RESEARCH PAPER | Biotechnology | Volume : 3 | Issue : 6 | June 2013 | ISSN - 2249-555X

Functional characterization of PGPR and its identification through 16 S rRNA sequencing

KEYWORDS: PGPR, 16s rRNA , Pseudomonas spp. and rhizosphere

Rashmin M. Dhingani
College of Food Processing Technology & Bio-Energy, Anand Agricultural University, Anand-388110, Gujarat

Manoj V. Parakhia
Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat.

Rukam S. Tomar
Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat.

Bipin J. Malviya
Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat.

B. A. Golakiya
Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat.

ABSTRACT *Plant growth promoting rhizobacteria are known to rapidly colonize the rhizosphere and suppress soil borne pathogens at the root surface. These organisms can also be beneficial to the plant by stimulating growth. Pseudomonas spp. have been studied mainly because of their widespread distribution in soil, their ability to colonize the rhizosphere of host plants and ability to produce a wide range of compounds inhibitory to number of serious plant pathogens.For performing this study, soil sample was collected from six different locations of Junagadh district.Total thirty six bacteria were isolated from the collected soil samples by using general microbiological media, among them ten prominent phosphate solubilizer were selected for studying other plant growth promoting potentials and biochemical characterization. Most efficient Plant growth promoting rhizobia was identified at their strain level by using 16 S rRNA sequencing technology.*

INTRODUCTION

The rhizosphere, representing the thin layer of soil surroundingplant roots and the soil occupied by the roots, supports largeactive groups of bacteria (Villacieroset al., 2003) known asplant growth promoting rhizobacteria (PGPR) (Kloepperet al.,1980).Plant growth promoting rhizobacteria are known torapidly colonize the rhizosphere and suppress soil bornepathogens at the root surface (Rangajaranet al., 2003). These organisms can also be beneficial to the plant by stimulatinggrowth (Bloemberg and Lugtenberg, 2001; Moeinzadehet al.,2010).

PGPR can affect plant growth by different direct and indirect mechanisms (Glick 1995; Gupta et al. 2000). Some examples of these mechanisms are (1) increased mineral nutrient solubilization and nitrogen fixation, making nutrients available for the plant; (2) repression of soil borne pathogens (by the production of hydrogen cyanide, siderophores, antibiotics, enzymes like chitinase and/or competition for nutrients); (3) improving plant stress tolerance to drought, salinity, and metal toxicity; and (4) production of phytohormones such as indole-3-acetic acid (IAA) (Gupta et al. 2000). The bacteria presenting one or more of these characteristics are known as plant growth promoting rhizobacteria. Pseudomonas spp. have been studied mainly because of their widespread distribution in soil, their ability to colonize the rhizosphere of host plants and ability to produce a wide range of compounds inhibitory to a number of serious plant pathogens (Weller 1988).

The soil bacteria that aggressively colonize the root zone and promote plant growth are generally termed as Plant Growth Promoting Rhizobacteriaand primarily Pseudomonas fluorescence s is identified as an important organism with ability for plant growth promotion and effective disease management properties (Mazzolaet al., 1992). Their applicability as biocontrol agents has drawn wide attention because of the production of secondary metabolites such as siderophore, antibiotics, volatile compounds, Hydrogen Cyanide, enzymes and phyto-hormones (Nagarajkumaret al., 2004). In vitro antibiosis of P. fluorescence strains towards the rice sheath rot pathogen S. oryzaedemonstrated that this pathogen is sensitive to P. fluorescence (Sakthivel and Gnanamanickam, 1987). The biocontrol mechanism to suppress fungal pathogens by Pseudomonas spp. normally involves the production of antibiotics and P. fluorescence s has a gene cluster that produces a suite of antibiotics, including compounds such as 2,4-diacetylphloroglucinol (DAPG), phenazine, pyrrolnitrin, pyoluteorin and bio-surfactantantibiotics (Angayarkanniet al., 2005). Fluorescence t Pseudomonas is uniquely capable of synthesizing many of these antibiotics, not only to enhance its own fitness but also to help in the maintenance of soil health and bioprotection of crops from pathogens (Gaur et al., 2004). Presently, there are number of commercial isolates of Pseudomonas available in the market.

MATERIALS AND METHODS

ISOLATION AND PURIFICATION

Collection of soil sample:

The soil sample was collected for the Isolation of plant growth promoting rhizobiafrom rhizosphere of Junagadh district fields of Gujarat. Intact root system was plowed out and the rhizospheric soil samples were carefully taken in sterilized plastic bags. Samples were collected from six different points within area and mix thoroughly; likewise six different locations were selected from the Junagadh district.

Isolation of bacteria:

For the isolation, 1 gram of each soil samples collected from the various collection sites was dissolved in 10 ml sterile distilled water and mix well for 10 minutes. Serial dilution was made from each sample and appropriate dilution was spread on Nutrient agar plate and incubated at 37±0.1 ^{0}C for 24hrs.

From the mixed population of microbes, microbial strains were isolated by single colony isolation from the nutrient agar medium. Out of total 57 microbial isolates, 36 isolates were selected for the test of phosphate solubilizing activity. Streak plate method was used for obtaining pure culture, as and when required.

Volume : 3 | Issue : 6 | June 2013 | ISSN - 2249-555X

PLANT GROWTH PROMOTING ACTIVITIES

Determination of Phosphatesolubilization:

Selected 36 isolates were plated on Pikovasky medium containing 5 g of tri-calcium phosphate [$Ca_3(PO_4)_2$] as sole phosphorus source for selectively screening of bacteria which have the ability to release inorganic phosphate from tri-calcium phosphate. After 3-days of incubation at 37±0.1 °C, phosphate solubilizing bacteria developed clear zones around colonies.

Out of 36 isolates, only 10 isolates were able to show zone of inhibition around the colony on plates containing Pikovasky medium. All 10 isolates having phosphate solubilizing activity were preserved in the laboratory as a pure culture for their further investigation.

Screening of isolates for other Plant Growth Promoting Activity

IAA Production:

Each isolates were inoculated into 5 ml of CzepakDox (CD) broth and incubated overnight at 37±1 °C. 100µl of this culture was inoculated to 50 ml of CD broth and incubated at 37±1 °C in dark for seven days. The amount of IAA produced by the isolates was estimated by following the method of Gordon and Paleg (1957). The intensity of pink colour developed was read in a spectrophotometer at 540 nm. The amount of IAA produced by the isolates was calculated from the standard graph developed from different concentrations of IAA solutions prepared using chemical grade Indole -3-acetic acid (Hi-Media).

HCN Production:

Isolates were streaked on nutrient agar medium containing 4.4 g per liter of glycine. A Whatman filter paper No. 1 soaked in 0.5% picric acid solution (in 2% sodium carbonate) was placed inside the lid of plate. Plates were sealed with parafilm and incubated at 37±0.1 °C for 4 days. Development of light brown to dark brown color was checked.

Ammonia Production:

This test is based on the production of urease which breaks urea into ammonia and which in turn increase the pH of the medium. In this test urea broth was inoculated with isolates and incubated at 37°C for 24 hrs. Next day change in color of the broth was checked.

Siderophore Production:

Isolates were screened for siderophore production by the Chrome AzurolSulfonate (CAS) plate assay. Spectrophotometric assay (Liquid CAS assay) was used for quantitative estimation of siderophore produced by the isolates (Schwyn B. and Neilands J. B., 1986).

Catalase activity:

A drop of 3% hydrogenperoxide was added to 48 hrs.old bacterial colony on a clean glassslide and mixed using a sterile toothpick. The effervescence was checked for catalase activity.

BIOCHEMICAL CHARACTERIZATION

Ten isolates which showed PGPR activity werefurther characterized by biochemical tests as perBergey's Manual of Systematic Bacteriology.Following tests were performed for the characterization of the isolates.

Starch hydrolysis:

This test was performed to determine the ability of the isolated strains to degrade starch by producing enzyme, amylase. Bacterial isolates were streaked on starch agar medium and incubated for two days. The plateswerethan flooded with 1 % iodine solution. A colorless zone around the growth and blue color in the rest of the plates showed utilization of starch by the microorganism.

Gelatin Hydrolysis:

This test was performed to determine the ability of the isolated strains to hydrolyze gelatin. Bacterial isolates were streaked on gelatin agar medium. The Petri dishes were incubated at 30°C for 2 days and then flooded with 12.5 % $HgCl_2$ solution. The development of yellow zone around the growth indicates utilization of gelatin (Stolpe and Godkeri, 1981).

Oxidase test:

The oxidase test identifies organisms that produce the enzyme cytochrome oxidase. The oxidase reagent contains a chromogenic reducing agent which changes color when it becomes oxidized. If the test organism produces cytochrome oxidase then oxidase reagent turns blue or purple within 15 seconds. This test was performed by using oxidase disc (Himedia manufactured). Blue color formation was checked after adding oxidase reagent.

Catalase enzyme activity:

This test was performed to determine the ability of the isolated strains to degrade hydrogen peroxide by producing enzyme, catalase or peroxidase. The test was performed by putting a drop of 3% hydrogen peroxide on a clean glass slide and emulsified by a 48 hrs.old bacterial culture in it. Presence or absence of air bubbles was checked.

Nitrate reduction test:

This differential test determines the ability of an organism to reduce nitrate or beyond nitrite to nitrogen gas. Nitrite production was checked in a nitrate broth added withsulphanilic acid and α-naphthylamine. Reduction in to nitrogen production was checked by adding zinc dust and observed for the color changes.

Sulfur reduction:

Certain bacteria have ability to reduce a sulfur metabolite into H_2S (a colorless gas that generates a black color in the agar medium). The iron ions (Fe^{2+}) have a high affinity (strong attraction) for sulfide ions. H_2S combines with the iron to make FeS, a black compound in the medium. Medium contains ferrous (iron) sulfate was inoculated with test organism. The inoculated tube is incubated at 37 °C for 24 hrs.

Citrate Utilization:

This defines the ability of bacteria to convert citrate into oxaloacetate. In this media, citrate is the only carbon source available to the bacteria. If it cannot use citrate then it will not grow. If it can use citrate, then the bacteria will grow and the media will turn a bright blue as a result of an increase in the pH of the media. Citrate slants was streaked with the isolates and incubated at 37 °C for 24 hours and color changes on slants were recorded.

Methyl Red-Vogues Proskauer(MRVP):

This test is used to determine two things. The MR portion is used to determine conversion of glucose to acidic products like lactate, acetate and formate. The VP portion is used to determine conversion of glucose to acetoin.

Two sets of glucose phosphate broth was inoculated with the isolates and incubated at 37°C for 48 hrs. One set was used for methyl red test, in which development of color was checked after adding 5 drops of methyl indicator. Second set was used for the Vogues Proskauer test, in which development of color was checked after adding 0.6 ml of α-naphthol and 0.2 ml of KOH solution in 1 ml culture broth.

Indole production:

This test is done to determine if bacteria can breakdown the amino acid tryptophan into indole. In this,tryptone broth was inoculated and incubated at 37°C for 48 hrs. Development of red/pink layer on top of the media was checked after adding Kovac's reagent.

Motility test:

This test can be used to check for the ability of bacteria to migrate away from a line of inoculation. To perform this test,

Volume : 3 | Issue : 6 | June 2013 | ISSN - 2249-555X

the bacterial sample was inoculated into motility media using a needle and incubated at 37°C for 24 hours and checked for the bacterialmigration.

Carbohydrate Utilization test:
Glucose fermentation test
A broth containing the glucose sugaris used for this test. Bacteria growing in this broth will produce acid if it uses the glucose. This test was done by inoculating glucose broth and incubated at 37°C for 48 hours. Acid and gas production was checked.

Lactose Fermentation Test
A broth containing the lactose sugar is used for this test. In this test isolates was inoculated in lactose broth and incubated at 37°C for 48 hours. Acid and gas production was checked.

MOLECULAR IDENTIFICATION
Out of the ten isolates, fifth isolates which shows comparatively highest PGPR activity as compare to other is further identified at species level by sequencing 16 S rRNAgenes.

Molecular characterization of the fifth isolates:
Genomic DNA isolation was carried out by using standard protocol as described by Sambrook and Russel (2001). PCR amplification of 16 S ribosomal RNA genes was carried out by using Fast MicroSeq ID kit of Applied Biosystems in the Applied Bio systems 9800 thermal cycler by following the manufacturer instruction.5 µl of amplified product was loaded to confirm on 1.2% agarose gel electrophoresis and run for 30 min at 70 V cm^{-1} in 1X TBE buffer.Amplified product was further purified by ExoSAP-IT (USB PN 78200) clean-up kit. Cycle sequencing was performed from the cleaned products by using Applied Biosystemcycle sequencing kit. Final product was purified by CentriSep column (Applied Biosystems PN 401763).Purified product was sequenced by 16 capillary sequencer (3130 XL, Genetic Analyzer, Applied-Biosystem). The BLAST program (www.ncbi.nlm.nih.gov) was used to find the identity and similarity of sequence and finally it was processed with the help of stand-alone software tool (sequin) developed by national center for biotechnology information (NCBI). Sequin processed sequence was submitted to the GeneBank.

RESULTS AND DISCUSSION
Total thirty six bacterial isolates were isolated from the six different rhizospheric soil samples from the Junagadh district. All were checked for phosphate solubilization activity.Among them ten isolates showedprominent phosphate solubilization zones which ranges from 3mm to 18 mm. Out of them four isolates showed sharp zones.Highest solubilization activity showed by fifth isolates(P-05)and remaining six showed hazy zone.Ten isolates which showed phosphate solubilization activity were further selected for the studying various PGPRs activities (Table 1). IAA production of the ten isolates were calculated which ranges from 06.23 µM/ml to 33.12 µM/ml, among them fifth isolates (P-05) shows highest IAA production. All of ten isolates showed negative for the ammonia production. Another trait of PGPR checked was HCN production, among ten isolates, P-02, P-03, P-04 and P-05 isolates showed positive result and remaining six shows negative result for HCN production. Siderophore activity of ten isolates were ranges from 48.27 µM/ml to 92.10 µM/ml, among them ninth isolate (P-09) shows highest activity however efficient P-solubilizer and IAA producer,fifth isolates (P-05) isolates shows 89.36 µM/ml activities. All the ten isolates showed the catalase positive.Morphological, cultural and biochemical characterization of these isolates has been done (Table 2). On the basis of this characterization the most efficient isolate which shows highest PGPR activity was identified as Pseudomonas. For the further identification at strain level, most efficient fifth isolate (P-05) was identified by 16 S rRNA sequencing. 16 S rRNA gene sequences compared with the available sequences in the databank with help of BLAST homology search. Fifth isolate (P-05) shows close homology with the Pseudomonas aeruginosa strain DSM 50071. Sequence of Pseudomonas aeruginosa strain was processed by sequin stand-alone software and deposited it in the GeneBank database with accession number JQ867395.

CONCLUSION
Plant growth promoting rhizobacteria are increasingly used for crop improvement and protection. In the same context, present study was focused for the isolation and characterization of PGPR from rhizosphere of the Junagadh district. Phosphate solubilization, Indole acetic acid production, Siderophore production, Hydrogen cyanide production and ammonia production was considered for the present study. Isolates with good plant growth promoting potentialities were characterized and most efficient isolate among them identified. The morphological, cultural, biochemical and molecular characterization of the most efficient isolate 5 revealed that it was Pseudomonas aeruginosa strain DSM 50071. PGPR characterizations shows that efficient isolate remain positive for catalase & HCN production and negative for ammonia production. IAA production remain 33.12 µM/ml, siderophore production found 89.36 µM/ml and phosphate solubilization activity remain highest among all. The results are promising for design of potentially active plant growth promoting P-05 strain based formulation which would be beneficial for crop improvement and crop protection. The potential of this strain could be investigated in detail and field application shall be studied for its biocontrol potential.

Table 1: Bacterial Isolates with different plant growth promoting activities

PGPR Isolates	Phosphate Solubilization	IAA Prod. µM/ml	Ammonia Prod.	HCN Prod.	Siderophore activity µM/ml	Catalase activity
P-01	+	06.23	-	-	48.27	+
P-02	+	12.46	-	+	56.73	+
P-03	+	19.88	-	+	63.34	+
P-04	+	14.35	-	+	60.07	+
P-05	+ (Highest)	33.12	-	+	89.36	+
P-06	+	23.96	-	-	59.21	+
P-07	+	29.58	-	-	60.34	+
P-08	+	25.29	-	-	61.65	+
P-09	+	09.19	-	-	92.10	+
P-10	+	21.99	-	-	60.53	+

Table 2: Biochemical characterization of bacterial isolates

Test	PGPR isolates									
	P-01	P-02	P-03	P-04	P-05	P-06	P-07	P-08	P-09	P-10
Oxidase	+	+	+	+	+	-	-	+	+	+
Catalase	+	+	+	+	+	+	+	+	-	+
Nitrate reduction	+	+	+	-	+	+	+	+	-	+
H_2S production	-	-	-	-	-	-	-	-	-	-
Citrate Utilization	+	+	+	+	+	-	+	+	+	+
Starch Hydrolysis	-	-	-	-	-	-	-	-	-	-
Pigment production	-	-	-	+	+	-	-	+	-	-
MR	-	-	-	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	+	+	+	+	-	-	+	-	-

Volume : 3 | Issue : 6 | June 2013 | ISSN - 2249-555X

Glucose fermentation	-	-	-	-	-	-	-	-	-	-
Lactose fermentation	-	-	-	-	-	-	-	-	-	-
Resistant to antibiotics										
Tetracycline	++	+	++	++	++	++	++	++	+	++
Gentamicin	++	++	++	++	++	++	+	++	+	++
Kanamycin	++	++	+	+	++	++	+	++	+	++
Co-Trimoxazole	-	++	+	++	+	++	-	++	-	+
Amikacin	++	++	++	++	++	++	++	++	+	++
Streptomycin	++	++	++	++	++	++	+	++	+	++
Chloramphenicol	+	+	+	-	-	+	++	+	+	-
Ampicillin	+	-	-	++	-	++	+	-	-	++

++ =Highly Susceptible, + = Susceptible, - = Resistance

REFERENCE

1. Villacieros, M., Power, B., Sanchez-Contreras, M., Loret, J., Oruzebal, R.I., Martin, M., Franandez-Pinas, F., Bouile, I., Whelan, C., Dowling, D.N., Rivilla, R. Colonization behaviour of Pseudomonas fluorescence and Sinorhizobiummeloti in the alfalfa (Medicago sativa) rhizosphere. Plant Soil, 2003; 251:47-54. | 2. Kloepper, J.W., Leong, J., Teintze, M., Schroth, M.N. Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. Nature, 1980; 268:885-886. | 3. Rangajaran, S., Saleena, L.M., Vasudevan, P., Nair, S. Biological suppression of rice diseases by Pseudomonas spp. under saline soil conditions. Plant Soil, 2003; 251:73–82. | 4. Bloemberg, G.V., Lugtenberg, B.J. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Current Opinion Plant Biology, 2001; 4:343–350. | 5. Moeinzadeh, A., Sharif-Zadeh, F., Ahmadzadeh, M., Heidari-Tajabadi, F. Biopriming of sunflower (Helianthusannuus) seed with Pseudomonas fluorescence for improvement of seed invigoration and seedling growth. Australian Journal of Crop Science, 2010; 4:564-570. | 6. Glick, B.R. The enhancement of plant growth by free living bacteria. Can J. Microbiol., 1995; 41:109–117. | 7. Gupta, A., Gopal, M., Tilak, K.V. Mechanism of plant growth promotion by rhizobacteria. Indian J. Exp. Biol., 2000; 38:856–862. | 8. Weller, D.M. Biological control of soil borne plant pathogens in the rhizosphere with bacteria. Ann. Rev. Phytopathol., 1988; 26:379–407. | 9. Mazzola, M., Cook, R.J., Thomashow, L.S., Weller, D.M., Pierson, L.S. Contribution of phenazine antibiotic biosynthesis to the ecological competence of Fluorescencepseudomonads in soil habitats. Appl. Environ Microbiol.,1992; 58: 2616-2624. | 10. Nagarajkumar, M., Bhaskaran, R., Velazhahan, R. Involvement of secondary metabolites and extracellular lytic enzymes produced by Pseudomonas fluoresces in inhibition of Rhizoctoniasolani, the rice sheath blight pathogen. Microbiology Research, 2004; 159(1): 73-81. | 11. Sakthivel, N. Gnanamanickam, S.S. Evaluation of Pseudomonas fluorescence for suppression of sheath rot disease and for enhancement of grain yields in rice (Oryza sativa).Applied Environmental Microbiology, 1887;53(1):2056-2059. | 12. Angayarkanni, T., Kamalakannan, A., Santhini, E., Predeepa, D. Identification of biochemical markers for the selection of Pseudomonas fluorescence against Pythium spp. Asian conference on Emerging Trends in Plant-Microbial Interactions. University of Madars, Chennai, 2005; Pp. 295-303. | 13. Gaur et al.Diacetylphloroglucinol producing Pseudomonas does not influence AM fungi in wheat rhizosphere. Current Science, 2004; 86: 453-457. | 14. Gordon, S.A., Paleg, L. Observations on the quantitative determination of indole acetic acid. Physiol. Plant, 1957; 10:39-47. | 15. Stolpe and Godkeri. Nonpathogenic members of genus Pseudomonas in the prokaryotes. Ed. Marthrier, Springer Verlag. New York, 1981; 719-741. | 16. Bergey's Manual of Systematic Bacteriology, 2nd Ed., 2, The Proteobacteia. Part C The Alpha-, Beta-, Delta- and Epsilonbacteria. |