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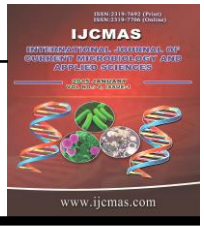

Original Research Article

Isolation and Characterization of bacterial isolates from Potato rhizosphere as potent plant growth promoters

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Indole acetic acid, Rhizobacteria, Enterobacter, Catalase, Potato

A B S T R A C T

Beneficial bacteria that colonize plant roots and enhance plant growth by a wide variety of mechanisms of interaction with the biological, physical and chemical properties of soil are plant growth promoting rhizobacteria (PGPR). PGPR directly promote plant development by nitrogen fixation, production of plant hormones, lowering of ethylene, enhance iron availability, and phosphate solubilization or indirectly by inducing resistance against plant pathogen, competing for nutrient and space. The use of PGPR in sustainable crop protection has drawn much attention in recent years. In the present study, we have isolated, screened and characterized the PGPR from the rhizosphere soil of potato field. Rhizobacterium isolated from rhizospheric soils of potato collected from Lokhandi potato field Bilaspur C.G, India, its name was tentatively designated as PGLO9. Isolate PGLO9 induced the production of siderophores, ammonia, catalase, and solubilize phosphorus. After characterizing the isolate PGLO9 for growth promoting activities, it was biochemicaly and molecularly characterized, by 16S rRNA sequencing and it was found that isolates shows 99 % similarity with *Enterobacter cloacae* strain AB2. The present study, therefore, explore microbe as PGPR that can be used as biofertilizers and may offers an attractive way to replace chemical fertilizers.

Introduction

Rhizobacteria are root colonizing microorganisms which are known to be in constant communication with plants roots and play an important role in promoting plant growth. Also, some strains of plant growth-promoting rhizobacteria can effectively stimulate the growth of plants by direct or indirect effects (Nelson 2004). The mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include: the ability to produce or change the concentration of the plant hormones indole acetic acid IAA (Maivannan *et al.*, 2012); gibberellic acid (Bottini *et al.*, 2004); asymbiotic N_2 fixation (Willey *et al.*, 2011); produce HCN, ammonia, siderophores, solubilise phosphate (Joseph *et al.*, 2007). Moreover modern agriculture relies on high input of agrochemicals which cause major environmental problems (Spiertz 2010). Feeding an increasing human population and

reducing the impacts on the environment urges for low input agricultural practices.

Microorganisms are important in agriculture in order to promote the circulation of plant nutrients and reduce the need of chemical fertilizers as much as possible (Rana *et al.*, 2011). The direct promotion by PGPR entails either providing the plant with plant growth promoting substances that are synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment. The use of PGPR as biofertilizers is one of the most promising biotechnologies to improve primary production with low inputs in fertilizers. To explore some more rhizobacteria that exhibit growth promoting activities in the present study, we have isolated rhizobacterium from potato rhizosphere soils which was collected from Lokhandi potato field Bilaspur C.G, India, it was later screened and characterized for growth promoting activites and identified it by 16S rRNA sequencing.

Materials and Methods

Soil sample collection and analysis

Soil samples were collected from rhizosphere of potato from Lokhandi potato field Bilaspur C.G, India. The rhizosphere was dugout with intact root system. The sample were placed in plastic bag and stored at 4°C in refrigerator for further study.

Isolation of rhizobacteria from potato root

Ten grams of collected rhizosphere soil were added in 100 ml of sterile triple de ionized water in flask separately. The flask was shaken for 25 min at 250 rpm on a rotary shaker. 1ml of suspension was added to 10 ml vial and shaken for 2 min. The suspension was serially diluted upto 12 fold. An aliquot (0.1 ml) of each dilution were spread on the plates of Luria-Bertany (LB) agar medium separately. Plates were incubated for 3 days at 28°C. The observations were recorded after 3 days of incubation. Each isolated colonies were transferred on LB slant for further investigation. The technique was perpetuated thrice and cultures were made single colony type.

Morphological and Biochemical characterization including Carbohydrate utilization test of isolates

Morphological characteristics of the colony of isolate were examined on LB agar plates, as method adopted by Manivannan *et al.*, (2012). 3 days old culture of isolated bacterial colony shape, size, elevation, surface, margin, colour, etc were recorded. The Gram's staining was performed to find out the gram positive and gram negative strain. Biochemical test and carbohydrate utilization test of potent strain was conducted using KB002 HiAssorted™ Biochemical Test Kit and KB009 HiCarbohydrate™ kit.

Characterization of the bacterium for growth promoting trait

Phosphate solubilisation

The isolates were screened for phosphate solubilization as per methodology adopted by Paul and Sinha (2013). In this experiment strain colony were transferred on Pikovskya's medium. The plates were incubated in an incubator at 28°C. The plates were then examined after 7 days of incubation and data were recorded. Visual detection of phosphate solubilizing ability of microorganism was done by plate screening methods that shows clear zone around the microbial colonies in media containing insoluble mineral phosphates (tricalcium phosphate or hydroxyapatite) as sole P source.

Determination of Indole Acetic Acid (IAA)

IAA production was detected by the modified method as described by Fischer *et al.,* (2007). Quantitative analysis of IAA was performed using the method of Loper and Scroth (1986). The bacteria culture incubated in the LB broth at 28°C. The bacterial cells were removed from the culture medium by centrifugation at 8000 rpm for 10 min. A 1ml of supernatant was mixed vigorously with 2ml of Salkowaski's reagent (4.5 gm of FeCl3 per liter in 10.8 M H_2SO_4) and incubated at room temperature in the dark for 30 min and observed the colour formation.

Ammonia production

Strain tested for the production of ammonia as described by Cappuccino and Sherman (2010). 12 hrs old bacteria culture were inoculated in peptone water (10 ml) in culture tube and incubated for 48-72 h at 36±20C. Development of brown to yellow colour after addition of Nesseler's reagent indicated positive test for ammonia, no colour change indicate negative test.

Siderophores production

Siderophore production was tested qualitatively using chrome azurol S medium (CAS-medium) method adopted by Manivannan *et al.,* (2012). The culture streaked on the surface of CAS agar medium and incubated at room temperature for 1 to 3 days. Siderophore production was indicated by orange halos around the colony after the incubation, and this test was done in three replications.

HCN production

Nutrient agar was amended with 4.4 g glycine/L and the culture streaked on modified agar plate (Lorck 1948). A Whatman filter paper (No. 1) soaked in 2% Sodium carbonate (in 0.5% picric acid) was placed at the top of the each isolated strain separately. Plates were sealed with parafilm and incubated at 36±2 °C for 4 days. Development of orange to red colour on the paper indicated HCN production.

Catalase production

One loopful culture of isolate were put on the slide and add few drop of H_2O_2 separately. The evolution of oxygen in the form of bubble indicate positive reaction (catalase production).

Molecular characterization

16s rRNA sequencing was performed by the commercial service provider, Chromous Biotech Pvt. Ltd., Bangalore, India. Potent Strain identification was carried out by amplifying 16S rRNA using (PCR) polymerase chain reaction. For this, Fragments from the 5′ and 3′ ends of 16S rRNA sequences from bacterial isolates were amplified and sequenced using16s Forward Primer: 5'AGAGTRTGATCMTYGCTWAC-3' and 16s Reverse Primer: 5'-CGYTAMCTTWTTACGRCT-3'.

Amplification and sequencing was done using 10µl Sequencing Reaction: Big Dye Terminator Ready Reaction Mix: 4µl, Template (100ng/ul): 1µl, Primer (10pmol/_): 2µl, Milli Q Water: 3µl. PCR condition selected was PCR Conditions: (25 cycles): Initial Denaturation: 96°C for 5 min, Denaturation: 96°C for 30 sec, Hybridization: 50 °C for 30 sec, Elongation: 60 °C for 1.30 min.

Following are the instrumentation and chemistry detail:-

Sequencing Machine: ABI 3130 Genetic

Analyzer, Chemistry Cycle sequencing kit: Big Dye Terminator version 3.1", Polymer &Capillary Array: POP_7 polymer 50 cm Capillary Array, Analysis protocol: BDTv3-KB-Denovo_v 5.2, Data Analysis Software: Seq Scape_ v 5.2, Reaction Plate: Applied Biosystem Micro.

Amplified sequences were compared with sequences from the NCBI database, using the blast algorithm (Altschul *et al.*, 1990).

Results and Discussion

Morphological characteristic of the culture

Morphologically colony of strain PGLO9 was white colour circular in shape, elevated umbonately with smooth and shiny surface as shown in Table 1.

Biochemical test

On the basis of biochemical test potent strain PGLO9 isolates was positive for citrate and orinithine utilization, nitrate reductase, glucose, arabinose and sorbitol production whereas negative for lysine utilization, urease, deamination, H_2S, adonitol, and lactose production, as shown in table 2.

Carbohydrate utilization test

On investigating carbohydrate utilization test of PGLO9 isolate, isolate was positive for xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, L-arabinose, mannose, inulin, glycerol, inositol, sorbitol, mannitol, α-methyl-D-glucoside, cellobiose, ONPG, esculin hydrolysis, D-arabinose, citrate utilization, and malonate utilization, whereas negative for lactose, sodium gluconate, salicin, dulcitol, adinitol, arabitol, erythritol, rhamnose, melezitose, α-Methyl-D-mannoside, xylitol, and sorbose, as shown in table 3.

Molecular Characterization

Rhizobacterium PGLO9 was molecularly characterized by means of 16S rRNA sequencing (Table 4). Based on BLAST searches on the NCBI data libraries for similarities of the 16S rRNA sequences, the microbe was found to be most similar to *Enterobacter cloacae* AB2 (NCBI accession No. JX188069.1, The next closest homologue was found to be *Enterobacter cloacae* strain RM20 (NCBI Accession No. KJ607605.1). Information about other close homologue for the microbe can be found from the phylogenetic tree (fig 1) and information regarding Alignment view and Distance matrix are shown in table 5.

Growth promoting characteristic

We investigated that PGLO9 out of 6 growth promoting parameter it showed production of siderophores, catalase, ammonia and phosphate solubilisation activity as mentioned in table 6.

Table.1 Morphological characteristic of isolates colony

Strain	Motility	Shape	Elevation	Edges	Surface texture	Colour	Gram staining
PGLO9	motile	circular	Umbonate	Entire	Smooth shiny	white	Gram negative

Table.2 Biochemical test of rhizobacterium isolates

S.No.	Biochemicals	PGLO9
1	Citrate utilization	+
2	lysine utilization	-
3	Ornithine utilization	+
4	Urease	-
5	Phenylalanine Deamination	-
6	Nitrate reductase	+
7	H_2S Production	-
8	Glucose	+
9	Adonitol	-
10	Lactose	-
11	Arabinose	+
12	Sorbitol	+

Fig.1 Show phylogenetic tree of PGLO9 constructed by Mega version 5.05

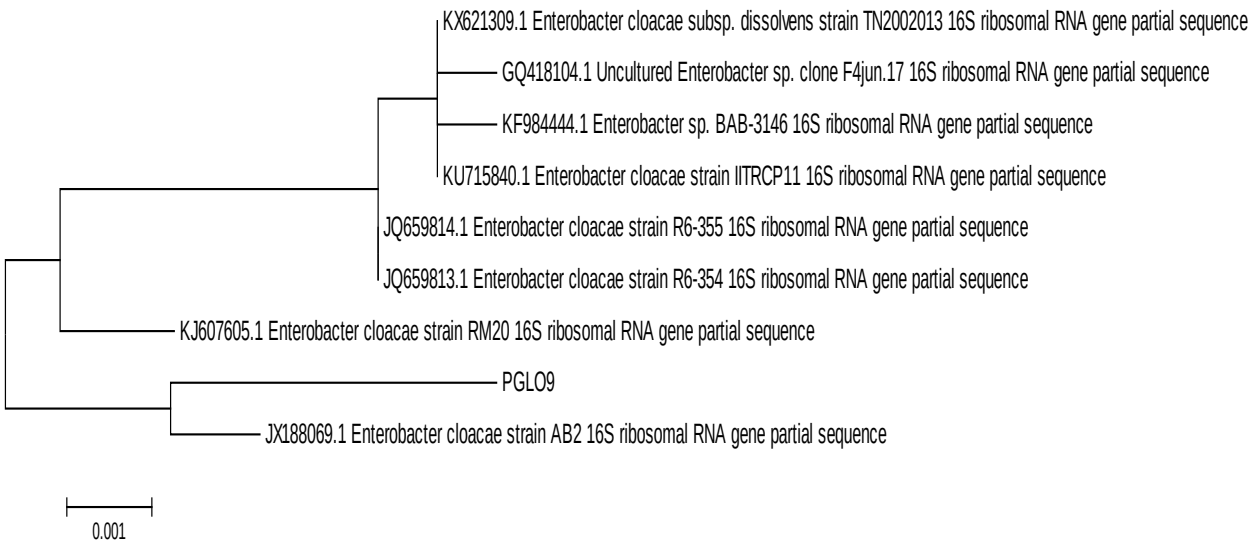

Table.3 Carbohydrate utilization test

S.No.	Carbohydrate	PGLO9
1	Lactose	-
2	Xylose	+
3	Maltose	+
4	Fructose	+
5	Dextrose	+
6	Galactose	+
7	Raffinose	+
8	Trehalose	+
9	Melibiose	+
10	Sucrose	+
11	L-Arabinose	+
12	Mannose	+
13	Inulin	+
14	Sodium gluconate	-
15	Glycerol	+
16	Salicin	-
17	Dulcitol	-
18	Inositol	+
19	Sorbitol	+
20	Mannitol	+
21	Adinitol	-
22	Arabitol	-
23	Erythritol	-
24	α-Methyl-D-glucoside	+
25	Rhamnose	-
26	Cellobiose	+
27	Melezitose	-
28	α-Methyl-D-mannoside	-
29	Xylitol	-
30	ONPG	+
31	Esculin hydrolysis	+
32	D-Arabinose	+
33	Citrate Utilization	+
34	Malonate utilization	+
35	Sorbose	-

Table.4 Molecular characterization of bacterial isolates by 16S rRNA analysis

Bacterial isolates	Number of rRNA sequenced bases	Maximum identity	Bacterial genus species	Accession No.
PGLO9	1500	99%	*Enterobacter cloacae stain AB2*	JX188069.1

Table.5 Alignment view and Distance matrix table

NCBI Accession No.	Organism name	Score
JX188069.1	*Enterobacter cloacae strain AB2*	99%
KJ607605.1	*Enterobacter cloacae strain RM20*	99%
JQ659814.1	*Enterobacter cloacae strain R6-355*	99%
JQ659813.1	*Enterobacter cloacae strain R6-354*	99%
KF984444.1	*Enterobacter sp. BAB-3146*	99%
KX621309.1	*Enterobacter cloacae subsp. dissolvens strain TN2002013*	99%
KU715840.1	*Enterobacter cloacae strain IITRCP11*	99%
GQ418104.1	*Uncultured Enterobacter sp. clone F4jun.17*	99%

Table.6 Plant growth promoting characteristics of rhizobacterial isolates

S.No	Rhizobacterium	HCN	Catalase	IAA	Siderophores	Ammonia	Phosphate solubilisation
1.	PGLO9	-	+	-	+	+	+

PGPR colonize roots of plant and promote plant growth and development through a variety of mechanisms. There are many papers related to the advantages and screening of PGPR from crop plants particularly rice, maize and sugar cane but few or none from potato. Little information about screening and using PGPR with Potato rhizosphere C.G, India is available. In present study, rhizobacterium was isolated from potato rhizosphere was screened for different plant growth promoting activities and identified it. Bacteria isolate was 99 % similar to *Enterobacter cloacae* strain AB2, Previously many workers has reported E. cloacae as plant growth promoting rhizobacteria in many different plants (English *et al.*, 2010; Ramesh *et al.*, 2014; suprapta *et al.*, 2014).

Enterobacter cloacae is a rod-shaped, motile, gram-negative bacteria from the Enterobacteriaceae family. *Enterobacter cloacae* lives in the mesophilic environment with its optimal temperature at 37 °C. In present work PGLO9 strain has been isolated from rhizophere of potato and it was found that isolate showed transparent halos zone of phosphate solubilization. It has been reported that higher concentrations of phosphate-solubilizing bacteria are commonly found in the rhizosphere soil as compared to non rhizospheric soil (Reyes and Valduz 2006).

Along with phosphate solubilisation, rhizobacterium showed significant production of siderophores, catalase, and ammonia production then other isolates and act an important plant growth promoting isolates. Siderophore is one of the biocontrol mechanisms belonging to PGPR groups under iron limiting condition. PGPR produces a range of siderophore which have a very high affinity for iron. Therefore, the low availability of iron in the environment would suppress the growth of pathogenic organisms including plant pathogenic fungi (Whipps 2001). Production of ammonia that indirectly influences the plant growth, bacterial strains showing catalase activity must be highly resistant to environmental, mechanical and chemical stress. In the present study isolate was found to be solubilized insoluble phosphorus, produced siderophores, ammonia, and catalase. Such type of study is necessary as it advocates that *Enterobacter cloacae* strain PGLO9 as inoculants or biofertilizers is an efficient approach to replace chemical fertilizers.

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