

Case study of the *P. fluorescens* as a biocontrol agent

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Introduction to microbiology

Microbiology is the study of microorganisms, bacteria, fungi, protozoa, algae, and viruses found in soil, water, air, and food. Some cause diseases and reduce crop yields (e.g., *Fusarium oxysporum*, *Ralstonia solanacearum*), while others benefit humans through food production (yogurt, bread, vinegar, alcohol), antibiotics, vaccines, composting, and sewage treatment. By isolating and studying microbes, we can maximize benefits and minimize harm. Agricultural microbiology examines microorganisms in farming and crop health. To increase sustainable farming, soil microbes aid:

- Nitrogen fixation (*Rhizobium* in legumes) to reduce fertiliser use.
- Decomposition (e.g., *Aspergillus*, *Bacillus*) to recycle nutrients for increased soil fertility.
- Organic manure production (*Trichoderma*) from waste.
- Biofertilisation (*Azospirillum*, *Rhizobacteria*) to boost nutrient uptake and disease resistance.
- Biocontrol to suppress soil borne pathogens and pests; focus of this study using *Pseudomonas fluorescens* against *F. oxysporum* and *R. solanacearum*, that cause losses for farmers.

Laboratory identification of bacteria vs fungi

- Bacteria (e.g., *P. fluorescens*) form smooth, glistening colonies and grow rapidly; UV light reveals fluorescence.
- Fungi grow more slowly, producing fuzzy, filamentous, or powdery colonies

Biological Properties of *Pseudomonas fluorescens*

P. fluorescens is a non-pathogenic, Gram-negative, rod-shaped bacterium that colonises plant roots. Under UV light, it emits a yellow-green glow due to pyoverdine, a siderophore pigment. Gram-negative bacteria have a thin peptidoglycan layer and outer membrane, so during Gram staining, the crystal violet–iodine complex is washed out during decolourisation, and safranin counterstains the cells pink/red. *P. fluorescens* acts as a biocontrol agent by producing antibiotics, siderophores, and lytic enzymes that suppress harmful soil-borne pathogens and improve plant growth through increased nutrient availability.

1. Nutrient competition – *Pyoverdine* binds iron in the soil, reducing availability to pathogens, lowering their growth.
2. Antibiotic production – Produces compounds that inhibit pathogens.

Against *Fusarium oxysporum* and *Alternaria solani*

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- DAPG (2,4-Diacetylphloroglucinol) inhibits spore germination and hyphal growth.
Chitinase breaks down chitin in fungal walls. ●
Glucanase digests fungal polysaccharides.

Against *Ralstonia solanacearum*

- Hydrogen cyanide inhibits respiratory enzymes.
- Siderophores limit iron availability.

Importance of this project

Soilborne pathogens such as *F. oxysporum* and *R. solanacearum* cause major yield losses by colonising xylem vessels, disrupting nutrient transfer, and eventually killing plants. *F. oxysporum* can also produce toxins that worsen damage. When infection is suspected, soil samples are analysed, plated, and CFUs counted—high CFUs mean higher disease risk due to high pathogen population. To address this, *F. oxysporum* is isolated, grown as pure culture, and tested against *P. fluorescens* strains using dual culture assays. Strains showing strong inhibition are considered good biocontrol agents. As pathogen traits vary with environment, locally effective strains must be recommended to farmers. This helps reduce pathogen populations, protect crops, and promote sustainable agriculture, avoiding heavy financial losses from pathogen-rich soils.

This report covers methods for isolating and identifying *P. fluorescens* from soil, subculturing in King's B medium, testing its biopotential, and using molecular characterisation to assess its agricultural and commercial value.

Objectives of the studies

- Introduction to Soil Microorganisms – understanding their role in sustainable agriculture.
- Microbial Isolation and Characterisation – methods for isolation, identification, and molecular characterization.
- Focused Case Study on *Pseudomonas fluorescens* – its biology and applications in agriculture.

Materials and methodology

Isolation Techniques - Serial dilution

Materials and Equipment: - Laminar air flow cabinet, 70% ethanol, Soil samples, Sterile distilled water, Micropipettes (1mL), Sterile pipette tips, Vortex mixer, Marker and tube stand Protocol

- Collect soil samples from field for microbial isolation.
- Prepare the water blanks with 9mL each for serial dilution, prepare the specific media and Sterilize in an autoclave at 121 °C, 15 psi for 15 min.
- Work aseptically in a laminar airflow cabinet cleaned with ethanol and UV-sterilized for 20min.

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Weigh 1g of soil in 9 mL sterile water (10^{-1} dilution) and vortex it for 5min.

- Transfer 1 mL from previous dilution into 9 mL fresh sterile water, using new tips each time, up to 10^{-5} dilution and transfer 1mL from each dilution into petri plate using pour plate technique.
- Cool down the agar media (King's Medium B) to 45–50°C before pouring into Petri dishes and after pouring mix well to spread the inoculum uniformly.
- Incubate at 28–30 °C for 1–2 days. (inverted)
- Record colony morphology and subculture fluorescent colonies on King's B agar for pure cultures.

Media Components

- Designed to enhance cell growth and support *P. fluorescens* via provision of nutrients.
- Contains peptone (nitrogen source), glycerol (carbon source), and magnesium sulphate (for fluorescent pigment production).
- Pigments can act as siderophores to help bacteria get iron in low-iron environments.

Fluorescence Detection Under UV

Objective - UV light can be used to identify whether *P. fluorescens* is present in the petri plate. This is morphological identification/characterization

Materials and Equipment: - UV transilluminator, Control King's B agar plate, and Incubated King's B petri plate with soil sample

Protocol

- After 24 h incubation, place *P. fluorescens* plates under 254 nm UV light to check for fluorescence.
- Use a control King's B plate and a pure *P. fluorescens* plate for comparison.
- Fluorescence is a key trait for identifying *P. fluorescens* species.
- Limit UV exposure to a few seconds to avoid pigment bleaching and safety risks.

Subculturing and Purification

Objective - To obtain pure colonies of *P. fluorescens* that have been detected in the Kings B media

Materials and Equipment: - Laminar airflow cabinet, 70% ethanol and sterile tissue wipes, Sterile inoculation loop, Bunsen burner or spirit lamp (for loop sterilization), King's B medium Petri plates (pre-poured and sterile), and *Pseudomonas fluorescens* colonies grown on King's B agar (source plate)

Protocol

- Wear gloves and sterilize an inoculation loop in a flame, letting it cool before use.

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Select a *P. fluorescens* colony identified under UV light and streak it onto fresh King's B agar using the Continuous streaking.

- Incubate plates inverted at 28°C for 24h to produce a pure culture, ensuring colonies remain viable and uncontaminated.

Note: if the sub cultured plates get contaminated then subculture again into new plate (fresh media). **Fungal subculturing**

Protocol

- Wear gloves and flame-sterilize tools before use.
- Pour PDA into sterile Petri plates and let it get solidify.
- Take a fully grown old culture plate and punch the culture (in fresh growing regions) using cork borer and transfer the disk in new fresh media (invertedly).
- Incubate at 28 °C to allow growth while keeping cultures uncontaminated for 5-7days.

Microscopic Identification Techniques – Gram Staining

Objective – to determine whether the bacteria is Gram-positive or Gram-negative based on difference in cell wall structure

Materials and Equipment: - Laminar air flow cabinet, 70% ethanol and tissue wipes, Sterile glass microscope slides, Inoculation loop, Bunsen burner or spirit lamp, Sterile distilled water (for smears from solid cultures), *P. fluorescens* pure culture (sub cultured plate), Crystal violet stain (primary stain), Gram's iodine solution (mordant), Decolorizer (acetone or 95% ethanol), Safranin stain (counterstain), and Distilled water (for rinsing)

Preparation

- Clean laminar airflow cabinet with ethanol and UV-sterilize for 20 min.
- Take grace free glass slide and wipe with alcohol and label with sample ID.
- Slide preparation by smear technique: - put a drop of water and take a loopful of pure culture and mix uniformly.
- Flood the smear with crystal violet for 1 min – this basic dye penetrates all bacterial cells, coloring them purple. Rinse gently with distilled water.
- Add Gram's iodine for 1 min – acts as a mordant, Rinse gently. • Rinse with Decoloulizer in order to remove excess stain • Counterstain with safranin for 1 min rinse and blot dry.
- Examine under the microscope: start at 20× and then 40×.
- (optional) use oil immersion at 100× for the clearest image.

Biochemical tests

1. Catalase Test

Purpose: To determine the ability of microorganisms to produce the enzyme catalase, which breaks down hydrogen peroxide (H_2O_2) into water and oxygen. Positive result → bubbling (release of O_2).

2. Urease Test

Purpose: To check whether bacteria can produce the enzyme urease, which hydrolyses urea into ammonia and carbon dioxide.

Positive result → pink colour due to alkaline pH.

3. Indole Test

Purpose: To determine if bacteria can break down tryptophan into indole using the enzyme tryptophanase.

Positive result → red/pink layer after adding Kovac's reagent.

4. Methyl Red (MR) Test

Purpose: To identify bacteria that perform mixed acid fermentation of glucose, producing stable acidic end products.

Positive result → red colour after adding methyl red indicator.

5. Voges-Proskauer (VP) Test

Purpose: To detect organisms that ferment glucose via the butanediol pathway, producing acetoin as an intermediate.

Positive result → red colour after adding VP reagents (α -naphthol + KOH).

6. Citrate Test

Purpose: To determine the ability of microorganisms to use citrate as the sole carbon source and ammonium salts as the sole nitrogen source.

Positive result → growth with a blue colour change in Simmon's citrate agar due to alkaline by-products

Antagonism Assay Against Pathogens - Antimicrobial testing

Objective – assessing the antimicrobial activity of *P. fluorescens* against *R. solanacearum* to evaluate potential as biocontrol agent.

Preparation of Pure Culture Broth

- Flame-sterilize an inoculation loop.
- Pick *P. fluorescens* and *R. solanacearum* inoculum and mix into nutrient broth.
- Incubate at 28 °C for 24 h to produce pure culture broth.

Spread Plate Technique – Protocol

- Prepare three nutrient agar plates in aseptic conditions.
- Spread 100 μL *R. solanacearum* culture evenly over each plate using sterile glass spreader (ethanol and flame) and put for drying.
- Make five equidistant wells (6mm) and add 0, 10, 30, 50, and 100 μL of *P. fluorescens* broth.
- Incubate at 28 °C for 24 h.
- Measure zones of inhibition in mm.

Dual Culture Assay

Materials and Equipment - Potato Dextrose Agar (PDA) medium, Sterile Petri plates (6 total), Laminar airflow cabinet (LAF), 70% ethanol and sterile tissue wipes, Gloves, Sterile micropipette and tips (also for disc formation), Sterile flamed needle or scalpel (for fungal disc transfer), *F. oxysporum* pure culture, *Alternaria solani* pure culture, *Pseudomonas fluorescens* pure culture, Incubator set at 28 °C

Protocol

- Prepare PDA plates for fungal growth; Take a fully grown old culture plate and punch the culture (in fresh growing region) using cork borer and transfer the disk in new fresh media (invertedly).
- Incubate at 28°C for 24hrs to allow initial fungal growth.
- After 24h streak fresh *P. fluorescens* (24hrs broth culture) on the opposite side of the fungal disc exclude a control plate with only the pathogen
- Incubate the treated plates for 7-8days until the control plate grow fully.
- Measure the percent inhibition of mycelial growth of pathogen by comparing with control and treated plates.

Percent inhibition of mycelial growth of pathogen = $\frac{C-T}{C} \times 100$

Molecular characterization

Objective – to extract bacterial DNA from pure culture

- Use overnight-grown culture of *Pseudomonas fluorescens*.

Protocol

- Transfer 1.5 mL pure culture into 2 mL Eppendorf tubes; prepare a replicate for centrifuge balance.
- Centrifuge at 6500 rpm for 5 min to pellet cells; discard supernatant.
- Resuspend 1 ml pure culture, centrifuge again.
- Add 0.5 ml TBE buffer (maintains pH, prevents DNA degradation), vortex for 5 min and centrifuge
- Add 1ml TBE buffer centrifuge and discard the supernatant ● Add 0.5 mL TBE and leave for 5 min at room temperature.
- Add 50 µL SDS (breaks cell membranes) and 25 µL Proteinase K (digests proteins).
- Incubate at 37 °C for 1 hr.

Deproteinization

- Add 100 µL 5 M NaCl to aid DNA–protein separation; incubate at 65 °C for 10 min.
- Add 700 µL Phenol: Chloroform: Isoamyl alcohol (25:24:1) to denature proteins and remove contaminants.
- Vortex, centrifuge at 9000 rpm for 10 min, and transfer the aqueous (upper) layer to a new tube without disturbing the interface.
- Repeat extraction 3× for maximum protein removal.

- Add 700 μL ice-cold propanol (precipitates DNA) + 50 μL potassium acetate; incubate at $-20\text{ }^{\circ}\text{C}$ overnight.
- Centrifuge at 10000 rpm for 10 min; discard supernatant.
- Add 100 μL 70% ethanol, centrifuge at 10000 rpm for 5 min (precipitates DNA).
- Dry pellet for 30-45 mins to remove ethanol.
- Add 50 μL TBE + 2 μL RNase (removes RNA); incubate at $37\text{ }^{\circ}\text{C}$ for 30 min.

Gel electrophoresis

Objective – To observe clear DNA bands to check whether the sample contains DNA or not. If DNA is present the bands will appear on the gel documentation unit.

- Take 1g of agarose and dissolve in 100ml of 0.5x TAE buffer, melt it using micro-oven (For one GEL run, we need 75ml of gel. So, take 0.75g Agarose in 75ml of I concentration TBE buffer in a conical flask and melt it in micro-oven for 30 seconds $\times$ 3times or 90 seconds)
- Add 2ml of ethidium dibromide mix thoroughly, and pour into the gel casting tray fitted with combs (Chemical present in refrigerator)
- The EtBr, should strictly kept in refrigerator immediate after use
- Allow the gel to get solidified (approx.35-40 min.) and then place the tray in the boat add 0.5x TAE buffer to the boat until the gel is completely immersed in it
- Take 1microliter of 6x gel loading dye and mix with 2 μl of DNA sample, and load the same into the well (next to DNA ladder well)
- The 6x gel loading dye, should strictly be kept in refrigerator immediately after use
- Always load the 1st well with 1 microliter of DNA ladder (1kb) for standard reference
- The DNA ladder (1kb), should strictly be kept in refrigerator immediately after use
- Switch on the gel electrophoresis unit and set ampere to 3mA, voltage to 60V and run the electrophoresis unit for 30-45 min (Device is set to 45mins)
- After completion of the gel run, document the image using Gel document instrument connected to the system [Clean the base of the gel documentation unit with sterile water and wipe thoroughly with tissue paper before using, and place the GEL at the center and close it properly Turn on system- open Bio vision software- take images of GEL- Save them- remove GEL and wipeout with tissue-close door-Turn off)
- Clean the base of the gel documentation unit with sterile water and wipe thoroughly with tissue paper after use
- After successful documentation discard the gel in the gel discarding tray
- A single gel can be reused for minimum of 2 times before final discarding

Observation and results

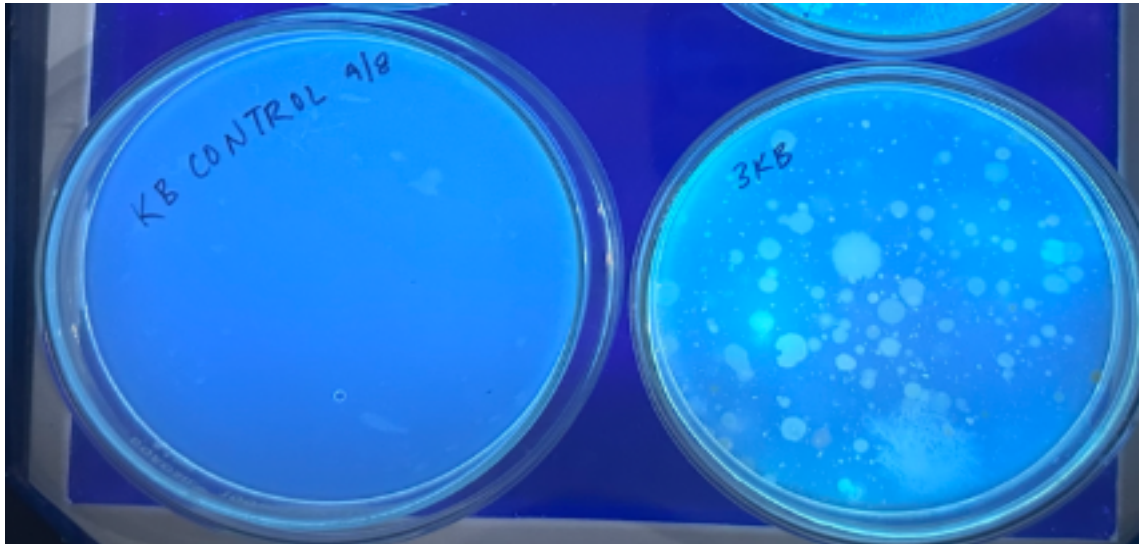


Fig: Fluorescent colony in King's Medium B under UV light at 256nm

The 10^{-4} King's B plate showed isolated yellow-green fluorescent colonies of *P. fluorescens*, with the medium enhancing pigment production for pure culture isolation. The colony morphology of the bacteria was round colonies, yellowish, circular, convex, smooth, flat edges, fluidity, and the secretion of greenish-yellow pigments on King's B media.

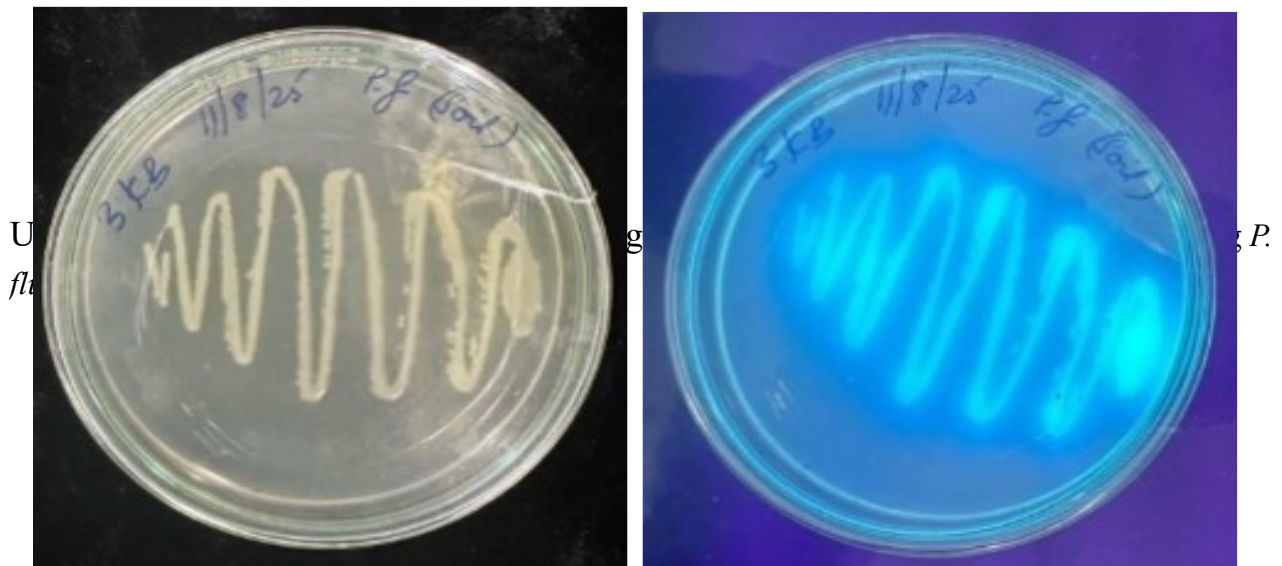


Fig: Pure culture of the colony in specific media(left) and under UV light (right)

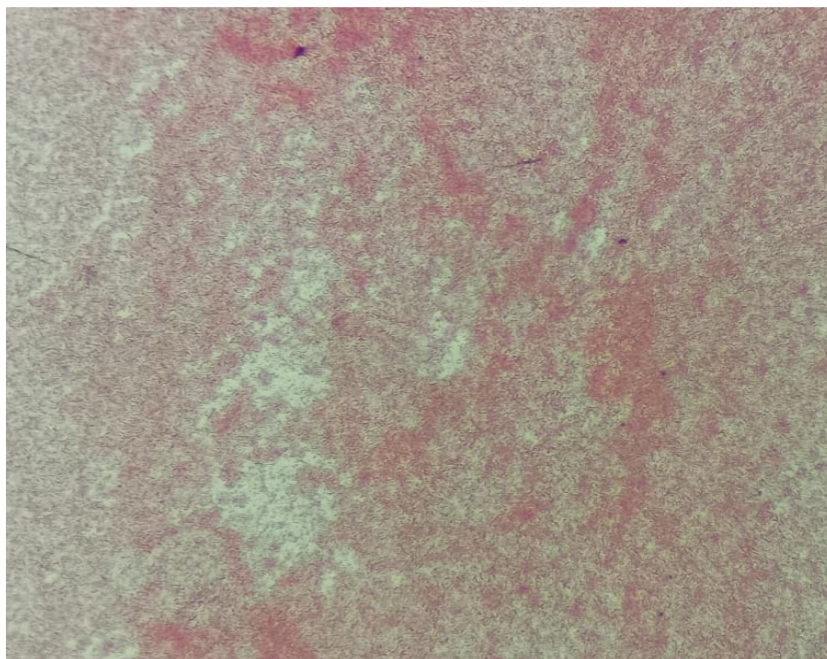


Fig: Gram's staining of *P. fluorescens* (grams -ve rod shape)

Under 40x magnification, cells appeared rod shaped arranged singly or in small groups. They were stained pink indicating gram-negative properties which is an expected characteristic of *P. fluorescens* due to thin peptidoglycan layer in cell wall. No violet stained cells observed, confirming this to be a pure gram-negative culture. **Observation after biochemical tests**

Test	Observation	Results
Catalase	Air bubble should appear	+ve
Urease	The media should develop pink colour	+ve
Indole	Absence of ring	-ve
MR	The broth medium doesn't change colour	-ve
VP	The broth medium doesn't change colour	-ve
Citrate	The media should from green to blue	+ve

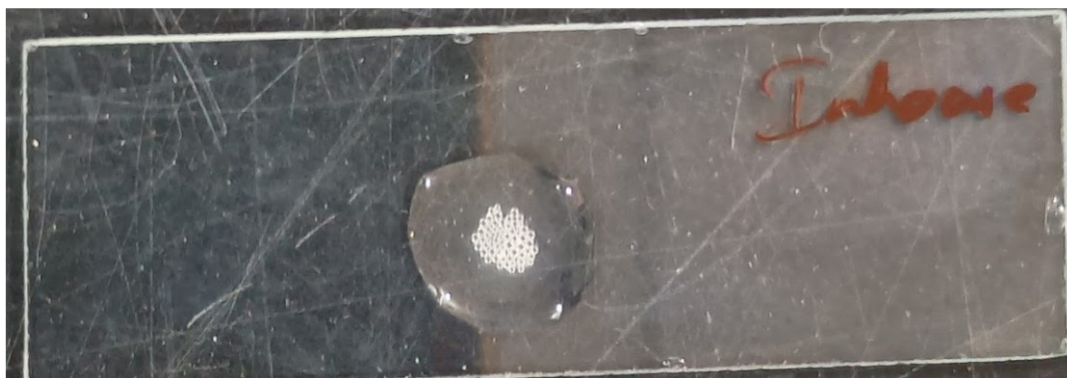


Fig: Catalase test

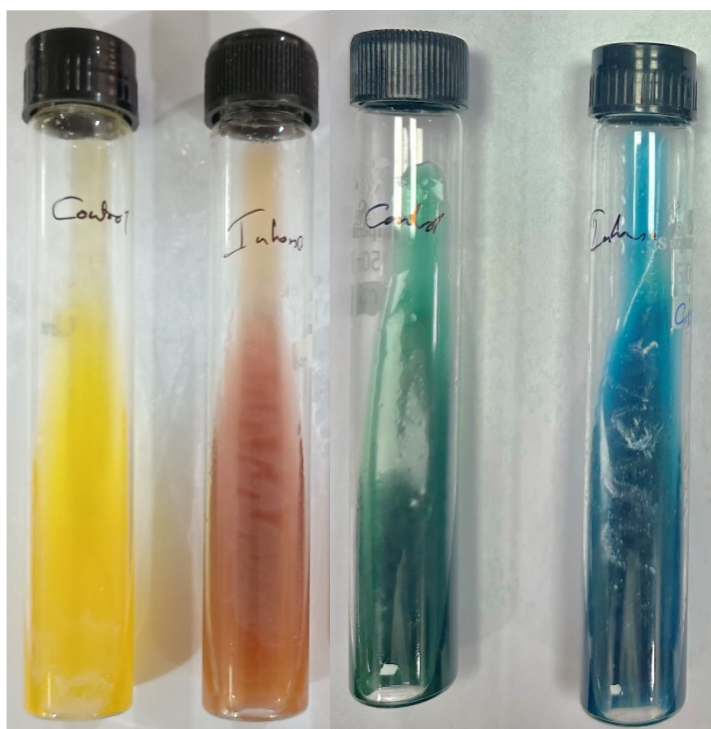


Fig: Biochemical test (Urease and Citrate)

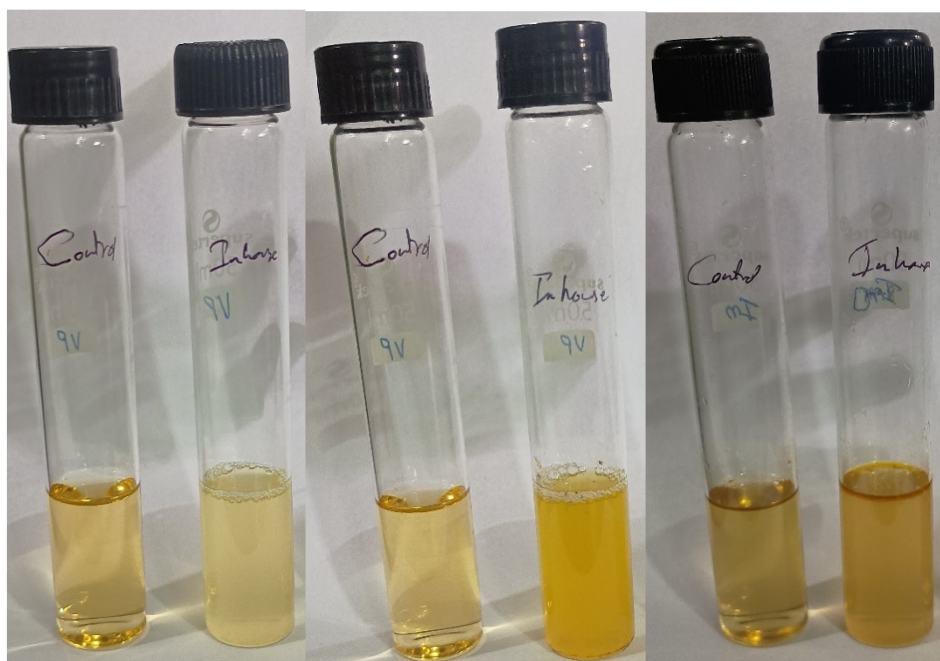
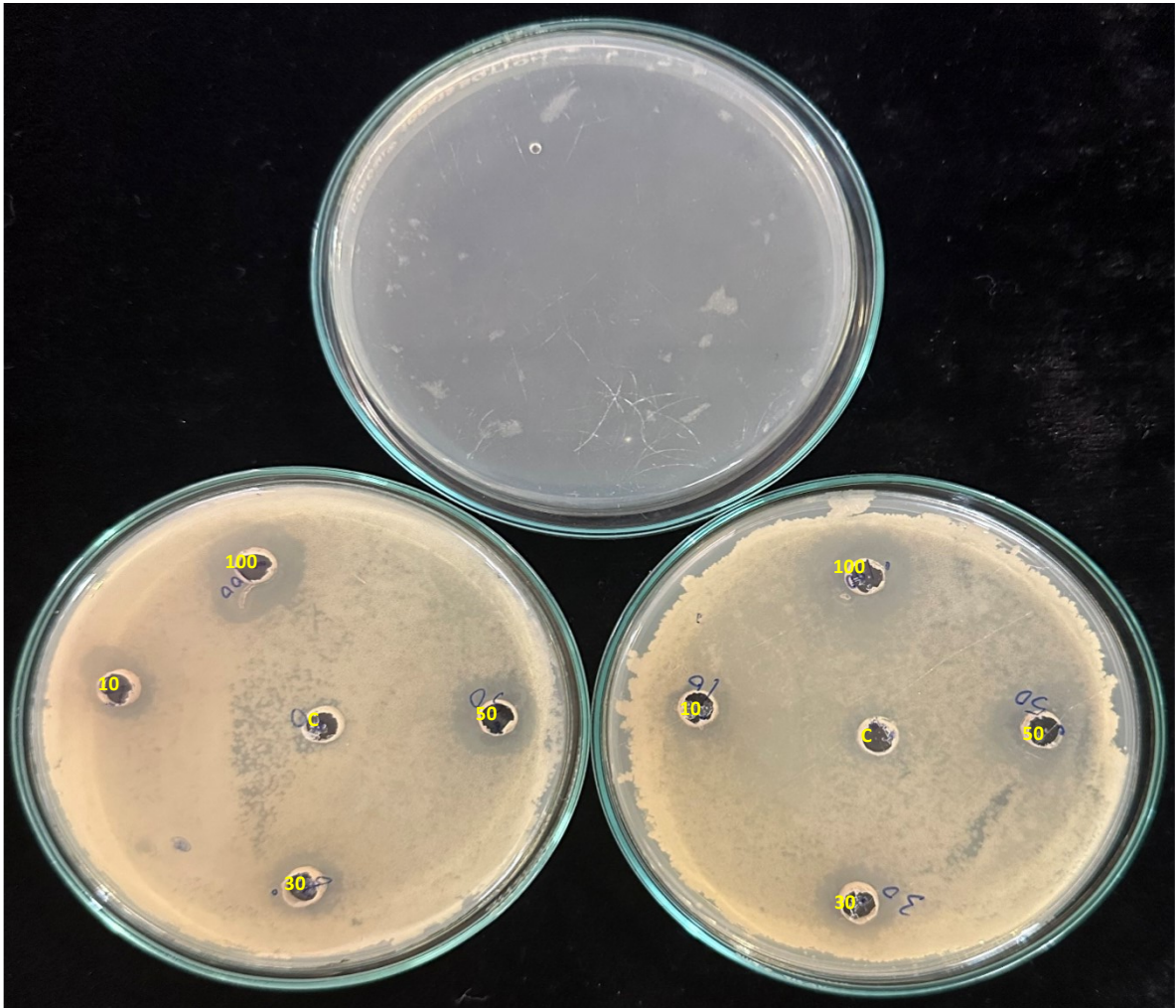


Fig: Biochemical test (MR, VP and Indole)

Table: Observation of Antimicrobial test of *P. fluorescence* against *R. solanacearum*

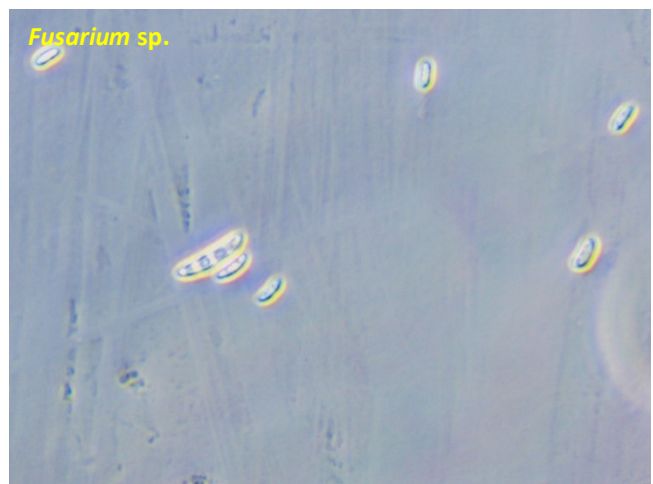
Test sample (mL)	Zone of inhibition (mm)
0	0
10	11
30	11.5
50	14
100	16.5



Pseudomonas fluorescence (inhouse) + *Ralstonia solanacearum*

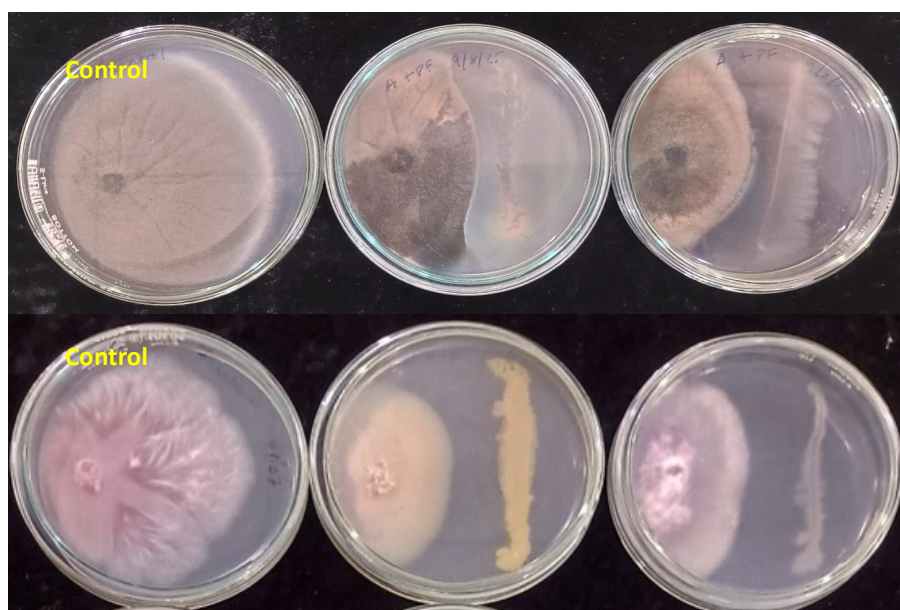


Alternaria sp.



Fusarium sp.

Fig: Microscopic view of Fungal pathogens

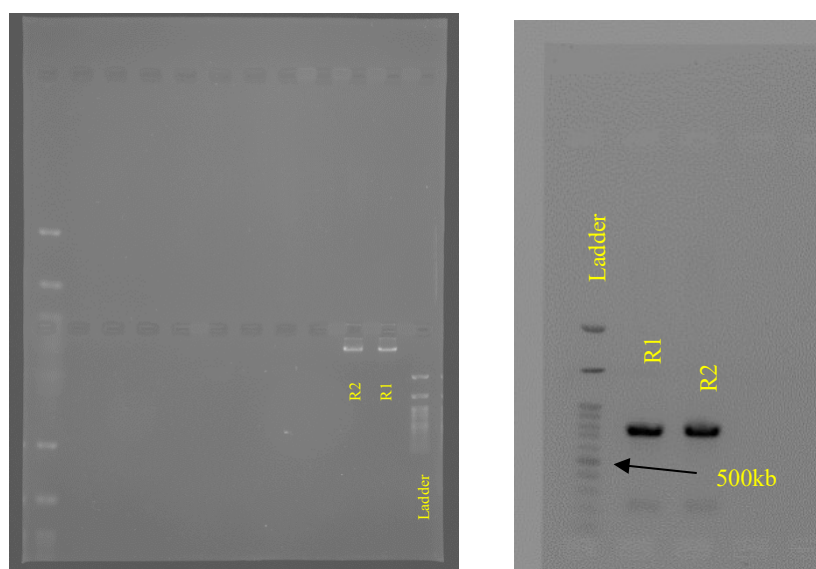


Pseudomonas fluorescens (inhouse) + *Alternaria* and

Table: % of mycelial inhibition of the pathogen against *P. fluorescens*

Test pathogen	In control mycelial growth	Treated mycelial growth	% Of mycelial inhibition
<i>Alternaria</i> sp.	7	4.3	38.5
<i>Fusarium</i> sp.	7	4	42.8

Molecular confirmation



Conclusion

The soil isolate of *Pseudomonas fluorescens* exhibited characteristic fluorescence under UV light at 256 nm and microscopic examination confirmed it as a Gram-negative, rod-shaped bacterium. In vitro studies revealed its strong biocontrol potential against major soil-borne pathogens such as *Fusarium oxysporum*, *Alternaria solani* and *Ralstonia solanacearum*, effectively suppressing their growth. Molecular characterization further supported its identity, with DNA

profiling showing a distinct band between 700–800 kb. These findings highlight the promising role of *Pseudomonas fluorescens* as a sustainable biocontrol agent in agriculture, offering an ecofriendly alternative to chemical pesticides while contributing to improved plant health and soil fertility.