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Production and optimization of microbial iron chelators (Siderophore) by*Pseudomonas* spp

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Abstract: **Siderophores are small protein molecules that can easily bind to ferric iron. As a chelating agent they transport iron molecules inside the bacterial cell for various biochemical reaction. At present studies characterization and Optimization of few siderophores producing bacteria from the soil samples collected from Salem district, Tamil Nadu. The siderophores production was assayed qualitatively and quantitatively through Chrome Azural S and the results showed positive for the strains PSF01 and PSA02 that grown in succinate medium. Further characterization and optimization results revealed that both the bacterium has the ability to yield siderophores in the optimum condition of P^H 6 at temperature 37° c with xylose as a carbon source and NH_4SO_4 as a Nitrogen Source. Estimation of siderophore production in different media using maximum % siderophore unit (85%) King's B Medium Fe content in different media using maximum % of siderophore unit (80%) in Nutrient broth medium. The antagonist activity of the siderophore producing *Pseudomonas* spp against fungal pathogen *Fusarium oxysporum.* This result showed that *Pseudomonas* spp is a good producer of siderophore which can be usefully exploited as a biocontrol agent.**

Keywords: **Biocontrol, *Pseudomonas* spp, siderophore, Optimization, *Fusarium oxysporum.***

1. INTRODUCTION

Plant growth promoting Rhizobacteria (PGPR) directly associate with plant roots and can exist within root tissue (as endophytes), on the surface of plant roots (the rhizoplane), or within the zone of soil specifically influenced by the root system (the rhizosphere). Characterized by their plant-growth promoting properties, PGPR are a diverse group of bacteria that produce a wide range of enzymes and metabolites, influence nutrient acquisition, modulate hormone levels, and ameliorate the negative impacts of biotic and abiotic stressors (Ngumbi and Kloepper 2016)

Pseudomonas are excellent candidates of PGPR and can produce hydrogen cyanide, siderophores, protease, antimicrobial compounds and possess phosphate solubilizing activity. (Noor *et al*.,2012). Microbes release siderophores to scavenge iron from these mineral phases by formation of soluble Fe^{3+} complexes that can be taken up by active transport mechanisms. Many siderophore are non-ribosomal peptides. (Hu *et al*., 2011). At present nearly 500 siderophores are reported from selected microorganisms. A great variation is seen in siderophores structures from one species to another (Alli and vidhale *et al*., 2013).

Biological control has emerged as a very popular alternative because it offers a way of controlling pathogens that does not involve the use of chemicals. Siderophores (derived from the Greek meaning 'iron carriers') are low molecular weight (below 1000Da), ferric –ion-specific chelating agents produced by bacteria and fungi to compact low iron stress (Ngamau *et al*., 2014).

Sesame (*Sesamum indicum L*.) seed has rich source of fatty acids and antioxidants, so it is used as healthy and nutritive additive to prepare several foods. The yield of sesame has been affected by several abiotic and biotic factors. The seedlings emergence, plant growth and the yield of *sesame* are inhibited up to 40%, during the effect of destructive pathogens of *Fusarium oxysporum* and *Macrophomina phaseolina*, which cause wilt and charcoal rot disease (Kumar *et al.*, 2011).

Siderophore producing PGPR play a vital role in Fe nutrition of plants and therefore in plant growth promotion leading to healthy plants, which are vital for increasing the crop and food yield. Siderophores of rhizobacteria provide iron nutrition to the plants and help in plant growth promotion. (Shaink *et al.,* 2014). They prevent the availability of iron to plant pathogens thereby restricting its growth, and this, help in biological control of phytopathogens.

2. MATERIALS AND METHODS

2.1. Isolation of soil microbes: Rhizosphere soils were collected from plant rhizosphere region Pagalpatti, Pannapatti. Poosaripatti (Omalur), Salem. TamilNadu. The collected soil sample was brought to the laboratory in sterile polythene bag under aseptic conditions the soil sample was air dried and subjected to the isolation of bacteria by spread plate technique.

2.2. Soil Analysis: The soil samples were air dried and stored in a refrigerator at 5ºC for analysis. The pH of the soil samples was measured with PH meter using 1:2.5 soil/wet system (Elico, India) and Electrical Conductivity (EC) was measured using an electrical conductivity bridge (Elico type CM82, India) and was expressed as dSm^{-}. The available nitrogen content in the soil was estimated by the alkaline permanganate method as described by (Subbiah and Asija 1956). Soil phosphorus was estimated by the method by the given by (Olsen *et al.,* 1954). Potassium content of the soil was estimated by the method of (Jackson, 1973).

2.3. Biochemical Characterization of soil bacteria

2.3.1. Gram Staining: Gram staining was detected by method described by **(Cappuccino and Sherman, 2002):**

2.3.2. Indole test (Morello, *et al*., 2002): Two ml of peptone water was inoculated with 5ml of bacterial culture and incubated for 48 hours. 0.5ml of Kovac's reagent was added shakes well and examined after 1 minute. A red colour in the reagent layer indicated Indole. In negative case there is no development of red colour

2.3.3. Methyl red (MR) and Voges Proskauer (VP) test (Morello, *et al*., 2002): MRVP broth was prepared and 5ml broth is transfer in culture tubes inoculated with bacterial strains and two test tubes were kept as control. The test tubes were incubated at 28ºc for 48h.Five drops of MR indicator were added to each test tube including the control and observed the change in colour similarly ten drops of VP-I reagent and 2-3 drops of VP-II reagent were added to other incubated test tubes and control also. Observed the test tubes for colour change and compared with control.

2.3.4. Citrate utilization test (Ruchhoft, *et al*., 1931): The isolates were carried out by inoculating the test organism in test tube containing Simon's citrate medium and this was inoculated for 24 hours to 72 hours. The development of deep blue colour after incubation indicates a positive result.

2.3.5. Catalase activity (Taylor and Achanzar, 1972): The isolates were streaked on yeast extract mannitol agar slant and incubated for 3 days at 28°C. The formation of effervescence upon addition of 1ml of 1% hydrogen peroxide was positive to catalase activity.

2.3.6. Oxidase production (Gaby and Hadley, 1957): The isolates were streaks on yeast extract mannitol agar plates and incubated for 3 days at 28°C. After incubation, a loop full of isolates was placed over oxidase disc (N, N – Tetra methyl-Para-phenyl diamine dihydro chloride). Development of blue (or) purple colouration was positive to oxidase production.

2.3.7. Urease activity (Mac Faddin, 2000): The isolates were streaked on Christensen's urea agar slants and incubated for 3 days at 28°C Observed the slant for a colour change at 6 hours, 24 hours, and every day for up to 6 days. Urease production is indicated by a bright pink colour on the slant.

2.4. IAA (Indole-3-acetic acid) production: (Ahmad *et al*., 2005). IAA (Indole-3-acetic acid) was detected by method described by Ahmad 2005.

2.5. Phosphate solubilization test (Chen *et al*., 2006): Phosphate solubilization ability of isolates was detected by spotting them on Pikovskaya's agar plates. The plates were then incubated at 28±1°C for 3 days and then observed for the appearance of clearing zones around the colonies (due to solubilization of inorganic phosphate by producing organic acid by bacteria).

2.6. Siderophores production: The *pseudomonas* isolates were inoculated in King's B broth and incubated for 15 days. After 15 days' incubation period the culture was centrifuged at 10,000rpm to 15min cell free supernatant were used for the following tests.

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2.6.1. Siderophore assay: Siderophore assay was carried out based on the CAS shuttle assay of (Payne 1994). The siderophore content in the aliquot was calculated by using following formula % of siderophore units= $\frac{(Ar-As)\times 100}{Ar}$ Where, Ar= absorbance of reference at 630 nm (CAS reagent) As= absorbance of sample at 630 nm

2.6.2. Ferric chloride test: To 0.5ml of culture filtrate 0.5ml of 2% aqueous ferric chloride solution was added. Appearance of reddish brown /orange colour was positive indication of siderophore production.

2.6.3. Arnow's test (Arnow, 1937): To 1ml of culture filtrate 1ml of 0.5N hydrochloric acid, 1ml of nitrite molybdate reagent and 1ml of 1N sodium hydroxide were added. The formation of red coloured solution was considered as indication of the presence of catechol type of siderophore.

2.6.4. Tetrazolium test (Snow, 1954): To a pinch of tetrazolium salt were added to 2drops of 2N sodium hydroxide and 0.1ml of the culture filtrate. Appearance of a deep red color was indication of hydroxamate type of siderophore.

2.7. Siderophore production in different media (Sayyed *et al* 2005) : Siderophore production occurs different media like NB and kings'B medium nutrient broth containing gL^{-1}:peptone 5.0;NaCl, 5.0;beef extract, 1.5;and yeast extract, 1.5;were separately inoculated and incubated to check their effect on growth and siderophore production by P^H 7.0 was inoculated with 1% of overnight inoculums and incubated at 30^0 c for 24-30 hours with constant shaking at 10,000rpm for 10min and the cell free supernatant was subjected to siderophore assay.

2.8. Estimation and siderophore production in Fe content in different media: Fe content of different media like, nutrient broth (NB) and King's B medium was determined on atomic absorption spectrometer (UNICAM, Model 969, UK).

2.9. Optimization of siderophore production: The bacterial isolates were allowed to grow in different fermentation conditions, such as pH, Temperature, nitrogen source, carbon source, iron concentration and organic acid were investigated in order to allow the utmost production of siderophore. (Fazary *et al*., 2016.)

2.10. Molecular Identification of Efficient Rhizosphere Isolate Using 16S rRNA Gene Sequencing: Molecular characterization of the efficient PGPR isolate was done by 16Sr RNA gene sequencing. DNA of the isolated was extracted by modified method of Sam brook and Russel. The gene was amplified using universal bacterial 16S rRNA gene primers under following conditions; Initial denaturation at 95ºC for 5mins, 30 cycles of denaturation at 95ºC for 30 secs, annealing at 52ºC for 30 secs, extension at 72ºC for 2mins and final extension for 10mins in Eppendorf thermos cycler. The amplified products were purified by electrophoresis in 1.2% agarose and extracted by QIA quick gel elution kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The purified PCR products were sequenced.

2.11. Isolation of fungal pathogen *Fusarium* spp: *Fusarium* species were isolated based on its colony and morphological characteristics (Singh *et al*., 1991). Isolated colonies were sub cultured onto potato dextrose agar (PDA) slants and stored at 4^0C. Six day old cultures were used throughout the study.

2.12. Antagonistic activity of *Pseudomonas* sp Bio control assay: Sterile Potato Dextrose Agar was prepared and solidified in petridishes. The well size 6mm diameter was made aseptically in the agar plate. Forty µ1 of partially purified siderophore was added to the well and allowed to diffuse radially for 1 hour at low temperature. The inoculums size of 6×10^3 spores of plant pathogenic fungi was swabbed over the surface of the PDA plates and incubated at room temperature for 48 hours. After incubation, the zone of inhibition of the mycelia growth was measure.

3. RESULTS

Rhizosphere soils were collected from Salem district, Tamil Nadu. The soil sample was analyzed for pH, Electrical conductivity, and NPK analysis (Table 1). Bacterial isolates was isolated by serial dilution method. The isolates were biochemical test (Table 2) and followed by genomic DNA identification. .*Pseudomonas aeruginosa* strain were submitted GenBank under Accession number Accession number MH128359.These isolates were conformed with *Pseudomonas aeruginosa*, *Pseudomonas fluorescence* they were named as from PSF01, PSA02, (Fig 2 AB). Phosphate solubilizing activity was shown by the isolates PSF01 with a zone of clearance 0.5mm (Fig 2 F). In PS01and PS02 IAA test was positive (Fig 3 E). The siderophores production was assayed qualitatively and quantitatively through Chrome Azural S and the results showed positive for the strains PSA01 and PSA02 that grown in succinate medium (Fig 2 D). Further characterization and optimization results revealed that both the bacterium has the ability to yield siderophores in the optimum condition of P^H 6 at temperature 37º c with xylose as a carbon source and NH_4SO_4 as a Nitrogen Source.

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Estimation of siderophore production in different media using maximum % siderophore unit (85%) King's B Medium Fe content in different media using maximum % of siderophore unit (80%) in Nutrient broth medium. (Fig 3).The antagonist activity of the siderophore producing *Pseudomonas* spp against fungal pathogen *Fusarium oxysporum.* (Fig 2 F) This result showed that *Pseudomonas* spp is a good producer of siderophore which can be usefully exploited as a biocontrol agent .

Table 1: Soil Characteristics

Location	pH	EC	N(mg/kg)	P(mg/kg)	K(mg/kg)
Pagalpatti	8.1	0.4	113	1.5	231
Pannapatti	7.9	0.3	125	4.5	185
Pusaripatti	7.3	0.2	98	3.5	85

EC = Electrical conductivity

Nitrogen =0 to 113 mg/kg-low, 113 to 181 mg/kg-medium, 181and above mg/kg-high

Phosphorus =0.0 to 4.5 mg/kg-low, 4.6 to 9.0 mg/kg-medium, 9.0 and above mg/kg-high

Potassium =0 to 46 mg/kg-low, 47 to 113 mg/kg-medium, 113 and above mg/kg-high

Table 2: Biochemical test

S.No	Test	PSA01	PSA02
1.	Gram staining	_	_
2.	Indole production	+	+
3.	Methyl Red	+	+
4.	Voges-proskaur	_	_
5.	Citrate utilization	+	+
Extra-cellular enzymes			
6.	Catalase activity	+	+
7.	Oxidase production	+	+
8.	Urea's activity	+	+

(+)Positive,(-)Negative

3. CAS Agar plate zone diameter

S.No	Culture Marking	CAS Agar Plate Zone diameter in (mm)
1	**PSFO1**	**10mm**
2	**PSAO2**	**7mm**

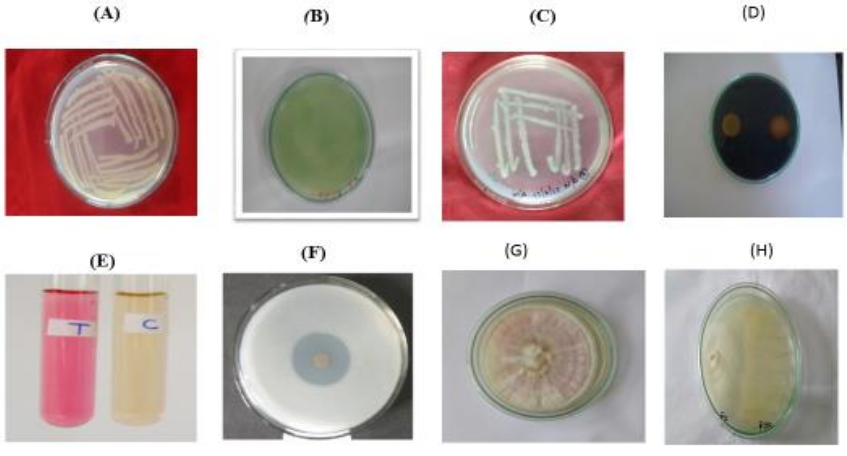

Fig 2: (A) *Pseudomonas aeruginosa*. (B)*Pseudomonas fluorescence*. (C)*Bacillus cereus*(D) Siderophore test. (E) IAA Test.(F) Phosphate solubilization test (G) *Fusarium* spp (H) Antagonistic activity

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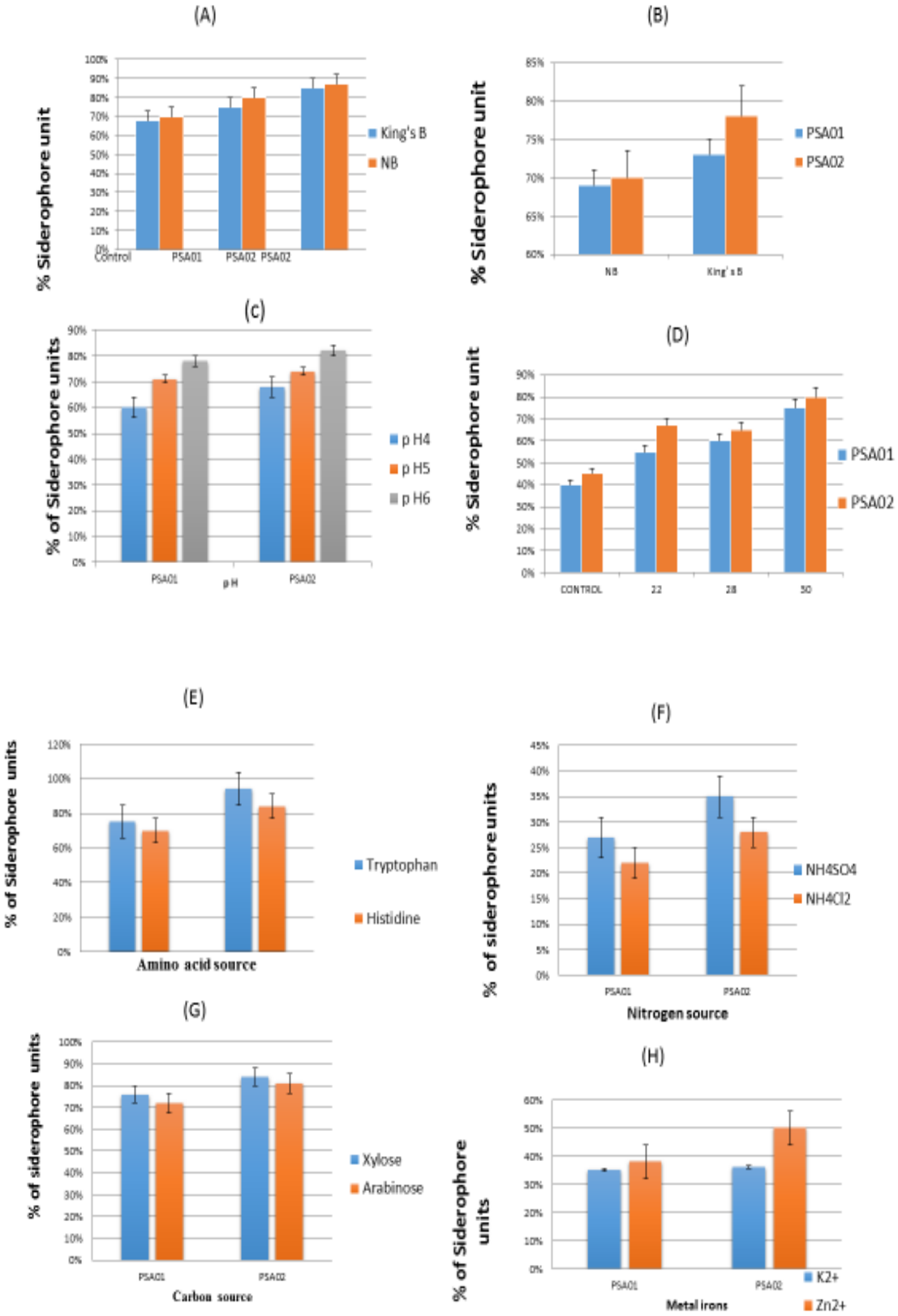

Fig 3: Optimization of siderophore production (A) Fe content in different medium (B) Siderophore production in different medium(C) Effect of p^H. (D) Siderophore production on different temperature. (E) Effect of amino acid. (F) Effect of nitrogen source. G) Effect of carbon source. (H) Effect of metal irons.

4. DISCUSSION

Pseudomonas gram negative, gram staining test it was confirmed that isolated bacteria belonged to genus *Pseudomonas*. In Gram's staining, the morphology of isolated *Pseudomonas* strains showed Gram-negative, pink colored, medium rod shaped appearance. These findings agreed with the findings reported by earlier researchers (Tripathi *et al*., 2011). In this study, the qualitative estimation of siderophores by *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* isolates showed that they were powerful producer of siderophores under limited iron on King's B medium. The production of siderophores by *Pseudomonas fluorescens and Pseudomonas aeruginosa* isolates indicated that these bacteria isolates can be used biocontrol against soil borne phytopathogens. Similarly, reported that *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* as siderophore producer on King's B medium. *Pseudomonas fluorescens* was able to give higher yields of siderophores under iron stress conditions (Sayyed *et al*., 2005). Different level of P^H tested production of siderophore by *pseudomonas aeurginosa* was optimum at P^H 7.0. The other nitrogen fixing bacteria Rhizobium strains isolated from root nodules of *sesbania sesban*.L produced siderophore in culture after 4h of incubation and at neutral P^H(Sridevi and Malaliah 2007).Since the production of siderophore depended largely on the P^H of the medium. It was very important to maintain the P^H of the fermentation broth between 7.0-7.5which is the optimum for siderophore production in *pseudomonas fluorescences.* The siderophore and cofactors produced by *Azotobacter* sp.is an iron chelating substance with the ability to bind to different metal ions was increased molybdenum and cadmium (Neilands 1974).Carbon source and its availability play a secondary but important role in regulating siderophore production. Siderophore production varied with the type of carbon source in *pseudomonas* sp. (Sayyed *et al*.,2005). In order to find out the effect of other minerals towards siderophore the production was carried out in mannitol medium amended with different mineral ions. The metal ions cu^{2+} in the medium enhanced the siderophore production. The other metal ions tested inhibitory effect on siderophore.

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