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Isolation and identification of *Pseudomonas syringae* from infected plants

Jaimini Patoliya[1], Khushali Thaker[1], Khushbu Rabadiya[2], Priyanshi Desai[1], Shruti Adeshara[1], Jigisha Prajapati[1], Nayan Jain[3], Rushikesh Joshi[1]*

[1]Department of Biochemistry & Forensic Science, University School of Sciences, Gujarat University, Ahmedabad, Gujarat, India.
[2]Department of Microbiology and Biotechnology, University School of Sciences, Gujarat University, Ahmedabad, Gujarat, India.
[3]Department of Life Sciences and Food Science and Nutrition, University School of Sciences, Gujarat University, Ahmedabad, Gujarat, India.
Email: rushikeshjoshi@gujaratuniversity.ac.in; rushikesh1987@gmail.com

ABSTRACT

Pseudomonas syringae is an ideal system for studying host-pathogen interaction. This study aims to isolate and identify Pseudomonas syringae from infected plant samples using microbial and biochemical tests. Infected leaves were collected, sterilised, and inoculated onto nutrient agar media. Morphological, microbial, biochemical and molecular tests were performed to identify the isolated bacterial strain. The results showed that the LF2 strain was gram-negative, produced fluorescent pigments under UV light, and could produce Levan and utilise citrate. hrp gene is also present in the bacterial strain. All characteristics showed that the strain is of P. syringae. This study shed light on the present infection, paving the way for the creation of potent agricultural Pseudomonas syringae control methods. The study emphasises the need to isolate and identify harmful bacteria to research plant diseases and create control strategies.

Keywords: *Pseudomonas syringae,* Bacterial Identification, Molecular Identification, *Pseudomonas* species, Plant-pathogen interaction, Host-pathogen interaction, Phytopathogens.

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INTRODUCTION

Microbial pathogens causing Plant diseases are a massive threat to agricultural ecosystems globally. From around 7100 species of plant pathogens, approximately 150 species are potentially disease-causing bacterial species. Bacteria cause significant crop damage among the diverse plant pathogenic microorganisms, which can impact the economy significantly [1]. It works with different approaches, such as: by extracting nutrients from one or more host plants for their growth; by secreting proteins and other molecules to specific locations on, in, and by using these proteins and other molecules, parasitic colonisation can be facilitated by altering or resisting plant defence machinery [2]. By invading host tissues, phytopathogenic bacteria cause plant disease resulting in symptoms like leaf and fruit spots, cankers, blights, vascular wilts, rots, and tumours are typical plant morphological symptoms of bacterial plant diseases [3,4]. Pseudomonas is one such pathogen, which is gram-negative bacteria. It is known to cause various plant diseases, including leaf spots, blights, wilts, and rot. The bacterium has several virulence factors that enable it to colonise and survive on plant surfaces, including the production of extracellular polysaccharides, adhesins, and toxins [5]. The bacterium's path is closely associated with its ability to colonise the plant surface and avoid the host defence response. *Pseudomonas syringae* can produce effector molecules that manipulate the plant immune system and suppress host defence mechanisms [6]. The bacterium can also exist as an epiphyte on plant surfaces without causing disease and can survive under harsh environmental conditions, such as drought and freezing [7,8]. The isolation and identification of pathogenic microorganisms are essential for studying plant diseases [9]. By taking infected plants from the

field and isolating microorganisms, researchers can gain insights into the pathogens responsible for plant diseases and develop strategies against them. Effective control measures for *Pseudomonas* infection in plants include using resistant cultivars, cultural practices, and applying biological or chemical control agents. Plant genetic engineering can also enhance plant resistance against *Pseudomonas syringae*. Understanding the molecular mechanisms of host-pathogen interactions is essential for developing effective control strategies against this pathogen [10]. In this study, *Pseudomonas syringae* has been isolated and identified from a group of microorganisms isolated from infected plant samples. It has been tested using several microbial and biochemical tests for its identification, which can further help develop effective agricultural control measures.

MATERIALS AND METHODS

Plant collection

For pathogen isolation, infected leaf samples were collected in the Ahmedabad region according to the area of necrotic leaf spots from the field. The infected leaves samples were cut using a sterilised surgical blade and then washed with distilled water [11].

1.1 Inoculation of sample

Infected leaves were cut into small pieces according to the area of necrotic leaf spots and black spots and put into Nutrient agar media. Then agar plates were incubated overnight at 37°C. After overnight culture, a sterile loop was taken to streak the bacteria on another nutrient agar plate and again incubated overnight at 37°C to obtain a pure culture of bacteria.

1.2 Morphological test

Nutrient agar plates were platted with isolated bacterial strains to observe their morphological characteristics using five sector streaking method [12].

1.3 Microbial tests

1.3.1 Gram staining

A fresh bacterial culture was spread on a clean glass slide during the Gram-staining process. The culture was then air-dried and fixed with mild heating. Then, the slide was flooded with crystal violet for 1 minute and rinsed with water. After that slide was saturated with gram's iodine for 1 minute and then rinsed with water. De-staining was done with 95% ethanol for 10-20 seconds to the point where no more stains run off the slide and then rinsed with water. Safranin was added to the slide for counterstaining for 1 minute and then rinsed with water, blotted dry and observed under a 100X light microscope [13].

1.3.2 Negative staining

At one end of a clean slide, just one drop of nigrosine was placed, and a loopful of the bacterial culture's inoculum was mixed using the aseptic method. Then, the drop of suspended organisms was expanded along the edge of the applied slide by positioning a slide at a 45° angle to the drop to make a thin smear and air-dried it. The slide was observed under an oil immersion lens [13].

1.4 Biochemical tests

1.4.1 Fluorescence under UV test

Selected gram-negative bacterial strains were platted on King's B agar medium. Plates were observed under UV light at 366nm after 24 hours [14].

1.4.2 Levan production test

Nutrient agar plates containing 5% sucrose were platted with selected gram-negative bacterial strains using five-sector streaking to observe its colony characteristics [15].

1.4.3 Simmon's citrate agar test

This test was done to determine the capability of a bacterium to utilise citrate as a side carbon source. A well-isolated colony was taken from a 24-hour culture with a sterile inoculating needle. The citrate agar tubes were inoculated by streaking the surface of the slant. The tubes were then incubated aerobically at 37°C for 48 hours. The test tubes were examined for colour change from blue to green [16].

1.4.4 MacConkey agar test

This test was used to isolate gram-negative enteric bacteria and differentiate lactose fermenting from lactose non-fermenting gram-negative bacteria. **Lactose fermenting strains were** grown **red or pink** and may be surrounded by a zone of acid-precipitated bile. MacConkey agar plates were platted with selected gram-negative bacterial strains and incubated overnight at 37°C [17].

1.4.5 Arginine dehydrolase test

This test was done to see if the microbe could use the amino acid arginine as a carbon and energy source for growth. Arginine dehydrolase enzyme uses arginine as a substrate. An inoculum from a pure culture was transferred aseptically to a sterile tube of arginine dehydrolase broth. The inoculated tube was incubated at 35-37°C for 24 hours, and the preliminary results were determined. Bacteria first used the

glucose in broth to cause a pH drop. This was indicated by the colour change from purple to yellow. The enzyme arginine dehydrolase was activated when the medium had been acidified. The culture was incubated for an additional 24 hours at 35 - 37°C for arginine utilisation by bacteria. Final results were obtained by observing the tube at 48 hours. A colour change from purple to yellow indicates a positive test. Failure to turn yellow at 24 hours or to revert to purple at 48 hours indicates a negative test [18].

1.4.6 Mannitol fermentation test

An inoculum from a pure culture was transferred aseptically to a mannitol agar plate containing a phenol red indicator. The inoculated plates were incubated at 37 C for 24 hours, and the results were determined. A positive test consists of a colour change from red to yellow, indicating a pH change to acidic [19].

1.4.7 Methyl red (MR) test

The test bacteria were grown in a broth medium containing glucose. If the bacteria can utilise glucose to produce a stable acid, the colour of the methyl red changes from yellow to red when added to the broth culture. The MRVP broth tubes were inoculated with the pure isolated colonies and incubated overnight at 37 °C. The results were observed after adding one or two drops of MR reagent [20].

1.4.8 Voges–Proskauer test

Bacterial colonies were inoculated in MRVP broth and incubated at 37 °C for 24 hours. The cultures were taken in a clear sterile Microcentrifuge tube, and VP reagents A and B were added to observe a pink colour within 30 minutes [20].

1.4.9 Indole test

In sterile test tubes containing tryptophan broth, bacterial colonies were inoculated and incubated for 24 hrs at 37°C. After the incubation, Kovac's reagent was added to observe the presence of a red colour ring [21].

1.5 Molecular identification by PCR

The PCR reaction was done by *hrp* gene-specific primers (F.P. 5' ATGCAGAGTCTCAGTC 3')(R.P. 5' TCAGGCCGCGGCCTGAT 3')for LF2 bacterial strain to check the presence of the *hrp* gene. PCR was done in 10 µl volume using TAKARA PCR Master mix in Veriti thermocycler with initial denaturation at 95 °C for 10 minutes, denaturation at 95 °C for 30 s, annealing by 55 °C for 30 s and extension at 74 °C for 30 s (35 cycles) and final extension at 74 °C for 5 minutes. The obtained PCR product was visualised on 1 % agarose gel by electrophoresis [22].

RESULTS

25 different bacterial strains were isolated from plant materials incubated on agar plates, as shown in Figure 1.

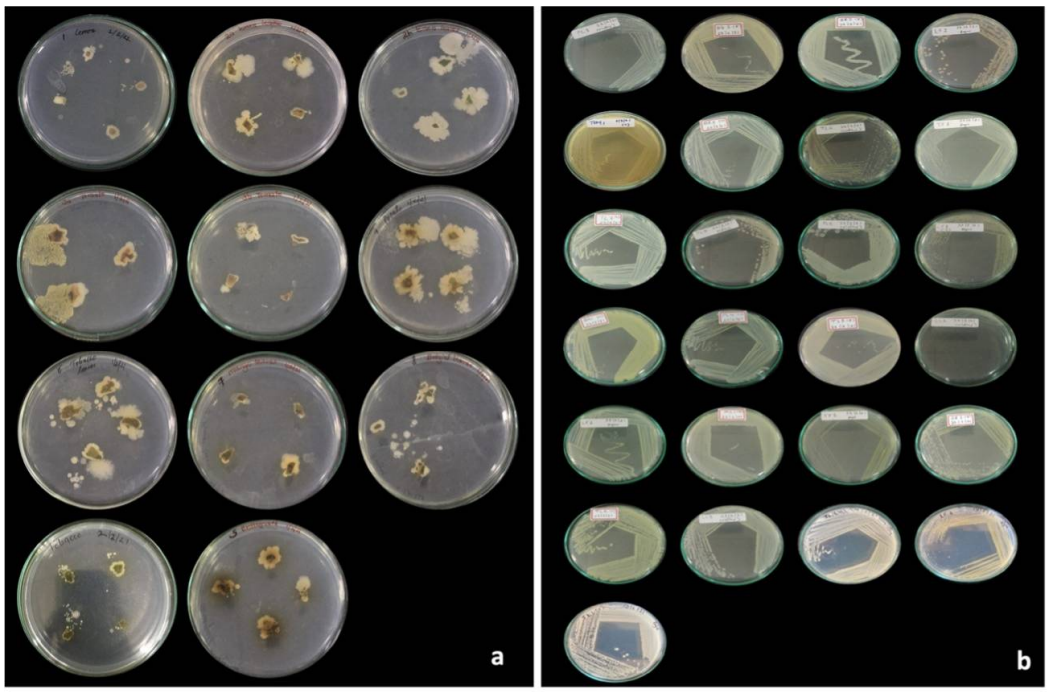

Figure 1: isolated bacterial strains from infected plant materials. a shows infected plant samples on nutrient agar plates. b shows different isolated bacteria inoculated from plates in a.

Different bacterial strains were analysed for their morphological characteristics, as mentioned in Table 1.

Table 1: Morphological characteristics of obtained strains

Number	Sample	Colour	Shape
1	PLO	Pink	Round
2	PL2	Milky white	Round
3	PL3	Milky white	Round
4	PL4	Milky white	Round
5	LF1	Milky white	Round
6	LF2	Pink	Round
7	LF3	Yellowish	Round
8	LF4	Yellowish	Punctiform
9	TL1(O)	Milky white	Round
10	TL1(W)	Milky white	Round
11	TL2	Milky white	Round
12	TL5	White	Round
13	TL6	Yellowish	Round
14	TL7	White	Round
15	BR1	White	Undulate
16	BR2	Milky white	Round
17	BR3	Milky white	Round
18	BG1	Yellowish	Round
19	BG2	Milky white	Round
20	TF1	White	Round
21	TF2	Yellowish	Round
22	LL2	Milky white	Round
23	LL3	White	Round
24	TOM1	Yellowish	Round
25	ML1	Milky white	Round

After the morphological tests, microbial staining was performed which includes gram staining and Negative staining. Negative staining of the strains has shown the size and shape of bacteria. While gram staining showed the gram's reaction of nine bacteria as gram-negative while others were gram-positive as it is shown in Figure 2. Based on this, only gram-negative strains were further tested with different biochemical tests.

These 9 strains were further checked under UV light in King's B agar media which showed that only 3 bacterial strains out of 9 bacterial strains were giving fluorescence, which is LF2, LL3, and PL0 as shown in Figure 3. These strains were further tested with different biochemical tests to identify the *Pseudomonas* species. The screening of biochemical tests of 3 isolated bacteria is shown in Figure 4 for different biochemical tests. All 3 strains are producing Levan as colonies are mucoid or slimy in appearance due to the exopolysaccharide matrix surrounding the bacterial cells. LF2 and PL0 are negative for the citrate

utilisation test. On MacConkey agar, all 3 are negative while for mannitol fermentation LF2 is negative and the other 2 are positive. LF2 is negative for methyl red, Voges-Proskauer test and arginine dehydrolase test, while LL3 and PL0 are positive. LF2 and LL3 have given negative Indole test while PL0 has given positive. The results of all Biochemical tests are given in Table 2.

Table 2: Biochemical test result of 3 strains.

Test	LF2	LL3	PL0
Fluorescence under UV test	+	+	+
Levan production test	+	+	+
Citrate utilization test	-	+	-
MacConkey agar test	-	-	-
Mannitol fermentation test	-	+	+
Methyl red test	-	+	+
Voges-Proskauer test	-	+	+
Arginine dehydrolase test	-	+	+
Indole test	-	-	+

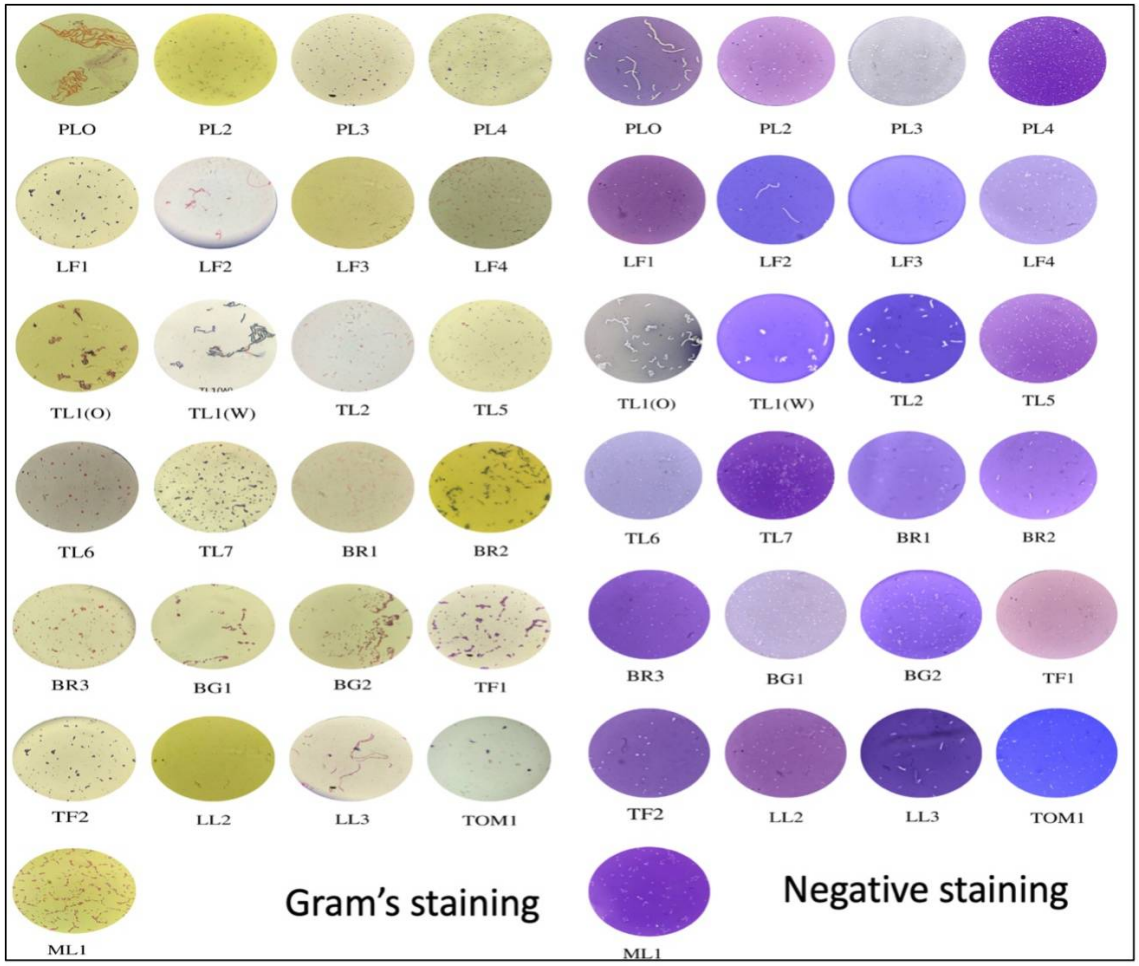

Figure 2: Grams staining and negative staining of all obtained 25 strains.

LF2 strain gives *hrp* gene band in agarose gel electrophoresis after amplification with gene-specific primers at different annealing temperatures. The gel electrophoresis result is shown in Figure 5.
According to the morphological and biochemical characteristics, LF2 bacterial strain was identified as *Pseudomonas syringae*.

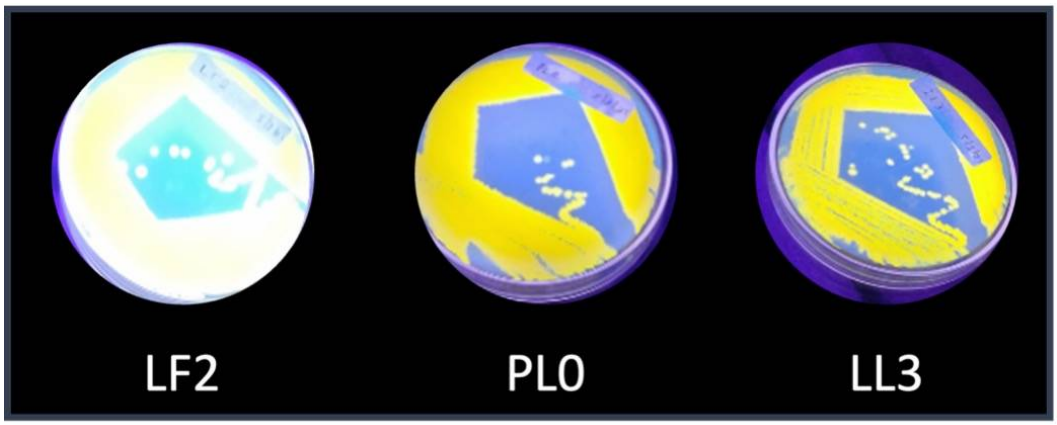

Figure 3: 3 bacterial strains showing fluorescence under UV light in King's B agar media.

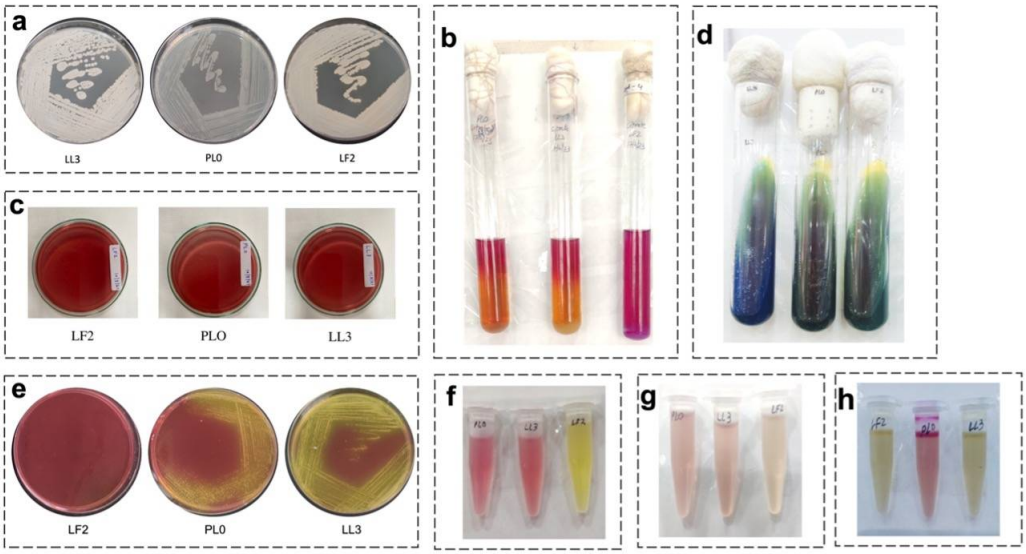

Figure 4: Various Biochemical screening of selected 3 strains. a Levan production test, b Citrate utilisation test, c MacConkey agar test, d Arginine dehydrolase test, e Mannitol fermentation test, f Methyl red test, g Voges-Proskauer test, h Indole test

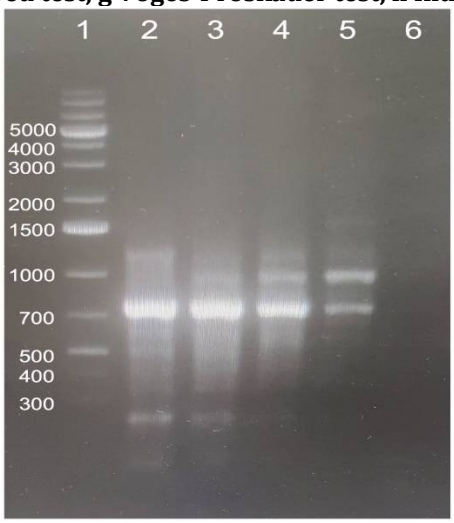

Figure 5: *hrp* gene-specific amplification of LF2. 1 molecular weight marker, 2 57 °C annealing temp, 3 60 °C annealing temp, 4 63 °C annealing temp, 5 66 °C annealing temp, 6 Negative control.

CONCLUSION

LF2 strain is cream, round, rod-shaped, and Levan-producing. It cannot ferment lactose and mannitol and glucose. Neither does it have arginine hydrolase activity, nor has citrate utilisation activity. LF2 does not produce stable acids and fluorescent pigments. It also has *hrp* gene which PCR confirms. All these characteristics lead to the conclusion that LF2 is *Pseudomonas syringae*. In this study, *P. syringae* bacterial strain was identified by the biochemical characteristics, isolated from the Ahmedabad region. It is one of the most frequently isolated plant pathogens which can cause devastated plant diseases. Isolating pathogenic strains from infected plants is important for understanding, controlling, and preventing the spread of plant diseases. Identifying this strain could lead to the development of control measures and the study of plant-pathogen interaction.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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