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Production of microbial iron chelators (siderophores) by fluorescent Pseudomonads

R Z Sayyed[1], M D Badgujar, H M Sonawane, M M Mhaske and S B Chincholkar*

Department of Microbiology, School of Life Sciences, North Maharashtra University, Jalgaon 425 001, India

[1]Department of Microbiology, Mooljee Jaitha College, Jalgaon 425 002, India

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Two fluorescent pseudomonads, *Pseudomonas fluorescens* NCIM 5096 and *P. putida* NCIM 2847 produced maximum yield of hydroxamate type of siderophore (87 & 83% units, respectively) in modified succinic acid medium (SM). $(NH_4)_2SO_4$ and amino acids were found to stimulate bacterial growth as well as siderophore production. However, optimum siderophore yield was obtained with urea. Increase in iron concentration up to 100 μ*M* favoured growth but drastically affected siderophore production in both the strains. Threshold level of iron ($FeCl_3$), which repressed siderophore production in both the strains, was 30 μ*M*. Sunflower oil proved to be suitable and cost effective defoaming agent for siderphore production in bioreactors. The results of shake flask level were found reproducible at scaled up conditions in bioreactors. Moreover, *P. fluorescens* NCIM 5096 inoculation enhanced seed germination, root length and shoot length of wheat (*Triticum aestivum*) under pot culture conditions.

Keywords: iron, optimization, Pseudomonads, siderophores, wheat growth
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Introduction

Rhizosphere is a dynamic environment, which harbours diverse groups of microbes. Some of the bacteria, which directly or indirectly stimulate plant growth, have been referred as plant growth promoting rhizobacteria (PGPR)[1-2]. PGPR promote growth of several annual crops by increased uptake of nitrogen[3], iron (through siderophores)[4] and phosphorus[5]; synthesis of phytohormones[6]; and by controlling plant diseases[7-8]. Recently, fluorescent pseudomonads are emerging as the largest and potentially most promising group of PGPR[9-10] involved in plant growth promotion and plant diseases control[11-14].

Siderophores producing *Pseudomonas* sp. play vital role in stimulating plant growth and in controlling several plant diseases[14]. They function as a biocontrol agent by depriving the pathogen from iron nutrition, thus resulting in increased yield of crop[11]. Growth and siderophore production by PGPR is attributed to organic acids, sugars, amino acids, minerals, enzymes and several other components of root exudates[15-16]. Effect of root exudation on microorganisms is of prime importance as it attracts beneficial organisms and has major influence on the diversity of bacteria in rhizosphere[16-17]. Any factor influencing either the growth or siderophore production by PGPR would greatly influence the efficacy of that PGPR in plant growth promotion and disease suppression[18].

Present work focuses on potential effects of cultural conditions on growth and siderophore production of fluorescent pseudomonads and effect of *Pseudomonas fluorescens* NCIM 5096 inoculation on seed germination and growth of wheat.

Materials and Methods

Source and Maintenance of Culture

P. fluorescens NCIM 5096 and *P. putida* NCIM 2847 were obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India. Cultures were routinely maintained on nutrient agar at 4°C and were used in further studies.

Screening of Culture for Siderophore Production

Both the cultures were screened for siderophore production by using spectrophotometric method described by Jalal *et al*[19], which was further confirmed by CAS agar method and Universal Chemical Assay [CAS][20-21].

Inoculum Development

A loopful of culture of *P. fluorescens* and *P. putida* from NA slant was separately inoculated in each 100 mL of iron deficient succinate medium (SM)[22] and

*Author for correspondence:
Tel: 91-257-2258417; Fax: 91-257-2258403
E-mail: chincholkar_sb@indiatimes.com

incubated for 24-30 h at 29°C with constant shaking at 120 rpm.

Production, Detection and Estimation of Siderophore

For siderophore production iron free SM medium[22] 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, *p*H 7.0 was used to inoculate 24 h old cultures of *P. fluorescens* and *P. putida* at the rate of 1% (v/v) inoculum. It was incubated for 24-30 h at 29°C with constant shaking at 120 rpm. Following the incubation, fermented broth was centrifuged (10,000 rpm for 15 min) and cell free supernatant was subjected to detection and estimation of siderophores. Siderophores produced in culture broth were detected by CAS assay as per Schwyn and Neilands[20].

Quantitative estimation of siderophores was done by CAS-shuttle assay[23]. In which 0.5 mL of culture supernatant was mixed with 0.5 mL of CAS reagent, and absorbance was measured at 630 nm against a reference consisting of 0.5 mL of uninoculated broth and 0.5 mL of CAS reagent. Siderophore content in the aliquot were calculated by using following formula:

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

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

Siderophore Production in Different Media

Different media preparations, like SM, Cas-amino acid medium (CAA)[22] containing g L^{-1}: Cas-amino acid, 5.0; K_2HPO_4, 1.180; and $MgSO_4.7H_2O$, 0.25, Barbhaiyya and Rao (BR) medium[24] containing g L^{-1}: K_2HPO_4, 0.1; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.2; $(NH4)_2SO_4$, 1.0; and succinic acid 4.0, and nutrient broth containing g L^{-1}: peptone, 5.0; NaCl, 5.0; beef extract, 1.5; and yeast extract, 1.5; were tried. Each medium was separately inoculated and incubated. Following the incubation, growth was measured and siderophore content was quantified as per Payne[23].

Siderophore Production as a Function of Time

P. fluorescens and *P. putida* were separately grown in SM by submerged fermentation method with constant shaking of 120 rpm at 29°C for 48 h. Samples were withdrawn after every 6 h intervals and were subjected for growth measurement and siderophore estimation.

Influence of Water

Growth and siderophore production was carried out in tap water based SM and was compared with that of SM prepared in deionized distilled water.

Optimization of Cultural Conditions

***p*H of Medium**

SM was prepared each with different *p*H in the range of 4-14 and separately inoculated with *P. fluorescens* and *P. putida* to check the effect of varying *p*H on growth and siderophore production.

Influence of Cell Mass Level

P. fluorescens and *P. putida* were separately added in SM in the range of 1-5 mg% of production medium (SM). Following the incubation at 29°C, growth and siderophore units were measured as described earlier.

Influence of Sugars, Organic Acids and Amino Acids

In order to examine the effect of different sugars, organic acids and amino acids on growth and siderophore production; in first set, each 100 mL of SM was externally supplemented separately with 1 g L^{-1} each of glucose, dextrose, sucrose and mannitol. Second set of SM was individually supplemented with 4.0 g L^{-1} each of malic acid, oxalic acid and citric acid. The third set of SM was separately fortified with 1 g L^{-1} each of serine, lysine, alanine, threonine, cystein, arginine, tyrosine and methionine. Each set was separately inoculated with *P. fluorescens* and *P. putida* and incubated. Following the 24 h incubation at 29°C and 120 rpm, each set was subjected for growth and siderophore quantification.

Influence of Nitrogen Sources

In this experiment, ammonium sulphates in SM was replaced separately by different concentrations of urea (commercial grade) in the range of 0.1-1.0 g L^{-1}, and sodium nitrate, corn steep liquor and soy flour at the rate of 1.0 g L^{-1}. Growth and siderophore production in these media was compared with that of SM containing ammonium sulphate.

Influence of Iron

In order to determine the threshold level of iron at which siderophore biosynthesis is repressed in fluorescent pseudomonads under study; both the cultures were grown in SM, externally supplemented with 1-100 μ*M* of iron ($FeCl_3.6H_2O$). Following the incubation at 29°C and 120 rpm, growth and siderophore content were estimated.

Influence of Other Metal Ions

For detecting the influence of different heavy metals on growth and siderophore production, both the cultures were separately grown in SM. Each of 100 mL of SM was separately supplemented with 10 μ*M* of different heavy metals, like cobalt ($CoCl_2$), molybdate ($MoCl_2$), magnesium ($MgCl_2$), manganese ($MnCl_2$), zinc ($ZnCl_2$), mercury ($HgCl_2$) and lead acetate ($PbCH_3CHOO$). Following the incubation at 29°C and 120 rpm, growth and siderophore content were estimated as per Payne[23].

Siderophore Production in Bioreactor and Influence of Antifoams

Modified SM was used for siderophore production in automated bioreactor (New Brunswick, USA, Model-BioFlo III) of 3.0 L capacity and semiautomated bioreactor (Navin Process Systems, Pune, India) of 10 L capacity. Process was carried out at 100 rpm with a sparger rate of 0.6 slpm. Siderophore production at different time intervals was analysed by CAS shuttle assay[23]. Various antifoam agents, such as sunflower oil, groundnut oil, glycerol and polyethylene glycol were separately added in SM to determine their effectiveness in suppressing foam formation and their effects on siderophore production. Following the separate inoculation with *P. fluorescens* and *P. putida* and incubation, each set was subjected for growth measurement and siderophore quantification as per Payne[23].

Influence of *P. fluorescens* on Wheat Germination and Growth

Wheat seeds (*Triticum aestivum,* variety Nirbhai NWH-1) were surface sterilized using 0.1% (w/v) $HgCl_2$ followed by three washing with sterile distilled water. Sterilized seeds were mixed/immersed for 10 min in siderophore (87% units) rich broth of *P. fluorescens* (8×10^7 cells mL^{-1}) grown in SM for 30 h. Control was prepared by adding 20 μ*M* of iron in siderophore containing SM to remove siderophores. Coated seeds were sown (5 seeds/pot) in pots containing sterile soil. Seeded pots were irrigated with sterile water after every 48 h to maintain the moisture necessary for the germination of seed. Observations, like increase in root length, shoot length and rate of germination, were recorded after 15 d of sowing.

Results and Discussion

Siderophore Detection and Production

After 24-36 h of incubation, development of green and red coloured pigment in SM by *P. fluorescens* NCIM 5096 and *P. putida* NCIM 2847, respectively indicated the production of siderophores. This was further confirmed by qualitative CAS test where instant decolorization of CAS reagent from blue to orange red was observed with both the cultures. *P. fluorescens* and *P. putida* produced 87 and 83% units of siderophores (hydroxamate type) in SM, respectively.

Influence of Media

While studying influence of media preparation, it was found that the development of orange red colour was faster in SM than in CAA and BR medium. However, there was no colour change in nutrient broth, indicating the absence of siderophore production in this medium. *P. fluorescens* and *P. putida* were found to yield maximum siderophore (87 & 83% units, respectively) in SM *vis-a-vis* CAA and BR medium (Tables 1 & 2). This indicated that SM is most suitable for siderophore production by *P. fluorescens* and *P. putida*.

Siderophore Production as a Function of Time

In the growth and siderophore production, as depicted in Tables 1 and 2, a lag phase of 6 h was observed. Siderophore production started after 12 h of incubation, which increased up to 24-30 h and declined thereafter. In case of *P. fluorescens* and *P. putida*, 24 and 30 h of incubation, respectively resulted in the maximum siderophore production.

Influence of Water

Tap water based SM gave better siderophore yields (87 & 83% units) in (*p*H 7.0) in contrast to deionized distilled water based SM (67 & 63% units) for both the pseudomonads.

Optimization of Cultural Conditions

***p*H of Medium**

*p*H plays important role in the solubility of iron and thereby its availability to the growing organism in the medium. From the various *p*H values (Table 1), it is evident that, at neutral *p*H (7.0), maximum siderophore yield (87 & 83% units) was obtained, which may be because bacteria grow better and iron is present in insoluble form at netural *p*H and, therefore, is not available to the bacteria. This stress of iron induces siderophore production. With increasing *p*H (towards alkalinity), siderophore production was found ceasing. This may be due to the fact that alkaline *p*H helps in excess solubilization of iron, which increases the iron content of the medium[25,20].

Table 1—Influence of various parameters on siderophoregenesis by *P. fluorescens* NCIM 5096

Media	Parameters and siderophore yield														
	% SU	Incubation (h)	% SU	*p*H	% SU	Inoculum (%)	% SU	Sugars (1 g L^{-1})	% SU	Organic acids (4.0 g L^{-1})	% SU	Amino acids (1 g L^{-1})	% SU	Nitrogen source (4.0 g L^{-1})	% SU
NB	-	6	-	4.0	-	1	72	Glu.	54	OAA	50	Ser	87	$(NH_4)_2SO_4$	85
BR	68	12	22	4.5	14	2	64	Suc.	48	CA	38	Lys	87	$NaNO_3$	81
CAA	66	18	36	5.5	72	3	53	Dex.	46	SA	72	Ala	87	SF	-
SM	72	24	60	6.5	77	4	48	Man.	36			Thr	89	CSL	-
		30	68	7.5	74							Cys	88	Urea	87
		36	58	8.5	70							Arg	86	(Urea g L^{-1})	
				9.5	50							Tyr	87	0.2	2
				10.5	—							Met	89	0.4	82
														0.6	84
														0.8	87
														1.0	82

SU, Siderophore units; -, No siderophore production; NB, Nutrient broth; BR, Barbhaiyya & Rao; CAA, Cas-amino acid; SM, Succinate medium; Glu, Glucose; Suc, Sucrose; Dex, Dextrose; Man, Mannitol; OAA, Oxaloacetate; CA, Citrate; SA, Succinate; Ser, Serine; Lys, Lysine; Ala, Alanine; Thr, Threonine; Cys, Cysteine; Arg, Argenine; Tyr, Tyrosine; Met, Methionine; SF, Soy flour; CSL, Corn steep liquor.

Table 2—Influence of various parameters on siderophoregenesis by *P. putida* NCIM 2847

Media	Parameters and siderophore yield												
	% SU	Incubation (h)	% SU	Inoculum (%)	% SU	Sugars (1 g L^{-1})	% SU	Organic acids (4.0 g L^{-1})	% SU	Amino acids (1 g L^{-1})	% SU	Nitrogen source (4.0 g L^{-1})	% SU
NB	-	6	-	1	60	Glu.	52	OAA	46	Ser	87	$(NH_4)_2SO_4$	80
BR	64	12	20	2	64	Suc.	38	CA	40	Lys	88	$NaNO_3$	76
CAA	61	18	32	3	50	Dex.	48	SA	68	Ala	87	SF	-
SM	68	24	50	4	46	Man.	44			Thr	88	CSL	-
		30	64							Cys	87	Urea	83
		36	52							Arg	86	(Urea g L^{-1})	
				-						Tyr	87	0.2	27
										Met	88	0.4	78
												0.6	85
												0.8	88
												1.0	84

SU, Siderophore units; -, No siderophore production; NB, Nutrient broth; BR, Barbhaiyya & Rao; CAA, Cas-amino acid; SM, Succinate medium; Glu, Glucose; Suc, Sucrose; Dex, Dextrose; Man, Mannitol; OAA, Oxaloacetate; CA, Citrate; SA, Succinate; Ser, Serine; Lys, Lysine; Ala, Alanine; Thr, Threonine; Cys, Cysteine; Arg, Argenine; Tyr, Tyrosine; Met, Methionine; SF, Soy flour; CSL, Corn steep liquor.

Influence of Cell Mass

Amount of cell mass determines the growth and, therefore, the production of secondary metabolites (siderophores). From various levels of cell mass, it is clear that 1.0 mg% (w/w) cell mass was optimum for maximum siderophore units (87% units) in *P. fluorescens* (Table 1), whereas 2 mg % (w/w) cell mass of *P. putida* (Table 2) was satisfactory for optimal siderophoregenesis (83% units). With increasing volume of cell mass, no significant increase in growth and siderophore production was observed.

Influence of Sugars, Organic Acids and Amino Acids

Among the various sugars tested, none of them were found to have stimulatory effect. On the contrary, all the sugars adversely affected the siderophoregenesis. Among organic acids, succinic acid was found suitable for optimum siderophoregenesis; 87 and 83% siderophore units

were obtained with *P. fluorescens* and *P. putida,* respectively. All tested amino acids positively affected the siderophore production. However, threonine resulted in the production of maximum siderophore units, i.e. 88.8 and 88.6% units for *P. fluorescens* (Table 1) and *P. putida* (Table 2), respectively. These results are in accordance with the results obtained by Dileep *et al*[15] who found that alanine, tyrosine, arginine, lysine and succinic acid were supportive for growth, fluorescence and siderophore production; while citric acid and sugars were not conducive for the production of siderophore.

Influence of Nitrogen Sources

Out of various nitrogen sources tried, optimum siderophore yield of 87 and 83% units by *P. fluorescens* and *P. putida*, respectively was obtained in SM supplemented with urea. Urea was proved to be the best utilizable nitrogen source; it gave maximum siderophore units within a short period of incubation (14 h), while $(NH_4)_2SO_4$ was found to boost growth. However, soy flour and corn steep liquor did not favour siderophore production because of the fact that these nitrogen sources are rich in iron content (Tables 1 & 2). Amongst the various concentrations of urea, a gradual increase in growth and siderophore production was observed with the increasing concentrations of urea; 0.8 g L^{-1} was found to be the optimum concentration for siderophore yields (Tables 1 & 2).

Influence of Iron

As depicted in Fig. 1, in both the cultures under study, growth of cultures increased with the increasing concentration of iron, whereas siderophore production repressed at higher concentrations of iron. Maximum siderophore units, i.e. 87 and 83% units in *P. fluorescens* and *P. putida*, respectively, were obtainable at 1 μ*M* of iron. Threshold level of iron,

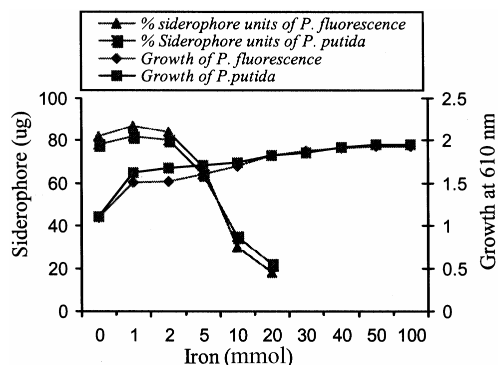

Fig. 1—Influence of iron level on siderophoregenesis by *P. fluorescens* NCIM 5096 and *P. putida* NCIM 2847

which repressed siderophore synthesis, was found to be 20 μ*M*. Increase in growth with increase in iron concentration reflects the iron requirement of organisms for cellular processes. No siderophore production at and above 20 μ*M* of iron suggests the acquisition of iron sufficiency in the organism. Dave and Dube[26] have reported the repression of siderophore production with increasing concentration of iron and reported 27 μ*M* of iron as threshold level at which fluorescent pseudomonads stopped siderophore production. Budde and Leong[27] and de Lorenzo *et al*[28] have reported that the transcription of iron regulated gene is under the negative control of *fur* protein (repressor) with Fe^{2+} as an essential co-repressor.

Influence of Other Heavy Metals

In case of heavy metals it was observed that the medium supplemented with Pb enhanced maximum siderophore production as well as growth of cultures, while Mn, Hg and Co showed inhibitory effect on both growth and siderophore production (Table 3). Page[29] have reported the inhibition of azotochelin and aminochelin synthesis in *Azotobacter vinelandii* by Mn^{2+} and Zn^{+}. It has been reported that Mn^{2+} and Zn^{2+} may substitute for Fe^{2+} in intracellular control of siderophore synthesis[30].

Siderophore Production in Bioreactor and Influence of Antifoams

During siderophore production in bioreactors (2.5 & 10 L capacity), similar siderophore yields (87 & 83%) as reported in experimental results were obtained with both the strains; biomass obtained was 1.96 g L^{-1} (dry weight). Amongst various antifoams, sunflower was proved to be suitable and cost-effective antifoaming agent because it gave maximum siderophore units (87 & 83% units) and was required in less quantity (0.5 mg L^{-1}) than other antifoam agents (Table 4).

Table 3—Influence of heavy metal ions on siderophoregenesis by fluorescent pseudomonads

Heavy metals (10 μ*M*)	% siderophore units	
	P. fluorescens NCIM 5096	*P. putida* NCIM 2847
Pb	85.7	84.2
Mg	81.1	80.5
Zn	75.1	73.2
Mo	66.8	46.5
Co	-	-
Mn	-	-
Hg	-	-

Table 4—Influence of various antifoam agents on siderophoregenesis by fluorescent pseudomonads

Antifoam agents	Amount required (mL)	% siderophore units	
		P. fluorescens NCIM 5096	*P. putida* NCIM 2847
Sunflower oil	0.5	68.0	64.0
Groundnut oil	2.5	62.0	61.0
Glycerol	3.0	66.0	59.6
Polyethylene glycol	4.0	64.0	60.5

Table 5—Influence of *P. fluorescens* NCIM 5096 inoculation on wheat germination and growth

Treat-ment	Root length		Shoot length		Germination	
	(mm)	(% increase in mm)	(mm)	(% increase in mm)	%	(% increase)
Control	5.90 (0.623)	-	7.4 (0.265)	-	80	-
Test	6.95 (0.840)	16.25	9.5 (0.693)	34.50	90	10

Values in the parenthesis indicate standard deviation
Values are the mean of three replicates

Influence of *P. fluorescens* Inoculation on Wheat Germination and Growth

A 10% increase in the rate of germination, 20% increase in the root length and 31% increase in the shoot length was evident in *P. fluorescens* NCIM 5096 inoculated wheat seeds over the control (Table 5). This is in accordance with the results obtained by Manwar *et al*[31].

Conclusion

Both pseudomonads, *P. fluorescens* NCIM 5096 and *P. putida* NCIM 2847 were able to give higher yields of siderophores (87 & 83% units, respectively) in modified SM and under iron stress conditions. At 30 µ*M* of iron, siderophoregenesis was repressed. No siderophore production was obtained in Zn^{2+} and Mn^{2+} containing SM, may be due to the substitution of Fe^{2+} by these metal ions in the intracellular control of siderophoregenesis. The experimental results also showed that siderophore producing microbes are involved in plant growth promotion of wheat.

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