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Isolation of Accdeaminase Producing Rice Rhizobacteria and Evaluation of Growth Promotingactivities

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Abstract

Twelve bacterial strains isolated from rice rhizosphere of different sites of Punjab, were screened for ACC deaminase production. These were further evaluated for plant growth promoting traits like phosphate solubilsation, ability to fix atmospheric nitrogen, IAA production and production of various hydrolytic enzymes. Biochemical characterization of these isolates were also performed. On the basis of mentioned PGPTs and ACC deaminase production, S5 strain was considered best asbiofertilizer. Present study concluded that S5 strain could be used as a biofertilizer in fields as it had considerable ACC deaminasepotential as well as growth promotion ability.

INTRODUCTION

Soil is an essential part of the ecosystem. Its maintenance is most important step towards many life processes. Soil microorganisms are vital to agro-ecosystem. Most soil contain an enormous diversity of microorganisms, less than 5% of total space is occupied by them and microorganisms are causing these 80-90 % of the processes in soil and these microorganisms play vigorous role in improving soil fertility and plant growth. In India, Punjab covers only 1.45% of the total area and most grown crop is rice. In rice root the presence of ACC deaminase was detected. ACC (1-aminicyclopropane -1 - carboxylic acid) is a precursor to plant ethylene level which ultimately exert beneficial effect on plant[1].

PGPR is a soil bacteria that are present in the rhizosphere of plant. Rhizosphere are rich in microbes and microbial activity and called as store house of microbes. Rhizosphereis area where the bacterial concentration is high than the rest of the soil. Interaction between plant and microorganisms play essential role in enhancing plant growth and productivity directly and indirectly. PGPR indirectly trigger plant growth by inhibiting harmful microorganisms[2] and

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directly PGPR produce a compound that is used by plant and synthesized by the bacteria of plant and help in plant growth development [3].

PGPR contain an essential enzyme ACC deaminase help metabolizing ACC into alpha - ketobutyrate and ammonia. ACC deaminase supports the plants for vigorous growth and development. PGPR involved in the synthesis of different hormones which further enhance plant growth there by contributing important role in growth promotion of crop plants [4]. Thus, present study is focused on isolation of ACC deaminase producing bacteria from Rice rhizosphere.

MATERIALS AND METHODS

Bacterial isolates

Bacterial cultures were isolated from different villages of four districts of Punjab and maintained at School of Bioengineering and Biosciences in Lovely Professional University, Punjab, India.

Isolation of rice rhizobacteria

Root soil samples were collected from four different location of Punjab, India i.e. Jalandhar, Ludhiana, Amritsar and Hoshiyarpur. The rice plant was carefully uprooted and stored at 4°C in polythene bags. The isolate of ACC deaminase rice rhizobacteria from rhizoshperesoil samples of rice (3cm to 4cm long primary root with secondary and tertiary roots along with the surrounding soil). Root soil samples were kept individually in air tight zip lock bag stored at 4°C. One gram of soil sample were separately enriched for ACC- utilizing bacteria by growing in nutrient broth and incubated on rotatory shaker for 24hours at 30°C, 200 rpm. Forth fold serial dilution of this culture were plated onto solid Dworkin and Foster (DF) minimal salt mediumcontaining ACC and incubated for 24 hours. Bacterial isolateswere selected, purified and kept at at 4°C till further use.

ACC deaminase assay [5]

1. Isolate were growing on TS (**Tryptic soy)** B medium at 28C and growth was ceased when stationary phase is achieved.

2. Collect the cell by centrifugation, perform washing with 0.1M Tris- HCl

3. Suspend the cell in 2ml of DF minimal medium and add 3mM ACC.

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4. Incubate for 36-72h at 28° C.

5. Collect bacterial cell by centrifugation at 3,000 rpm and wash cell by 0.1M Tris- HCl maintain pH-7.5 and suspend in 200ul of 0.1M tris- HCl (ph-8.5) and add 5%toulene followed by vortex for 30s.

6. 50 microliter of labelized cell was incubated in 5ul of 0.3M ACC at 28C for 30min and the negative control including 50ul of labelized cell without ACC.

7. In blank add 50ul of 0.1M Tris-HCl and also add 5ul of 0.3M ACC and mixed sample thoroughly by adding 500ul of 0.56N HCl by vortexing and remove cells by centrifugation at 12,000rpm for 5min.

8. A 500ul supernatant was added to a new eppendroff and mixed with 400ul and 150ul of DNF solution and the mixture was incubated at 28° C for 30min.

9. Before the absorbance at 540nm was measured add 2N NaOH.

Screening of isolates for other plant growth promoting activities [6]

Indole acetic acid Production

Minimal medium amended with 500 µg/ml L-tryptophan, 0.065% SDS and 1% glycerol was inoculated with selected isolates for quantitative determination of IAA production.

Nitrogen fixation

Rhizobacterial colonies were streaked on Jensen's medium and incubated at 37°C for 72h. Bacterial isolates showing growth on medium were considered as nitrogen fixer.

Ammonia production

For the production of ammonia, strains were incubated into 5ml peptone medium 48hours at 37° C. Add Nessler's reagent to the tubes drop by drop. Yellow color was considered as positive test for ammonia production.

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Phosphate Solubilization

Rhizobacterial isolates were spot inoculated on on the PVK agar plates containing known amount of tri-calcium phosphate ($Ca_3(PO_4)_2$). Plates were incubated at 37ºC for 48h. Bacterial isolates with yellow coloured zones were considered P- solubilizers.

Cellulase production

For cellulolytic and lipase activity, spot inoculation with 24h old bacterial culture was done on Czapek mineral salt agar medium for 48-72h at 37°C, respectively. Flood the plates containing bacterial culture with Congo red solution and wash with 1M NaCl. Clear zones formation around the growth considered cellulase producers.

Amylase production

Spot inoculation with 24h old bacterial culture was done on starch hydrolysis agar plate to screen amylolytic activity in antagonistic isolates, respectively. After incubation of 24-48h at 37ºC, clear zone produced around the spot after the addition of iodine were considered positive for the amylolytic activity.

Other biochemical tests suchas catalase, methyl red, H2S production, Gelatin, casein Hydrolysis andindole test were performed for functional characterization of rice rhizobacteria.

RESULTS

Isolation of ACC deaminase rice rhizobacteria

The rice rhizosphere soil samples are collected from different district of Punjab i.e. Jalandhar, Banga, Amritsar and Hoshiyarpur. Overall 12 isolates were isolated. Out of these, 3 isolates from Jalandhar, 2 from Banga, 5 from Amritsar and 2 from Hoshiyarpur.

Morphological evaluation of ACCdeaminaseproducingrhizobacteria of rice growing in different location of Punjab

Out of 12, three isolates were obtained from site Jalandhar. Out of these three, two were (S1, S2) were positive and one were (S3) negative for gram staining. However all three isolates were round shape (S1, S2, S3). On DF salt minimal media, elevated, circular, transparent and slimy colonies were observed for all isolates.

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Out of 12 isolate, two were isolated from site Banga. Out of these one were gram positive (S4) and other were (S5) gram negative. However, S4 is round in shape and other one (S5) is rod in shape. On DF salt minimal media, elevated, circular, transparent and slimy colonies were observed for all isolates.

Among 12 isolate, four were isolated from sites of Amritsar. Out of these five, two are gram positive (S7, S8) and three are gram negative (S6, S9, S10). Out of these four, all are round in shape. On DF salt minimal media, elevated, circular, transparent and slimy colonies were observed for all isolates.

Among 12 isolates, two were isolated from sites of Hoshiyarpur. Out of two, both are gram positive (S11, S12) and round in shape. On DF salt minimal media, elevated, circular, transparent and slimy colonies were observed for all isolates.

Table 1: MORPHOLOGICAL CHARACTERIZATION OF RICE RHIZOBACTERIAL ISOLATES OF PUNJAB

District	Isolate	Gram's reaction	Shape	Morphology
Jalandhar	S1	+	Round	Transparent , elevated, slimy
	S2	+	Round	Transparent , elevated, slimy
	S3	-	Round	Transparent , elevated, slimy
Banga	S4	+	Rod	Transparent , elevated, slimy
	S5	-	Round	Transparent , elevated, slimy
Amritsar	S6	-	Round	Transparent, elevated, slimy
	S7	+	Round	Transparent, elevated, slimy
	S8	+	Round	Transparent, elevated ,slimy
	S9	_	Round	Transparent, elevated, slimy
	S10	_	Round	Transparent, elevated, slimy
Hoshiyarpur	S11	+	Round	Transparent ,elevated, slimy

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	S12	+	Round	Transparent, elevated, slimy

ACC deaminase activity of rhizobacterial isolates of rice of Punjab

The ACC deaminase activity was assayed for all 12 isolates to quantify the amount of alpha-ketobutyrate.All the 12 isolates were screened for ACC deaminase activity out of 12 isolates, Isolate S5 showed greater amount of AAC deaminase activity i.e. 0.0088 mg M/ml/h of alpha ketobutyrate. While the other isolate such as S2 0.0005, S4 0.0019, S7 0.0017, S12 0.0078 mg M/ml/h and In S1, S3, S6, S8, S9, S10, S11 isolates no activities were detected.

Table 2: ACC DEAMINASE ACTIVITY OF RHIZOBACTERIAL ISOLATES OF RICE OF PUNJAB.

Isolates	ACC Activity (mg/ml/h)
S1	Not detected
S2	0.0005
S3	Not detected
S4	0.0019
S5	0.0088
S6	Not detected
S7	0.0017
S8	Not detected
S9	Not detected
S10	Not detected
S11	Not detected
S12	0.0078

Functional characterization of rhzobacterial isolates of Rice of Punjab

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Bacteria that produce gas bubble interacting with hydrogen peroxide are known as catalase positive bacteria. In present study 4 isolates were showing catalase positive and 8 were showing catalase negative.In indole test, Kovac's reagent is added to the bacterial culture (broth) for identification. Cherry red layer formation shows positive result. Out of 12, 8 isolates were showing positive result with the formation of cherry red colour on the top of the culture broth i.e. indole positive and 4 isolates were showing indole negative. In present study, 5 isolates were showing clear zones in contact with iodine solution and 7 isolates were showing starch negative result. Addition of Congo red solution on bacterial culture growing on CMC agar medium identifies bacteria that can hydrolyse cellulose and form clear zones. This clear zones show positive result. Out of total 12 isolates, 3 were cellulase producers and 9 were negative.Bacteria grow on casein medium show clear zones are hydrolysing casein i.e. casein positive. Out of 12 isolates, 8 were capable of hydrolysing casein and rest four were casein negative. Bacteria that are capable of hydrolysinggelatinemeans they can hydrolyse gelatin into amino acid. Out of 12 isolates, 6 isolates were capable of hydrolyse gelatin into amino acid and 6 were found negative. Hydrogen sulphide when produced react with the metal salt forming visible insoluble black ferrous sulphide precipitates were hydrogen sulphide positive. All isolates were found negative for hydrogen sulphide production. Bacteria possess the ability to utilise glucose and convert it to a lactic acid as the end result are methyl red positive test. Out of 12 isolates, all 12 isolates were capable of producing lactic acid as end result.

Table3: FUNCTIONAL CHARACTERIZATION OF RHZOBACTERIAL ISOLATES OF RICE OF PUNJAB.

Isolate Name	Catalase Test	Indole Production	Amylase test	Cellulase production	Casein hydrolysis	Gelatin hydrolysis	Hydrogen Sulphide Production	Methyl red test
S1	-	+	-	-	+	+	-	+
S2	+	+	-	-	+	-	-	+
S3	+	+	+	-	+	+	-	+
S4	-	+	+	-	+	-	-	+
S5	-	-	_	-	-	+	-	+
S6	+	+	+	-	-	+	-	+

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S7	-	+	+	+	+	_	-	+
S8	-	+	+	-	+	+	-	+
S9	+	+	-	+	+	_	-	+
S10	_	_	-	-	+	+	-	+
S11	_	_	-	-	-	-	-	+
S12	_	_	-	+	-	-	-	+

Plant growth promoting activities of rhizobacterial isolates of rice of Punjab

Phosphate solubilizing bacteria form insoluble compound and zone of clearance around the isolates. Out of 12 isolates, 11 isolates were capable of forming clear zones considered as positive for phosphate solubilisation. It means that they are solubilizing phosphorus and 1 isolates were negative for phosphate solubilisation.

In indole acetic acid out of 12 isolates, 4 isolates were showing positive result for indole acetic acid by forming reddish colour and 8 isolates were showing negative for indole acetic acid.

Nitrogen fixation bacteria are capable of transforming atmospheric nitrogen into fixed nitrogen and isolate streak on nutrient agar capable of showing growth are considered as positive for nitrogen fixation. Out of 12 isolates, 6 isolates were showing positive result for nitrogen fixation and 6 were showing negative result for nitrogen fixation.

Bacteria have the ability to utilize the ammonia and form yellow precipitates are positive for ammonia. Out of 12 isolates, all 12 isolates were showing positive result for ammonia.

Table4: PLANT GROWTH PROMOTING ACTIVITIES OF RHIZOBACTERIAL ISOLATES OF RICE OF PUNJAB

Isolates	Phosphate solubilisation	Indole acetic acid	Nitrogen Fixation	Ammonia test
S1	+	+	-	+
S2	+	-	-	+
S3	+	+	+	+
S4	+	-	+	+

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S5	-	-	-	+
S6	+	-	-	+
S7	+	-	-	+
S8	+	+	+	+
S9	+	+	+	+
S10	+	-	+	+
S11	+	-	-	+
S12	+	-	+	+

Discussion

In present study, the isolation of rhizobacteria was done for the root sample of rice roots from Punjab area. The rice roots is a surprisingly nutritious food. It contain high amount of vitamin B and C that helps body to from new cells. Regarding to some Public health centres rice having iron, magnesium. The samples were collected from the different villages of different districts of Punjab viz. Jalandhar, Amritsar, Banga, Hoshiyarpur [7].

There are different well known mechanisms performed by PGPR which can stimulate the growth of plants [8]. There are more than one mechanisms for plant growth promotion and is very complex phenomenon, exhibited by plant- associated bacteria [9]. The bacterial isolates inhabited a large number of PGP traits associated to the plant growth promotion: ability to grow on nitrogen free medium, Phosphoroussolubilisation, IAA production, and production of lytic enzymes which are according to the results of Almoneafyet al.[10].

Nitrogen fixation is a process that enhances the soil fertility and helps in the growth of plants. The atmospheric nitrogen which is the most abundant componentof the atmosphere cannot be used by plants for their survival. Mainly soil rhizosphere uses as a nitrogen fertilizer there than that roots and leaves of nitrogen fixing plants are also used as a nitrogen fertilizer. The nitrogen fixing bacteria, directly convert this nitrogen to ammonia. The bacteria that are found in roots and leaves for their nitrogen fixing[11].

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From the root samples of Rice, twelve isolates were grown in Dworkin and foster salt minimal medium (N- free medium) which lead to the formation of transparent, slimy, elevated growth after 4 days of incubation at 37C. The isolate had different biochemical activity. This difference show the genetic diversity of 12 isolates, in Gram stating test 7 isolates were Gram positive whereas rest 5 were Gram negative. This study shows that most of the isolates are gram positive. In case ofcatalase test four isolates (S2, S3,S6,S9) were showing positive results whereas rest eight isolates(S1,S4,S5,S7,S8,10,S11,S12) were showing negative result. In case of indole test eight isolates(S1,S2,S3,S4,S6,S7,S8,S9) were showing positive results whereas rest four isolates (S5,S10,S11,S12) were showing negative result. In case of amylase test five isolates(S3,S4,S6,S7,S8) were showing positive results whereas rest seven isolates(S1,S2,S5,S9,S10,S11,S12) were showing negative result. In case of cellulose test three isolates (S7,S9,S12)were showing positive results whereas rest nine isolates (S1,S2,S3,S4,S5,S6,S8) wereshowing negative result. In case of casein eight isolateswere showing positiveresults(S1,S2,S3,S4,S7,S8,S10,S11) whereas four eight isolates (S5,S6,S11,S12) were showing negative result. In case of gelatine six isolates (S1,S3,S5,S6,S8) were showing positive results whereas rest six isolates (S2,S4,S7,S9,S11,S12)were showing negative result. In case of hydrogen sulphite test all the isolates were showing positive results.

In plant growth promoting activates like phosphate solubilisation, indole test, nitrogen fixation test and ammonia test by accdeaminase rice rhizobacteria isolates were showing different diversity. In case of phosphorus solubilisation test (S1,S2,S3,S4,S6,S7,S8,S9,S10,S11,S12) isolates were showing positive results where as one (S5) isolates were showing negative result. In case of indole test (S1,S3,S8,S9) four isolates were showing positive results and rest eight isolates(S2,S4,S5,S6,S7,10,S11,S12) were showing negative result. In nitrogen fixation test five isolates (S3,S4,S8,S9,S10) were showing positive result and rest seven were showing negative result (S1,S2,S5,S6,S7,S12). In case of ammonia test all isolates were showing positive results.

It is already well known fact that plant roots promote the growth of selective group of rhizobacterial population. This may be the reason of isolation of mostly gram positive bacteria in a total bacterial population from different plant rhizospheres[12]. Different plant species showed the dominance of Gram positive bacteria in theirrhizosphere[13,14]. Among rhizobacterialpopulations, *Bacillus* is most potential spore forming genera. thereby increasing the adaptation of *Bacillus* strains to commercial formulation and field application [10].

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ACC deaminase producing rhizobacteria has been reported from different plant species and enhanced nodulation has also been reported from such plant hosts. [15].Rhizobacteria supported mechanisms result in production of soluble nutrients which were then by diffusion followed by absorption process improved plants growth[16].

Penrose and Glick [5] also reported that plant rhizosphere is rich in ACC utilizing bacteria. All such bacteria have ability to show other plant growth promoting activities. They also reported their ability to elongate roots of canola seedlings under gnotobiotic conditions. There was decrease in ethylene concentration because of treatment of plant seeds or roots with these bacteria. In present study, all the 12 isolates were screened for ACC deaminase activity .Isolate S5 showed greater amount of ACC activity (0.00884mgM/mg protein/h of alpha ketobutyrate).While the other isolate such as S2 0.00050, S4 0.00188, S7 0.00169 , S12 0.0078 mg M/ml/h and in S1,S3,S6,S8,S9,S10,S11 isolates no activities were detected. Thus, strain S5 can utilized for plant growth promotion in pot and field conditions.

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