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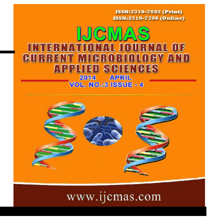

Original Research Article

Isolation and characterization of potential plant growth promoting rhizobacteria from non-rhizospheric soil

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A B S T R A C T

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Potential bacterial strains with multiple plant growth promoting attributes were isolated and characterized. Plant growth promoting traits were evaluated by determining the P-solubilisation efficiency, Indole acetic acid production, HCN and Siderophore production. All the four isolates were gram positive, rod shaped showed growth from 5-40°C (optimum 28°±2°C) with a pH range of 6-12, and tolerate upto 10% (w/v) salt concentration. 16S rRNA gene sequencing provided confirmation of isolates to Arthrobacter sp. with which they shared >98% sequence similarity. Under in-vitro conditions all four isolates were found to produce indole acetic acid, P-solubilization and hydrogen cyanide. Phosphate solubilization was accompanied by a decrease in pH from 7.0<3.0. Hence the four Arthrobacterial strains are promising plant growth promoting isolates showing multiple PGPR properties. Studies on these isolates provide further basis for further formulation and can be used for field applications.

Introduction

Large quantities of fertilizers are applied annually to augment the availability of phosphorous in the soil. Chemical fertilizers impart potential negative effects on the environment, leading to research for supplementation with microbial inoculants benefitting plant growth by improving the nutrient status of soil (P. Rahi *et al.*, 2009). The microorganisms with multiple plant growth promoting activities could be highly effective as microbial inoculants in agriculture. Beneficial effects of PGPR were demonstrated for many crops, though inconsistency in their field performance attributed mainly to poor rhizosphere competence and lacked multiple PGPR activities is the major limiting factor in releasing the potential of the microorganisms (Arvind Gulati *et al.*, 2009). Many phosphate solubilising bacteria (PSB) belongs to *Pseudomonas, Bacillus, Enterobacter, Serretia, Pantoea, Azospirullum, Azotobacter, Rhizobium, Burkholderia, Flavobacterium* and to the fungal genera *Aspergillus* and *Penicillium* (Deepa *et al.,* 2010).

The immense potential of microorganisms in these areas remains unexplored and untapped. Screening of PGPR appears to be good strategy for the selection of potent strains to apply in certain condition environments for sustainable agriculture (Arvind Gulati *et al.,* 2009). In this report isolation of plant growth promoting bacteria from the North Eastern non-rhizospheric soil and their characterization on the morphological and physiological studies and also by 16s rRNA sequence analysis is presented.

Materials and Methods

Soil samples for the bacterial isolation were collected from non-rhizosphere soil from North East India at an altitude of 1965 meters above the sea level. The soil samples were processed and diluted serially, and were subjected to spread plate technique on ISP4 medium and incubated at 30°C for 48 h. A total of 97 strains were isolated and these purified cultures were stored on ISP-4 medium at 4°C. These isolates were screened and selected on the basis of clear zones around the colonies on Pikovaskaya's medium. The isolates were assessed for morphology, physiology and Gram's reaction and other characterization.

Characterization, identification and phylogenetic analysis

The isolates were differentiated by Gram reaction, salt tolerance, biochemical characterization and microscopic observation. The ISP medium 4 (Inorganic salt starch agar) containing (gL^{-1}) Starch 10, $CaCO_3$ 2, K_2HPO_4 1, $(NH_4)_2$ SO_4 2, $MgSO_4.7H_2O$ 1, NaCl 1, $ZnSO_4.7H_2O$ trace, $FeSO_4.7H_2O$ trace, $MnCl_2$ trace and Agar 20 was used in the screening process. The ability of the isolates to grow at different salt concentrations were carried by inoculating the cultures on ISP-4 plates supplemented with 7-12% (w/v) NaCl and incubated 30°C for 72 h. The ability of the isolates to grow in alkaline or acidic medium was tested on ISP-4 medium in which the pH was adjusted from 4.0 to 12.0 (increments of 1.0 unit) using different buffers. To check the ability of the isolates to grow at different temperature ranges, the isolates were inoculated onto ISP-4 medium and incubated at 10°C - 45°C (increments of 5°C).

Phenotypic characterization of isolates was carried out based on their colony morphology, microscopic observation and biochemical tests.

Phylogenetic analysis

The universal primer 27F and 1492R was used for the partial sequencing of the 16s rRNA gene (1091, 1096, 1126, 1104) sequence analysis was done at the RDP-11 (Ribosomal Database Project, Michigan State university MI, USA) using Seqmatch version 3 (Cole *et al.,* 2005). Similarity scores were obtained by the similarity rank analysis function at RDP data version 9.50. Nucleotide sequences were aligned using Clustal X 1.81 algorithm (Thompson *et al.,* 1997). Phylogenetic and molecular evolutionary analysis was conducted using mega version 4.0 (Kumar *et al.,* 2004). The phylogentic tree was constructed by the neighbour joining method (Saitou and

Nei 1987) using the distance matrix from the alignment. Distances were calculated using (Kimura, 1980).

Quantitative estimation of phosphate solubilisation

Initially the qualitative estimation of P-solubilizing activity of the isolates were carried out on Pikovaskaya agar (1948) followed by qualitative estimation of P-solubilization as per standard methodology (Mehta and Nautiyal 2001) by inoculating 1ml of bacterial suspension (1×10^9 cells per ml) in 50ml of NBRIP Broth in 150ml Erlenmeyer flask incubating it for 15 days. Every 3 days the cell suspension was centrifuged at 10,000 rpm for 10 minutes and Phosphate content in the supernatant was spectrophotometrically estimated by the method of Murphy and Riley, 1962**.**

Quantification of Indole acetic acid

The isolates were grown in LB Broth supplemented with a filter sterilized solution of 1gm of L-Tryptophan. The liquid medium was inoculated with the bacterial culture adjusted to optical density 0.5 measured at 600nm in a spectrophotometer. The inoculated tubes were incubated at 30°C for 24 to 48 h. Then the bacterial broth was centrifuged at 5000 rpm for 15 minutes to obtain cell free extract. Auxin was detected in 1ml of supernatant with Salkowski reagent. A standard curve was drawn for comparison for the determination of the auxin production.

Quantitative estimation for Siderophores and HCN production

Siderophore productions by isolates were detected by CAS assay. The siderophore production was tested on the petri dishes containing CAS agar. The CAS blue solution for this assay was done according to Schwyn and Neilands (1987). The isolates were stab inoculated with tooth picks and incubated at 28°C ±2 for 2 weeks in the dark. The colony showing orange zones were considered as siderophore producing strains. The uninoculated control plate was incubated under the same conditions, and no change in colour was observed.

The isolates were plated on ISP-4 medium and incubated for 48 hours and were screened quantitatively for the production of cyanide by using picrate or sodium carbonate saturated vapour fixed to the underside of the petri dish lids (Bakker and Schipper 1987) which were sealed with parafilm before incubation at 28°C. Colour change of the filter paper from yellow to light brown, or reddish brown was noted at 4, 24 and 48 hours as indication of weak, moderate or strong cyanogenic potential respectively. Incubated reaction from the inoculated plate was compared with the uninoculated plates.

Results and Discussion

Isolation and characterization of the bacterial isolates

Four potent strains producing about 15–20 mm zone of phosphate solubilisation were seen after incubation on modified pikovaskaya agar for 5 days. The bacterial colonies were circular, smooth, convex and entire.

The strains were Gram positive, motile and was able to grow over a wide range of pH 6-12 with a pH optimum 7.0 ±0.5. The isolates showed good growth at temperature ranging from 5- 45°C and showed tolerance to Nacl at a concentration of 7% (w/v). All the 4 isolates were catalase positive, reduced nitrates, ONPG negative except for one isolate. All four isolates hydrolyzed starch and casein except for one strain. All the properties are tabulated in Table-1. Molecular analysis based on 16s rRNA gene sequencing revealed that the isolates showed maximum similarity with the genus *Arthrobacter* available in the public domain. The phylogenetic tree constructed with 16S rRNA gene sequences show that JUA5 and JUM65 form a separate clade with *Arthrobacter nicotianae* DSM 20123 and *Arthrobacter arilaitensis* CIP 108037T whereas JUA29 and JUA14 separate with *A.mysoreans* LMG16219T and *A.rhombi* F983HR69T.

IAA biosynthesis is not limited to higher plants; microorganisms are also capable of producing physiologically active IAA that may have great effect on plant growth development. 80% of the microorganisms isolated from rhizosphere soil are capable of IAA production (Costacurta and Varduley 1995). L-Tryptophan was also considered as IAA precursor in bacteria, as in plates. Its addition to the bacterial broth increases IAA production greatly (Park *et al.*, 2005, Tsavkelova *et al.*, 2007). Root exudates are natural sources of L-Tryptophan for rhizosphere micro flora which may enhance auxin production (Kravchenko *et al.*, 1991, Martens and Frankenberger 1994).

The present work aims to highlight the plant growth promoting potential of strains JUA5, JUA14, JUA29 and JUA65 which belong to the genus *Arthobacter*. The strains isolated from the North East India possessed multiple plant growth traits like phosphate solubilisation, 200-1200 µg of tri calcium phosphate/ml per day. It is well known that improved phosphorus nutrition influences plant growth and root development overall (Jones and Darrae 1994). Siderophore production by the isolates was significant for iron nutrition of plants grown under iron deficient conditions (Picterse *et al.,* 2001). HCN production by non-rhizospheric bacteria is viewed variably while it is considered effective from bio-control point of view.

Studies have been taken up to understand the nature and properties of microbes which possess potential plant growth promoting traits. With increasing awareness about chemical fertilizers based agricultural practices it is important to search for region-specific microbial strains which can be used as potential plant growth promoters to achieve desire products (Ahmed 1995). In present work, the differences in the plant growth promoting attributes of the isolates can be due to their rhizospheric competence. Further work is required to prove the PGPR activity with pot experiments for further utilization of these isolates for field applications.

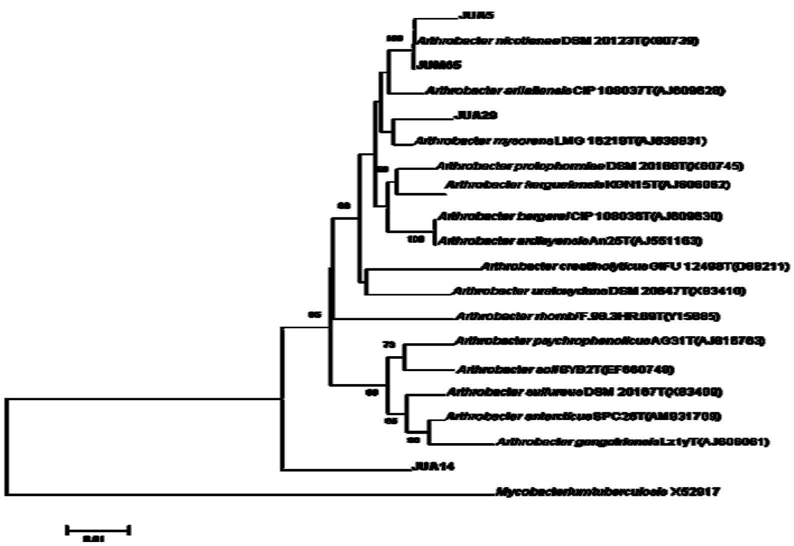
P-solubilization activity and IAA production

The isolates were seen to solubilise phosphate significantly at 30°C, though the zone of solubilization around the bacterial colony on Pikovaskaya agar after 72 h of incubation. Quantitative estimation of phosphate solubilization after incubation for 15 days at 3, 5, 7, 10, day intervals. Maximum solubilisation was observed at 30°C. At this temperature maximum solubilisation 200-1200 µg/ml was seen after 10^{th} day of incubation. The pH of the broth also decreased in each case. A decrease in pH showed efficient phosphate solubilisation(pH 7.0 to 4.0-3.0)

Table.1 Various attribute of the *Arthrobacter* strains

Character	**Isolates**			
	JUA5	**JUA14**	**JUA29**	**JUM65**
Morphological				
Gram stain	+	+	+	+
Cell shape	Rods	Rods	Rods	Rods
Colony colour	Pale pink	Pale pink	Pale yellow	Pale yellow
motility	Motile	Motile	Motile	Motile
ONPG	-	-	-	+
H□S production	-	-	-	+
Nitrate production	+	+	+	+
Urease	+	-	+	+
Voges Proskauer's	-	-	-	-
Indole	-	+	-	-
oxidase	+	+	-	+
Methyl red	+	+	+	-
Casein	-	-	-	+
Starch	+	+	+	+
Plant growth promoting traits				
P-solubilisation ($\mu g^{-1} ml^{-1} day^{-1}$)	200	1000	900	1200
IAA Production (µg-1ml-1day-1)	110	50	60	100
HCN production	-	+	-	-
Siderophore production	-	+	-	+
pH decline	7.0-4.0	7.0-4.0	7.0-4.0	7.0-4.0

Fig-4 Phylogenetic tree of the isolates



in all isolates tested (Table-1). Maximum IAA production from 50-110 µg/ml/day in tryptophan amended media with different isolates at 30°C after 48 hours incubation.

Appearance of clear zones around the bacterial colonies on PVK agar indicated phosphate solubilisation by the bacterial strains. The results on the solubilisation of inorganic phosphates by *Arthrobacter* strains support the earlier reports that rock, iron and aluminium phosphates are less responsive to phosphate solubilisation (Pradhan and Sukla, 2005). Also a significant decline in the pH of medium was recorded during solubilisation of different phosphate substrates, which suggested secretion of organic acids by the bacterial strains. The endogenous regulators of many aspects of plant growth and development are the plant hormones. Auxins are the most extensively studied hormones which regulate cell division, cell elongation, cell differentiation in plants (Berleth & Sacrs, 2001). Although the indole acetic acid is not limited to plants, microorganisms do produce physiologically viable auxins which may have a positive effect on plant growth and its development. L-tryptophan as it a precursor in plants does the same in microorganisms (Park *et al.,* 2005).

The present study has generated information on the genetic variations among the plant growth promoting strains with different tolerance levels to temperature, alkalinity, salinity and calcium salts. The results are of importance in developing plant growth promoting inoculants which can find applications in harsh conditions and for development of microbial formulations which may have wide applications. Thus the results reveal that the isolates from North East India are acid or alkali tolerant where the isolates can be applied for field trials.

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