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Isolation and Characterization of Multifarious Plant Growth Promoting Bacteria *Enterobacter ludwigii* PGP 19 Isolated from Pearl Millet

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Abstract: ***A multifarious plant growth promoting bacteria designated as PGP-19 was isolated from rhizosphere of Pennisetum glacum. Based on 16S rRNA gene sequencing isolate was identified as Enterobacter ludwigii. Isolated strain exhibited various plant growth promoting characteristic features like ACC deaminase production, Indole acetic acid (IAA) production, solubilization the inorganic phosphate etc. Strain PGP-19 could also tolerate a wide rangs of pH, salt (NaCl) and temperature stress conditions. In addition Enterobacter ludwigii PGP-19 also exhibited nitrogen fixation potential, HCN production, chitinase activity and sensitivity towards antibiotics. Production and HCN and chitinase activity illustrated the biocontrol ability of strain. A significant increased in various growth parameters of plant such as shoot length, root length, fresh weight and dry weight was observed following inoculation of isolate PGP-19 in in-vitro pot study. The study indicated the potential of isolate PGP-19 for development of bacterial inoculum production and used as a biofertilizers for enhancing growth of pearl millet and other major crops.***

Keywords: PGPR, ACC deaminase, IAA, PCR

1. Introduction

Plant growth promoting rhizobacteria are a hetrogenous group of bacteria that closely associated with the rhizosphere region and promotes the growth of plants. Term PGPR was first introduced in 1978 by Kloepper and colleagues to a group of beneficial bacteria that promote the plant by production of phytohormones, phosphate solubilization, nitrogen fixation etc.(Compant et al., 2005). Several studies have reported that PGPR can significantly improves the growth of economically useful plants (Shoebitz et al., 2009). Use of fertilizers and other chemical supplements are depleting the availability of nutrients in soil hence the scientific community focusing in the field of biofertilizer technology includes the exploitation of plant growth promoting rhizobacteria (PGPR) for improving plant growth promotion, prevention of soil erosion and biological control (Vessey, 2003). Use of PGPR as inoculants can fulfill beneficial interactions in plants to solutions for sustainable and environment friendly agriculture (Bhattacharyya and Jha, 2012).

Pearl millet (*Pennisetum glacum* L.) is a important staple food for poor in semiarid regions of Asia and Africa. In India it stands as the fifth major crop and grown as a rain-fed or irrigated crop on 10 million hectares producing 7.01 million tons (Bhatnagar et al., 2002). Therefore exploitation of efficient PGPRs from the rhizosphere of *Pennisetum glacum* is of great importance for enhancing the growth and yield of agricultural crops.

2. Materials and Methods

Isolation

1 g rhizospheric soil of *Pennisetum glacum* was mixed with 50 ml of PBS buffer and spread plating was done on DF-agar medium. Bacterial colony grown on media was further streaked on DF-agar plate supplemented with 3mM ACC as unique nitrogen source. 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 physiological characterization.

Genetic Identifiction

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′-TACGGCTACCTTGTTACGAC-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 (Fig.1) was send to Xcelris genomics labs ltd (Xcelris Ahemdabad, India) for sequencing.

3. Test for plant growth promoting traits

ACC deaminase assay

For enzymatic activity assay, strain was grown in tryptic soya broth up to late log phase at 30 °C in an orbital shaker at 150 rpm for 24h. The obtained 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 standard curve of α-ketobutyrate was determined at 540 nm to standard curve of α-ketobutyrate ranging between 0.1 to 1.0 µmol.

Phosphate solubilization assay
Screening for P- solubilization ability of isolate was done using NBRIP medium 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.

Siderophore production
Assay for siderophore production of the isolate was carried out by spot inoculating on chrome azurole S agar (CAS-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).

IAA production
The IAA production was estimated in tryptophan (100µg/ml) supplemented media by growing the isolate in Nutrient broth (Himedia, India) for 72 h at 30 °C and kept on shaking at 180 rev/min. After 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).

Ammonia and nitrogen fixation test
For ammonia production, strain PGP-19 was inoculated into 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 and observation of brown to yellow color was observed as positive test for ammonia production. Nitrogen fixation ability was evaluating by streaking the isolate 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 estimation of HCN production isolate was streaked on nutrient agar plate supplemented with 4% glycine and 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.

Stress tolerance
Isolate PGP-19 was screened for their ability to tolerate the abiotic stress particularly salt and pH and temperature. Bacterial culture was inoculated into nutrient broth amended with 0.5% to 10% salt. After 24 h, absorbance of the culture was determined at 600 nm. The ability of bacteria 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 (Himedia, India). Similarly bacteria was cultured in nutrient broth and incubated a wide range of temperature (30 °C to 60 °C). 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.

Biochemical tests
Isolate was screened for indole, methyl red (MR), voges-proskauer (VP) test as per standard protocols (Prescott and Harley, 2002). For citrate utilization ability 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. Strain PGP-19 was freshly streaked on clean glass slide and a 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). Lipolytic activity was carried out on trimethoprim plate and observation of halogen was considered as positive for lipase production.

Protease production
Protease production of the strain was checked following the suitable protocol (Prescott and Harley, 2002). 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. 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.

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 isolate PGP-19 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).

Antagonistic test

Biocontrol ability of the isolate was evaluated by fungal mycelial inhibition by well diffusion method against the fungal pathogens *Fusarium oxysporum*, *Fusarium moniliforme*, *Fusarium graminearum* and *Penicillium citrium*. After the solidification of potato dextrose agar (PDA, Himedia, India) 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.

Inoculum preparation and seed treatment

Preparation of bacterial inoculum (OD 0.15) was performed according to Penrose and Glick, (2003). Pearl millet (*Pennisetum glacum* 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. Bacterized seeds were sown in plastic pots filled with soil in triplicates in a growth chamber with 16:8 photo-period up to 21 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. The experiment was conducted for 21 days after the seed germination. Root and shoot length, fresh and dry weight of five randomly selected seedlings from each replication were recorded.

4. Result

Identification

Based on 16S rRNA gene sequence analysis, the strain was identifies as *Enterobacter ludwigii*. The 16S rRNA gene sequence of the strain was deposited in the Genebank database under accession no KJ950720.

Plant growth promoting (PGP) test

The ACCD activity of strain was found to be 231.90±14.40 nmoles of α-KB/mg pr·h^{-1}. Isolate was further tested for IAA production and based on quantification IAA production was found to be 0.414±0.0310 µgml^{-1}. Strain was able to solubilise inorganic phosphate 7.110±1.140 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 (Table 1).

Biochemical characterization

Strain was observed positive for catalse, lipase, urease, cellulase, nitrate reductase whereas negative for indole, methyl red (MR), voges-proskauer (VP), amylase. 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 10. Strain was found to be sensitive for amplicillin, chloramphenicol, gentamycin and resistant for kanamycin, erythromycin, tetracycline and vancomycin (Table 2).

Crbohydrate utilization test

Strain was able to utilize lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, L-arabinose, mannose, inulin, glycerol, salicin, sorbitol, mannitol, adonitol, arabitol, rhamnose and cellobiose (Table 3).

Antagonistic test

Isolated strain showed the certain degree of antagonism to fungal strain *Fusarium moniliforme and Fusarium oxysporum* only. Among pathogenic bacterial strain tested, it was found to be effective against *Escherischia coli*, and *Erwinia carotovora.*

Plant growth promotion test

Inoculation with isolate strain PGP-19 significantly increased the various growth parameters of plant tested. Significantly increased in shoot length (36.23%) was observed after 21 days of bacteria treatments. The significant increase in other growth parameters like root length 29.20%, fresh weight 34.73% and dry weight 39.86% was observed (Table 4).

5. Discussion

Rhizosphere of plants is a preferential niche for various types of microorganisms in the soil. The growth promotion of plants is believed to occur by polyphasic mode of action. In the present study the isolate showed various plant growth promoting traits that supports the plant growth and increased the biomass. The indole acetic production by various bacterial isolates from the rhizosphere of different crops, i.e., peanut, maize, wheat, and rice had already been reported in number of studies that enhances the root growth. The ability of bacterial strains to solubilize insoluble phosphate and convert it to plant available form is an important characteristic under conditions where phosphorus is a limiting factor for crop production. The soil phosphate solubilizing strains can increase the availability of phosphorus to plant by converting inorganic phosphorus into more available form. Moreover, the results of nitrogen fixation could be beneficial to improve nitrogen nutrition of crop plants. Previous study has reported that production of HCN and chitinase activity control fungal diseases in various crop plants. The capacity of some bacterial isolates to produce ammonia also enhances plant growth. Certain microorganisms contain an enzyme ACC-deaminase that hydrolyses ACC into ammonia and α-ketobutyrate (Mayak et al., 2004) that serves as carbon and nitrogen sources for plants growth. In conclusion, the isolate significantly improved the growth of host plant. Therefore application of

such multifarious plant growth promoting bacteria could be useful for development of biofertilizers for crop plants.

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References

[1] Abo-Aba S, Soliman E, Nivien A (2006) Enhanced productio n of extracellular alkaline protease in *Bacillus circulance* through plasmid transfer. Res Agric Biol Sci 2(6), 526 -530.
[2] Bhatnagar, S.K., Khaiwal, I.S. and Prateek, S., 2002. Pearl millet nucleus and breeder seed production. Technical Bulletin. Project Coordinator (Pearl Millet) All India Coordinated Pearl Millet Improvement Project (AICPMIP), Jodhpur, India.
[3] Bhattacharyya PN, Jha DK (2012) Plant growth promoting rhizobacteria (PGPR): emergence in agriculture. World J Microbiol Biotechnol 28, 1327–1350.
[4] Compant, S., Duffy, B., Nowak, J., Cle´ment, C., Ait Barka, E., 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Applied Environmental Microbiology 71, 4951–4959.
[5] Gordon, S.A., Weber, R.P. (1951) Colorimetric estimation of indole acetic acid. Plant Physiol 26, 192–195.
[6] Kloepper JW, Schroth MN (1978) Plant growth promoting rhizobacteria on radish. In: Proceedings of the 4th International Conference on Plant Pathogenic Bacteria, vol 2. Station de Pathologie Vegetale et Phytobacteriologie, INRA, Angers, France, pp 879–882.
[7] Marinetti, G.V. (1962) Chromatographic separation: identification and analysis of phospholipides. Journal of Lipid Research 3, 1–20.
[8] Mayak, S. et al., 2004. Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. Plant Physiol Biochem 42, 565–572.
[9] Penrose, D.M. and Glick, B.R., 2003. Methods for isolating and characterizing ACC deaminase containing plant growth-promoting rhizobacteria. Physiolog Plantarum 118, 10-15.
[10] Prescott, L., Harley, J., 2002. Laboratory Exercises in Microbiology.
[11] Rorth, M., and P. K. Jensen. 1967. Determination of catalase activity by means of the Clark oxygen electrode. Biochim. Biophys Acta 139, 171-173.
[12] Saleh, S.S., Glick, B.R. (2001) Involvement of gasS and pros in enhancement of the plant growth-promoting capabilities of *Enterobacter cloacae* CAL2 and UW4. Can J Microbiol 47, 698–705.
[13] Schwyn, B., Neilands, J.B. (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160, 47-56.
[14] Shoebitz M, Ribaudo CM, Pardo MA, Cantore ML, Ciampi L, Cura JA (2009) Plant growth promoting properties of a strain of *Enterobacter ludwigii* isolated from *Lolium perenne* rhizosphere. Soil Biol Biochem 41:1768–1774.
[15] Vessey JK (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255:571–586.

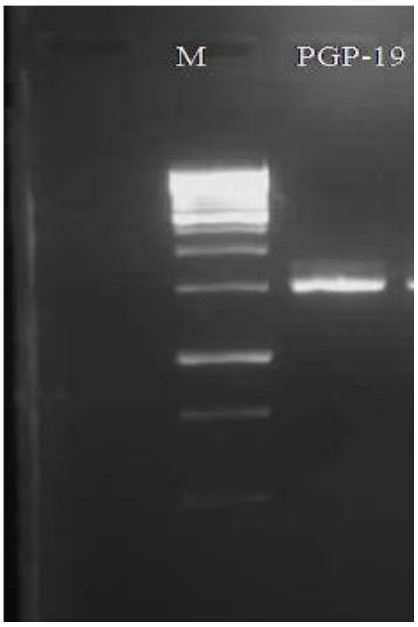

Figure 1: 16S rRNA image of isolate PGP-19

Table 1: PGP traits of strain PGP-19

Plant growth promoting traits	Activity
ACCD activity (nmoles of α-KB/mg prh^{-1})	231.90±14.40
IAA production (µg ml^{-1})	0.414±0.0310
Phosphate solubilization (µg ml^{-1})	7.110±1.140
Growth on N-free medium	+
Siderophore index	-
Ammonia production	+
HCN production	+
Chitinase activity	+

Table 2: Biochemical characteristic feature of strain PGP-19

Characteristic (s)	Activity
Catalase	+
Indole	-
MR	-
VP	-
Protease	-
Lipase	+
Urease	+
Nitrate reductase	+
Temp. tolerance (°C)	50
Salt tolerance (%)	6%
pH tolerance	10
Antibiotic sensitivity test	
Amplicillin	+
Erythromycin	++
Chloramphenicol	+
Tetracyclin	++
Kanamycin	++
Gentamycin	+
Vancomycin	++
+, sensitive: ++, resistant	

Table 3: Carbohydrate utilization 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	-

Table 4: Plant growth promotion test: an in-vitro pot study

Treatment	Shoot lg. (cm)	Root lg. (cm)	Fresh wt. (g)	Dry wt. (g)
Control	23.90±3.45	17.09±2.08	4.75±0.57	1.48±0.31
PGP-19	32.56±2.60*	22.08±1.95*	6.40±0.28*	2.07±0.18*