Isolation, Production and Optimization of Siderophores (Iron Chelators) from *Pseudomonas fluorescens* NCIM 5096 and *Pseudomonas* from Soil Rhizosphere and Marine Water

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**ABSTRACT**

Two *Pseudomonas* spp. are isolated from different natural sources *i.e.* soil rhizosphere and marine water on selective media like cetrimide agar base and King’s B medium. *Pseudomonas fluorescens* NCIM 5096 and other two isolated of *Pseudomonas* produced siderophore with the yield 71%, 72.33%, 33% of hydroxamate type, in iron free succinate medium (SM). Nutrient medium inhibited the growth of siderophore while the succinate medium gave the maximum production of siderophore. pH has important role in production of siderophore *i.e.* at neutral pH 7; maximum siderophore yield while at acidic pH production was found ceasing. Succinate medium supplemented with Pb showed enhancement while other supplemented with Co showed inhibition of production of siderophore. Isolated siderophore showed the antagonists effects against human pathogenic *Pseudomonas aeruginosa* and on phytopathogenic fungi.

**Keywords**

Siderophores, Phytopathogens, *Pseudomonas* sp.

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**Introduction**

The role of siderophores is to scavenge iron from the environment and to make the mineral, which is almost always essential, available to the microbial cell. Important groups of siderophores include hydroxamate siderophores, catecholate (phenolates) siderophores and carboxylate siderophores. In Open Ocean, the iron content is low because of its solubility nature in sea water. When iron is scarce, marine bacteria (and possibly some kinds of microalgae) make siderophores. Siderophores are chemically diverse and feature either catecholate, hydroxamic acid or citric acid based Fe (III) binding groups. Proteins located at the bacterial cell surface or outer membrane recognize the soluble Fe(III)-siderophore complex with high specificity and actively transport the complex into the cell with the Fe ultimately released in the cytoplasm. Siderophore producing *Pseudomonas* sp. Play vital role in stimulating plant growth and in controlling several plant diseases. They function as a biocontrol agent by depriving the pathogen like phytopathogens from iron nutrition. A different strain, *Pseudomonas fluorescens* NCIM 5096 produces an iron binding fluorescent pigment, studied by Chincholkar *et al.*, (2004) who found it as most promising group of plant growth promoting rhizobacteria (PGPR) involved in plant growth promotion and disease control. Our
work began as an effort to isolate \textit{Pseudomonas} from soil rhizosphere and marine water and their identification. In this study it was demonstrated that siderophore production varies with different conditions provided; also antagonistic effects on pathogenic \textit{Pseudomonas aeruginosa} and phytopathogenic fungi. Hence the present study carried out UV visible Spectrophotometric and Fourier transform infrared spectrometric characterization of produced siderophores.

**Materials and Methods**

**Source and isolation**

\textit{Pseudomonas fluorescence} NCIM 5096 was obtained from National Chemical Laboratory (NCL), Pune, India. \textit{Pseudomonas} was isolated from soil rhizosphere by serial dilution method on selective medium like Kings agar B, Cetrimide agar base and Dettol agar (Hi-Media). Another \textit{Pseudomonas} was isolated from marine water sample from Juhu beach, Mumbai on marine nutrient agar. Pathogenic \textit{Pseudomonas aeruginosa} was collected from Pravara Medical Trust (PMT), Pravaranagar.

**Identification and maintenance**

The identification of isolated bacteria was done at PDPVVP’s Medical College, Ahmednagar. All the four strains were maintained at 4\(^\circ\)C on nutrient agar and were used in further studies.

**Inoculum development**

Loopful culture of \textit{P. fluorescence} 5096 and both isolated \textit{Pseudomonas} from nutrient agar were separately inoculated in 250 ml of iron deficient Succinate medium (SM) and incubated at 24-30\(^\circ\)C for 48 hrs with constant shaking at 100 rpm.

**Production of siderophore, U.V. Spectrophotometric analysis and % siderophore unit**

For production of siderophore \textit{Pseudomonas} iron deficient SM was used which consists of g L\(^{-1}\): K\(_2\)HPO\(_4\), 6.0; KH\(_2\)PO\(_4\), 3.0; MgSO\(_4\) 7H\(_2\)O, 0.2; (NH\(_4\))\(_2\)SO\(_4\), 1.0 and Succinic acid 4.0, pH 6.8 – 7. 24-30 h old cultures of \textit{Pseudomonas fluorescence} NCIM 5096 and other two isolated \textit{Pseudomonas} were inoculated and incubated for 24-30 h at 28\(^\circ\)C with constant shaking at 100 rpm. After incubation period cultures were centrifuged separately at 10000 rpm for 15 min and cell free supernatant was subjected for U.V. Spectrophotometric analysis and estimation of siderophore.

UV Spectrophotometric analysis was done by using CAS reagent in equal volume with cell free supernatant at 200-800 nm range.

Percent siderophore unit was calculated by using following formula:

\[
\% \text{ siderophore unit} = \left( \frac{A_r - A_s}{A_r} \right) \times 100
\]

Where, \(A_r = \) absorbance of reference at 630 nm (CAS reagent)
\(A_s = \) absorbance of sample at 630 nm.

**Siderophore production on different media**

Two different media were used like

Nutrient broth consists of g L\(^{-1}\): Peptone, 10.0; Beef extract, 5 gm; NaCl, 10.0; pH, 7.2 ±0.5
Succinate media (SM) consists of g L\(^{-1}\): K\(_2\)HPO\(_4\), 6.0; KH\(_2\)PO\(_4\), 3.0; MgSO\(_4\) \(7\)H\(_2\)O, 0.2; (NH\(_4\))\(_2\)SO\(_4\), 1.0 Succinic acid 4.0, pH 6.8 – 7.

All three species were inoculated in both media and incubated at RT for 48 hrs. After incubation U.V. spectra was taken and siderophore content was quantified by formula.

**Optimization of cultural conditions**

**Influence of pH on growth of siderophore**

Iron free SM was prepared each with two different pH as 4.5 and 7. Each of three flasks was incubated separately by three samples to check the effect on growth and siderophore production.

**Influence of heavy metals**

All the three cultures were separately grown in SM. Each of 50 ml SM was separately supplemented with 10 µM of cobalt chloride (CoCl\(_2\)) and lead acetate (PbCH\(_3\)CHOO). Following the incubation at 28\(^0\)C with constant shaking at 100rpm growth and siderophore production was estimated out.

**Extraction and FTIR analysis**

After centrifugation the cell free supernatant of each sample was mixed with equal volume of ethyl acetate separately (1:1) in separating funnel. After 24 h organic phase was separated in another sterile iron free bumper tube and stored at 4\(^0\)C. FTIR analysis of extracted samples was carried at KTHM College, Nashik.

**Effect of siderophore on growth of pathogenic *Pseudomonas aeruginosa* and phytopathogenic fungi**

**Antibacterial assay**

Mueller- Hinton agar (MHA) consisting of g L\(^{-1}\): Beef infusion solids, 4.0; Starch, 1.5; Casein hydrolysate, 17.5; Agar, 15.0; pH, 7.2 – 7.4 was prepared by spread plate technique for growth of pathogenic *Pseudomonas aeruginosa*. Sterile Whatmann’s paper discs were dipped into three supernatant separately for half an hour. Three discs from samples were placed at 3 different opposite places on solidified MHA plate on which pathogenic *Pseudomonas aeruginosa* was already spread. Plates were incubated at 37\(^0\)C for 24 h and zone of inhibition was observed.

**Phytopathogenic assay**

Potato-Dextrose agar (PDA) consists of g L\(^{-1}\): Potato, 200.0; Dextrose, 20.0; Agar, 15.0 was prepared and distributed in 5 flasks. Autoclaved medium when slightly cooled spores of different fungi such as *Aspergillus flavus*, *Monila*, *Humicola*, *Aspergillus niger*, *Periconia*, *Geophila*, *Candida albicans* were added in different flasks. The media was poured in Petri dishes. Sterile Whatmann’s paper discs were dipped into three supernatant separately for half an hour. Three discs from samples were placed at 3 different opposite places on solidified media. Plates were incubated at room temperature for 48 h. Zone of inhibition was observed and diameter was calculated.

**Results and Discussion**

**Isolation and identification of *Pseudomonas* from soil and marine water**

Work had started with the intention to isolate *Pseudomonas* from rhizosphere soil and marine water. First, serial dilution (10\(^{-4}\)) suspension was streaked on the king’s B medium. After 24 hr it has shown the crowded colonies. These were then purified on to Cetrimide agar plate. Colony characteristics were noted down, further identification was carried out at PDVVP’S medical college, Ahmednagar. As the bacteria
were Gram negative rods with semitransparent in appearance. With the help of biochemical tests it was confirmed that organisms were *Pseudomonas* spp.

![Image]

**a) Pseudomonas fluorescence NCIM 5096 b) Pseudomonas spp from soil rhizosphere c) Pseudomonas aerugiosa from marine water**

**Production % spectrophotometric detection and estimation of siderophore**

After 24-36 hr of incubation, development of green coloured pigment in SM by marine *Pseudomonas* and also pH changed to 9 in all three broths indicated the production of siderophore. This was further confirmed by qualitative CAS test where instant decolorization of CAS reagent from blue to orange red was observed with three cultures. All three cultures produced 71%, 72.33%, 33% units of siderophores (hydroxamate type) in SM, respectively.

**Optimization parameters**

**Influence of media**

While studying influence of media preparation, it was found that development of green colour and pH change was only observed in SM and not in nutrient broth. As there was no colour change in nutrient broth indicating the absence of siderophore production in the medium. *Pseudomonas fluorescence NCIM 5096*, sample 1 (*Pseudomonas* spp), sample 2 (*Pseudomonas aeruginosa*) gave maximum siderophore unit in SM than in nutrient media (Table 1). This indicated that SM is most suitable for siderophore production from all 3 *Pseudomonas* sps.

**Influence of heavy metals**

In case of heavy metals it was observed that the medium supplemented with Pb in the form of lead acetate enhanced the siderophore production as well as growth of culture while media supplemented with Co show reduced production and growth (Table 1) of *Pseudomonas* fluorescence NCIM 5096 and soil *Pseudomonas* isolate. Marine water isolate did not show Co inhibitory effect. Maximum siderophore production was detected by Marine *Pseudomonas* isolate (84.66%)

**FTIR**

FTIR is more sensitive; the optical throughput
is much higher which results in much lower noise levels. By carrying out FTIR of three samples we observed that all the three samples were having common functional groups (Table 2) present which were: N-H (Amines), -OH alcohol C-O stretch, C=O stretch. These groups are present in the siderophore structures. Hence by combining the total effect of U.V. spectrophotometer and FTIR analysis the presence of siderophore in sample was confirmed.

**FTIR analysis: FTIR of standard sample**

![FTIR chart](image1)

**FTIR of standard extracted sample**

![FTIR chart](image2)
Pseudomonas fluorescence NCIM 5096 showed λ max at 252 (Pyochelin), 401, 407 (Pyoverdin) and 359 (Phenazine). Soil Pseudomonas sp showed λ max at 275 (Catechole), 360 (Phenanzin) and 401 (Pyoverdin).

Marine Pseudomonas sp- showed λ max at 247 (Catechole), 401, 405, 407, 406 (Pyoverdin), 366 (Phenanzine pigment) and 386, 391, 387, 396 (Hydroxymate)

Phytopathogenic activity

In case of both assays it was proved that siderophores were acted as an antagonist against pathogenic Pseudomonas aeruginosa and phytopathogenic fungi. The zone of inhibition (Table 3) was more in case of Aspergillus niger indicates that siderophore can be used as a biocontrol agent.

Antifungal

Siderophore’s antibacterial activity was tested against pathogenic Pseudomonas collected from Pravara Medical college. Zone of inhibition (A = 1.4 cm, B = 1.33 cm, C = 1.4 cm) on MHA agar

* A = Standard sample Pseudomonas fluorescence NCIM 5096
* B = Pseudomonas spp (Soil sample)
* C = Pseudomonas aeruginosa (Marine water).

In conclusion, all three Pseudomonas cultures produced 71%, 72.33%, 33% units of siderophores (hydroxamate type) in SM, respectively. Lead in the form of lead acetate enhanced the siderophore production as well as growth of culture while media supplemented with CO show reduced production growth except Marine isolate. Pseudomonas from marine water showed better antifungal activity against Aspergillus flavus, Aspergillus niger, Periconia and Candida albicans while as Pseudomonas from soil showed better activity against Aspergillus flavus, Geophila and Candida albicans than standard strain.
Table 1 Optimization of Siderophore production

| Parameters used | % siderophore unit | Pseudomonas fluorescence NCIM 5096 (Std.) | Pseudomonas spp (soil isolated) | Pseudomonas aeruginosa (marine isolated) |
|-----------------|-------------------|------------------------------------------|---------------------------------|----------------------------------------|
| Media | Nutrient broth | -15 | < 0 | < 0 |
| | SM | 71 | 72.66 | 33 |
| pH | 4.5 | 22.66 | -5 | -29.5 |
| | 7 | 71 | 72.33 | 33 |
| Heavy metal | Cobalt chloride (CoCl₂) | 20.66 | 31.33 | 79.66 |
| | Lead acetate (PbCH₃CHO) | 56.83 | 73 | 84.66 |

Table 2 Peaks and respective groups observed by FTIR analysis

| Sample | Peak | Group |
|--------|------|-------|
| Standard sample Pseudomonas fluorescence NCIM 5096 | 3576.14 | N-H |
| | 3417.98 | N-H / -OH |
| | 2985.91 | -OH (carboxylic acid) |
| | 2941.54 | C-H stretching |
| | 1741.78 | C=O stretching |
| | 1643.41,1633.76 | C=O stretching |
| | 1446.66 | C-O stretching |
| | 1373.36 | Alkyl C-H stretching |
| | 1300.07 | C-O stretch |
| | 1242.20 | C-O stretch |
| | 1047.38 | C-O stretch |
| Soil sample Pseudomonas spp | 3578.07 | -OH (alcohol) |
| | 3458.48 | N-H (Amines) |
| | 3441.12 | N-H (Amines) |
| | 2985.91 | -OH (carboxylic acid) |
| | 2910.68 | -OH (carboxylic acid) |
| | 1743.71 | C=O stretch/ester |
| | 1639.55 | Amids/ C=O stretching |
| | 1373.36 | Alkyl C-H stretching |
| | 1242.20 | C=O stretching |
| | 1047.38 | C-O stretch |
| Marine sample Pseudomonas aeruginosa | 3458.48 | N-H (Amines) / -OH alcohol |
| | 3435.34 | N-H (Amines) / -OH alcohol |
| | 3423.76 | N-H (Amines) / -OH |
Table 3  Antifungal activity of siderophore on phytopathogens

| Sr. No. | Name of fungus       | Zone of inhibition (cm) |
|---------|----------------------|-------------------------|
|         |                      | A  | B   | C   |
| 1       | Aspergillus flavus   | 0.73| **2.1**| 2  |
| 2       | Monila               | 0.6 | 0.63| 0.7 |
| 3       | Humicola             | 2.2 | 1.3 | 1.46 |
| 4       | Aspergillus niger    | 2.6 | 1.83| **3.53**|
| 5       | Periconia            | 2.36| 2.26| **2.53**|
| 6       | Geophila             | 0.7 | **1.73**| 1  |
| 8       | Candida albicans     | 1.7 | **3.1**| **3.13**|

The zone of inhibition is more in case of *Aspergillus niger* indicates that siderophore can be used as a biocontrol agent. We could isolate different types of siderophores from Standard *Pseudomonas* strains, soil *Pseudomonas* isolate and marine *Pseudomonas* as observed by Alison Butlerand *et al.*, at from Oceanography, have isolated and characterized several siderophores from marine microorganisms. These siderophores are structurally quite different from known terrestrial siderophores.

In Martha Páez *et al.*, (2005) showed that siderophore producing *Pseudomonas* acts as antagonists against pathogenic *rhisoctonia solani* and *botrytis cinerea*. Dr Rachel Codd from Bosch Institute (Pharmacology) had worked on the siderophore. They have studied bacterial metal-management strategies which have implications for treating iron overload disease and for better understanding bacterial pathogenic virulence.

In this experiments siderophores showed Pyochelin, Pyoverdin, Phenazine, Catechol and Hydroxymate groups. Soil isolate and *Pseudomonas fluorescence NCIM 5096* Sokol, *et al* - Azurechelin was distinct from the other siderophores of *P. cepacia*, having absorbance maxima at 210, 250 and 310nm, whereas those of pyochelin are at 240 and 310 nm and those of cepabactin are reported to be 330 and 440 nm.

The zone of inhibition is more in case of *Aspergillus niger* indicates that siderophore can be used as a biocontrol agent.

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