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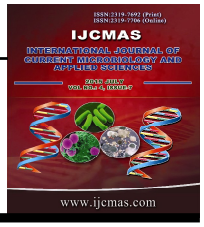

Original Research Article

Plant Growth Promoting Potential of ACC Deaminase Rhizospheric Bacteria Isolated from *Aerva javanica*: A Plant Adapted to Saline Environments

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ACC deaminase, IAA, HCN, PGP

A B S T R A C T

The present study demonstrates the plant growth promoting features of a bacterial isolate isolated from rhizospheric roots of *Aerva javanica*, commonly growing around the Sambhar salt lake Rajasthan. Isolate was able to produce the indole-3-acetic acid and solubilize the inorganic phosphate. Based on 16SrRNA gene sequencing bacterial isolate belongs to *Enterobacter cloaceae*. Among the other PGP traits isolates were found to positive for ACC deaminase activity, nitrogen fixation and ammonia production. The strain has shown the tolerance to NaCl concentrations up to 6% (w/v), growing ability up to pH 11 and temperature of 50 °C. Isolate was showed production of industrially important enzymes that make it suitable for various biotechnological and agricultural applications. Moreover strain AJS-15 showed antibacterial and antifungal activities against certain pathogenic microorganism, illustrating its biocontrol ability. Therefore use of bacterium with multifarious traits could be used as biofertilizers for ameliorating the salt stress for the plants.

Introduction

Salinity is a major deleterious abiotic stress factor severely affecting the crop productivity worldwide (Allakhverdiev *et al.,* 2000). According to FAO (2008) report more than 800 million hectares of land is affected by salinity across the globe. Several biochemical and physiological processes like protein synthesis, lipid metabolism, photosynthesis, ionic homeostasis and nitrogen fixation are severely affected by salinity (Parida and Das, 2005). Approaches are in progress to ameliorate the salinity stress by development of salt tolerant transgenic plants with especial attention to use of plant growth promoting rhizobacteria (Dimkpa *et al.,* 2009). Term PGPR was first introduced in 1978 by Kloepper and colleagues to a group of beneficial bacteria that promote the plant by enhancing the acquisition of nutrients, production of phytohormones, phosphate solubilisation, nitrogen fixation, production of ammonia, siderophore production, and indirectly by antibiotic production and through secretion of lytic enzymes. The organisms that are able to survive in hyper-saline and alkaline

condition are halophilic microorganisms and the natural habitat for these microorganisms are hyper-saline lake, soda lake, evaporation pond, saline soil etc. Thus, the utilization of haloalkalophilic bacterium for reducing the effect of salinity and alkalinity has drawn considerable attention to promoting the plant under various level of salinity stress. The salinity and alkalinity of the soil is increasing day by day because of the extensive use of chemical fertilizer, monoculture and irrigation with surface saline water. Hence, the halotolerant bacteria adapted to that environment and possessed the ability to produce plant growth hormones, phosphate solubilization, ACCD activity and other growth promoting activity could be an option to ameliorate salt stress.

Therefore use of these beneficial bacteria can significantly increase the plant growth and productivity and could be used as alternatives to chemical fertilizers and other agricultural supplements. In addition, production of ACC deaminase by PGPR has an additional benefit on plants by reducing the stress inducible ethylene level in host plants (Karthikeyan *et al.,* 2012). Therefore production of ACC deaminase hydrolytic enzyme could a useful tool to mitigate the plant stress caused by adverse environmental stresses including salinity. PGPR are known to employ one or more direct and indirect mechanisms of action to improve plant growth and productivity, although the major mode of action of many PGPRs is through increasing the availability of nutrients for the plant in the rhizosphere region (Glick, 1995). Several studies have reported the influence of PGPR is sometimes crop or niche specific, or their benefits are limited due to the climatic variability and inconsistency of soil (Khalid *et al.,* 2004; Ryu *et al.,* 2003).

The other potential biotechnological applications associated with halophilic microorganism like production of industrially useful enzymes drawn a considerable attention in past decade. Therefore, the present study was conducted to isolate and evaluate the potent halophilic microorganism in terms of certain plant growth promoting activity, production of industrially important enzymes and able to inhibit the phytopathogens.

Materials and Methods

Isolation and screening for ACC deaminase activity

For the isolation, 1 g rhizospheric soil of *Aerva javanica* was mixed with 50 ml PAF media (Composition: per litre, 10 g proteose peptone, 10 g casein hydrolysate, 1.5 g anhydrous $MgSO_4$,1.5 g k_2HPO_4, and 10 ml glycerol) and incubated in a shaker at 200 rpm at 30 °C for 48 h. The processed water sample was serially diluted and spread plating was done on DF-agar medium. Bacterial colony grown on above media was further streaked on DF-agar plate supplemented with 3mM ACC as unique nitrogen source. For confirmation of ACC utilizing as nitrogen source, bacterial colonies were subcultured many times on DF-ACC plate. Isolated bacterial strain was quantified for ACC deaminase activity and other PGP traits. Strain was maintained in 15% glycerol at -80 °C and was screened for their biochemical and other physiological characterization.

ACC deaminase assay

For ACC deaminase activity isolated strain was grown in tryptic soya broth up to late log phase at 30 °C in an orbital shaker at 150 rpm for 24h. After centrifugation cell pellet was washed with 0.1M Tris-HCl (pH

7.6) and finally suspended in DF minimal salt medium with 3 mM ACC as sole nitrogen source. The enzyme activity in cell free extract was determined by measuring the amount of α-ketobutyrate generated by enzymatic hydrolysis of ACC (Saleh and Glick, 2001). The protein concentration of suspension was determined by Lowry method (Lowry, 1951). The standard curve of α-ketobutyrate was determined at 540 nm to standard curve of α-ketobutyrate ranging between 0.1 to 1.0 µmol.

Genetic identification and phylogenetic analysis

Identification of isolated bacterial strain was done by 16S rRNA gene sequencing. Genomic DNA of bacterial isolate was amplified with universal primer 27F1 (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1494Rc (5′-TACGGCTACCTTGTTAC GAC-3′) in a 25 µl reaction mixture containing 10 X buffer (with 2.5mmol l^{-1} $MgCl_2$) 2.5 µl, 20 pmole forward and reverse primer each 2.0 µl, dNTP mixture (2.5mM) 3.0 µl, 0.5 µl of Taq DNA polymerase (2.5 U), nuclease free water and 50 ng of DNA template. DNA samples were amplified on DNA thermalcycler (T 100, BioRad, India).

The PCR condition were as follows: initial denaturation for 3 min at 94 °C, 30 cycles each consisting of denaturation for 1 min at 94 °C primer annealing for 1 min at 54 °C and extension at 72 °C for 5 min and a final elongation of 5 min at 72 °C. Amplified product was then purified using Qiaquick PCR purification kit (Qiagen, USA). The purified amplified PCR product of 1.5 kb was send to Xcelris genomics labs ltd (Xcelris Ahemdabad, India) for sequencing. Phylogenetic and molecular evolutionary analysis was done by using software MEGA 6.0 (Tamura, 2013).

Test for plant growth promoting traits

Phosphate solubilization assay

Screening for P- solubilization ability of isolate was done using NBRIP medium (Composition:g/l 10, glucose; 5, $Ca_3(PO_4)$; 1, $MgSO_4.7H_2O$; 0.2, KCl; 1, NaCl; 5, NH_4Cl; and 2% agar, pH 7.0) following the method of Mehta and Nautiyal (2001). Release of free phosphate was quantified according to the method of Marinetti (1962). Standard curve was prepared using various concentration of K_2HPO_4.

IAA production

The IAA production was estimated by growing the isolate in Nutrient broth (Himedia, India) containing 100µg/ml Tryptophan for 72 h at 30 °C and kept on shaking at 180 rev/min. Following incubation culture was centrifuged at 8,000 g and 1 ml of supernatant was mixed with 2 ml of Salkowsky's reagent and kept at room temperature for 20 min (Gordon and Weber, 1951).

Optical density was measured spectrophotometrically at 530 nm using a Jasco-630 UV-visible spectrophotometer (Jasco Corporation, Japan). The concentration of IAA in each sample was determined from the standard curve of IAA. Un-inoculated media was used as a control

Siderophore production

Assay for siderophore production of the isolate was carried out by spot inoculating test organism (1µl) on chrome azurole S agar plates and incubated at 30 °C for 4-5 days in dark. Appearance of orange halogen around the colony was considered as positive for siderophore production Schwyn

and Neiland (1987). Assay on solid media was performed in triplicate.

Ammonia test

For ammonia production strain was inoculated into 5 ml peptone medium and incubated for 48 h at 37 °C. After the bacterial growth, Nessler's reagent (0.5 ml) was added to tube in 2:1 ratio.

Development of brown to yellow color was observed as positive test for ammonia production. Uninoculated medium was used as reference.

Nitrogen fixation

Nitrogen fixation ability was evaluating by growing on N- free JNFb and LGI medium. Bacterial isolate was streaked on semi solid JNFb / LGI medium and incubated at 28 °C for 7 days. Observation of bacterial growth on plate was observed as qualitative evidence of atmospheric nitrogen fixation (Dobereiner *et al.*, 1995).

Production of HCN and chitinase

For qualitative estimation of HCN, isolate was streaked on nutrient agar plate supplemented with 4% glycine.

A whatman filter paper soaked in a solution of 2% Na_2CO_3 in 0.5% picric acid was placed between base and lid of petriplate and incubated at 28 ± 2 °C in inverted position for 96 h and observed for color change from yellow to orange brown.

For the chitinase test spot inoculation of isolated strain was made on chitin agar plate amended with 2% phenol red and incubated for 120 h at 28 ± 2 °C. Presence of clear zone around the streaked line indicates the chitinase activity.

Biochemical tests

Gram staining

For the gram staining, smear was prepared from 1-2 drops of culture on clean slide and heat fixed. 1-2 drops of crystal violet solution A was applied on the fixed smear for 1 min and then washed with sterile distilled water. Gram's iodine solution B was applied for 1 min and then washed with 95%alcohol. Finally stain the smear with counter stain safranin for 30 seconds again washed with sterile distilled water. The smear was air dried and examined under light microscope by using oil immersion. The Gram positive bacterial cells appeared violet while gram negative bacteria turned pink to red (Vincent, 1970).

IMVIC and citrate utilization test

Isolate was screened for indole, methyl red, Voges-Proskauer test as per standard protocols (Prescott and Harley, 2002). The microorganism having ability to utilize the citrate as a carbon and energy source for the growth and ammonium salts as the source of nitrogen. The Simmons citrate medium was prepared and the pH was set at 6.8. The plates were streaked with different bacterial cultures and incubated at 30±2°C for 48 hour and observed for color change.

Catalase test

Strain AJS-15 was freshly streaked on clean glass slide and mixed using a sterile tooth pick. Few drops of 3% H_2O_2 were added to the streaked culture. Appearance of bubbles confirmed the presence of catalase activity (Rorth and Jensen, 1967).

Protease production

Protease production of the strain was checked following the suitable protocol

(Abo-Aba *et al.,* 2006). Isolated microorganism was spot inoculated on skim milk agar plate and kept for incubation for 24 h. Appearance of halogen around the colony was considered as positive for protease production.

Amylase assay

The amylase assay of strain was evaluated by starch hydrolysis test (Budi *et al.,* 2000). An inoculum from a fresh culture was streaked on the starch agar plate and incubated at 37 °C for 24 h. The incubation plate was flooded with gram's iodine to produce a blue colored starch-iodine complex. Presence of clear halogen around the streaked colony was considered as positive for amylase production.

Lipase test

Lipolytic activity was carried out on trimethoprim plate and observation of halogen was considered as positive for lipase production.

Oxidase

Oxidase test of strain AJS-15 was done by following the protocol of (Kreig and Holf, 1984). Fresh culture of strain was grown in 4.5 mL of nutrient broth for overnight. After the overnight growth, 0.2 mL of 1% α-naphthol and 0.3mL of 1% *p*-aminodimethylaniline oxalate was added. Appearance of color change was observed after few mins.

Urease test

Bacteria growing naturally in an environment are exposed to the urine and with the help of enzyme Urease they decompose the urea. The plates were streaked with different bacterial cultures and incubated at 30±2°C 4 days. The purple-pink color in the test tubes indicated the positive result for the test.

Carbohydrate utilization test

Carbohydrate utilization efficacy of AJS-15 against various carobohydrates such as lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, L-arabinose, mannose, inulin, glycerol, salicin, sorbitol, mannitol, adonitol, arabitol, rhamnose, cellobiose, ONPG, esculin hydrolysis, D-arabinose etc was evaluated by carbohydrate utilization test kit (KB 009, Himedia).

Antibiotic sensitivity test

Isolate bacterial strain AJS-15 was tested for its resistance against standard antibiotics by Antibiotic sensitivity kit (HTM 002, Himedia) against the antibiotics namely gentamicin (30 µg), ampicillin (10 µg), erythomycin (10 µg), kanamycin (5 µg), tetracyclin (10 µg), vancomycin (25 µg), and chloramphenicol (10 µg) by the antibiotic sensitivity assay. Briefly, the bacterial culture was swabbed onto NA media plates. The standard antibiotic disc (6 mm) was placed over the media surface and the plates were incubated at 37 °C for 24 h. The experiment was done in triplicate. The results were interpreted on the basis of the diameter of inhibition zone using the zone size interpretative chart supplied by the manufacturer (Himedia, India).

Stress tolerance

Isolate AJS-15 was screened for their ability to tolerate the abiotic stress particularly salt and pH. A 20µl of overnight grown culture was inoculated into nutrient broth amended with 0.5% to 10% salt. After 24 h, absorbance of the culture was determined at

600nm. The ability of the isolate to sustain the pH was tested by growing in a varying degree of pH from pH 5.0 to pH 13.0 in the nutrient broth medium. Following the growth of the isolate for 24 h, the absorbance of the culture was taken at 600nm using uninoculated broth as a blank. Isolate was inoculated in triplicate sets.

Antagonistic test

Biocontrol ability of the isolate was evaluated by fungal mycelial inhibition by well diffusion method against the fungal pathogens *Aspergillus flavus*, *Fusarium oxysporum*, *Fusarium moniliforme*, *Fusarium graminearum* and *Penicillium citrium*. After the solidification of potato dextrose agar (PDA) 100µl of fungal spore suspension in 0.85% sterile saline was spread on the solidified plate. Well diameter of 6mm was made by metallic borer and filled with overnight grown culture of isolate (1×10^8cfu) and kept for incubation at 28 °C for seven days. Antagonistic activity against certain bacterial pathogens such as *Bacillus cereus*, *Erwinia carotovora*, *Escherichia coli*, and *Staphylococcus aureus* were also determined by incubating the plate at 37 °C for 24 h.

Preparation of bacterial inoculums and seed treatment

The halotolerant bacteria was selected for plant growth promoting activity on wheat plant (*Triticum aestivum* L.) in a controlled environment maintained in a plant growth chamber. Preparation of bacterial inoculum (OD 0.15) was performed according to Penrose and Glick (2003). Wheat (*Triticum aestivum* L.) seeds were surface sterilized by treating with 2.0 % sodium hypochlorite (NaOCl) solution for 3 min followed by three consecutive washing using sterile water to remove all trace of sodium hypochlorite. The treatment of seed was done as: Sterile 0.03 M $MgSO_4$ control and bacterial suspension in sterile 0.03 M $MgSO_4$ (OD of 0.15 at 600nm) (Positive control). Bacterized seeds were sown in plastic pots filled with soil in triplicates in a growth chamber with 16:8 photo-period up to 15 days after seed germination at 28 ± 2°C.

Sterilization of soil was done by autoclaving at 121°C for 1 h for three consecutive days to kill the entire microorganism and their spores. The experiment was conducted for 15 days after the seed germination. Root and shoot length, fresh and dry weight of five randomly selected seedlings from each replication were recorded.

Results and Discussion

Identification and Biochemical characterization

Based on 16S rRNA gene sequence analysis, the strain was identifies as *Enterobacter cloacae*. The 16S rRNA gene sequence of the strain was deposited in the Gene bank database under accession no KJ950716 Based on staining strain AJS-15 was found to be gram (-ve) negative. Strain was observed positive for catalse, Voges-Proskauer (VP), lipase, urease, whereas negative for indole, methyl red (MR), amylase, oxidase and protease activity.

Strain was able to grow up to 6 % (w/v) of NaCl concentration while the optimum growth was observed in media supplemented with 4% salt and and tolerate the alkalinity up to pH 11. Strain was found to be sensitive for amplicillin, chloramphenicol, tetracycline, gentamycin and resistant for kanamycin, erythromycin and vancomycin (Table 1).

PGP traits

The plant growth promoting trait of strain AJS-15 was evaluated in vitro based on the ACC deaminase activity, phosphate solubilization, ability to produce IAA, siderophore production and HCN production. The ACCD activity of strain was found to be 191.90 ± 16 (nmoles of α-KB/mg $pr \cdot h^{-1}$). Isolate was further tested for IAA production and based on quantification IAA production was found to be 0.531±0.050 µg ml^{-1}. Strain was able to solubilise inorganic phosphate 8.612±2.148 µg ml^{-1}. Strain was able to grow on nitrogen free JNFb- media, indicating nitrogen fixation ability and positive for ammonia production. Strain was found to be siderophore negative and positive for HCN production and chitinase activity (Table 2).

Antagonistic and carbohydrate utilization test

The bio-control ability of the isolate was evaluated based on the in vitro antibacterial and antifungal activity. Isolate was found to inhibit the growth of pathogenic gram negative bacterium *Escherichia coli and Erwinia carotovora*. Among the tested fungal strains, it was found to be inhibitory against *Fusarium graminearum* and *Fusarium oxysporum only* (Table 3).

Isoate was found to be positive for utilizing carbohydrates like xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, L-arabinose, mannose, glycerol, inositol, sorbitol, mannitol, rhamnose, cellobiose, ONPG, D-arabinose and negative for lactose, inulin, sodium gluconate, salicin, dulcitol, adonitol, arabitol, erythritol, α-methyl-D-glucoside, melezitose, α-methyl-D-mannoside, xylitol and esculin hydrolysis (Table 4).

Plant growth promotion test

Inoculation with the isolate increases the various growth parameters of wheat plant. Based on *in-vitro* plant growth promotion test, isolate AJS-15 increase the shoot length 11.78% as compared to control. Significantly increase (p= 0.05) in root length 21.51% and fresh weigh 24.22% was observed with respect to control. Increase in dry weight was 10.77% (Table 5).

The use of microorganism with beneficial traits to increase productivity of agricultural crops growing under stress conditions is becoming a strong biotechnological alternative to use of chemical fertilizers and pesticides that have a negative effect on environment (Saharan and Nehra, 2011). Besides of ACC deamianse activity, isolate was also able to produce IAA and solubilize inorganic phosphate that is important contributors to plant growth (Glick, 1995). Microbes having bio-control ability are able to generate induce systemic resistance (ISR) in several plant species thereby protecting plants against various diseases (Meziane *et al.,* 2005). PGPR able to produce IAA have a significant advantage to enhance root growth and development that enhance the nutrient uptake (Yang *et al.,* 2009). According to previous reports IAA and ACCD could work synergistically and promote plant growth, especially root elongation (Glick, 2014; Noreen *et al.,* 2012). Therefore results of present study illustrated that bacterium, isolated from extreme environment of salinity have unique property to grow in environment of high pH and high NaCl, showing the plant growth promoting traits. Bio-control ability towards pathogens has a promising application to be used as an alternative to chemicals, which can ultimately benefit the agricultural sector to a greater extent. In summary, the results illustrate that isolation of strain from saline

environment with multifarious beneficial traits could be used as low cost bio-based technology to combat saline stress for the plants growing around salt rich regions. Thus, with the isolation of strain from saline environment with multifarious beneficial traits could be used as low cost bio-based technology in agriculture sector.

Table.1 Biochemical and physiological characteristic feature

Characteristic (s)	Activity
Gram reaction	-
Catalase	+
Indole	-
MR	-
VP	+
Amylase	-
Oxidase	-
Lipase	+
Urease	+
Optimal growth temp. (°C)	37
Salt tolerance (%)	6%
pH tolerance	11
Antibiotic sensitivity test	
Amplicillin	+
Erythromycin	++
Chloramphenicol	+
Tetracyclin	+
Kanamycin	++
Gentamycin	+
Vancomycin	++

Table.2 Plant growth promoting traits of strain AJS-15

Plant growth promoting traits	Activity
ACCD activity (nmoles of α-KB/mg $pr \cdot h^{-1}$)	191.90 ± 16
IAA production (µg ml^{-1})	0.531±0.050
Phosphate solubilization (µg ml^{-1})	8.612±2.148
Growth on N-free medium	+
Siderophore index	-
Ammonia production	+

Table.3 Test of antagonistic activities of AJS-15 against bacterial and fungal pathogens

Bacteria	**Activity**
Escherichia coli	++
Staphylococcus aureus	--
Bacillus Cereus	--
Erwinia Carotovora	++
Fungal species	
Fusarium oxysporum	++
Fusarium moniliforme	--
Fusarium graminearum	++
Aspergillus flavus	--
Penicillium citrium	--

-- negative, + poor, ++ good; ±denote standard deviation; NA no activity

Table.4 Carbohydrate utilization test of strain AJS-15

Carbohydrates	**Test**
Lactose	-
Xylose	+
Maltose	+
Fructose	+
Dextrose	+
Galactose	+
Raffinose	+
Trehalose	+
Melibiose	+
Sucrose	+
L-Arabinose	+
Mannose	+
Inulin	-
Sodium gluconate	-
Glycerol	+
Salicin	-
Dulcitol	-
Inositol	+
Sorbitol	+
Mannitol	+
Adonitol	-
Arabitol	-
Erythritol	-
α-Methyl-D-glucoside	-
Rhamnose	+
Cellobiose	+
Melezitose	-
α-Methyl-D-mannoside	-
Xylitol	-
ONPG	+
Esculin hydrolysis	-
D-Arabinose	+

+ indicates a positive reaction, - indicates a positive reaction

Table.5 Effect of bacterial isolate AJS-15 on different growth parameters of wheat plant grown under salt stress condition

Treatmet	SL (cm)	RL (cm)	FW (g)	DW (g)
Control	26.57±1.59	20.08±2.11	2.56±1.07	0.297±0.028
AJS-15	29.70±1.26*	24.40±1.40*	3.18±0.60*	0.329±0.010

Growth was measured at 15 days after seed germination. SL shoot length, RL root length, FW fresh weight, DW dry weight. The values are mean±SD (n=15). * represent significant difference from respective control according to Duncan's multiple range test (p= 0.05)

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