Production, Purification and Characterization of Siderophore from *Pseudomonas fluorescence*

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In the present study plant growth promoting rhizobacterial strains belonging to fluorescent pseudomonads were isolated from rhizosphere soil and screened for siderophore production. Among these isolates, the potent strain was identified by biochemical and 16S ribosomal DNA gene sequencing and the isolate was confirmed as *Pseudomonas fluorescence*. Optimized production of siderophore was carried out utilizing various sources such as carbon, nitrogen, organic acids, amino acids and pH (5-9). Siderophore production was optimum during 24-30 h incubatory span at pH 7, 29°C temperature. Other parameters investigated were sugars, nitrogen sources, organic acids, amino acids, metal ions and iron (FeCl₃). The siderophore was purified using Amberlite XAD-4 column chromatographic technique. The purified siderophore was characterized by FTIR spectral analysis.

Key words: Siderophore, Optimized production, Pseudomonas fluorescence, Succinate medium.

Plant Growth Promoting Rhizobacteria (PGPR) are of agronomic importance. Indeed, they produce metabolites such as plant growth regulators, which directly promote growth and facilitate nutrient uptake by plants. Plant growthpromoting rhizobacteria (PGPR) are free-living soilborne bacteria that colonize the rhizosphere, and enhance seed germination and plant growth¹. PGPR plays an important role in producing siderophores which enhance the availability of soil iron to higher plants^{2,3}. Siderophores are secreted under low iron stress, that act as a specific ferric iron chelate agent and due to their potential in the biological control against fungal and bacterial parasites they have been studied widely in recent years^{4,5}. They stimulate plant growth directly by increasing the availability of iron in the soil rhizosphere or indirectly by competitively inhibiting the growth

of plant pathogens with less efficient iron uptake system⁶. Most of the microorganisms exhibiting PGPR activity, belong to the Gram negative group. The biosynthesis of a yellow-green fluorescent, water soluble pigment 'pyoverdin'produced by Pseudomonas fluorescence occur when the bacteria are iron deficient^{7, 8}. Siderophore production of PGPR is influenced by calcium, nitrogen and minerals9. The factor that influences growth or siderophore production by PGPR would influence the efficacy of PGPR in plant growth promotion and suppression of disease¹⁰. An alternative to synthetic chemical fungicides having negative effects and inducing pathogen resistance is the use of microorganisms or their antibiotics for managing the plant diseases.

Present work focuses on isolation and identification of *Pseudomonas fluorescence* from rhizosphere soil, utilizing *Pseudomonas fluorescence* for the production and optimization of siderophore production to investigate the optimal parameters required for the production.

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MATERIALSAND METHODS

Isolation of siderophore producing bacteria

Rhizospheric soils were collected from healthy plants in and around Chennai from different fields and bacteria were isolated by serial dilutions using sterile nutrient agar. The isolates showing different colony morphology were grown in King's B medium. Colonies that showed fluorescence at 365 nm under UV light on a transilluminator were selected, purified by repeated streaking on the same medium and used for further studies.

Screening of cultures for siderophore production

The Cultures were screened for siderophore production using blue agar plates containing the dye Chrome Azurol S agar (CAS) method ¹¹.

Identification of the isolated potential strain

Isolated potential strain was subjected to identification on the basis of colony morphology, Gram's staining and motility. Further identification by biochemical test was done based on the standard methods of Bergey's manual of Systematic Bacteriology. In order to identify the bacterial strain the16S rRNA gene sequence analysis was performed by PCR and the sequence of the isolate was compared with its related gene sequences available at the NCBI/Genbank database for sequence homology using BLAST (Basic Local Alignment Research tool) online service (Accession #: KC913193). Multiple sequence alignment was done by CLUSTAL W and phylogenetic tree was constructed using PHYLIP software.

Siderophore production

For siderophore production Iron free succinate medium consisting of g L-1; K_2HPO_4 , 6.0; KH_2PO_4 , 3.0; $MgSO_4$, $7H_2O$, 0.2; $(NH_4)_2SO_4$, 1.0 and Succinic acid, 4.0; pH, 7.0 was inoculated with 24 h old bacterial cultures at the rate of 1% (V/V) inoculum. It was incubated at 29°C on a rotary shaker at 120 rpm for 24-30 h. Following incubation the fermented broth was centrifuged at 10,000 rpm for 15 minutes and cell free supernatant was subjected to detection and estimation of siderophores.

Quantitative estimation of siderophore

A 0.5ml of culture free supernatant was mixed with 0.5ml of CAS assay solution. A reference was prepared using 0.5ml of uninoculated succinate

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medium mixed with 0.5ml of CAS solution. After equilibrium was reached the absorbance was measured at 630nm using UV-vis spectrophotometer.

Siderophore content in the aliquot was calculated by using following formula:

% Siderophore units =
$$\frac{dr - ds}{dr} \times 100$$

Where, Ar = absorbance of reference at 630nm (CAS reagent) and As = absorbance of sample at 630nm.

Optimization of cultural conditions for the production of siderophores

The fermentation medium was treated with the following parameters, and incubated at 29°C on a rotary shaker at 120 rpm for 24-30 h, growth and siderophore content were estimated to inspect their influence on the production of siderophores.

Fermentation span

The culture was grown in iron free succinate medium by submerged fermentation method with constant shaking at 120 rpm at 29°C for 48 hours. Samples were withdrawn after every 6 hours interval and were subjected for growth measurement (Optical Density 600nm) and siderophore production (Optical Density 630nm). Hydrogen ion concentration

The pH of the medium was set to a broad range. Acidic pH comprised 5 and 6, neutral pH comprised the value of 7 and alkaline pH comprised 8 and 9. These were adjusted using standardised concentrations of 0.1N hydrochloric acid and 1N sodium hydroxide solutions.

Carbon supplements

Four carbon sources had been externally supplemented to succinate medium which included dextrose, glucose, sucrose and mannitol at a concentration stated 1g/L.

Nitrogen supplements

Iron free succinate medium was optimized by using various nitrogen sources which included ammonium nitrate, corn steep liquor, soya flour tryptone and urea at concentration stated 1g/L. **Organic acids**

Fermentation medium was optimized by using different organic acids such as citric acid, malic acid, oxalic acid and succinic acid at a concentration of 4g/L.

Amino acids

Succinate medium was separately fortified with 4 amino acids, which included arginine, cystine, lysine and tyrosine, at concentration stated 1g/L.

Metal ions

Five metal ions had been externally supplemented to succinate medium which included cobalt (CoCl₂), lead acetate (PbCH₃CHOO), magnesium (MgCl₂), manganese (MnCl₂) and zinc (ZnCl₂) at concentration 10μ M/100ml.

Iron concentration

In order to determine the threshold level of iron at which siderophore biosynthesis is repressed, succinate medium had been supplemented at concentrations of 1-100 μ g/mL of iron (FeCl₂.6H₂O).

Purification of Siderophore

Pseudomonas fluorescence grown in succinate medium for 48 h in a rotary shaker at 120 rpm was centrifuged at 12000 rpm for 20 min. The supernatant was then passed through a column packed with Amberlite XAD-4 resin. The adsorbed column was washed three times with distilled water and then eluted with methanol. The eluted fraction was subjected to CAS test. The fraction was lyophilized and used for further studies.

Characterization

Thin layer chromatography

The purity of the eluted fraction was assured on silicagel TLC plate using solvent system n-butanol/water/acetic acid 4:1:1 (v/v/v) as mobile phase. Separated spots were located by spraying with 2MFecl₃ solution .The colour and Rf values of the spots were determined.

Fourier-Transform Infrared (FTIR) analysis of purified siderophore

The purified product was subjected to Fourier Transform Infrared Spectroscopy (FTIR) in JASCO FT/IR 4100 series instrument, recorded from 4000 to 400 per cm; for the determination of functional groups present in the purified product in Department of Chemistry, Indian Institute of Technology (IIT), Madras, Chennai.

RESULTS AND DISCUSSION

In recent years, fluorescent *Pseudomonas* has drawn attention worldwide because of their production of secondary

metabolites such as siderophores, antibiotics, enzymes and phytohormones.

Isolation, screening, identification and characterization of the strain

In the present study, the Plant Growth Promoting Rhizobacteria was isolated from rhizospheric soil and screened for siderophore production. Screening for siderophore production was done using Chrome Azurol S agar plate technique; change in the colour of CAS agar from blue to orange confirmed the ability of isolates to produce siderophore. One isolate was selected as potential strain for siderophore production which produced greater zone of orange halos and used for further studies. A sensitive chemical method for the detection of siderophores, which is based on their affinity for iron (III)¹¹. Biochemical characterization of the isolated potential strain was carried out according to Bergeys manual of Systematic Bacteriology and confirmed by 16S rDNA gene sequencing. The potential strain was Gram negative, motile, rod shaped and produced yellowish green pigment on King's B medium which fluoresced under UV light on a transilluminator. BLAST analysis of partial 16S rDNA gene sequences showed that the isolate was closely affiliated with members of the genus Pseudomonas. The isolate exhibited 100% similarity to that of Pseudomonas fluorescens strain IAM 12022 (Acc # NR043420) and 99% similarity to Pseudomonas sp AH2 (Acc # AF451275) Therefore, the potential strain was identified as Pseudomonas fluorescens. A soil isolate Pseudomonas fluorescens CV6 identified by 16S rDNA gene sequence analysis¹². *Pseudomonas* discriminated into genus, species and isolate levels based on electrophoretic pattern using ERIC-PCR has been identified as Pseudomonas aeruginosa and Pseudomonas fluorescens¹³.

Siderophore production

Iron free succinate medium was used for siderophore production^{8,14,15}. The change in the colour of succinate medium from colourless to green indicated siderophore production. The green colour of succinate medium indicated development of green coloured pigment and production of siderophores¹⁶.

The growth of *Pseudomonas fluorescens* in standard succinate medium (which contained no added iron) is accompanied by excreation of pyoverdine, excretion ceased as the culture entered the stationary phase⁷. Siderophore production has been reported to be maximum (92.25%) when Alcaligenes faecalis was grown in succinate medium9.

Effect of various physico-chemical parameters on the production of siderophores

Fermentation span

Siderophore production started after 12 h of incubation. Maximum amount of siderophore (80%) is produced during 30 h of incubation (Fig.1). Thereafter siderophore production and growth decreased. Highest production of siderophore occurred at 24 and 30 h of incubation^{16,17}. Rhizobium strains started siderophore production at 8-9 hours post inoculation, with maximum production at 24 hours¹⁸. Initiation of siderophore production occurred in first quarter of exponential growth phase ¹⁵.

Hydrogen ion concentration

90

80

70 60 Maximum siderophore yield (80% units)

was obtained at pH 7 (Fig.2). Iron is present in insoluble form at neutral to alkaline pH and at alkaline pH growth was favoured, while at slight acidic pH (6.5) siderophore yield was maximum²⁴. It has been reported that alkaline pH helps in excess solubilization of iron, which increases the iron content of the medium¹⁹.

The pH of the medium increased from 7 to 8.5 during the growth period in accordance with the siderophores concentration which suggested that alkalinity is important to avoid siderophore destruction²⁰.

Influence of sugars, organic acids and amino acids

Among the various sugar tested at a concentration of 1g/L, sucrose was found to have the maximum effects (Fig.3). Nature of carbon compound determines the Fe requirement of cell and thus regulates siderophore production²¹. Addition of 1% sucrose greatly increased the production of siderophores¹⁸. Among organic acids; the yield was more with malic acid (85%





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siderophore units). But the production was high with succinic acid (Fig.4.) present in the control medium. This is in accordance with the result obtained by others^{9, 16, 17}.

Different amino acids were checked for the utilization as carbon and nitrogen source (Fig.5). Tyrosine resulted in the production of maximum siderophore units (78%) followed by arginine, cysteine and lysine. All the tested aminoacids positively supported the siderophore production and growth.

Organic acid (Succinic acid), sugar (Sucrose) and amino acids (Tyrosine) boosted the growth while enhancing siderophore production. Root exudates containing organic acids, sugars, amino acids or essential constituents in rhizosphere which influences growth and metabolite production and root colonization of PGPR²².

Nitrogen sources

Out of various nitrogen sources, urea

found to be the highest nitrogen source with a yield of 79%. Utilization of urea by the isolate suggested its possible exploration for bioremediation of alkaline soil, by reducing the excess amount of urea present in the soil.¹⁶. Siderophore production of 85% units with good growth was obtained with succinate medium supplemented with ammonium sulphate present in the control medium (Fig. 6). Tryptone, corn steep liquor, ammonium nitrate, soyfluor also supported the growth as well as siderophore production considerably followed by urea. Arginine and proline utilized as nitrogen sources stimulated growth and siderophore production in *Rhizobium* strains¹⁸. **Metal ions**

Lead acetate gave the highest siderophore units (82%). Magnesium chloride is the next heavy metal which gave the maximum siderophore value followed by zinc sulphate .All these metal ions enhanced the siderophore production without altering the growth. (Fig. 7).

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Fig. 6. Effect of Nitrogen Sources on

Siderophore Production

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Fig. 8. Effect of Iron on Siderophore Production

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While Manganese chloride and Cobalt chloride showed inhibitory effect on both growth and siderophore production. When medium was supplemented with lead, it enhanced maximum siderophore production as well as growth of cultures. While manganese, and cobalt showed inhibitory effect on both growth and siderophore production¹⁶. The metal ions Mg²⁺ and Zn²⁺ in the medium enhanced the siderophore production of about 75% and 72% respectively¹⁷. Inhibition of azotichelin and aminochelin was inhibited by Mn²⁺ and Zn²⁺ in *Azotobacter vinelandii*²³. **Iron**

Growth of the cultures increased with the increasing concentration of iron in succinate medium.Siderophore production decreased after 10µg/ml of iron (Fig. 8). Maximum siderophore units of 80% were obtained with 10µg/ml of FeCl₃. This result is in accordance with the result obtained by other authors. Increase in growth with increase in iron concentration reflects the iron requirement of organisms for cellular processes^{16, 24}. Concentration of iron >10µM had a negative effect on siderophore production²⁰. Siderophore production is inversely proportional to iron concentration whereas growth is directly proportional to iron concentration¹⁵.

Characterization

Yellowish green fluorescent spot was detected on TLC plates under UV light. The Rf value calculated was 0.72. These result was found similar to that detected by other workers^{25,26,27}.

Functional group analysis of the purified siderophore done by FTIR spectra showed a broad peak at 3444.24 cm⁻¹ indicating the presence of an alcoholic group. The result of this study is in accordance with ^{28,29,30} observed a similar hydroxyl peak at 3415 m⁻¹, 3357.21 cm⁻¹, 3351 cm⁻¹ and 3462 cm⁻¹ respectively. Appearance of peak at 1730.32 cm⁻¹ indicated C=O stretch, while 1649.32 cm⁻¹ revealed the presence of C=C stretch and a peak at 1644.5 cm⁻¹ showed an N-H bend. Functional chemical group resembling ferrichrome siderophore were observed in *Pseudomonas fluroscence*³¹.

CONCLUSION

A myriad of environmental factors are essential for siderophore production, including pH, iron concentration, supply of carbon source, nitrogen source, organic acid, aminoacid,

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incubation span etc., Besides acting as iron chelates, siderophore are also known to have large potential as bio control agent of soil borne plant pathogen. Fluorescent *Pseudomonas* has high ability to promote plant growth and utilization of them in stable agriculture is suggested as replacing chemical compounds. Therefore the exploitation of rhizobacteria *Pseudomonas fluroscence* has significance since it can be used in the production of siderophore in a commercial scale. Further studies on the effect of the strain on plant growth are under study

REFERENCES

- 1. Kloepper, J.W., Schroth, M.N. Relationship of *in-vitro* antibiosis of plant growth promoting rhizobacteria to plant growth and the displacement of root microflora. *Phytopathol.*, 1981; **71**:1020-1024.
- Bar-Ness, E., Hadar, Y., Chen, Y., Romheld, V., Marschner, H. Short term effects of rhizosphere microorganisms on Fe uptake from microbial siderophores by maize and oat. *Plant Physiol.*, 1992; 100:451-456.
- Yang, C-H., Crowley, D. E. Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Appl Environ Microbiol.*, 2000; 66: 345-351.
- Neilands, J.B. Siderophores : structure and function of microbial iron transport compounds. *J Biolog Chem.*, 1995; 270 (45):26723-26726.
- Abd-Alla, M.H. Nodulation and Nitrogen fixation of *Lupinus* species with *Brady rhizobium* (lupin) strains in iron –deficient oil. *Biol.Fertil.soils.*, 1999;28: 407-15.
- 6. Marek-Kozaczuk, M., Deryto, M., Skorupska, A. The insertion mutants of *Pseudomonas* sp.267 defective in siderophore production and their effect on clover (*Trifolium pratense*) nodulated with *Rhizobium leguminosorum* bv.trifollii. Plant and soil., **179**:269-74.
- Meyer, J.M., Abdallah, M.A. The fluorescent pigment of *Pseudomonas Fluorescens*: Biosynthesis, Purification and properties. *J Gen Microbiol.*, 1978; 107: 319-328.
- Rachid, D. and Ahmed, B. 2005. Effect of iron and growth inhibitors on siderophores production by *Pseudomonas fluorescens*. *Afr J Biotechnol.*, 4(7): 697-702.
- Sayyed, R.Z., Gangurde, N.S., Patel, P.R., Joshi, S.A., Chincholkar, S.B.. Siderophore production by *Alcaligenes faecalis* and its application for growth promotion in *Arachis hypogaea*. *India J*

Biotechnol., 2010; 9: 302-307.

- Chincholkar, S. B., Chaudhari, B. L., Talegaonkar, S. K., Kothari, R. M. Microbial iron chelators : A tool for sustainable agriculture, in Biocontrol potentials and their exploration in crop disease management., 2007; (1): 49-70.
- 11. Schwyn, R. and Neilands, J.B. Universal chemical assay for the detection and determination of siderophores. *Anal Biochem.*, 1987; **160**:47-56.
- 12. Maleki, M., Mostafaee, S., Mokhtarnejad, L., Farzaneh, M. Characterization of *Pseudomonas fluorescens* strain CV6 isolated from cucumber rhizosphere in varamin as a potential biocontrol agent. *Aust J Crop Sci.*, 2010; 4(9):676-683.
- Pastor, N.A., Reynoso, M.M, Tonelli, M.L., Masciarelli, O., Rosas, S.B., Rovera, M. Potential biological control *Pseudomonas* sp. PC12 against damping-off of tomato caused by *Sclerotium rolfsii. J Plant Pathol.*, 2010; 92(3):737-745.
- Rakh, R.R., Raut, L.S., Dalvi, S.M., Manwar, A.V. Biological control of *Sclerotium rolfsii*, causing stem rot of groundnut by *Pseudomonas* cf. *monteilii* 9. *Rec Res Sci Technol.*, 2011; 3(3):26-34.
- Sarode Prashant, D., Rane Makarand, R., Chaudhari Bhushan, L., Chincholkar Sudhir, B. Siderophoregenic *Acinetobacter calcoaceticus* isolated from wheat rhizosphere with strong PGPR activity. *Malay J Microbiol.*, 2009; 5(1): 6-12.
- Sayyed, R.Z., Badgujar, M..D., Sonawane, H.M., Mhaske, M.M., Chincholkar, S.B. Production of microbial iron chelators (Siderophores) by fluorescent *Pseudomonads*. *Ind J Biotechnol.*, 2005; 4:484-490.
- Kanimozhi, S., Perinbam, K. Siderophore production by *Pseudomonas fluorescens* Lp1 and its application as biocontrol agent. *J Pharma Res.*, 2011; 4(90):3175-3179.
- Sridevi, M., Mallaiah, K.V. Production of Hydroxamate – Type of Siderophores by *Rhizobium* strains from *Sesbania sesban* (L.) Merr. *Inter J Soil Sci.*, 2008; 3(1):28-34.
- Bolton, H. J., Fredrickson, J.K., Elliot, L. F. Microbial ecology of the rhizosphere, *Soil Microb Eco.*, 1993; 27-63.
- Villegas, M.E.D., Pilar Villa., Alina Frias. Evaluation of the siderophore production by *P. aeruginosa* PSS. *Revista atinoamericana de Microbiol.*, 2002; 44:3-4.
- 21. Deelip, C., Deelipkumar, B. S. Influence of

aminoacids,organic acids,and sugars on growth, fluroscence and siderophore production of *Fluroscent pseudomonads. Ind J Exper Biol.*, 1998; **36**: 429-431.

- 22. Kunc, F., Macura, J. Mechanism of adaptation and selection of microorganisms in soil. *Soil Microb Assoc.*, 1988; 281-299.
- Page, W. J. The effect of manganese oxides and manganese ions on growth and siderophore production by *Azotobacter vinelandii*, *Biometal.*, 1995; 8: 30-36.
- 24. Dave, B.P., Dube, H.C. Regulation of siderophore production by Fe (III) in certain fungi and fluorescent *Pseudomonas. Ind J Exper Biol.*, 2000; **38**:297-299.
- Hassanein ,W.A., Awny, N.M., El-Mougith, A.A, Salah El-Dien,S.H.The antagonistic activities of some metabolites produced by *Pseudomonas aeruginosa* Sha8. J Appl Sci Res., 2009; 5 (4):404-414.
- Kumar, S.R., Ayyadurai, N., Pandiaraja, P., Reddy, A.V., Venkateswarlu, Y., Prakash, O., Sakthivel, N.Characterization of antifungal metabolite produced by a new strain *P. aeruginosa* PUP a3 that exhibits broad spectrum antifungal activity and biofertilizing traits. *J. Pure Appl Microbio.*, 2005; **98**: 145-154.
- Aditi Bhattacharya. Siderophore mediated metal uptake by *Pseudomonas fluorescence* and its comparison to iron (III) chelation. *Cey.J.Sci.* (*Bio.Sci.*)., 2010; **39**(2):147-155.
- Murugappan, R.M., Aravinth., A., Karthikeyan., M. Chemical and structural characterization of hydroxamate siderophore produced by marine *Vibrio harveyi. J Ind Microbiol Biotechnol.*, 2011; 38: 265–273.
- 29. Patel, A.K., Deshattiwar, M.K., Chaudhari, B.L. and Chincholkar, S.B. Production, purification and chemical characterization of the catecholate siderophore from potent probiotic strains of *Bacillus* spp. *Biores Technol.*, 2009; **100**: 368–373.
- Shirishkumar, V.S., Ajay, M. S., Avinash, A. R. Catechol Siderophore Produced by *Klebsiella pneumoniae* Isolated from Rhizosphere of *Saccharum officinarum L. Intern J Sci Res.*, 2013; 2(5): 423-425.
- 31. Neelam Tank, Narayanan Rajendran, Baldev Patel, Meenu Saraf. Evaluation and biochemical characterization of a distinctive pyoverdin from a *Pseudomonas* isolated from chickpea rhizosphere. *Braz J. Microbiol.*, 2012; 639-648.