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The effect of vinasse as a carbon source on the activity of urease-producing bacteria in the microbially induced calcite precipitation (MICP) approach

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ABSTRACT

One of the most important causes of soil desertification is wind erosion. Deserts are known for their poor physical, chemical, and biological properties. In order to solve the problem of loose sand, many solutions have been thought and used so far. Today, scientists have greatly benefited from the capabilities of MICP in the field of sand dune stabilization, which seems to be an environmentally friendly solution. In Khuzestan, a lot of "vinasse" enter the environment every year, which is considered a pollutant. Combining it with beneficial native microorganisms can turn it into a valuable ingredient. The objectives of this study were to investigate the microbial activity of bacteria isolated from the sand dunes of Khuzestan in Iran using vinasse as a carbon source for growth. In order to eliminate the effect of competition and investigate the activity of the studied bacterial isolates (*Bacillus licheniformis* MZ057843, MZ057842, OP329211, and *Sporosarcina pasteurii*), the samples of sand dunes collected from Khuzestan were first sterilized. Then, inoculated vinasses were added to them, and the prepared samples were incubated for five weeks. The experiments (counting bacteria by plate count technique, Soil catalase by the method of titration with $KMnO_4$, and Soil microbial respiration by measuring the amount of CO_2) were measured after the first, third and fifth weeks of incubation time. Then, the data were analyzed by split plot in time design. The current study showed significant effects of vinasse, bacteria, time and their interaction in all three tests ($p < 0.0001$). Isolate B1 was able to cause the highest amount of microbial population and microbial respiration, and isolate B3 produced the highest amount of catalase enzyme in the soil. Among the three types of vinasse, V3 had the highest amount of catalase and microbial population, and V2 had the highest soil microbial respiration rate. The best incubation time for the microbial respiration and population tests was the first week, and third week of incubation for the catalase test. Generally, native *Bacillus* bacteria grew in vinasse as a carbon source and perhaps the potential of vinasse as a mulch can be explored. However, more research is needed to use it in soil stabilization operations.

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1. Introduction

Factors such as erosion, earthquakes, subsidence and industrial activities can weaken the mechanical properties of soil, and produce sand and unstable structures subsequently, on the surface of the earth in all parts of the world (van Paassen *et al.*, 2010). One of the most important causes of soil desertification and sandification is wind erosion, which disrupts agricultural activities and destroys the ecological environment through damage to the soil structure (Wang *et al.*, 2019). Deserts are known for their high temperature, pH, salinity, evapotranspiration, lack of organic matter in the soil, and low rainfall (Wall and Virginia, 1999). In desertification, vegetation removal occurs, and the fixed sand dunes are gradually transformed into moving dunes (Zhang *et al.*, 2004; Zuo *et al.*, 2008). This issue has affected 20% of the lands of Khuzestan and put additional pressure on the environment and life of that area (Hashemimanesh and Matinfar, 2012). The most sustainable way to control wind erosion is revegetation on sand dunes (Cordoba *et al.*, 2001; Zhang *et al.*, 2004; Gong *et al.*, 2020). However, due to the difficulty of establishing plant species, it is better to carry out preliminary practices such as mulching (Azoogh *et al.*, 2018). Depending on the conditions of the area, cheap, available, and sustainable mulches should be applied (Jamili *et al.*, 2015; Khalili Moghadam *et al.*, 2016). Other ways to stabilize sand dunes include fences (Gumaa *et al.*, 1998), windbreaks (Vacek *et al.*, 2018), or a combination of them (Fulbright *et al.*, 2006). Today, apart from chemicals and methods been widely used so far, biological methods are considered an emerging phenomenon in improving soil properties and stabilization. The most recent of them is biocementation through MICP (Microbially induced calcite precipitation) (Gowthaman *et al.*, 2019; Vail *et al.*, 2020; Sun *et al*., 2020; San Pablo *et al*., 2020). It would be a great achievement if it is possible to provide conditions that microorganisms are added to the sand dunes in a substrate of a disposable material and connect the sand grains together by producing calcium carbonate. In the most well-known MICP pathway, urea hydrolyzing bacteria release carbonate ions by breaking down the urea that is available to them, which reacts with calcium in the environment and produces $CaCO_3$ (Maleki *et al.*, 2016; Gowthaman *et al.*, 2019; Zehner *et al.*, 2020; Tang *et al.*, 2020). Additionally, agricultural wastes are increasing due to human population growth, so it is crucial to find ways to convert them into functional and valuable products (Wu *et al.*, 2021). Sugarcane is cultivated as an economic crop in tropical and subtropical regions of many world countries (Dlamini, 2021). About 130,000 ha of farms in Khuzestan province in Iran have been dedicated to cultivating this crop (Khalili Moghadam *et al.*, 2016). Alcoholic distillation of sugarcane molasses produces a large amount of vinasse as wastewater (Soto *et al.*, 2021). Researchers are looking for ways to make beneficial use of vinasse, including energy production, vinasse recycling in fermentation, fertilizer-irrigation, and feeding livestock (Christofoletti *et al.*, 2013). For this purpose, there are two categories of techniques: aerobic treatment and anaerobic digestion (Carvalho *et al.*, 2023). Using vinasse as a fungal and bacterial inoculum causes more microbial growth and also improves the characteristics of raw vinasse in terms of toxicity and low pH, and fertilizing with it reduces its harmful environmental effects (Torres *et al.*, 2023). The idea of using vinasse as a substrate for plant growth-promoting bacteria and improving the properties of vinasse for fertigation has also been positively reported (Silva *et al.*, 2020). The application of vinasse as a microbial culture medium for the production of biomass, enzymes and metabolites has given an essential role to vinasse in the circular economy (Torres *et al.*, 2022). Vinasse mulch was recommended to stabilize sand dunes of Ahvaz due to increasing water retention in the subsurface layers and greater resistance to erosive forces (Jamili *et al.*, 2015). In addition, vinasse is readily available in this region and does not have the same disadvantages as petroleum mulch.

The present study is conducted on ureolytic bacteria isolated from the sand dunes of

Khuzestan in Iran to investigate their survival and microbial activity in different types of vinasse produced from sugarcane and alcohol factories as the bacterial growth media during a certain period. Moreover, this research is an overture to investigating the microbiologically induced calcite precipitation (MICP) process of the native isolates, therefore, *Sporosarcina pasteurii* was used as a well-known bacterium for MICP to obtain a better comparison.

2. Materials and methods

2.1. Preparation of samples

Isolate *Bacillus licheniformis* MZ057843 (strain 1D1), isolate *B. licheniformis* MZ057842 (strain 1S5) and isolate *B. licheniformis* OP329211 (strain 1D2) were isolated from sand dunes of Khuzestan province and *Sporosarcina pasteurii* was purchased from Iranian biological resource center (Tehran, Iran). They were inoculated on the nutrient broth already sterilized by autoclaving at 121°C for 20 min. For *S. pasteurii* 2% filtered urea solution was added to the culture medium. Then, 10% overnight culture of each isolate was inoculated in Erlenmeyer flasks containing 500 ml of vinasse which was sterilized in an autoclave at 120°C for 20 min. The lids of the containers were covered with cotton and kept in a shaker incubator at 30°C for three days and 120 rpm to reach an acceptable population of each bacterium. Nutrient broth (Scharlau) and vinasse were used for bacterial growth. Three types of vinasse were applied in this study, which were obtained from alcohol, yeast and a mixture of alcohol and yeast vinasse in a ratio of 50:50 (Table 1).

Table 1. Physicochemical composition of sugarcane vinasse

Parameter	Alcohol	Yeast	a mixture of alcohol and yeast vinasse in a ratio of 50:50
pH	4.15	5.32	4.58
EC ($dS.m^{-1}$)	50.20	34.70	41.40
COD ($mg.L^{-1}$)	74280	19420	56490
BOD ($mg.L^{-1}$)	39740	9830	28420
TDS ($g.L^{-1}$)	25.90	13.37	22.20
Common characteristics among all three types of vinasse			
Density ($g.cm^{-3}$)	1.28		
OM (%)	37.51		
OC (%)	19.04		
Cl (%)	2.20		
Na (%)	1.47		
S (%)	18.10		
NO_3^- (%)	<0.1		
Ca (%)	<0.1		
Mg (%)	<0.1		
NH_4^+ (%)	<0.1		
N (%)	1.22		
P (%)	<1		
K (%)	5.20		
Protein (%)	7.65		
Folic acid (%)	1.69		

In this study, the galvanized steel trays with dimensions of 105* 35* 2cm were used. Sand samples were collected from Ahvaz sand dunes (Hamidiyeh region) in Jan-Feb 2021 were sterilized by dry heat sterilization method for three hours at a temperature of 180°C in an oven. Then, they were placed in trays and their surface was smoothed using a ruler, and prepared for spraying. samples prepared from vinasse containing 10% bacterial suspension was uniformly sprayed on the sand trays so that it penetrated to a depth of one centimeter. Then, the trays were left aseptic at room temperature (25±2°C). Experiments were conducted at one, three, and five weeks after spraying.

2.2. Experimental design and statistical analysis

A split plot in time design including 11 variables was performed: 1- Without of bacteria (B0), 2- *B. licheniformis* MZ057843 strain 1D1 (B1), 3- *B. licheniformis* MZ057842 strain 1S5 (B2), 4- *B. licheniformis* OP329211 strain 1D2 (B3), 5- *S. pasteurii* (B4), 6- Alcohol vinasse (V1), 7- Yeast vinasse (V2), 8- A mixture of alcohol and yeast vinasse in a ratio of 50:50 (V3), 9- The first week of incubation (T1), 10- The third week of incubation (T2), and 11- The fifth week of incubation (T3). In general, 15 types of MICP samples were considered in three repetitions. The mean comparison test was proceed using Duncan test method at the 1% level. The statistical calculations of the results were done by SAS software and the graphs were drawn by Excel.

2.3. Counting bacteria by plate count technique

Determining the population of bacteria by counting them is the most appropriate method for this category of soil microorganisms. In plate count technique method, most viable bacteria are counted only, and a colony usually consists of more than one individual cell and is therefore expressed by the term CFU (colony forming units) (Schinner *et al.*, 2012).

The samples were diluted 1:10 using physiological saline solution (NaCl 0.9%), and shaken at room temperature for 60 minutes at 160 rpm. Following two hours of remaining stagnant, serial dilution was prepared, and 50µl (microliter) of the supernatant was cultured on nutrient agar (Conda pronadisa) in two replications, and incubated at 30°C until bacterial colonies appeared. Then, the bacterial colonies were counted and using the following formula, the bacterial population of each sample was obtained based on CFU per gram of soil:

$$\mathrm{CFU} = \frac{\text{number of colonies}}{\text{dilution used} \times \text{volume plated(ml)}} \quad (1)$$

2.4. Soil catalase

Catalase activity is used to determine the microbial activities of non-waterlogged soils because it is present in aerobic organisms and can convert the toxic H_2O_2 produced by metabolism into H_2O and O_2 and prevent its toxic effects on organisms (Schinner *et al.*, 2012; Guangming *et al.*, 2017). To measure catalase activity in soil, the rate of oxygen release from hydrogen peroxide or the residual hydrogen peroxide recovery over time is done (Trevors, 1984). In this experiment, the residual hydrogen peroxide recovery method was titrated with $KMnO_4$ in the presence of H_2SO_4 (Trasar-Cepeda *et al.*, 1999).

Two grams of the sample was poured into a 250 ml Erlenmeyer flask, 40 ml of distilled water, and then 5 ml of 0.3% H_2O_2 were added. The samples were shaken for 20 minutes and filtered after adding 5 ml of 3 N sulfuric acid. Then, the obtained solution was titrated with 0.1 M $KMnO_4$ to change color to pink. A control without a sample was also considered for calculations (Johnson and Temple, 1964). In this method, the unused H_2O_2 is measured, not the

produced oxygen. The activity of soil catalase is expressed as the consumed $KMnO_4$ 0.1 N per gram of soil per hour which is equivalent to decomposed H_2O_2.

2.5. Soil microbial respiration

One of the best indicators of microbial activity in soil is respiration, which can be evaluated by measuring the amount of carbon dioxide produced over time. A higher respiration rate in the soil indicates more activity of microorganisms (Asgari *et al.*, 2015).

About 20 grams of moist sample was weighed in a container. A beaker containing 20 ml of 0.05 M NaOH was placed on the on soil sample in the container so that the CO2 in the sample was in contact with the beaker containing NaOH. The container was sealed and incubated at 25°C for 24 hours. After that time, the beaker containing NaOH was picked up from the closed container. Then, 2 ml of 0.5 M $BaCl_2$ was added to the NaOH until it became milky. Then 3-4 drops of phenolphthalein reagent were added to turn purple. Finally, the solution was titrated with 0.1 M HCl until the milky color returned (Schinner *et al.*, 2012). A sample without soil sample was considered as a control. The respiration rate of the sample was obtained according to the following formula:

$$\frac{(C-S)\times 2.2\times 100}{SW\times \%dm} = mgCO_2 \cdot g^{-1}dm \cdot 24h^{-1}$$ (Equation 2, Schinner *et al.*, 2012)

C: mean volume of HCI consumed by controls (ml)
S: mean volume of HCI consumed by samples (ml)
2.2: conversion factor (l ml of 0.1 M HCI corresponds to 2.2mg CO_2)
SW: initial soil weight (g)
$100 \cdot \%^{-1}$ dm: factor for soil dry matter

3. Results

The results of the variance analysis of the effect of bacterial isolates, vinasse type, and incubation time on bacterial population (CFU) are shown in Table (2). The independent and mutual effects of vinasse type, bacteria, and time on the microbial population were significant ($p < 0.0001$). It means that the bacterial isolates using three types of vinasse as carbon sources to act differently during the incubation time and caused their microbial population to be different to each other in sterile sand dunes. Isolate B1 (*B. licheniformis* MZ057843 strain 1D1), in V3 (a mixture of alcohol and yeast vinasse in a ratio of 50:50) and T1 (the first week of incubation time) had the highest bacterial population by 2.1×10^7 cfu. g^{-1}, and isolate B4 (*S. pasteurii*) in T3 (the fifth week of incubation time) and the same vinasse type (V3) had the lowest by 6×10^4 cfu. g^{-1}. As expected, no bacterial cell growth was observed in isolate B0.

The results of the comparison of the average microbial population under the influence of different types of treatment through Duncan's multi-domain test showed that the average treatments were in different classes. Treatment B1, V3 in T1 had the highest and after B0 (Without of bacteria) as a control, treatments B4 and B2 (*B. licheniformis* MZ057842 strain 1S5), V2 (yeast vinasse) in the fifth week of incubation time (T3) had the lowest bacterial population, respectively (Table 3). According to the graph in Figure 1, in the first week of incubation, the population of bacteria was at the highest level compared to the third and fifth weeks. Among the isolates, B1 in alcohol and the mixed vinasse followed by B2 in yeast vinasse, and B4 in the mixed vinasse had the highest population in the first week. Overall, the population of all bacteria declined sharply over time to less than 5.4×10^5.

Table 2. Analysis of variance (ANOVA) of Vinasse (V), Bacteria (B), Repetition (R) and Time effects on the Bacterial population (CFU), Soil microbial respiration (SMR) and Soil catalase (SC).

Source of variance	Df	Mean Square		
		CFU ($.g^{-1}$)	SMR ($mgCO_2.g^{-1}dm.24h^{-1}$)	SC ($KMnO_4.g^{-1}.h^{-1}$)
V	2	2.97×10^{13} **	0.005**	0.35**
B	4	9.78×10^{13} **	0.36**	35.96**
V*B	8	2.77×10^{13} **	0.01**	0.46**
R*V*B	30	8.7×10^{10} ns	0.00001216ns	0.0004614ns
Time	2	1.2×10^{15} **	0.23**	12.78**
Time*V	4	2.73×10^{13} **	0.04**	0.68**
Time*B	8	8.76×10^{13} **	0.12**	5.42**
Time*V*B	16	2.75×10^{13} **	0.01**	0.50**
Error	60	8.17×10^{10}	0.00001265	0.0002390
CV%	-	8.989414	1.536797	0.651416
R^2	-	0.998867	0.999772	0.999937

ns, * and ** stand for non-significant, significant at 0.05 and 0.01 probability levels, respectively.

Table 3. Mean comparisons of the effects of Vinasse (V1, V2, and V3), Bacteria (B0, B1, B2, B3 and B4), and Time (T1, T2 and T3) on the Bacterial population (CFU), Soil microbial respiration (SMR) and Soil catalase (SC).

Treatments		CFU ($.g^{-1}$)	SMR ($mgCO_2.g^{-1}dm.24h^{-1}$)	SC ($KMnO_4.g^{-1}.h^{-1}$)
Bacteria	B0	0^{d}	0.0662727^{e}	0.361080^{e}
	B1	5135556^{a}	0.3560214^{a}	3.006758^{b}
	B2	3569444^{c}	0.1621008^{d}	2.695267^{c}
	B3	3756389^{b}	0.2687645^{c}	3.229161^{a}
	B4	3440278^{c}	0.3040554^{b}	2.575054^{d}
Vinasse	V1	3201833^{b}	0.2299752^{b}	2.297233^{c}
	V2	2357500^{c}	0.2430040^{a}	2.351700^{b}
	V3	3981667^{a}	0.2213497^{c}	2.471458^{a}
Time	T1	9140000^{a}	0.3119298^{a}	1.858327^{c}
	T2	269500^{b}	0.1705211^{c}	2.922829^{a}
	T3	131500^{c}	0.2118779^{b}	2.339235^{b}

According to Table (2), the independent and interaction effects of vinasse type, bacterial isolates, and incubation time on soil catalase activity were significant ($p < 0.0001$) as the bacterial population. Quantitatively and case-wise, the highest amount of catalase activity was obtained for isolate B3 (*B. licheniformis* OP329211 strain 1D2) in V1 (alcohol vinasse) and T2

(the third week of incubation time) with a value of 4.361 $KMnO_4.g^{-1}.h^{-1}$ and the lowest after the samples without bacteria (B0) was obtained for isolate B1 (*B. licheniformis* MZ057843 strain 1D1) in V1 and T3 with a value of 1.053 $KMnO_4.g^{-1}.h^{-1}$. Table (3) shows the results of comparing the average soil catalase activity in five classes for bacterial isolates and three classes for types of vinasse and incubation times. Treatment B3, V3 in T2 had the highest soil catalase activity, followed by B0 (without bacteria) as a control, and treatments B4 in V1 at the first week of incubation time (T1) had the lowest enzyme activity. According to the graph in Figure 2, the enzyme activity of B3 was higher in all three types of vinasse. All isolates and all three types of vinasse had the highest catalase activity in the third week of incubation (T2). The analysis of the tripartite interaction effect on soil catalase activity showed that in T1, the highest enzyme activity was related to B1. In T2, B1 and B3 had constant enzymatic activity in all three types of vinasse (the enzymatic activity of both isolates at this time was higher than in the first and fifth week of incubation). In T3, B3 performed much better than other isolates.

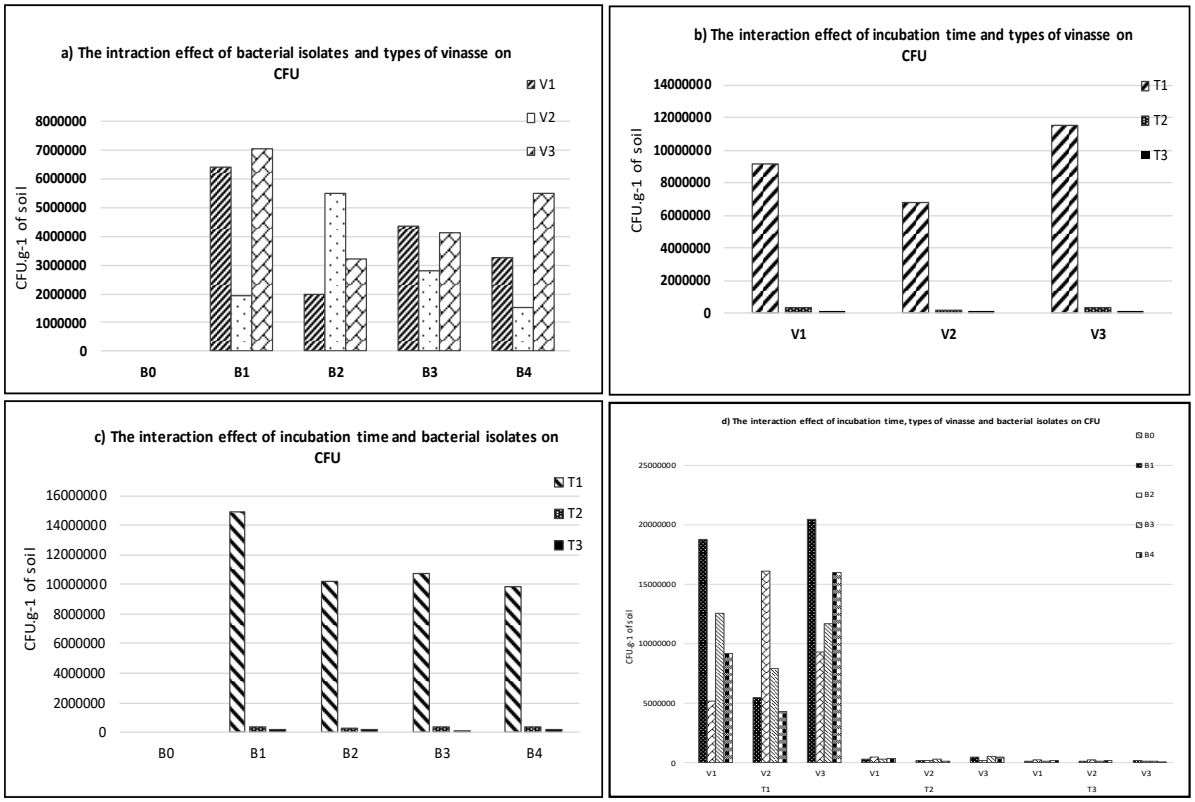

Fig. 1. Diagrams of the effects of interactions on microbial populations (CFU):
a) The interaction effect of types of vinasse and bacterial isolates on CFU
b) The interaction effect of incubation time and types of vinasse on CFU
c) The interaction effect of incubation time and bacterial isolates on CFU, and
d) The interaction effect of incubation time, types of vinasse and bacterial isolates on CFU.

The results of the variance analysis of the effect of bacterial isolates, vinasse type, and incubation time on soil microbial respiration (SMR) are shown in Table (2). As CFU and SC, the independent and interaction effects of vinasse type, bacteria, and time on the microbial respiration were significant ($p < 0.0001$). Quantitatively, the highest amount of microbial respiration was related to the isolate B3 in V2 and T1 by 0.601 $mgCO_2.g^{-1}dm.24h^{-1}$, and the lowest after B0 was related to the isolate B2 in V1 and T2 by 0.090 $mgCO_2.g^{-1}dm.24h^{-1}$.

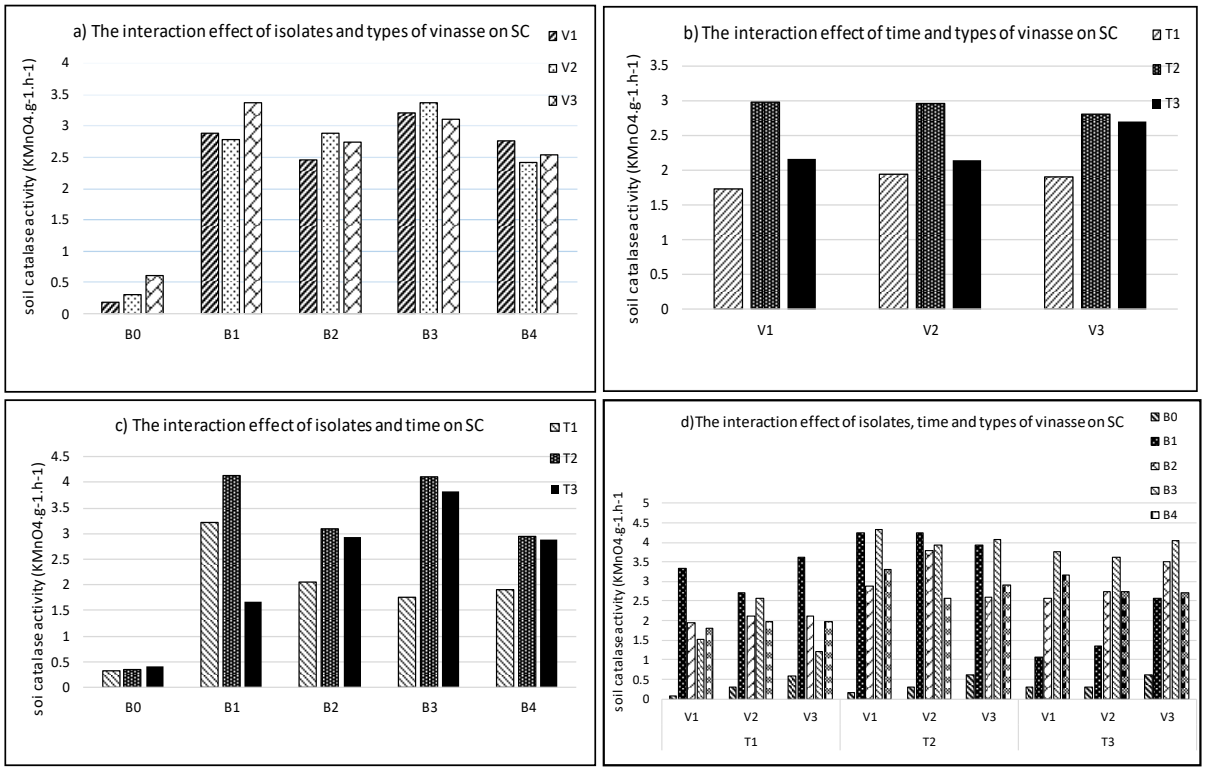

Fig. 2. Diagrams of the effects of interactions on soil catalase activity (SC):
a) The interaction effect of types of vinasse and bacterial isolates on SC
b) The interaction effect of incubation time and types of vinasse on SC
c) The interaction effect of incubation time and bacterial isolates on SC, and
d) The interaction effect of incubation time, types of vinasse and bacterial isolates on SC.

According to Table (3), the results of the comparison of the average soil microbial respiration showed treatment B1 (*B. licheniformis* MZ057843 strain 1D1), V2 (yeast vinasse) in the first week of incubation time (T1) had the highest amount, followed by B0 (Without of bacteria) as a control, treatment B2 (*B. licheniformis* MZ057842 strain 1S5), V3 (a mixture of alcohol and yeast vinasse in a ratio of 50:50) in the third week of incubation time (T2) had the lowest. As shown in Figure (3), B1 had a better performance than the other isolates in all three types of vinasse, but it had the highest rate of microbial respiration in V3. All three types of vinasse had better microbial respiration at T2. All isolates had the highest microbial respiration at T2. The analysis of the tripartite interaction effect on soil microbial respiration showed that in T1, the highest respiration was related to B1 in three types of vinasse. In T2, B1 and B3 had constant performances in all three types of vinasse. In T3, B3 performed much better than other isolates.

4. Discussion

Biological activity in soil is critical for plant growth and soil health (Jabran *et al.*, 2019), regulated by bacterial communities as biogeochemical engineers by controlling nutrient cycling, gas flow, and soil fertility (Wang *et al.*, 2024). Counting soil bacteria is an indicator of soil biological activity, and one of the goals of inoculating vinasse with bacteria and spraying on a bed of sterile sand was to investigate the viability and survival of the inoculated bacteria. In standard methods for inoculation, the number of organisms was in the range of 10^8 cfu.ml^{-1} (Baker *et al.*, 1983). It has been variable in different reports between 10^6-10^9 cfu.ml^{-1} (Duquenne *et al.*, 1999; Hale *et al.*, 2014; Xu *et al.*, 2016; Sharma and Reynnels, 2016;

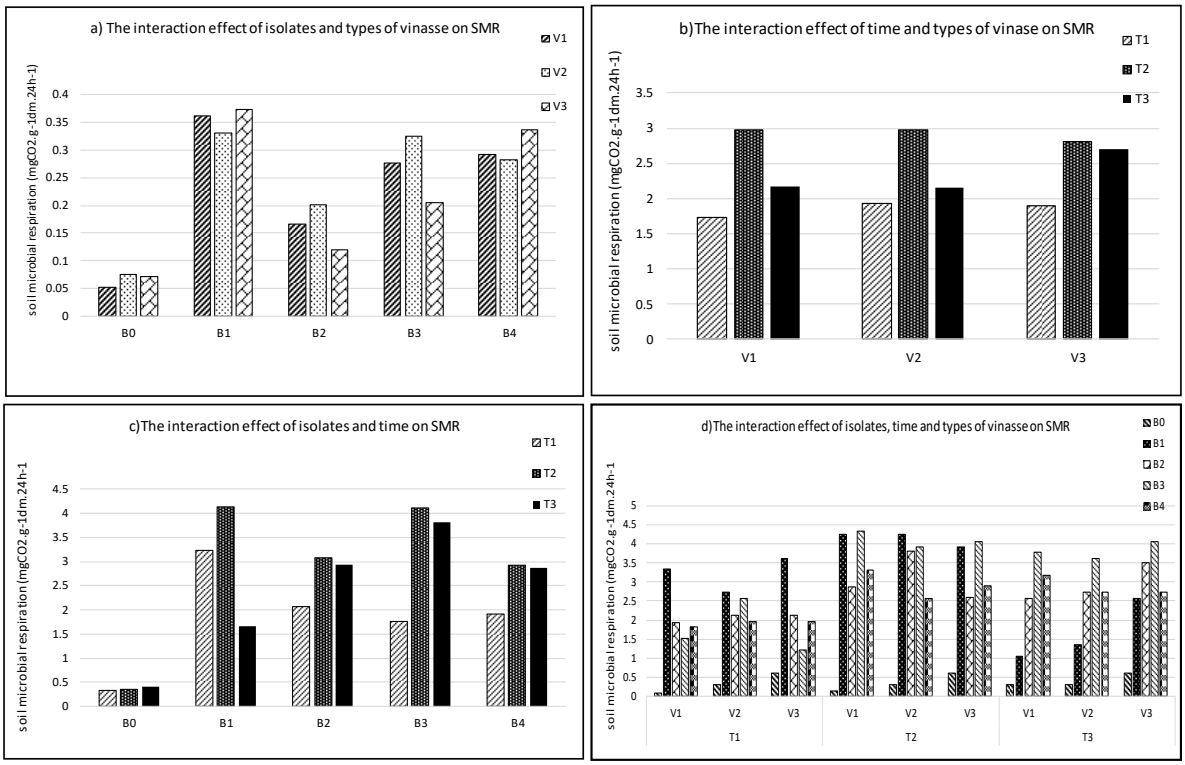

Fig. 3. Diagrams of the effects of interactions on soil microbial transpiration (SMR):
a) The interaction effect of types of vinasse and bacterial isolates on SMR
b) The interaction effect of incubation time and types of vinasse on SMR
c) The interaction effect of incubation time and bacterial isolates on SMR, and
d) The interaction effect of incubation time, types of vinasse and bacterial isolates on SMR.

Kim *et al.*, 2018; Wu *et al.*, 2021). Therefore, it seems that the highest bacterial population obtained in this study (2.1×10^7 cfu. g^{-1}) is favorable after one week of the incubation period, and the lowest population was obtained at the end of the fifth week (6×10^4 cfu. g^{-1}) which is consistent with the results of Nasser *et al.*, (2022). They counted the population of *Bacillus pasteurii* and *Bacillus sphaericus* inoculated into cement during a period, which decreased from 10^7-10^8 on the third day to 10^4-10^5 after 35 days with an almost similar pattern, and attributed it to the reduction of nutrients. Identifying the effective factors in the persistence of bacteria in the soil is vital for appropriate management measures in soil protection and erosion (Micallef *et al.*, 2023). According to the results presented in Figure 1, After a week of spraying, while the surface of the sample was not completely dry and hardened, the population of isolates was at its highest level. Micallef *et al*., (2023) showed that, mulches can enable the persistence of bacteria and pathogens in the soil. With the passage of incubation time, the population of bacteria has faced a sharp decrease from the first week to the third week. This issue probably shows the effect of moisture on the survival of bacteria. According to the research of Moreira *et al.*, (2023), the main advantage of mulches is maintaining soil moisture and increasing the survival of microorganisms. Microorganisms in crops grown with mulch survive longer than crops grown in bare soil, which is related to higher humidity and warmer temperatures. Jamil *et al.*, (2005) also pointed that soil microorganisms are active in all kinds of mulches, especially on the surface of the soil where both soil and mulch are wet. Some studies have shown that drip irrigation of mulch causes the activation of nutrients and the growth of microorganisms in the surface soil (Wang *et al.*, 2023). Micallef *et al.*, (2023) concluded that, the short-term use of mulch cannot change the conditions

of the substrate in such a way that the effect of bacteria on the substrate lasts longer than when they are covered by the mulch. So, in the short term, the microclimate created during mulch application is probably more related to the bacteria than the mulch material. In Xu *et al.*'s study (2016), the number of inoculated bacteria in different mulches decreased from 1 to 14 days. Our results showed that, among all three types of vinasse, the highest bacterial population was found in the V3 which contained 50% alcoholic vinasse and 50% yeast vinasse. The difference between alcohol vinasse and yeast vinasse is in the amount of their compounds. Alcohol vinasse contains much alcohol, and its sugar content is high. Because a large amount of yeast is cultivated in yeast vinasse to increase the cell mass, the amount of biomass and protein compounds are high. The third type of vinasse (a combination of alcohol and yeast vinasse) was probably able to provide a balanced amount of nutrients bacteria need for growth and survival and was the preferred source of bacteria. The results showed that, among the isolates, *B. licheniformis* MZ057843 strain 1D1 (B1) had more population. *Bacillus* bacteria are common in soil and plants (Zhou *et al.*, 2023). *B. licheniformis* is one of the rod-shaped and gram-positive bacteria with the ability to form endospores (Cho and Chung, 2020). This bacterium is widely used in food industry, agricultural waste treatment, and raw material processing due to its specific metabolic and genetic activity (He *et al.*, 2023). Omeroglu *et al.*, (2024) introduced *B. licheniformis* as a suitable thermophilic candidate for microbial fermentation of molasses to Butanediol in non-sterile processes. As a result of the sucrose-alcohol cycle, vinasse is obtained from the fermentation of sugarcane juice (molasse) that is rich in sucrose, and is considered a biofertilizer due to its high content of minerals and organic substances (Balakrishnan, 2024). Lakra *et al.*, (2023) isolated the thermophilic *B. licheniformis* from the hot spring, and by examining its exopolysaccharide production, they found that the highest production efficiency was in the sucrose carbon source and pH = 6. In the study by Xu *et al.*, (2023), changes in the sucrose catabolism pathway in molasses by *B. licheniformis* were successfully made for the biosynthesis of phenylethanol. In some other studies, the ability of *B. licheniformis* to use the sucrose carbon source compared to other carbon sources has been reported (Huang *et al.*, 2013; Klaewkla *et al.*, 2020). As mentioned above, *B. licheniformis* is highly resistant to chemical and physical treatments such as heat, radiation, and chemicals due to their ability to produce spores (Cho and Chung, 2020). Therefore, it can be reasonable to use this spore-forming bacterium in Khuzestan weather conditions. With low-metabolic activity, some strains produce extremely long-life spores viable for up to 200 years (Feng *et al.*, 2021).

Catalase enzyme (hydrogen-peroxide oxidoreductase) is one of the most essential intracellular enzymes in facultative and obligate anaerobic microorganisms. It deals with hydrogen peroxide as a toxic substance and oxidative stress for cells by breaking it down into oxygen and water. Various factors can produce H_2O_2, including photosynthesis and aerobic respiration, or environmental factors, such ultraviolet radiation (Chabot *et al.*, 2020). Catalase activity in soil indicates the aerobic activity of microorganisms, which depends on two factors: soil fertility and the number of aerobic microorganisms (Trasar-Cepeda *et al.*, 1999). The amount of catalase activity in this study was 1.053 – 4.361 $KMnO_4.g^{-1}.h^{-1}$ (regardless of the amount of control without bacteria). Li *et al.*, (2014) investigated the activity of catalase enzyme in different land uses and reported its values between 1.8 (natural forest) and 2.1 (slope field into terrace) $KMnO_4.g^{-1}.h^{-1}$ without any significant change among them. Johnson and Temple (1964) obtained catalase activity in different soils with or without organic matter between 3.5 (Bozeman acid soil) - 9.8 $KMnO_4.g^{-1}.h^{-1}$ (Bowdoin clay). Zheng *et al.*, (2019) reported the catalase activity of different mulches in different rice growing seasons between 7 (control in maturity stage) and 12 (straw with chemical fertilizer) $KMnO_4.g^{-1}.h^{-1}$. Shi *et al.*, (2022), by investigating the effect of four mulching patterns on soil catalase enzyme under potato cultivation in a semi-arid region, found that the lowest amount was related to the

treatment flat plot without mulch by 17 and the highest was related to the treatment ridge planting with full mulch by 30 $KMnO_4.g^{-1}.h^{-1}$. In this study, the activity of catalase in sterile sandy soils is related to the inoculated isolates, so their activity can be positively evaluated compared to the control treatments without bacteria with values of 0- 0.608 $KMnO_4.g^{-1}.h^{-1}$. According to Chabot *et al.*'s study (2020), indigenous microbial communities in areas with high oxidative stress have higher basal intracellular catalase concentrations. Oxidative environmental stresses include high temperatures, air pollutants, salinity, drought, and UV Radiation (Xie *et al.*, 2019). Our results showed the lowest level of catalase activity in the first week, and over time, the activity of this enzyme increased in the third week and then decreased in the fifth week of incubation. An increase in catalase activity means more production of H_2O_2 in the growth environment of bacteria. According to the study conducted by Ferreira *et al.*, (2020), a strong increase in catalase occurred on the 6th, 9th, and 12th days of *Pleurotus sajor-Caju* incubation in the vinasse culture medium, which was attributed to the presence of phenolic and resistant color compounds and degraded products produced by ligninolytic enzymes. In this study, the highest catalase enzyme activity was obtained in treatments inoculated with *B. licheniformis* OP329211 strain 1D2 (B3). *Bacillus*, *Panibacillus*, and *Geobacillus* strains contain many catalases (Yuan *et al.*, 2021). Among the bacteria, *Bacillus* and *Pseudomonas* species are usually considered suitable in mixed bio-inoculations to promote plant growth (Torres *et al.*, 2022). By secreting antioxidant enzymes, Bacillus destroys active oxygen species and helps in antioxidation (Huang *et al.*, 2023). Among *Bacillus* genus that produce catalase, many studies have been conducted on *B. subtilis*. Due to the high production of this enzyme, especially at high temperatures and high stability, it is known as a candidate for industrial use (Abdelhamid *et al.*, 2020; Shaeer *et al.*, 2021). Ni *et al.*, (2001; 2002), introduced *Bacillus sp.* TEl24 is a relatively stable catalase producer. Paar *et al.*, (2001) used catalases from three thermoalkaliphilic *Bacillus* to treat bleaching effluent. Chu *et al.*, (2021) reported a catalase enzyme production from *B. licheniformis* Cas02. The inoculation of peaches with *B. licheniformis* increased the resistance of the fruit against the disease with the ability to remove free radicals by increasing the production of catalase enzyme (Wang *et al.*, 2023). Sen *et al.*, (2011) performed cloning, expressing and purifying catalase from thermoalkaliphilic *B. licheniformis*, and introduced it for industrial applications. In recent years, the production of the antioxidant enzyme catalase by *B. licheniformis* has identified it as a growth-promoting bacterium even in animal diets (Xu *et al.*, 2021; Qin *et al.*, 2023). According to our results, the highest activity of catalase occurred in treatments by V3 (A mixture of alcohol and yeast vinasse in a ratio of 50:50). Some reports indicate an increase in catalase enzyme activity in soils irrigated with vinasse and an increase in yield and growth of sugarcane seedlings (Wang *et al.*, 2006). Ferreira *et al.*, (2020) investigated the effect of oxidative stress based on the activity of antioxidant enzymes including catalase during the treatment of vinasse with *Pleurotus sajor-caju* and found it suitable for sugarcane wastewater treatment.

The release of carbon dioxide from the soil is called respiration, which has three origins: soil microbes, plant roots, and the dissolution of carbonates in the soil solution (Parkin *et al.*, 1996). Soil respiration is also known as the process of carbon mineralization because, during it, soil organic matter is decomposed and turned into mineral forms (Parkin *et al.*, 1996). Factors affecting soil respiration include temperature, humidity and soil carbon content (Jozedaemi and Golchin, 2024). In our study, the highest amount of soil microbial respiration was 0.601, and the lowest was 0.090 $mgCO_2.g^{-1}dm.24h^{-1}$. Compared to the values related to the control without bacteria (0- 0.082 $mgCO_2.g^{-1}dm.24h^{-1}$), the value related to the lower number is not much different. However, the larger value is almost seven times larger than it, and it seems valuable. Alves *et al.*, (2019) obtained results that were almost similar to ours. They investigated the

microbial respiration of tropical agricultural soils amended with sugarcane vinasse and reported the values to be around 1 (for the lowest concentration of vinasse) to 5 $mgCO_2.g^{-1}.24h^{-1}$ (for the highest concentration of vinasse). In another study, soil microbial respiration in different land uses was measured as 1.709 for pristine forest and 3.570 for grazed pasture $mgCO_2.g^{-1}.24h^{-1}$ (Kelliher *et al.*, 2005). Buckley *et al.*, (2022) reported the amount of soil microbial respiration in soil rich in organic matter as 0.72 and in the same soil with the addition of sucrose and litter as 2.4 $mgCO_2.g^{-1}.24h^{-1}$. Jafari *et al.*, (2013), reported the microbial respiration of soil inoculated with *Bacillus subtilis* and soil without bacteria about 0.57 and 0.4 $mgCO_2.g^{-1}.24h^{-1}$, respectively, at a salinity level of 8 $dS.m^{-1}$ (similar to the electrical conductivity of our studied soil). Hashempour *et al.*, (2020) obtained the highest amount of microbial respiration in the soil inoculated with native *Bacillus* with 0.51 in 4% of the refinery plant-solid waste and the lowest for the soil inoculated with *Bacillus persicus* with 0.27 $mgCO_2.g^{-1}dm.24h^{-1}$ in 0% of that. Our results showed the highest level of soil microbial respiration in the first week, and over time, the activity of this enzyme decreased in the third week and then increased in the fifth week. The high rate of respiration in the first week is due to the consumption of microorganisms from sufficient organic carbon (Ghorbanzadeh *et al.*, 2018). In environments where the amount of carbon is high, soil microorganisms release more carbon through respiration, digestion, and secretion, then, reduce carbon immobilization (Wang *et al.*, 2023). In a study, the rate of respiration was measured by adding sucrose to the soil, and the result was that due to the presence of high carbon in the first seven days, the rate of respiration was significant and caused the loss of carbon through respiration (Buckley *et al.*, 2022). Among the other reasons for the high respiration activity of bacteria in the first week, we can mention the appropriateness of the conditions in terms of soil moisture (Cruz-Paredes *et al.*, 2021). Because the surface of the sand bed had not yet dried. In the third week, a sharp decrease in microbial respiration occurred. Due to the decrease in the number of bacteria (CFU) compared to the first week, this decrease was predictable. In the last incubation period, i.e., the fifth week, a re-increase in soil microbial respiration was observed. An increase in respiration of agricultural soils due to vinasse has been reported between 30-60 days (Senatore *et al.*, 2023). Vaclavik *et al.*, (2004) stated that, sometimes other mechanisms are responsible for respiration and maybe dead bacteria become a carbon source for other microorganisms and thus increase their respiration. Differences may become more obvious over longer periods (Buckley *et al.*, 2022). The highest soil microbial respiration was related to *B. licheniformis* MZ057843 strain 1D1 (B1). The effect of inoculation of some species of *Bacillus* to the soil has been reported to investigate microbial respiration. Adding native *Bacillus sp.* and *Bacillus persicus* to the agricultural waste caused a decrease in microbial indicators such as soil respiration (Ghorbanzadeh and Farhangi, 2021). Our results showed that the highest microbial respiration was associated with yeast vinasse (V2). Tejada *et al.*, (2009) claimed that the application of beet vinasse had harmful effects on the soil, probably due to the presence of high Na^+ that destabilized the soil. Since wastes with a high percentage of organic matter and easy to decompose, such as vinasse, immediately after adding to the soil, cause a strong increase in respiration, vinasse can be considered an organic and ready-to-use fertilizer (Grigatti *et al.*, 2010). According to the research conducted by Senatore *et al.*, (2023), vinasse is more effective on the microbial activity of the soil than on the microbial population. It can be quickly decomposed and incorporated into the biomass.

5. Conclusion

The ability of *B. licheniformis* has been proven in many research studies related to using soil microbial potential in industrial applications. On the other hand, due to the climatic conditions prevailing in Khuzestan, the sporulation of this bacterium is an advantage that can guarantee its

survival in harsh environmental conditions. The use of wastes and effluents from sugarcane factories (vinasse), which many lands in Khuzestan are dedicated to their cultivation, has already been proven to stabilize sand dunes, but creating conditions that can improve the properties of this valuable material and its durability as much as possible will lead to greater efficiency.

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