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A Sustainable and Eco-Friendly Approach to Sand Dunes Stabilization Using Vinasse and Indigenous Bacteria in MICP Technology

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ABSTRACT

Among emerging solutions to wind-erosion–driven sand dunes, stabilizing sand grains through an environmentally friendly MICP process offers a distinct, bio-based mechanism for enhancing sand stability relative to conventional methods. This study aimed to identify indigenous ureolytic bacteria suitable for Microbial Induced Calcium Precipitation (MICP) and to evaluate vinasse as a substrate to support MICP in arid-sand dune environments of southwestern Iran. Sand dunes were sampled; Bacteria were isolated and screened for urease activity and identified by 16S rRNA sequencing. The effects of various parameters on bacterial growth, urease activity, and carbonic anhydrase production were assessed. Isolates' growth in vinasse was tested, and $CaCO_3$ precipitation was confirmed by XRD and FESEM. Seven *Bacillus* isolates were selected based on urease activity. Strain 1S5 showed the highest specific urease activity in response to Ni^{2+} and remained active across high urea concentrations across all incubation periods. Early $CaCO_3$ precipitation was observed for strains 5D1, 1D2, 1D1, and 8S1, indicating carbonic anhydrase activity. All isolates grew in varying vinasse concentrations, and $CaCO_3$ precipitation was observed in all strains, with the highest yields for indigenous *Bacillus licheniformis* strains 1S5, 1D1 and 1D2. The indigenous *Bacillus* strains, particularly strain 1S5, exhibit robust urease activity and $CaCO_3$ precipitation under vinasse-containing conditions, supporting vinasse as a feasible substrate for MICP applications. Further optimization and field-relevant testing are warranted.

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1. Introduction

Deserts occupy area of about 907,293 km^2 in Iran, of which 2,614 km^2 are located in Khuzestan in the form of sand dunes (Abbasi *et al*, 2019). This area located in the world's dry belt and its evaporation is almost 13 times more than precipitation (<250 mm/yr.) because of high temperatures (Azoogh *et al*, 2018). The need to control sand dunes seems necessary due to the creation of many problems not only in nearby affected areas, but even on a larger scale (Indoitu *et al*. 2012), including climate processes (Middleton. 2017), reduction the nutrient content of soils (Powell *et al*, 2015), loss of soil productivity (Luo *et al*, 2018), human impacts (Indoitu *et al*. 2012), sand dune movement (Shojaei *et al*. 2019) and consequently salt deposition (Kheirfam and Asadzadeh. 2020), destruction of farms, houses and being a source of pollution through formation of dust (Moradi *et al*, 2017). There are different solutions to control this global problem. The most sustainable method to combat wind erosion is biological restoration using drought-resistant and sand-loving plants, but the initial establishment of these plants requires the use of sand-stabilizing materials such as mulches (Abtahi and Khosroshahi, 2015; Azoogh *et al*, 2018). Mulching is the most common method for sand dunes stabilization (Douzali joushin *et al*, 2018; Naghizade Asl et. al, 2019). Mulch, which can contain organic or inorganic substances, protects the soil and water, or if there are plants in the soil, from the wind (Shojaei *et al*, 2019). It seems that oil mulch is the most popular mulch. Mulching was started in Iran in 1967-68 (Refahi, 2006). After about half a century, more than two million hectares of activated desert lands and sand dunes in Iran have been restored and stabilized through the implementation of the aforementioned methods. Due to the investigation of researchers on oil mulch, consequences such as groundwater pollution due to the presence of heavy metals in it (Vaezi, 2011; Khalili Moghadam *et al*, 2015) or increasing the daily price of oil (Khalili Moghadam *et al*, 2015), caused the idea of alternative mulches to be proposed. Other mulches that have been used for sand dunes stabilization include: clay (Majdi *et al*, 2006), steel slag (Alipour *et al*, 2018; Safaei Qahnoye *et al*, 2012), Gravel–sand (Li, 2003), polymer stabilizers (Rauch *et al*, 2003; Petry and Little, 2002; Santoni *et al*, 2002), Polylatice polymer (Rezaei, 2009), Soil stabilization resin (Jafari, 2014), polyacrylamide (Rabiee *et al*, 2010; Genis *et al*, 2013), polyvinyl acetate (Crowley *et al*, 2008), Mineral salts such as $MgCl_2$ and $CaCl_2$ (Goodrich *et al*, 2009; Edvardsson, 2010).

Previously, the presence of oil and gas reserves in Khuzestan province made the use of oil mulch common. But recently, the need for alternative and environmentally friendly materials is increasing (Jamili *et al*, 2015; Ramdas *et al*, 2020; Sukumaran and Poulose, 2018). On the other hand, it is better to use materials in the composition of mulches that are actually disposable, and in this way, it will be a great help in solving the problem of waste or sewage of those materials. More than 130,000 ha of Khuzestan province are under sugarcane cultivation (Khalili Moghadam *et al*, 2015), and in fact, this province is one of the main sugar and alcohol production areas of the country, which annually produces 800,000 m^3 of Vinasse which is currently the wastewater of Khuzestan's sugarcane cultivation and industry. (Jamili *et al*, 2015). 10-15 liters of vinasse are produced to produce one liter of ethanol, which varies depending on the distillation equipment in each factory and researchers are looking for ways to use vinasse beneficially, things like energy production, vinasse recycling in fermentation, fertilizer-irrigation, concentration by evaporation and feeding livestock are among them. (Christofoletti *et al*, 2013). Vinasse is an acidic substance with pH: 3.5-5, dark brown color with high organic content (COD: 50-150 g/L), unpleasant smell for humans (Montiel-Rosales *et al*, 2022) and it's not inert and not dangerous (Nikseresht *et al*, 2020). It's rich in K, Ca and Mg, and its N and P are moderate. It also does not contain toxic compounds (Jamili *et al*, 2015). Jamili *et al*. (2015) prepared a mulch containing vinasse, filter cake and clay. Then, sprayed it on the bed of sand

dunes and found that it increases the water holding capacity as well as nutrients like N, P, K, Fe and Cu in the subsurface layers and creates more heat in the soil due to its dark color. In addition, it is easily available and does not have the same disadvantages as petroleum mulch.

When any environmentally friendly method to improve and modify soil in the field of soil biology discussed, the potential of soil microflora and soil microfauna is also considered to solve the problem. There are kinds of microbes compared with the other microbial habitats because of soil nutrients and some stored liquid in its pore space. They quickly adapt to different genetic and environmental conditions and for this reason they have been around for more than 3.5 billion years (Stutzky, 1997). There are about 10^9-10^{12} organisms per Kg of near-surface soil (Umar *et al*., 2016). Bacteria are the most abundant soil microbes. (Michel and Santamarina, 2005). Most of these bacteria are spore-forming and can withstand harsh environmental conditions such as sub-zero temperatures to boiling temperatures, dryness and high pressure. The dominant surface charge of bacteria is negative and they are able to bind with ions, and because bacteria are indigenous to the soil, they do not have negative effects on the soil (Umar *et al*., 2016).

An option that can lead to the preparation of mulch that utilizes the potential of microorganisms to stabilize sand dunes is called biomineralization. Biomineralization means the formation of minerals by living organisms is carried out by all living organisms (from prokaryotes to humans) (Krajewska, 2018). This is done in the cellular or extracellular matrix in the form of crystal which is well known in microbes (Portugal *et al*, 2020). Calcite, magnetite, greigite and amorphous silica are among the products of biomineralization (DeJong *et al*, 2010) and among all minerals produced by biomineralization, calcite ($CaCO_3$) is the most important that many microorganisms like fungi and bacteria can precipitate it (Kumari *et al*, 2016). There are two different mechanisms for biomineralization: 1) biologically-controlled (active precipitation) such as: magnetite formation in magnetotactic bacteria and 2) biologically-induced (passive precipitation) like: calcium carbonate production) (Krajewska, 2018; Muynck *et al*, 2010). According to Castanier *et al* (1999) there are two general pathways for the production of $CaCO_3$ particles by bacteria: 1- The autotrophic pathway and 2- The heterotrophic pathway (Castanier *et al*, 1999). The most widely used path that has been used for the precipitation of calcium carbonate has been based on hydrolysis or urea degradation related to the heterotrophic pathway (Krajewska, 2018; Sun *et al*, 2021). Microbial induced calcium precipitation (MICP) is a newfound biomineralization technique (Yang *et al*, 2022; Wang[1] *et al*, 2022; Wang[2] *et al*, 2022) which is eco-friendly, sustainable (Tian *et al*, 2022), very cost-effective (Ivanov and Chu, 2008) and like conventional chemical methods, doesn't have toxicity, environmental harm and limitation by injection distance (Karol *et al*., 2003). The applications that have been reported for MICP technology in different fields include: bioremediation (Sovljanski *et al*, 2022), removal of heavy metals (Sheng *et al*, 2022; Xue *et al*, 2022), remediation applications and stabilization of mine waste (Proudfoot *et al*, 2022), Self-healing cement (Yang *et al*, 2022; Wiktor and Jonkers, 2011), Protection and restoration of historical monuments (Tiano *et al*, 1999), dust suppressant agent in silty and clay soils (Bang *et al*, 2011; Meyer *et al*, 2011). In MICP process, generally two steps are involved (1 and 2 reactions) (Cheng and Cord-Ruwisch, 2012; Harkes *et al*, 2010; Landa-Marban *et al*, 2021; Meng *et al*, 2021):

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

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

Urea is hydrolyzed by urease of bacterial cells and broken into carbonate and ammonium ions. In the next step, if there is calcium in the environment, carbonate ions will react with it and produce $CaCO_3$ crystals. Finally, this calcium carbonate acts as a cement agent between the sand particles and connects them together (Cheng and Cord-Ruwisch, 2012; Rowshanbakht *et al*, 2016; Sun *et al*, 2021). In addition to plants, urease enzyme activity in soil is also related to microorganisms (Ciurli *et al*, 1996) and microorganisms in environments such as soil and concrete provide nucleation sites for the formation of $CaCO_3$ (Tian *et al*, 2022). On the other hand, sandy soil has good permeability and is a suitable option for microbial treatments (Wang *et al*, 2022).

Portugal *et al*., (2020) based on other researches included Whiffin *et al*., 2007; Van Paassen, 2011; Cheng and Cord-Ruwisch, 2012; Yasuhara *et al*., 2011; Shahrokhi-Shahraki *et al*., 2015, believes that more research should be focus on the identification of microorganisms with high $CaCO_3$ precipitation, suitable soil conditions to increase the rate of bacterial growth and produce more ureolytic activity, and the difference between indigenous microorganism in terms of the rate of bacterial growth and the production of ureolytic activity. Therefore, this study was conducted in the sand dunes of southwestern Iran: i) to identificate and evaluate of indigenous and exotic urease-producing bacteria, ii) to determinate of enzyme strength, identity and ability to tolerate environmental condition of microorganisms, and iii) to determinate of the ability to use vinasse as a substrate and production of $CaCO_3$ for MICP.

2. Materials and methods

2.1. Soil collection

To isolate the bacteria, soil samples were taken from a depth of 0-30 and 30-60 cm in sterile containers and kept at 4°C until the experiments (Paul, 2014). The studied area includes the public citadel west of the Karkheh River, which is one of the western regions of Khuzestan province and is located between the cities of Sosangerd, Bostan and Shush and has the following coordinates: 31° 28' 6″ N - 31° 53' 8″ N and 48° 15' 18″ E - 48° 23' 55″ E.

2.2. Isolation and purification of bacteria

The samples were diluted 1:10 using physiological saline solution (NaCl 0.9%), and shaked at room temperature for 60 minutes at 160 rpm. 50µl of the suspension supernatant were cultured in petri dishes containing nutrient agar culture (conda pronadisa) medium in quadrant streak pattern and incubated at 28-30°C until bacterial colonies appear. After different bacteria were grown in the isolation step, a complete loop of each bacterial colony grown on the petri dish was sub-cultured several times to grow pure colonies (Omoregie *et al*, 2016; Omoregie *et al*, 2017; Su *et al*, 2017). Phenotypic tests for isolates were performed following Harrigan and Cance (1976). The tests included colony morphology, Gram staining, spore staining, and catalase activity.

2.3. Screening for urease-producing bacteria

Identification of urease-producing bacteria was done through culture on urea agar (Ali *et al*, 2020; Ghanbari *et al*, 2020; Fan *et al*, 2020) and urea broth (Bahmani *et al*, 2019). Isolates that changed the color of the culture medium from yellow to pink were considered as urease-producing isolates (Omoregie *et al*, 2016; Vahabi *et al*, 2013; Su *et al*, 2017; Sarayu *et al*., 2014).

The isolates that showed a positive response in the in the previous step were quantitatively evaluated in terms of urease enzyme production power so that isolates with higher enzyme

capacity could be identified. For this purpose, ammonium production by the isolates was tested with the modified Nessler method. (Whiffin *et al*., 2007). First, overnight culture of bacteria was prepared and read using a spectrophotometer at a wavelength of 600 nm and the OD600 parameter of the samples was obtained. Then, the sample was diluted with deionized water to be in the range of 0.5-0.5 mM concentration. 2 ml of the sample was mixed with 100 µl of Nessler's reagent, after 1 minute they reacted together and the color of the sample changed to yellow or orange, the sample is read with a spectrophotometer at a wavelength of 425 nm. The absorbance data were calibrated with several NH_4Cl standards (concentrations of 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mM) measured under the same conditions.

2.4. Examining the ability to tolerate environmental conditions by isolates

Different treatments including growth temperature range, pH, carbon source, salinity, were applied to the isolates and their viability was tested. Isolates that can tolerate adverse environmental conditions and survive or form spores are selected as resistant isolates.

2.4.1. Temperature

Bacterial colonies were cultivated linearly on plates containing nutrient agar culture medium with pH =7 using a sterile loop and incubated at temperatures of 28°C and 45°C. The appearance of a colony on the culture medium in these conditions is considered as a positive response.

The growth of bacteria in sand dunes at low temperature was also investigated. For this purpose, 100µl of sand suspension were inoculated in tubes containing 4 ml of sterile nutrient broth medium (Scharlau) and after mixing, they were placed at 4–5°C for 21 days. Finally, the samples were compared for their turbidity with the control (no suspension inoculation); growth was assessed qualitatively by visual inspection for turbidity, indicating bacterial growth at 4–5°C.

2.4.2. pH

Bacterial colonies were cultivated linearly on plates containing nutrient agar culture medium with acidic, neutral and alkaline pH. pH was adjusted to 6.5, 7.5, and 8.5 with NaOH and HCl 1M and the plate are incubated at 28°C. Colony emergence was used as an indicator of bacterial growth on each pH condition.

2.4.3. Carbon source

B_4 culture medium (Seifan *et al*., 2016) was used, but glucose in this culture medium was replaced by sucrose because the dominant sugar in sugarcane is sucrose, and if the bacteria could use this carbon source, it was a confirmation for the use of vinasse as a substrate. After the culture medium was prepared and autoclaved at 121°C for 15 minutes, sucrose, which was sterilized with a 0.22 µm filter, added to the mixture and distributed in the plates. Then, 50 µl of the overnight culture of bacteria were inoculated into the plates and the plates kept for 2 weeks at 37°C and finally the growth of the bacteria in the plate was checked. A bacteria-free control was also used alongside the samples. Colony emergence was interpreted as evidence of utilization of the sucrose carbon source.

2.4.4. Salinity

Two methods were used to determine the survival of bacteria in different concentrations of salt, in the first method, three salts were used with the usual ratio in the soil, and in the second method, only one salt was used.

2.4.4.1. Using a mixture of three salts

To investigate the growth of bacteria in different salinity concentrations of 3 salts NaCl, $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$ with a ratio of 3:2:1 respectively at 5 levels of 100, 200-, 400-, 600- and 800-mM salt with Keeping SAR≤ 13 was used. As a positive control, *Staphylococcus sp.* was considered. The culture medium used was nutrient agar. strains were cultured in plates contain nutrient agar with different concentrations of salts. The inoculated plates were incubated in 28-30 °C for 24-48 hours. Colony growth on the plate was considered as a positive response (Jafari *et al*, 2015).

2.4.4.2. Use of NaCl

The procedure was done exactly like the previous section, but only NaCl salt is used in concentrations of 800 mM to check the resistance of bacteria to salinity in highest concentration (Jafari *et al*, 2015).

2.5. Using other sources of Nitrogen

For this purpose, instead of urea (merck), industrial urea and two nitrogen sources, NH_4NO_3 and $NaNO_3$, were used separately in MMSM medium. After preparing two culture media, these media were added to each tube in the amount of 4 ml and autoclaved for 15 minutes at 121°C. Then, 100 µl of overnight culture of bacteria was added to it, and the inoculated tubes were kept in an incubator at 30°C for 5-7 days. Results were reported as positive or negative turbidity or lack of visible turbidity.

2.6. Use of other sources of calcium

CCP culture medium with different calcium source was considered for bacteria, where calcium sources were: $Ca(NO_3)_2.4H_2O$ and $Ca(CH_3COO)_2.H_2O$ with pH= 8.5 and 0.5 M of calcium. Overnight culture of bacteria was prepared and 100 µl of it was inoculated linearly on plates containing CCP medium with different calcium sources and the plates were kept in an incubator at 28°C for 7 days. After the incubation period, the plates are examined for the presence of calcium carbonate crystals under a stereoscope or a microscope at a magnification of 4 (Zhang, 2015, Wei *et al*., 2015, Chahal *et al*., 2011).

2.7. The effect of nickel chloride on urease enzyme production

First, a stock solution of 1 mM of $NiCl_2.6H_2O$ was prepared as a source of Ni^{2+} and sterilized using a 0.22 µm filter. Then, a volume of 4 ml was considered for each tube, which contained nutrient broth, 2% urea, and concentrations of 0, 100 and 200 µM Ni^{2+} from $NiCl_2.6H_2O$ stock solution (Xu, 2017). Then, 100 µl of the overnight bacterial suspension were inoculated into the tubes and they were incubated for 48 hours at 30°C on a rotary shaker at 120 rpm. Finally, the samples were read using a spectrophotometer.

2.8. Investigating the resistance of bacteria to urea in different concentrations

First, overnight cultures were prepared from bacteria. Then 100 microliters of them were inoculated into nutrient broth medium containing different concentrations of urea (20, 40, 60, 80 and 100 g/liter) separately. Three replicates were considered for each isolate, and then they were placed in a rotary shaker at 120 rpm with a temperature of 30°C. At the end of 24, 48 and 72 hours of incubation, the OD600 of the samples was read by a spectrophotometer (Xu, 2017).

2.9. Activity of carbonic anhydrase enzyme in bacteria qualitatively

B4 culture medium was prepared according to (Seifan *et al*., 2016) in such a way that 2.68 (g/L)

of $CaCl_2.2H_2O$ and 4 (g/L) of yeast extract were mixed in a 250 ml Erlenmeyer flask and autoclaved at 121°C for 15 min. Then 10 (g/L) of filter sterilized glucose, was added and distributed in the plates. The amount of 50 µl of the overnight bacterial culture was spread on the plates. Then the plates were sealed using parafilm. The inoculated plates were incubated in 37°C for two weeks. At the end of each week, the plates were examined under a microscope at magnification 4 to checking $CaCO_3$ crystals.

2.10. Molecular identification of the isolates

Molecular identification was confirmed by determining the sequence of the 16S rRNA gene and comparing it with the data available in the gene bank of the NCBI database (Weisburg *et al*., 1991). For the molecular identification of the isolates, the following steps are performed. 16S rRNA general forward and reverse primers were:

FD1 (5'-CCCGGGATCCAAGCTTACGGTTACCTTGTTACGACTT-3'),

RD1 (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3').

2.11. Determination the ability to use the Vinasse substrate

The strains that had a better response to urea hydrolysis and were preferably spore-forming and showed a stronger response than the other strains in the qualitative test were selected and inoculated in different percentages of vinasse.

2.11.1. Preparation of MMSM medium

The contents of MMSM medium were (per liter): NH_4NO_3 4 g, K_2HPO_4 4 g, KH_2PO_4 6 g, $MgSO_4.7H_2O$ 0.2 g, trace mineral solution 1 ml (The contents of the solution were (per liter): $CaCl_2.2H_2O$ 1 g, $FeSO_4.7H_2O$ 1 g, EDTA 1.4 g). After preparing the MMSM medium and the solution of trace elements in two different 250 ml flasks, the pH of the solution of trace elements was adjusted to 7 using two solutions of NaOH and HCl 1 normal, and then these two are combined together.

2.11.2. Preparation of Vinasse concentrations

Four types of vinasses obtained from sugarcane factory, which were: vinasse from alcohol factory, vinasse from yeast factory, vinasse mixed with alcohol and yeast factories, and concentrated vinasse in 3 concentrations: 10%, 50%, and 100%. The concentrations of 10 % and 50% were prepared with the use of modified mineral salts medium (MMSM). For each strain, 12 test tubes were considered.

2.11.3. Bacterial inoculation to Vinasse

200 microliters of overnight bacterial culture were added to each of the tubes containing Vinasse. As controls, tubes containing the substances were not inoculated. Then, the tubes were kept in shaker incubator at 28 for 5 days and 120 rpm.

2.11.4. Cultivation of Vinasse inoculated tubes in nutrient agar medium

Due to the nature of vinasse, which has a very dark brown color, it is very difficult to detect the growth of bacteria. Therefore, after five days of incubation, the tubes containing vinasse, one loop from each of them was inoculated into plates containing nutrient agar medium and the cultured plates were transferred to the incubator and kept 28°C for 48 hours. Then, the appearance of the colony on the plate was checked and considered as a positive response.

2.12. Investigation of calcium carbonate production by isolates

First, the formation of calcium carbonate crystals was qualitatively checked by bacteria, and then the production amount of this material was quantified in the constituent isolates. For investigation of production of calcium carbonate by isolates qualitatively, according to the method of Wei *et al*. (2015) and Chahal et (2011), calcium carbonate precipitation media (CCP) culture medium was prepared and overnight culture of bacteria were cultured in plates containing CCP culture medium and incubated for one to two weeks at 28°C. Then, the plates were viewed under a stereoscope or microscope with magnification 4.

Another immediate method was used to prove the presence of calcium carbonate crystals in the culture medium, in this way, the overnight culture of bacteria was prepared with 2% calcium chloride. After 18-24 hours, when the bacterial population reached a suitable level, 2% filtered urea solution was added to the tubes containing the overnight culture of bacteria and calcium chloride, and after a few minutes, crystal production was observed in the tubes.

For quantification of calcium carbonate produced by isolates, according to the method of Zaghloul *et al*. (2020), 90 ml of 0.5 M calcium chloride was combined with 90 ml of sterilized 1 M urea with a filter and 20 ml of overnight culture of each bacterium was added to it. A sample without bacteria was used as a negative control. The prepared samples were incubated at 37°C for 48 hours. Calcium concentration was measured using EDTA titration (Zaghloul *et al*., 2020).

2.13. XRD and FESEM analysis of isolates

Calcium carbonate production was performed for the isolates by culturing in a nutrient broth medium containing 2% urea and 2% $CaCl_2$. 30 ml of medium were inoculated with 2% overnight bacterial culture and incubated at 30°C with shaking at 130 rpm for 7 days. The precipitated $CaCO_3$ was filtered (Whatman filter paper) and dried at 60°C for 3 h (Krishnaprya, 2015). Dried samples were stored in sterile vials and transported to the Sharif University of Technology Laboratory Services Center for analyses. FE-SEM analyses were performed using a Tescan Mira3 to assess surface morphology and topology. X-ray diffraction (XRD) analyses were conducted on the same precipitated $CaCO_3$ samples using a PANalytical X'Pert Pro MPD diffractometer. All procedures followed the facility's standard operating procedures for sample preparation, measurement, and analysis. Finally, for contextual comparison, FE-SEM images were compared with published $CaCO_3$ crystal images, and XRD peaks were cross-validated against reference patterns in the XPert HighScore Plus software to phase identification targeted crystalline phases such as calcite, vaterite, and aragonite.

3. Results and discussion

3.1. Soil collection

In total, 16 samples of sand dunes were taken from the studied area, 8 of which were from the surface part of the soil and the other 8 samples were from deeper parts up to the depth of root activity (0-30 and 30-60 cm from the soil surface). Sampling was done in such a way that the distribution of the bacterial population was well represented. Isolation of ureolytic bacteria from this region because of poor granularity and poor in organic matter (Khalili Moghadam *et al*, 2015), and the extreme weather conditions prevailing in Ahvaz, i.e. the hot climate with more than 45°C during the hot seasons of the year classifies Ahvaz as a hot region (Abbasi *et al*, 2021), as well as the lack of rainfall and dryness of the region (Chitsazan and Akhtari, 2009), could be a way to find resistant strains for the production of biological mulch and ultimately prevent the movement of sand dunes due to wind erosion towards the surrounding cities and villages (Omid Bakhsh *et al*, 2003). The soil texture of this area was sandy. The pH was neutral

and equal to 6.98-7 which is normal for Khuzestan soils that receive relatively little rainfall throughout the year (Abbasi *et al*, 2019). The soil EC of these areas is high and is around 7.5 $mS.cm^{-1}$, which according to Richards (1954) can limit the productivity of many plants. The high salinity of the soil in these areas, despite its light texture, can be due to the low potential for mineral leaching due to low rainfall (Azoogh *et al*, 2018).

3.2. Isolation of ureolytic bacteria

A number of 108 isolates were isolated from the soil, which 45.3% of them were from surface soil and 54.7% were from deeper soil. It is normal for the bacteria to seek refuge in the subsurface layers of the soil for survival due to the conditions of the sampling area. In all types of soil, there is a large population of bacteria, which is usually estimated at 1 million per gram of soil (Anyadoh *et al*, 2017). There are studies that show that microorganisms isolated from extreme environments can produce stable diverse enzymes (Anyadoh *et al*, 2017). Then, all the strains were investigated for the urease activity by growing on urea culture media. The activity of urease enzyme in bacteria allows them to use urea as the only nitrogen source (Olivera-Severo *et al*, 2006). 63 strains (58.33%) were able to change the color of the culture medium from yellow to pink. Lloyd and Sheaffe (1973), reported that 17-30% of the isolated bacteria from the six types of studied soils were able to hydrolyze urea. Change in the color of urea culture media occurs because of urease activity of isolates that phenol in these media transforms to pink due to creation of an alkaline environment (Ghezelbash and Haddadi, 2018; Sheng *et al*, 2022). After that, the amount of urease activity of isolates was measured. It is possible to measure urease enzyme activity with these methods: (Liu *et al*, 2021) 1) pH increment, 2) Titration, 3) Nash (Nessler) reagent method and (Omoregie *et al*, 2016) 4) conductivity method. Nessler's method was used in this research (Harkes *et al*, 2010). Nesslerization reaction is a suitable method when high sensitivity or interference of cellular components is not considered because it is a quick and easy method (Mobley and Hausinger, 1989). As a result, seven isolates (1S5, 1D1, 1D2, 2D2, 5D1, 7D3, 8S1) with high urease activity (6.51, 9.08, 5.87, 5.37, 4.58, 7.46, 8.33 mM hydrolyzed urea. min^{-1}. OD^{-1} respectively) were screened. The results are shown in Fig (1) and Tab (1). All seven isolates were positive in catalase production, gram and spore staining. The use of some species of *Bacillus* to repair limestone buildings is probably the presence of their spores as a site for the nucleation of $CaCO_3$ (Schwantes-Cezario *et al*, 2017).

Isolates 1S5, 1D1, 1D2 and 7D3 could create pink color in the both urea broth and urea agar, strongly and the amount of their urease activity was 6.51, 9.08, 5.87, and 7.46 mM hydrolyzed urea. min^{-1}. OD^{-1} respectively. 1D1 and 8S1 showed highest urease activity. Despite, the response of 8S1 to urea hydrolysis was weak and it couldn't change the color of urea. Urea broth is a very buffered culture medium that bacteria must produce large amounts of ammonia to increase the pH to change its color from yellow to pink. In case, the urea agar culture medium contains reduced buffer that supports the growth of more urea hydrolyzing bacteria (Brink, 2010).

Urease is an important enzyme in MICP (Ivanov *et al*, 2019), if bacteria can produce more amount of this thus more calcite will make finally (Jalilvand *et al*, 2020). Ureolytic bacteria are found in many climates with high level of salts and even high level of heavy metals in soils (Proudfoot *et al*, 2022).

Sporosarcina pasteurii was used as a positive control and the amount of its specific urease activity was about 18 $mM.min^{-1}$. OD^{-1}. In many studies *Sporosarcina pasteurii* is used due to high ability in hydrolysis of urea and biocementation (Ghorbanzadeh *et al*, 2021). The amount of urea hydrolysis of this is reported: 12-17 mM urea hydrolyzed/min/OD (Omoregie *et al*, 2016), 187 mM urea hydrolysed per hour per unit OD600 (about 3.1 mM per min) (Dubey *et*

al, 2021), 19.98- 23.97 mM /min/OD (Omoregie *et al*, 2017), 5- 5.5 mM /min (Marin *et al*, 2020) and 16.5 mM /min/OD (Kahani *et al*, 2020).

Table 1. Some physiological characteristics of 7 Indigenous urease-producing isolates

Isolate	Urea agar	Urea broth	Colony					Gram	Catalase	Spore
			Shape/edge	Color	Elevation	size	texture			
1S5	++	++	Irregular/ lobate	white	Umbonate	Moderate	mucoid	+ bacillus	+	+
1D1	++	++	Irregular/undulate	white	Rose	Large	mucoid	+ bacillus	+	+
1D2	++	++	Irregular/undulate	white	Rose	Large	mucoid	+ bacillus	+	+
2D2	+/-	-	Irregular/undulate	white	Flat	Moderate	opaque	+ bacillus	+	+
5D1	+/-	-	Irregular/ lobate	white	Flat	Small	dry	+ diplobacillus	+	+
7D3	++	++	Rhizoid/ rhizoid	white	Flat	Large	mucoid	+ bacillus	+	+
8S1	+/-	-	Irregular/ curled	white	Flat	Moderate	opaque	+ bacillus	+	+

Description: ++: strong positive, +: medium positive, -: negative, +/-: The top of the tube is positive and pink, and the bottom is negative and without color change.

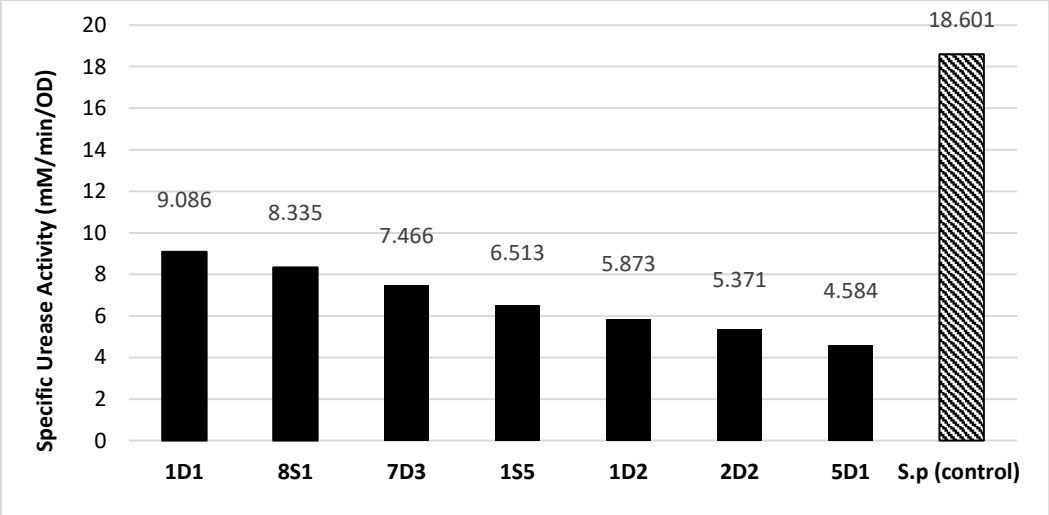

Fig. 1. Comparison of specific urease activity (mM urease hydrolyzed/min/ OD) among the ureolytic isolates and *Sporosarcina pasteurii* (S.p) as a positive control. The specific urease activity of bacterial isolates was much lower than that of *S. pasteurii*. The highest activity among native isolates was related to *Bacillus licheniformis* strain 1D1.

The research conducted on *Bacillus* species has estimated their urease activity as 3.3 to 8.8 $mM.min^{-1}$. OD^{-1} (Ali *et al*, 2020). The results obtained regarding isolates 1D1, 1S5, and 1D2, which were identified as *Bacillus licheniformis*, showed good urease activity with values of 9.08, 6.5, and 5.8 $mM.min^{-1}$. OD^{-1}, respectively. In the study conducted by Ali *et al* (2020), *B. licheniformis* had about 2 $mM.min^{-1}$. OD^{-1} urease activity. Tabalvandani *et al* (2023) and Krishnapriya *et al*. (2015) also identified *B. licheniformis* as a bacterium with strong urease

activity. The specific activity of urease enzyme for isolate 5D1 identified as *Bacillus subtilis*, was 4.58 $mM.min^{-1}$. OD^{-1}, which is similar to the result reported by Neha *et al* (2021), which reported the amount of urease activity for *B. subtilis* as 4.139 $unit.ml^{-1}$. The amount of production of this enzyme by the same bacteria was reported as 6 U/ml in the study of Khadim *et al* (2019). Isolates 2D2 and 8S1 by specific activity of urease enzyme about 5.37 and 8.33 $mM.min^{-1}$. OD^{-1}, respectively were identified as *Bacillus mojavensis* and Isolate 7D3 by specific activity of urease enzyme about 7.46 $mM.min^{-1}$. OD^{-1} was identified as *Bacillus piscis.* It should be noted that so far, there has been no report on the urease activity of any of these three strains. The ability to hydrolyze urea in some *Bacillus* species has been proven in some studies (Khaim *et al*, 2019; Rajabi Agereh *et al*, 2019; Wang *et al*, 2022; Smitha *et al*, 2022; Jalilvand *et al*, 2020; Bahmani *et al*, 2019). There has been no report on urease activity of *Bacillus piscis* and *Bacillus mojavensis*.

3.3. Examining the ability to tolerate environmental conditions by isolates

According to Tab (2), all the isolates, except isolate 8S1, were able to grow in acidic, neutral and alkaline pH at two temperatures of 28°C and 45°C. Isolate 8S1 could not grow at 45°C and alkaline pH. Temperature is an important factor in the urease activity of bacteria, which varies in different organisms (Omoregie *et al*, 2017). Bahmani *et al* (2019) examined the growth rate of several *Bacillus* species that had been isolated at temperatures 4-45°C and found that the most appropriate temperature was 28-37°C. Omoregie *et al*. (2017), showed that *Sporosarcina pasteurii* can grow in the temperature range of 20-45 °C and can hydrolyze urea. Cuzman *et al*. (2015) considered the effect of pH on the enzyme activity of *S. pasteurii* to be very effective and considered pH values above 10 to be critical for it. *S. pasteurii* has the maximum specific urease activity in the pH range of 6.5-8, and in some studies, *S. pasteurii* and some species of *Bacillus* have grown as alkali-tolerant bacteria in the range of 7-9.5 (Omoregie *et al*, 2017). Urease activity and cell growth (OD) of *S. pasteurii* was the highest at pH=8.5 and the lowest at pH=5 (Omoregie *et al*, 2019).

Table 2. Qualitative assessment of isolates' tolerance to environmental conditions (pH, temperature, carbon source, and salinity)

Isolate	pH and Temperature						Carbon source		EC (dS/m)	
	28ºC			45ºC			Sucrose		3NaCl: $2CaCl_2.2H_2O$: $1MgCl_2.6H_2O$	NaCl
	6.5	7.5	8.5	6.5	7.5	8.5	1ST week	2nd week	100-800 mM	800 mM
S. pasteurii	+	+	+	+	+	+	+	+	+	+
1S5	+	+	+	+	+	+	+	+	+	+
1D1	+	+	+	+	+	+	++	++	+	+
1D2	+	+	+	+	+	+	-	-	+	+
2D2	+	+	+	+	+	+	+	+	+	+
5D1	+	+	+	+	+	+	++	++	+	+
7D3	+	+	+	+	+	+	-	-	+	+
8S1	+	+	+	+	+	-	+++	+++	+	+
Staphylococcus sp.	-	-	-	-	-	-	-	-	+	+

Description: *Staphylococcus sp.* was used as a reference strain for growth in saline media.

All the isolates except isolates 1D2 and 7D3 were able to grow in the culture medium with

sucrose carbon source instead of glucose (Tab 2). According to the research conducted by Lapierre *et al*. (2020), monosaccharides and disaccharides such as glucose, fructose, sucrose and lactose improved the growth of *Sporosarcina pasteurii* for MICP. The results of this experiment also evaluated the cost-effectiveness of the culture mediums obtained from the waste products of industrial factories, such as molasses from sugar refineries containing sucrose.

In the salinity test, all the isolates were able to survive the saline medium containing three salts up to the highest concentration (800 mM) and after that they were also able to grow in the high concentration of NaCl salt. Hosseini *et al* (2017) found that most of the halophilic bacteria they isolated from a Salt Lake in Iraq belonged to the bacilli class. Rathakrishnan and Gopalan (2022) reported the growth of *Bacillus subtilis* and *Bacillus piscis* in culture medium containing 15-20% salt. Orhan and Gulluce (2015), in their study, assessed *Bacillus* species including: *Bacillus subtilis* as salt tolerant due to their growth in 15% salt. James *et al*. (2023) isolated *Bacillus licheniformis* from saline soils that could tolerate up to 10% salt. In the research of Ali *et al*. (2020), *Bacillus licheniformis* was able to grow in NaCl 10%. The ability to salt tolerance (18% NaCl) in *B. subtilis* (Minami *et al*, 2003) and salt tolerance (18% NaCl) and high temperature (above 60°C) in *B. licheniformis* (Bindal and Gupta, 2016) has been proven by isolating the relevant enzyme (γ-glutamyl transpeptidase). *Sporosarcina pasteurii* as a modest halophilic bacterial strain expressing osmosis regulating proteins with tolerance of 10% NaCl salt can grow in saline environment and function without disrupting the MICP process (Dikshit *et al*, 2022). The strategy of halophilic or halotolerant bacteria is to accumulate a high concentration of organic solutes outside their cytoplasm (Ghezelbash and Haddadi, 2018).

According to the evidence of the presence of bacteria on the planet three and a half billion years ago, their adaptation to different environmental conditions for physiological and genetic reasons can be true (Almajed *et al*, 2021). The spore-forming capacity of some *Bacillus* species allows them to cope with environmental stress (Coelho *et al*, 2022, Seifan *et al* 2016). Bacteria are the most abundant microorganisms among microbial communities with the highest survival rate in variable environmental conditions on earth (Portugal *et al*, 2020). The use of indigenous bacteria in biocementation because of their adaptation to local conditions is effective and recommended for the same place (Anyadoh *et al*, 2017).

3.4. Using other sources of nitrogen

Since no published studies have been reported on the use of indigenous microorganisms and ammonia sources other than urea in real bioslurries (Akiyama and Kawasaki, 2012), it is better to investigate the hydrolysis of the other sources with ammonium, as well as industrial urea instead of laboratory and pure urea (Merkh). All 7 isolates were able to grow in culture medium containing other nitrogen sources (NH_4NO_3, $NaNO_3$ and Industrial urea (2%). It is better that this growth rate is quantified in future studies and compared with each other to obtain the most suitable nitrogen source for more urease production. Akiyama and Kawasaki (2012) used amino acids asparagine, glutamine and urea as a source of nitrogen for the growth of indigenous bacteria and investigated the pH changes in them.

3.5. Use of other sources of calcium

Among calcium sources, all isolates were able to grow in calcium chloride. Isolates 1D1, 7D3, and 8S1 were not able to grow in the source of calcium acetate, and isolates 1D2 and 8S1 were unable to grow in the source of calcium nitrate. Research has shown that urease activity can depend on the type of calcium used (Liang *et al*, 2022). Whereas, some reports indicate that

calcium, due to the difference in ionic radius or charge density between nickel, urease and calcium, has little or no effect on urease enzyme activity (Gorospe *et al*, 2013).

Xiang *et al*. (2022) among three carbon sources (Ca $(NO_3)_2$, Ca $(CH_3COO)_2$ and $CaCl_2$) for biocementation, introduced Ca $(CH_3COO)_2$ as the best source of calcium due to better distribution and greater abundance and strength of $CaCO_3$ produced in the sample, more biomass and urease activity, and less NH_3 emission. Zhang *et al*'s study (2015) showed that Ca $(CH_3COO)_2$ as a calcium source by using the *Sporosarcina pasteurii* bacteria has positive effects on the uniaxial compressive strength and tensile strength of microbial mortar and also provides a more uniform distribution pore structure treated with calcium acetate. However, Helmi *et al*. (2016) found that the amount of calcium carbonate precipitation is lower in the medium containing calcium acetate as a source of calcium and because calcium acetate is hydrolyzed to calcium and acetic acid and acetic acid reduces the pH and then reduces the production of urease enzyme and calcium carbonate. Xue *et al* (2022) explained that carbonates such as calcium carbonate nucleate and encapsulate bacteria under the influence of calcium source on urea hydrolyzing bacteria. In the process of urealysis, crystallization is usually done on the cell wall of bacteria, which act as nucleation sites and because they have a negative charge, they absorb calcium ions (Krajewska, 2018). The results of Amiri and Bundur (2018) showed the effect of the type of calcium source used on precipitation morphology in such a way that Ca $(NO_3)_2$ caused vaterite precipitation and $CaCl_2$ caused calcite precipitation. While the crystal type of Ca $(CH_3COO)_2$ is mostly aragonite (Zhang *et al*, 2014).

3.6. The effect of nickel chloride on urease enzyme production

As shown in the diagram of Fig (2), the highest amount of urease production in isolates 1S5, 7D3 and 1D1 occurred at the highest Ni^{2+} concentration (200 µM) with values of 13.86, 6.73 and 4.74 mM hydrolyzed urea per min per OD, respectively. In S.p (*Sporosarcina pasteurii* as positive control), the highest amount of urease activity was related to 100 µM of Ni^{2+} by 11.52 mM hydrolyzed urea.min^{-1}.OD^{-1}. In isolates 1D2, 8S1 and 2D2, the highest production of urease was 4.36, 3.56 and 1.9 mM.min^{-1}.OD^{-1} at 50 µM Ni^{2+} concentration. In the case of isolate 5D1, the highest urease enzyme production (2.45 mM.min^{-1}.OD^{-1}) occurred when the Ni^{2+} concentration was 0 µM. Bachmeier *et al*. (2002) investigated the effect of adding nickel (concentrations of 0-1000 µM) on calcite precipitation by recombinant *E. coli* and *Bacillus pasteurii* and found that calcite precipitation rate increased with the addition of 5-100 µM of nickel and higher concentrations Ni^{2+} (500 µM) not only prevents urease activity but also inhibits cell growth. Nickel ions are present in the structure of urease enzyme and are important for maintaining the structure and activity of this enzyme (Al-Thawadi, 2011; van Vliet *et al*, 2002). The urease enzyme in *Sporosarcin* and *Bacillus* is nickel-dependent and similar, and therefore, the presence of nickel in minimum concentration affects the function of this enzyme (Sovljanski *et al*, 2022). The last stage of organic nitrogen mineralization by urease is catalyzed by the hydrolysis of urea with the help of a divalent nickel center (Xu *et al*, 2017).

3.7. Investigating the resistance of bacteria to urea in different concentrations

5 concentrations (20, 40, 60, 80 and 100 g/L) of urea were considered so that in three 24-hour incubation periods the isolates are able to grow or survive in that.

According to Fig (3), isolate 1S5 (*B. licheniformis*) had the highest urease enzyme activity at the highest urea concentration (100 g/L) in all three incubation periods compared to other isolates, but its activity was higher in the second period. Isolate 1D1 (*B. licheniformis*) was more active in the highest concentration of urea in the first period, and in the second period at

the same concentration, its activity was higher than even the first period, and in the third period, its highest activity was at the concentration of (60 g/L) of urea. Isolate 1D2 (*B. licheniformis*) was more active in the first and third period with the highest urea concentration, and in the second period, the highest urease activity was related to the concentration of 60 g/L, and in general, the highest amount of urease activity was in the first period. Isolate 7D3 (*B. piscis*), like isolate 1S5, had the highest activity in all three periods with the highest concentration, and its maximum activity was in the second period with a slight difference from the first and third periods. The urease activity of isolate 2D2 (*B. mojavensis*) was at the highest concentration in the first period, and in the second period at the lowest urea concentration (20 g/L), it had a quantitatively higher activity than the first and third periods, and in the third period, it was active at the highest urea concentration. 5D1 (*B. subtilis*) had almost constant urease activity in all three periods at a concentration of 80 (g/L), although it had the same activity in the first and second period and less activity in the third period. Isolate 8S1 (*B. mojavensis*) had more activity at the concentration of 20 (g/L) at first period and at the concentration of 60 (g/L) in the second and third periods, and in total, its amount was higher in the second period. *Sporosarcina pasteurii* in the first period, with increasing urea concentration, the amount of urease activity gradually increased from 2 to 7.36 mM of hydrolyzed urea but in the second and third period, the highest amount of urease activity was observed at the concentration of 60 (g/L) of urea with values 33.14 and 24.21 mM of hydrolyzed urea, respectively. Omoregie *et al*. (2017), reported the maximum specific urease activity for *Sporosarcina pasteurii* isolates in concentrations of 6 to 8% urea with an amount of 25 to 39 mM hydrolyzed urea. min^{-1}. OD^{-1}. Urea enters the bacterial cell through active transport and undergoes urealysis, which is a substrate-dependent reaction catalyzed by urease (Sridhar *et al*, 2021).

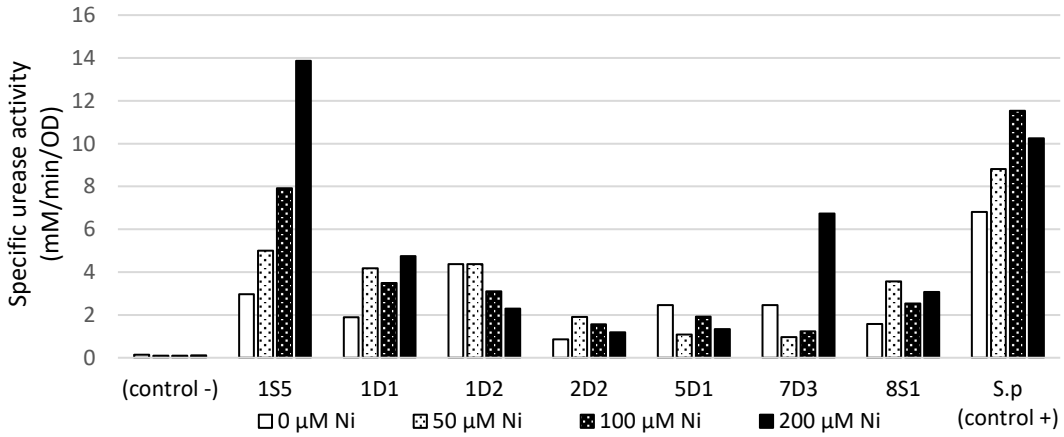

Fig. 2. The results of specific urease activity (mM urease hydrolyzed/min/ OD) in the medium containing Ni^{2+} in different concentrations (0- 200 µM) after 48 hours. (Control -) is an un-cultured sample, and (control +) is cultured with *Sporosarcina pasteurii* (S.p). *Bacillus licheniformis* strain 1S5 shows high urease activity, almost comparable to the control strain (S.p) in the highest Ni^{2+} concentration (200 µM).

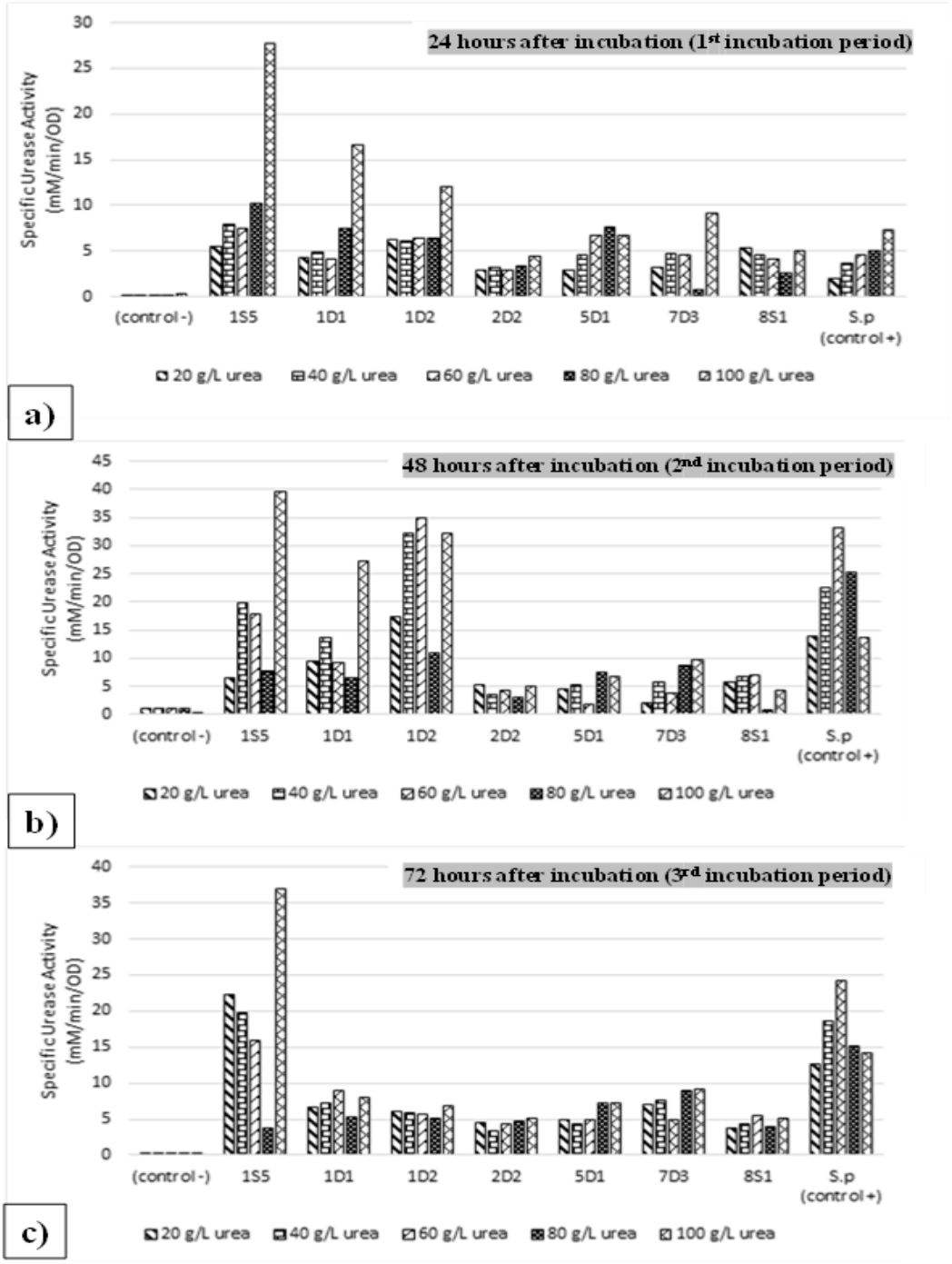

Fig. 3. The results of specific urease activity (mM urease hydrolyzed/min/OD) of isolates in different urea concentrations (20-100 g/L): **a)** after 24 hours (1st incubation period). **b)** after 48 hours (2nd incubation period). c) after 72 hours (3rd incubation period).

Although the increase in urea concentration due to the production of ammonia may have toxic effects on the cytoplasm of bacteria, on the other hand, it provides more energy in the form of ATP for the enzymatic activity of urea hydrolyzing bacteria and it causes less energy to be spent on the growth, reproduction and biomass of bacteria. (Omoregie *et al*, 2017). According to Naji *et al*., a fraction of ammonium ions is used as nitrogen or energy source by

bacteria and some of it is released outside the cell and regulates the pH (Sridhar *et al*, 2021).

Urease activity makes bacteria use urea as the only source of nitrogen (Olivera-Severo *et al*, 2006). The relationship between urea concentration and urease activity determines what concentration of urea is desirable to obtain the highest enzyme activity in the desired bacteria (Xu *et al*, 2017). It is also important to note that the amount of ammonium produced does not exceed the permissible limit of its pollution (Safdar *et al*, 2021). With the increase of urea concentration, the growth rate of the strains decreases due to high concentration of urea and cell senescence on cell proliferation (Anyadoh *et al*, 2017).

3.8. Activity of carbonic anhydrase enzyme in bacteria qualitatively

Isolates 1D1, 1D2, 5D1, 8S1 and *Sporosarcina pasteurii* were able to precipitate calcium carbonate in the first week. In the second week, isolate 2D2 was able to produce $CaCO_3$ too. The strongest response was related to isolate 5D1 (*Bacillus subtilis*), followed by isolate 1D2 (*B. licheniformis*) (in the first week) and isolate 1D1 (*B. licheniformis*) and *S. pasteurii* (in the second week). Oviya *et al* (2012), reported of the purification, characterization, and immobilization of a carbonic anhydrase enzyme secreted by *Bacillus subtilis* VSG-4. Urease and carbonic anhydrase enzymes are two enzymes involved in MICP process (Liang *et al*, 2022). The study on carbonic anhydrase enzyme has not been done as much as urease enzyme in the MICP. Carbonic anhydrase is a metalloenzyme with the presence of Zn^{2+} in its structure, and with the presence of this enzyme during the process, after the production of HCO^{-3}, H^{+} ions strengthen the precipitation of $CaCO_3$ in the form of calcite (Portugal *et al*, 2020). This enzyme is actually a biocatalyst and catalyzes the reversible hydration of CO_2 to bicarbonate and is present in many living organisms such as archaea, prokaryotes and eukaryotes (Abdelsamad *et al*, 2022). Orhan and Akincioglu's results confirm the existence of carbonic anhydrase enzyme in Gram positive bacteria including *Bacillus pumilus*, *Bacillus horikoshii*, *Bacillus patagoniensis*, *Bacillus sp*. (Orhan and Akincioglu, 2020). *Sporosarcina pasteurii*, *Bacillus pumilis* and *Bacillus megaterium* are produce two enzymes: urease and carbonic anhydrase (Dhami *et al*, 2016). Some *Bacillus* species with carbonic anhydrase enzyme can be mentioned in some conducted research: *Bacillus cohnii* (Liang *et al*, 2022), *Bacillus halodurans* (Faridi and Satyanarayana, 2016), *Bacillus mucilaginosus* (Xiao and Lian, 2016; Zheng and Qian, 2020), *Bacillus sp.* SS105 (Maheshwari *et al*, 2019), *Bacillus* sp.(Sundaram and Thakur, 2018) They found that the carbonic anhydrase enzyme is vital in prokaryotes that have this enzyme because it affects important physiological processes such as survival, growth and reproduction and cyanate degradation (Orhan and Akincioglu, 2020). One of the ways to absorb, store and stabilize carbon dioxide is to use the carbonic anhydrase enzyme as an environmentally friendly enzyme that does not cause any secondary pollution (Effendi and Ng, 2019).

3.9. Identification of the isolates

The results of identifying the isolates can be seen in Tab (3). The 16S rRNA gene sequence of strains 1S5, 1D1, 1D2, 2D2, 5D1, 7D3 and 8S1 showed to be similar, respectively, by 98%, 99%, 100%, 100%, 96%, 99% and 100% to *Bacillus licheniformis***,** *Bacillus licheniformis***,** *Bacillus licheniformis*, *Bacillus mojavensis*, *Bacillus subtilis*, *Bacillus piscis* and *Bacillus mojavensis*, respectively. Their 16S rRNA sequences were deposited in GenBank under the accession numbers of MZ057842, MZ057843, OP329211, MZ057844, MZ057845, MZ057846, MZ057847, respectively. Then, the genetic affinity of the isolates with the bacteria recorded in NCBI was compared by drawing a phylogeny tree using Mega 11 software Fig (4). Among bacteria, more attention has been paid to *Bacillus* species in the MICP technique

(Portugal *et al*, 2020).

Table 3. Molecular identification of the isolates

Isolate	Accession number	Query cover	Per ident.	Molecular identification
1S5	MZ057842	98	96.56	*Bacillus licheniformis*
1D1	MZ057843	99	96.99	*Bacillus licheniformis*
1D2	OP329211	100	99.09	*Bacillus licheniformis*
2D2	MZ057844	100	98.05	*Bacillus mojavensis*
5D1	MZ057845	96	98.11	*Bacillus subtilis*
7D3	MZ057846	99	98.03	*Bacillus piscis*
8S1	MZ057847	100	99.59	*Bacillus mojavensis*

Fig. 4. Phylogenetic tree (Neighbor-joining) based on the partial 16S rRNA gene sequences data from different isolates of the current study and sequences available in the GenBank database. The tree was constructed using Molecular Evolutionary Genetic Analysis (MEGA) version 11 (Tamura *et al*., 2021). Bar 0.5 substitutions per nucleotide.

3.10. Determination the ability to use the Vinasse substrate

All seven isolates grew in 4 types of vinasse (A=alcohol, Y=yeast, M=mixed alcohol and yeast, C=concentrated) and in all percentages (10%, 50% and 100%) after 48 hours in the incubator. It should be noted that the growth of these bacteria was positive but weak after 15 hours of incubation, but after 48 hours they were able to show a strong positive response. In MICP, inexpensive culture mediums are very important for the production and growth of bacteria with

high urease activity (Omoregie *et al*, 2019). The researchers used different materials to supply the carbon needed by the bacteria in the MICP process, and among them, the use of corn-steep liquor (CSL) (Amiri and Bundur, 2018), lactose mother liquor (LML) (Achal *et al*, 2009) instead of yeast extract has been successful.

3.11. Investigation of calcium carbonate production by isolates

As the primary source of carbon, $CaCO_3$ is one of the most abundant minerals in geological history (Abdelsamad *et al*, 2022). At first, the investigation of calcium carbonate crystals for 7 isolates that had urease activity, with two different concentrations of $CaCl_2$ was done qualitatively. Isolates 7D3 and 8S1 precipitated $CaCO_3$ crystals in both concentrations of $CaCl_2$ to a good extent. Isolates 1D1, 2D2, and 5D1 acted in both concentrations almost equally but less than the previous two isolates. Isolate 1D2 and 1S5 in a medium with a lower concentration of calcium chloride and isolate 2D4 in a medium with a higher concentration performed better. According to graph in Fig (5), isolates 1S5, 1D2 and 1D1 (All three of them were *Bacillus licheniformis*) had the highest amount of $CaCO_3$ precipitation (g. L^{-1}) with values of 0.36, 0.22 and 0.13, respectively. Krishnapriya *et al*. (2015) also estimated the amount of calcium carbonate precipitation by *Bacillus licheniformis* to be about 0.8 grams per liter. Sovljanski *et al* (2022) found that *B. licheniformis* can precipitate calcium carbonate at the rate of 3.14 grams per 100 ml under optimal conditions. The amount of 1.33 g/L of calcium carbonate by *B. licheniformis* was also reported by Vahabi *et al* (2013). Safdar *et al*. (2021) reported the successful use of native soil bacteria called *Bacillus licheniformis* for biocementing in organic soils with increased $CaCO_3$ content. Seifan *et al* (2016) reported the highest amount of calcium carbonate precipitation by *Bacillus licheniformis* and *Bacillus sphaericus* at the rate of 33.78 g. L^{-1}.

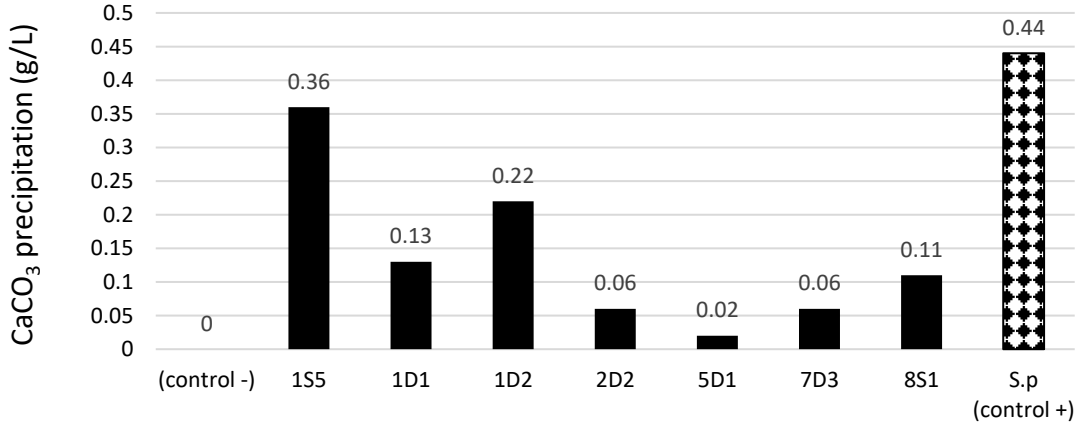

Fig. 5. Calcium carbonate precipitation (g/L) results by the isolates. Among the native isolates, 1S5 had the highest amount of precipitated $CaCO_3$, followed by 1D2 and 1D1, all of which were *Bacillus licheniformis*. *Sporosarcina pasteurii* (S.p) caused the highest $CaCO_3$ precipitation as a positive control.

Isolates 8S1 and 2D2 (identified as *Bacillus mojavensis*) were ranked after the first three isolates with the amount of calcium carbonate production of 0.11 and 0.06 g. L^{-1} respectively. There has been no report on the production of calcium carbonate by this bacterium so far. Isolates 7D3 (identified as *Bacillus piscis*) also has the same amount of calcium carbonate as isolate 2D2, i.e. 0.06 g.$(100ml)^{-1}$, similarly, there is no report about this strain it in this field.

Isolate 5D1 (identified as *Bacillus subtilis*) had the lowest production among the isolates with $CaCO_3$ precipitation equal to 0.02 g. L^{-1}. Feng *et al* (2021) successfully used the alkali-resistant *Bacillus subtilis* to biocement self-healing concrete. Pérez and García (2020) investigated the biomineralization of $CaCO_3$ by *B. subtilis*. Feng *et al*. (2021) used *B. subtilis* to precipitate calcium carbonate in self-healing concrete samples. Hoffman *et al*. (2021) found that the cell surface of *B. subtilis* may be highly negative, making it susceptible to the MICP process.

As can be seen from the graph in Fig (5), the highest amount of calcium carbonate production in this study was related to *Sporosarcina pasteurii* with a value of 0.43 g/liter. Eryürük (2022) reported the amount of calcium carbonate deposition by *S. pasteurii* between 4 and 28 mg, which was directly related to the number of bacterial cells. Saricicek *et al*. (2019) compared the ability of *Sporosarcina pasteurii* and *Bacillus licheniformis* in the MICP process and found that *S. pasteurii* is definitely more successful in $CaCO_3$ precipitation. In natural environments such as fresh and salt water, soils and carbonate structures, there are bacteria that cause precipitation of carbonates (Anyadoh *et al*, 2017). The ability of many species of *Bacillus* in biocementation has been proven, and for this reason they are called cementing bacteria (Li *et al*, 2017). The use of indigenous *Bacillus* species to $CaCO_3$ production in order to increase sands resistance is efficient, economical and recommended (Almajed *et al*, 2021; Burbank *et al*, 2013). Bahmani *et al*. (2019) reported the amount of precipitated $CaCO_3$ for indigenous *Bacillus* species about 2.2 - 3 g/L.

3.12. XRD and FESEM analysis of isolates

According to the FESEM images (Fig. 6, left side), *B. licheniformis* strains 1S5 (a), 1D1 (b), 1D2 (c), and *B. piscis* strain 7D3 (e) were able to precipitate vaterite and calcite crystals, although XRD analysis (Fig. 6, right side) of these isolates showed vaterite peaks in 1S5, 1D1, and 7D3, and vaterite, calcite, and aragonite peaks in 1D2. FESEM images and XRD analysis confirmed the formation of vaterite crystals in *B. subtilis* strain 5D1 and calcite crystals in *B. mojavensis* strain 8S1. FESEM of *B. mojavensis* strain 2D2 showed calcite particles, while its XRD pattern revealed both calcite and aragonite peaks. Overall, the MICP process resulted in the formation of $CaCO_3$ polymorphs, including calcite, vaterite, and aragonite. The result of process MICP is the production of calcium carbonate polymorphs such as calcite, vaterite and aragonite, of which calcite is the most stable (Ghezelbash and Haddadi, 2018). Many factors can affect the morphology of calcium carbonate including: bacterial metabolic activity, cementation reagents, extracellular polymeric matter, and abiotic elements (Omoregie *et al*, 2020). Polymorphs of calcium carbonate are seen in the form of calcite (square and rhombic), aragonite (needle-shaped) and vaterite (spherical) (Kalantary and Kahani, 2015). Vaterite and aragonite are formed at low pH and high temperature, while calcite is formed at high pH and low temperature and is the most stable form of calcium carbonate (Faridi and Satyanarayana, 2016).

4. Conclusions

This study demonstrates that indigenous *Bacillus* isolates from the sand dunes of Khuzestan can drive MICP under local conditions, with *B. licheniformis*, *B. mojavensis*, *B. subtilis*, and *B. piscis* showing robust urease activity and $CaCO_3$ precipitation. The principal novelty is the first-time report of urease activity in *B. piscis* and *B. mojavensis* under our experimental conditions, underscoring the regional microbial potential for dune stabilization. When compared with *Sporosarcina pasteurii*, the indigenous strains offered competitive performance while providing the advantage of environmental compatibility and potential cost savings through the use of waste-derived substrates (vinasse) as a carbon source.

These findings lay groundwork for field-oriented development of MICP-based sand dunes

stabilization using locally adapted bacteria and sustainable substrates. Future research should focus on in situ validation, scale-up considerations, and optimization of substrate deployment for practical implementation.

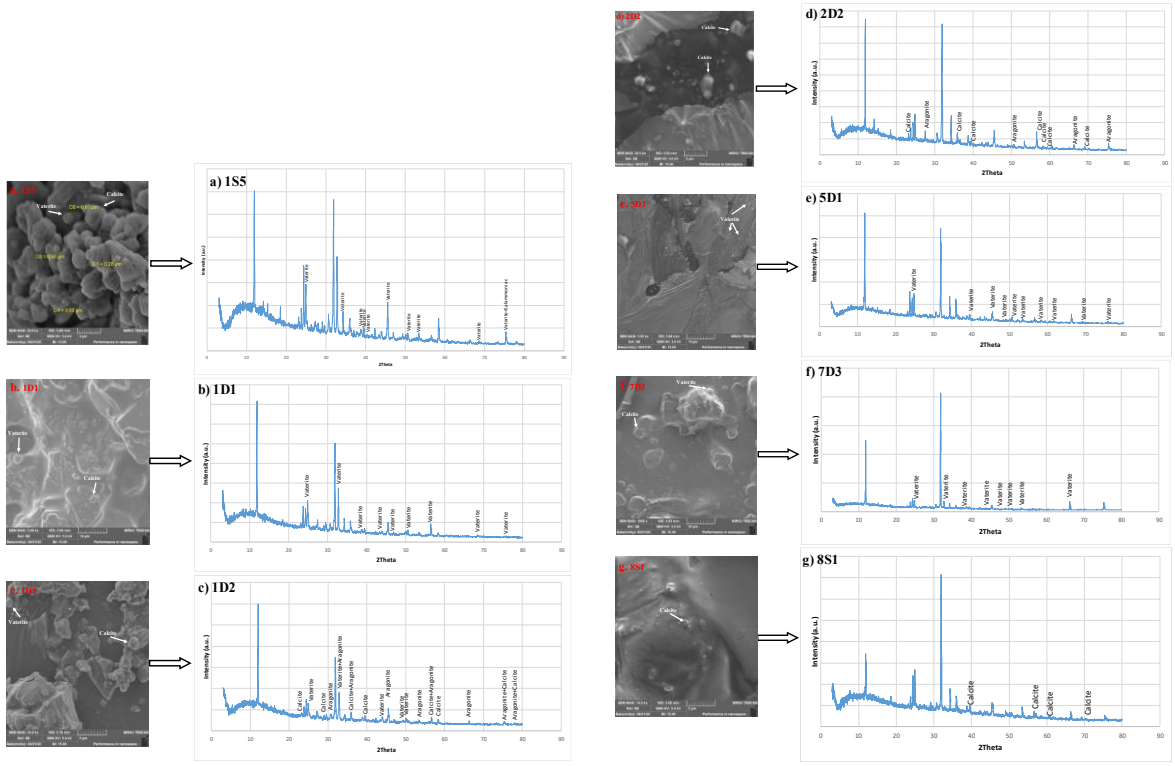

Fig. 6. On the left, FESEM images show the type of calcium carbonate crystals produced by the MICP process in 1S5 (a), 1D1 (b), 1D2 (c), 2D2 (d), 5D1 (e), 7D3 (f), and 8S1 (g). On the right, graphs of XRD analysis related to the same isolates.

Data Availability Statement

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

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Ethical considerations

The authors confirm that data were collected, analyzed, and reported honestly, without fabrication, falsification, or misrepresentation.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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