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Original Article

Measurement of ACC-Deaminase Production in Halophilic, Alkalophilic and Haloalkalophilic Bacterial Isolates in Soil

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Strains with 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity can cause plant growth on stress condition. In the presence of bacteria with the ability of ACC deaminase production in the rhizosphere, it is possible to convert the ethylene precursor (ACC) to α-ketobutyrate and ammonium, reducing the ethylene levels in host plants. In order to measure the power and level of ACC deaminase production in halophilic, alkalophilic and haloalkalophilic microbial isolates, soil samples were collected from six different areas of Khorasan Razavi Province and bacterial isolates were separated and purified. The haloalkalophiles isolates showed the maximum ACC deaminase production (0.4374 mM) among the three groups of isolates, followed by alkalophiles isolates (0.241 mM) and halophiles (0.0848 mM). Equations to predict the concentration of ACC deaminase production were only significant in the halophilic isolates (probability level= 1.0) under the effect of electrical conductivity and pH at 0.01 probability level. Also, the multiple regression analysis for predicting ACC deaminase production by isolates had significant performance in low concentrations, while at higher concentrations of the enzyme other factors were effective in ACC deaminase production. The higher ACC deaminase productivity power of haloalkalophilic isolates makes them interesting for basic biotechnology studies.

Keywords: Stressed ethylene, salinity stress, ACC deaminase, haloalkalophilic bacteria

Proper utilization of saline soils in order to achieve maximum crop yield has always been a major challenge for the agricultural sector. Cultivated plants in these soils are affected by salinity and alkalinity in different ways, and do not achieve their maximum growth and yield. Ethylene is one of the most important plant growth regulator hormones that plays an important role in fruit ripening, photosynthesis, respiration, transpiration, embryogenesis, rooting processes, and many other features of the plant. In addition to the positive effects of ethylene, it also has inhibitory effects on the growth of plants. Ethylene may prevent roots and stems elongation, and plantsflowering. It also accelerates the ageing process in plants.The amount of ethylene under stress conditions significantly increases in plants, causing their premature ageing (1). 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme is able to convert ACC which is a precursor for ethylene production in plants, into ammonia and α-ketobutyrate, and thereby reduce the amount of ethylene production under stress conditions. Ethylene accumulation in root is one way of salt stress damage to some plants. Ethylene accumulation reduces root growth, and consequently causes plant yields loss. In the

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presence of bacteria with the capacity of ACC deaminase production in the rhizosphere, it will be possible to convert the ethylene precursor (ACC) to α-ketobutyrate and ammonium, and therefore reduce ethylene levels in host plants. In this process, ammonia can be used as the nitrogen source for the bacteria (2). According to Penrose and Glick (3) each bacteria pocessing ACC deaminase activity and producing more than 20 nmol α-ketobutyrate per mg per hour is a plant growth promoting rhizobacteria (PGPR), and can increase the growth indexes of plants. Plants inoculation with bacteria that are able to produce the ACC deaminase enzyme can be effective in ethylene levels reduction upon stress occurrence in plants, and thus can reduce its negative effects. Bacteria with this ability can preserve plants against harmful effects of environmental stresses including heavy metals, waterlogging, plant pathogens, drought and salinity. Considering a significant development of saline areas, it is essential to isolate and identify PGPR bacteria from these areas (4).

Halophilic soil microorganisms are a group of microorganisms that can thrive in environments with high salt concentration (5). Alkalophilic bacteria maintain their internal pH around 9.5 in the external pH range from 9 to 11. These bacteria continue their activities with proton transfer systems in the cytoplasmic membrane (ATP and sodium replacement with proton pump) (6). Another group of bacteria that can grow in alkaline conditions and in the presence of salt is known as haloalkalophiles. This dual characteristic of saline and alkaline-friendly, make them interesting for basic studies on different aspects of biotechnology (7-8). Isolation of these bacteria was performed from area such as alkaline lakes, saltern lakes, salt brines, carbonate springs and sea salt (9). Sadat et al. reported that salinity stress reduced root growth in wheat by increasing the amount of ethylene , while wheat inoculation with PGPR, increased plant growth and the availability of micronutrients in salinity condition via siderophore and chelating agents production, due to ACC deaminase production and decreasing ethylene levels (10). Shaharoona et al. (11) studied the role of ACC deaminase producing bacteria on the growth of wheat, and found that *Pseudomonas fluorescens* ACC50 was the most effective strain among five studied isolates, and produced the highest yield, length and weight of roots in a pot. They announced that the presence of the ACC deaminase enzyme is the most important parameter for the selection of PGPR. Moreover, the study of the role of Rhizobium bacteria generating ACC deaminase in wheat, showed that wheat plants inoculated with rhizobia strains producing ACC deaminase have higher root length, stem length, root and shoot dry weight than controls, with a significant increase in root length (12).

The aim of this study was to evaluate ACC deaminase production potential in halophilic, alkalophilic and haloalkalophilic native isolates in some Khorasan Razavi province soils, in order to further use ACC deaminase producers to reduce the adverse effects of salinity in the vicinity of the plant.

Materials and methods

Samples collection

For quantitative determination of ACC deaminase production by halophilic, alkalpphilic and haloalkalophilic isolates, soil sampling was carried out from six different areas of Khorasan Razavi, Iran saline soils from a depth of 0-25 cm (Table 1). Then samples were transferred to the lab in sterile tubes with area recording by GPS, and in less than 48 h. The samples were kept at 4 °C during the transfer, and were further stored at 4 °C in the lab.

Bacteria isolation

Isolation and purification of halophilic, alkalophilic and haloalkalophilic isolates was done using their specific culture media (Table 2). For isolation, a 1:1 suspension of soil to water (1 g of soil for 1 ml of sterile distilled water) was prepared.

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One drop of this suspension was spread on the specific agar media cultures for isolation of halophilic (13), alkalophilic (14), and haloalkalophilic (15) bacteria. Media cultures were incubated at the proper temperature (35 to 37 °C) for 3 to 7 days depending on the type of microorganisms. After isolation, subculture was done several times to ensure purity of isolates. Purified isolates were preserved in liquid nitrogen (6).

ACC deaminase activity assessment

ACC deaminase activity was measured according to Penrose and Glick (3) which is the modified method of Shimonura and Honma (16). This method evaluates the amount of α-ketobutyrate produced by ACC deaminase in bacterial extracts. In this method, each bacterial isolate was inoculated in a 50 ml Erlenmeyer flask containing 20 ml of appropriate fresh medium. Then inoculated flasks were shook for 24 h on a shaker with 180 rpm at 28 °C. Then 50 ml of the resulting bacterial suspension was transferred into 100 ml Erlenmeyer flask containing 30 ml of fresh medium and was placed for another 12 h on a shaker with 180 rpm at 28 °C. The contents of the flasks were transferred to centrifuge tubes and were centrifuged for 10 min at 8000 rpm at 4 °C. After centrifugation, pellets were washed twice with 5 ml Dworkin-Foster (DF) salts minimal medium. Then the remaining cells were resuspended in 7.5 ml DF medium, and transferred into 50 ml sterile flasks. Then 45 µl of 0.5 M ACC solution (Sigma) was added to the bacterial suspension. The bacterial suspension was placed on a shaker with 180 rpm at 30 °C for 24 h. Then, the bacterial suspension was centrifuged for 10 min at around 8000 rpm at 4 °C. The supernatant was discarded and the remaining cells were washed twice with 5 ml 0.1M Tris-HCl pH 7.6, and were finally resuspended in 1 ml 0.1 M Tris-HCl pH 7.6 and transferred into 10 ml centrifuge tubes, and were centrifuged for 5 min at 16000 rpm at 4 °C. The pellets were suspended in 600 µl 0.1 M Tris-HCl pH 8.5. Then, 30 µl toluene was added, and the bacterial suspension was vortexed intensely for 30 s. Then, 200 µl of the suspension was transferred separately into three new 10 ml centrifuge tubes (a tube as a blank and two tubes for two replication of each isolate). 20 ml of 0.5 M ACC solution was added to each of the two tubes containing 200 µl of toluene cells and the same volume of distilled water was added to the third control tube. The tubes were vortexed and kept at 30 °C for 15 min. Then, 1 ml of 0.56 M HCl was added to each tube. After that, each final solution was mixed by vortex and was centrifuged at 16,000 rpm at room temperature for 5 min. Then, 1 ml of supernatant was picked up from each centrifuge tube and was vortexed in the presence of 800 µl of 0.56 M HCl in a small test tube. 300 µl of 2, 4-dinitrophenylhydrazine solution (0.2 percent 2, 4-dinitrophenylhydrazine solution in 2 M HCl) was added to each tube and was vortexed. The content of each tube was kept for 30 min at 30 °C. Then, 2 ml NaOH 2M was added to the mentioned solution and was mixed to be uniform. At the end, the amount of absorbance was read at a wavelength of 540 nm by a spectrophotometer (PG 9000). The amount of α-ketobutyrate produced in each sample was calculated by comparing the amount of its absorbance with the standard curve. α-ketobutyrate standard solutions were prepared from a 100 mM stock solution of α-ketobutyrate (Sigma), in 0.1 M Tris-HCl pH 8.5. Immediately before standard solutions preparation the stock solution was diluted 10 times with the same buffer to obtain a 10 mM α-ketobutyrate solution. Then, using this 10 mM solution, α-ketobutyrate standard solutions were prepared at 0.1, 0.2, 0.5 and 1 µmol concentrations in 200 µl (in two repeats) into the test tubes. Then 300 µl of 2, 4-dinitrophenylhydrazine was added to each tube, and vortexed, tubes were kept for 30 min at 30 °C. After this period, 2 ml of NaOH 2N was added to each tube and after shaking, the amount of standard solutions absorbance was read at 540 nm, and standard curves were drawn accordingly.

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Table 1. Geographic data of sampling locations of Khorasan Razavi

No.	Location of sampling	coordinate of sampling Geografic					Height (m)	Number of sample
		Second	Minute		Degree	Aspect		
1	Bardaskansaltern	47	36	03	35	■N	859	2
		60	08	53	57	△E		
2	Kale shour Aref Abad Kashmar	89	51	5	35	N	933	1
		78	37	38	58	E		
3	Tounis and Merendiz of Bajestan	30	50	47	35	N	798	1
		18	22	27	58	E		
4	Astan Ghodse Razavi Gardens-Gonabad(1)	84	33	36	34	N	908	1
		63	56	56	58	E		
5	Astan Ghodse Razavi Gardens-Gonabad(2)	89	30	26	34	N	884	1
		51	27	58	58	E		
6	Kale shourEshgh Abad Nayshabour	276	-	05	36	N	1105	1
		643	-	41	58	E		

East direction E△, North direction N■

Table 2. Specific media cultures of halophilic, alkalophilic and haloalkalophilic bacterial isolates

Compounds	Amount (g/L)		
	Halophile■	Alkalophile	Haloalkalophile
Glucose	1	10	-
Poly Peptone	-	5	-
Yeast extract	10	5	10
Di potassium hydrogen phosphate	-	1	-
Magnesium sulphate seven H_2O	9.6	0.2	1
Sodium carbonate	-	*10	18.5▲
Sodium Chloride	81	-	200
Magnesium chloride two H_2O	7	-	-
Calcium chloride	0.36	-	-
Potassium chloride	2	-	2
Sodium hydrogen bicarbonate	0.06	-	-
Sodium bromide	0.026	-	-
Protease Peptone	5	-	-
Casino acid	-	-	7.5
Tri sodium citrate	-	-	3
Manganese(II) chloride	-	-	0.00036
Ferrous sulfate	-	-	0.05
Agar	15	20	20

■pH was adjusted with KOH 1 N to 2.7 before the medium culture sterilization. ▲Was sterile from other materials separately and was added to culture medium before isolates cultivation.

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Results

Overall 15 halophilic, 19 alkalophilic, and 14 haloalkalophilic isolates were obtained.

The comparison of the average concentration of ACC deaminase production by halophilic isolates (Figure 1) showed that the ACC deaminase production was significantly different in various isolates at 5% level. ACC deaminase production in isolates varied between zero and 0.5732 mM.

Maximum ACC deaminase concentration was observed in H2, H6, H8, H11, H9 and H14 halophilic isolates.

Results of ACC deaminase production for alkalophiles showed that maximum ACC deaminase producing concentration was observed in A5, A14, A16, A11, A1, A12, A4, A10, and A19 isolates with significant difference (Fig 2). The remaining isolates did not produce ACC deaminase (Fig.1). The amount of ACC deaminase varied between zero and 0.9740 mM among isolates.

The concentration of ACC deaminase produced by haloalkalophilic isolates was significantly different (P<0.05) (Fig 3). Maximum and minimum ACC deaminase concentrations were observed in HA1 and HA6 with 0.8942 and 0.06188 mM, respectively.

Results of multiple regression analysis of ACC deaminase concentration for each group of isolates (Table 3) also showed that only the equation of ACC deaminase production forecasting in halophilic isolates (the first equation of Table 3) was significant under the effect of electrical conductivity and pH at 1% level (P< 0.0001). Results of multiple regression analysis were not statistically significant for other equations and regression coefficients (Table 3).

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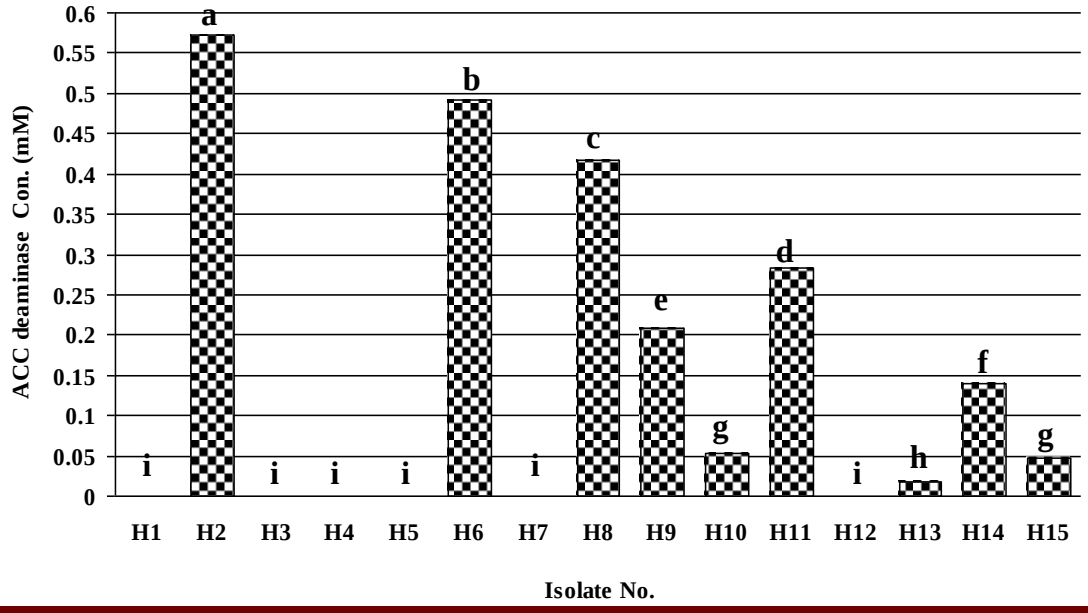

Figure 1. Mean ACC deaminase concentrations (mM) produced by halophilic isolates.

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Table 3. Multiple regression analysis of ACC deaminase concentration in halophilic, alkalophilic and haloalkalophilic bacterial isolates

Type of isolate	Independent variables		The coefficient of correlation between independent variables		Significant independent variables		Equation	$(R)^2$ The regression coefficient	(R^2_{adj}) Adjusted regression	$(R^2_{multiple})$ multiple	Significant equation
Halophile	pH	EC (dS/m)	1.01×10^{-19}	1.45×10^{-16}	0.331	0.000	ACC deaminase=0.89EC+ 2.05×10^{-9} pH	1.0	1.0	1.0	**0.000
Alkalophile	pH	EC (dS/m)	-1.99×10^{-2}	4.71×10^{-2}	0.125	0.273	ACC deaminase = -0.245EC+0.36pH - 0.498	0.152	0.046	0.389	0.269
Haloalkalophile	pH	EC (dS/m)	-3.12×10^{-4}	9.27×10^{-7}	0.508	0.827	ACC deaminase = -2.14×10^{-4} EC- 0.45pH+4.51	0.074	-0.095	0.271	0.657

EC: electrical conductivity; ACC: 1-aminocyclopropane-1-carboxylate; **Significant at 1% probability level.

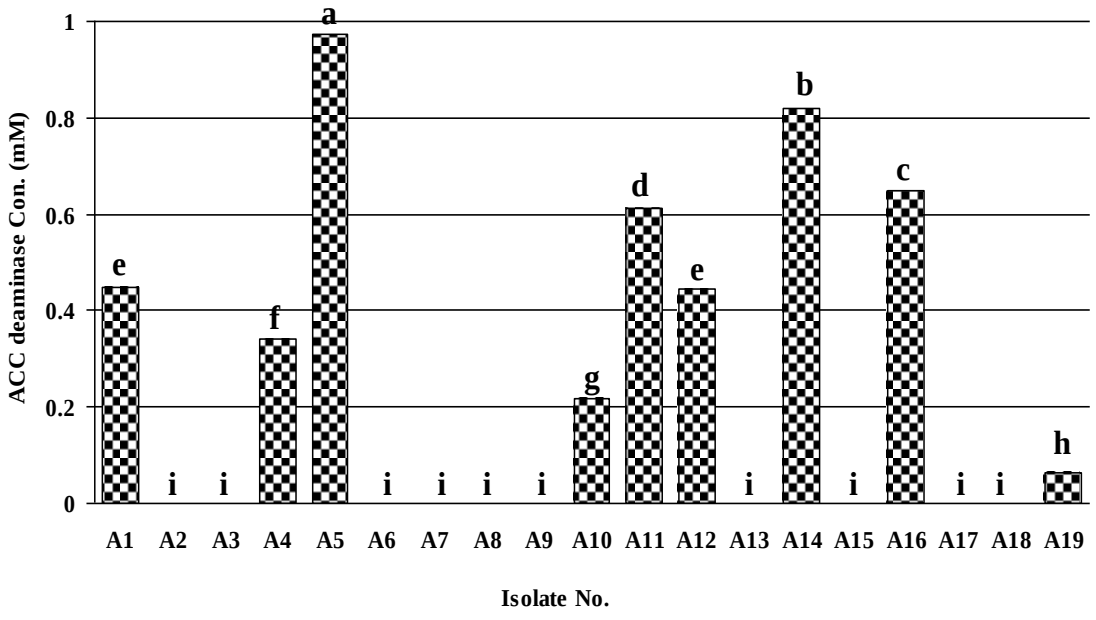

Figure 2. Mean ACC deaminase concentrations (mM) produced by alkalophilic isolates.

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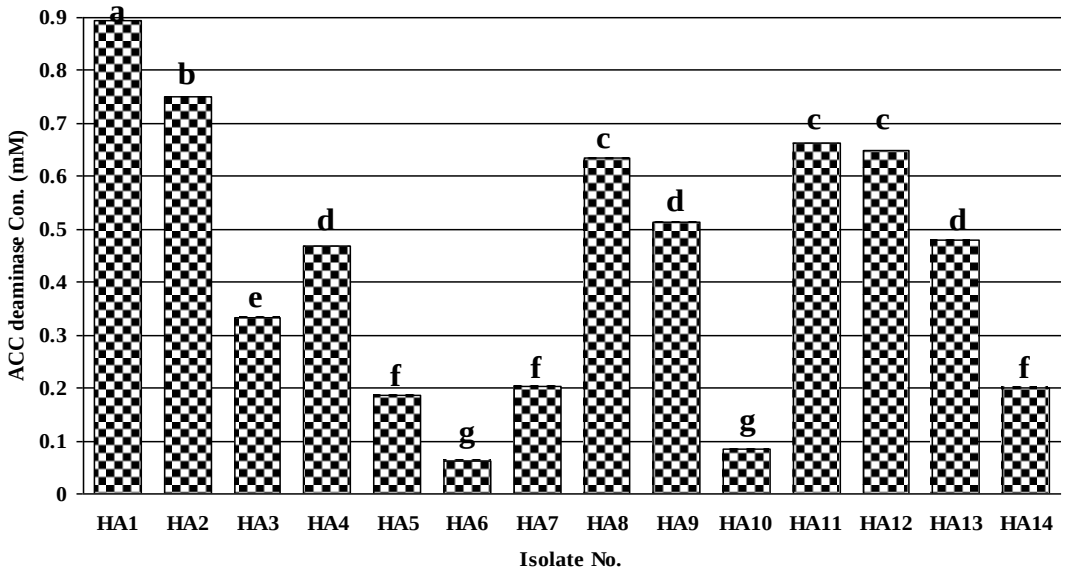

Figure 3. Mean ACC deaminase concentrations (mM) produced by haloalkalophilic isolates.

Discussion

Our results showed that all three groups of bacteria isolated from saline-alkaline soils, were ACC deaminase producers. According to Penrose and Glick (3) every bacterium with ACC deaminase activity producing more than 20 nmol α-ketobutyrate per mg per hour, is PGPR and it can increase plant growth indices. In fact, most of these isolates improved plant growth. To study the effect of inoculation of the 8 ACC deaminase producing isolates, rhizobacteria were placed on germinated canola seeds and increased trend of root lengthening was observed. Also shoot length has increased in most strains in comparison with control strains (17). Moreover, ACC deaminase production was investigated by Ramezaniyan Bajgiran et al. (18) in 100 indigenous soil rhizobia strains of different genus and species, on RMM culture in the presence of two different nitrogen sources (ACC and ammonium chloride), and results showed that 65 out of 100 strains had the ability to produce ACC deaminase either in strong, medium, or weak manner based on the diameter of colonies formed on the culture media. Based on this grouping all strong and most medium strains of bacteria belonged to *Sinorhizobium meliloti* genus. Other isolates were mostly in the poor group (18). In another study, 25 out of 60 isolates belonging to the species of *rhizobium leguminosarum biovar phaseoli*, *rhizobium leguminosarum biovar viciae*, and *Sinorhizobium meliloti* showed ACC deaminase producing ability (19).

Isolation of bacteria resistant to salinity and drought and study of compatibility of isolates to alkaline conditions showed high correlation between salinity and drought resistance of isolates to alkaline conditions (20). Alkalophilic halotolerant bacteria (*Planococcus rifietoensis*) was indole acetic acid (IAA) producer (264 µg/ml) and had mineral phosphate solubilization ability (16.7 µg/ml) as well as ACC deaminase activity (utilization of AAC as a source of nitrogen in the presence of 100 to 300 mM NaCl), and had the potential to improve the growth of wheat (21). Inoculation of bacterium to wheat increased by 37% the wheat growth under salt stress, 63 percent of the dissolution of calcium phosphate, and induced more than 60% ACC deaminase activity in the presence of ACC (21).

In the present study, the maximum ACC deaminase concentration was observed in haloalkalophilic isolates (0.4374 mM). This amount

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was about two times of its average in alkalophilic isolates (0.241 mM) and 5.5 times of that observed in halophilic isolates (0.0848 mM).

Studies have shown that the use of natural or recombinant bacteria producing the ACC deaminase lead to coping with stressed ethylene caused by flood conditions, drought conditions, soil salinity stress, increased resistance to pathogens and more tolerance to heavy metals (22-23). Nadim et al. investigated ACC deaminase activity of different bacterial strains under different salinities (4, 8 and 12 dS m) on yield and yield components of rape seed. They found that even in the salinity of 12 dS/m, growth and yield of oil seed rape increased, reducing ethylene levels in roots, increasing potassium to sodium ratio and consequently increased the amount of chlorophyll (24). Wagner et al. studied the effect of inoculating ACC deaminase containing bacteria on growth and yield of wheat, and found a significant grain yield, straw yield, root weight, root length increase. Also, tillers number, nitrogen, phosphorus and potassium uptake increased in grain and straw in comparison with control. All these effects were attributed to reduced ACC ethylene level in plants (25).

It was reported that bacterial rhizospheres containing ACC deaminase may stimulating plant growth. They can help plants by reducing the level of ethylene, and therefore producing longer roots and increased residue seedlings after planting, especially in the first few days, and under various stresses (26).

In conclusion, among the three groups of ACC deaminase producing isolates, haloalkalophilic isolates were superior both in terms of productivity power (all of them were producers) and the amount of production. This dual haloalkalophile characteristic makes them interesting for basic studies on different aspects of biotechnology, particularly in reducing salinity damage in agriculture. The produced ACC deaminase showed perfect correlation only in halophlic isolates with electrical conductivity and pH values.

Conflict of interest

The authors declared no conflict of interest.

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