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Original Article

Siderophore production by *Pseudomonas aeruginosa* FP6, a biocontrol strain for *Rhizoctonia solani* and *Colletotrichum gloeosporioides* causing diseases in chilli

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ABSTRACT

Siderophores are compounds secreted under low iron stress, which act as specific ferric iron chelating agents. Owing to their potential in the biological control of fungal phytopathogens, they may be used as an alternative strategy to chemical control. *Pseudomonas aeruginosa* FP6, previously isolated from rhizospheric soil samples was screened for its siderophore production on a chrome-azurol S agar plate. Change in the colour of the chrome-azurol S agar from blue to orange red confirmed the siderophore producing ability of *P. aeruginosa* FP6. The effects of various physicochemical parameters on siderophore production were studied. The maximum siderophore production was obtained in succinate medium (125 μM) followed by King's B medium (105 μM). The presence of sucrose and mannitol increased the siderophore production. Yeast extract proved to be the most suitable nitrogen source. Media supplemented with Pb^{2+}, Mn^{2+} and Mg^{2+} showed appreciable siderophore production as well as growth of cultures. An increase in the iron concentration favoured growth but substantially reduced siderophore production. The strain when tested for its *in-vitro* antagonistic activity against *Rhizoctonia solani* and *Colletotrichum gloeosporioides* on King's B media, with and without $FeCl_3$, showed a significant reduction in *R. solani* growth with $FeCl_3$ supplementation compared to the control (without $FeCl_3$), suggesting the role of siderophore mediated antagonism of *R. solani.* Antifungal activity was not influenced by $FeCl_3$ in the case of *C. gloeosporioides*, suggesting the presence of other antagonistic mechanisms.

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Introduction

Chilli (*Capsicum annuum* L.) belongs to the family Solanaceae and is one of the important vegetable-cum-spice crops cultivated in India, which, after China, is not only largest producer but also the largest exporter of chilli in the world, with a cultivated area of 0.81 million ha in 2010–2011 producing 1.22 million t of green chilli (Anonymous, 2011). Andhra Pradesh is the largest chilli-producing region in India, contributing about 26% to the total area, followed by Maharashtra (15%), Karnataka (11%), Orissa (11%) and Madhya Pradesh (7%) with other states contributing nearly 22% to the total area under chilli cultivation (Jagtap et al., 2012).

Chilli is known to suffer from as many as 83 different diseases, of which more than 40 are caused by fungi (Rangasami, 1988). Anthracnose (fruit rot) and damping off are the two major fungal diseases incited by *Colletotrichum gloeosporioides* and *Rhizoctonia solani* which often cause high yield loss (Than et al., 2008). With the ever-increasing awareness of the negative health-implications and environmental concerns regarding prolonged chemical usage there is a burgeoning demand for the development of alternative and safer methods of disease management.

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 1000 Da), ferric-ion-specific chelating agents produced by bacteria and fungi to combat low iron stress (Ngamau et al., 2014).

For several fluorescent *Pseudomonades*, it has been suggested that the siderophore-mediated competition for iron with soil borne pathogens is an important mechanism for biological control as most of these plants are able to use bacterial iron siderophore complexes as a source of iron from the soil with a competitive

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advantage under iron stress and thereby restrict proliferation and root colonization by phytopathogens (Decheng et al., 2005). Siderophore-producing rhizobacteria are also known to impart induced systemic resistance to plants and suppressiveness to the soil and have been implicated in the biocontrol of several plant diseases (Akhtar et al., 2011).

The presence of heavy metals—even in traces—is toxic and detrimental to both flora and fauna (Priyadarshini and Rath, 2012). Plant growth-promoting rhizobacteria capable of growing in the presence of a variety of heavy metals are seen as potent bio-inoculants (Nilanjana et al., 2008). Siderophores are also found to complex with heavy metals like cadmium, lead, nickel, arsenic (III, V), aluminium, magnesium zinc, copper, cobalt and strontium as well as iron (Sayyed and Chincholkar, 2010).

Siderophore-based biological control agents are gaining commercial significance as they are safer, do not lead to bio-magnification and also provide iron nutrition to the crops thereby promoting plant growth (Sayyed et al., 2007). Therefore, the present study was undertaken to study the effects of culturing conditions on the siderophore production of *Pseudomonas aeruginosa* FP6 and its effect on biocontrol activity against *C. gloeosporioides* and *R. solani*.

Materials and methods

Iron decontamination

All glass ware was soaked overnight in 6 M HCl and rinsed with distilled water several times to remove any traces of iron.

Bacterial strain

The *P. aeruginosa* FP6 strain used in this study was isolated from a rhizospheric soil sample (Bangalore) and was identified as *P. aeruginosa* (Sasirekha et al., 2013a). The nucleotide sequence of the 16S rRNA of *P. aeruginosa* FP6 has been deposited in the GenBank database under the accession number JN861778. The organism was maintained on King's B agar at 4 °C.

Screening for siderophore production

Siderophore production was qualitatively assayed as described by Schwyn and Neilands (1987). Briefly, overnight culture of *P. aeruginosa* FP6 was spot inoculated onto a chrome azurol S (CAS) agar plate and incubated for 18–24 h at 28 °C. The basic principle underlying the test is that when a strong ligand L (for example, siderophore) is added to a highly coloured dye-Fe^{3+} complex, the iron-ligand complex is formed and the release of free dye is accompanied by a colour change.

Siderophore estimation

An amount of 100 μL of 24 h broth culture (1×10^8 colony forming units (cfu) per mL) was inoculated into 100 mL of iron-free succinic acid broth medium. The flask was incubated at 30 °C for 24 h in a rotary shaker (120 revolutions per minute; rpm). Then, the broth culture was centrifuged for 10 min at 10,000 rpm. The amount of siderophore produced was determined by measuring the absorbance of the supernatant at 400 nm. The concentration was calculated using the absorption maximum ($\lambda = 400$ nm) and the molar extinction coefficient ($\varepsilon = 20{,}000/M/cm$) according to Rachid and Bensoltane (2005).

Spectrophotometric characteristics of siderophore

P. aeruginosa FP6 was inoculated into 100 mL of iron-free succinic acid broth medium. The flask was incubated at 30 °C for 24 h in a rotary shaker (120 rpm). Then, the broth culture was centrifuged for 10 min at 10,000 rpm. After centrifugation, the supernatant was scanned for peaks in ultraviolet–visible spectrum range (420–450 nm for hydroxamate and 490–515 nm for catecholate) using a spectrophotometer (UV-2450; Shimadzu; Tokyo, Japan). Siderophore quenching was studied by adding 10 μL of Fe^{3+} solution to 3 mL of crude culture supernatant to achieve a final concentration of 3.3 mM.

Siderophore production in different media

Siderophore production occurs only under iron-deficient conditions; hence, different iron-deficient media like King's B, succinate, glucose and glutamate medium (Table 1) were separately inoculated. Samples were withdrawn every 6 h and assayed for their siderophore content as described above. The media supporting the maximum siderophore production was used for further studies (Sayyed et al., 2005).

Optimization of physiochemical parameters for siderophore production

Various physico-chemical parameters were optimized for siderophore production using succinate media. The parameters tested were: carbon source, nitrogen source, iron and heavy metal.

Effect of different carbon and nitrogen sources on siderophore production

In order to examine the effect of different carbon sources on siderophore production, 100 mL of succinate broth was supplemented with 1 g/L of different carbon sources—glucose, sucrose, lactose, maltose, mannitol and starch. Each flask was incubated at 37 °C for 24 h in a rotary shaker (120 rpm). Growth and siderophore production were estimated. In order to determine the effect of different nitrogen sources on siderophore production, 100 μL of 24 h bacterial broth (1×10^8 cfu/mL) was inoculated into 100 mL of succinate broth containing 1 g/L of different nitrogen sources—yeast extract, peptone, sodium nitrate, potassium nitrate, ammonium nitrate, ammonium chloride and urea (replaced with ammonium

Table 1
Composition of various culture media used for siderophore production.

Component (g/L)	Medium			
	King's B	Succinate	Glucose	Glutamic acid
K_2HPO_4	–	6	0.56	–
KH_2PO_4	–	3	–	–
$(NH_4)_2 \cdot SO_4$	–	1	–	–
$MgSO_4 \cdot 7H_20$	–	0.2	–	–
Succinic acid	–	4	–	–
Glycerin	10	–	–	–
Peptone	20	–	–	–
$MgSO_4$	1.5	–	–	–
Glucose	–	–	10	–
Urea	–	–	0.85	–
Glutamic acid	–	–	–	1
$(NH_4)_2 \cdot NO_3$	–	–	–	1
Na_2SO_4	–	–	–	0.02
NaCl	–	–	–	0.02

sulphate in succinate medium). Each flask was incubated at 37 °C for 24 h in a rotary shaker (120 rpm). Growth and siderophore production in these media were compared with those of succinate medium containing ammonium sulphate (Tailor and Joshi, 2012).

Influence of iron and heavy metals on siderophore production

The threshold level of iron which represses siderophore biosynthesis was determined by externally supplementing succinic media with 0–250 μM of iron ($FeCl_3 \cdot H_2O$). Following incubation at 37 °C and rotary shaking at 120 rpm, growth and siderophore production were estimated. To determine the influence of different heavy metals on siderophore production, each 100 mL of succinate broth was separately supplemented with 10 μM of different heavy metals—cobalt ($CoCl_2$), magnesium ($MgCl_2$), manganese ($MnCl_2$), mercury ($HgCl_2$) and lead ($PbCH_3CHOO$). Following the incubation at 30 °C for 24–48 h, the siderophore content was estimated.

Siderophore-mediated antifungal activity

Potato dextrose agar supplemented with $FeCl_3$ (100 mg/mL) was seeded with an agar plug (4 mm diameter) of actively growing fungal culture of *C. gloeosporioides* and *R. solani*. Simultaneously, *P. aeruginosa* FP6 was streaked 3 cm away from the agar plug on the side towards the edge of the Petri plates. The plates were incubated at 30 °C for 24–48 h. The same experiment was repeated in the absence of $FeCl_3$ and was used as the control (Ramesh et al., 2002).

Plasmid curing

Curing of the plasmid from the *P. aeruginosa* FP6 strain was done by exposing the overnight grown culture of *P. aeruginosa* to ethidium bromide (500 μg/mL). The derivatives of *P. aeruginosa* which were cured of the plasmid were selected on the basis of their inability to grow on culture media supplemented with kanamycin (1000 μg/mL) unlike the parent strain, which is kanamycin resistant. The treated culture of *P. aeruginosa* was initially plated on a complete medium without kanamycin. The colonies of *P. aeruginosa* obtained were picked and spotted on medium containing kanamycin. Colonies that were unable to grow on the medium containing kanamycin were derivatives of *P. aeruginosa* that were cured of the plasmid and were designated as *P. aeruginosa* PC 1 to PC 100. The plasmid-cured strains were screened for siderophore production (Naidu and Yadav, 1997).

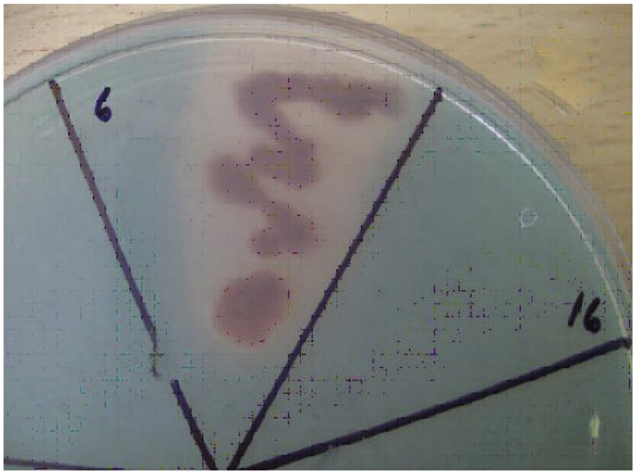


Fig. 1. Siderophore production on chrome azurol S medium.

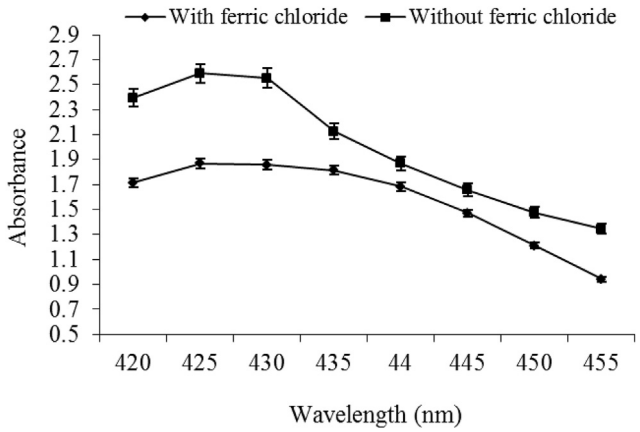
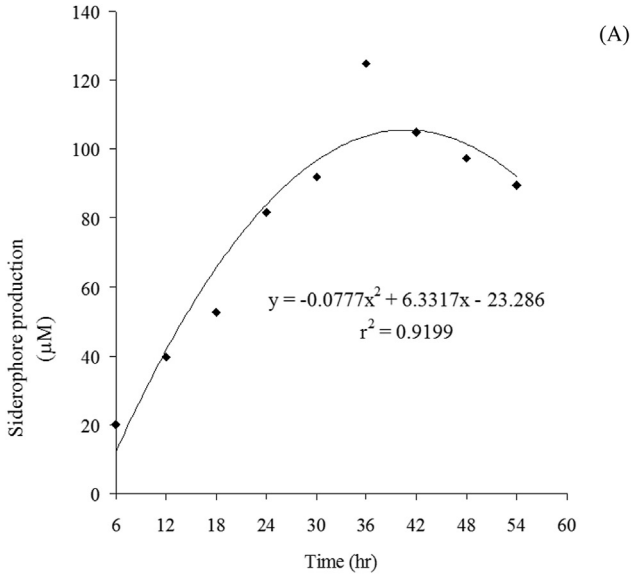


Fig. 2. Absorption characteristics of *Pseudomonas aeruginosa* FP6 culture filtrate confirming the hydroxamate nature of siderophores.



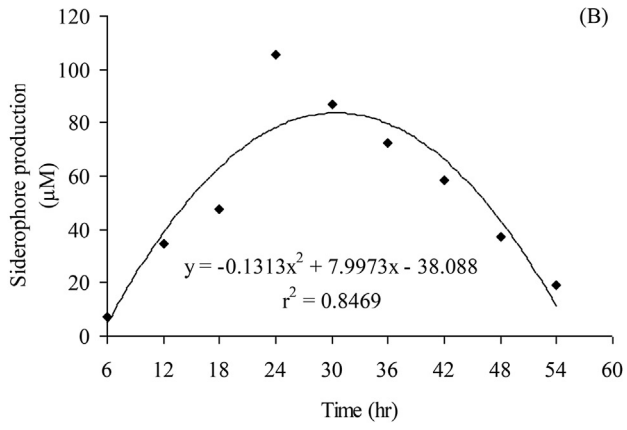


Fig. 3. Correlation and equation (r^2 is the coefficient of determination) between siderophore production and time on: (A) succinate medium; (B) King's B medium.

Plasmid DNA isolation

The presence of plasmid in the plasmid-cured strains (*P. aeruginosa* PC 1 to PC 100) and the parent strain (*P. aeruginosa* FP6) was detected using the alkaline lysis method (Davis et al., 1986). The purified plasmid DNA was visualized by resolving on 0.8% agarose gel electrophoresis.

Results and discussion

The siderophore production detected using CAS agar showed orange-coloured colonies after 24 h of incubation due to the siderophoral removal of Fe from the dye which primarily indicated the ability of *P. aeruginosa* FP6 to produce siderophore (Fig. 1). Several researchers (Sayyed et al., 2005; Omidvari et al., 2010) have reported the production of siderophore by various microorganisms. *P. aeruginosa* FP6 produced 85.7 μM siderophore in succinate medium. Spectrophotometric analysis of the culture in standard succinate medium showed a peak between 420 nm and 450 nm (Fig. 2) indicating the presence of siderophore of a ferric hydroxamate nature in *P. aeruginosa* FP6 (Ali and Vidhale, 2011). The amount of hydroxamate type of siderophore produced was 18.18 mg/L whereas Gull and Hafeez (2012) reported 15.5 μg/mL of hydroxamate siderophore in *Pseudomonas fluorescens* Mst 8.2. Chandra et al. (2007) reported 32 μg/mL of the hydroxamate type of siderophore by *Mesorhizobium loti* after 48 h of incubation.

Siderophore production in different media

Among the different iron deficient media tested, the glucose and glutamate media supported the growth of FP6 but showed low siderophore production. The maximum siderophore production

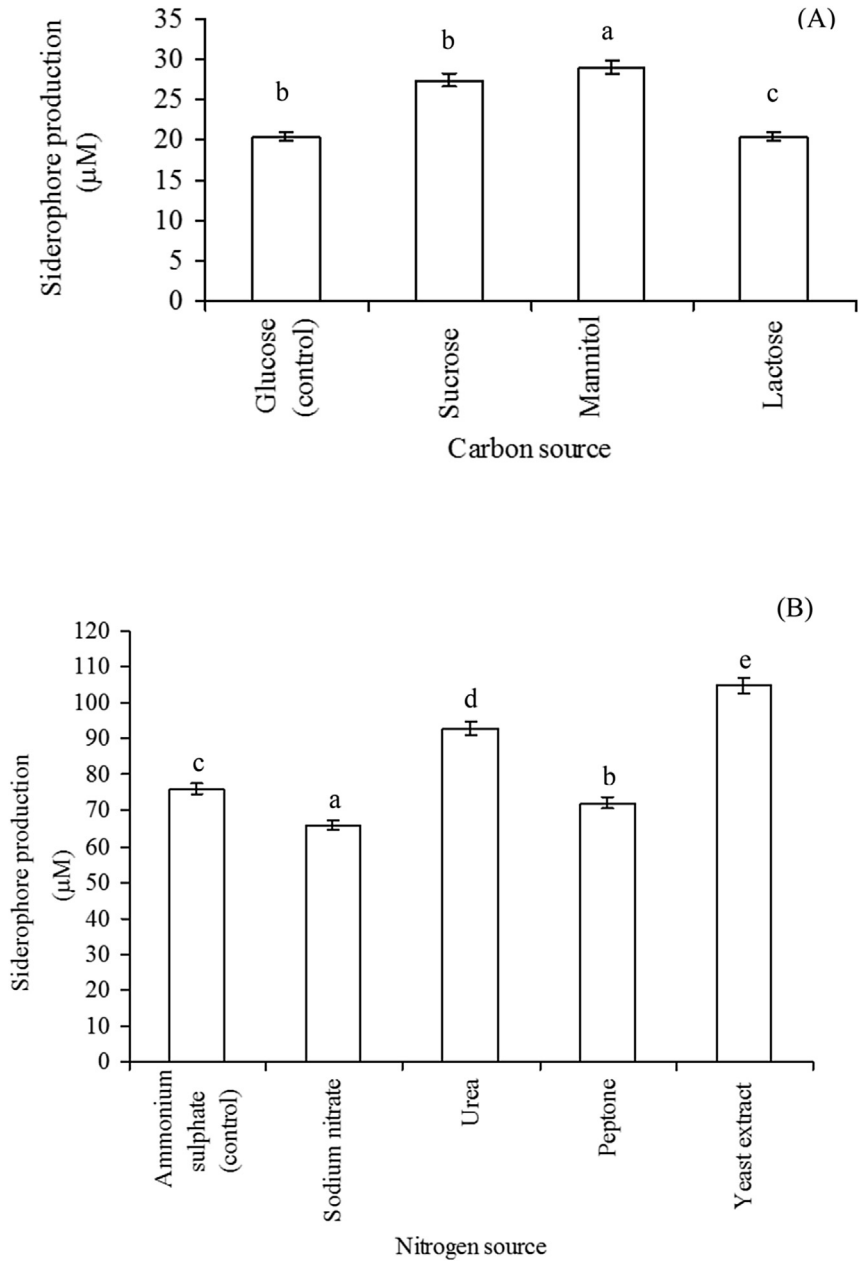


Fig. 4. Effect of source on siderophore production: (A) carbon; (B) nitrogen. Different lowercase letters above each column indicate significant differences within sources, according to least significant difference ($p < 0.05$). Error bars show mean ± SE.

was obtained on succinate medium (125 μM) followed by King's B medium (105 μM). Increased siderophore production in succinate media can be substantiated by the structure of pyoverdines in which the three amino moiety of the chromophore is substituted with various acyl groups derived from succinate, malate, α-keto-glutarate (Linget et al., 1992).

The time course study revealed that siderophore production commenced after a lag period of 6 h after inoculation. Maximum siderophore production was observed at 36 h of incubation, after which, it started to decrease as the culture had reached its stationary phase in the succinate medium (Fig. 3A). King's B medium supported maximum siderophore production from 24 h of incubation (Fig. 3B). Statistically, the results showed a positive correlation between siderophore production and time in both succinate (coefficient of determination, $r^2 = 0.91$) and King's B ($r^2 = 0.84$) medium. This was in contrast to the work by Tailor and Joshi (2012) who reported maximum siderophore production at 24 h, and Sayyed et al. (2005) who reported maximum siderophore production between 24 h and 30 h in modified succinate medium using fluorescent *Pseudomonas* spp. Prashant et al. (2009) reported that *Acinetobacter calcoaceticus* produced optimum siderophore at 36 h of incubation, similar to the results in the current study.

Effect of carbon and nitrogen sources on siderophore production

The 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). Along similar lines, FP6 showed a varied response to the presence of different sugars, with mannitol and sucrose supporting increased growth and siderophore production (Fig. 4A).

Of the various nitrogen sources tested, the optimum siderophore yields of 104.8 μM and 92.9 μM were obtained in yeast extract and urea supplemented media, respectively (Fig. 4B). Urea was used with the view that the unutilized part of it serves as N fertilizer when the post-fermentation broth is used for field application. Utilization of urea by the isolate in the current study also suggested its possible exploitation for bioremediation of alkaline soils by reducing the excess amount of urea present in the soil (Sayyed et al., 2010).

Influence of iron and heavy metal on siderophore production

As shown in Fig. 5A, the growth of FP6 increased with an increasing concentration of iron tested (1–250 μM). However, an Fe concentration ranging from 5 μM upward repressed siderophore production. An amount of 100 μM Fe in the media completely repressed siderophore synthesis by *P. aeruginosa* FP6. Statistically, the results showed a positive correlation ($r^2 = 0.94$) between the iron concentration and siderophore production (Fig. 5B). The increase in growth with an increase in the iron concentration reflected the iron requirement by the strain for cellular processes. It has been reported that the siderophore transport system contains several gene products, all of which are negatively regulated by Fe-binding protein (Fur) for ferric uptake regulation (Klaus, 2002).

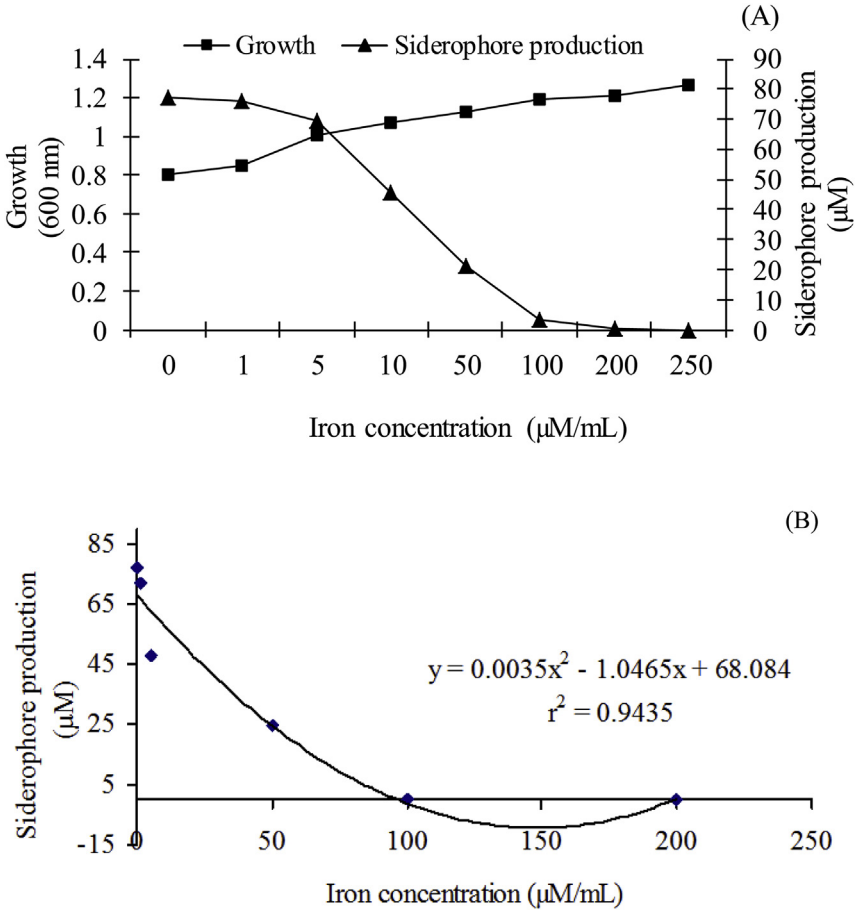


Fig. 5. Graphs of (A) influence of iron level on siderophorogenesis and growth by *P. aeruginosa* FP6; (B) correlation between iron concentration and siderphore production and equation (r^2 is the coefficient of determination).

Table 2
Influence of heavy metals on siderophore production.

Heavy metal (10 μM)	Siderophore (μM)[a]
Control	105.00 ± 0.02
Hg	5.75 ± 0.05
Pb	88.21 ± 0.04
Co	10.35 ± 0.12
Mn	78.80 ± 0.019
Cd	7.69 ± 0.037
Mg	79.20 ± 0.026

[a] Values are mean ± SE of triplicates.

Table 3
Inhibition of *Colletotrichum gloeosporioides* and *Rhizoctonia solani* by *Pseudomonas aeruginosa* FP6 in either the presence or absence of $FeCl_3$.

Fungal pathogen	Inhibition zone (%)[a]	
	Without $FeCl_3$	With $FeCl_3$
R. solani	72.25 ± 0.065	12.22 ± 0.078
C. gloeosporoides	72.12 ± 0.057	61.40 ± 0.086

[a] Values are mean ± SE of triplicates.

Similar observations have been made by Sayyed et al. (2005) who reported that an increase in the iron concentration up to 100 μM favoured growth but affected the siderophore production of *P. fluorescens* NCIM 5096 and *Pseudomonas putida* NCIM 2847. Prashant et al. (2009) have shown that the biosynthesis of siderophores in *A. calcoaceticus* was suppressed in the presence of 20 μM free Fe, emphasizing the role of iron in siderophore production.

Baysse et al. (2000) reported that siderophore can form complexes with metals with lower affinity other than Fe^{3+}. In the current study, media supplemented with lead, manganese and magnesium salts supported siderophore production but this was reduced compared to the control (Table 2). Metals like Cd^{2+}, Hg^{2+} and Co^{2+} showed an inhibitory effect on both growth and siderophore production which may have been due to the competitive binding of the metals, thereby elevating oxidative stress in the bacterial cells (Dimkpa et al., 2009). Chelation of metal ions may reduce their toxicity and hence allow pigment production. It has been reported that Mn^{2+} may substitute for Fe^{2+} in the intracellular control of siderophore synthesis (Williams, 1982).

Siderophore mediated antifungal activity

P. aeruginosa FP6 inhibited *R. solani* in the absence of $FeCl_3$ (72.25%), which was reduced to 12% in the presence of $FeCl_3$, suggesting that siderophore mediated antagonism whereas the strain showed an inhibitory effect against *C. gloeosporioides* in either the presence or absence of $FeCl_3$, which may have been due to the production of other antifungal metabolites (Table 3).

Plasmid curing

Plasmid curing was carried out to determine whether the siderophore genes are located on plasmid or chromosomal DNA. The antibiotic susceptibility test of the wild strain showed sensitivity to most of the antibiotics except kanamycin (1000 μg/mL), and rifampicin (500 μg/mL); therefore kanamycin was used as a marker (Van den Broek et al., 2003; Sasirekha et al., 2013b). In total, 100 colonies were obtained, of which 88 colonies were able to grow on Luria Bertanni broth (LB) medium but not on LB medium supplemented with kanamycin. Thus, those colonies which grew on LB medium were selected for further studies. The plasmid curing efficiency was 88%.

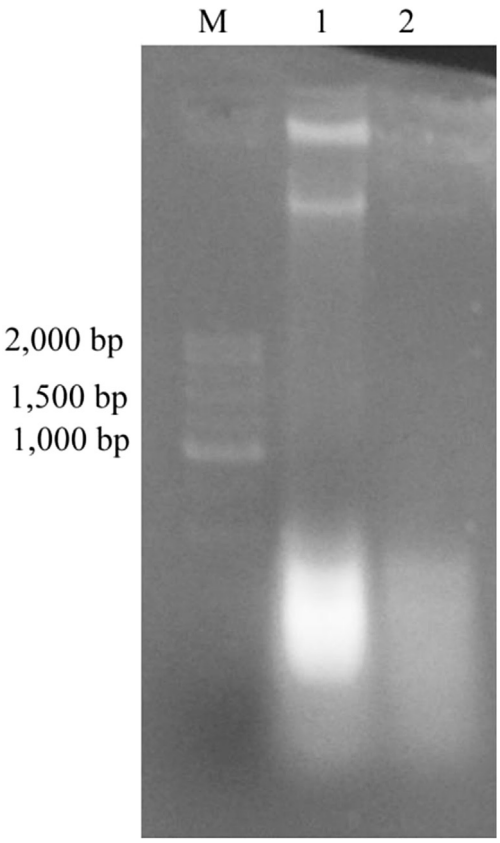


Fig. 6. Photograph showing absence of plasmid in cured strain (lane 2) compared to wild strain (lane 1) and 1000–5000 bp ladder (lane M).

Plasmid analysis of the wild strain showed the presence of four bands suggesting the presence of one or more plasmids in this strain (Fig. 6, lane 1). All cured clones showed the loss of plasmid bands (Fig. 6, lane 2) which were present in the original wild strain, which clearly showed the relationship between the loss of plasmid and the loss of kanamycin resistance but no loss in siderophore production, indicating the chromosomal location of the gene.

P. aeruginosa FP6 was able to produce a higher yield of siderophore. Siderophore production by *P. aeruginosa* FP6 was high under the iron-stressed conditions, suggesting that siderophore chelates Fe from soil and there is a lignand exchange with plants and thereby negatively affecting the growth of several fungal pathogens—an important feature in plant disease suppression. The appreciable siderophore production observed in the presence of lead, magnesium and manganese can be attributed to the ability of rhizobacterial strains to mitigate the toxic effects of metals besides their role in providing plants with the sufficient amounts of growth promoting substances.

Conflict of interest

No conflict of interest.

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