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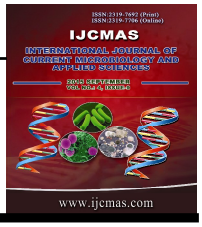

Original Research Article

Screening and Characterization of Diazotrophic Bacterial isolates for Plant Growth Promoting Properties

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A B S T R A C T

Total ten unknown bacterial isolates were collected from the different research stations of Andhra Pradesh. Further cultural and biochemical characterization of isolates were identified. Among ten isolates three were *Rhizobium* (PGP-1,2,3),three were *Azotobacter* (PGP-4,5,6) and four were *Azospirillum*(PGP - 7,8,9,10) are seen and pure cultures preserved for analysis. The rate of nitrogenase enzyme activity based on acetylene reduction assay showed variation in these isolates which ranged from 3.57 to 9.25 µmol C_2H_4 formed/mg protein/h. Their plant growth promoting characters were also analysed. It was observed that of them showed IAA production, ranging from 3.02 to 7.76(µg ml^{-1}), phosphate solubilization 50 %, siderophore production 60%.

Introduction

Plant growth-promoting rhizobacteria (PGPR) are root colonizing and nonpathogenic beneficial soil rhizobacteria which play a key role in plant growth , development and nutrition by a number of mechanisms. These include direct biological nitrogen fixation by freely or symbiotically, solubilization of rock phosphates, the production of phytohormones, especially indole-3-acetic acid (IAA), and the presence of the enzyme 1-aminocyclopropane-l-carboxylic acid (ACC) deaminase that hydrolyzes ACC, the immediate precursor of the phytohormone ethylene (Glick et al., 1997) and indirect mechanism of plant growth enhancing by the synthesis of siderophores that can solubilize iron in the soil and make it available to the plant, leads to plant pathogenic fungal suppression. PGPR are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots. In last few decades, a large array of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligens*, *Arthobacter*, *Burkholderia*, *Bacillus* and *Serratia* have been isolated and reported to enhance plant growth. Present investigation was screening of selected rhizobacteria for the search of

efficient diaztotrophic PGPR have been done. Keeping this objective in mind the present investigation was performed and their plant growth promoting abilities were evaluated after screening of diazotrophic activity by acetylene reduction assay method.

Materials and Methods

Collection of bacterial isolates

Bacterial isolates are collected from different resource laboratories of Andhra Pradesh and these isolates were tested for their purity and preservation in Dept.of Agricultural Microbiology & Bioenergy, College of Agriculture, Rajendranagar, Hyderabad (Table.3.1)

Morphological characterization

All the ten isolates were checked for their purity and then studied for the colony morphology and pigmentation. The cell shape and gram reaction were also recorded as per the standard procedures given by Barthalomew and Mittewar (1950) and Anonymous (1957). Cultural characterization of isolates observed by different characteristics of colonies such as shape, size, elevation, surface, margin, color, odor, pigmentation, etc were recorded.

Motility test

This test was done using the hanging drop method. A drop of the test organisms in asaline suspension was placed on a cover slip. The cover slip was inverted and placed on cavity slide, this was viewed under the microscope.

Pellicle test

The active *Azospirillum* isolates were inoculated at subsurface level in screw cap tubes containing sterilized semisolid N- free malate medium(Okon *et al*.,1977) under aseptic conditions.The tubes were incubated at 30^0C for a period of one week and observed for growth of *Azospirillum* as subsurface pellicle.

Cyst formation

Azotobacter sp have ability to form cysts under adverse conditions.Presence of cyst is as one of the criterion for identification of these isolates. The *Azotobacter* isolates were grown om Waksman No.77 N free agar medium for 7 days. These isolates were stained with a mixture of neutral red and light green SF yellowish , observed under oil immersion.

Biotin requirement:

Two sets of test tubes containing 10 ml of sterile nitrogen free semisolid medium were prepared One with biotin(100mg l^{-1}) other set without biotin were inoculated with 0.1ml of the standard inoculms of *Azospirillum* isolates, incubated at 37^0C for three days and observed the tubes for growth of *Azospirillum* isolates. Incase were growth occurred in the medium without biotin, a second transfer was made to fresh medium without biotin and biotin requirement was confirmed.

Biochemical and physiological characterization

Different biochemical tests performed and the protocols followed are briefly outlined below.

Physiological and biochemical characterizations of the bacterial isolates such as catalase, oxidase, gelatin

liquefaction, H_2S test, Starch hydrolysis nitrate reduction, IMViC and carbon source utilization were examined according to the standard methods. The isolates were identified accordance with the Bergey's manual of determinative bacteriology.

Screening of isolates for plant growth promoting properties

Pure isolates were isolated by streaking isolates on respective media plates and screened for following Plant growth promoting properties.

Phosphate solubilization

For this test sterilized Pikovskaya's agar was poured as a thin layer on to the sterilized petri plates and incubated for 24h . After incubation the Pikovskaya's plates were spot inoculated with sixteen isolates and incubated at 28±1^{0}C for 4-5 days. Formation of a clear zones around the colonies were considered as positive result for phosphate solubilization.

PSE (Phosphate Solubilization Efficiency) = Z / C x 100

Z- Clearance zone including bacterial growth
C- Colony diameter

Nitrogen fixation efficiency by Acetylene reduction assay (ARA)

The nitrogen fixing capacity of the test organisms were evaluated by using acetylene reduction assay (ARA) following the standard procedure (Bergersen,1980).Twent five ml of semisolid N-free sodium malate medium (*Azospirillum*), JNFb- medium (*Azotobacter*) were prepared in 100 ml vials. The vials were inoculated with 25µl of respected PGP isolates (PGP-1 to PGP-10) and incubated in an incubator at 28±1^{o}C . After 5 days of growth, cotton plugs were replaced by Suba-seal septa and tightened with aluminium cap. The air in the vial was replaced with nitrogen gas. Ten percent (v/v) of the inert gas was removed and ten percent pure acetylene gas was injected. The vials were incubated for 24h at room temperature . After incubation,1 ml of gas sample was withdrawn and injected into the gas chromatograph(Agilent 7820 A ,India) fitted with Porapak R column and Flame ionization detector(FID). The column temperature was maintained at 80^{o}C . Nitrogen gas was used as carrier gas at the flow rate of 20ml.min^{-1})

The acetylene reduction activity of the strains was calculated using the formula:

$$\frac{\text{Sample peak length of ethylene (mm)} \times \text{Attenuation} \times \text{volume of gas phase of flask} \times 0.0006}{\text{Incubation time (h)} \times \text{volume of gas sample injected into gas chromatograph (ml)}}$$

The acetylene reduction activity of the sample was expressed as nmoles of ethylene formed mg of protein h^{-1}. At the end of experimental period the cell protein content of the cultures were determined following the method described by Lowery *et al*.(1951)

Indole acetic acid production

Indole acetic acid production was tested according to Gorden and Weber (1951). The active culture of each test isolate was raised in 5ml respective broth tubes and incubated at determined temperature and time. After incubation these cultures were centrifuged at recommended rpm and time. Two drops of O- phosphoric acid was added to 2ml of supernatant and incubated for 30 min to

develop the colour. Development of pink colour considered as positive for IAA production.

Siderophore production

Siderophore production was estimated qualitatively. 0.5% of cell free culture supernatant was added to 0.5ml of 0.2% aqueous Ferric chloride solution. Appearance of orange or reddish brown colour indicated the presence of siderophore (Yeole and Dube 2000)

Results and Discussion

Plant root colonizing bacteria can function as harmful, deleterious rhizobacteria (DRB) or beneficial, plant growth promoting rhizobacteria (PGPR). PGPR colonize roots of monocots and dicots, and enhance plant growth by direct and indirect mechanisms. Modification of root system architecture by PGPR implicates the production of phytohormones and other signals that lead, mostly to enhanced lateral root branching and development of root hairs. PGPR also modify root functioning, improve plant nutrition and influence the physiology of the whole plant.

The cell morphology, colony morphology, Gram reaction was studied for ten plant growth promoting isolates. All PGP isolates were gram negative reaction. All isolates colony morphology and cultural characters were studied on basic nutrient agar medium. Similarly all isolates colony morphology was studied on king's B agar medium, *Azotobacter* agar (Jensen's agar), *Azospirillum* agar (N- free sodium malate medium) and yeast extract mannitol agar (YEMA) medium. All isolates were shown different colony morphology on different specific media.

The bacterial isolates PGP-1, PGP-2, PGP-3 were the isolates were formed whitish mucilaginous translucent colonies on yeast extract mannitol agar medium (Vincent, 1970). PGP-4, PGP-5, PGP-6, PGP-7 were growth recorded on Jensen's agar medium (Jenson, 1954, Norris and Chapman, 1968).The isolate PGP-8, PGP-9, PGP-10 growth was fully recorded in N-free sodium malate medium (Okon *et al*.1977) with whitish growth and light pellicle type colonies observed. The isolates were examined for biochemical characterization. All the PGP isolates tested for different biochemical tests like IMVIC tests, catalase test, oxidase test Starch hydrolysis test, Gelatin liquefaction test, H_2S production test, Carbohydrate utilization test and denitrification test.

Acetylene reduction assay

Results of ARA method for nitrogenase enzyme activity aim for better or efficient nitrogen fixing activity were shown values ranging from 5.58µmol C2H4mgprotien/h to 8.55µ mol C2H4mgprotien/h. Maximum nitrogensae enzyme activity shown by isolate PGP-10*(Azosprillum* spp) followed by PGP-7(*Azospirillum)* and *Rhizobium* (PGP-1) (Table 1).

Similar kinds of results reported by Shrivastava (2013) isolated diazotrophic bacteria were isolated from the rhizosphere of rice plants. The rate of nitrogenase enzyme activity based on acetylene reduction assay showed remarkable variation in these isolates which ranged from 0.69 to 1.63 nmol. C_2H_4 mg protein^{-1}h^{-1}.

Similar results obtained by Murumkar *et al*. (2013) isolated 93 *Azospirillum* isolates and reported the *A. lipoferum* isolates exhibited a higher average nitrogenase activity compared to *A. brasilense* isolates (105.9 vs. 20.8 nmol C_2H_4 mg protein^{-1}h^{-1}, respectively), similar results recorded by Gothwal *et .al.*(2007) and Azin *et al.* (2005).

Table.1 Screening for Nitrogenase activity (Acetylene Reduction Assay) of bacterial isolates

S.No	Isolates	Nitrogenase activity (nmol C_2H_4/mg protein/h)
1.	*Rhizobium* (PGP-1)	8.26
2	*Rhizobium* (PGP-2)	7.64
3	*Rhizobium* (PGP-3)	7.84
4	*Azotobacter* (PGP-4)	8.61
5	*Azotobacter* (PGP-5)	5.58
6	*Azotobacter* (PGP-6)	6.58
7	*Azospirillum* (PGP-7)	8.67
8	*Azospirillum* (PGP-8)	6.51
9	*Azospirillum* (PGP-9)	4.41
10	*Azospirillum* (PGP-10)	9.31
	CD 5%	**0.270**
	S(Ed)	**0.128**
	CV	**2.140**

Plate.1 Plant growth promoting characteristics of PGP-10(*Azospirillum*) isolate

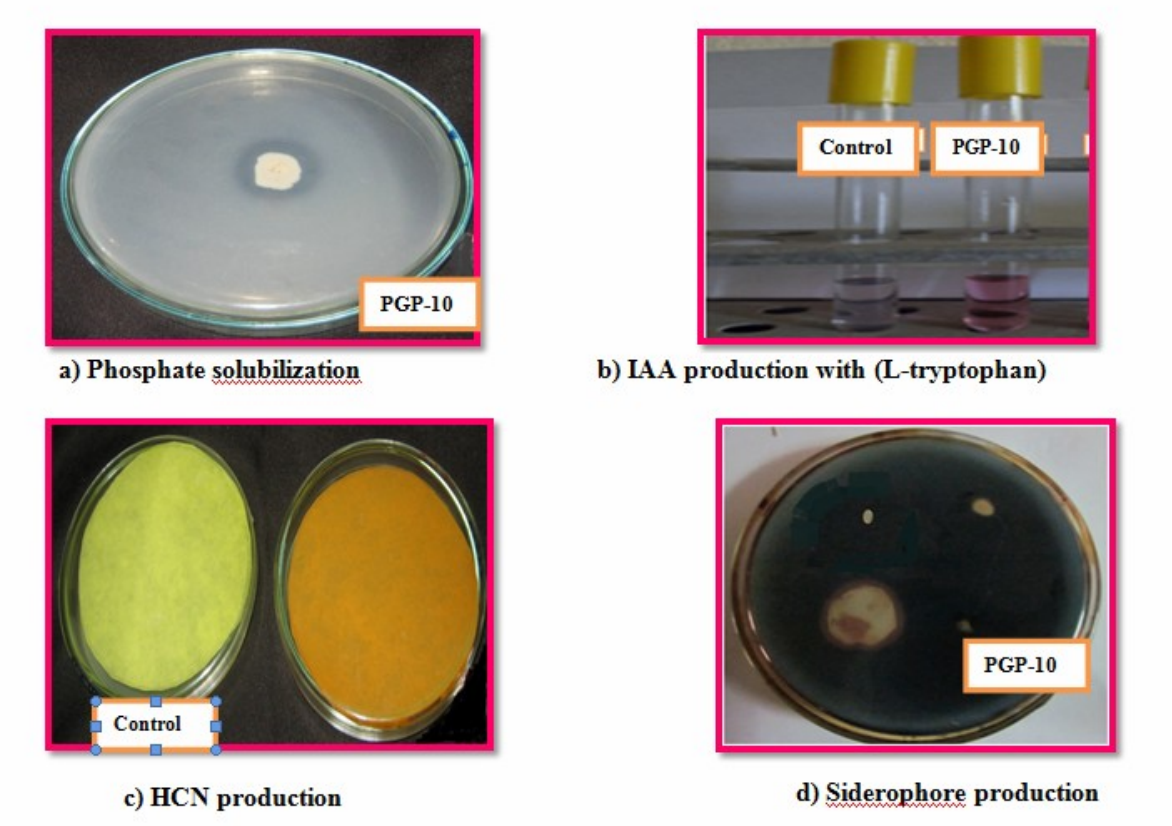

Table.2 Screening for plant growth promoting properties

S.No	Bacterial isolates	Phosphate solubilization			IAA production(µg/ml)	Siderophore production
		Zone diameter(mm)	Culture diameter(mm)	Solubilization efficiency(%)		
1.	*Rhizobium* (PGP-1)	0.00	0.00	0	0	+
2.	*Rhizobium* (PGP-2)	0.00	0.00	0	0	-
3.	*Rhizobium* (PGP-3)	11.66	7.00	164.18	3.15	+
4.	*Azotobacter* (PGP-4)	10.00	6.00	168.22	4.9	++
5.	*Azotobacter* (PGP-5)	8.00	6.00	136.11	7.73	++
6.	*Azotobacter* (PGP-6)	9.00	6.00	152.33	5.76	+
7.	*Azospirillum* (PGP-7)	20.66	11.00	186	6.03	+
8.	*Azospirillum* (PGP-8)	10.00	6.00	168.55	3.65	+++
9.	*Azospirillum* (PGP-9)	13.05	9.00	148.83	3.20	+
10.	*Azospirillum* (PGP-10)	20.30	5.00	200.33	7.75	+++
	CD 5%			**0.210**	**0.391**	
	SE(d)			**0.100**	**0.186**	
	CV			**3.183**	**5.400**	

Plant growth promoting activities

Plant growth promoting properties like indole acetic acid, phosphate solubilization and siderophore production results were shown as considerable positive results. The isolate pgp-10(*Azosprillum*) shown maximum IAA production i.e 7.76µg/ml followed by pgp-5(azotobacter) i.e 7.51,other isolates were shown considerable range of without supplementation of tryptophan precursor(Table.2).

The ability of phosphate solubilization was measured by seeing phosphate solubilization efficiency on pikovskaya's agar media. Results were observed,the isolate pgp-7(*Azospirillum*) shown maximum p-solubiization efficiency i.e 190.9%,followed by PGP-15(*Azospirillum*) i.e 167%.

IAA. High Gibberillic acid production was also detected in *Azotobacter* (71.42%) isolates and Sudha *et* In the present study IAA production and phosphate solubilization by PGPR isolates were in agreement with the earlier reports are available on PGPR strains which were isolated from wheat showed IAA production ranging from 5.5-31.0 µg ml^{-1} and (Abbasi *et al*. 2011). Hussain and Srinivas (2013) isolated 35 isolates *Pseudomonas* and *Azotobacter* each from rhizosphere of *Acacia nilotica* and *Albizia lebbeck* and reported 70% of the isolates of *Azotobacter* and *Pseudomonas* produced *al.,* (2012) (Table.2).

In conclusion, Diazotrophic bacterial isolates having efficient nitogenase enzyme activities and having plant growth promoting properties. This is advantage for single bacterium consists of multiple beneficial activities for bioinoculants preparation.

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