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Full Length Research Article

Biocontrol Efficiency of Siderophore Produced by *Pseudomonas fluorescens* against Phytopathogenic Fungi

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Abstract

Siderophores are secondary metabolites camouflaged in the presence of low iron conditions, which may act as ferric iron chelating agents, and their potentiality to control phytopathogenic fungi as well as bacteria that study have been inspired in the modern centuries. Siderophores secreted by Pseudomonas species have been extensively used as biocontrol agents and it is a substitute to control phytopathogenic microorganism in the field of agriculture. In the present study, *Pseudomonas fluorescens* strain secreted extracellular siderophores when it has been grown in Kings'B medium under low iron conditions. The optimal medium composition for the secretion of siderophore was 10 µM iron concentration, 0.1% maltose and glycine concentrations, temperature 30°C, incubation time 72 hours, and pH 7.0. The siderophores produced under optimal conditions showed better antagonistic effects to fungal pathogens like *Fusarium oxysporum* and *Sclerotium rolfsii*. Hence *Pseudomonas fluorescens* strain was considered as an efficient biocontrol agent for fungal pathogens through siderophore metabolite secretion.

***Key words*:** *Pseudomonas fluorescens,* Siderophore, *Fusarium oxysoporum, Sclerotium rolfsii*

Beneficial microbes like *Pseudomonas spp*. colonize plant roots and protect the plants from phytopathogens by the secretion of plant growth-promoting constituents, antimicrobial compounds, and pathogen-associated proteins [1-2]. Several *Pseudomonas fluorescens* strains display a promising role as biocontrol agents of plant pathogens particularly root pathogens. Fluorescent Pseudomonas spp. have become conspicuous paradigms for rhizosphere green studies and analysis of bacterial secondary metabolites, and nowadays the knowledge of their plant-beneficial attributes has been substantially improved by spreading the attention beyond the phytopathogenic fungi targeted antagonism. Siderophore-producing bacteria may have a significant role in the control of various fungal infections that result in plant illnesses. The important phytopathogens are *Fusarium oxysporum* and *Sclerotium rolfsii.* They are fairly common and have an adverse impact on a number of field crops, ornamental plants, and vegetables. In addition to this, it also affects the crown, roots, and bottom portion of plant stems.

The bacterial secondary metabolite siderophores are low-molecular-weight compounds with a great Fe^{3+} chelating attraction [3] liable for the solubilization and transportation of this element into bacterial cells. A few bacterial species produce hydroxamate-type of siderophores, whereas, others produce catecholate-type of siderophores [4]. Siderophores produced by bacterial species have been engaged powerfully as biocontrol agents against several soil-borne phytopathogens. Siderophores significantly lower the number of ferric ions available to some rhizosphere microbes and impede the growth of the fungus [5]. Siderophores are beneficial in the field of agriculture for disease management in plants as well as plant growth development [6]. Therefore, it has been expanding attention among microbiologists to use advantageous microorganisms as an alternative to the extraordinary usage of harmful pesticides [7-9]. Hence, the present study was proposed to scrutinize the siderophore-producing *Pseudomonas fluorescens* strain for phytopathogens antagonistic study.

MATERIALS AND METHODS

Chemicals and glassware

All chemicals used in the present study were of analytical grade and purchased from Hi-Media Laboratories Pvt. Ltd

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(Mumbai, India. All glassware used was cleaned in 20% HCl to remove residues and sluiced in deionized water.

Collection of culture and maintenance

The bacterial and fungal strains used in the present experiment were *Pseudomonas fluorescens*, MTCC 665; *Fusarium oxysporum*, MTCC 2087; and *Sclerotium rolfsii*, MTCC 2156. The strains were procured from the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. The purchased strain was maintained on the Pseudomonas phage agar and Potato Dextrose agar medium respectively.

Siderophore detection assay

Siderophore produced by the Pseudomonas fluorescens strain was detected on the Chrome Azurol Sulphonate (CAS) agar as portrayed by Schwyn and Neilands [10].

Preparation of CAS indicator solution

Initially, 60.5mg of chrome azurol S was dissolved in 50 ml of double distilled water. 10ml of Fe III solution (27mg $FeCl_3$. $6H_2O$ and 83µL concentrated HCl in 100ml double distilled water) was added, along with 72.9mg cetrimide dissolved in 40ml double distilled water. The cetrimide solution was added slowly while stirring, resulting in a dark blue solution (100ml), which was then autoclaved.

Preparation of basal agar medium

In a 250ml flask, 3g 3- (N-morpholino) propane sulfonic acid (MOPS) (0.1 M), 0.05g NaCl, 0.03g KH_2PO_4, 0.01g ammonium chloride (NH_3Cl), and 0.05 g L-asparagine were dissolved in 83ml double distilled water. The pH of the solution was adjusted to 6.8 using 6M NaOH. The total volume was brought to 88ml using double distilled water, and 1.5g agar was added to the solution while stirring and heating until melted and then autoclaved.

Preparation of CAS agar plate

The autoclaved basal agar medium was cooled to 50ºC in a water bath. The CAS indicator solution was also cooled to 50ºC, along with a 50% solution of glucose. Once cooled, 2ml of the 50% glucose solution was added to the basal agar medium with constant stirring, followed by 10ml of the CAS indicator solution, which was added carefully and slowly along the walls of the flask with constant stirring, but at an appropriate speed so as not to generate any bubbles. Once mixed thoroughly, the resulting solution (100ml) was poured into sterile plastic plates, in such a way that each plate received approximately 20 ml of CAS blue agar.

A well was created by using a cork borer on the CAS agar plate. 10µl of culture supernatant was added to the well and the plate was incubated at room temperature to develop a zone. A maximum of 8hrs was given for any color change to develop. If a siderophore was produced, a yellow/orange halo would be visible on the plate. In addition to using supernatant from the uninoculated medium as a control, was also added to separate well to ensure that the medium alone does not cause a color change.

Quantitative assay for siderophore production

The experimental bacterial strain was grown in Luria-Bertani (LB) broth at 30°C and 120 rpm for 48–72 h and the siderophore production was quantified spectrophotometrically. The amount of siderophores produced was calculated using the formula % Siderophore = Ar –As/Ar∗100 [11], where Ar is the absorbance of the reference (CAS reagent) and As is the absorbance of the sample at 630nm and represented as % siderophore units. The quantitative assay was carried out in five individual replicates.

Characterization of siderophores

By applying a 2% aqueous solution of $FeCl_3$ to 1 ml of cell-free supernatant, the Neilands spectrophotometric assay was used to identify the hydroxamate type of siderophore [12]. A peak formed between 420 and 450 nm was identified as a hydroxamate type of siderophore. A peak at 495 nm was observed after adding a 2% aqueous solution of $FeCl_3$ to 1 ml of cell-free supernatant which indicated a catecholate type of siderophore [13].

Optimization of siderophore production conditions

Different attributes such as pH (6-10), temperature (25-45 °C), incubation time (24-120 hrs), and iron concentration (25-125 µM) were optimized for the production of siderophore superiorly.

Effect of temperature

Upon the production of siderophores, the bacterial culture was inoculated to the King's B broth and incubated at varying temperatures such as 25, 30, 35, 40, and 45 °C (with 5-point frequency) for 72 hours. After that, 1ml of the CAS blue solution and 1ml of the culture filtrate was added and mixed thoroughly. The absorbance was read at 630 nm and siderophore units were calculated accordingly.

Effect of pH

The impact of altering pH (6–10, with a 1-point frequency) in the siderophore production medium on the formation of siderophores was investigated. The log-phase bacterial culture was separately added to the King's B broth at various pH levels and incubated at 35°C for 72 hours. After that, 1ml of the CAS blue solution and 1ml of the culture filtrate were added and mixed thoroughly. The absorbance was read at 630 nm and siderophore units were calculated consequently.

Effect of incubation time

The production of siderophores, the bacterial culture was inoculated to the King's B broth and incubated at varying hours of incubation such as 24, 48, 72, 96 and 120 hours. After that, 1ml of the CAS blue solution and 1ml of the culture filtrate were added and mixed thoroughly. The absorbance was read at 630 nm and siderophore units were calculated suitably.

Effect of iron concentration

In order to identify the optimum concentration of iron that influences siderophore formation, the King's B broth was added with various quantity of iron. Separate inoculations of the log-phase bacterial culture to varying quantities of $FeCl_3 \cdot 6H_2O$ (10, 20, 30, 40, and 50 µM) and were then incubated at 35°C for 72 hours. After that, 1ml of the culture filtrate was added to 1ml of CAS solution, and the absorbance at 630nm was measured and the siderophore yield was calculated appropriately.

Effect of carbon sources

To study the impact of carbon sources to influence siderophore production, the King's B medium was supplemented with 0.1% concentration of sucrose, dextrose, maltose, lactose, fructose, and galactose and were then incubated at 35°C for 72 hours. After that, 1ml of CAS solution was added to the culture filtrate, and the absorbance was read at

630nm. Siderophore units were used to calculate the siderophore yield.

Effect of nitrogen sources

To study the influence of different nitrogen sources on siderophore production, the King's B broth was supplemented with 0.1% concentration of ammonium nitrate, ammonium chloride, potassium nitrate, glycine and urea. After that, the log-phase culture was inoculated and then incubated at 35°C for 72 hours. Then, 1ml of CAS solution was added to the culture filtrate, and the absorbance was read at 630nm. Siderophore yield was quantified as Siderophore Units in percentage.

In vitro antagonistic assay

The experimental strain *Pseudomonas fluorescens* was exposed for antagonistic activity against phytopathogenic strains *Fusarium oxysporum* and *Sclerotium rolfsii* using dual culture method as portrayed by Foldes *et al.* [14] with few alterations. The fungal strains were point inoculated in a potato dextrose agar plate and incubated at 28°C. Nearly 4 cm from the actively growing mycelium, the experimental strain was inoculated with a zig-zag streak. After streaking, the petri dishes were incubated at 28°C for three days. Then the plates were periodically observed for phytopathogenic growth inhibition in a visual manner. The control Petri dishes with test fungal strains were seeded without being inoculated with the *Pseudomonas fluorescens* strain. The percent mycelium inhibition was calculated as follows:

$$\text{Percent inhibition of mycelia} = (dc - dt/dc) \times 100$$

Where dc is the average diameter of the fungal colony in the control group and dt is the average diameter of the fungal colony in the treatment group [15].

Statistical analysis

Experimental data obtained were analyzed using GraphPad Prism software version 6.0 and expressed as Mean ± SEM. The one-way ANOVA and post hoc analysis were carried out using Minitab software version 17. The statistically significant inferences were expressed as $p<0.001$.

RESULTS AND DISCUSSION

Siderophore detection and characterization

Siderophore produced by the *Pseudomonas fluorescens* strain was confirmed by the development of a yellow to orange coloured zone on a blue CAS agar plate. The siderophore removal of Fe from the dye triggered the colour change from blue to orange (Fig 1), which is similarly suggestive of a hydroxymate type of siderophore [16]. After 72 hours of incubation, the uninoculated plates exhibited no colour change, as anticipated. Numerous researchers have observed similar findings [17-18]. A peak between 420 and 450 nm (Fig 2) from a spectrophotometric study of the culture in normal King's B medium suggested the existence of siderophores of ferrate hydroxamate nature.

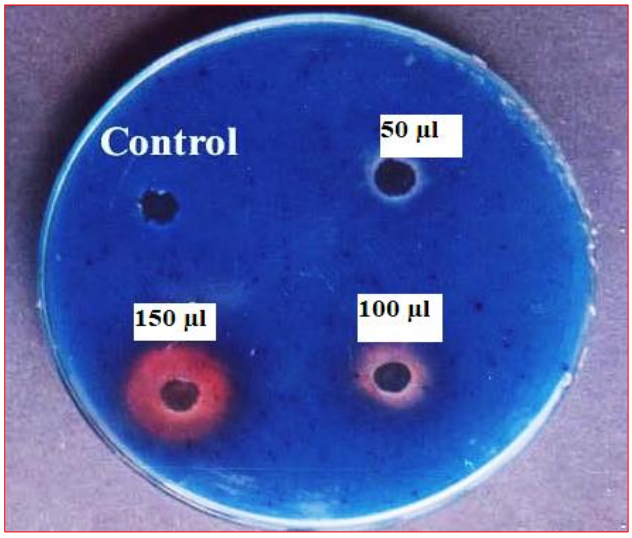

Fig 1 CAS detection assay for siderophore production by *Pseudomonas fluorescens*

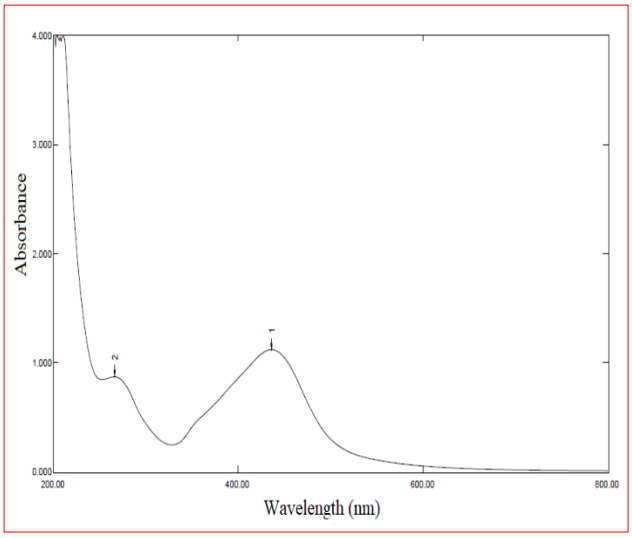

Fig 2 UV spectrum of siderophore produced by *Pseudomonas fluorescens*

Optimization of physicochemical parameters for siderophore production

The obtained results for physicochemical parameters optimization for siderophore production are depicted in (Fig 3). The optimized siderophore production conditions by the test organism were attained while it was grown in King's B medium at pH 7 and incubated at 35°C for 72h under shaker conditions using 10 µM iron concentration with 0.1% maltose as sole carbon source and 0.1% glycine as an exclusive nitrogen source along with an initial optical density of 0.6 at 600nm.

The generation of siderophores at various temperatures under static and shaker conditions is shown in (Fig 3a). The maximum siderophores production (49.58 ± 0.95 %SU) was obtained at 35°C. The regression analysis revealed that the highly significant R^2 value observed was 0.97. The one-way ANOVA followed by the post hoc Tukey test revealed the observed data were statistically significant ($p<0.001$). Previous literature reported that the optimum temperatures for *P. aeruginosa* strains to produce siderophore were found to be 30°C and 27°C [18]. Similarly, most of the cited literature reports that temperatures between 28°C and 37°C are ideal for producing siderophores in Enterobacter sp. Contrary to this *Bacillus* VITVK5, *Bacillus* VITVK6, and *Enterobacter* sp. have been found to be producing siderophores at high levels over a wide temperature range varied from 25 to 45°C [19-21]. Similarly, it has been demonstrated that *P. aeruginosa* and *Candida albicans* strains cannot produce siderophores at elevated temperatures (over 30°C) [22-23].

The impact of pH on siderophore synthesis using *P. fluorescens* is shown in Fig. 3b. In our investigation, it was discovered that the best condition for siderophore formation was a neutral pH (66.77 ± 0.77 %SU). The general tendency of

bacteria to favour neutral pH for their growth and survival may be responsible for this siderophore production discovery. The regression analysis revealed that the highly significant R^2 value was 98%. The one-way ANOVA followed by the post hoc Tukey test showed the observed data were statistically significant (p<0.001) and *Azotobacter* sp. have both been found to produce siderophores in neutral pH, which is consistent with earlier reports [24-25]. The solubilization of iron at a lower pH, which increases iron availability, may be the cause of the decrease in siderophore synthesis under acidic pH [26]. On the contrary, a previous study reported siderophore synthesis by an unidentified strain of *P. aeruginosa* upon a broad range pH (5-11), with an optimum pH of 9 [18]. Likewise, *P. stutzeri* AS22 and *P. oxalicum* exhibited an optimum pH of 8.0 and 4.5 for siderophore production respectively [27-28].

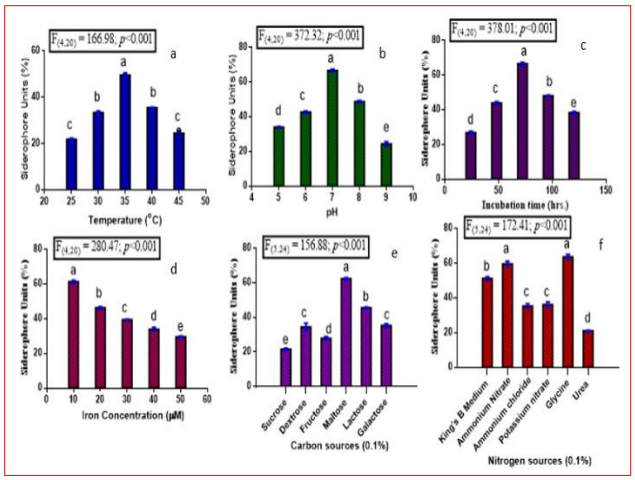

Fig 3 Effect of various culture conditions for siderophore production by *Pseudomonas fluorescens*. Each value is the mean ± SEM of five individual replicates
(a) temperature; (b) pH; (c) Incubation time; (d) Iron concentration; (e) Carbon sources @ 0.1%; (f) Nitrogen sources @ 0.1%. Different superscript alphabets are statistically significant from each other (One-way ANOVA followed by Post hoc Tukey test; p<0.001)

The synthesis of siderophore was determined to be greater after 72 hours of incubation (66.59 ± 0.83 %SU), as shown in (Fig 3c). The regression analysis revealed that the highly significant R^2 value observed was 0.98. The one-way ANOVA followed by post hoc Tukey test explained the recorded data were statistically significant (p<0.001). According to Colombowala and Aruna [29], the siderophore production by *P. aeruginosa* isolate gradually increased from 72 to 96 hours after which the production declined up to 120h of incubation. Earlier studies have shown that siderophore production increases between 3 and 12 hours after inoculation for *P. aeruginosa* PB19 and *Azotobacter* sp., optimum production was reached after 30 hours, for an unidentified strain of *P. aeruginosa*, it took 12 hours [30-32]. The impact of iron concentration on siderophore synthesis using experimental strain is displayed in (Fig 3d). As anticipated, the highest siderophore production of 68.35±0.86% SU was seen in the low iron supplemented medium. This might be due to the 'Fur' proteins negative transcriptional regulation, in which Fe^{+2} functions as a co-repressor. Because siderophores are iron-specific compounds, they are secreted when there is an iron deficiency, and numerous studies have demonstrated that iron deficiency increases siderophore production [21], [33- 35]. In contrary to these reports, *P. fluorescens* NCIM 5096 and *Pseudomonas putida* NCIM 2847 demonstrated siderophore production up to 100mM iron concentration, but few studies have reported tolerance to moderate concentrations of iron [36]. The regression analysis revealed that the highly significant R^2 value was 97%. The one-way ANOVA followed by the post hoc Tukey test indicates the observed data were statistically significant (p<0.001).

The effect of carbon and nitrogen sources on siderophore production by the test organism is depicted in (Fig 3e-f). The siderophore yield was maximum (62.31±0.66 and 63.42± 59.62 %SU) while using maltose as the sole carbon source and ammonium nitrate as a good nitrogen source. The less siderophore production (21.01±0.41 %SU) was recorded while using urea as a nitrogen source. The regression analysis revealed that the highly significant R^2 value observed was 0.97. The one-way ANOVA followed by the post hoc Tukey test revealed the observed data were statistically significant (p<0.001). Similarly, Colombowala and Aruna [29] reported that the *Pseudomonas* sp. P_1, P_2, and P_3 yielded the greatest amount of siderophore production in succinate medium supplemented with 0.1% urea, whereas supplementation of sugars resulted in decreased siderophore production [37]. In contrast to this study, urea was also found to be efficient for siderophore production in *Pseudomonas* sp. PB19 at 0.6 g/L concentration [38].

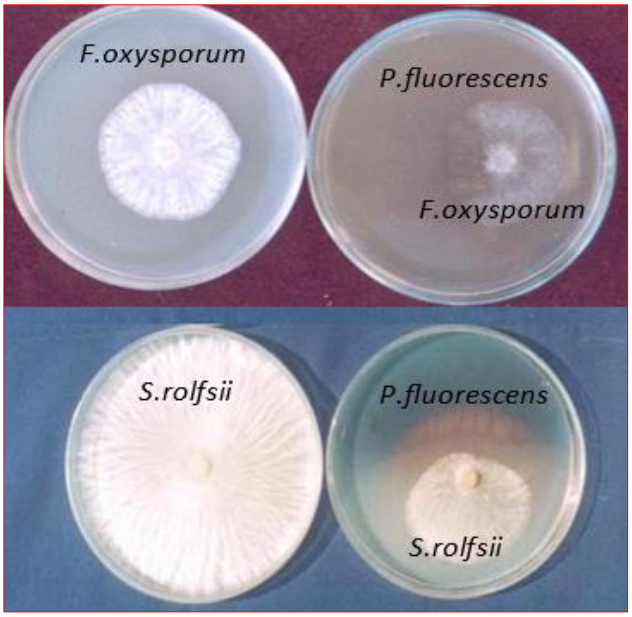

Fig 4 *In vitro* antagonistic assay of siderophore produced by *Pseudomonas fluorescens* against *Fusarium oxysporum* and *Sclerotium rolfsii*

In vitro antagonistic activity

The *in vitro* antagonistic activity of experimental bacterial strains towards soil-borne fungal strains such as *Fusarium oxysporum* and *Sclerotium rolfsii* significantly suppressed the growth in a dual culture assay. The obtained inhibitory effect varied from species to species. The percentage inhibitory effect recorded was 42.64% (*Fusarium oxysporum*) and 64.25% (*Sclerotium rolfsii*) respectively (Fig 4). The pathogenic mycelia did not cover the surface of the inhibitory ring during the incubation period, indicating that the antagonism was quite potent [14]. According to the outcomes of an antagonistic test, the experimental strain had a better inhibiting effect on the examined phytopathogens. Antagonistic tests revealed that the confluent growth of bacteria inhibited the development of fungal phytopathogens. The antifungal secondary metabolite siderophore inhibited the mycelial diameter of *Fusarium oxysporum* and *Sclerotium rolfsii*. Singh *et al.* [39] found that *Pseudomonas* shows fungal growth

inhibition by mechanisms like antibiosis, site competition, HCN production, fluorescent pigments, antifungal volatiles metabolites, and siderophore production.

CONCLUSION

Due to their effectiveness in inhibiting pathogenic fungal strains *in vitro* research opinions to the possible use of indigenous strains of *Pseudomonas fluorescens* that may produce hydroxamate-type siderophores as biocontrol agents. The produced siderophores were strongly antagonistic to the phytopathogenic fungi *Fusarium oxysporum* and *Sclerotium rolfsii*. Furthermore, based on the research findings the utilization of *P. fluorescens* as potential antagonistic to control diseases caused by the phytopathogens in plants, which will enable as the development of affordable biocontrol products for agricultural sustainability, and research in this area appears to be promising one.

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