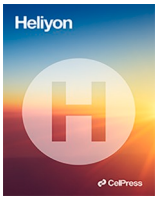
Heliyon 10 (2024) e35813

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Evaluation of microbial-induced calcite precipitation performance for soil surface improvement and toxicity assessment of the biostabilizer

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ARTICLE INFO

Keywords:
Biocementation
Biogeochemistry
MICP
Soil stabilization
Toxicity
Wind erosion control

ABSTRACT

Microbial-induced calcite precipitation (MICP) is an environmentally friendly process that can be used to enhance soil surface stability against wind erosion. In this study, the performance of the MICP process on soil surface improvement was investigated using *Staphylococcus warneri* IR-103 bacteria. The biostabilizer, containing *S. warneri* suspension and a cementation solution consisting of 0.5 mM $CaCl_2$ and 1.5 mM urea, was sprayed on fine-grain soil to induce a surface MICP reaction. Soil surface strength was measured using a penetrometer test, and wind tunnel tests were conducted to evaluate the soil surface's resistance to wind erosion. Scanning electron microscopy (SEM) analysis of the treated soils was conducted to visualize carbonate crystal formations within and on the soil particles. Additionally, X-ray diffraction (XRD) was used to confirm the presence and identify the crystal structures. The ecotoxicological assessment of the biostabilizer was carried out by performing phytotoxicity and oral/dermal/ocular *in vivo* acute toxicity experiments due to a few case reports of *S. warneri*'s harmfulness and virulence of coagulase-negative staphylococci, highlighting the need for safety measures for workers and end-users. Mixing cementation solution with bacterial suspension in yeast-ammonium chloride medium increased soil strength and durability. The biostabilizer did not harm the seed germination of *Agropyron desertorum*, and the soil surface remained resistant to wind erosion. Rat oral/dermal acute toxicity tests revealed no adverse effects during the 14-day observation period. The LD_{50} (median lethal dose) cut-off value of the biostabilizer in oral and dermal administrations was 5000 and 1000 mg/kg body weight, respectively. Ocular administration of a 0.1 mL drop did not induce eye irritation in rabbits. In conclusion, the use of the biostabilizer for wind erosion control appears to be technically and environmentally feasible and justifiable.

1. Introduction

Biocementation is a process in which various microorganisms generate crystalline calcium carbonate under specific conditions [1,

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https://doi.org/10.1016/j.heliyon.2024.e35813
Received 20 April 2024; Received in revised form 3 August 2024; Accepted 5 August 2024
Available online 5 August 2024

2], a phenomenon known as microbial-induced calcium carbonate precipitation (MICP) [3–6]. By bonding soil particles together, biocementation significantly enhances the mechanical properties of soil [7]. MICP has several applications, including concrete reinforcement and healing [8], as well as countering wind erosion and stabilizing the soil [9]. In fact, MICP has been reported to improve the mechanical properties of porous materials [10]. Biocementation has the capability to increase the shear strength and stiffness of the soil while maintaining its permeability [11,12].

Biocementation through MICP can proceed through various bacterial metabolic pathways, including denitrification, ureolysis, sulfate reduction, and iron reduction. The ureolytic pathway is particularly prominent among these routes, due to its simplicity, efficiency, rapidity, and absence of excessive proton production. Within the microbial ureolytic pathway, urea undergoes hydrolysis catalyzed by urease to yield ammonia and carbon dioxide (**Equations 1,2**) [13–16]:

$$CO(NH_2)_2 + 2H_2O \rightarrow 2NH_4^+ + CO_3^{2-} \quad (1)$$

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3(s) \quad (2)$$

Kucharski et al. (2005) pioneered this field by patenting a microbial soil stabilizer using urease-producing bacteria. Their innovative approach resulted in cement boasting a compressive strength of 5 MPa [17]. A summary of recent studies on surface treatment are listed in Table 1 and deliberated in the following paragraphs. Kalantari and Kahani investigated the influence of culture medium and different temperatures on calcium carbonate precipitation using *Sporosarcina pasteuri*. They showed that a two-step treatment of soil with microbial suspension and reaction solution within an hour could enhance the soil's compressive strength to over 400 kPa. Interestingly, lowering the temperature below ambient conditions further boosted the compressive strength from 80 kPa to 230 kPa [18,19]. Schwantes et al. investigated the effect of *Bacillus subtilis* on the mechanical properties of the concrete. The researchers added the bacterial spore to the mortar in two forms. In the first mode, they mixed the spore with water, and secondly, they tested the spore-containing solution immersed in saturated mortar. The results showed that the compressive strength of concrete increased by 31 % through immersion in the bacterial spore solution. The mentioned study highlighted the potential of biocementation of $CaCO_3$ to

Table 1
A summary of recent studies on surface treatment.

Study characteristics	Main outcomes	Ref.
Moosazadeh et al. found that *Staphylococcus warneri* IR-103 was a high urease producer bacterium, which could grow on YN medium containing 20 g/L of yeast extract and 10 g/L of NH_4Cl medium without staying viable for long following the biocementation process and did not alter the soil microbial flora.	Therefore, this recently isolated strain from the soil in National Institute of Genetic Engineering and Biotechnology (NIGEB) showed the potential to be used in the geotechnical improvement of soil for large-scale field purposes	[7]
Kucharski et al. patented a microbial soil stabilizer using urease-producing bacteria.	A cement with a compressive strength of 5 MPa.	[17]
Kalantari and Kahani used *Sporosarcina pasteuri* to investigate the effect of culture medium and different temperatures on the formation of calcium carbonate precipitates.	It was shown that the compressive strength of soil increased to over 400 kPa if the microbial suspension and reaction solution were treated in two steps within 1 h. In addition, decreasing temperature relative to ambient increased the soil compressive strength from 80 to 230 kPa.	[18, 19]
Schwantes et al. investigated the effect of *Bacillus subtilis* on the mechanical properties of the concrete. The researchers added the bacterial spore to the mortar in two forms. In the first mode, they mixed the spore with water, and secondly, they tested the spore-containing solution immersed in saturated mortar.	The results showed that the compressive strength of concrete increased by 31 % through immersion in the bacterial spore solution. They also demonstrated that the biocementation of calcium carbonate in cracks led to the recovery and increased strength of the concrete.	[20]
Cheng et al. used *Bacillus sphaericus* DSM-23526 in a medium containing 20 g/L yeast extract, 0.17 mM ammonium sulfate, and 0.1 mM nickel chloride. Nickel is present in the active site of the urease enzyme.	Their results showed that the soil strength increased with decreasing soil saturation percentage of cementation solution and bacterial suspension.	[24]
Kim et al. investigated the impact of adding bacterial metabolites and montmorillonite on crack healing and biocementation in sandy soil during the MICP process.	The results demonstrated that after five cycles of treatment with a spray containing living cells/microbial metabolites, the ratio of surface cracks decreased significantly. Living cells resulted in a 71 % reduction, while microbial metabolites led to an 80 % reduction. In contrast, water and cementation solution alone had lower crack reduction ratios of 28 % and 48 % respectively. Introducing montmorillonite to sandy soil during MICP increased soil strength, with higher levels of montmorillonite leading to greater stabilization through colloid formation and adhesion between sand grains.	[28]
A study by Pan et al. developed a prediction model using the response surface method to determine the optimal nutrient concentrations in the medium. By using the model, the researchers were able to compare the effectiveness of the optimized medium with other media through biocementation tests.	The study found that the prediction model was accurate and effective in determining an optimized culture media (20.0 g/l yeast extract, 10.0 g/l polypeptone, 5.0 g/l ammonium sulfate, and 10.0 g/l NaCl) that significantly enhanced biofilm growth. The use of this optimized medium led to increased strength and calcium carbonate content in sand column structures.	[29]
A study by Idris et al. sought to identify potential bacterial isolates from rock samples in an arid region that could induce $CaCO_3$ precipitation and analyze the precipitate they produce. Bacterial isolates with high ureolytic activity were then studied further for their ability to produce $CaCO_3$ precipitation.	The finding revealed that isolate M 2.6 from five tested bacterial isolates was recognized as the best isolate, able to induce the highest amount of $CaCO_3$ precipitation at 2.6 g/L, and was later identified as *Mesobacillus campisalis*. The $CaCO_3$ precipitate produced by the isolates ranged from 1.4 g/L to 2.6 g/L.	[30]

repair cracks and enhance the overall strength of concrete structures [20]. Another research study achieved a specific urease activity of 177.55 U/mg after single-step purification using a diethylamine ethyl sepharose® column. The kinetic parameters of the urease were analyzed so that the maximum rate of reaction was 0.27 μmol/min and the Michaelis constant was equal to 61.5 mg/mL [21].

Yeast extract culture medium has been used in a majority of the research studies concerning the field of biocementation for bacterial culture and production of urease [3,22,23] used *Sporosarcina pasteurii* ATCC-1859 and *Proteus vulgaris* in 20 g/L yeast extract and 75 mM ammonium sulfate solution to examine enzyme activity and bacterial viability in a non-sterile medium. It was found that *S. pasteurii* had a high potential urease capacity (29 mM urea/min; OD) which was sufficient for the biocementation process [3]. Cheng et al. explored the use of *Bacillus sphaericus* DSM-23526 in a medium containing 20 g/L yeast extract, 0.17 mM ammonium sulfate, and 0.1 mM nickel chloride. Nickel is present in the active site of the urease enzyme. Their results showed that the soil strength increased with decreasing soil saturation percentage of cementation solution and bacterial suspension [24]. *Staphylococcus pasteuri* was grown in Luria–Bertani broth containing yeast extract, tryptone, and NaCl and evaluated for its potential to strengthen sands by MICP. The unconfined compressive strength of the soil reached about 2.3 MPa at the strain rate of 0.005 mm/s. The hydraulic conductivity of soil columns treated with this bacterium was reduced from 13 to 7.5 m/day [25,26].

In a study focused on cost-effective biocementation, Bhutange et al. utilized soil-derived urea-hydrolyzing bacteria and a growth medium containing table sugar, meat extract, and lentils. This approach successfully increased the soil's compressive strength by 20 % while simultaneously reducing water uptake by 17 % [27].

According to the findings of recent studies, *Staphylococcus warneri* is a type of bacteria commonly found on the skin of healthy individuals. While usually harmless, it can occasionally cause infections in vulnerable populations, particularly those with weakened immune systems, the elderly, and those with medical devices. Infections can manifest in various forms, including bloodstream infections, bone infections, and infections related to implanted devices, therefore evaluation of biostabilizers from this bacterium should be considered [31–35].

Although studies have investigated the effects of different media containing yeast extract, urea, and ammonium chloride on *Staphylococcus* sp. IR-103 growth and urease production, there is limited research on the evaluation of MICP performance for soil surface improvement and toxicity assessment of the biostabilizer. The main objective of this study was to assess the performance of the MICP process using *S. warneri* IR-103 on surface stabilization of loose, dust-prone soils, particularly in large-scale field applications. Considering the necessity for environmental friendliness, cost-effectiveness, and practicality, the biostabilizer must demonstrate these characteristics. *S. warneri* IR-103, being non-spore-forming, reduces the risk of fluctuating soil microbial flora over time. The study initially assessed the influence of various carbon sources in the bacterium's culture medium and factors affecting soil strength in the MICP process with a formulated biostabilizer, aiming to identify optimal conditions. Furthermore, we evaluated the biostabilizer's impact on *Agropyron desertorum*'s seed germination and its acute oral/dermal toxicity toward rats and ocular toxicity in rabbits. Assessment of acute oral toxicity of a soil biostabilizer *in vivo* can be conducted to investigate the putative health risks and hazards attributed to the substance for mammals and the environment based on recent case reports. Also, these toxicological findings can assist in establishing guidelines for the safe handling, storage, and application of the soil biostabilizer, as well as inform the development of appropriate personal protective equipment and safety measures for workers and end-users.

2. Materials and methods

2.1. Bacterial strain and culture media

We used *Staphylococcus warneri* IR-103 (accession number LT853888.1) which was previously isolated and identified using molecular methods in NIGEB [7]. To assess the effects of different carbon sources, we used MY medium, which contained 7.5 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 mL/L trace element solution, 20 g/L yeast extract, and 10 g/L NH_4Cl. Glucose, galactose, and fructose were used as monosaccharides at a concentration of 10 g/L, while sucrose and lactose were used as disaccharides at a concentration of 5 g/L. Each carbon source was prepared separately, autoclaved, and then added to a flask containing MY medium. The bacterium was cultured at 37 °C with shaking at 180 rpm for 14 h. Cell concentration and urease activity were measured at 2-h intervals. In order to culture in the fermenter and inoculum preparation, yeast-ammonium chloride medium (YA medium) was used comprising 20 g/L yeast extract and 10 g/L NH_4Cl.

2.2. Measurement of urease activity

One unit of urease activity is defined as the amount of enzyme that can hydrolyze 1 μmol of urea per minute. The Nessler method [36] involves quantifying the release of ammonia following the hydrolysis of urea at different growth intervals. Assessments were performed under specific parameters: using a spectrophotometer at a wavelength of 425 nm, maintaining a temperature of 25 °C, and ensuring a pH of 7.34. Urease activity is expressed as U/min, representing the quantity of urease enzyme in 1 mL of culture medium that hydrolyzes 1 μmol of urea per minute. The determination of urease activity data was principal in identifying the most suitable culture medium for MICP. These data provided insights into the efficiency of *S. warneri* IR-103 bacteria in converting urea into ammonia and bicarbonate ions, a critical process in MICP, across different culture media. By comparing urease activity levels among the various culture media, we selected the medium that exhibited the highest urease activity as the optimal choice for MICP. The findings related to urease activity could significantly contribute to establishing the ideal culture medium [37].

2.3. Soil specifications

The tested soil was collected from a desert area in Garmsar, Semnan Province. The soil has fine grains and a uniformity coefficient of 1.81, indicating a low dispersion of particle sizes according to the ASTM D4320-based classification. It contains a maximum of 3 % particles smaller than 0.075 mm, with 40 % of the particles in the size range of 125 μm and 51 % in the range of 250 μm. These particle size distributions proposed a susceptibility to dust formation and a high potential for wind erosion. According to the Allied Classification (ASTM D4320), this soil was classified as poorly graded sand. Both untreated and MICP-treated soil samples were subjected to direct shear tests following the ASTM D3080 standard. To further assess the effectiveness of a novel culture medium in promoting the precipitation of carbonate crystals within and on the surface of soil particles, scanning electron microscopy (SEM) imaging and X-ray diffraction (XRD) analyses were performed on the treated samples.

2.4. Fermentation

A fermenter (Triton, France) with a total volume of 5 L was used for bacterial culture. The working volume of the fermenter was set to 2 L. Two liters of YA medium were prepared and sterilized in the fermenter vessel after calibrating the pH probe to a pH of 7.0. The inoculum was prepared by incubating 100 mL of YA medium at 37 °C overnight and then transferring it to the fermenter. Batch fermentation was carried out at a temperature of 37 °C, an agitation speed of 240 rpm, and a dissolved oxygen tension of 40 % air saturation. For fed-batch fermentation, a feed solution containing 500 g of glucose was prepared and added to the fermenter at flow rates of 10 and 20 mL/h.

2.5. Investigation of different factors on soil strength

The effects of various factors, including inoculation percent, cementation solution composition, the volume ratio of cementation solution to microbial suspension, and temperature variations, on soil strength were investigated in this study.

To identify the most effective bacterial concentration for enhancing soil strength, experiments were conducted using 50 mL of YA culture medium inoculated with four different percentages (2.5 %, 5 %, 10 %, and 15 %) of bacteria. Following a 24-h incubation at 37 °C, each suspension was mixed with an equal amount of a cementation solution (containing equal parts $CaCl_2$ and urea) and applied to the soil. Soil strength was measured after the samples were thoroughly dried.

To explore the effect of different concentrations of components within the cementation solution, calcium chloride, and urea solutions were prepared at 0.5 M, 1 M, and 1.5 M. Nine distinct combinations of these molarities were tested by mixing them with the bacterial suspension and spraying the mixture onto soil, followed by strength measurement.

To ascertain the ideal ratio for effective soil stabilization, three different volume ratios (0.5, 1, and 1.5) of cementation solution to microbial suspension were examined. Soil strength was assessed post-application of these ratios.

To investigate the stability of biomineralized soil under varying temperatures, treated soil samples (with a biostabilizer made from bacterial suspension and cementation solution) were incubated at 37 °C for 24 h before being subjected to cycles of 60 °C and −20 °C for another 24 h. This thermal cycling was repeated over a week, with a control sample kept at a constant 37 °C throughout. Soil strength measurements were taken at the end of the experiment.

2.6. Measurement of soil stabilization

To investigate the mechanical strength of the soil, an 8 cm-long plate was used. Initially, eight holes were created at the bottom of the plate using a perforator and were then filled with soil of the desired topography. According to the created surface area, 20 mL of a stabilizer solution was sprayed onto the surface. A sprinkler-like device was used for uniform spraying, ensuring the soil surface was completely covered with the biostabilizer solution. After spraying, the samples were placed in an incubator set at 37 °C until their surfaces were completely dry. The biostabilizer comprised a bacterial suspension and a cementation solution. Once the soil samples were completely dried, their compressive strength was measured using a penetrometer. To measure soil surface strength, the penetrometer was employed vertically on the soil surface, and pressure was applied till the device reached the breaking point of the soil surface, signifying the maximum strength.

For studying soil erosion resistance, a subsonic wind tunnel was used. A 25 × 13 cm glass container was designed to fit within the wind tunnel chamber and was filled with topographic soil. The surface area of the soil samples was measured, and subsequently, 72 ml of the cementation solution was sprayed uniformly onto the soil surface using the sprinkler-type device. The samples were then incubated at 37 °C for 15 days to ensure complete drying. After drying, the sample was placed in the wind tunnel, and airspeeds of 5, 10, 15, and 20 m/s (equivalent to 18, 36, 54, and 72 km/h, respectively) were applied in series. To quantify soil particle loss owing to wind erosion, the samples were weighed before and after exposure to each wind speed using precision scales.

2.7. Evaluation of biostabilizer's impact on seed germination

Seed germination of *Agropyron desertorum* commonly known as desert wheatgrass, sourced from the International Desert Research Center, Tehran University, was studied under different NaCl concentrations of 0, 5, 10 g/L, and biostabilizer concentrations of 50, 75, and 100 % (v/v) as shown in Table 2. Seeds were first cleaned by washing with distilled water and treated with a 2 % sodium hypochlorite solution for 2 min. Afterward, they were washed and rinsed two times with distilled water carefully. Eleven plastic pots,

each filled with 1 kg of pre-sterilized soil collected from the desert area in Garmsar, received 7 seeds of *Agropyron desertorum* seeded at a depth of 5 cm, placed equidistantly. The pots were treated with 50 mL of different concentrations of NaCl and biostabilizer. Germination experiments were conducted in a germinator (Sinjders Scientific, The Netherlands) under constant conditions of 20 ± 1 °C, 12 h/day of illumination (day and night), and watered with tap water every 5 days. Seed germination was monitored every three days and germination percent was recorded for 30 days. All treatments were replicated three times and the data were presented as mean ± SE.

2.8. In vivo acute toxicity evaluation

In this part of the study, 12 female Wistar albino rats, aged 8–12 weeks old and weighing 200 ± 15 g were obtained from Pasteur Institute of Iran. The animals were housed under controlled conditions: 12-h light/dark cycles, humidity maintained at 55 ± 15 %, and a temperature range of 22–24 °C. They had ad libitum access to food and water. The experiments were conducted in accordance with the ethical standards of the National Institute of Genetic Engineering and Biotechnology (NIGEB) and Zist Azma Arya Vizhen (ZAAV) Company, Tehran, Iran (IR.NIGEB.EC.1394.8.10). Each rat was allowed to acclimate to these environmental conditions for 7 days before initiating the toxicity testing.

The assessment of acute toxicity often begins with determining the lethal dose (LD_{50}), which represents the dose that causes the death of 50 % of the test animal population. For this study, both acute oral and dermal toxicity of the biostabilizer were evaluated in accordance with OECD guidelines.

To analyze the acute oral toxicity, 6 female Wistar albino rats were divided into two groups. The control group received an oral administration of 5000 mg of tap water per kg body weight, while the treatment group was administered 5000 mg of biostabilizer per kg body weight. All procedures adhered to OECD protocols (OECD guidelines no. 425) [38]. The number of awakenings was 10–20 times/h, and illumination was on for 12 h/day. The animals were observed for 14 days.

For acute dermal toxicity analysis, another set of 6 female Wistar albino rats were divided into two groups, each consisting of three rats. The fur in the dorsal area of each animal's trunk was shaved, creating a circular exposed area of approximately 3 cm in diameter. The control group received a dermal application of 1000 mg of tap water per kg body weight, while the treatment group received 1000 mg of biostabilizer per kg body weight on the shaved skin. The site was observed at 24, 48, and 72 h after removing the test substance, following the Draize criteria for evaluating skin irritation. The animals were monitored for 14 days, in accordance with OECD guidelines no. 402 [39].

Following the administration of the biostabilizer and control treatments, all animals were closely monitored for clinical signs of toxicity and mortality. This involved vigilant observation within the first 4 h post-administration and daily examinations for the following 14 days. The weight of the animals was recorded on the first, seventh, and fourteenth days of the observation period. After the 14-day monitoring period, the study concluded, and necropsy examinations were performed two weeks after the initial treatments. All animals were anesthetized with isoflurane and then euthanized by cervical dislocation. Various organs, including the brain, liver, spleen, heart, kidney, sexual organs, bladder, muscle, skin, intestine, stomach, and pancreas, were collected and subjected to macroscopic examination for lesions or abnormalities. The relative weight of each organ was calculated by dividing the organ weight by the corresponding body weight of the animal [40].

2.9. Acute eye irritation evaluation

In order to investigate acute eye irritation of the biostabilizer, we used 3 female New Zealand white rabbits, aged 8–12 weeks old and weighing 1650 ± 50 g were obtained from the Pasteur Institute of Iran. The animals were housed under controlled conditions in individual ventilated cage systems: 12-h light/dark cycles, humidity maintained at 55 ± 25 %, and a temperature range of 18–25 °C. They had ad libitum access to food and water. The experiments were carried out in accordance with protocol IR.NIGEB.EC.1394.8.10. Each rabbit was allowed to acclimate to these environmental conditions for 7 days before the test was conducted. Before the test performance general anesthesia was applied by injection of ketamine hydrochloride 10 % (100 mg/kg, i.p.) and xylazine 2 % (10 mg/kg, i.p.). Then, the test solution was applied intraocular (0.1 mL drop, single dose). The observation of adverse effects was conducted on each animal during the first 30 min and followed periodically during the first 4 h, and daily thereafter up to 21 days (OECD no. 405) [41].

Table 2
Factors and their levels for evaluating phytotoxicity of biostabilizer in the presence of salinity.

Trial No.	1	2	3	4	5	6	7	8	9	10	11	12
Factors												
NaCl conc. (g/L)	0	0	0	5	5	5	10	10	10	5	10	0
Biostabilizer conc. (%v/v)	50	75	100	50	75	100	50	75	100	0	0	0

*Trial #12 was considered as control.

2.10. Data analysis

Each experiment was repeated twice and the samples were measured in triplicate. Utilizing SPSS software version 22 for Windows, a one-way analysis of variance (ANOVA) followed by Tukey's test was conducted to compare the collected data. The findings were reported as mean ± SE. A statistical significance threshold of $p \leq 0.05$ was established for all analyses.

3. Results and discussion

3.1. The effect of different carbon sources on bacterial growth and urease production

MY and YA media are simple and low-cost, containing only a few ingredients. Given that urease plays a pivotal role in the bio-cementation process and its activity is linked to biomass, optimizing bacterial growth conditions is crucial. Previous studies have indicated that *S. warneri* can utilize a diverse range of carbon sources [42]. To identify the best carbon source for *S. warneri* IR-103 growth, a bacterial growth curve was monitored over 14 h in YA and MY media, each with different carbon sources. *S. warneri* IR-103 exhibited varying levels of sugar consumption and growth depending on the carbon source provided. As shown in Fig. 1, the highest growth was observed in the medium containing galactose, with an $OD_{600nm} = 14$ of approximately.

After the fourth hour of culture, no significant increase in the activity of the urease was observed despite the increase in the cell population. As depicted in Fig. 2, urease enzyme activity ranged from 0.35 to 0.53 μmol/min, which was not significantly different under various conditions.

3.2. Influence of different carbon sources in culture medium on soil stabilization of MICP process

Equal volumes of the cementation solution were combined with each microbial suspension, which had been cultured in media containing various carbon sources. The resulting biostabilizers were then sprayed onto the soil samples. Visible white precipitates of varying degrees were observed on the surface of the plates, particularly those treated with the microbial suspension prepared in the presence of galactose (Fig. 3(A–E)). However, mechanical strength analysis revealed that the sample with the most pronounced precipitate (galactose-treated) had not undergone effective immobilization. The measured strength of the other samples, in kPa, was as follows: glucose (450 kPa), fructose (300 kPa), lactose (50 kPa), and sucrose (150 kPa). Notably, the biostabilizer prepared in YA media yielded the highest mechanical strength, exceeding 500 kPa.

To identify the chemical composition of the precipitates, X-ray diffraction (XRD) analysis was performed. The XRD results revealed that no calcium carbonate precipitate formed in the sample treated with the galactose-containing medium; instead, the precipitate was attributed to ammonium chloride salt. In contrast, distinct peaks corresponding to calcium carbonate were observed at 2θ diffraction angles of 44 and 25° in the XRD pattern for the sample treated with the microbial suspension prepared with YA and glucose-containing media (Fig. 4(A–C)). The peak of area 24 indicated the presence of calcium carbonate and the peak of area 34 showed ammonium chloride. The comparison of peak 24 in the XRD curve showed that the production of calcium carbonate crystal using the microbial suspension prepared from the YA medium was more than the MY + glucose medium, which confirmed the choice of this medium to continue this research. As seen in Fig. 4C, the medium containing galactose had only an ammonium chloride peak. The result of this experiment established that despite the high growth rate of bacteria and also the high activity of urease enzyme in this environment, calcium carbonate crystal was not formed and finally this environment was not able to stabilize the soil. The soil stabilized with

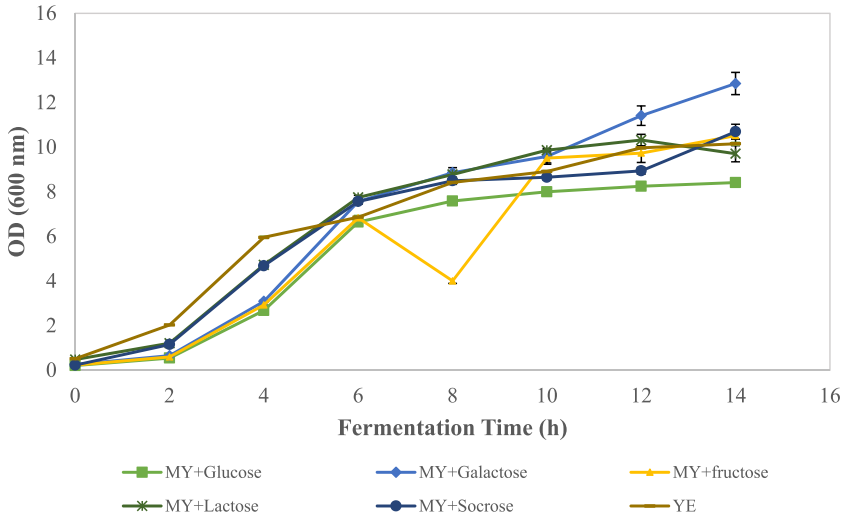


Fig. 1. Bacterial growth curve in culture media with different carbon sources at various fermentation times.

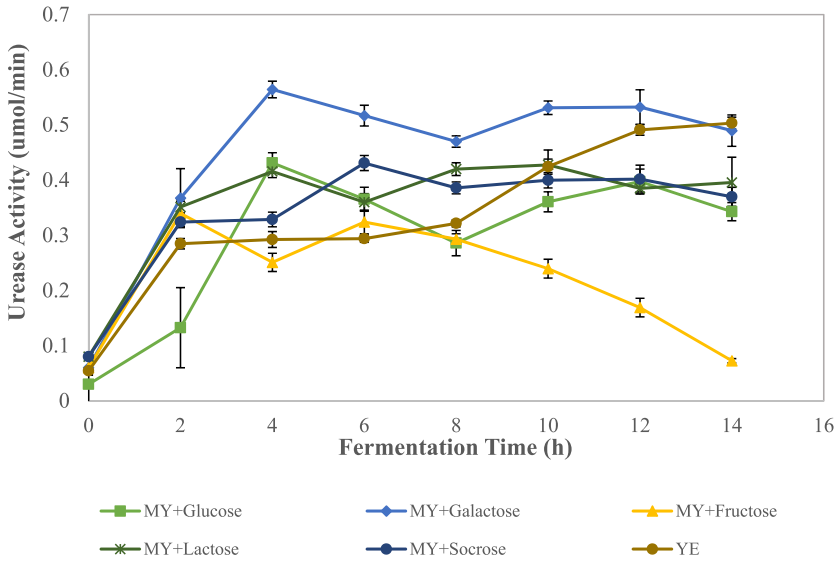


Fig. 2. Urease activity in media supplemented with different carbon sources at various fermentation times.

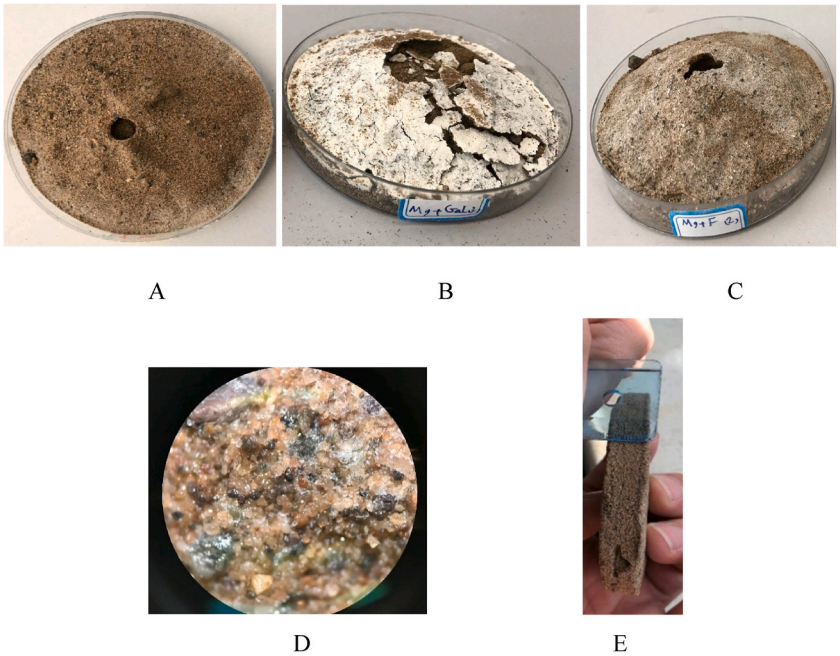
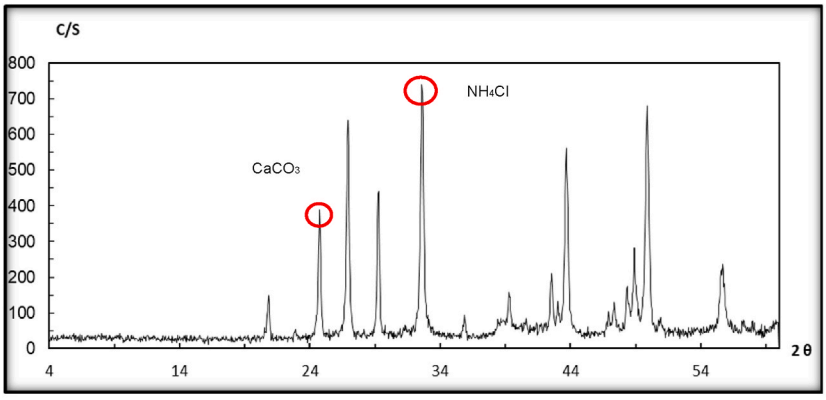


Fig. 3. Precipitate patterns on soil samples treated with biostabilizers containing different carbon sources: (A) MY + glucose, (B) MY + galactose, (C) MY + fructose, (D) Stereo microscope image of the treated soil surface, and (E) Depth of stabilized soil in a Petri dish. The holes in images A and C were created during mechanical strength testing with a penetrometer.

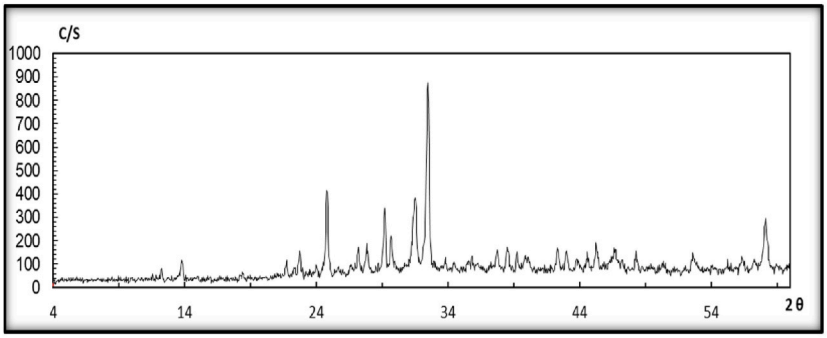
galactose-containing medium was not able to strengthen the soil surface in any of the repetitions and broke with the lowest pressure. Also, the medium containing lactose was not able to strengthen the soil surface.

SEM imaging provided further insights into the calcium carbonate precipitation process. As shown in Fig. 5(A and B), SEM images of soil samples stabilized with the microbial suspension prepared in YA and MY media containing glucose revealed the presence of calcium carbonate precipitates effectively binding the soil particles together in both samples. No significant difference was observed between SEM micrographs of two samples prepared in both culture media.

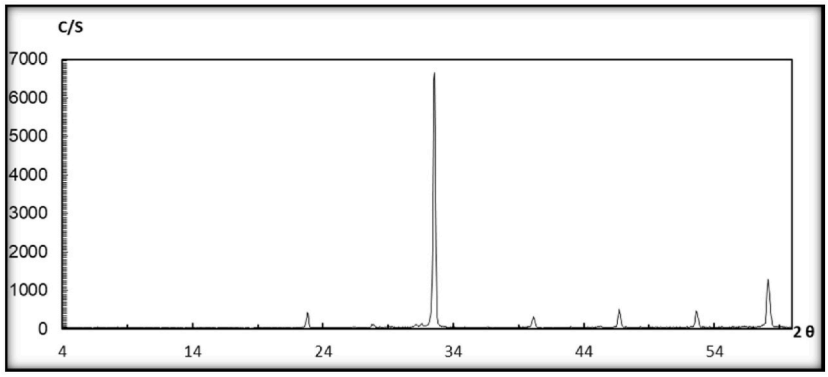
As can be seen, calcium carbonate was precipitated where the soil particles were very close to each other and there was a small distance between soil grains. This might be due to bacteria that would rather attach themselves in those areas and form biofilm on the



A



B



C

Fig. 4. The XRD patterns confirming the presence of calcium carbonate precipitates formed by the MICP reaction: (A) YA medium, (B) MY + glucose medium, (C) MY + galactose medium.

surface of soil grains that were not easily detached. The nucleation of calcium carbonate crystals was the main result of metabolic activity of bacterial colonies in those critical zones which build bridges between soil particles.

3.3. 3.3. Formulation of biostabilizer

Bacterial cells showed good growth and proper production of urease in the YA medium, and the soil treated under these conditions

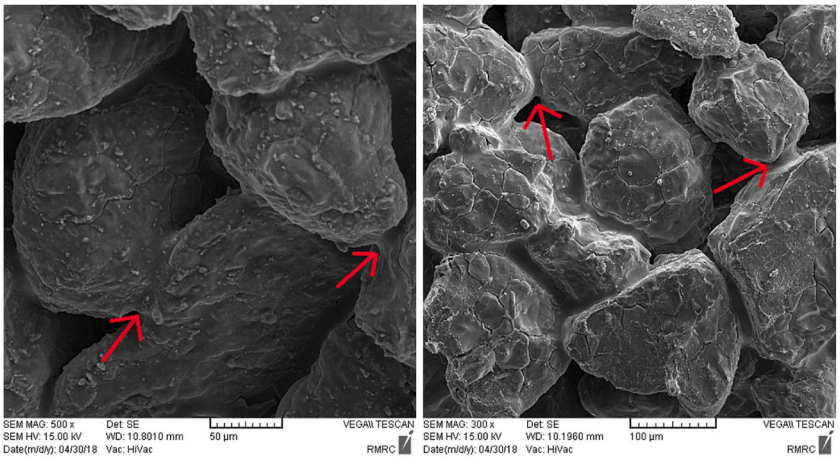


Fig. 5. SEM images illustrating calcium carbonate precipitation between soil particles: (A) MY + glucose medium and (B) YA medium. Arrows indicate calcium carbonate precipitates.

had acceptable strength. Additionally, YA is a simple, inexpensive, and easy-to-prepare medium. To determine the effect of initial inoculation on soil strength, the bacteria were inoculated into YA fermentation medium at 2.5, 5, 10, and 15 % values. The results showed that the soil strength for 2.5 % inoculation was around 300 kPa, while 5–15 % inoculation completely immobilized the soil, with a strength of >500 kPa. It has been suggested that the initial cell population could play an important role in the stabilization of soil, possibly related to the size and type of calcium carbonate crystals formed. Previous studies have shown that the increasing concentration of bacteria in the medium increased the size of formed crystals. They used four dilutions of microbial suspension and indicated that the size of calcium carbonate crystals increased concomitantly with the growth of the bacterial population [17,43].

The study also examined how the concentration of certain substances in the cementation solution affected soil strength. The results revealed that soil strength exceeded 500 kPa when the concentrations of calcium chloride and urea were 0.5 mM and 1.5 mM, respectively, or when both were at a concentration of 1.5 mM. Due to the similar outcomes in these experiments and for cost-efficiency, the cementation solution was formulated with 0.5 mM calcium chloride and 1.5 mM urea. It was noted that the samples containing more calcium chloride had less strength in the long run and that their surface became loose due to the absorption of ambient moisture. This is likely attributed to the hygroscopic properties of calcium chloride, which can attract air moisture and decrease soil strength [44]. Qabany and colleagues found that the chemical effectiveness of calcium carbonate precipitation ranged from 70 % to 100 % when using 0.25 mM and 0.5 mM concentrations of urea and calcium chloride, but dropped to less than 20 % when 1 mM concentrations were used, likely due to the formation of different types of calcium carbonate crystals [45]. The effect of the volume ratio of cementation solution to the bacterial suspension on soil strength was also investigated. The volume ratios of 1:1, 1.5:1, and 1.5:1.5 of cementation solution and bacterial suspension caused the highest mechanical strength in the soil, while ratios below 1 did not produce the necessary strength for soil stabilization.

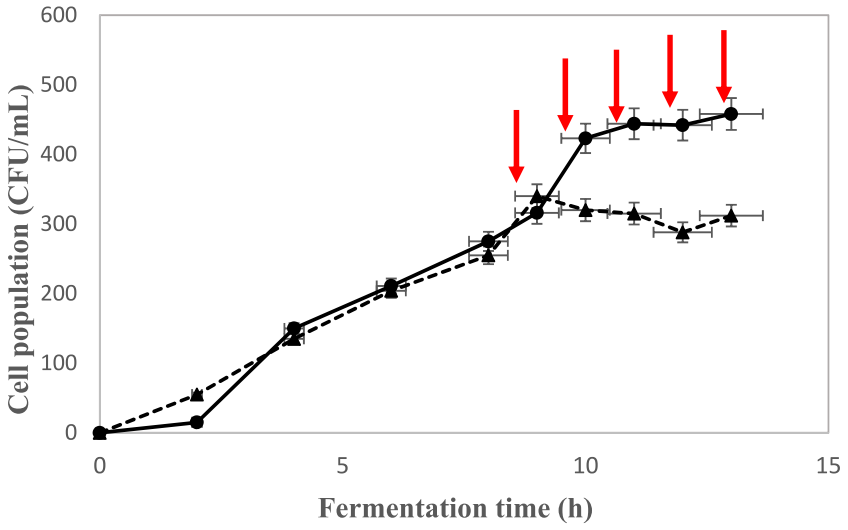
The samples transferred from 37 to 4 °C had a perfectly resistant and uniform surface, while samples moved from 37 to 60 °C and 37 to −20 °C showed cracks despite having a strength of 400 and 450 kPa, respectively. The harsh temperatures and severe temperature differences led to cracks on the surface of the treated soil, likely due to increased expansion and volume changes.

Finally, the samples prepared at 5 % inoculation, 0.5 and 1.5 mM calcium chloride and urea concentrations, and a 1:1 vol ratio of cementation solution and microbial suspension prepared in YA medium, were examined for wind erosion in a wind tunnel. The results showed that none of the treated soil samples experienced weight decrease even at 20 m/s (72 km/h) speed for 5 min, indicating high resistance to wind erosion.

3.4. 3.4. cell population growth by fed-batch fermentation

A batch culture of *S. warneri* IR-103 in YA medium showed that the bacterium was fast growing, its specific growth rate of 2.5 h^{-1}, and bacterial doubling time was calculated at 16 min. According to the results of the fermentation process (data not shown), the bacteria grew to the end of the logarithmic phase after 8 h. Feeding with a 500 g/L glucose solution was initiated after 8 h, using a pulse feeding strategy at 10 and 20 g/h to boost the bacterial population. The findings of bacterial culture and urease production in the fed-batch fermentation process are shown in Fig. 6(A and B).

A comparison of bacterial growth showed that at a 20 ml/h feeding rate, the maximum cell population reached 4.58×10^9 CFU/mL, which was approximately 45 % higher than that of fermentation with a feeding rate of 10 mL/h likely due to nutrient availability. A comparison of urease enzyme production at both feeding rates showed that the maximum enzyme activity was 0.61–0.65 U/mL. However, the activity of the enzyme produced in the fermenter increased by 18 % compared to that in the shake flask, which was a



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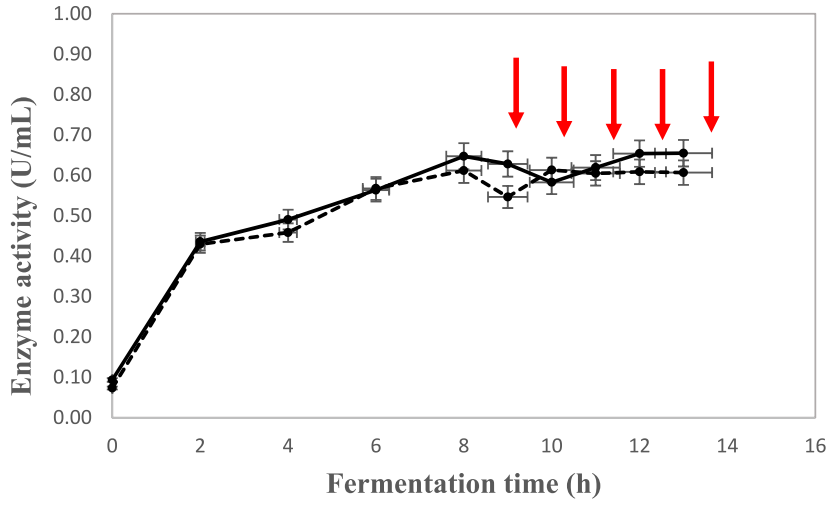


B

Fig. 6. (A) Bacterial growth curve in fed-batch with 20 ml/h (●) and 10 ml/h (▲) feeding rate. (B) Urease activity in YA medium with 20 (●) and 10 (▲) ml/h feeding rate. Arrows indicate the feeding time.

maximum of 0.55 U/mL in yeast extract-ammonium chloride medium and may be related to the process of bacterial growth as well as activation of bacteria under controlled conditions of the fermenter. Despite the growth of the cell population after feeding, the enzyme activity in the batch process did not significantly differ from that of the batch process, and then remained constant after 8 h in the batch process. To evaluate the effect of cell population growth on the MICP process and soil stabilization, sampling was performed every 2 h during fermentation and every hour after feeding. The microbial suspension samples were mixed with the cementation solution after its preparation and sprayed onto the soil. The results of soil strength measurements are presented in Table 3. As can be seen, in the batch fermentation process, increasing bacterial growth has enhanced the activity of the urease enzyme, leading to an increase in the mechanical strength of the soil. The rising microbial population in the biostabilizer has reinforced its effect on soil strength, which was also confirmed in the inoculation test. However, unexpectedly, no significant difference in soil strength was observed in fed-batch fermentation despite a higher bacterial cell population, presumably due to the limited increase in enzyme activity after feeding. In other words, the urease activity was not directly due to the increasing biomass population. The key to effective MICP lies in increasing the active cell population capable of urease production. It should be noted that extraction and purification of the enzyme are expensive, so using whole bacterial cells containing active enzymes offers a remarkable cost saving to the biocementation process. Therefore, the batch fermentation process is advantageous over that in the mentioned feeding conditions given the optimum results in terms of soil

Table 3
Effect of cell population on mechanical strength of soil in the fermentation process.

Time of fermentation (h)	Fed-batch fermentation with 10 mL/h feeding			Fed-batch fermentation with 20 mL/h feeding		
	Mechanical strength of soil (kPa)	Enzyme activity (U/mL)	Cell population (CFU/mL)	Mechanical strength of soil (kPa)	Enzyme activity (U/mL)	Cell population (CFU/mL)
4	300	0.45	1.35×10^9	300	0.49	1.5×10^9
6	400	0.57	2.04×10^9	350	0.56	2.11×10^9
8[a]	500<	0.61	2.55×10^9	500<	0.65	2.75×10^9
9[a]	500<	0.55	3.4×10^9	500<	0.63	3.16×10^9
10[a]	500<	0.61	3.2×10^9	500<	0.58	4.23×10^9
11[a]	500<	0.6	3.15×10^9	500<	0.62	4.44×10^9
12[a]	500<	0.61	2.88×10^9	500<	0.65	4.42×10^9
13	500<	0.61	3.12×10^9	500<	0.65	4.58×10^9

[a] pulse feeding was carried out each hour.

surface reinforcement as well as saving time and cost.

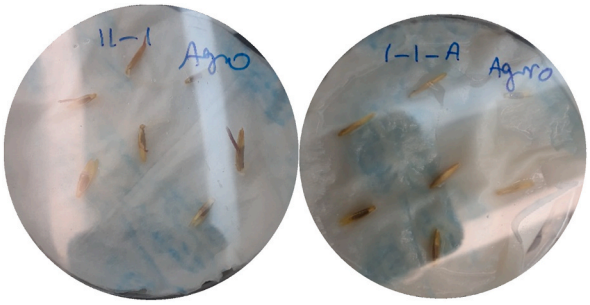
3.5. Phytotoxicity test

The highest seed germination percentage of 80 % was observed in the control pot treated with tap water, followed by the second control treated with 5 g/L NaCl, with a germination rate of around 70 %. The study found that seed germination of *A. desertorum* was delayed for 3 days compared to the control and decreased with rising NaCl and biostabilizer concentrations. The highest biostabilizer concentration with and without NaCl showed a substantial reduction in seed germination. Maximum germination in the presence of 50 and 75 % of biostabilizer reached 60 % after 21 days, which was 25 % less than the control (Table 4) (Fig. 7(A and B)). It means that germination was affected by the biostabilizer; nevertheless, seed germination occurred in all pots. So, it can be concluded that there is no toxicity effect caused by biostabilizer. Statistical analysis of seed germination percent indicated that salinity had no significant effect on seed germination (Table 5). Possibly due to *A. desertorum*'s natural adaptability to dryland conditions and some salinity levels [46]. The purpose of adding NaCl to the experiment was to simulate the effects of salinity on seed germination. Salinity is a common environmental stress that plants face in arid and semi-arid regions, where the soil might have high salt concentrations. By studying the effects of NaCl on seed germination, the researchers could gain insights into how *A. desertorum*, a species adapted to dry environments, responded to salt stress and how it might be affected by increasing salinity levels in its natural habitat [47,48]. Biostabilizer concentration had a significant effect ($p < 0.05$) on seed germination. No interaction effect between the two factors was observed. Tukey's test for pairwise comparison of different concentrations of biostabilizer showed that means of 100 % biostabilizer are significantly different from others.

Petroleum mulch, a widely used dune stabilization material, consists of a range of heavy petrochemical compounds, including saturated naphthene, polar aromatics, and asphaltenes. The toxicity of petroleum substances to microorganisms and plants has been summarized. Aliphatic hydrocarbons are chemically inert and, therefore are of no risk to the environment; however aromatic hydrocarbons are highly toxic and common pollutants of soil and groundwater. Petroleum substances not only retard the growth of plants or stop seed germination but also affect the uptake of many macro- and micronutrients. Their influence on plants depends on the type and rate of petroleum substances and plant species [49].

Table 4
Seed germination percentage of *A. desertorum* as influenced by different salinity and biostabilizer concentrations during 30 days.

Trial No.	NaCl (g/L)	biostabilizer (% v/v)	Seed germination (%)										
			day 1	day 3	day 6	day 9	day 12	day 15	day 18	day 21	day 24	day 26	day 29
1	**0**	**100**	0	0	0	0	5	10	19	19	24	29	29
2		**75**	0	0	0	0	0	5	24	57	57	57	57
3		**50**	0	0	0	0	19	33	43	52	52	57	57
4	**5**	**100**	0	0	0	0	5	5	5	29	29	29	29
5		**75**	0	0	0	0	10	33	33	43	43	43	52
6		**50**	0	0	0	0	5	5	14	29	29	33	43
7	**10**	**100**	0	0	0	0	0	0	0	10	10	19	24
8		**75**	0	0	0	0	5	19	24	43	43	48	48
9		**50**	0	0	0	0	10	43	48	57	57	62	67
10	**5**	**0**	0	0	0	0	43	57	57	57	57	57	57
11	**10**	**0**	0	0	0	0	29	38	52	52	52	52	52
12	**0**	**0**	0	0	0	24	71	81	81	81	81	81	81



A B

Fig. 7. The seed germination of *Agropyron desertorum* in the A) control sample and B) the sample with biostabilizer.

Table 5
Statistical analysis (ANOVA) of seed germination of *A. desertorum* in the presence of biostabilizer and saline.

Source	Type III Sum of Squares	Df	Mean Square	F-value	Sig. (p-value)
Corrected Model	5735.021[a]	8	716.878	3.728	0.010
Intercept	64906.404	1	64906.404	337.526	0.0001
Salt	1054.703	2	527.352	2.742	0.091
biostabilizer	3679.516	2	1839.758	9.567	0.001
salt * biostabilizer	1000.802	4	250.201	1.301	0.307
Error	3461.409	18	192.301	–	–
Total	74102.835	27	–	–	–
Corrected Total	9196.430	26	–	–	–

3.6. Toxicity evaluation in rats and rabbits

Experimental results showed that the biostabilizer had no significant effect on mortality and change of body weight in rats while they orally received 5000 mg biostabilizer/kg body weight over a period of 14 days. The biostabilizer also did not cause specific clinical signs (Table 6). The histopathological examination of the organs did not reveal any abnormalities. Moreover, no skin irritations, redness, or inflammation were observed on the shaved skin of rats treated with biostabilizer after administration, up to the endpoint date (Fig. 8(A and B)). No eye sensitivity, irritation, and/or redness was observed after the test. Based on the results and the Global Harmonized Classification system for acute toxicity categories, the acute eye irritation of the tested biostabilizer was considered non-sensitive and non-irritable.

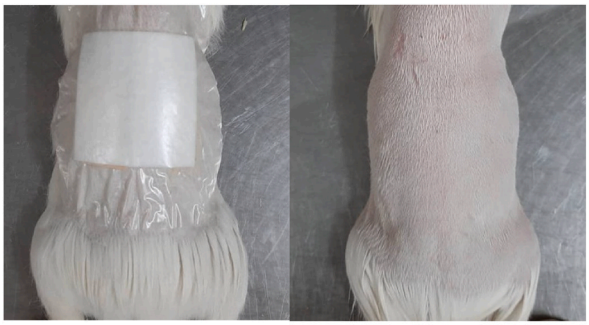
According to a previous study, the polymer-based stabilizers, vinyl acetate fraction. Hydrocarbon stabilizers, typically derived from crude oil or refined paraffin, can exert anesthetic effects at high concentrations and are flammable, occasionally causing dermatitis. Polysaccharide stabilizers, composed mainly of sugar and starch, showed limited inhalation toxicity but could also cause dermatitis [50]. To mitigate environmental concerns, it is essential to formulate an eco-friendly and biodegradable soil stabilizer with minimal or no adverse effects on live organisms.

4. Conclusion

Various techniques have been developed for managing wind erosion by employing soil stabilizers. In our study, *S. warneri* IR-103 has been used as a biostabilizer to stabilize the soil surface through the MICP process. This environmentally compatible technique has demonstrated cost-saving, high efficiency, and performance for soil surface improvement, prevention of soil erosion, and dust control, without significant adverse effects on flora and fauna. The biostabilizer containing *S. warneri* suspension and cementation solution was sprayed on fine-grain soil, inducing a surface MICP reaction. The treated soil exhibited increased surface strength, as evidenced by penetrometer tests and wind tunnel experiments, which confirmed its resistance to wind erosion. SEM and XRD analyses unveiled the formation of carbonate crystal structures within and on the soil particles, contributing to enhanced soil surface stability. To address any potential concerns regarding the safety of *S. warneri* and the necessity for implementing safety protocols for workers, ecotoxicological assessments were conducted. The biostabilizer did not exhibit phytotoxicity, as demonstrated by the successful seed germination of *A. desertorum*. Furthermore, oral, dermal, and ocular acute toxicity tests on rats and rabbits revealed no adverse effects, with the LD_{50} cut-off values for oral and dermal administrations being 5000 and 1000 mg/kg body weight, respectively. Ocular administration did not induce eye irritation in rabbits. In conclusion, the use of the *S. warneri* IR-103 biostabilizer for wind erosion control through the MICP process appears to be technically and environmentally feasible and justifiable, offering an effective and sustainable solution for improving soil surface and preventing erosion.

Table 6
Results of body weight changes in oral toxicity test on rat administrated by biostabilizer.

Group No.	Animal ID	Day (0)		Day (7)		Day (14)	
		BW(g)	change	BW(g)	change	BW(g)	change
Group I	I-1	185	0	187	+2	189	+4
	I-2	190	0	193	+3	195	+5
	I-3	205	0	207	+2	210	+5
Group II	II-1	185	0	188	+3	191	+6
	II-2	200	0	202	+2	206	+6
	II-3	190	0	194	+4	197	+7



A B

Fig. 8. Dermal toxicity test of biostabilizer on the rat. (A) Shaved skin treated with biostabilizer was covered for observation. (B) No irritation or inflammation was observed on the treated skin after dosing until the endpoint date, indicating the biostabilizer's favorable safety profile.

5. Application potentials and limitations of the current study

This investigation demonstrated the encouraging potential of MICP as an eco-friendly alternative for soil stabilization and wind erosion mitigation. Utilizing *Staphylococcus warneri* IR-103 bacteria in combination with a cementation solution notably enhanced soil surface strength and wind erosion resistance, lacking any harmful effects on plant growth or mammalians' health. Consequently, the subsequent paragraphs outline numerous noteworthy practical implications associated with the outcomes of our research.

The successful outcomes of wind tunnel experiments indicate that MICP could serve as a valuable asset in arid and semi-arid regions where wind erosion presents a notable ecological hazard. MICP has the capacity to enhance soil composition and moisture retention, potentially resulting in augmented agricultural productivity.

There exist certain limitations associated with our investigation. Prolonged use of bacterial strains in MICP might cause bacterial resistance, attenuating the efficiency of the process over time. Our study was carried out at a laboratory scale, necessitating further investigation to evaluate the viability and cost-effectiveness of MICP application in expansive field settings. Additional research is imperative to refine the MICP technique, encompassing the selection of microorganisms, concentrations of cementation solutions, and application methodologies.

Funding

This research was financially supported by National Institute of Genetic Engineering and Biotechnology, Tehran, Iran (Project #103) and is greatly appreciated.

Compliance with ethical standards

All the animal experiments were conducted according to the ethical guidelines of NIGEB and ZAAV, Tehran, Iran (IR.NIGEB.EC.1394.8.10).

Data availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Sarah Khalaj: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Hamidreza Naseri:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marjan Talebi:** Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation. **Rouzbeh Almasi Ghale:** Writing – review & editing, Methodology, Investigation. **Fatemeh Tabandeh:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Authors cordially thank A. Kazemi, A. Behrooz, and Dr. E. Hashemi for their great assistance in toxicity tests.

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