of microorganisms. High salt concentration leads to the destruction of cell membranes and the deactivation of many enzymes, which can be fatal for the microorganisms in the environment (Kargi and Dinçer 2000). Growth and activity of halophilic bacteria in high salt concentrations is possible, while halotolerant bacteria have better growth and activity at lower concentrations and their growth decreases by an increase in the salt concentrations (Lanyi 1974; Margesin and Schinner 2001; Ventosa et al. 1998).

Zheng et al. (2009), investigated the effect of salt on a type of halotolerant bacteria. Their results showed that the maximum growth of this bacteria occurs in concentrations of 0–3% of salt, and with an increasing salt percentage from 7 to 10%, the growth of bacteria decreases significantly. An increase in salt content can cause adverse effects on the activity of microorganisms through osmotic pressure in the environment. In halotolerant bacteria, there are mechanisms that allow them to pump excess salt ions outside the cell and provide a good balance between the concentration of salt inside the cell and the outside (Moradi et al. 2011).

Considering the increasing importance of preventing the wind erosion of saline sandy lands and the benefits of improving the microbial-induced calcite precipitation, in particular the simpler and less costly method of using native bacteria instead of augmented bacteria to stabilize susceptible sand to wind erosion, this research attempted to assess the effects of salinity and culture medium components by stimulation of the native bacteria. In this regard, different salt contents (0%, 1%, 3%, 5%, 10%, 20%, and 30%) were used. The effect of the presence or absence of urea in the culture medium was also evaluated. In order to quantitatively and qualitatively evaluate the samples, Unconfined Compressive Strength test, calcimeter and scanning electron microscopy (SEM) apparatus were used. Standard plate count, urease and PCR tests were also used to isolate and identify native urease-positive bacteria in the soil.

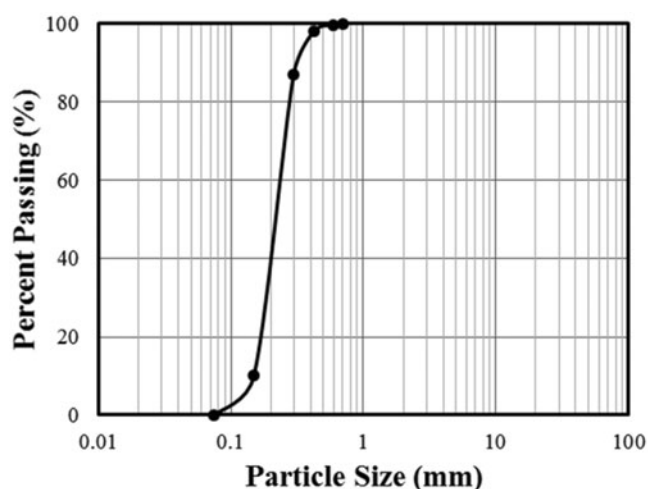


Figure 1. Soil particle size distribution curve.

Table 1. Soil characteristics.

Description	S
Coefficient of uniformity, C_u	1.60
Coefficient of curvature, C_c	0.90
Effective grain size, D_{10} (mm)	0.15
D_{30} (mm)	0.18
D_{60} (mm)	0.24
Maximum dry density (kN/m^3)	14.90
Minimum dry density (kN/m^3)	17.60
D_r (%)	35

Table 2. Specifications of the culture media.

Type of culture media solution	Composition	Concentration (g/L)	pH
Yeast extract + Urea	Yeast extract	20	8.5
	NH_4Cl	10	8.5
	$\text{CH}_4\text{N}_2\text{O}$	20	8.5
	NaOH	0.4	8.5
Yeast extract	Yeast extract	20	8.5
	NH_4Cl	10	8.5
	NaOH	0.4	8.5

Materials

Soil

A cohesionless sandy soil (classified as SP), was used in this study. The particle size distribution curve of the soil sample used in the tests is shown in Figure 1. The summary of the physical and mechanical properties of the soil according to the respective ASTM standards, are presented in Table 1. Sodium chloride with 99.2% purity was used to alter the medium's salinity.

Ingredients of the culture medium and cementation solution

In this study, two types of liquid culture media, one with urea and one without urea, with the specifications listed in Table 2, were used.

The so-called cementation solution was a mixture of calcium chloride with urea (Table 3).

Table 3. Specifications of the cementation solution.

Cementation solution	Composition	Concentration (g/L)	pH
Calcium chloride + Urea	CaCl ₂	147.02	6.5
	CH ₄ N ₂ O	60.06	6.5

Preparation and testing procedures

Preparation of culture media and cementation solution

Preparation of the culture medium can be carried out in a variety of ways. This work has been carried out in accordance with the instructions given on the containers of compositions of the culture medium, as expressed by the manufacturing companies. The culture medium should be prepared at the indicated pH and bacteria should be cultivated in it. Urease-positive bacteria are alkaline and perform better in high pH medium (Keykha et al. 2017). In this paper, a medium with pH = 8.5 was used.

Like the culture medium, the preparation of the cementation solution is also carried out according to the instructions given on the containers of compositions of the cementation solution.

Sample preparation

As a criterion for assessing the resistance of the soil, an Unconfined Compressive Strength test was used on the made sample with geometric characteristics similar to the ASTM D2166 2016 standard.

The sample was prepared as follows:

1. The mold was cleaned and its inner surface was slightly lubricated.
2. The interior surface of the mold was covered with a very thin layer of talc so that it would be easy to remove the sample from the metal mold after the completion of the injection.
3. At the bottom of the mold, a layer of gravel and on top of that a layer of sponge was placed to act as a filter. The thickness of the two layers was 1 cm in total.
4. Inside the mold was filled with sand with an approximate density of 1.5 g/cm³ ($D_r = 35\%$).
5. Similar to the bottom of the mold, in the upper part, a layer of sponge and a layer of gravel were placed and the sample was completely sealed.

In [Figure 2](#), the various stages of preparation of the sample are shown.

Injection

In this research, a total of 33 soil samples with 0%, 1%, 3%, 5% of salt without urea and 0%, 1%, 3%, 5%, 10%, 20%, 30% of salt with urea in a culture medium was made (3 sand blocks are made and used for each treatment). The injections were made Bolus, gravity, and top-down. The schematic drawing of the injection is shown in [Figure 3](#).

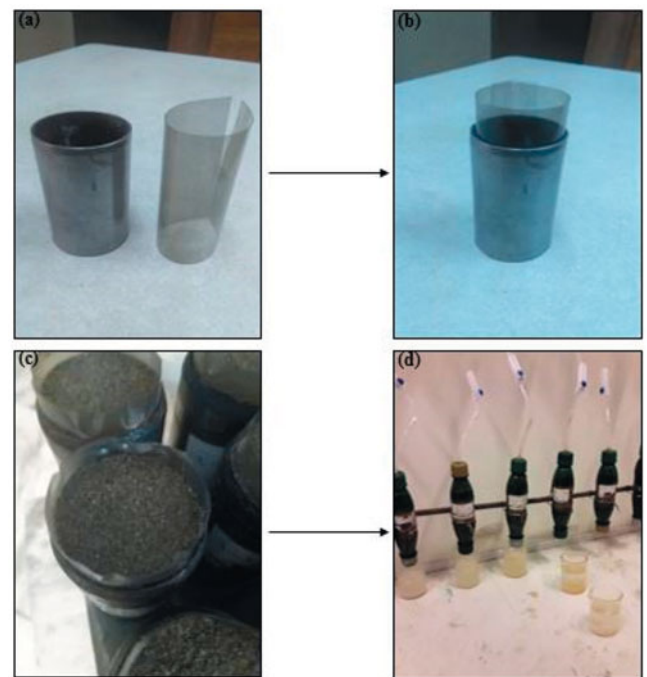


Figure 2. Sample preparation stages; (a) Lubrication of moulds, (b) covering the mould with a thin layer of talc, (c) filling the mould with sand, and (d) placing layers of sponge and gravel on top and bottom of the mould.

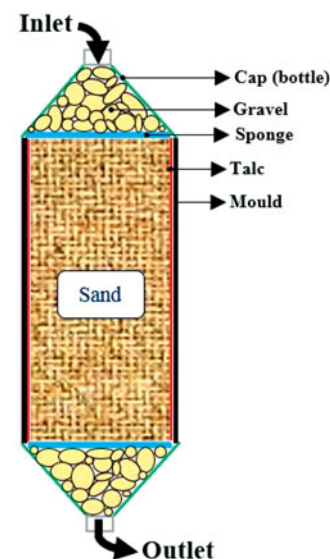


Figure 3. Schematic drawing of the injection.

Injections of the culture medium and cementation solution were performed separately. At first, the medium with the dose of 30 ml was injected into the soil samples in a 6-day period with a 24-h interval and after that, the samples were left for 1 day. Then the injection period of the cementation solution with the dose of 30 ml was started and performed at a 24-h time interval for 6 days.

Samples were stored at room temperature (25 °C) for 1 day at the end of the injection period of the culture medium and the cementation solution. Subsequently, according to [Figure 4](#), the samples were brought out from the inside of the mold and transferred to the oven to dry

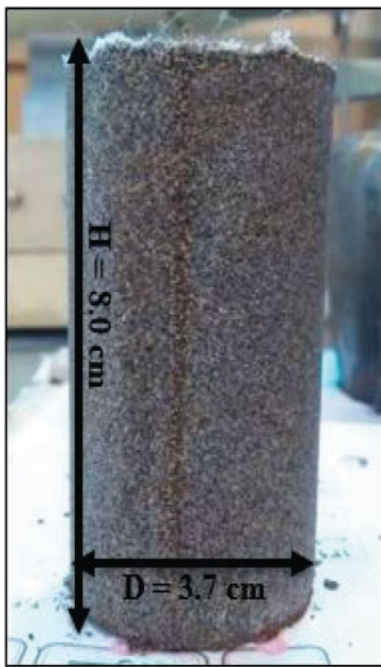


Figure 4. Sample dried at oven.

out at 110°C for 24 h and to prepare for an Unconfined Compressive Strength test.

Experimental program

After preparation, injection and drying out the samples, for quantitative and qualitative evaluation, the Unconfined Compressive Strength and calcimeter tests were carried out on the samples. Finally, scanning electron microscopy (SEM) was used to produce images of the samples.

Unconfined compressive strength test

The Unconfined Compressive Strength test is commonly used for clay samples, but this test can also be used for cemented samples. In this test, an axial displacement rate of 0.5 mm/min was used throughout until the failure of samples (strain control).

Calcimeter test

In order to determine the percentage of calcium carbonate sedimentation formed due to the microbiological sedimentation, Bernard Calcimeter apparatus was used. Bernard Calcimeter is an apparatus that can measure the amount of CO₂ gas resulted from the reaction of the calcium carbonate and diluted hydrochloric acid, and measure the amount of the resulted sedimentation (Calcium Carbonate). (Krumbein and Pettijohn 1938).

Scanning electron microscopy (SEM)

The scanning electron microscope is a type of electron microscope that produces images of a sample by scanning

the surface with a focused beam of electrons. The SEM analysis provides information on the topography of the sample and its surface characteristics, morphology, shape, size, arrangement of the particles on the surface of the object and the chemical composition of the sample phases (Echlin, 2011; Goldstein et al. 2017).

SEM analysis was used for visual observe the shape and distribution of the sediment between sand particles in pre and post improvement conditions.

Isolation and identification tests for the urease-positive bacteria of soil

In order to isolate bacteria in the soil, the method of Standard Plate Count through preparation of serial dilution is used. In this method, a certain volume of the sample is added to the prepared solid culture medium (Nutrient Agar) and then spreads uniformly in a sterile condition with the L shape spreader on the medium (Willey et al. 2008).

In this method, 0.1 ml of the sample with prepared dilution is cultured on a plate by a spreader. It will be placed for 24 h in standard conditions in incubation and then plates with colonies of 30–300 will be used for counting. Colonies counting unit is Colony Forming Units (cfu).

In this study, the samples were diluted 7 times each.

1. Seven test tubes containing 9 ml distilled water were sterilized.
2. 1 g of soil was transferred to the first tube under sterile conditions. The contents of the tube were slowly blended to obtain a consistent dilution.
3. From the first tube, 1 ml was transferred to the second tube. This was repeated for six other tubes as well.
4. From each tube, 0.1 ml was transferred to Nutrient Agar solid plates.
5. Plates were incubated for 24 h and the number of colonies was counted.
6. Then plates with standard colonies were used for purification.

Urease test was also performed to detect the ability of the organisms to hydrolysis urea. Urea is a product of decarboxylation of amino acids, when urea is hydrolyzed it generates carbon dioxide and ammonia, the production of ammonia causes the ambient to become alkaline and changes color from yellow or orange at pH = 7 to red or pink at pH = 8. Urease-positive organisms can change the color of the entire ambient to pink within 24 h. For poorly positive organisms, this color change may take several days. Negative organisms do not cause color change or the ambient remains yellow.

In order to identify and detect the type of urease-positive bacteria, the Polymerase Chain Reaction (PCR) was used. The PCR *in vitro* allows the reproduction of a certain sequence of DNA between two distinct sequences, this is accomplished with the aid of a thermocycler in order to reach the sufficient amount of DNA to allow for electrophoresis, agarose gel, polyacrylamide, and probe

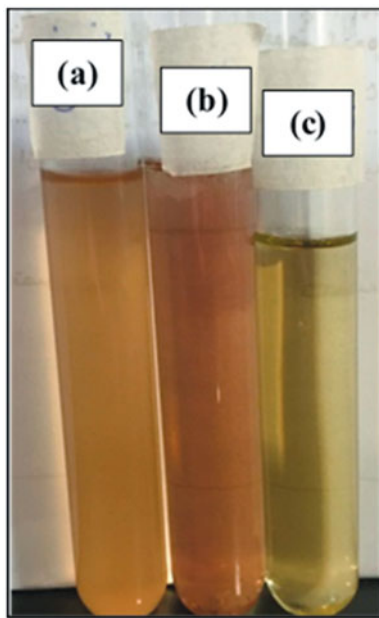


Figure 5. Urease test for isolated bacteria; (a) Strain with no urease activity, (b) urease positive (c) blank.

hybridization tests. This method is scientifically similar to DNA replication.

Results

Results of isolation and identification of the urease-positive bacteria of soil

After three stages of purification, 9 strains were isolated from the soil. The strains that were pure and free of contamination were used for urease testing. Of the 9 strains tested, 4 strains were urease-positive and changed the ambient color to pink (Figure 5). Also, to investigate the effect of salinity the growth of all 4 strains of urease-positive bacteria in saline and salt-free ambient was checked, it was determined that all four strains were of halotolerant bacteria.

The results of PCR are as follows:

- Strain 1 is 100% similar to *Streptomyces flaveolus*.
- Strain 2 is 99.65% similar to *Streptomyces coelicoflavus*.
- Strain 3 is 100% similar to *Streptomyces erythrogriseus*.
- Strain 4 is 99.68% similar to *Streptomyces cavourensis*.

Performance evaluation of the native urease-positive bacteria

Calcium carbonate can be formed in several crystal types of Calcite, Vaterite, and Aragonite. Calcite is the most resistant of all (Van Paassen, 2009). In appearance the Calcite has polygons form (diamond, square, rectangular, etc.), Vaterite has spherical and planar forms, and Aragonite has a needle-shaped form (Dana 1869; Deer et al. 1992; Effenberger et al. 1981; Klein et al. 1993).

Images obtained through SEM are shown in Figures 6 and 7. Figure 6, is for the uncemented sample, and Figure 7 for the cemented samples. It is seen in Figure 6 that there is

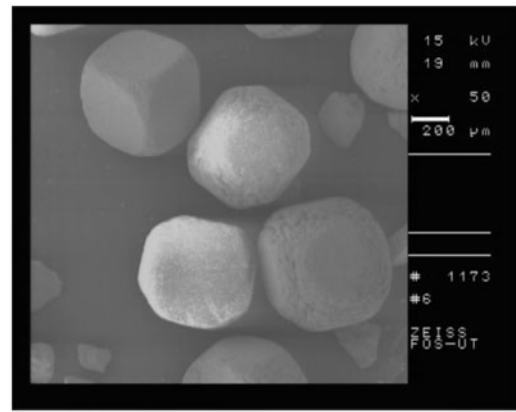


Figure 6. SEM graph of uncemented sand (50× magnification).

no sediment or other adhesive factors between the sand particles and the empty space between the grains is quite evident.

In Figure 7(a), the small-scaled image of the improved sample is shown. Even on this scale, the adhesion between the particles is clear. In Figure 7(b), which is a larger scaled image than Figure 7(a), bonding and adhesion between the sand grains and the filled space between them are more visible. In this image, a layer of carbonate sedimentation surrounding the sand particle is noticeable. In Figure 7(c), a thin layer of sediments around the grains is clearly visible. The outer surface of the grains and the space between the grains are also filled with calcium carbonate sediments.

Figure 7(d) focuses on one of the grains and shows how the particle is covered by precipitates, which, in this figure, the sediments are almost visible and the adhesion created between the sand grains can be seen. Figure 7(e,f) shows the shape of calcium carbonate crystals, which are often polyhedral. Therefore, as previously mentioned, polyhedral sediments are calcite sediments that have the highest resistance among the three calcium carbonate sediments and have created significant adhesion between the sand grains.

Effect of culture medium urea on improvement

In this research, in order to investigate the effect of the urea presence in culture medium on cementation, 24 samples with different percentages of salt (3 sand blocks for 0%, 1%, 3% and 5% salt), once without adding urea in the culture medium, and once with the addition of urea in the culture medium, are made and tested. Vertical stress–axial strain curves of the urea-free and urea-containing improved samples variation are shown in Figures 8 and 9, respectively, and the comparative plot of the maximum compressive strength is shown in Figure 10. According to Figure 10, the highest resistance in both types of culture media is for 5% salt, and the least resistance is for the salt-free specimen.

The comparative amount of the produced sediment (calcium carbonate) percentage of urea-free and urea-containing specimens with percentages of 0%, 1%, 3%, and 5% salts obtained from the calcimeter test is shown in Figure 11.

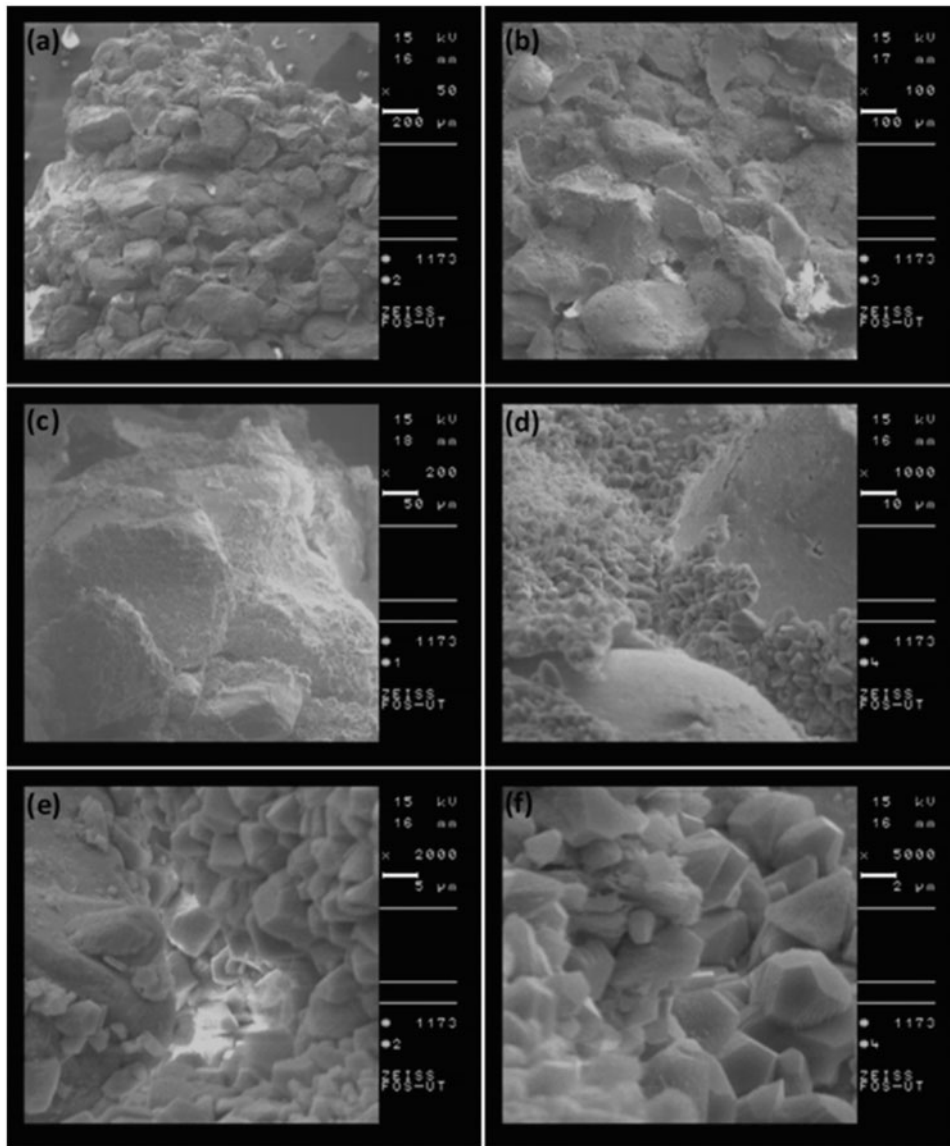


Figure 7. SEM graphs of cemented sand with different scales, (a) 50× magnification, (b) 100× magnification, (c) 200× magnification, (d) 1000× magnification, (e) 2000× magnification, and (f) 5000× magnification.

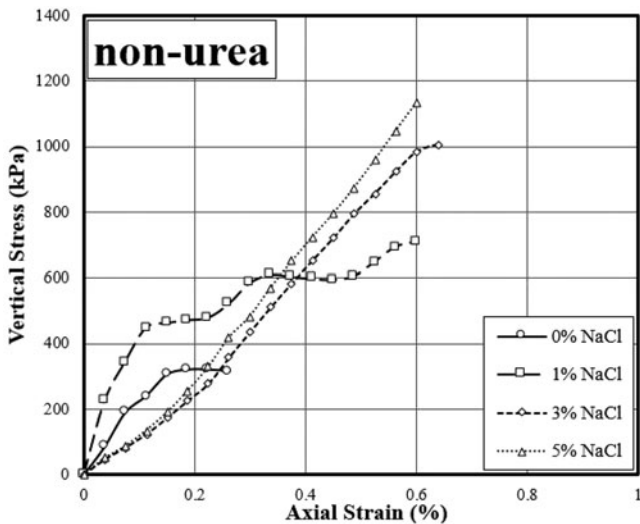


Figure 8. Vertical Stress versus Axial Strain curves for improved samples with absence of urea in the culture medium.

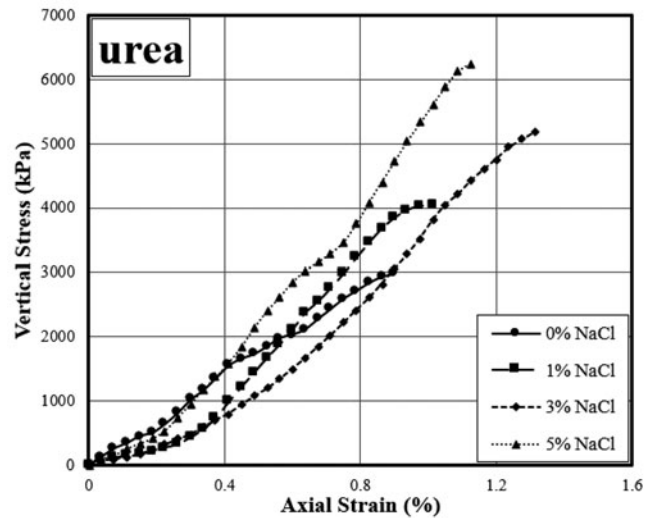


Figure 9. Vertical Stress versus Axial Strain curves for improved samples with presence of urea in the culture medium.

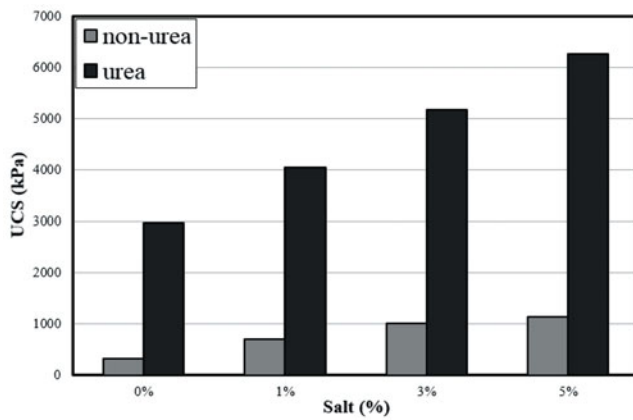


Figure 10. Comparative UCS for improved samples with presence and absence of urea in the culture medium.

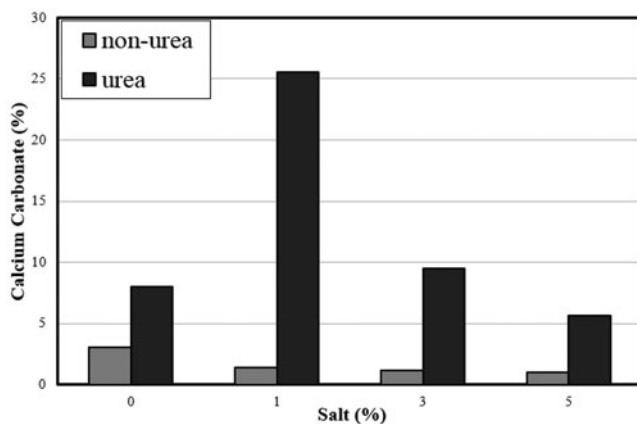


Figure 11. Comparative sediment for improved samples with presence and absence of urea in the culture medium.

According to Figures 10 and 11, it is seen that the maximum compressive strength and the percentage of calcium carbonate sediment produced by the improved specimen urea-containing culture medium were significantly higher than that of urea-free culture medium. Due to the fact that the preparation and testing conditions of all these samples are completely identical, it can be concluded that the increase in compressive strength and the percentage of sediment produced is due to the better and higher activity of the urease-positive bacteria.

The presence of urea in the culture medium is necessary because the bacteria in the preparation and cultivating stage should be familiar with the composition of urea as a nutritious substance, so that in the next stage, when the bacteria is in a favorable population, placed in a rich urea and without protein environment, it attempted to hydrolyze urea and not deal with it as a foreign agent. On the other hand, the number of native bacteria in the soil is limited and started to grow and multiply by injecting urea into the culture medium using this substance. Therefore, the lack of urea causes practically the lack of proper hydrolysis of urea by the bacteria at the injection phase of the cementation solution to the soil (Bachmeier et al. 2002; Hata et al. 2013; Stocks-Fischer et al. 1999; Xu et al. 2017).

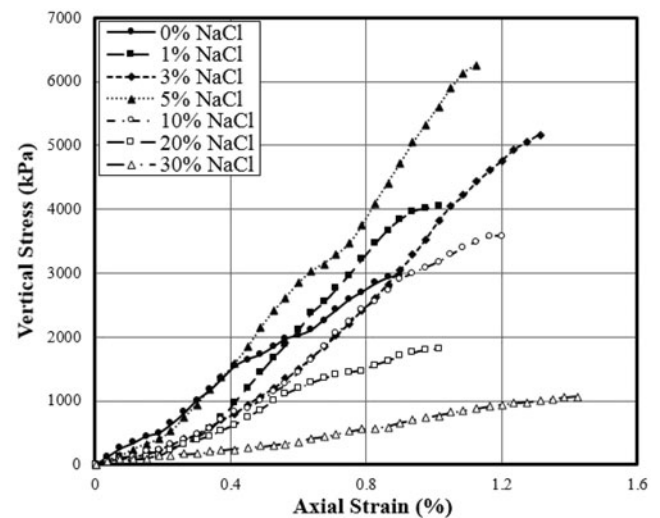


Figure 12. Vertical Stress versus Axial Strain curves for improved samples with different salt percentage levels.

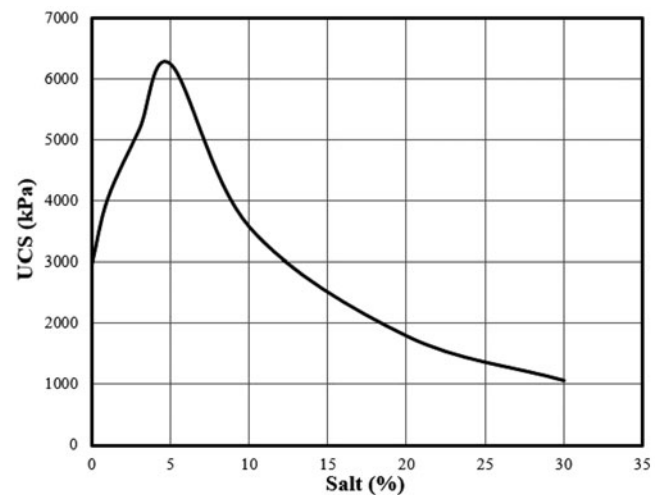


Figure 13. UCS variations versus Salt percentage levels curves.

The effect of salt content

The effect of salt content on compressive strength

In Section Effect of culture medium urea on improvement, it was found that injection of urea is very important in the culture medium so that its injection increases compressive strength and the percentage of calcium carbonate sedimentation. As a result, samples made with urea in a culture medium with 0%, 1%, 3%, 5%, 10%, 20%, and 30% salt percentages were used as a criteria to compare the effect of salt percentage and the results of samples made with the urea-free culture medium have been discarded.

Vertical Stress–Axial Strain of improved samples with different salt percentages and maximum compressive strength variations in salt percentages are shown in Figures 12 and 13, respectively.

Figures 12 and 13 show that the maximum compressive strength value initially increases with increasing salt content up to 5% and then decreases with increasing salt content. The rate of reduction of resistance (after increasing salt percentage to more than 5%) initially is high and gradually

decreases with increasing salt content, and almost reaches a constant of 1 MPa.

Because native urease-positive bacteria in the sandy soil is of the halotolerant type, they have caused the maximum compressive strength to occur in 5% salt. On the other hand, drying the salt creates some adhesion in the specimens and increases their compressive strength. Consequently, according to Figure 13, it is concluded that by increasing the salt percentage by up to 5%, the maximum compressive strength of the specimens has increased due to the bacterial activity and the adhesion resulting from the drying of the salt. By increasing the salt content to more than 5%, the activity and effect of the bacteria initially decreased so that even the adhesion obtained from the drying of the salt cannot prevent the loss of resistance. As a result, the maximum compressive strength of the samples is reduced. In high percentages of salts (20% and 30%), this decrease and increase in strength are almost in equilibrium and the maximum compressive strength will be 1 MPa.

Effect of salt content on the amount of sediment

The variation in the percentage of sediment produced by salt percentage and the results of scanning electron microscopy with the same scale for salts percentage of 0%, 5% and 30%, are shown in Figures 14 and 15, respectively.

According to Figure 14, the highest amount of sediment produced is for 1% salt and the lowest amount is for 5%

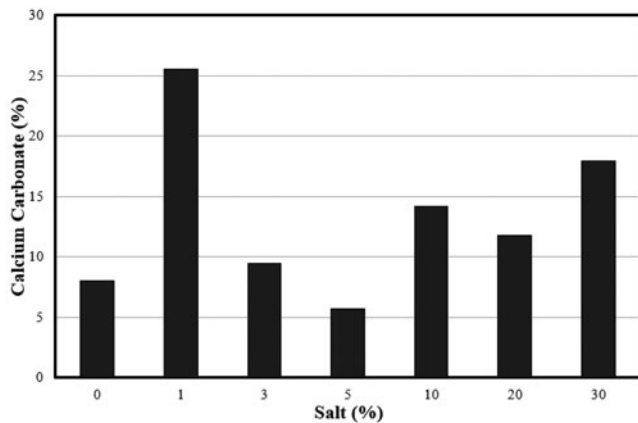


Figure 14. The percentage variations of produced sediment for improved samples with different salt levels.

salt. It can be concluded that there is no relationship between the amount of produced sediment and the maximum compressive strength. Therefore, 5% salt with the highest resistance value has the lowest amount of sediment.

However, it cannot be concluded from Figure 15 about the form, type, mode, and amount of sediments with the salt percentage, but it can be concluded that by increasing the salt percentage from 0% to 30%, the activity of the bacteria did not stop and the operation of the sediment formation has continued. As a result, biological improvement based on native bacteria is possible not only in low salinity soils, but also in high-salinity soils.

Effect of salt percentage on modulus of elasticity

The modulus of elasticity as one of the mechanical properties of materials is equal to the stress–strain line slope.

The modulus of elasticity and the compressive strength of the samples to the salt percentage is shown in Figures 16 and 17, respectively. Figure 17 shows that with increasing salt percentage up to 5%, the modulus of elasticity increases, and with increasing salt content to more than 5%, the modulus of elasticity has decreased.

By comparing the values of the modulus of elasticity and the compression strength of the improved samples with different salt percentages (Figures 16 and 17), it is shown that the salt content in both parameters has the same effect, this means that the values of both parameters increase with an increase in salt percentages up to 5% and

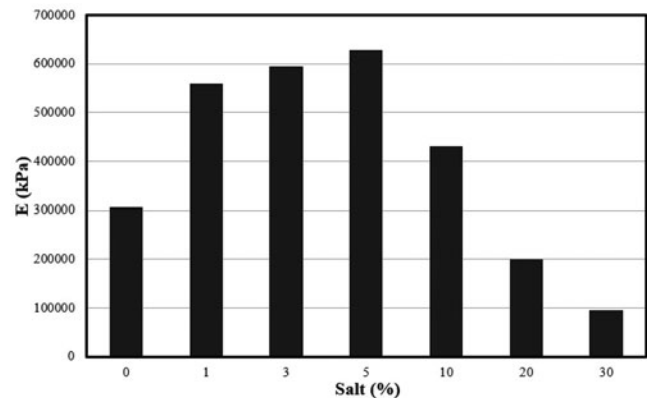


Figure 16. Elastic modulus of improved samples with different salt percentage levels.

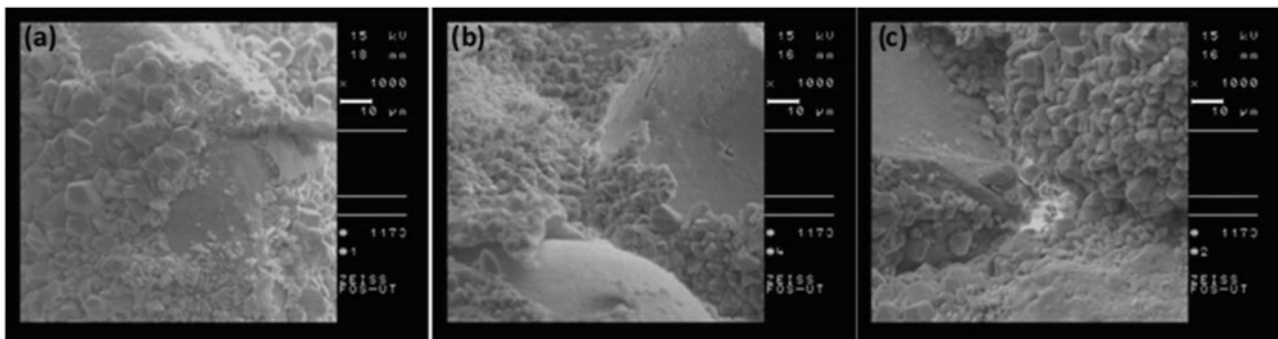


Figure 15. SEM graphs of cemented sand with the same scale: (a) 0% salt, (b) 5% salt, and (c) 30% salt.

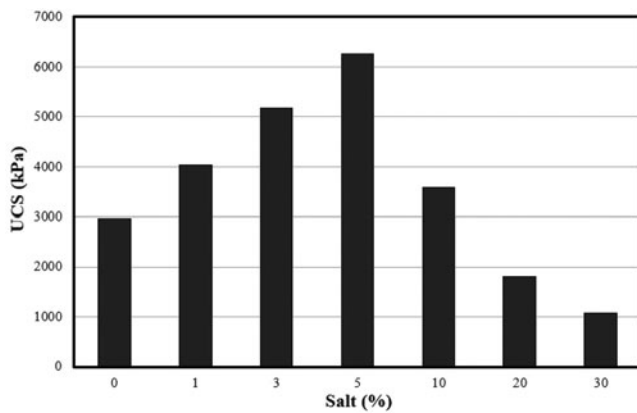


Figure 17. USC for improved samples with different salt percentage levels.

the corresponding amounts decrease with the increasing in salt content.

Conclusion

In this investigation, a series of tests were performed to study the effect of factors such as salinity (0%, 1%, 3%, 5%, 10%, 20% and 30% of salt) and culture medium components (presence or absence of urea in the culture medium) on stimulation of native urease-positive bacteria, which is a simpler and cheaper method than foreign bacteria. This was done by using Unconfined Compressive Strength, Calcimeter, Standard plate count, urease and PCR tests and SEM in biological improvement. The results are as follows:

- There are many microorganisms in the soil that some of these microorganisms can hydrolyze urea. In this study, nine strains of bacteria were identified in the soil, four of which were halotolerant urease-positive bacteria: *Streptomyces flaveolus*, *Streptomyces coelicoflavus*, *Streptomyces erythrogriseus*, and *Streptomyces cavourensis*.
- The formation of calcium carbonate sedimentation and the acquisition of the compressive strength show the high ability of biological improvement by native bacteria to stabilize sandy soils prone to wind erosion.
- Adding urea to the culture medium and introducing it as a nutrient to native urease-positive bacteria had a significant effect on bacterial urea hydrolysis so that it increases the compressive strength by more than 5 times.
- Biological improvement of saline soils and susceptibility to wind erosion is strongly influenced by salinity percentages. The compressive strength and modulus of elasticity increase up to 5% increase in the salt content and then decrease.
- The upward trend of the compressive strength of the specimens up to 5% of the salt and then its descending order, is due to the halotolerant native urease-positive bacteria in the sandy soil.
- Sand salinity has no effect on the amount of sediment produced so that the amount of sediment production to the salt percentage does not follow a specific pattern.

Disclosure statement

No potential conflict of interest was reported by the authors.

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