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To study of isolation and characterization of *Pseudomonas fluorescens* from rhizospheric soil samples

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Abstract
Production of the crop is affected by deficiency of fertilizers and low number of plant growth promoting rhizobacteria in soil. At the present study, Pseudomonas fluorescens isolates possess a variety of promising properties which make it a good plant growth promoting traits. A total of twenty-five *Pseudomonas fluorescens* isolates were obtained and characterized on the basis of their colony morphology, microscopy and biochemical test. Isolates growing *P. fluorescens* isolates on the King's B medium at 25ºc to 30ºc for 48 hours. The plates were exposed to UV light for 30 seconds and the isolate with pigment were exhibiting the fluorescence. All the isolates showed creamy translucent, mucoid, and circular shape colony morphology. Colonies having *Pseudomonas* like morphology were microscopically analyzed and those depicting rod shaped, gram negative bacteria were selected. All *Pseudomonas* isolates were further characterized by different biochemical test. It showed positive results in all the biochemical tests (Citrate utilization test, Methyl red test, Catalase test, Oxidase test, Nitrate reduction test, Urease test). Further, antibiotic sensitivity profiling of these isolates was done all the isolates were found resistant to Amoxyclav and Erythromycin and all were inhibited by the Ciprofloxacin by forming a clear zone of 15mm. These *Pseudomonas* isolates were tested for physiological efficiency on different pH (6, 7, and 8). All isolates grew well on alkaline medium of pH value 8. *P. fluorescens* for plant growth promoting activities in green house showed a positive result. These isolates can be used as potential biofertilizers and plant growth promoter.

Keywords: Growth Promoter, Isolation, Characterization, P fluorescens, Chemical fertilizers

Introduction
Conventional agriculture plays a significant role in meeting the food demands of a growing human population, which has also led to an increasing dependence on chemical fertilizers and pesticides. Chemical fertilizers are industrially manipulated, substances composed of known quantities of nitrogen, phosphorus and potassium, and their exploitation causes air and ground water pollution by eutrophication of water bodies. In general, 60% to 90% of the total applied fertilizer is lost and the remaining 10% to 40% is taken up by plants. In this regard, microbial inoculants have paramount significance in integrated nutrient management systems to sustain agricultural productivity and healthy environment. Recent efforts have been channelized towards the production of 'nutrient rich high quality food' to ensure bio-safety. The innovative view of farm production attracts the growing demand of biological based organic fertilizers as promising alternative to agro-chemicals. In agriculture, encouraging alternate means of soil fertilization relies on organic inputs to improve nutrient supply and conserve the field management. Such organic inputs will be of crucial importance to popularize organic farming including enhanced biodiversity of soil rhizosphere. **(**Adesemoye, A.O. and Kloepper, J.W. 2009) [1]

Among the biofertilizers nitrogen fixing microorganisms plays a crucial role to reduce the dependency on inorganic nitrogen sources. P. fluorescens has multiple flagella. It has an extremely versatile metabolism, and can be found in the soil and in water. It is an obligate aerobe, but certain strains are capable of using nitrate instead of oxygen as a final electron acceptor during cellular respiration. Some *P. fluorescens* strains present biocontrol properties, protecting the roots of some plant species against parasitic fungi such as Fusarium or the oomycete Pythium, as well as some phytophagous nematodes. (Araujo, A.S.F. and Santos, V.B. (2008) [2]. It is not clear exactly how the plant growth-promoting properties of P. fluorescens are achieved; theories include:

- The bacteria might induce systemic resistance in the host plant, so it can better resist attack by a true pathogen.
- The bacteria might outcompete other (pathogenic) soil microbes, e.g., by siderophores, giving a competitive advantage at scavenging for iron.
- The bacteria might produce compounds antagonistic to other soil microbes, such as phenazine-type antibiotics or hydrogen cyanide.

Materials and Methods

Collection of Soil Samples

4 Soil samples were collected from different locations of Motherhood University, Roorkee, Bhagwanpur, (Uttarakhand) from cultivated land. Soil Samples were collected from a depth of 0-10 c.m. in polyethylene bags, stored at field moisture level and room temperature. The reference *Pseudomonas* strain was procured from Microbiology division, PBRI, Haridwar, Uttarakhand.

Isolation of *Pseudomonas*

The pure *Pseudomonas* isolates were obtained on King's B Medium. Single colonies were picked up and were re-streaked on King's B Medium. Slants of same medium were prepared for the long time preservation of the isolates to be further utilized.

Colony and Cell Morphology

The morphological characteristics were studied for all isolates including the PBRI reference strain. The isolates were evaluated for different colony morphology creamy, white, transparent, translucent, mucoid, circular shape etc. All the colonies of different isolates were subjected to stain by the method of Gram's staining, and observations under microscope at 100X**. (**Gomare, K.S., Mese, M. and Shetkar, Y. 2013) [3].

Biochemical Characterization

The samples were examined for 10 different biochemical characteristics namely, Triple sugar iron agar test, Citrate utilization test, Methyl red test, Voges-Proskauer test, Catalase test, Oxidase test, Nitrate reduction test, Urease test, Starch hydrolysis test and Motility test with typical procedure. Triple sugar iron agar test is used to determine whether gram negative bacilli utilize glucose and lactose or sucrose fermentative and produce H2S. Phenol red and ferrous sulphate serves as indicator for acidification of medium and H2S production respectively. Citrate Utilization test is used to detect the ability of an organism to utilize sodium citrate as a sole source of carbon and ammonium salt as a sole source of nitrogen. Methyl Red (MR) test determines whether the microbe performs mixed acids fermentation when supplied glucose. Voges-Proskauer involves observation of red color reaction produced by appropriate culture media after treatment with potassium hydroxide. The active product in the medium formed by bacterial metabolism is acetyl methyl carbinol, a product of the butylenes glycol pathway. Catalase test was used to determine the presence of catalase, an enzyme that catalyses the release of O2 and H2O from hydrogen peroxide (H2O2). **(**Jimenez, D.J., Montana, J.S. and Martinez, M.M. (2011) [4]. The oxidase test was used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome c oxidase oxidizes the reagent (tetramethyl-p phenylenediamine) to (indophenols) purple color end product. Nitrate reduction test is used for the differentiation of members of Enterobacteriaceae on the basis of their ability to produce nitrate reductase enzyme that hydrolyze nitrate (NO3–) to nitrite (NO2–) which may then again be degraded to various nitrogen products like nitrogen oxide, nitrous oxide and ammonia (NH3) depending on the enzyme system of the organism and the atmosphere in which it is growing. **(**Megali, L., Glauser, G. and Rasmann, S. (2013) [5].

Urease test involves hydrolysis of urea with the release of ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink. Starch hydrolysis test involves the bacterial ability to hydrolyse starch, by the amylase enzyme. While the starch forms dark blue color with iodine, its hydrolyzed products do not acquire such dark blue color with iodine. Motility test was used to detect the ability of an organism to move by itself by means of propeller like flagella unique to bacteria or by special fibrils that produce a gliding form of motility. Sulphide Indole Motility (SIM) medium is a combination differential medium that tests three different parameters, Sulfur Reduction, Indole Production and Motility. (Mishra, D.J., Singh, R., Mishra, U.K. and Kumar, S.S. (2013) [6] This media has a very soft consistency that allows motile bacteria to migrate readily through them causing cloudiness in the stabbed area.

Antibiotic sensitivity profiling test

In Antibiotic sensitivity test, the disc diffusion method was used to identify the sensitivity for Ciprofloxacin (Cf), Cloxacillin (Cx), Co- Trimoxazole (Co), Tetracycline (T), Amoxyclav (Ac), Cephalexin (Cp), Clindamycin (Cd), Tetracycline (T) and Erythromycin (E) antibiotic.

pH Tolerance Test

For pH stress tolerance capacity, the *Pseudomonas* isolates were tested on King's B medium agar plate with pH (6.0, 7.0, and 8.0) by spot inoculation from log phase culture. Plates were incubated at 25˚-30˚C for 48 hours. (Raja, N. (2013) [7].

Table 1: Studies on various biochemical tests for *Pseudomonas* isolates.

sample code	Biochemical Tests									
	TSI	CU	MR	VP	CT	OT	NR	UT	SH	MT
A-1	+	+	+	+	_	+	_	+	+	+
A-2	+	+	+	+	+	+	+	+	+	+
A-3	_	+	_	_	_	+	_	_	_	+
A-4	+	+	+	+	_	+	+	_	+	+
A-5	+	+	+	+	+	+	_	_	_	+
A-6	_	+	_	_	_	+	+	_	+	+
A-7	+	+	+	+	_	+	_	_	_	+
A-8	+	+	+	+	+	+	+	+	+	+
A-9	+	+	_	_	_	+	_	_	_	+
A-10	_	+	_	+	_	+	_	_	+	+
A-11	+	+	+	+	_	+	_	+	+	+
A-12	+	+	+	+	+	+	_	_	+	+
A-13	+	+	_	_	_	+	+	_	_	+
A-14	_	+	_	_	_	+	+	_	_	+
A-15	+	+	+	+	_	+	+	_	_	+
A-16	+	+	+	+	+	+	+	+	+	+
A-17	+	+	+	_	+	+	_	_	_	+
A-18	_	+	_	_	+	_	+	_	_	+
A-19	+	+	+	+	+	+	_	_	_	+
A-20	+	+	_	_	+	+	+	+	_	+
A-21-PBRI	+	+	_	_	+	+	_	_	_	+

TSI= Triple sugar iron agar test, CU= Citrate utilization test, MR= Methyl red test, VP=Voges-Proskauer, CT= Citrate test, OT= Oxidase test, NR= Nitrate reduction test, UT= Urease test, SH= Starch hydrolysis test, MT= Motility test.

Table 2: Growth of different *Pseudomonas isolates* at different pH medium.

S.No	Sample codes	Growth Medium		
		pH 6	pH 7	pH 8
1.	A-1	+	++	++
2.	A-2	+++	++	+++
3.	A-3	+	++	++
4.	A-4	++	++	++
5.	A-5	+++	+++	+++
6.	A-6	++	++	++
7.	A-7	+++	+++	+
8.	A-8	+++	++	++
9.	A-9	++	+	+
10.	A-10	+	++	+++
11.	A-11	++	++	+++
12.	A-12	+++	++	++
13.	A-13	++	_	++
14.	A-14	+	++	+
15.	A-15	_	+	++
16.	A-16	++	++	++
17.	A-17	++	_	+++
18.	A-18	+	+	++
19.	A-19	_	_	+
20.	A-20	_	_	+
21.	PBRI Strain	+++	+++	+++

+++ =Best Growth, ++ = Good Growth, + = Less Growth, - = No

Results and Discussion

A total of Twenty five soil samples were cultured on King's B Medium. The pure *Pseudomonas* isolates were identified based on their colony morphology. The creamy/ white transparent, shining, mucoid, smooth and circular colonies was selected as *Pseudomonas* isolates traits as presented in Fig.1. Subsequent identification of twenty five different isolates was studied based on their cell morphology and Gram's staining reaction under microscope. All the isolates were gram negative with pink colored and rod shaped cells as shown in Fig.2. Studies of Santos, V.B., Araujo, S.F., Leite, L.F., Nunes, L.A. and Melo, J.W. (2012) [8]. also reported similar colony and cell morphology, signifying that *Pseudomonas* is a Gram negative, rod shaped bacteria.

Biochemical characterizations were made of all isolates including the PBRI reference strain. Isolates A-2, A-8, A-16, A-23, A-24 and A-28 showed positive results in all the biochemical tests, whereas 24 other isolates showed variable results for different biochemical tests. It indicates that isolates collected from various locations were distinct. All the isolates were inhibited by Ciprofloxacin (Cf) and Co- Trimoxazole (Co) as evident by formation of clear zone around the antibiotic disc. The largest clear zone formed by Ciprofloxacin was 15mm. The isolates were less inhibited by other antibiotics used in the studies as evident by formation of smaller clear zone around the antibiotic disc as compared to Cf and Co. (Fig. 3). All the isolates were found resistant to Amoxyclav (Ac), and Erythromycin (E). Studies of Sindhu, S.S., Grover, V., Narula, N. and Lakshminarayana, K. (1989) [9] also reported similar All the isolates (A-1 to A-30) including PBRI reference strain showed good growth at pH 8. At pH 7, isolates A-13, A-17, A-19 and A-20 showed no growth. Results showed that isolates namely A-5, A- 7, A-27 A-29 and PBRI strain had maximum growth at pH 7. At pH 6, all isolates grew well except A-15, A-19 and A-20. These results showed that the isolates are distinct. Keeping the above results in view it will be worthwhile to further characterize the above isolates at molecular level and their efficiency for nitrogen fixation in various field crops.

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