BIOCONTROL POTENIAL OF A SIDEROPHORE PRODUCING PSEUDOMONAS AERUGINOSA

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Abstract: Bacteria that inhabit the rhizosphere may influence plant growth by contributing to a host plant's endogenous pool of bioactive compounds such as phytohormones, antibiotics, siderophores. These bacterial groups are well-known as Plant Growth Promoting Rhizobacteria (PGPR). PGPR can exhibit a variety of characteristics responsible for influencing plant growth. PGPR promote plant growth directly or decrease the growth of phytopathogens and other deleterious microorganisms. *Pseudomonas* sp has been reviewed for its biofertilizer, phytostimulator and phytopathogen biocontrol activities. Thus in the current study we isolated *Pseudomonas* sp. from a Creek water sample, screened its ability for siderophore production & assessed its antifungal activity against different plant pathogenic fungi viz. A*spergillus niger* NCIM 1025 and *Fusarium oxysporum* NCIM 1008 *in vitro*.

Keywords: Pseudomonas, Siderophores, Antifungal , Iron.

Introduction: Siderophores are iron-scavenging ligands synthesized under low iron stress for the solubilization and transport of iron (Fe III) inside the microbial cell [1]. Siderophore producing PGPR have been widely used for plant growth promotion in various crops [2–5] and for the biocontrol of phytopathogenic fungi [6–9].They exhibit requisite hydrophilic, lipophilic and hydro-lipo-phile properties for chelating the extracellular iron respectively from aqueous environment, through the lipoproteinaceous membrane receptors of the cell and from fatty environment [7].Till date hundreds of different siderophores have been characterized and all of them contain either hydroxamate (C=O, N-(OH) or catecholate (derivates of 2,3 dihydroxy benzoic acid) groups [1]. Great variation occurs in physico-chemical properties of siderophores. One organism may produce a variety of siderophores therefore for the extraction of different siderophores several recovery methods and identification approaches have been used [10, 11].

Materials & Methods: A bacterial isolate, obtained from a local Creek water sample of Mumbai, was screened for siderophore production by Chrome Azurol Sulphonate(CAS) agar method [13] and Universal Chemical Assay [14].

Biochemical Characterization and Molecular Identification of Isolate:

For the partial identification, isolate was subjected to various biochemical tests as mentioned in Bergey's Manual of Systematic Bacteriology [15]. This partially identified culture was subjected to confirmation using 16S r RNA identification at National Centre for Cell Science, Pune.

Siderophore Production, Detection and Estimation:

Siderophore production was carried out by submerged process at two levels namely shake flask and bioreactor level. *P.aeruginosa* (6×10^7cells ml^{-1}) was inoculated in succinic acid medium (SM) consisting of gl^{-1} K_2HPO_4: 6, KH_2PO_4: 3, $MgSO_4 \cdot 7H_2O$: 0.2, NH_4SO_4: 1, succinic acid: 4 [13], incubated at 28 ± 2°C with constant shaking at 120 rpm for 24 h. Followed by centrifugation (15 min 4000×*g*) and subjecting cell free supernatant to CAS assay for the detection of siderophores [14]. For determining the hydroxamate and catecholate nature of siderophores, Csaky [17] and Arnow tests [18] were performed respectively.

Recovery of Siderophore:

CAS positive cell free supernatant was concentrated (10×) on rotary vacuum

evaporator at 35°C at 100 rpm, pH of the concentrated supernatant was set to 6.0 with 12 N HCl and it was loaded on XAD [10]. CAS positive fractions obtained from the columns were evaporated to dryness.

Study of antifungal activity of Siderophore extract:

Two separate 25 ml of Czapeck's-Dox agar medium adjusted at pH 6.0 was inoculated with 0.1ml of 10^6 spores ml^{-1} suspensions each of A*spergillus niger* NCIM 1025 and *Fusarium oxysporum* NCIM 1008 respectively and poured into Sterile Petri plates. Agar well diffusion method was carried out to determine the antifungal activity of the culture supernatant and extracted siderophore (Undiluted, 1:2, 1:5 & 1:10 dilutions were used). 100 µl of the extracted siderophore was added in the wells (6mm diameter).All the plates were incubated at RT for 3 days, and diameter of the inhibition zones (mm) were measured.

Effect of pH & iron concentration on the activity of siderophore as an antifungal agent:

1ml of different concentrations of the extracted siderophore (Undiluted, 1:2, 1:5 & 1:10 dilutions) were added separately to Czapeck's- Dox broth medium adjusted at different pH values ranging from 4.0-8.0. Similarly, for examining the effect of iron on the activity of siderophore, 1ml of different concentrations of the extracted siderophore as mentioned earlier were added separately to Czapeck's- Dox broth medium adjusted at different iron concentrations ranging from 10-100 micromoles/ml. Buffered broth medium without siderophore served as control. Each treatment was inoculated with 1ml of 10^6 spores ml^{-1} suspension of 3 days old A*spergillus niger* NCIM 1025 and *Fusarium oxysporum* NCIM 1008 respectively and incubated at room temperature for 3 days under shaking condition. After incubation period, fungal dry weight was determined for each treatment.

Results and Discussion:

Screening for Siderophore Production:

After 24 h incubation at room temperature, change in color of CAS agar from blue to orange red confirmed the ability of organism to produce and excrete siderophores **(Fig1.a)**

Biochemical Characterization and Identification of Isolate:

Microscopically, the 24 h old culture of isolate was found to be moderately motile, aerobic, Gram negative, nonsporulating, short rod. Culturally on nutrient agar it showed bluish green colonies (0.8 mm) having irregular margin . Further, on basis of the biochemical tests (Koneman, 1992), it was found to be a lactose non fermentor, oxidase positive, lysine decarboylase positive, with an ability to utilize gelatin. The presence of fluorescein and pyocyanin indicated that the isolate belonged to the family *Pseudomonadaceae* indicating possibility of being *Pseudomonas aeruginosa,, P. putida,, P. flourescens, or P. syringae*. A table of 16S rRNA gene sequence was edited using the MicroSeq microbial identification software version1.0 that enabled the assembly of each forward and reverse sequence into a consensus sequence. On further editing to the resolve base pair ambiguities between two strands by evaluation of electropherograms, it was identified as *Pseudomonas aeruginosa LMG 1242(T)*Accession:Z76651

Similarity: 99.55% using SeqScape sequence analysis software version 1.0 (Applied Biosystems, USA). The details of these microbes were searched on http://www.ncbi.nlm.nih.gov website.

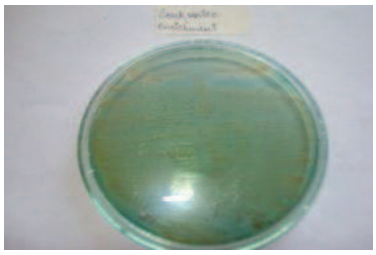
Sequence of blast performed:

CGGATTACTGGGCGTAAGCGCGCGTAGGTGG
TTCAGCAAGTTGGATGTGAATACTCCCCGGGC
TCAACCTGGGAACTGCATCCAAAACTACTGAG
CTAGAGTACGGTAGAGGGTGGTGGAATTTCCT
GTGTAGCGGTGAAATGCGTAGATATAGGAAG
GAACACCAGTGGCGAAGGCGACCACCTGGAC
TGATACTGACACTGAGGTGCGAAAGCGTGGG
GAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGTCGACTAGCCGTTGGG
ATCCTTGAGATCTTAGTGGCGCAGCTAACGCG
ATAAGTCGACCGCCTGGGGAGTACGGCCGCA
AGGTTAAAACTCAAATGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCG
AAGCAACGCGAAGAACCTTACCTGGCCTTGAC
ATGCTGAGAACTTTCCAGAGATGGATTGGTGC
CTTCGGGAACTCAGACACAGGTGCTGCATGG
CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGT

TAAGTCCCGTAACGAGCGCAACCCTTGTCCTT
AGTTACCAGCACCTCGGGTGGGCACTCTAAAG
GAGACTGCCGGTGACAAACCGGAGGAAGGTG
GGGATGACGTCAAGTCATCATGGCCCTTACGG
CCAGGGCT

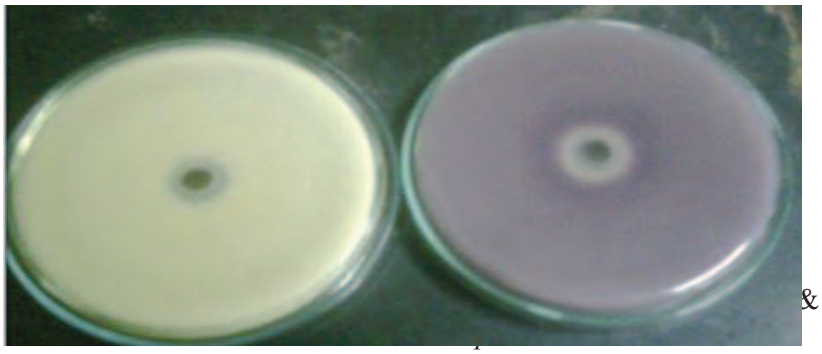
Siderophore Production, Detection and Estimation.

Siderophore production was carried out at two levels namely shake flask and bioreactor level. In the shake flask studies change in the color of SM from colorless to golden yellow and an instant change in the color of CAS reagent from blue to orange red after the addition of cell free supernatant revealed the presence of siderophores in SM. (**Fig1.a and 1.b**)



1.a Orange halo by Sid^+ isolate

postitive test by isolate



&

Recovery of Siderophore:

From XAD column two CAS positive fractions were obtained with a λ_{max} at 224 (major fraction) and 264 nm (minor fraction). Major fraction was found to contain hydroxamate type while minor fraction contained catecholate type of siderophore. It is known that hydroxamate type siderophores are comparatively stable, strong iron chelators and possesses antifungal activity [13]. Siderophore yield obtained from these fractions was **217** and **60** respectively mg l^{-1}

Study of antifungal activity of Siderophore extract:

During preliminary experimentation using plate assay, both the test cultures used showed growth zone inhibition ranging from 22 to 55% (as compared to control, by the siderophore extract (undiluted and 1:2 diluted) at pH 6.0. On the basis of these results, for further analysis, pH range from 4.0 – 8.0 and siderophore extract (undiluted and 1:2 diluted was selected for broth medium quantitative assay

Effect of pH & iron concentration on the activity of siderophore as an antifungal agent:

Regarding the effect of different pH values on the activity of siderophores, (**Figure** 2.a) and (**Figure** 2.b) it was found that pH 5.0-6.0 was suitable for siderophore activity as indicated by decrease in the fungal dry weight, for both the test cultures. With increase in pH, the antifungal activity was retained. The results indicated that *Fusarium oxysporum* NCIM 1008 was more sensitive to inhibition than that of *Aspergillus niger* NCIM 1025. Maximum antifungal activity was observed at pH 7 in presence of undiluted siderophore samples thereby reducing the fungal biomass to 0.42gm%, followed by reduction of fungal biomass to 0.63 gm% observed at pH 7 by 1:2 diluted siderophore sample.

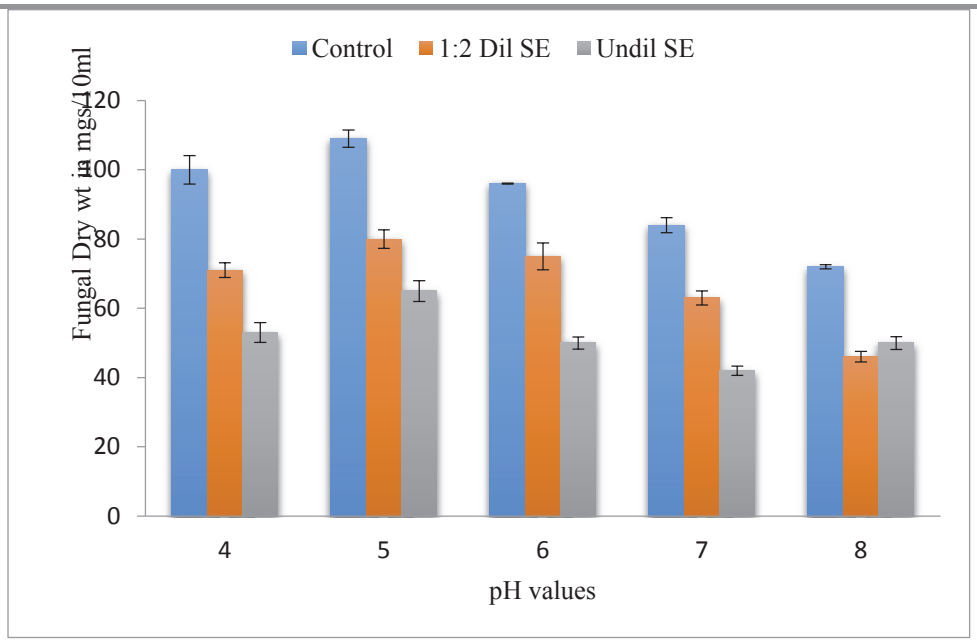


Fig 2.a: Effect of different pH values on the activity of siderophore as an antifungal agent against *Aspergillus niger*: NCIM 1025

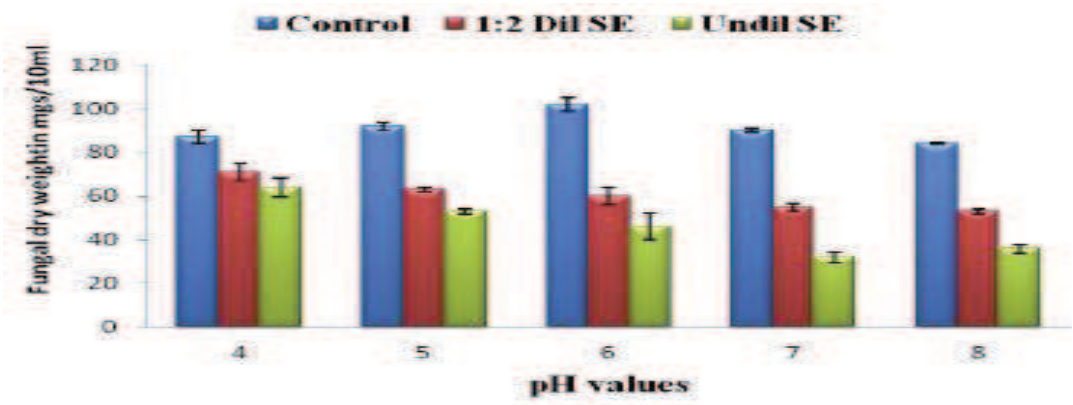


Fig 2.b: Effect of different pH values on the activity of siderophore as an antifungal agent against *Fusarium oxysporum* NCIM 1009

Through this research it was found that iron (one of the most important nutritional factor) concentration had a great effect on antifungal activity of siderophores produced by *Pseudomonas aeruginosa*. (**Fig3.a** and **Fig3.b**) indicated that the maximum percentage of inhibition (maximum siderophore activity) in dry weight of both fungi was achieved at low iron concentration (10 micromole/ml iron).

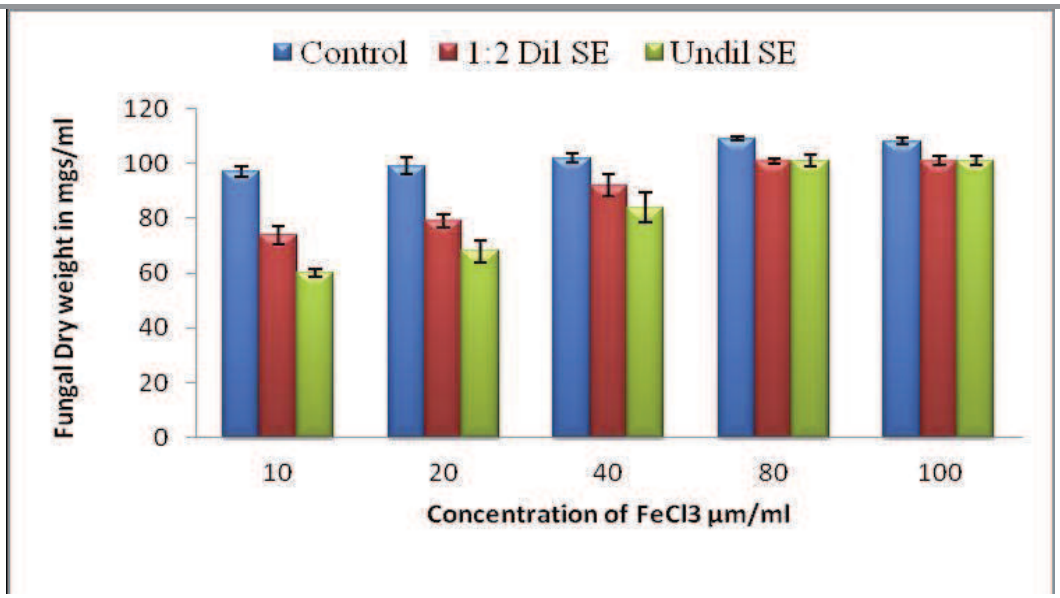


Fig 3.a: Effect of different iron concentration on the activity of siderophore as an antifungal agent against *Aspergillus niger*: NCIM 1025

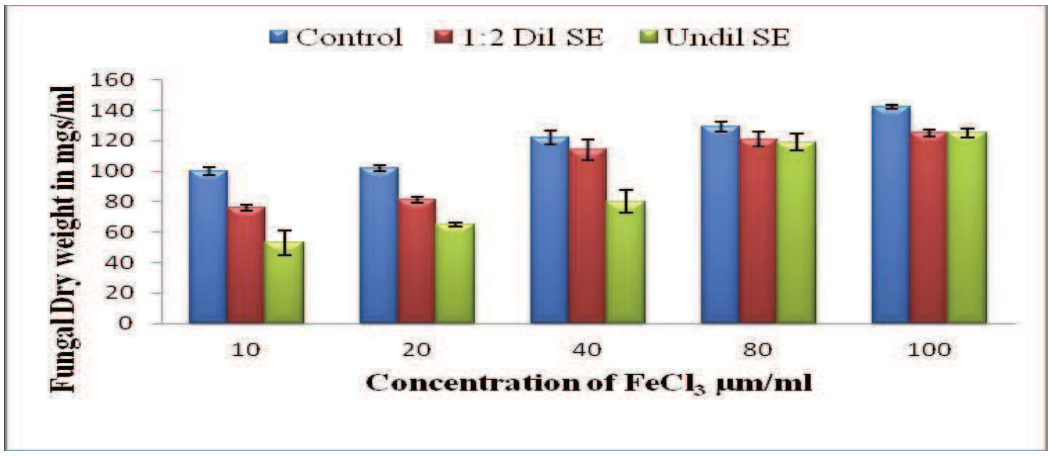


Fig 3.b: Effect of different iron concentration on the activity of siderophore as an antifungal agent against *Fusarium oxysporum* NCIM 1009.

As the iron concentration increased, the antifungal activity was found to decrease The results indicated that *Fusarium oxysporum* NCIM 1008 was more sensitive to inhibition than that of *Aspergillus niger* NCIM 1025.Maximum antifungal activity was observed at 10 µM/ml concentration of iron giving a yield of 0.6gm% in presence of undiluted siderophore sample.

Conclusion:

The presented data exhibit the antifungal activity of *Pseudomonas* strains and indicate the possibility of using *Pseudomonas aeruginosa* as a biological control agent of some plant pathogenic fungi. However, this requires further screening of a large number of *Pseudomonas* strains from different regions of India. The antimicrobial activity of *Pseudomonas* may be attributed to the various phytochemical constituents that have even more potency with respect to the inhibition of microbes.

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