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Bio-efficacy of *Pseudomonas fluorescens* isolated from chickpea fields as plant growth promoting rhizobacteria

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Abstract

Chickpea is an economically important food crop, which is subjected to infection by a host of fungal, viral and bacterial pathogens. Thirty isolates of *Pseudomonas fluorescens* were isolated from the rhizosphere of Chickpea fields. These were tested against *F. oxysporum* in dual culture method. Among these, four (Pf 1, Pf 3, Pf 5 and Pf 8) isolates were showed bright fluorescence under UV light were further tested. All the cultural and biochemical studies confirmed them to be *P. fluorescens*. The isolates also showed positive response for siderophore production and plant growth promoting activity on Chickpea cultivar Bital 98. Among these isolates Pf 3 and Pf 5 shown significant results by increasing root length and shoot length. Both the Pf 3 and Pf 5 isolates were found significantly superior than other isolates in increasing the shoot length (12.7 cm) and root length (24.5 cm) over control. The isolates Pf 3 was recorded high vigor index (3830) followed by Pf 5 (3648). The least vigor index was recorded by Pf 1 (2631).

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Introduction

Biological control of plant pathogens by antagonistic microorganisms is a potential non-chemical means (Harman, 1991) and is known to be a cheap and effective eco-friendly method for the management of crop diseases (Cook and Baker, 1983). The use of biological control agents as an alternative to fungicides is increasing rapidly in the present day agriculture due to the deleterious effects of chemical pesticides. Members of the genus *Pseudomonas* and *Trichoderma* have long been known for their potential to reduce the plant disease caused by fungal pathogens and they have gained considerable importance as potential antagonistic microorganisms (Pant and Mukhopadhyay, 2001). Among these the bacterial antagonists have the twin advantage of faster multiplication and higher rhizosphere competence hence, *P. fluorescens* have been successfully used for biological control of several plant pathogens (Ramamoorthy *et al*., 2002) and biological control using PGPR strains especially from the genus Pseudomonas is an effective substitute for chemical pesticides to suppress plant diseases (Compant *et al*., 2005).

The soil bacteria that aggressively colonize the root zone and promote plant growth are generally termed as Plant Growth Promoting Rhizobacteria (PGPR) and primarily *Pseudomonas fluorescens* is identified as an important organism with ability for plant growth promotion and effective disease management properties (Mazzola *et al*., 1992). Their applicability as biocontrol agents has drawn wide attention because of the production of secondary metabolites such as siderophore, antibiotics, volatile compounds, HCN, enzymes and phytohormones (Nagarajkumar *et al*., 2004). In vitro antibiosis of *P. fluorescens* strains towards the Chickpea sheath rot pathogen *F. oxysporum* demonstrated that this pathogen is sensitive to *P. fluorescen* (Sakthivel and Gnanamanickam, 1987). The biocontrol mechanism to suppress fungal pathogens by *Pseudomonas* spp. normally involves the production of antibiotics and *P. fluorescens* has a gene cluster that produces a suite of antibiotics, including compounds such as 2,4-diacetylphloroglucinol (DAPG), phenazine, pyrrolnitrin, pyoluteorin and biosurfactant antibiotics (Angayarkanni *et al*., 2005). Fluorescent *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. However, the native isolates of certain biocontrol agents showed superiority over other isolates for the management of crop diseases (Dubey and Patel, 2001).

Therefore to exploit the potential of native strains of *P. fluorescens*, the present study was aimed at isolation, characterization, and *in-vitro* screening of rhizhosphere isolates of *P. fluorescens* from Chickpea fields against *F. oxysporum*.

Materials and methods

Isolation of Pseudomonas fluorescens

Isolation of *P. fluorescens* was made from rhizhosphere of Chickpea fields in Punjab district of Pakistan. The 10 cm rhizosphere soil particles loosely adhering to the roots were gently teased out and the roots were cut into small pieces and mixed well. The soil thus obtained was crushed in a sterile mortar and pestle and shaken with 100 ml of sterile distilled water for 10-20 min. to obtain standard soil suspension. Isolation of *P. fluorescens* was made by following the serial dilutions and pour plate method using the specific King's B medium.

Pour plate method

King's B medium, a selective one (Kings *et al*., 1954) was used for the isolation of *P. fluorescens*. One ml of soil suspension from aliquot dilutions (10^5 to 10^8) was aseptically added to sterile Petri plates containing twenty ml of sterile medium and incubated at 28±2°C for 48 h. After incubation, well separated individual colonies with yellow green and blue white pigments were marked and detected by viewing under UV light.

The individual colonies were picked up with sterile loop and transferred to fresh King's B slants and the pure cultures so obtained were stored in refrigerator at 4°C for further use.

Morphological characterization

Pure cultures of the selected isolates were streaked on King's B agar Petri plates separately for colony development. The individual colonies were examined for shape, size, structure of colonies and pigmentation.

Biochemical tests for P. fluorescens

For the identification of *P. fluorescens*, certain biochemical tests were conducted according to Bergey's Manual for Determinative Bacteriology (Breed *et al.*, 1989).

Gram staining

A loopful bacterial culture was transferred on a clean slide and a smear was made which was air dried and heat fixed. The smear was flooded for one min. with ammonium oxylate crystal violet. Excess strain was poured off and the slide was washed in a gentle stream of water. Lugol's iodine solution was applied and allowed to remain for one min. decolorized with 95 per cent ethyl alcohol. The smear was washed in gentle stream of water and counter stained with safranin for 30 seconds. The Gram negative cells appeared red in color and Gram positive cells appeared violet in color (Cyrabree and Hindshill, 1975).

Starch hydrolysis

Filter paper was dipped in a dry old culture suspension and was placed on Petri dishes containing starch agar medium and incubated for two days. The plates were than flooded with one per cent iodine solution. A colorless halo around the growth and blue color in the rest of the plates showed utilization of starch by the microorganism (Stolpe and Godkeri, 1981).

Gelatin liquefaction

Filter paper discs were dipped in a day old culture suspension and were placed on Petri dishes containing gelatin nutrient agar medium. The Petri dishes were incubated at 300C for two days and then flooded with 12.5 per cent HgCl2 solution. The development of yellow halo around the growth indicates utilization of gelatin (Stolpe and Godkeri, 1981).

Fluorescent pigment

The test tubes containing sterilized Kings B medium were inoculated with the isolate of *Pseudomonas* sp. incubated for five days and observed. Yellowish green fluorescent pigment observed under UV light (365 nm) indicated positive results.

Estimation of IAA

Indole acetic acid (IAA) in the methanol fraction was determined by employing Salper reagent (Gordon and Paleg, 1975). To 1.5 ml of distilled water in a test tube 0.5 ml of methanol residue was mixed, four ml fresh Salper reagent was rapidly added, kept in complete darkness for one hour and read in spectrophotometer at 535 nm. From a standard curve prepared with known concentration of IAA, the quantity of IAA in the filtrate was calculated (1 division =0.307 µg of IAA)

Extraction of Siderophore from the medium

The spent culture fluid was separated from the cells by centrifugation at 7000 rpm for 15 min. The supernatant was concentrated to one fifth of the original volume by the flash evaporation at 45 °C. Catechol type phenolates were extracted with ethyl acetate from the culture supernatant twice with an equal volume of solvent at pH 2.0. The ethyl acetate layer was removed and evaporated to dryness and the residues were dissolved in a minimum quantity of distilled water, while hydroxamate types were measured from the untreated culture supernatant.

HCN Production

Production of HCN was determined as per Wei *et al.* (1996). Bacteria were grown on TSA supplemented with 4.4g/l of glycine, white filter paper strips soaked in picric acid solution (2.5 g of Na_2CO_3 and 1 lit. of water) were placed in the lid of each Petri dishes,

sealed with parafilm and incubated for two to three days at 28±2 °C. After incubation HCN production was indicated by the presence of a coloured zone around the bacteria.

Testing the Antagonism of P. fluorescens

Dual Culture Technique

The antagonistic activity of *P. fluorescens* against *F. oxysporum* was tested by dual culture technique (Dennis and Webster, 1971). Bacterial isolates were streaked at one side of Petri dish (one cm away from the edge) containing PDA. 9 mm mycelial disc from four days old PDA culture of *F. oxysporum* was placed at the opposite side of Petri dishes perpendicular to the bacterial streak and incubated at 28±2 °C for 5-7 days. Petri dishes inoculated with fungal discs alone served as control. Three replications were maintained for each isolate. Observation on width of inhibition zone and mycelia growth of test pathogen was recorded and per cent inhibition of pathogen growth was calculated by using the formula proposed by Vincent (1927).

Per cent inhibition (I) = C-T/C ×100

Where, C- mycelial growth of pathogen in control

T- mycelial growth of pathogen in dual plate.

Effect of P. fluorescens on Plant Growth Promotion (in vitro)

Preparation of Inoculum of the Antagonists

The isolates of *P. fluorescens* was grown in conical flasks (250 ml) containing 100 ml of King's B broth for 48 h on a rotary shaker (150 rev min^{-1}) at 28± 2 °C. Cells were removed by centrifugation at 8000 rpm for 10 min at 4 °C and washed in sterile water. The pellet was resuspended in small quantity of sterile dist. water until to obtain bacterial colonies of 10^8 cfu/ml measured by dilution plate technique.

Seed Treatment with antagonist

Seeds of Chickpea (ADT 36) were surface sterilized with two per cent sodium hypochlorite for 30 sec, rinsed in sterile dist. water and dried overnight. One gram of seeds was soaked for 2 h in ten ml of antagonist inoculum taken in Petri dish and added with 100 mg of carboxyl methyl cellulose (CMC).

Plant Growth Promotion (in vitro) Roll Towel Method

Plant growth-promoting activity of the antagonists was assessed based on the seedling vigour index by the standard roll towel method (ISTA, 1993). Twenty five Chickpea (ADT 36) seeds treated with antagonist were kept over the presoaked germination paper. The seeds were held in position by placing another presoaked germination paper strip over it and gently pressed. The sheets along with seeds were then rolled and incubated in growth chamber for 10 days. Three replications were maintained for each treatment. The root length and shoot length of individual seedlings were measured and the per cent germination of seeds was also calculated. The seedling vigour index was calculated by using the formula as described by Abdul Baki and Anderson (1973).

Vigor index (VI) = (Mean root length + Mean shoot length) × Germination (%)

Results and discussion

Isolation of P. fluorescens

Thirty isolates of *P. fluorescens* were isolated from the rhizhosphere soil of different localities. All the isolates were found effective against *F. oxysporum*. However, out of the thirty isolates, four isolates were found significantly superior to other isolates in inhibiting *F. oxysporum*. Hence, these four isolates were further subjected to morphological and biochemical characterization and plant growth promotion activity.

Dual culture technique

The results of the dual culture technique indicated that all the isolates inhibited growth of test fungus significantly (Table 1). A maximum inhibition 93.3% was recorded by PF 3 and minimum of 68.2% was recorded with the isolate Pf 13. Pf 1 (87.8%), Pf 3 (95%), Pf 5 (88.7%) and Pf 8 (84.9%) were significantly superior to other isolates against *F. oxysporum*. Similar to the present observations Reddy *et al.* (2007) also reported that fluorescent pseudomonad isolates effectively inhibited the mycelial growth of *F. oxysporum*.

Table 1. Antagonist activity of *Pseudomonas fluorescens* isolates against *Fusarium oxysporum*. (Dual culture technique).

Isolates	F. oxysporum	
	Mycelial growth (mm)	Growth inhibition (%)
Pf 1	19.9h	87.8
PF 2	24.6h	69.5
Pf 3	5.9a	94.0
Pf 4	24.3h	69.4
Pf 5	08.9b	88.7
Pf 6	24.9h	68.2
Pf 7	26.7h	68.9
Pf 8	12.8c	84.9
Pf 9	25.9h	69.8
Pf 10	26.8h	69.7
Pf 11	28.0h	68.0
Pf 12	27.7h	67.8
Pf 13	26.7h	65.7
Pf 14	27.6h	67.9
Pf 15	26.5h	74.8
Pf 16	27.5h	68.9
Pf 17	25.3h	71.2
Pf 18	26.9h	69.4
Pf 19	25.9h	69.9
Pf 20	28.4h	64.8
Pf 21	25.4h	70.5
Pf 22	25.9h	69.8
Pf 23	26.9h	68.8
Pf 24	26.3h	69.8
Pf 25	25.3h	69.8
Pf 26	25.4h	71.0
Pf 27	26.1h	69.8
Pf 28	29.6h	68.9
Pf 29	27.8h	68.5
Pf 30	27.3h	69.0
Control	89.0	0.0

Fungal Root pathogen *F. oxysporum* is highly sensitive to *P. fluorescens* (Sakthivel *et al*., 2007) and several authors have also reported that *P. fluorescens* inhibited several pathogens including *F. oxysporum* (Sakthivel and Gnanamanickam 1987; Tiwary *et al.,* 2007; Reddy *et al*., 2007; Upadhyay *et al*., 2008; Prasanna kumar *et al*., 2009; Prakash Nathan *et al*., 2011).

Morphological and Biochemical Characterization

Based on the antagonistic potential and other characteristics, four isolates of *P. fluorescens* were studied in detail for colony, colour, growth type, fluorescence, and cell shape. It was evident from the observations that all the four isolates viz., Pf 1, Pf 3, Pf 5 and Pf 8 produced round shaped colonies and rod shaped cells (Table 2).

Table 2. Cultural characteristic of different isolates of *Pseudomonas fluorescens*.

Isolates	Cell shape	Colony type	Colour of colony	Type of growth	Reaction to UV light-fluoresce emission
Pf 1	Rod	Round	Yellowish	Fast	Bright
Pf 3	Rod	Round	Yellowish white	Fast	Bright
Pf 5	Rod	Round	Yellowish	Fast	Bright
Pf 8	Rod	Round	Dull Yellowish	Fast	Bright

Table 3. Biochemical characterizations of different isolates of *Pseudomonas fluorescens*.

S. No	Parameters	Isolation of Pseudomonas fluorescence			
		Pf 1	Pf 3	Pf 5	Pf 8
1	Gram staining	Negative	Negative	Negative	Negative
2	Gelatin liquefaction	Positive	Positive	Positive	Positive
3	Catalase test	Positive	Positive	Positive	Positive
4	Oxidase test	Positive	Positive	Positive	Positive
5	Starch hydrolysis	Negative	Negative	Negative	Negative
6	Fluorescent pigment	Positive	Positive	Positive	Positive
7	Estimation of IAA (µg/ml)	3.6	3.2	3.0	2.9
8	Siderophore production (Hydroxamate)	0.86	0.80	0.87	0.84
9	Hydrogen cyanide production	8.13	7.72	8.22	7.98

The results of the biochemical tests performed for the identification of the effective native isolates of *P. fluorescens* showed that all the isolates produced similar results with regard to gram staining (negative), starch hydrolysis (negative), gelatin liquefaction (positive), catalase test (positive), oxidase test (positive) and fluorescent pigmentation (positive). All the isolates showed positive results in IAA production. Among the isolates Pf 5 produced more quantity (3.7) of IAA followed by Pf 1, Pf 3, Pf 5 and Pf 8 (3.6, 3.2, 3.0 and 2.9 respectively) in the decreasing order of merit.

Table 4. Plant growth promoting activity of *P. fluorescens* isolates on chickpea Cultivar (Bital-98) Roll Towel Method.

Isolates	Root Length Mean(cm)	Shoot Length Mean(cm)	Germination (%)	Plant Vigor Index
Pf 1	23.0a	11.9a	94a	3590b
Pf 3	23.9b	11.9b	91b	3825b
Pf 5	24.5b	12.7a	92b	3639b
Pf 8	24.3b	12.2b	93b	3470b
Control	17.8	5.9	81	1890

All the isolates recorded positive results with regard to hydrogen cyanide production. The isolate Pf 5 recorded maximum production of siderophore (Table 3).

All the isolates were found to be green fluorescent on King's B medium under ultraviolet light at 365 nm.

The biochemical tests i.e gelatin liquefaction, starch hydrolysis, catalase test, oxidase test, IAA production, siderophore production and hydrogen cyanide production further confirmed the isolates to be *P. fluorescens* as reported by earlier workers (Tiwary *et al.*, 2007; Reddy *et al.*, 2007; Prasanna-kumar *et al.*, 2009; Prakash-Nathan *et al.*, 2011).

Plant growth promotion

Among the four isolates the isolate Pf 5 and Pf 3 were on par showing shoot length (12.7cm and 11.9 cm) and root length (24.5 cm and 23.9cm). The isolate Pf 3 (3825.0) recorded the highest plant vigor index followed by Pf 5 (3639.0), Pf 1 (3590.0) and Pf 8 (3470.0) respectively in the decreasing order of merit (Table 4).

The culture filtrates of selected isolates also increased the germination of Chickpea seeds and induced the plant growth promotion under *in vitro* conditions. Among the four isolates Pf 1 recorded the maximum germination per cent, increased the shoot length; root length and vigour index.

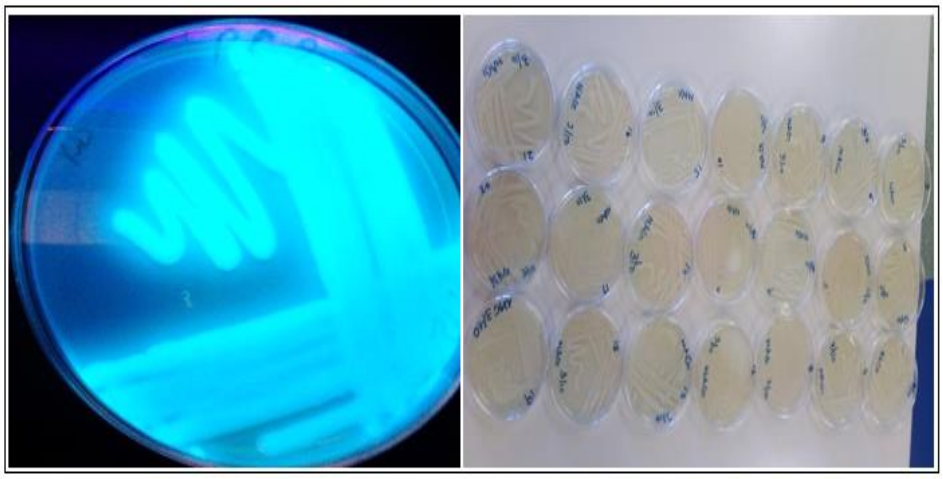

Fig. 1. Fluorescence under UV light.

The growth promoting substance produced by *P. fluorescens* might have exerted a synergistic action and enhanced the growth promotion of rice. *Pseudomonas* spp. were reported to produce amino acids, salicylic acid and IAA (Sivamani and Gnanamanickam, 1988; O'Sullivan and O'Gara, 1992) which might have improved the plant growth and seedling vigour.

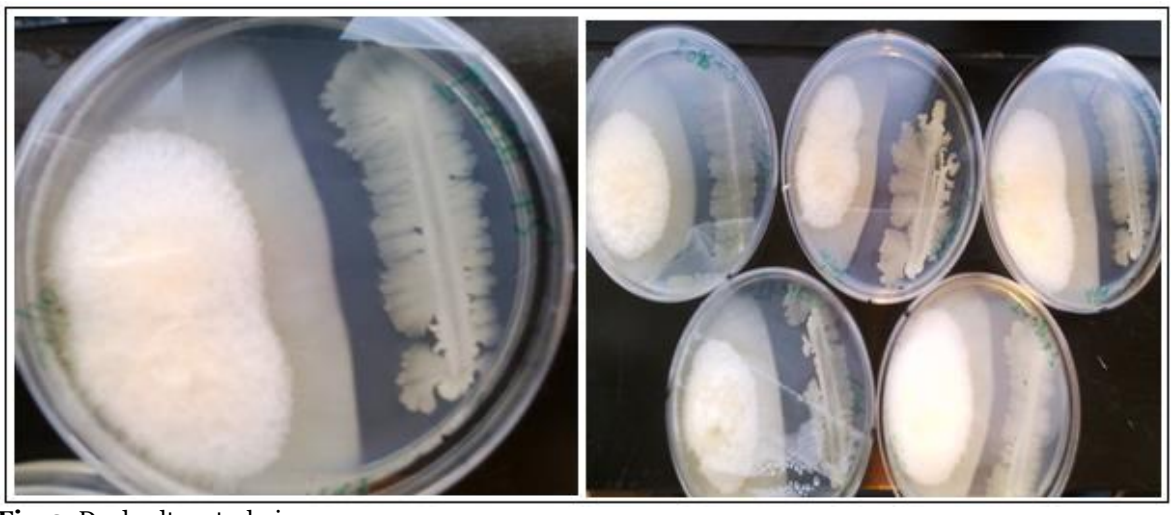

Fig. 2. Dual culture technique.

Production of indole acetic acid by the strains of *Pseudomonas* spp. responsible for increasing root elongation was also reported (O' Dowling and O' Gara, 1994).

Thus, the studies have clearly indicated the effectiveness of the native *P. fluorescens* isolates against *F. oxysporum* and these isolates could be explored further under field condition.

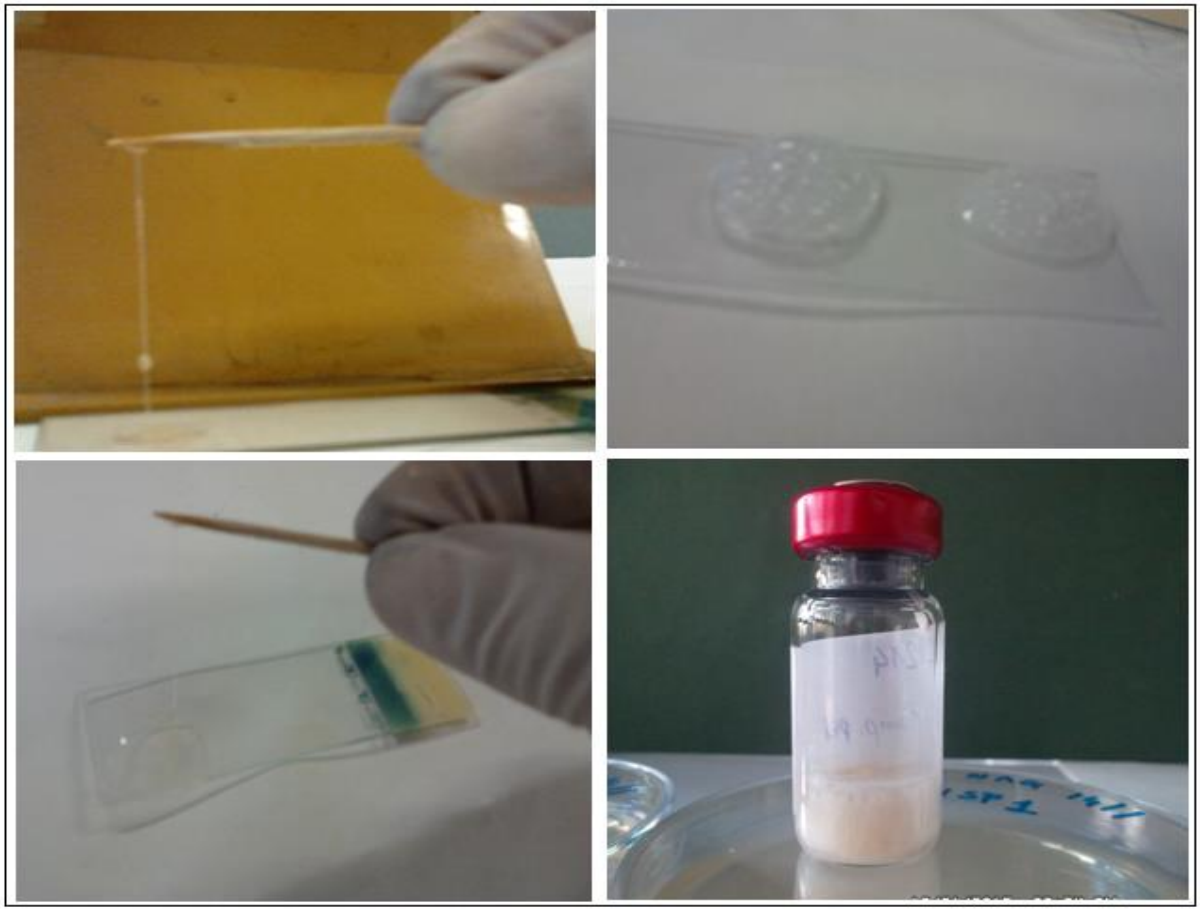

Fig. 3. Biochemical identification of *Pseudomonas fluorescence*.

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