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# Characterization of Fluorescent *Pseudomonas:* Potential Candidates for Liquid Bioinoculant

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***Abstract* – The rhizospheric species of fluorescent *Pseudomonas* are the eminent biological control agents. These are not only known for alternative to agrochemicals for controlling plant diseases but also promote plant growth. Amongst the PGPRs, fluorescent *Pseudomonas* have emerged as the largest and potentially the most promising group of PGPR, because of their potential to act as a biocontrol and biofertilizing agents. In the present study fluorescent *Pseudomonas* (FP) isolates FP-1, FP-2 and FP-3 were isolated from the rhizospheric soil of Cotton fields of village Shivasawanga located in Nagpur district, Maharashtra, India. The isolates were subjected to standard morphological, microbiological, biochemical profiling and further characterized for molecular analysis. On the basis of 16S rRNA analysis, nucleotide homology and phylogenetic analysis the strains FP-1, FP-2 and FP-3 were identified as *Pseudomonas fluorescens* strains. The study aims at developing liquid biofertilizer by using some competent strains of fluorescent *Pseudomonas.* By formulating such liquid biofertilizer, the better yield of crops in the Vidarbha region can be achieved.**

***Keywords* – Biological Control, *Fluorescent Pseudomonas*, PGPR, Rhizobacteria, Antagonism.**

## I. INTRODUCTION

Biological method for control of plant pathogens offers a highly effective, economical and environmental friendly alternative to the use of synthetic fertilizers [1]-[2]. The intensive use of chemical fertilizers for protection of crops against phytopathogens which are developing resistance in plant pathogens as well as demolishing the soil health, hence the use of biological methods is a significant approach in modern agriculture practices [3]-[4]. The naturally occurring soil organisms including fluorescent *Pseudomonas* promote the growth of host plant by a variety of mechanisms. Fluorescent *Pseudomonas* belongs to plant growth promoting rhizobacteria (PGPR), which actively colonize plant roots and promote plant growth by direct or indirect mechanism [5]. Direct mechanism of plant growth includes production of phytoharmones [6], solubilization of phosphate and other minerals [7], while their indirect mechanism includes production of antibiotics [8] and siderophore [9] which are suppressive to phytopathogens. Fluorescent *Pseudomonas* achieve biocontrol by production of antibiotics such as 2,4-diacetylphloroglucinol (PHL), pyoluteorin (PLT), pyrrolnitrin (PRN), phenazine-1-carboxyclic acid (PCA), 2- hydroxy phenazines and phenazine-1-carboxamide (PCN) [10]. These antibiotics play a critical role in disease management as they inhibit the phytopathogens at low concentration. In addition, to produce antimicrobial agents they also induce a systemic resistance in plants [10].

The use of biological control agents for controlling plant diseases is one of the most promising method for sustainable agriculture development. Fluorescent *Pseudomonas spp.* offer an excellent biocontrol against a range of phytopathogenic bacteria and fungi. It is noted that rhizospheric bacteria of a particular crop may be the efficient diseases controller than bacteria originally isolated from other plant species [11]-[5]. Therefore, screening of locally adapted biocontrol strains is essential [5]. The present study is based on isolation, characterization and evaluation of potential strains of fluorescent *Pseudomonas spp*. for their intensive use in commercial world.

## II. MATERIALS AND METHODS

### *2.1. Soil sampling and isolation of fluorescent bacteria*

Root adhering soil samples were collected from Cotton plant rhizospheric region grown in field of Shivasawanga which is located near Nagpur. A soil suspension was prepared by shaking 1g of soil sample having 2-3 cm undamaged root pieces with tightly adhered soil in 100 ml of sterile distilled water and kept for 1hr on a rotary shaker to release the rhizoplane bacteria. The processed samples were serially diluted from $10^{-1}$ to $10^{-6}$ and 0.1 ml of the suspension was spread on to King's medium B ( KMB ) agar plate and incubated at 28°C for 48h. The occurrence of fluorescent *Pseudomonas* was examined under UV light (356 nm) by using Spectroline Ultravoilet Transilluminator. On these preliminary identification basis three isolates namely FP1, FP2, FP3 were identified as fluorescent *Pseudomonas* which fluorescenced under UV-light ( fig. 1).

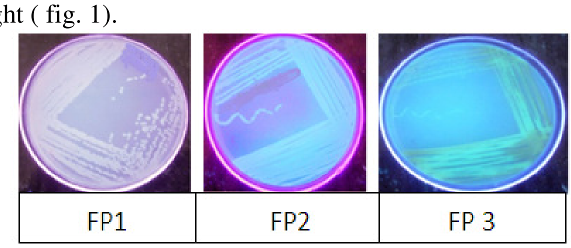


Fig.1. Fluorescent Pseudomonas isolates under UV light

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*2.2. Purification and Preservation of isolated bacterial culture*

Colonies showing fluorescence under UV-transilluminator (365 nm) were picked up and enriched in KMB broth. Thereafter these were streaked on KMB plates and preserved on KMB slants. The slants were covered with mineral oil and preserved in the refrigerator at 4°C for further use. Again the colonies were enriched in KMB broth and preserved in 20% glycerol at -20$^0$ C for further use.

*2.3. Preliminary Characterization of isolates*

Standard microbiological and biochemical tests were performed for rapid identification of isolates. For oxidase test, bacterial cultures on KMB agar plate was placed on Himedia's bacteriological differentiation Oxidase disc for Oxidase testing. Oxidase reaction was carried out by touching and spreading a well isolated colony on oxidase disc. The reaction was observed within 5-10 seconds. For catalase test, bacterial inoculum on the KMB agar plate was placed on glass slide and suspended in a drop of 3% $H_2O_2$. Gelatin hydrolysis was performed by the Nutrient Gelatin stab method as describe by ASM MicrobeLibrary. The heavy inoculums of an 18-24 hours old test bacteria was picked up and stab-inoculated into tubes containing Nutrient Gelatin. The inoculated tubes and an uninoculated control tubes were incubated at the test bacterium's optimal growth temperature for up to 1 week, checking everyday for gelatin liquefaction. To confirm that liquefaction was due to gelatinase activity, the tubes were immersed in an ice bath for 15-30 minutes. Afterward tubes were tilted to observe if gelatin has been hydrolyzed. Nitrate reduction test was performed by using Himedia's Nitrate reduction disc.

*2.4. Optimization of different media for growth and fluorescence*

The growth and fluorescence pattern of isolates – FP1, FP2, FP3 were analyzed further on different media such as King's B media, Pseudomonas Agar ( for fluorescein ), Pseudomonas Agar Base, Nutrient agar, Citrimide agar with their respective pH and incubated at 28$^0$ C to determine optimum medium constituent at which they show maximum growth and fluorescence.

*2.5. Bacterial DNA extraction, PCR amplification, sequencing and identification*

Bacterial DNA was isolated by using HiPurA$^{TM}$ Bacterial and Yeast Genomic DNA Purification Spin Kit HIMEDIA. Amplification of 16s r DNA genes were performed by using universal primer as 16SF: 5' AGA GTT TGA TCC TGG CTC AG 3' and 16SR: 5' ACG GCT ACC TTG TTA CGA CTT 3' for all the three isolates ( FP1,FP2,FP3 ). Amplification was performed in Bio-RAD My Cycler$^{TM}$ thermal cycler (PCR) machine and amplification cycle was kept as follows: The initial denaturation (2 min at 94 °C) was followed by 30 PCR cycles (94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s) and a final extension at 72° C for 10 min. The amplified products of each isolate were electrophoresed on a 0.7% agarose gel in 1x TAE at 100V for one hour. The gel was stained with Ethidium Bromide and the PCR products were visualized by using UV transilluminator. PCR products were purified further using commercial kit (Axygen PCR purification kit), then purified PCR products were send for sequencing. Further sequences were subjected to BLASTN search algorithm and identified for genus and species. The Phylogenetic trees were constructed using the MEGA software.

*2.6. Screening the isolates for phosphate solubilization activity*

Phosphate solubilization activity of all the three isolates were checked on Pikovskaya Agar plates by agar well diffusion method. Bacterial cultures were grown in KMB broth and 50 ul were poured in well and incubated the plates at 28$^0$ C. Phosphate solubilization was determined by clear halo formation after 3 days of incubation.

*2.7. Screening of isolates for indol acetic acid production:*

Determination of indol acetic acid production (IAA) was performed according to method described by Ramyasmruthi S. *et al.* Briefly, Bacterial cultures were grown in KMB broth supplemented with Tryptophan (5mg/ml) for 24 hrs at 28$^0$ C. Fully grown bacterial cultures were centrifuged for 30 min at 3000 rpm. The culture filtrate 2 ml mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M FeCl3 solution).

## III. Growth Curve

The activity of isolates FP 1, FP 2, FP 3 were observed at different time interval. After every 30 min of duration, the O.D. at 600 nm was taken by using Halo DB-20S V-VIS double beam spectrophotometer.

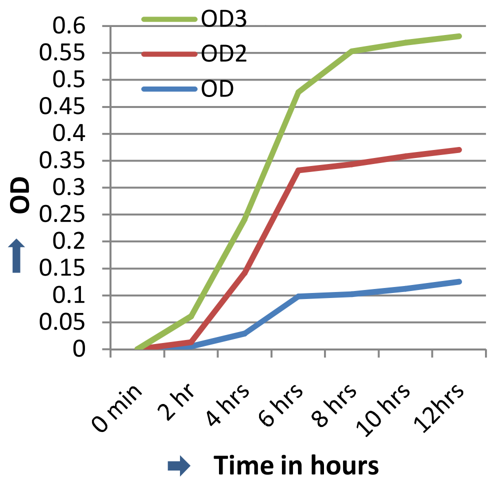


Fig.2. Growth curve of isolates

## IV. Antibiotic Sensitivity Assay

Isolates were screened for their antimicrobial susceptibility test by using Himedia Icosa Pseudo-1 disc against 20 antibiotics (table 3).

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## V. Results and Discussion

*Isolation, purification and characterization of fluorescent pseudomonas isolates*

The fluorescent *Pseudomonas spp.* are the ecologically important group of bacteria in the field of agriculture and their effectiveness for supporting the plant growth and control over the phytopathogens has been proved in numerous studies [12]-[13]-[4]. The proper isolation of bacteria from rhizosphere is necessary, for the identification of potential biocontrol and biofertlizing agents [14]-[4]. The objective of this study was to isolate and characterize the fluorescent *Pseudomonas* bacteria from rhizospheric soil of selected plants in and around Nagpur region.

In the present study, three bacterial strains were isolated from the cotton plants rhizospheric soil collected from agricultural field of Shivasawanga, district Nagpur, Maharashtra, India. The isolates were found to be large, circular, convex with an entire margin when observed on King's B media. These isolates were further purified and characterized, all were found to be fluorescent, gram-negative, rod shaped (fig.3), produced pigments on King's B media and Pseudomonas agar (for fluorescin).

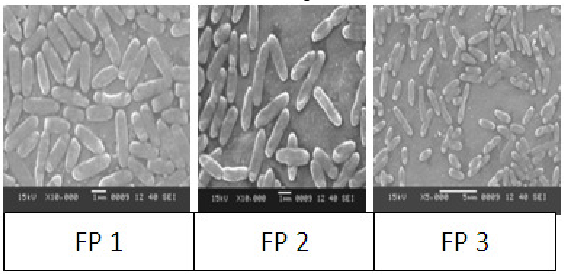


Fig.3. SEM analysis of fluorescent Pseudomonas isolates

The representative isolates were denoted as FP1, FP2, FP3. The isolates FP 1, FP 2 and FP 3 showed positive results when subjected to Oxidase test. After application of bacterial culture Oxidase disc turned deep purple blue. Also all the isolates gave positive results for catalase test as the bubble formation was observed after addition of 3 % $H_2O_2$. Isolates which were found motile, catalase and oxidase positive, confirming them to be *Pseudomonas* spp. [15]-[16]. According to Joseph, B. *et al*, it is stated that the bacterial strains which show catalase positive activity, must be highly resistant to environmental, mechanical and chemical stress [17]. Gelatin hydrolysis was absent in FP2 while FP1 and FP3 showed hydrolysis. The hydrolysis of gelatin in the isolates FP1 and FP3 was due to presence of gelatinases which were secreted extracellularly and digested gelatin. Because of the absence of gelatinase, no hydrolysis of gelatin was observed in FP2. The nitrate reduction test was performed, to determine the ability of the isolates to reduce nitrate to nitrite and further to the free nitrogen gas. All the isolates were found to be nitrate positive, when characterized for nitrate reduction test.

The growth and fluorescence pattern of isolates were observed by using different media such as King's B media, Pseudomonas Agar (for fluorescein), Pseudomonas Agar Base, Nutrient agar, Citrimide agar. The greater growth and fluorescence was observed on King's B media and Pseudomonas Agar (for fluorescein), intermediate on Pseudomonas Agar Base, Nutrient agar and low on Citrimide agar (table 2).

Table I. Characterization of isolates

| Characteristics | FP1 | FP2 | FP3 |
|---|---|---|---|
| Gram's staning | -ve | -ve | -ve |
| Motility | + | + | + |
| Fluorescent ( F ) | F ( + ) | F ( + ) | F ( + ) |
| Oxidase | + | + | + |
| Catalase | + | + | + |
| Gelatin hydrolysis | + | - | + |
| Nitrate reduction | + | + | + |
| Phosphate solubilization | + | + | + |
| Indol acetic acid production | + | + | + |
| Molecular identification as | *P. fluore-cens strain FP18* | *P. fluore-cens strain CB32* | *P. sp. PcFRB0 39* |

Table II. Optimization of diffent media for observing growth and fluorescence of isolates

| Media | FP1 (G & F) | FP2 (G & F) | FP3 (G & F) |
|---|---|---|---|
| King's B | + + + | + + + | + + + |
| Pseudomonas Agar ( for fluorescein ) | + + + | + + + | + + + |
| Pseudomonas Agar Base | + + + | + + | + + + |
| Nutrient Agar | + + + | + + | + + |
| Citrimide Agar | + | + | + + |

G : Growth ; F : Fluorescence
For highest G & F : + + + ,
For medium G & F : ++ ,
For lowest G & F : +

### *16s rDNA sequence analysis*

For the identification of soil bacteria, 16S RNA analysis was performed. PCR amplicons of all the three isolates were electrophoresed on 0.7% agarose gel in 1x TAE at 100V for one hour. Using aligner software consensus sequence of 736 bp, 1142 bp, 1063 bp rDNA genes were generated by using forward and reverse primer ( fig. 4 ).

### *Phylogenetic analysis*

To understand the Phylogenetic relationship, sequence analysis of 16 S rDNA genes were done to carry out BLAST with NCBI GeneBank database. Using Multiple Sequence Alignment ClustalW software, sequences were selected which showed similarity with closely related strains and aligned. Further Phylogenetic trees were constructed using MEGA 4 (fig.5). Based on nucleotide homology and Phylogenetic analysis the isolates were identified as *Pseudomonas fluorescens strain FP18, Pseudomonas fluorescens strain CB32*, *Pseudomonas sp. PcFRB03*.

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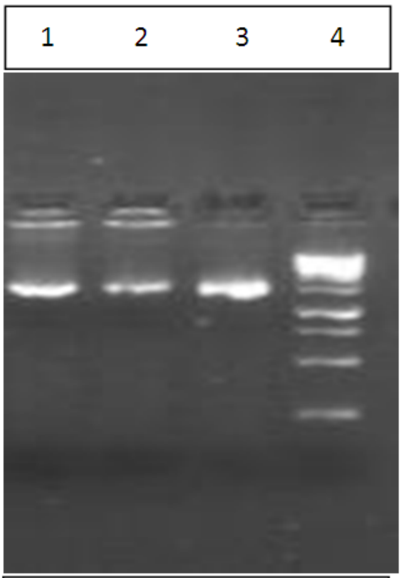


Lane 1:16S rDNA gene (FP 1)
Lane 2: 6S rDNA gene (FP 2)
Lane 3:16S rDNA gene (FP 3)
Lane 4: 3000 bp ladder

Fig.4. 16S RNA analysis of isolates

*Plant growth promoting rhizobacteria (pgpr ) traits of isolates*

The isolates (FP1, FP2, FP3) when screened for IAA production and phosphate solubilization were found to be positive for this PGPR traits. Isolates FP1, FP2 and FP3 were found to be phosphate solubilizing bacteria which showed clear zone on Pikovskaya agar plate after three days of incubation at $28^0$ C ( fig. 6). When screened for IAA production, isolate FP2 was found powerful IAA producer as compared to FP1 and FP2 (fig.7).

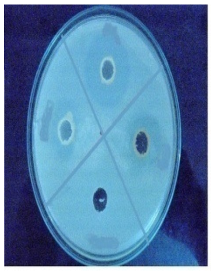

Fig.6. Isolates showing phosphate solubilization

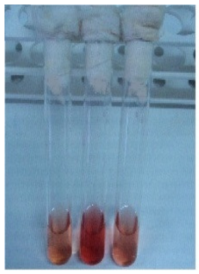

Fig.7. Isolates showing indol acetic acid production

The isolates have been characterized for its ability to produce IAA with and without use of L- tryptophan in medium. Higher amounts of IAA were yielded in the media with tryptophan for all the three isolates after 24h and 48h of incubation. Results from this study revealed that the level of IAA production was higher with the use of tryptophan (5 mg/ml) than without tryptophan. Isolate FP2 was found foremost IAA producer as compared to FP1 and FP2. This result was found similar to that described by M. Maleki, 2010. From the decomposition of roots, tryptophan can be produced in the rhizosphere and the amount of tryptophan produced can vary strongly among plant species [4]-[19]. Indol acetic acid producing bacteria, is found to be responsible for growth of roots, increases root surface area and root length which support the plants to absorb nutrients from soil [20]-[21]. *Pseudomonas spp.* were identified as efficient phosphate solubilizing rhizobacteria [22]. In the present study, all the three isolate showed phosphate solubilization on Pikovskaya agar medium and isolate FP2 showed higest efficacy for phosphate solubilization as compared to FP1 and FP3. The phosphate solubilization in these isolates was due to secretion of the organic acid and phosphatase, which was responsible for conversion of insoluble form of phosphorus into soluble form that can be utilized by plants.

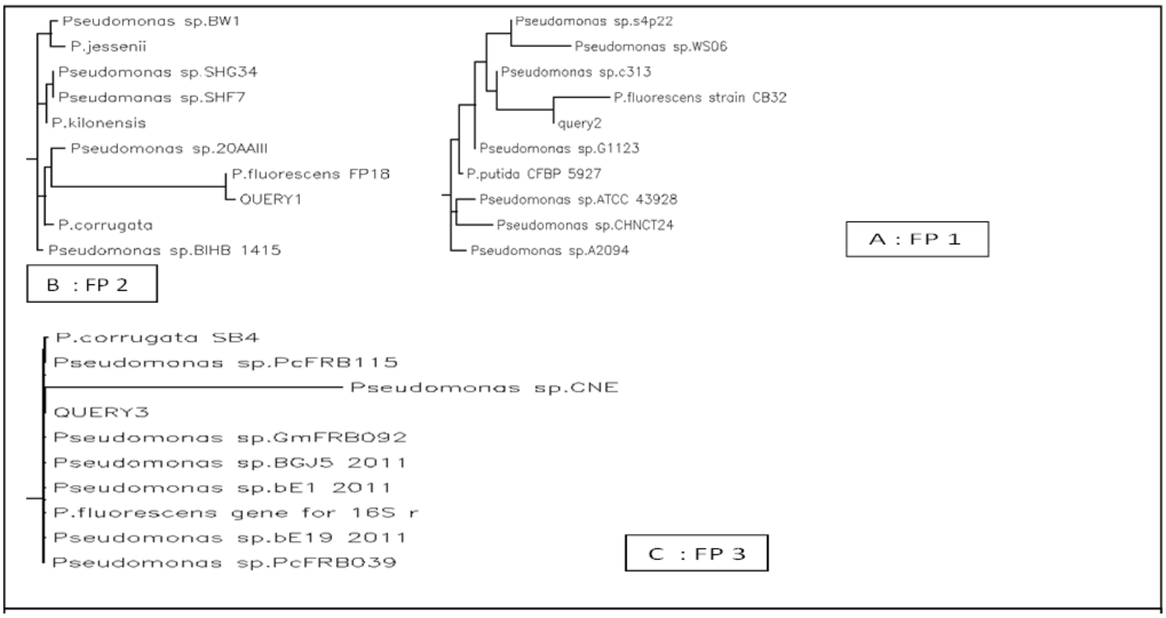


Fig.5. Phylogenic analysis of isolated fluorescent Pseudomonas strains based on 16S rDNA by using Clastal W software

Table III. Antimicrobial susceptibility test

| Antibiotics | Conc. | Zone of Inhibition (in mm) | | | Susceptible(S)/ Intermediate(I)/Resistant(R) | | |
|---|---|---|---|---|---|---|---|
| | | FP 1 | FP 2 | FP 3 | FP 1 | FP 2 | FP 3 |
| Amikacin | 10 mcg | 22 | 20 | 19 | I | I | I |
| Gentamicin | 10 mcg | 25 | 22 | 15 | S | S | S |
| Imipenem | 10 mcg | 40 | - | 20 | S | R | S |
| Carbenicillin | 100 mcg | 20 | 10 | 30 | S | R | S |
| Pipercillin | 100 mcg | 20 | 6 | 25 | I | R | S |
| Aztreonam | 30 mcg | 20 | - | 20 | I | R | I |
| Tobramycin | 10 mcg | 28 | 24 | 20 | S | S | S |
| Norfloxacin | 10 mcg | 25 | 19 | 22 | S | S | S |
| Ciprofloxacin | 5 mcg | 27 | - | 25 | S | R | S |
| Ticarcillin | 75/10 mcg | 28 | - | 10 | S | R | R |
| Cefoperazone | 75 mcg | 30 | 9 | 7 | S | R | R |
| Azlocillin | 75 mcg | 24 | 18 | 20 | S | S | S |
| Levofloxacin | 5 mcg | 30 | 10 | 30 | S | R | S |
| Ticarcillin | 75 mcg | 23 | 8 | 14 | S | R | R |
| Ofloxacin | 5 mcg | 30 | 22 | 8 | S | S | R |
| Mezlocillin | 75 mcg | 22 | 18 | 2 | S | S | R |
| Gatifloxacin | 5 mcg | 25 | - | 3 | S | R | R |
| Piperacillin | 100/10mcg | - | - | 10 | R | R | R |
| Ceftriaxone | 30 mcg | 10 | 25 | 10 | R | S | R |
| Netillin | 30 mcg | 24 | 12 | 23 | S | R | S |

## VI. Conclusion

In order to control over various plant diseases biological methods are advantageous than the use of harmful pesticides and chemicals. Among PGPR, fluorescent *Pseudomonas s*pp. not only have plant growth promotional capacity but also they have phytopathogenic activity against various plant pathogenic bacteria and fungi. So, through the isolation and characterization of fluorescent *Pseudomonas* potential candidates, one can be effectively used them as a substitute for chemical fertilizers in order to achieve sustainable agriculture approach. In present investigation, three strains of fluorescent *pseudomonas spp.* were isolated which may represent biological alternative to the destructive chemical fertilizers. They showed phosphate solubilization , nitrate reduction and indol acetic acid production which may promote plant growth directly or indirectly and hence due to their potential these bacterial strains could be further used as effective biofertilizing agents in the agricultural practices. Further, the field demonstration trials of these isolates would be taken up for their possible prospective applications as a liquid biofertilizer.

## Acknowledgement

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