RESEARCH PAPER

Estimation and Production of Siderophores from *Pseudomonas fluorescens* From the Phyllosphere of the Paddy Plants

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Abstract

Siderophores are iron-chelating compounds produced by microorganisms, playing a crucial role in plant growth promotion and biocontrol of phytopathogens. This study focuses on the estimation and production of siderophores from *Pseudomonas fluorescens* isolates obtained from the phyllosphere and rhizosphere of rice (Oryza sativa L.). The study includes screening of isolates for siderophore production, extraction, and quantitative estimation. Results indicate that siderophore production varies among isolates and is influenced by environmental conditions and iron availability.

Keywords: Siderophore, *Pseudomonas fluorescens*, Plant Growth-Promoting Rhizobacteria (PGPR), Iron Chelation, Biocontrol

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.Introduction

Iron is an essential micronutrient for plant growth and microbial metabolism, but its availability in soil is often limited due to its insolubility. Siderophores, produced by fluorescent Pseudomonads, enhance iron uptake by plants and suppress phytopathogens through competitive iron acquisition. This study investigates siderophore production in Pseudomonas fluorescens isolates and their potential application in sustainable agriculture.

Plant Growth-Promoting Rhizobacteria (PGPR), including Pseudomonas fluorescens, are known to enhance plant growth through various mechanisms, including siderophore production. Siderophores improve iron availability in the rhizosphere, facilitating plant growth and suppressing pathogens (Neilands, 1981; Glick, 1995). Studies have shown that siderophore-producing Pseudomonas strains effectively control fungal pathogens like *Pyricularia oryzae* in rice (Suslow, 1982; Weller and Cook, 1986).

Materials and Methods

Bacterial Strains and Culture Conditions

Pseudomonas fluorescens isolates were obtained from the phyllosphere and rhizosphere of rice (IR-50 variety) grown in Chengalpattu district, Tamil Nadu, India. The isolates were maintained on King's B medium (KMB) at 28±2°C.

Isolation of *Pseudomonas fluorescens* from phyllosphere of rice

To collect representative rice plant samples from a specific location, the entire leaves of each selected plant were removed, cut into 1 cm fragments, and combined into a single sample weighing 10 g. This sample was placed in a 100 ml tube containing 50 ml of phosphate buffer (pH 7.0). The tube was then subjected to sonication for 7 minutes, followed by vortexing for 20 seconds to dislodge microbial cells.

Serial dilutions of the resulting leaf washings were prepared using sterile distilled water. A 1 ml aliquot of the diluted sample was transferred aseptically into sterile Petri dishes, followed by the addition of King's B medium (KMB). To inhibit fungal contamination, cycloheximide (100 µg/ml) was incorporated into the medium. The mixture was evenly spread by rotating the plates in both clockwise and counterclockwise directions before allowing it to solidify.

The prepared plates were incubated at 28 ± 2°C for 48 hours. After incubation, colonies displaying fluorescent pigment production were isolated, transferred to King's B agar slants, and stored at 4°C for future use.

Isolation of *Pseudomonas fluoresces* from rhizosphere of rice

A 10 g sample of air-dried rice rhizosphere soil was placed into 90 ml of sterile distilled water in a 250 ml Erlenmeyer flask. The mixture was then incubated on a rotary shaker at 100 rpm for 30 minutes at room temperature to ensure proper suspension. The resulting solution was serially diluted up to 10^{-7} dilution using sterile distilled water.

From the 10^{-5} and 10^{-6} dilutions, 1 ml of each was carefully transferred aseptically into sterile Petri plates. Next, 15-20 ml of selective King's B medium (KMB) was added to each plate. The medium and bacterial suspension were thoroughly mixed by rotating the plates both clockwise and counterclockwise before allowing them to solidify.

The prepared plates were then incubated at 28 ± 2°C for 48 hours. After incubation, fluorescent pigment-producing colonies were isolated, transferred to King's B agar slants, and stored at 4°C for further analysis.

Siderophore Production Assay

The isolates were screened for siderophore production in iron-deficient KMB broth. The cultures were incubated for five days under static conditions, and cell-free supernatants were obtained by centrifugation (7000 x g, 15 min).

Extraction and Estimation of Siderophores

Siderophore production by *Pseudomonas fluorescens* isolates

1 ml culture of *Pseudomonas fluorescens* isolates, prepared in phosphate buffer (pH 6.5) as previously described, was inoculated into 100 ml of synthetic King's B broth in 250 ml sterilized Erlenmeyer flasks. The experiment was carried out using an iron-deficient medium to assess siderophore production.

Each isolate was aseptically introduced into the broth, and the flasks were incubated under static conditions at 28 ± 2°C for 5 days. After the incubation period, bacterial growth in iron-deficient King's B broth was evaluated by measuring absorbance at 420 nm using a Spectronic-20 colorimeter.

Extraction of siderophore from the medium (Modi *et al*., 1985)

The spent culture was subjected to centrifugation at 7000 × g for 15 minutes to separate bacterial cells. The resulting supernatant was then concentrated to one-fifth of its initial volume using flash evaporation at 45°C.

To extract catechol-type phenolates, the culture supernatant was mixed with ethyl acetate at pH 2.0 and extracted twice using an equal volume of solvent. The ethyl acetate layer was then carefully removed, evaporated to dryness, and the remaining residues were dissolved in a small amount of distilled water. Meanwhile, hydroxamate-type siderophores were analyzed directly from the untreated culture supernatant.

Preparation of Hathways reagent

An 1 ml solution of 0.1 M Ferric chloride prepared in 0.1 N HCl was mixed with 100 ml of distilled water. Subsequently, 1 ml of 0.1 M Potassium ferric cyanide was added to the mixture.

Estimation of phenolate siderophores (Reeves *et al*., 1983)

One volume of Hathway's reagent was mixed with an equal volume of the sample. The appearance of a wine-colored solution indicated the presence of phenolate-type siderophores. Absorbance measurements were taken at 560 nm for salicylates using sodium salicylate as the standard, and at 700 nm for dihydroxyphenols with 2,3-dihydroxybenzoic acid (DHBA) as the standard. A concentration of 1.0 µmol of sodium salicylate resulted in an absorbance of 0.5, whereas 1.0 µmol of 2,3-DHBA produced an absorbance of 0.75.

Estimation of hydroxamate siderophores (Gibson and Magrath, 1969)

To 0.5 ml of culture supernatant, an equal volume of 6 M H_2SO_4 was added, and the mixture was autoclaved in a glass tube. After autoclaving, 1 ml of sulfanilic acid (1% v/v) in 30% acetic acid (v/v) and 0.5 ml of 1.3% iodine in 30% acetic acid (w/v) were introduced. Any excess iodine was neutralized by adding 1 ml of a 2% (w/v) sodium arsenate solution. Subsequently, 1 ml of α-naphthylamine solution (0.3% in 30% acetic acid) was added, and the final volume was adjusted to 10 ml using distilled water. After allowing the reaction to proceed for 30 minutes, the absorbance was recorded at 526 nm. Hydroxylamine hydrochloride served as the standard, with 1.0 µmol of the compound yielding an absorbance of 0.1.

Catechol-type Siderophores

Ethyl acetate was used to extract catechol-type siderophores from the culture supernatant. The extract was evaporated, and the residue dissolved in water. Hathway's reagent was used for colorimetric estimation at 560 nm (Reeves et al., 1983).

Hydroxamate-type Siderophores

Hydroxamate siderophores were quantified using the Gibson and Magrath (1969) method, involving the addition of acidic ferric chloride and measuring absorbance at 526 nm.

Statistical Analysis

All experiments were performed in triplicates, and results were analyzed using ANOVA to determine significant differences among isolates.

Results and Discussion

Screening of Pseudomonas fluorescens Isolates

Twenty isolates were screened for siderophore production, of which 12 exhibited high siderophore activity. The highest production was observed in rhizosphere isolates PFR-6 and PFR-8.

Siderophore Quantification Quantitative estimation revealed that catechol-type siderophores ranged from 0.5 to 2.3 µmol/ml, while hydroxamate-type ranged from 0.4 to 1.9 µmol/ml. The results indicate that siderophore production varies significantly among isolates and is influenced by iron availability.

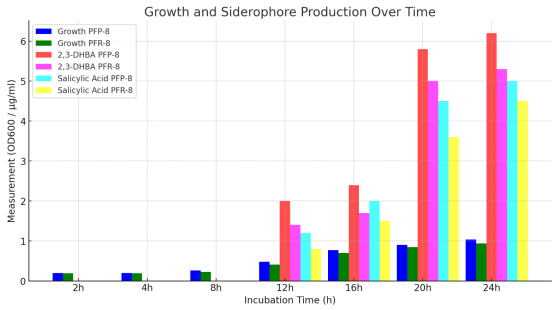
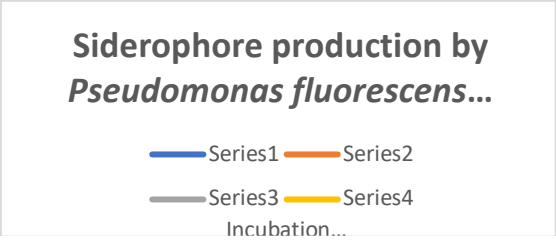


Table 1 -Siderophore production by *Pseudomonas fluorescens* isolates at various growth intervals

Incubation time (h)	Growth		Siderophore production (2,3-DHBA)* (□g ml^{-1})		Siderophore production (Salicylic acid) □g ml^{-1}	
	PFP-8	PFR-8	PFP-8	PFR-8	PFP-8	PFR-8
2	0.201	0.190	-	-	-	-
4	0.201	0.190	-	-	-	-
8	0.265	0.230	-	-	-	-
12	0.480	0.411	2.0	1.4	1.2	0.8
16	0.770	0.700	2.4	1.7	2.0	1.5
20	0.904	0.850	5.8	5.0	4.5	3.6
24	1.040	0.940	6.2	5.3	5.0	4.5

* - Absorbancy measured at 420 nm
** - 2,3 - DHBA - Dihydroxybenzoic acid



Biocontrol Potential

In vitro assays demonstrated that high siderophore-producing isolates inhibited the growth of *Pyricularia oryzae*, suggesting a potential role in biocontrol.

Conclusion

This study confirms the role of *Pseudomonas fluorescens* in siderophore-mediated plant growth promotion and biocontrol. The findings support the application of siderophore-producing PGPR in sustainable rice cultivation.

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