

Original Research Article

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

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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 fluorescence* 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.

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 fluorescence* 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 *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 *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

Pseudomonas fluorescence NCIM 5096 was obtained from National Chemical Laboratory (NCL), Pune, India. *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 *Pseudomonas* was isolated from marine water sample from Juhu beach, Mumbai on marine nutrient agar. Pathogenic *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°C on nutrient agar and were used in further studies.

Inoculum development

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

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

For production of siderophore *Pseudomonas* iron deficient SM was used which consists of g L⁻¹: K₂HPO₄, 6.0; KH₂PO₄, 3.0; MgSO₄ 7H₂O, 0.2; (NH₄)₂SO₄, 1.0 and Succinic acid 4.0, pH 6.8 – 7. 24-30 h old cultures of *Pseudomonas fluorescence* NCIM 5096 and other two isolated *Pseudomonas* were inoculated and incubated for 24-30 h at 28°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 CAS shuttle assay in which 0.8 ml of CAS reagent was mixed with 0.8 ml of cell free supernatant giving orange color and absorbance was measured at 630 nm against reference consisting of 0.8 ml of uninoculated broth/ millipore water with equal volume of CAS reagent.

Siderophore content in aliquot was calculated by using following formula;

$$\% \text{ siderophore unit} = \frac{Ar - As}{Ar} \times 100$$

Where, Ar = absorbance of reference at 630 nm (CAS reagent)

As = 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: K₂HPO₄, 6.0; KH₂PO₄, 3.0; MgSO₄ 7H₂O, 0.2; (NH₄)₂SO₄, 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 (CoCl₂) and lead acetate (PbCH₃CHOO). Following the incubation at 28⁰C 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 4⁰C. 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 37⁰C 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.

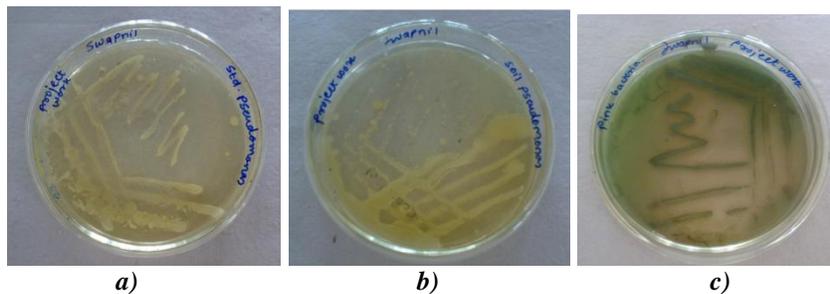
Results and Discussion

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 PDVVPF'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.*



a) *Pseudomonas fluorescence* NCIM 5096 b) *Pseudomonas spp.* from soil rhizosphere c) *Pseudomonas aeruginosa* 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.

p^H of medium

pH plays important role in the solubility of iron and thereby its availability to the growing organism in the medium. For the two pH values (Table 1) it was evident that at neutral pH (7), maximum siderophore yield (71%, 72.33%, 33%) was obtained which may be because bacteria grow better and iron was present in insoluble form at neutral pH and therefore was not available to the bacteria. This stress of iron induces siderophore production. With increase in acidity siderophore production was found ceasing.

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%)

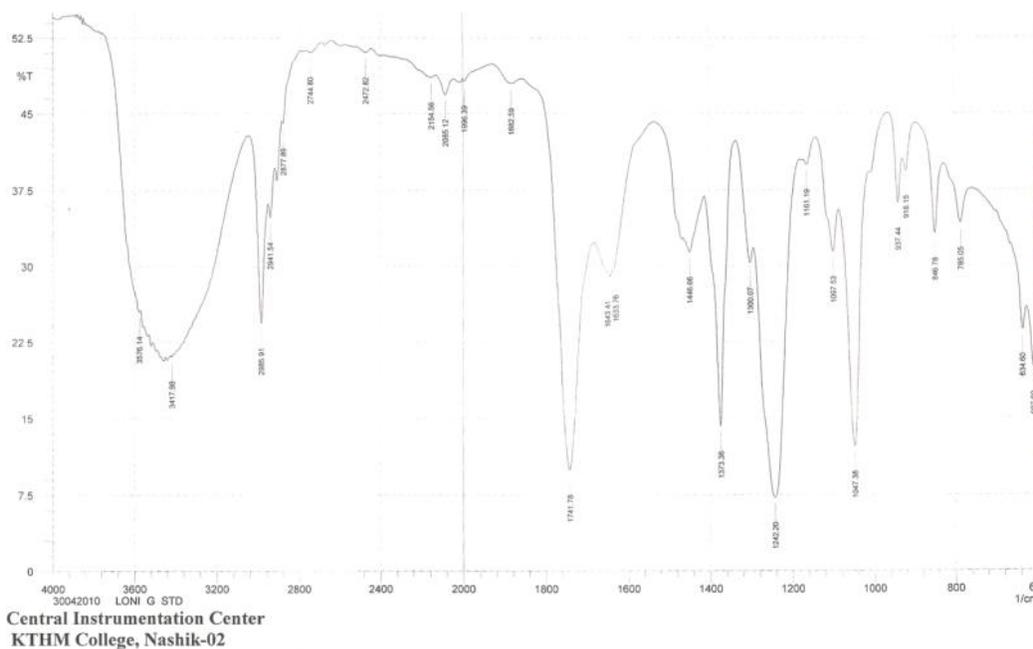
FTIR

FTIR is more sensitive; the optical throughput

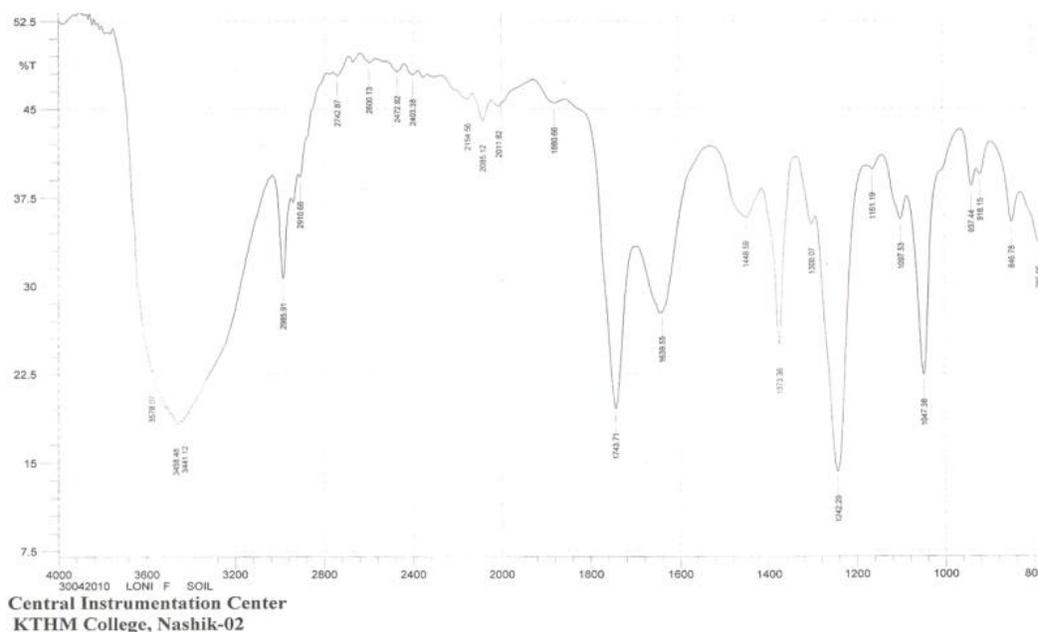
is much higher which results in much lower noise levels. By carrying out FTIR of three samples we observed that all the three samples were having common functional groups (Table 2) present which were; N-H (Amines),-OH alcohol C-O stretch, C=O

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.

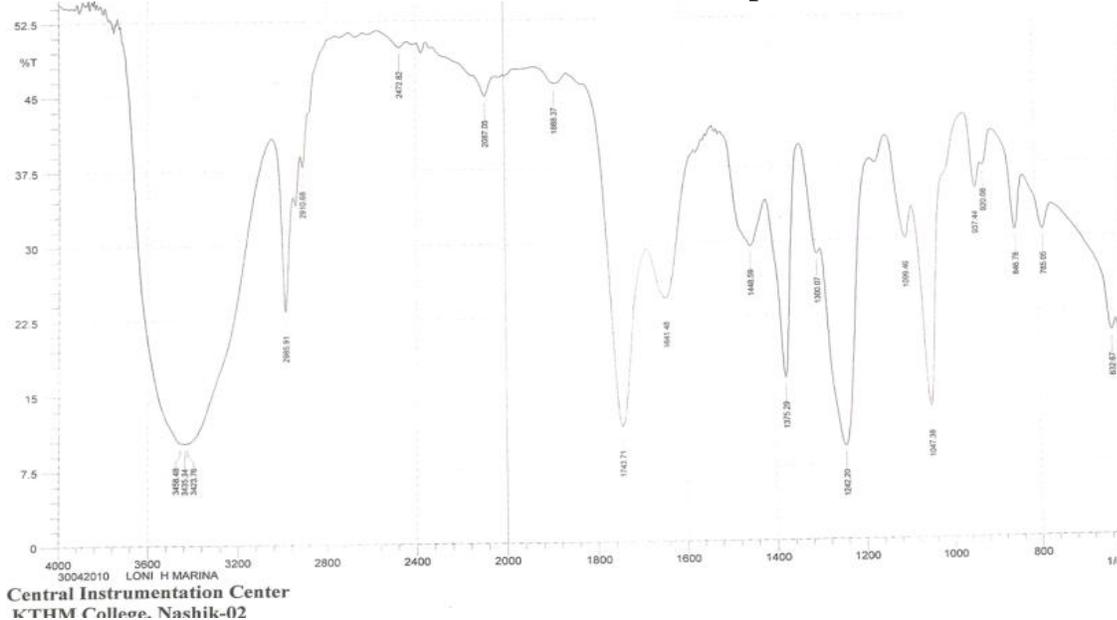
FTIR analysis: FTIR of standard sample



FTIR of standard extracted sample



FTIR of extracted sample 1



FTIR of extracted sample 2

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 (Phenazine 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= Standadr 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 flaus*, *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		
		<i>Pseudomonas fluorescence</i> NCIM 5096 (Std.)	<i>Pseudomonas spp</i> (soil isolated)	<i>Pseudomonas aeruginosa</i> (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 (CoCl ₂)	20.66	31.33	79.66
	Lead acetate (PbCH ₃ CHOO)	56.83	73	84.66

Table.2 Peaks and respective groups observed by FTIR analysis

Sample	Peak	Group
Standard sample <i>Pseudomonas fluorescence</i> 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
Soil sample <i>Pseudomonas spp</i>	1047.38	C-O stretch
	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
Marine sample <i>Pseudomonas aeruginosa</i>	1047.38	C-O stretch
	3458.48	N-H(Amines)/ -oh alcohol
	3435.34	N-H(Amines)/ -oh alcohol
	3423.76	N-H(Amines)/ -oh

		alcohol
	2985.91	-OH(carboxylic acid)
	2910.68	-OH(carboxylic acid)
	1743.71	C=O stretch
	1641.48	Amids/ C=O stretching
	1375.29	C=O stretch
	1242.20	C=O stretch
	1047.38	C-O stretch

Table.3 Antifungal activity of siderophore on phytopathogens

Sr. No.	Name of fungus	Zone of inhibition (cm)		
		A	B	C
1	<i>Aspergillus flavus</i>	0.73	2.1	2
2	<i>Monila</i>	0.6	0.63	0.7
3	<i>Humicola</i>	2.2	1.3	1.46
4	<i>Aspergillus niger</i>	2.6	1.83	3.53
5	<i>Periconia</i>	2.36	2.26	2.53
6	<i>Geophila</i>	0.7	1.73	1
8	<i>Candida albicans</i>	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 Butler and *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, Pyoverdine, Phenazine, Catechol and Hydroxamate 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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