Isolation and identification of *Pseudomonas fluorescens* from the soil of *Dulbergia sisso*

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Abstract
Pseudomonas fluorescens (PGPR) beneficial bacteria that settle the plant roots and improve the plant growth by an extensive variety of mechanisms. The use of PGPR gradually increasing in agriculture microbiology and offers an attractive way to replace chemical fertilizers and pesticides. In this study, an attempt was made to collect rhizospheric soil sample isolation and record of native *Pseudomonas* population from rhizospheric sample and to identify the native *Pseudomonas fluorescens* strains, an intoxicating bio control agent as well as PGPR in the rhizosphere under UV light and further characterize them morphologically and culturally. Maximum *P. fluorescence* strains showed positive PGPR activity. The study showed that *Pseudomonas* as an effective plant growth promoting bacterium.

Keywords: Bio fertilizer production, enhance the plant growth, *Pseudomonas fluorescens*, biological control, P-solubilizing activity

Introduction
Pseudomonas fluorescens includes a group of common, nonpathogenic saprophytes that colonize soil, water and plant surface environments. It is a common gram negative, rod-shaped bacterium. As its name indicates, it secretes a soluble greenish fluorescent pigment called fluorescein, particularly under conditions of low iron availability. It is an obligate aerobe, except for some strains that can utilize NO3 as an electron acceptor in place of O2. It is motile by means of multiple polar flagella. (Pandey R, Chavan PN, Walokar NM) *Pseudomonas fluorescens* has simple nutritional requirements and grows well in mineral salts media supplemented with any of a large number of carbon sources. Because they are well adapted in soil, *P. fluorescens* strains are being investigated extensively for use in applications that require the release and survival of bacteria in the soil. Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that actively colonize plant root and increase plant growth by production of various plant growth hormones, P-solubilizing activity, N2 fixation and biological activity. Few strains from genera such as Pseudomonas, *Azospirillium, Azotobacter, Bacillus, Burkholderia, Enterobacter, Rhizobium, Erwinia and Flavobacterium* are well known PGPRs. (Bhatia S, Maheswari DK, Dubey RC) Pseudomonas sp. is ubiquitous bacteria in agricultural soils and has many traits that make them well suited as PGPR chief among these are bio control of pathogens in agriculture and bioremediation of various organic compounds. Certain members of the *P. fluorescens* have been shown to be potential agents for the bio control which suppress plant diseases by protecting the seeds and roots from fungal infection. (VanRhijn P and Vanderleyden J. 1995) They are known to enhance plant growth promotion and reduce severity of many fungal diseases. This effect is the result of the production of a number of secondary metabolites including antibiotics, siderophores and hydrogen cyanide. *P. fluorescens* control pathogenic microorganisms in detail. Competitive exclusion of pathogens as the result of rapid colonization of the rhizosphere by *P. fluorescens* may also be an important factor in disease control. The present review discusses the occurrence, distribution, growth requirements of *P. fluorescens* and diseases controlled by the bacterial antagonist in different agricultural and horticultural crops. (Chen Y.P., Rekha P.D., Arunshen A.B)

Objectives of the study

1. Soil sample collection
2. Pseudomonas fluorescens isolates from rhizosphere soil
   - Preparation of soil dilution
   - Preparation of spread plates
3. Identification and characterization of bacterial isolates
   - Morphological characterization
   - Cultural Characterization
   - Biochemical and Physiological characterization

Soil Sample collection

Soil samples were collected from the location of Motherhood University, Roorkee, Bhagwanpur, (Uttarakhand) from cultivated land of rhizospheric soil *Dalbergia sissoo*. 40gm soil samples was collected up to the depth of 10 to 15cm from the rhizosphere of *Dulbergia Sisso.* The soil intimately adhering to the roots was collected and mixed to provide a composite. Soil sample collected in polyethylene bags, stored at field moisture level and room temperature. The reference Pseudomonas strain was procured from Microbiology division, PBRI, Haridwar, Uttarakhand.

Pseudomonas fluorescens isolates from rhizosphere soil

Preparation of Soil Dilutions

Firstly weigh out 20 g of soil sample in flask and add to 100ml of distilled water. Shake the suspension well and give label as "A".

Prepare six 9ml water blank and give the label as B to G, before the soil settles, remove 1 ml of the suspension with a sterile pipette from suspension A and transfer it to a 9-ml distilled water blank. Shake it well and give label as "B".

Repeat this dilution step five times, each time with 1 ml of the previous suspension and 9-ml distilled water blank. Label these sequentially as tubes C, D, E, F and G. This results in serial dilutions of 10^{-1} through 10^{-6} grams of soil per ml. (Deshwal VK, Punkaj Kumar)

Preparation of Spread Plates for Bacterial Culture

For the growth of bacterial colonies, take 6 pre-prepared Kings B medium plates and label them as B, C, D, E, F and G. Vortex samples B, C, D, E, F and G and pipette 0.1 ml onto each plate.

After this process, dip a glass spreader into ethanol. Place the spreader in a flame for a few seconds to ignite and burn off the ethanol. This will sterilize the spreader.

Hold the spreader above the first plate until the flame is extinguished. Open the plate quickly, holding the lid close by. Touch the spreader to the agar away from the inoculum (Inoculum = cells used to begin a culture) to cool, and then spread the drop of inoculum around the surface of the agar until traces of free liquid disappear. Replace the plate lid.

Re-flame the spreader and repeat the process with the next plate, working quickly so as not to contaminate the plates with airborne organisms.

Incubate the bacteria plates at room temperature for some time. Make sure the plates are inverted during the incubation to prevent drops of moisture from condensation from falling onto the agar surface. (Deshwal VK, Vig K, Amisha DM)

Enumeration

The plates incubated for a day at 30 ± 1°C were observed for the growth of *pseudomonas* colonies on KB plates and the colonies are enumerated manually and recorded. Results are presented in the Table1.

S.No	Plates replicates	*Pseudomonas fluorescens* (x 10 ^{6}cfu / gm soil)
1	P.P-1	4
2	P.P-2	3
3	P.P-3	3
4	P.P-4	2
5	P.P-5	2
6	P.P-6	2.65

Table 1: Microbial population in the rhizosphere soil of *dulbergia sisso*

Identification of Bacterial Isolates

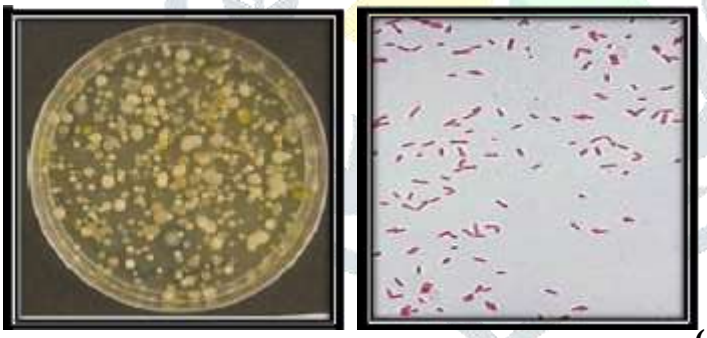
Morphological Characterization

All the 6 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 Akhtar M.S. and Siddiqui Z.A. (2009)

Cultural Characterization

Morphological characteristics of the colony of each isolate were examined on Nutrient agar and specialized medium and incubated for according to isolate. Cultural characterization of isolates observed by different characteristics of colonies such as shape, size, elevation, surface, margin, color, odour, pigmentation etc. were recorded as per Bergey's Manual of Determinative Bacteriology (Holt JG).

Plate 1: Plant growth promoting rhizobacterial growth on medium & Microscopic observation



(A) Pseudomonas colonies on Kings B agar medium **(B)** Microscopic Observation of Pseudomonas

Biochemical and Physiological Characterization

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

Starch Hydrolysis

Sterile starch agar plates were spotted with 10±l overnight broth cultures of the isolates and incubated at 28ºC for 24-48 h. After incubation, the plates were flooded with iodine solution. The formation of a transparent zone around the colony was taken as positive reaction for the test.

Hydrogen Sulfide Test

Sterilized Hydrogen sulfide- Indole-Motility agar stabs were inoculated along the wall of the tubes with overnight cultures of the isolates and incubated for 48 h at 28ºC. Visualization of black color along the line of inoculation indicated a positive reaction for the test.

Indole Production

Sterilized SIM agar slants were inoculated with the overnight cultures of the isolates and incubated for 48 h at 28ºC. Following incubation, 10 drops of Kovac's indole reagent were added to each tube. The isolates showing production of red color were recorded as positive for indole production.

Catalase Test

This test was performed to study the presence of catalase enzyme in bacterial colonies. Fresh cultures of Pure isolates were taken on glass slides and one drop of H_2O_2 (30 %) was added. Appearance of gas bubble indicated the presence of catalase enzyme.

Oxidase Test

The overnight cultures of the test isolates were spotted on plates poured with sterile trypicase soy agar and the plates were incubated for 24 h. at 28ºC. After incubation, 2-3 drops of N, N, N', N'- tetramethyl- p-phenylenediamine dihydrochloride (Wurster's reagent) were added onto the surface of growth of each test organism. The isolates showing change of color to maroon were noted as oxidase positive.

Gelatin liquefaction

The overnight cultures of the test isolates were inoculated to sterilized nutrient gelatin deep tubes and incubated for 24 h at 28ºC. Then the tubes were kept in the refrigerator for 30 min at 4ºC. The isolates showing liquefied gelatin were taken as positive and those which resulted in solidification of gelatin on refrigeration were recorded as negative for the test.

Methyl Red Test

Sterilized glucose- phosphate broth tubes were inoculated with the test culture and incubated at 28±2 ^{0}C for 48h. After incubation five drops of methyl red indicator was added to each tube and gently shaken. Red color production was taken as positive and yellow color production was taken as negative for the test.

Voges Prausker's Test

To the presterilized glucose-phosphate broth tubes, test cultures were inoculated and incubated at 37 ^{0}C for 48h. After incubation ten drops of Baritt's reagent A was added and gently shaken followed by addition of 10 drops of Baritt's reagent B. Development of pink colour in the broth was taken as positive for the test.

Citrate Utilization

Isolates were streaked on Simmon's citrate agar slants and incubated at 28±2 ^{0}C for 24h. Change in colour from green to blue indicates the positive reaction for citrate utilization.

Denitrification test

Sterilized nitrate broth tubes inserted with Durham's tube in inverted position were inoculated with overnight grown cultures of the test organisms and incubated at 25 ºC for 10-15 days. After incubation, the isolates which showed accumulation of gas in the Durham's tubes were scored as positive for denitrification.

Carbohydrate Utilization

All pure bacterial isolates were screened for the carbohydrate fermentation abilities using 4 different carbohydrates (lactose, sucrose, dextrose and mannitol) in Peptone broth medium. Bacterial isolates were inoculated in broth containing specific carbohydrate. The change in colour of Peptone broth was observed for utilization of particular carbohydrate present in broth.

Isolation of rhizobial *pseudomonas fluorescens*

The microbial population in the rhizospheric soil of *dulbergia sisso* was collected. Maximum population of *pseudomonas fluorescens* was found in the university farm. The *pseudomonas* population ranged between 1-6.0 x 10^6 cfu /g soil.

Cultural and Morphological Characterization

Pseudomonas fluorescens based on their colony morphology on different media, cell morphology and Gram reaction. The bacterial isolates were named according to the crop and cultural characters presented in Table 2 *Pseudomonas fluorescens* (6 isolates), based on their colony morphology on different media, cell morphology and Gram reaction presented in the Table 2.

Table 2: Labelling of isolates according to crop and cultural characters

	Pseudomonas fluorescens
Isolate name	**Soil sample/plant**
PP-1	*Dulbergia sisso*
PP-2	*Dulbergia sisso*
PP-3	*Dulbergia sisso*
PP-4	*Dulbergia sisso*
PP-5	*Dulbergia sisso*
PP-6	*Dulbergia sisso*

All the isolates developed small to medium, smooth, glistening colonies, out of the 8 isolates 5 isolates showed yellowish green color with light green pigmentation and the remaining isolates showed dull white colonies with no pigmentation. These isolates were Gram negative, small, single isolated rods without sporulation when observed under microscope and presented in the Table 3

Table 3: Cultural and morphological characteristics of *Pseudomonas fluorescens* isolates on King's B medium

S.No	**Characteristics of isolates**	**size**	**Colony shape**	**Color**	**Elevation**	**Surface**	**Margin**	**Pigmentation**	**Gram reaction**	**Shape**	**Sporulation**
1.	PP-1	Small	Round	Yellowish green	convex	Smooth	irregular	Yellowish green	Negative	Rod	Negative
2.	PP-2	Small	Round	white	Convex	Smooth, shiny	Regular	Bluish green	Negative	Rod	Negative
3.	PP-3	Small	irregular	Dull white	Convex	Smooth	Irregular	Light green	Negative	Rod	Negative
4.	PP-4	Small	Round	Yellowish green	Convex	Smooth, Shiny	Regular	yellowish green	Negative	Rod	Negative
5.	PP-5	Small	Round	White	Raised	Smooth, mucoid	Regular	Light green	Negative	Rod	Negative
6.	PP-6	Medium	irregular	Yellowish green	Convex	Smooth, shiny	Irregular	yellowish green	Negative	Rod	Negative

Biochemical and Physiological Characterization

After the study of cultural and cell morphology, the isolates of the *Pseudomonas fluorescens* (6 isolates) were tested for different biochemical test viz., IMVIC test, oxidase test, catalase test, carbohydrate fermentation, denitrification, H2S production, starch hydrolysis, gelatin liquefaction etc. All the 6 isolates of *Pseudomonas fluorescens* showed positive results for catalase test and oxidase test whereas they were negative for Voges Prausker's test. For methyl red test PP-1, PP-2, PP-3, PP-6 isolates showed positive results. Out of 6 isolates 4 isolates showed positive results for starch hydrolysis, only 2 isolates showed positive results for gelatin liquefaction, 6 isolates showed positive results for citrate utilization, only 5 isolates showed positive results for H2S test, denitrification, PP-1, PP-2, PP-3, PP-4 isolates respectively showed positive results. Results are presented in the Table 4.

Table 4: Biochemical and physiological characteristics of *Pseudomonas fluorescens* isolates

S. N.	Isolates	Indole test	MR	Vp test	Citrate utilization Test	Catalase test	Oxidase tset	Starch hydrolysis test	Gelatine liquefaction test	Denitrification	H2 S test	Carbohydrate Utilization			
												Lactose	Sucrose	Dextrose	Mannitol
1	PP-1	-	+	-	+	+	+	+	+	-	+	+	-	+	+
2	PP-2	-	+	-	-	+	+	+	+	+	+	+	-	+	+
3	PP-3	-	+	-	+	+	+	+	+	-	+	+	+	+	+
4	PP-4	-	-	-	+	+	+	-	+	-	+	+	-	-	+
5	PP-5	-	-	-	+	+	+	-	+	+	-	-	-	-	-
6	PP-6	-	+	-	+	+	+	+	+	-	-	-	-	+	-

Conclusion

Future study with these isolates using them in pot cultures and followed by field experiments will help in establishing their potential to be used as bio fertilizers. The data obtained in the present study suggest that *Pseudomonas flourescence* isolates would be ideal organisms for further study in pot culture and field experiments to exploit their PGPR potential for a good bio fertilizers production.

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