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ORIGINAL PAPER

# Bio-improvement of dune sand by bacterial medium cultures using *Sporosarcina urea* and *Bacillus subtilis*

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**Abstract**

Sand storms are catastrophic geological challenges worldwide in arid and semiarid areas with growing intensity. Engineering geologists and geotechnical engineers are always trying to monitor the desertification phenomenon and stabilize weak soils with dust storms origin through environmentally friendly procedures. It is assumed that erosion resistance can be improved by improving the strength of soils. In the past few years, microbial-induced calcite precipitation (MICP) has been utilized as a sustainable ground improvement technique. In this method, urea is fractured by bacterial urease enzymes, and the biochemical reaction network is then completed by adding cementation solution and instigating calcite precipitation which improves the mechanical and physical properties of the soil. In the presented research, a single culture of *Sporosarcina urea* (*S. urea*) and a mixed culture of *Sporosarcina urea* and *Bacillus subtilis* (*S. urea* + *B. subtilis*) mediums were used for the bio-cementation of typical dune sand. Firstly, the medium culture was gravitationally injected into the soil specimens in one cycle. Secondly, the cementation reagent (urea + calcium chloride) was injected into the specimens in two cycles. After a specific curing period, the specimens were evaluated using unconfined compression strength (UCS), permeability, direct shear, and California bearing ratio (CBR) tests. The tests outcomes revealed a considerable enhancement in strength and stiffness and a significant reduction in permeability for all treated soil specimens. Finally, the improvement of stabilized specimens was verified by quantifying calcite content, scanning electron microscopy (SEM) images, and X-ray diffraction (XRD) analyses on selected treated specimens.

**Keywords** MICP · Ground improvement · Sustainable geotechnics · Permeability · Unconfined compressive strength

## Introduction

Wind-driven dunes can cause several environmental issues such as desertification, fine dust, farmland degradation, and respiratory ailments. The weak geotechnical characteristics of dune sand, such as looseness, instability, high settlement, low cohesion, and bearing capacity, have led to high maintenance costs, environmental pollution, and less efficiency in civil projects. Thus, it is necessary to stabilize dunes to address environmental challenges. Several methods have been used to tackle engineering geological problems of weak soils and create a strong cohesion among soil particles. (DeJong et al. 2010; Sheikhhosseini Lori et al. 2021; Amoroso et al. 2020; Toufigh et al. 2023). Biological cementation or microbial-induced calcite precipitation (MICP) is an eco-friendly method in which bio-grouting is employed to create bio-cementation within soil mass (Cheng and Shahin 2019; Liu et al. 2020, 2021; Zhao et al. 2023). In this method, typical native or non-native bacteria capable of

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hydrolyzing urea are used to instigate calcite precipitation while calcium ions are present (Karimian and Hassanlourad 2022; Zhao et al. 2021; Khaleghi and Rowshanzamir 2017).

The mechanism of the MICP method is shown in Fig. 1. In the MICP procedure, urease bacteria or pure enzymes such as *Bacillus thuringiensis*, *Helicobacter pylori*, *Stenotrophomonas ginsengisoli*, and *Sporosarcina pasteurii* can be used for the hydrolysis of urea and the production of ammonium and carbonate ions (Van Paassen et al. 2009; Wath and Pusadkar 2019; Salifu et al. 2016). The urease micro-organism activity within the soil disintegrates the urea into ammonia and carbon dioxide (Fig. 1; part 1). These materials are transmitted through the bacteria's cell wall and enter the surrounding solvent phase. In the next stage, due to the presence of water in the surrounding medium, ammonia changes to ammonium (Fig. 1; part 2), and carbon dioxide is balanced with carbonic acid, carbonate, and bicarbonate (Fig. 1; part 3). While a calcium chloride solution is present, the calcium ion is absorbed into a bacterium with a negative charge. During the production of ammonium, due to the production of negative hydroxyl ions, which are more than the positive calcium ions, the alkaline medium dominates, and the conditions for calcite precipitation are provided (Fig. 1; part 4). Finally, the voids between the soil particles are filled with calcium carbonate, which bonds the particles, confines their movement, and improves soil geotechnical properties (Harkes et al. 2010; Ng et al. 2012; Zhao et al. 2014).

Bacteria are the most common species of micro-organisms in the soil (Carmona et al. 2016; Kim et al. 2001). Improving the soil properties through the MICP is affordable since some micro-organisms used in the MICP are naturally present in the ground and underground water and induce the least disruption in soil and environment (Fujita et al. 2000; Lloyd and Sheaffe 1973). Researchers have mentioned MICP as a sustainable technique due to its low production cost and low demand for energy, water, and natural resources (Dubey et al. 2023; Konstantinou and Wang 2023; Zhang et al. 2021). The influential factors on the efficiency of MICP are recognized as nutrients, type of bacteria, bacteria geometric compatibility, concentration of bacterial cell, bacteria distribution within the soil, temperature, reagent's concentration, pH, and the injection technique (Ng et al. 2012; Sharma et al. 2021; Khaleghi and Heidarvand 2023).

The concentration of the treatment solution has a significant effect on the biochemical network of the MICP system (Rivadeneyra et al. 2004). An increase in the concentration of components (i.e., urea and calcium chloride) increases the amount of calcite precipitation (Okwadha and Li 2010; De Muynck et al. 2010). A lower concentration of urea and calcium chloride causes the decomposition of urea to a suitable level of ammonia. Also, the urease and biological activity of bacteria might be decreased with high salinity (Rivadeneyra et al. 2000; Nemati et al. 2005).

Stabinkov et al. (2013) assessed the capability of the MICP method by *Bacillus pasteurii* bacterium for stabilization and wind erosion resistance to the sand dust's movement and its chemical and bacteriological pollutants. After the sand bio-stabilization, the escape of particles to the atmosphere reduced compared with the reference by 99.8% for dust, 92.7% for phenanthrene (chemical pollutant), 94.4% for lead nitrate (chemical pollutant), and 99.8% for *Bacillus megaterium*'s bacterial cells (bacteriological pollutant) (Stabnikov et al. 2013). Biological calcite precipitation increases the sand dust particle size and creates cohesive bonds between particles, preventing sand and dust emission into the atmosphere. Thus, the MICP method can be a suitable and eco-friendly approach for sand and dust

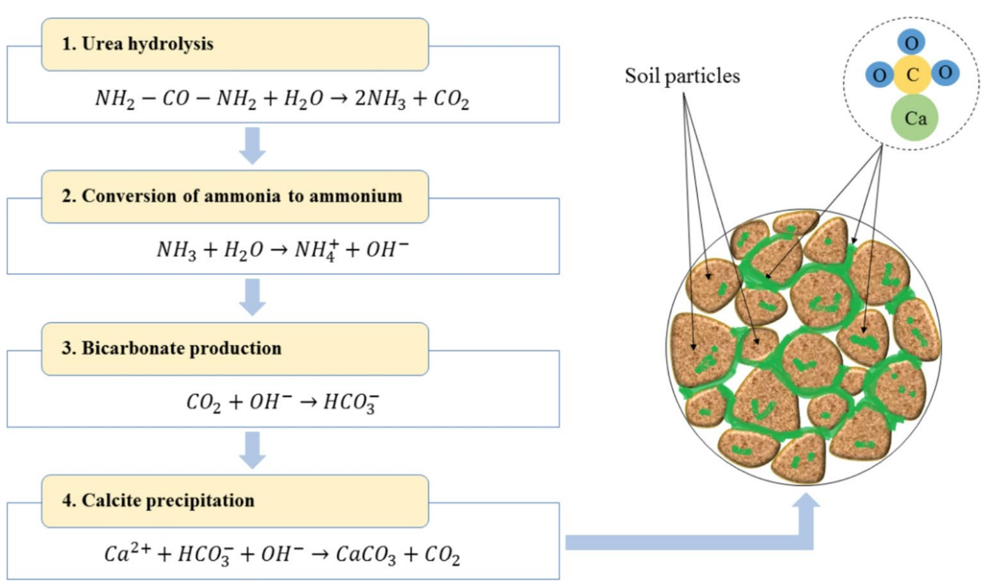



**Fig. 1** The microbially induced calcite precipitation formation process and biochemical reactions in soil improvement

stabilization (Anderson et al. 2014; Gomez et al. 2016; Maleki et al. 2016).

Gowthaman et al. (2021) studied improvement of Toyoura silica sand by calcium phosphate bio-cement using bone meal and acid urease, and the unconfined compression strength (UCS) increased up to 1.5 MPa (Gowthaman et al. 2021). Yang et al. (2020) investigated soil stabilization using wastewater treatment sludge through bacteria cultivation. The results indicated that the unconfined compressive strength of the soil experienced a growth to 2.7 MPa and the permeability decreased to $10^{-6}$ m/s. Thus, it could be considered an efficient stabilizer for practical applications, including dust control (Yang et al. 2020). Another study revealed that the MICP has a positive effect on the shear strength of soil (Portugal et al., 2020). A study on engineering properties of marginal soil showed that the California bearing ratio (CBR) of silty sand could increase by 92.5% using the biochemical improvement method (Mohammed and Abdulfatah 2018). Gat et al. (2014) investigated the impact of interactions between ureolytic and non-ureolytic bacteria on MICP in natural soils. The results indicated that the precipitation of dissolved calcium as $CaCO_3$ occurred more quickly in the mixed culture, even though the mixed culture had less favorable chemical conditions, such as lower pH and $CO_3^{2-}$ concentration. *B. subtilis* grew at a much faster rate than *S. pasteurii*, leading to a higher density of bacterial cells in the mixed culture (Gat et al. 2014). In a study conducted by Zhao et al. (2014), the influence of curing conditions and reaction time on the UCS of sand cemented by MICP was investigated through submerging technique. The research indicated that the UCS values were 0.56, 0.98, and 1.36 MPa for 3, 5, and 7 days of reaction time, respectively, which indicated the importance of this factor (Zhao et al. 2014).

Researchers investigated the potential of MICP in mitigating wind erosion by performing field tests on sandy land in Ulan Buh Desert, Ningxia Hui Autonomous Region, China. To increase the efficiency of MICP, all treatments were conducted near sunset to minimize evaporation caused by the high sand temperature and intense sun exposure. The results showed that MICP could substantially improve bearing capacity and wind erosion resistance of loose and cohesionless desert soil in arid and semi-arid areas (Meng et al. 2021). Tao et al. (2025) addressed the gap between laboratory research and real-world applications by studying the efficiency of MICP in sand improvement in the Taklimakan Desert using Sporosarcina pasteurii under the harsh desert environment. The results revealed MICP as an environmentally friendly alternative to conventional techniques, offering excellent mechanical performance for mitigating desertification in arid areas. For better long-term performance of MICP, they suggested occasional water spraying for reactivating dormant bacteria, and applying biodegradable mulch on the surface for UV protection and long bacterial survival (Tao et al. 2025).

Compared to conventional cementation practices, one advantage of MICP is its easy permeation through soil media due to the solution's water-like viscosity. Recent research has shown that MICP-treated soils can effectively suppress wind erosion, with a maximum of 3.5% and a minimum of 1.6% mass loss recorded. These values were found to decrease with the treatment period, indicating that MICP could be an effective and eco-friendly means of controlling airborne fugitive dust (Dubey et al. 2023; Osinubi et al. 2020). The dust and sand storms (DSS) phenomena occur in the Kerman desert annually due to the extensive fine-grained sediments, lack of vegetation, prolonged drought, and strong winds. Thus, DSS are one of the most critical environmental issues in this region and need to be investigated in more detail.

Currently, there are no published studies available on the improvement of dune sand by MICP technique through single and mixed bacterial medium cultures using *Sporosarcina urea* (*S. urea*) and *Bacillus subtilis* (*B. subtilis*). Therefore, given this gap and the importance of environmental concerns, this research aims to investigate the feasibility of bio-stabilization on dune sand collected from near Kerman desert on a laboratory scale. First, treated dune sands specimens with single (*S. urea*) and mixed (*S. urea* + *B. subtilis*) bacterial medium cultures were evaluated comparatively using UCS and permeability tests. Then, best best-performing medium culture was selected to be studied in more detail using direct shear and CBR tests. Finally, the calcite precipitations were evaluated in detail by acid leaching, scanning electron microscopy (SEM), and X-ray diffraction (XRD) methods. All tests were also carried out on the untreated soil as a reference. The key innovation in the presented research is applying mixed culture (i.e., two bacteria) to enhance the MICP's efficiency.

## Materials and methods

### Soil

According to the USGS (United States Geological Survey), Dunes are formed when wind accumulates sand on top of each other, eventually creating a small mound. In the presented research, dune sand was collected from a dune field adjacent to the Kerman in the southeast of Iran. Figure 2 displays the location of Kerman desert and the used soil. Figure 3 shows the sieve analysis carried out based on

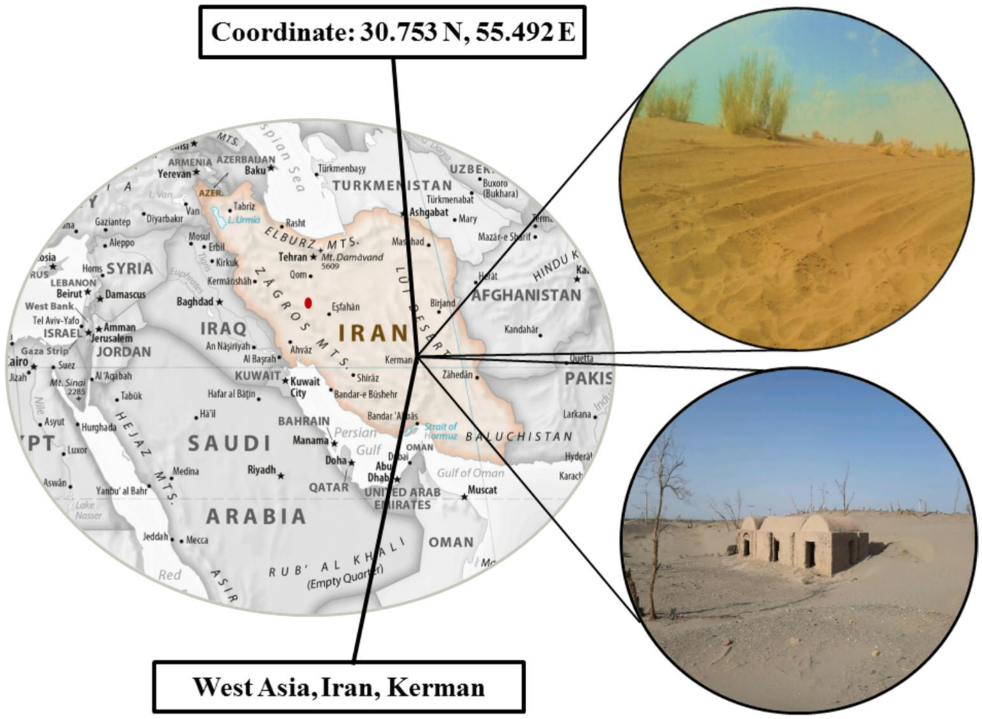



**Fig. 2** A picture of Kerman desert and the location of studied soil

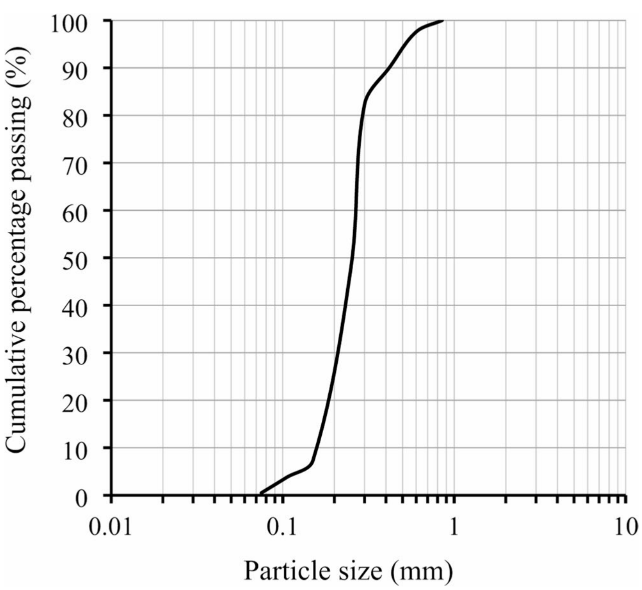



**Fig. 3** The particle size distribution of dune sandy soil

ASTM 175 D422. Based on the particle size distribution, the soil is composed of nearly 98% sand with a fines content of approximately 2% (particles passing the No. 200 sieve, 0.075 mm) and it was classified as SP (poorly graded sandy soil) according to ASTM D2487. The soil maximum density and minimum void ratio were determined according to ASTM D 4253–00. The soil minimum density and maximum void ratio were also determined according to ASTM D 4254–00. The properties of the used soil are displayed in Table 1.

**Table 1** Characteristics of Dune sandy soil

| Properties | Value |
|---|---|
| $D_{10}$ (mm) | 0.18 |
| $D_{30}$ (mm) | 0.21 |
| $D_{60}$ (mm) | 0.27 |
| Coefficient of uniformity ($C_u$) | 1.50 |
| Coefficient of curvature ($C_c$) | 0.92 |
| Optimum water content (%) | 16 |
| AASHTO classification system | A-3 |
| Unified classification system | SP |
| Specific gravity (Gs) | 2.70 |
| Max. dry density $(g/cm^3)$ | 1.74 |
| Max. void ratio | 0.78 |
| Min. void ratio | 0.60 |

## Bacterial strains

Two bacterial strains, *Sporosarcina urea* (*S. urea*) ATCC 13,881 (American Type Culture Collection) as ureolytic gram-positive bacteria and *Bacillus subtilis* (*B. subtilis*) ATCC 6633 (American Type Culture Collection) as a non-ureolytic component, were used in this study. Figure 4 shows microscopic photos of the mentioned bacteria at 1000x magnification. Similar to natural procedures within the soil mass in nature, processes such as MICP may be influenced by the reaction between the ureolytic and non-ureolytic bacteria (Gat et al. 2014a). Thus, combining these micro-organisms was used for the microbial solution. This research only studied two types of bacteria for simplicity of analysis, which is a limitation.

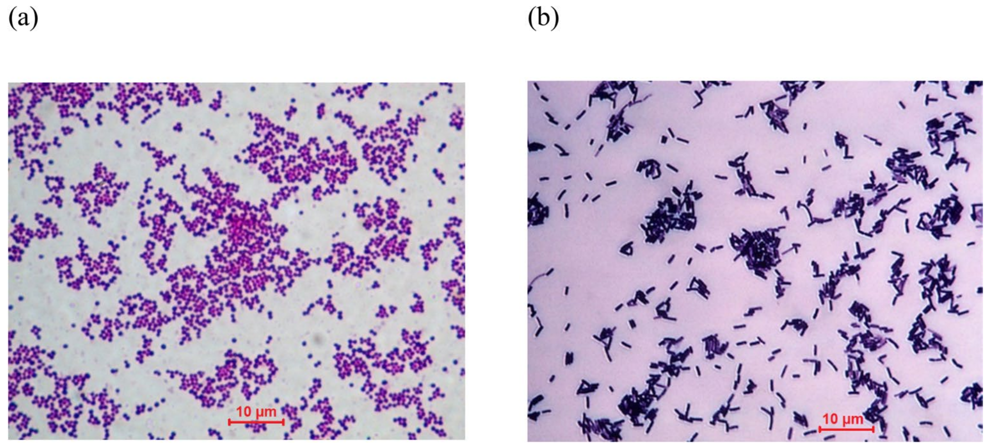



**Fig. 4** Microscopic photos of micro-organism in gram staining test, (**a**) *S. urea* as the ureas bacterium (**b**) *B. subtilis* as the non-ureas bacterium

Urease activity analysis was carried out in urea-free and urea-included culture mediums, according to Table 2. The culture mediums were made by dissolving all components except urea in water and steam-sterilized at 121 °C for 15 min. Then, the filter-sterilized urea solution was added aseptically post-autoclaving to the cooled medium. In all culture mediums prepared for testing, bacterial suspension was added to both urea-free and urea-included mediums, and then urea hydrolysis was explored.

Figure 5 shows the results of urease activity tests for *B. subtilis* and *S. urea*. As can be seen, the color of the culture tube containing *B. subtilis* remained yellow, revealing that *B. subtilis* could not hydrolyze the urea and was non-ureolytic. However, the color of the culture tube containing *S. urea* turned red, which indicated it could hydrolyze the urea and was ureolytic.

## Bacterial suspension

For preparing the bacterial suspension, stock cultures were made by mixing a pure culture grown overnight at 28 °C for *B. subtilis* and 37 °C for *S. urea* in LB broth medium (Merck, Darmstadt, Germany) supplemented with 25% glycerol as cryoprotectant and kept frozen at 80 °C. To obtain a fresh inoculum, strains were subcultured in 10 mL of broth culture medium at 37 °C overnight. This preculture was transferred (1% v/v) to the value volume of the fermentation medium and incubated at a proper temperature overnight. After cultivation, the cells were collected by centrifugation at 8000 g for 20 min (4 °C) and washed twice with sterile phosphate buffer saline (PBS). Then they were used for a bacterial suspension containing 3 g of NB, 20 g of urea, 10 g of ammonium chloride ($NH_4Cl$) and 2.12 g of sodium bicarbonate ($NaHCO_3$) per liter of distilled water.

**Table 2** The proportion of urea-free and urea-included culture mediums for urease activity test

| Material | Chemical Formula | Amount (g/L) |
|---|---|---|
| Urea | $CH_4N_2O$ | 20 |
| Sodium chloride | NaCl | 5 |
| Monopotassium phosphate | $KH_2PO_4$ | 2 |
| Peptone | $C_{13}H_{24}O_4$ | 1 |
| Glucose | $C_6H_{12}O_6$ | 1 |
| Phenol red | $C_{19}H_{14}O_5S$ | 1.74 |

Bacterial concentration is one of the factors that influences the process, so as the concentration increases, the calcite precipitate increases (Okwadha and Li 2010). Bacterial concentration was determined using a spectrophotometer device by measuring the optical density of the suspension at a wavelength of 600 nm, OD600. In this study, the optical density of bacteria was constant and equal to 1.2. Also, Based on the preliminary tests, it was observed that hydrolysis occurs faster in a slightly acidic environment. Thus, the pH value of bacterial suspension was adjusted to 5.8.

## Treatment solution

In this research, the concentration of urea (60 g/mol) and calcium chloride (i.e., $CaCl_2$) (111 g/mol) was considered approximately based on stoichiometry 2 M and 1 M, respectively. According to the components molecular weight of the treatment solution, the urea powder and the calcium chloride granular were weighed and dissolved in distilled water through a magnetic stirrer for 30 min.

## Preparation and curing of specimens

The specimens were prepared according to the primixig approach presented by Cheng and Shahin (2016), where the soil was mixed with bio-slurry and then the cementation solution was injected to the mixture, resulting in a uniform $CaCO_3$ precipitation (Cheng and Shahin 2016). Since the studied soil is windblown, it should be injected and improved under the same conditions as the field. Thus, the specimens were made at a dry density of 1.63 g/cm$^3$ which is equal to the relative density of 67%. For specimens

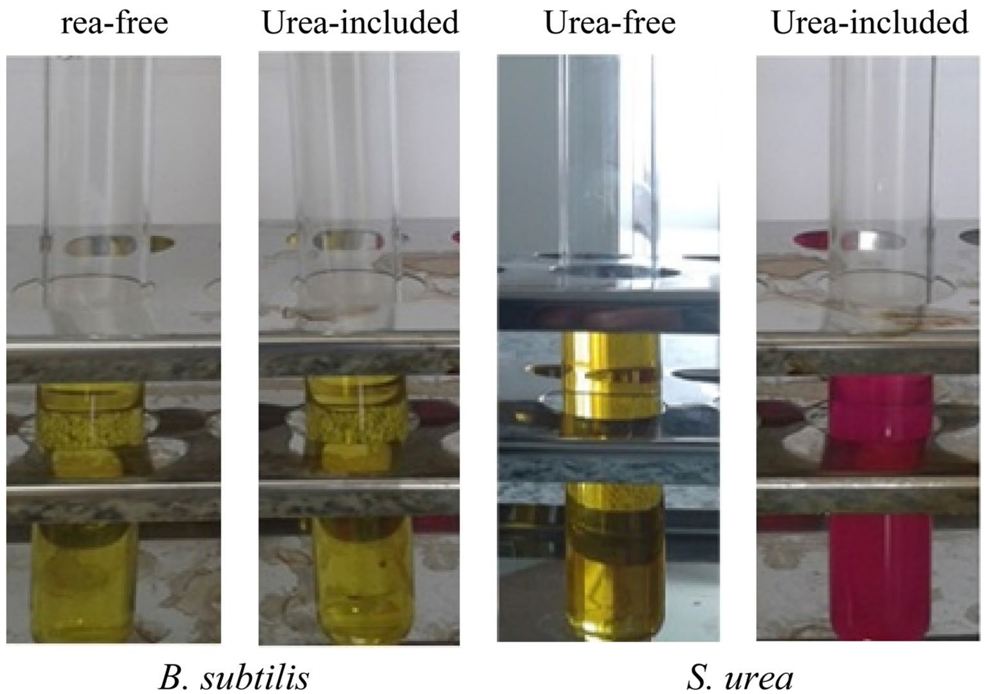



**Fig. 5** The urease activity of *B.subtilis* and *S. urea*

preparation, first, the required mass of dry soil for the mold volume was calculated. The corresponding pore volume ($V_v$) for this soil mass at 1.63 g/cm$^3$ density was then determined. A volume of bacterial suspension precisely equal to the $V_v$ of soil mass was thoroughly mixed with the dry soil, as this amount of bacterial suspension showed the best workability according to the preliminary experiments. This mixture was then carefully placed and compacted in three layers into the sample mold.

The Polytetrafluoroethylene (PTFE) mold was made in a height-to-diameter ratio of 2.1 (47 mm diameter and 100 mm height) for UCS and permeability tests. Figure 6 shows the PTFE mold and all of its components. In order to prepare specimens for the UCS and permeability tests, the combination of the soil and the bacterial suspension was put in the mold in 3 layers by gently thumping each layer to achieve the determined density. Finally, the mold was sealed through an o-ring gasket and prepared for injecting of treatment solution. For the direct shear test, a steel mold was made in 63 mm diameter and 20 mm height. Then, the mixture (dune sand + bacterial suspension) was poured into the steel mold in 3 layers which were tapped gently to achieve the required compaction (1.63 g/cm$^3$ equal to 67% of relative density).

The injection setup was designed according to Fig. 6, which does not need to utilize a peristaltic pressure pump. The injection procedure is an essential parameter for achieving uniform calcite precipitation within the dune sand sample. In order to enhance the uniformity of the treatment and mitigate the challenges associated with gravitational injection, a controlled, multi-cycle procedure was employed. The treatment solution was injected while the outlet valve was initially closed for 15 min. Subsequently, the outlet valve was opened to achieve a controlled, slow flow rate, thereby increasing the solution's residence time within the specimen. This staged, controlled-flow approach was specifically designed to minimize preferential flow. Also, the treatment was performed in two distinct 12-hour cycles, as the cyclic injection procedure causes more uniform calcite precipitation than the continuous injection procedure (Huat 2006; Martinez et al. 2011; Barkouki et al. 2011).

Based on the designed injection procedure, the treatment solution with an amount of 100% of $V_v$ was poured into the reservoir at the top of the specimen. The specimen was injected gravitationally while the injection rate was adjusted to approximately 90 mL/h through the valve. The injection process was precisely duplicated after 12 h. The specimens made for direct shear tests were injected through a simple dropper pipe with two alternate 12 h cycles. The bacteria's cell is negatively charged, causing them to bond strongly to the surface of the positively charged soil particles (DeJong et al. 2010). This makes the cementation treatment solution, which is a neutral and stable solution, unable to wash down bacteria during injection.

The specimens were impounded inside the injection setup for 24 h so as to start the calcite precipitation. Then, the specimens were demolded and cured for seven days at room temperature (25 ± 2 °C). MICP is a time-consuming complex process, and a near-complete chemical reaction occurs over time (Rajasekar et al. 2017). Thus, during the curing period, the amount of produced calcite is likely to increase until it reaches a stable condition. After this stage, the increase in calcite amount would be inconsiderable.

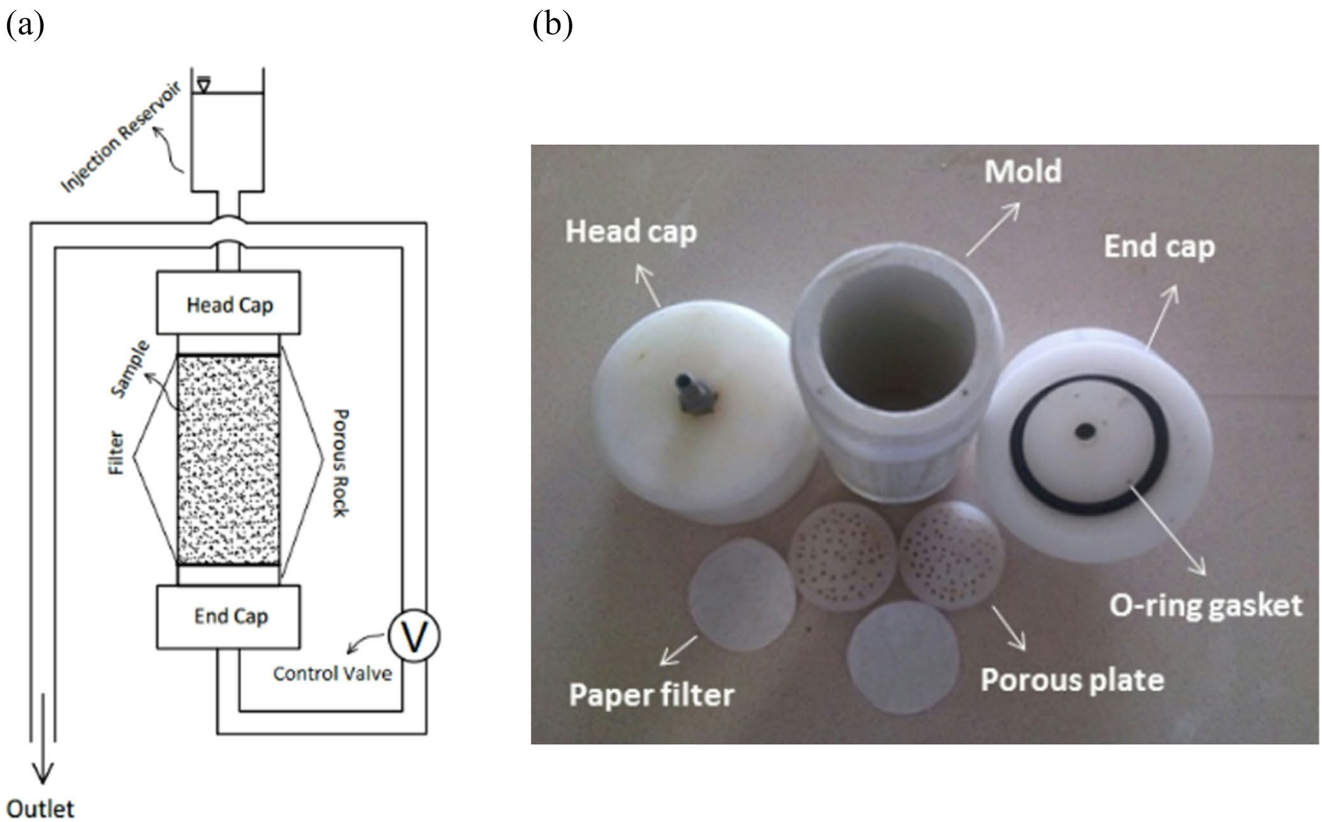



**Fig. 6** Design of injection apparatus: (**a**) the injection setup, (**b**) parts of the PTEF mold

Previous research showed the least curing time is 4–7 days for reaching the acceptable level of the MICP (Nemati et al. 2005; Sidik et al. 2015). Furthermore, curing a vast geotechnical site is not an easy task. Therefore, in this research after 24 h of reaction time, the specimens were placed at room temperature (25 ± 2 °C) for seven days (curing period) in order to investigate and evaluate the possibility of easy field use of the MICP technique and used materials. Finally, the specimens were put in an oven at 70 °C for one day to stop the calcite precipitation process into specimens.

To ensure the accuracy of the results, a set of three identical specimens of each combination was prepared, cured, and tested. The intermediate value of each set of tests was reported. A threshold of ± 10% deviation from the median was assumed to be acceptable for triplicate specimen results. In a few instances where this variation exceeded the set limit, the specimens were reprepared and retested.

## Tests

At the first phase of the research, the primary goal was to comparatively evaluate the mechanical performance and void-filling potential of single versus mixed bacterial cultures after a short curing period of 7 days, which was an effective basis for identifying the most promising culture. For this purpose, UCS and permeability tests, as widely used characterization experiments, were selected. Thus, the unconfined compression strength (ASTM D1633) with a 1 mm/min vertical displacement rate of loading and permeability (ASTM D5856-15) tests were performed on all 7-day cured specimens to discover the best-performing medium culture.

In the second phase, the research focused on the best-performing culture identified in the phase 1. The objective was to conduct a more comprehensive assessment of the engineering properties. This involved measuring shear strength parameters and CBR. Consequently, these tests were conducted over multiple, longer curing periods. The direct shear (ASTM D3080) with a 1 mm/min horizontal displacement rate of loading and California bearing ratio (ASTM D1883) with a 1.27 mm/min vertical displacement rate of loading tests were conducted on the best-performed culture after different curing periods to assess the mechanical properties of stabilized specimens. At the end, SEM and XRD analyses were conducted on untreated soil, and the best-performing treated specimens to study micromorphological and microstructural changes due to MICP in more detail. Also, the precipitated calcite content of the best-performing bio-treated specimens using single and mixed cultures was quantified using the acid leaching method in order to investigate the effect of using 2 cultures.

## Results and discussion

In this research, *S. urea* as the single medium and *S. urea*+*B. subtilis* as the mixed medium were utilized to improve dune sand specimens. The experimental results evaluated the efficiency of single and mixed mediums. The mechanical, hydraulic, and physical characteristics of the MICP-stabilized soil specimens are explained and discussed in the subsequent sub-sections.

### Unconfined compressive strength (UCS)

USC is a common and widely used test to evaluate the effectiveness of soil improvement. However, untreated dune sand is a cohesionless soil and conducting a UCS test is inapplicable and invalid. Thus, the compressive strength of untreated dune sand was forcibly examined using the triaxial compression test at a very low confining pressure. Although this inconsistency might raise a concern about the validity of the comparison between untreated and treated specimens, this approach has been taken and accepted in previous studies (Khaleghi and Rowshanzamir 2019). Figure 7 illustrates the stress-strain curve of bio-treated and untreated dune sand derived from the unconfined compression strength test and triaxial test, respectively. By using the stress-strain curve, the specimens' stiffness was calculated as a secant modulus ($E_s$) at a stress level equal to half of the failure stress. As shown in Fig. 8a, the bio-treatment of dune sand has a significant effect on the UCS and stiffness values that are 727.8 kPa and 93.3 MPa for *S. urea*+*B. subtilis* culture and 527.7 kPa and 69.4 Mpa for *S. urea* culture, respectively.

It is clear that both the mixed and single cultures improve the UCS of dune sand, whereas the mixed culture results in higher strength than the single culture. Table 3 shows some previous MICP studies on sandy soil that agree with this investigation's results. It is worth mentioning that the reported discrepancies in the amount of the UCS might be ascribed to the soil type, bacteria, nutrients, bacteria cell concentration, geometric adaptability of bacteria, temperature, and reagents concentration (Ng et al. 2012). According to Fig. 7, the failure strain of untreated dune sand and bio-treated specimens through the single and mixed culture is 3.1%, 0.8%, and 0.7%, respectively. Due to the dryness and the brittle failure of cementation bonding among soil particles in bio-treated mediums, the failure strain of untreated dune sand is higher than bio-treatment specimens.

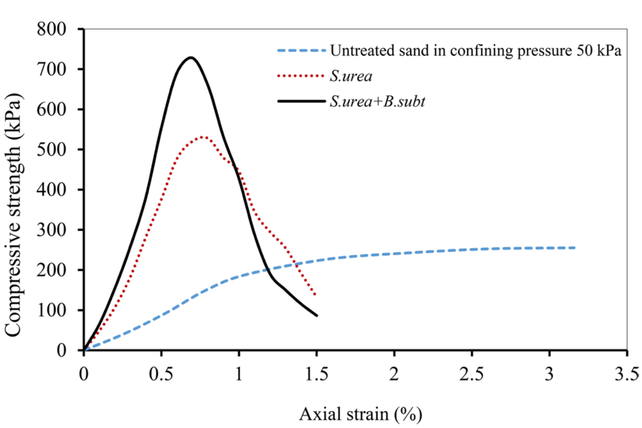



**Fig. 7** Stress-strain diagram of bio-treated specimens and untreated dune sand

Figure 8b shows the nominal energy absorption of the untreated and bio-treated specimens. Nominal energy absorption of the samples was determined by calculating the area under the curve of the stress-strain diagram of each specimen (Ranjbar et al. 2016). The energy absorption of mixed culture is more than single culture, and both of them are less than that of untreated dune sand because of the presence of confining pressure in the triaxial test. The higher strength, stiffness, and energy absorption of the bio-treated specimen by *S. urea*+*B. subtilis* medium compared with that of *S. urea* medium could result from the existence of *B. subtilis* in the mixed medium, which increases the pace of the calcite precipitation rate.

### Hydraulic conductivity

The permeability of specimens was conducted in the PTFE mold (shown in Fig. 6) by the falling head permeameter. Prior to the measurement, the specimen was saturated by introducing a slow, upward flow of de-aired water. Saturation was considered complete once no further air bubbles were observed emerging from the sample outlet. This procedure ensured continuous water flow paths through the soil matrix, which was essential for accurate permeability testing. The permeability coefficients of the untreated dune sand and bio-treated through *S. urea*+*B. subtilis* (mixed culture) and *S. urea* (single culture) are shown in Fig. 9. The permeability coefficients of untreated and bio-treated dune sand with single and mixed culture are $7.40\times10^{-3}$ cm/s, $3.66\times10^{-3}$ cm/s, and $2.99\times10^{-3}$ cm/s, respectively. The permeability coefficient of untreated dune sand is significantly higher than that of bio-treated specimens. Also, these results agree with the previous research (Whiffin et al. 2007; Yasuhara et al. 2011; Soon et al. 2013). The decrease in permeability coefficient of dune sand treated by the used single and mixed culture could be attributed to precipitated calcite that has filled the voids in the soil.

It is important to acknowledge the limitations of the experimental setup used in this research. The permeability of specimens was evaluated using a rigid-wall permeater, which has some drawbacks, including the possibility of

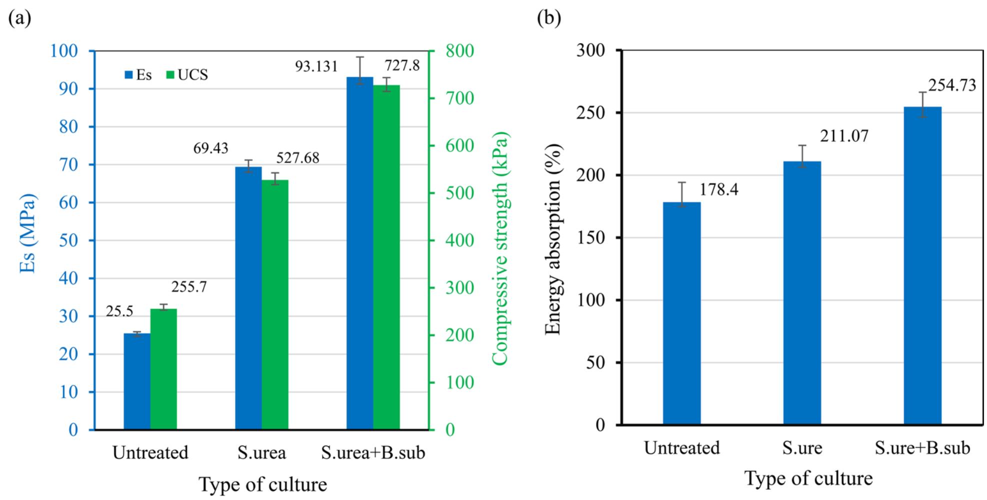



**Fig. 8** (**a**) the compressive strength and $E_s$, and (**b**) energy absorption of untreated and bio-treated dune sand with single and mixed cultures

**Table 3** Results of bio-treated sand in comparison with some previous MICP investigations

| Reference | Soil type | $CaCl_2$/Urea ratio | Number of injection cycles | UCS: kPa | Cohesion: kPa | Internal friction angle: ° | CBR |
|---|---|---|---|---|---|---|---|
| Whiffin et al. (2007) | Itterbeck sand, $D_{50}$ = 0.165 mm | 1.1 | 1 | 0–600 | NR | NR | NR |
| Yasuhara et al. (2011) | Sand | 0.5, 1.0 | 4–8 for UCS 1–4 for permeability | 373–1500 | NR | NR | NR |
| Chou et al. (2011) | Quartz sand, $D_{50}$ = 0.46 mm | 0.14 | 1 | NR | 5.5 | 43 | NR |
| Palmen (2013) | Quartz sand, $D_{50}$ = 0.85 mm | NR | 1–5 | 200–2600 | NR | NR | NR |
| Soon et al. (2013) | Sandy silt, $D_{50}$ = 0.031 mm | 0.25 | 8 | 12.3–69.2 | NR | 55.5 | NR |
| Montoya and DeJong (2015) | Ottawa sand, $D_{50}$ = 0.22 mm | 0.15 | 6–16 | $q/p$ = 1.9 (triaxial test) | Tiny | 43.7 | NR |
| Saffari et al. (2017) | Quartz sand, $D_{50}$ = 0.46 mm | NR | mixing | NR | 49.5 | 29.8 | NR |
| Azadi et al. (2017) | Quartz sand, $D_{50}$ = 0.27 mm | 0.67 | 2 | NR | 736 | 65 | NR |
| Pakbaz et al. (2018) | Quartz sand, $D_{50}$ = 0.18 mm | 1 | 4 | NR | 21.3 | 39.6 | NR |
| (Mohammed and Abdulfatah 2018) | SM | 0.75 | NR | 94.86 | NR | NR | 30.8 |
| (Hasriana et al. 2018) | CH | 0.2 | NR | 382 | NR | NR | 72.03 |
| Current investigation | Dune sand, $D_{50}$ = 0.25 mm | 0.5 | 2 | 527.7 (single culture) 727.8 (mixed culture) | 159.9 | 54.8 | 98.26 (unsoaked) 85.77 (soaked) |

* NR, not reported.

preferential flowpaths and a lack of confining stress. As a result, reported values of permeability might be higher than the true values under field conditions.

## Shear strength

Due to the mixed culture's better performance than the single culture in increasing the UCS, direct shear experiments were also performed on the bio-treated specimens with mixed culture to achieve more information on their shear strength. After 7, 14, and 21 days of curing bio-treated specimens with mixed culture, the treated specimens were placed inside the box of the direct shear test. For saturated conditions, after the specified curing period, each specimen was fully submerged in water for 24 h before being placed in the shear box. This immersion method is a common practice to achieve a high degree of saturation. The direct shear tests were conducted at normal stresses of 50, 100, and 150 kPa on both dried and saturated specimens to compare their shear strength with untreated specimens at the same density. The values of internal friction angle and cohesion were measured using the Mohr-Coulomb failure model according to the highest shear stress acquired from direct shear experiments on the untreated soil and bio-treated with mixed medium specimens. The outcomes are displayed in Fig. 10.

As seen in Fig. 10, the maximum shear strength of bio-treated samples is significantly higher than that of untreated dune sand. Bio-treated specimens' cohesion and friction

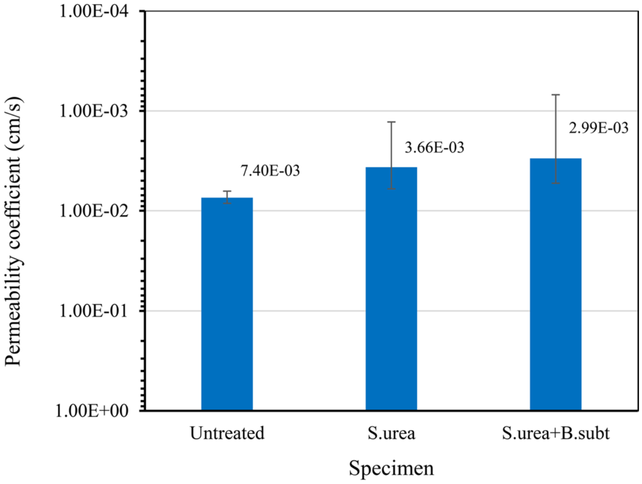


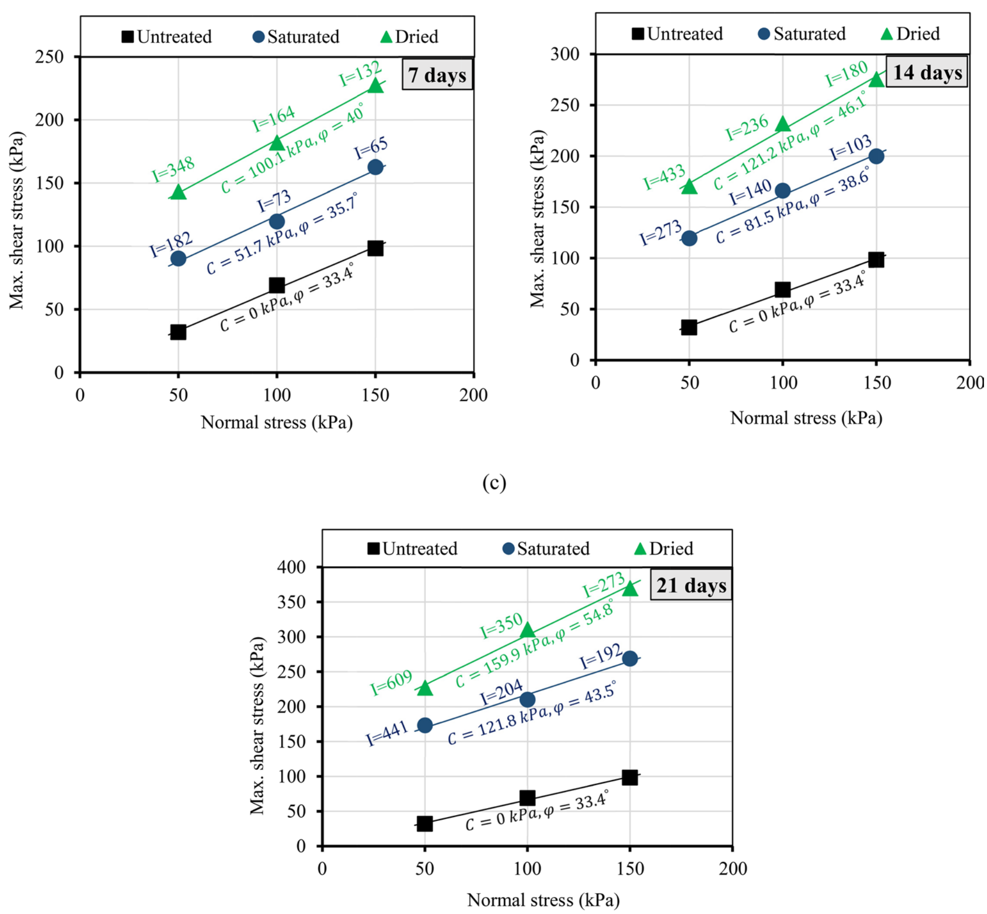

**Fig. 9** Permeability of untreated and treated dune sand with single and mixed cultures

angle are significantly higher than the untreated specimen. As shown in Fig. 10, cohesion and friction angle increase with curing duration. The friction angle and cohesion of bio-stabilized specimens are much higher in treated dried samples than that of saturated ones. The considerable rise in the values of internal friction angle and cohesion could be ascribed to cementation among the soil particles caused by *S. urea* in the mixed medium with similar behavior as demonstrated in earlier investigations employing single culture (Ramachandran et al. 2001; Rodriguez-Navarro et al. 2003). The formation of calcium carbonate crystals during the 4th step of the MICP process, which was mentioned in the Introduction section, is likely to result in bonding and adhesion in soil particles and filling voids. Also, due to the angularity and irregular corners of calcite crystals, these crystals could increase soil surface roughness and friction angle. It seems that the higher cohesion and friction angle in the dried condition is due to calcite crystals precipitation, which is unstable in water presence (Trushina et al. 2014; Khaleghi and Rowshanzamir 2019). This calcite precipitation can be confirmed with XRD analysis. The obtained results agree with numerous previous works, even though the achieved strength values are considerably greater due to the usage of mixed medium (Table 3).

## California bearing ratio (CBR)

The CBR test was performed on the untreated soil and the bio-treated specimens with mixed culture, considering the decent performance of the mixed culture. After 7 and 21 days of

**Fig. 10** Mohr-coulombs graph, cohesion and friction angle values of untreated and bio-treated dune sand with mixed culture at the period **(a)** 7, **(b)** 14, and **(c)** 21 days in dried and saturated conditions; C: cohesion (kPa), ϕ: friction angle (°), I: approximate improvement percentage compared to untreated soil (%)



curing bio-treated specimens with mixed culture, they were evaluated using the CBR test device. The results of the CBR experiments are displayed in Table 4. The CBR value of the soil saw a considerable growth of 83.21% and 75.45% using the MICP stabilizing method through the mixed medium for unsoaked and soaked conditions, respectively.

The rise in CBR can be explained by the reaction between bacteria and the cementation reagent, which results in calcite production. As a result, this calcite binds soil particles efficiently, fills the voids, and increases the soil's strength.

## Microstructural analysis

In order to present more proof and verification of the calcite precipitation process, SEM and XRD were conducted on the untreated and the best-performed bio-treated dune sand specimen. Figure 11 shows the SEM images of untreated dune sand and bio-treated dune sand using *S. urea* and *S. urea* + *B. subtilis*. These images indicate the calcite crystals' formation in treated dune sand compared to untreated dune sand. The images illustrated two distinct forms of calcite precipitation: (1) surface calcite crystals, which have formed on the surfaces of the sand grains, and (2) calcite bonds, which were accumulations of calcite crystals that span the inter-particle voids, providing the primary soil cementation effect by bonding the particles. As a result, the strength of the soil has been improved and the permeability coefficient has been reduced. The results are closely similar to previously reported works (Chou et al. 2011; DeJong et al. 2006). Also, it can be observed that more accumulated calcite precipitation exists in dune sand particles treated with mixed culture than in the treated dune sand using a single culture. According to the SEM images, the mixed medium probably accelerates the calcite precipitation rate by developing excess nucleation areas for the precipitation. Moreover, the XRD analysis was carried out to investigate the calcite crystals' formation. Figure 12 illustrates the XRD patterns of untreated dune sand and bio-treated dune sand using a mixed culture of *Sporosarcina urea* and *Bacillus subtilis*, ranging from 2θ = 20° to 2θ = 90°. As can be seen, the untreated soil was predominantly composed of quartz and lacked natural cementitious minerals, such as calcite. However, the pattern exhibited the formation of new peaks corresponding to calcite crystals after improvement of the soil through MICP. The most intense peak appeared at approximately 2θ = 29°, showing the predominance of calcite, which is consistent with previous research (Kariminia et al. 2025). The XRD results demonstrated the efficacy of MICP and the mixed culture in calcite precipitation and forming calcite crystals, which resulted in the bonding of sand particles and improvement of compressive strength and shear parameters. Thus, the XRD analysis corroborated the results discussed in the preceding sub-sections of the research.

**Table 4** CBR values of the untreated soil and the MICP-stabilized soil through the mixed medium

| Specimen | Unsoaked CBR (%) | Soaked CBR (%) |
|---|---|---|
| Soil | 15.05 | 10.32 |
| Stabilized soil through the mixed medium after 7 days of curing | 79.68 | 60.14 |
| Stabilized soil through the mixed medium after 21 days of curing | 98.26 | 85.77 |

## Precipitated calcite content

One mole of urea produces one mole of carbonate ions ($CO_3^{2-}$), and one mole of calcium ions ($Ca^{2+}$) from calcium chloride will react with one mole of carbonate ions to form one mole of calcite ($CaCO_3$) based on the MICP process and biochemical reactions (shown and explained in Fig. 1 and the Introduction). Thus, according to the used PTEF mold and volume of specimens, stoichiometrically 13.6 g of calcite was expected to be produced in the 2 cycles of treatment in ideal conditions.

The actual amount of precipitated calcite was determined by conducting the acid leaching method on the best-performed bio-treated specimens (Putra et al. 2016; Yasuhara et al. 2012). The outcomes showed that 8.96 g and 10.37 g of calcite were precipitated through MICP in bio-stabilized specimens using single and mixed cultures, respectively. These results indicated that using the mixed culture resulted in more calcite content than the single culture, justifying the results in previous subsections.

## Impact of mixed culture

The results showed that the UCS, cohesion, and friction angle in bio-treated specimens through mixed culture are higher than that of bio-treated specimens through single culture. Also, the permeability reduction in the mixed medium treated specimens is higher than that of single culture specimens. The better performance in bio-treated specimens using mixed culture emphasizes the influential role of *B. subtilis* in the mixed medium that provides an excess nucleation environment in the MICP system (Gat et al. 2014a). Also, another role of *B. subtilis* is enhancing the calcite precipitation pace. In more detail, the growing *S. urea* in the presence of *B. subtilis* cells has been likely to increase calcite formation through the creation of extracellular polymeric substances (EPS) and the development of biofilm. This increase in calcite formation allowed the cation to be efficiently absorbed into dune sand particles and shape mineral deposits, as noted in earlier studies (Beveridge et al. 1997).

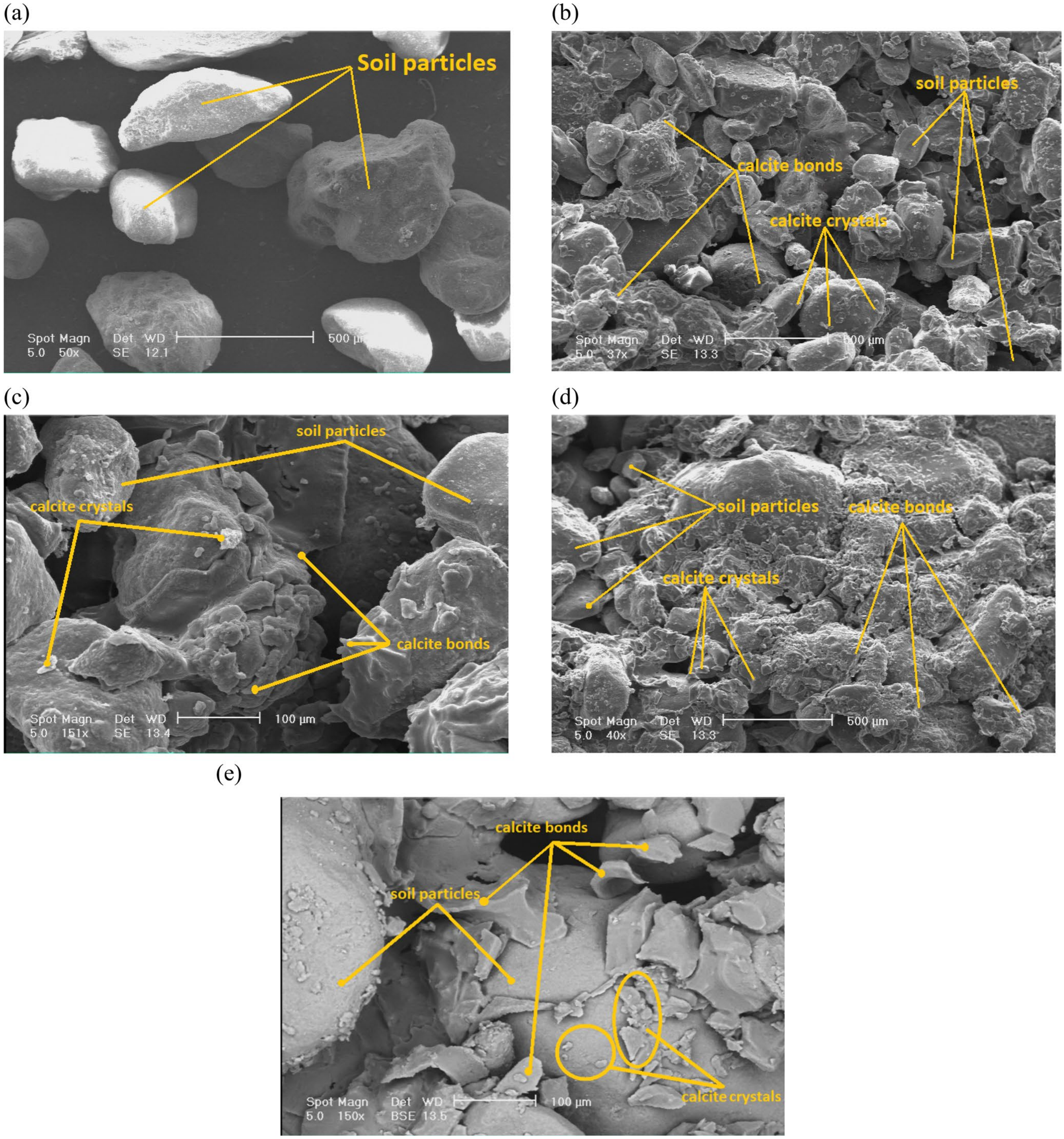



**Fig. 11** The SEM photos of (**a**) untreated sand, (**b**) bio-treatment sand by single culture at 37x magnificaion, (**c**) bio-treatment sand by single culture at 151x magnification, (**d**) bio-treatment sand by mixed culture at 40x magnificaion, and (**e**) bio-treatment sand by mixed culture at 150x magnification

## Limitations and potential future research

MICP is considered an eco-friendly technique in soil improvement. However, the bacterial strains used in this study, *Sporosarcina urea* and *Bacillus subtilis*, are not native to Kerman dune sand. The introduction of non-native bacteria could potentially disrupt local microbial ecosystems. In addition, MICP may raise concerns including ammonia emissions and long-term durability. Therefore, future research should focus on investigating long-term environmental

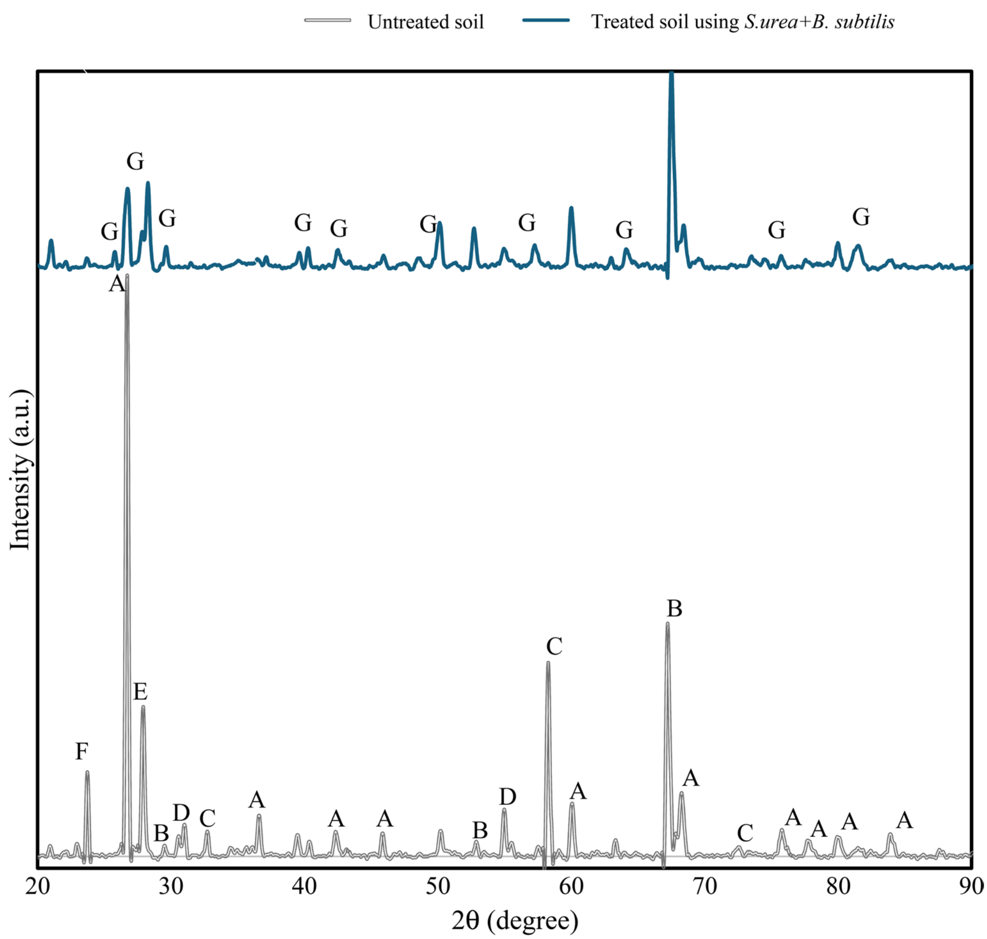



**Fig. 12** XRD spectrum of the untreated dune sand (grey line) and the bio-treated dune sand through the mixed culture (blue line); **A**: $SiO_2$, **B**: $TiS_2$, **C**: $InO_3$, **D**: $Ba_3Ta_2ZnO_9$, **E**: $Rb_6Te_2O_9$, **F**: $Li_9KNb_{10}O_{30}$, **G**: $CaCO_3$

impact of used materials in the field at large scale and optimizing the process to achieve a balance between operational proficiency and environmental sustainability.

Another limitation of the current study was that the soil salinity was not quantified. The soil used was predominantly sand (approx. 98%), and sandy soils typically have lower salinity levels due to high leaching potential and low Cation Exchange Capacity (CEC) (Xing et al. 2025; Brady et al. 2008). Thus, the salinity was assumed to be low. Also, the experiments in the research were carried out under controlled laboratory conditions. This approach was taken to minimize variables and establish a baseline for the efficiency of the MICP process, which is consistent with the laboratory-scale scope of this research.

However, the controlled settings do not fully replicate the dynamic nature of a field environment like the Kerman Desert, where parameters such as salinity, pH, temperature, and moisture fluctuate significantly. These environmental factors are known to have a substantial impact on microbial activity and the overall efficiency of calcite precipitation (Mortensen et al. 2011). Therefore, before this technology can be considered for field-scale application, further research should be conducted on simulating realistic environmental conditions to evaluate the feasibility and robustness of MICP treatment using mixed medium culture of *Sporosarcina urea* and *Bacillus subtilis* under the variable parameters found in situ.

## Conclusions

This paper explains a research study on the efficiency of MICP bio-treatment for stabilizing dune sand. The main emphasis is to use this technique as an environmentally friendly alternative stabilizer instead of conventional chemical-based additive stabilizers such as cement. Besides, new mixed cultivations were used for biological treatment purposes. Several criteria, including the UCS, direct shear, permeability, SEM, and XRD tests, were utilized to evaluate the mechanical, hydraulic, and physical properties of bio-treated dune sand specimens over an untreated dune sand specimen. To sum up, the bio-treating procedure seems to be an appropriate ground improvement technique for stabilizing dune sands which can address geological and geotechnical challenges of this type of soil. The following conclusions may be outlined from this study:

- The UCS values of the bio-treated dune sand through single and mixed cultures saw a considerable increase of 2.1 and 3 times more than that of the untreated specimen, respectively. Thus, specimens treated with mixed culture presented about 1.4 times greater strength than single culture ones.
- The soil stiffness also showed significant enhancement due to bio-treatment. The stiffness values of bio-stabilized specimens were 2.7 (for single culture) and 3.7 (for mixed culture) times of untreated specimens. It can be interpreted that the stiffness of bio-treated samples through mixed culture is about 1.3 times that of samples treated through single culture.
- The permeability of the bio-treated specimen experienced a reduction of about 50% than that of untreated soil.
- The dune sand shear strength showed a significant increase due to bio-treatment with mixed culture procedure, where the soil cohesion and friction angle increased from zero and 33.4° to 159.9 kPa and 54.8°, respectively.
- The CBR value of the soil significantly increased by bio-treatment through the mixed culture from 15.05% to 98.26% after 21 days of curing.
- By using MICP method, 8.96 g and 10.37 g of calcite were precipitated in bio-treated specimens using single and mixed cultures, respectively.
- The efficiency of the MICP technique for sandy soil stabilization was verified using microstructural studies, the SEM and XRD analyses.

Overall, the MICP showed considerable potential to be used as an efficient improvement technique for sandy soils in a laboratory scale. However, it is essential to address the concerns regarding the durability and environmental impact of the MICP method, particularly due to the production of ammonia byproducts, in order to develop a practical and sustainable approach for field-scale applications. Therefore, further research is needed to investigate and address these issues thoroughly.

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**Data availability** Data will be made available on request.

## Declarations

**Competing interest** The authors have no competing interests to declare that are relevant to the content of this article.

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