Chapter 3

Studies on siderophore production by the selected isolates

P. aeruginosa TMR2.13 and

B. amyloliquefaciens NAR38.1
3.1 Introduction

Siderophores produced by rhizosphere bacteria enhance plant growth by increasing the availability of Fe near the root or by inhibiting the colonization of roots by plant pathogens or other harmful bacteria (Alexander and Zuberer 1991). Plant growth promotion by microorganisms may be direct or indirect. Direct promotion is by providing the plant with growth promoting substances like indole acetic acid (phytohormone) or by making nutrients available through phosphate solubilization or siderophore production. The indirect effect is seen if plant growth promoting rhizobacteria (PGPR) lessen or prevent the deleterious effects of one or more phytopathogenic microorganisms, for example by producing hydrogen cyanide (HCN) (Voisard et al. 1989, Adam and Zdor 2001). The two isolates *P. aeruginosa* TMR2.13 and *B. amyloliquefaciens* NAR38.1 obtained during this study were screened for other plant growth promoting properties like phosphate solubilization, production of HCN and production of ammonia.

Siderophores, the iron chelating agents produced by microorganisms, have been classified into three main types: hydroxamates, catecholate and carboxamate types depending on the iron ligation groups (Wandersman and Delepelaire 2004). The most extensively studied iron chelators are catechol and hydroxamate siderophores produced by Gram-negative bacteria, in particular, pseudomonads, enterobacteria, agrobacteria and related species, *Yersinia* and vibrions. Hydroxamates have been primarily studied from Gram-positive microorganisms, whereas catechols are identified from a relatively smaller number of representatives of this group (Temirov et al. 2003). Amongst the *Bacillus* spp. siderophores from *Bacillus anthracis* (Petrobactin and Bacillibactin) (Wilson et al. 2010), *B. megaterium* (Schizokinen), *B. subtilis* (Bacillibactin) (Zawadzka et al. 2009) and *B. licheniformis* (2, 3-dihydroxybenzoyl-glycyl-threonine) (Temirov et al. 2003) have been well studied. In the present study, the type of

Although, iron is the key factor in regulating siderophore production as increase in iron concentration decreases siderophore production (Villegas et al. 2002), other factors such as pH, temperature, carbon source and other metals also play an important role (Saha et al. 2012). The pH of an econiche determines the type of siderophore produced by the microorganisms inhabiting it. Hydroxamate siderophores are stable over a wide pH range as compared to the catecholate type which are stable at neutral to alkaline pH. Trihydroxamates are stable over a pH range of 2 to 8; however, ferric complexes of catecholates are unstable below pH 5. Many bacteria which produce only catechol type of siderophores can grow mainly under neutral to alkaline conditions. For an example, *Azotobacter* and *Agrobacterium* produce azotochelin, protochelin and aminochelin (Winkelmann 2004).

The effect of pH, NaCl and Fe concentration on siderophore production by these isolates was determined and further, binding of siderophores to different forms of Fe was evaluated. Results obtained are discussed in this chapter along with the characterization of the functional group of the siderophore and the plant growth promoting characteristics of the selected isolates.

3.2 Materials and methods:

3.2.1 Plant growth promoting properties

i) Phosphate solubilization: The isolates were streaked on Pikovskaya’s medium and incubated for 24 hours at 28 °C (Appendix A). Zone of clearance indicating phosphate solubilisation activity was observed (Gupta et al. 1994).
ii) HCN production: The isolates were streaked on growth medium with 4.4 g/L glycine (Appendix A). Filter papers dipped in picric acid solution were placed on the lid of each plate. HCN production was determined from change in color of the filter paper strip from orange to red (Bakker and Schippers 1987).

iii) Indole acetic acid production: Isolates were grown in nutrient broth containing 0.5% tryptophan for 24 hours and the culture broth was centrifuged. 2 ml Salkowsky’s reagent (Appendix B) was added to 1 ml of supernatant. The mixture was incubated for 30 minutes at 28 ºC and the absorbance was recorded at 530 nm (Viswanathan 1999).

iv) Ammonia production: Freshly grown culture was inoculated in 10 ml of peptone water (Appendix A) and incubated for 48 hours at 28ºC. 0.5 ml of Nessler’s reagent (Appendix B) was added to each tube and the development of a yellow brown color, indicating ammonia production, was observed (Cappuccino and Sherman, 1992).

3.2.2 Spectrophotometric and spectrofluorimetric characteristics of siderophore produced by the isolate TMR2.13

TMR2.13 was inoculated in MSM with 0.2% glucose, and the flask was incubated at 28ºC at 150 rpm for 24 hours. The culture broth was centrifuged, and the supernatant was scanned for peaks in UV-Vis range using spectrophotometer (Shimadzu UV-2450). Spectrofluorimetric analysis was carried out with RF-5301 PC Shimadzu spectrofluorometer at an excitation wavelength of 400 nm and the emission was recorded.

3.2.3 Determination of the functional group of siderophore

The isolates were inoculated in a mineral salts medium (MSM) without FeSO₄. The flasks were incubated on shaker at 150 rpm at 28 ºC for 24 hours after which the broth was
centrifuged at 10,000 rpm for 10 minutes and the supernatant was subjected to the following tests for determining the functional group:

i) Hydroxamate

a) Neiland’s spectrophotometric assay: To 1 ml of cell - free supernatant, 1.5 ml of freshly prepared 2% aqueous FeCl₃ solution was added. The resultant mixture was scanned between 400-600 nm. A peak between 420-450 nm indicates the hydroxamate nature of the siderophore (Neilands 1981).

b) Tetrazolium salt test: To 0.5 ml of cell - free supernatant, a pinch of tetrazolium salt and a few drops of NaOH were added. Instant appearance of a deep red color indicated the presence of a hydroxamate siderophore (Snow 1954).

c) Csaky assay: The cell free supernatant (1ml) was hydrolyzed with 1 ml of 6 N H₂SO₄ in a boiling water bath at 130 °C for 30 min. The solution was buffered by adding 3 ml sodium acetate solution and 1 ml sulfanilic acid solution followed by the addition of 0.5 ml iodine solution. After 3-5 min, excess iodine was destroyed with 1ml sodium arsenite solution. To this, 1 ml α - naphthylamine (Appendix B) solution was added and the volume was made upto 10 ml with water. The absorbance of the color developed after 20-30 minute was measured at 526 nm (Gilliam 1981).

ii) Catecholate

a) Neiland’s spectrophotometric assay: 1.5 ml of freshly prepared 2% aqueous FeCl₃ was added to 1 ml of the test sample. The wine colored complex formed that absorbed maximally at 495 nm, indicated the catecholate nature of the siderophore (Neilands 1981).

b) Arnow’s assay: To 1 ml of supernatant, the following reagents were added: 1 ml 0.5 M HCl, 1 ml nitrite molybdate (Appendix B) and 1 ml 1 M NaOH. After the addition of each reagent, the tubes were vortexed. The colour change from yellow to red indicated the presence of catechol in the reaction mixture (Arnow 1937).
iii) Carboxylate

a) Vogel’s chemical test: Few drops of 5N NaOH were added to water along with 3 drops of phenolphthalein solution to give a light pink colour. Disappearance of pink color on addition of the test sample, indicated the presence of carboxylate type of siderophores (Yeole et al. 2001).

b) Spectrophotometric assay (Shenker’s test): To 1 ml of cell - free supernatant, 1 ml of 250 µM CuSO₄ and 2 ml of acetate buffer (pH 4) (Appendix B) were added. The copper complex formed was scanned from 190 to 800 nm and the absorption maxima was noted between 190-280 nm (Shenker 1992).

3.2.4 Determination of the effect of pH on growth and siderophore production

*Pseudomonas aeruginosa* TMR2.13 and *Bacillus amyloliquefaciens* NAR38.1 were streaked on TYG and NA respectively. The plates were incubated at 28ºC for 24 hours and the cultures were inoculated in MSM with pH 7 and 0.2% glucose as the carbon source. The flasks were incubated at 28ºC and 150 rpm for 24 hours. 5% of 24 hour old culture was inoculated in MSM with 0.2% glucose as the carbon source. The pH of the medium ranged from 5 to 9 (adjusted using 1 N HCl or 1 N NaOH). The flasks were incubated at 28 ºC at 150 rpm for 24 hours. 4 ml sample was removed from all the flasks after 24 hours to determine growth and siderophore production. Growth was determined as increase in absorbance at 600 nm on a UV- Vis spectrophotometer (Shimadzu UV-2450). Siderophore (Pyoverdine) produced by *Pseudomonas aeruginosa* TMR2.13, was quantified in cell-free supernatant by noting the absorbance at 400 nm. Siderophore concentration was calculated as described by Gupta et al. (2007). Siderophore produced by *B. amyloliquefaciens* NAR38.1 was quantified by Arnow’s assay using catechol as the standard (Appendix C).
3.2.5 Determination of the effect of NaCl on growth and siderophore production in the selected isolates

_Pseudomonas aeruginosa_ TMR2.13 and _Bacillus amyloliquefaciens_ NAR38.1 were streaked on TYG and NA respectively. The plates were incubated at 28 ºC for 24 hours and the cultures were inoculated in MSM with pH 7 and 0.2% glucose as the carbon source for inoculum build-up. The flasks were incubated at 28 ºC at 150 rpm for 24 hours. 5% of the 24 hour old culture was inoculated in MSM with different NaCl concentrations (0, 1, 2, 3, 4 and 5%) and 0.2% glucose as the carbon source. The flasks were incubated at 28 ºC and 150 rpm for 24 hours. 4 ml sample was removed from the flasks after 24 hours to determine growth and siderophore production as mentioned in section 3.2.4.

3.2.6 Growth and siderophore production in presence and absence of iron

The isolates _Pseudomonas aeruginosa_ TMR2.13 and _Bacillus amyloliquefaciens_ NAR38.1 were streaked on TYG and NA respectively. The plates were incubated at 28 ºC for 24 hours and the cultures were inoculated in MSM with 60 mg/L FeCl₃ and without added iron. 0.2% glucose was used as the carbon source for inoculum build-up. The flasks were incubated at 28 ºC at 150 rpm for 24 hours. 5% of 24 hour old culture was inoculated in MSM with 60 mg/L FeCl₃ and without added iron and 0.2% glucose as the carbon source. 4 ml of sample was removed under aseptic conditions every 8 hours upto 72 hours for _Pseudomonas aeruginosa_ TMR2.13 and every 4 hours upto 48 hours for _Bacillus amyloliquefaciens_ NAR38.1. Growth and siderophore production were determined as mentioned in section 3.2.4.

3.2.7 Effect of iron concentrations on growth and siderophore production by

_Pseudomonas aeruginosa_ TMR2.13 and _B. amyloliquefaciens_ NAR38.1
Flasks containing MSM with 0.2% of glucose as the sole carbon source, supplemented with Fe$^{+2}$ (FeSO$_4$) and Fe$^{+3}$ (FeCl$_3$), in increasing concentrations were inoculated with 5% of exponential cells grown in the respective medium. The culture flasks were incubated at 150 rpm at 28 ºC. 5 ml sample was removed after 24 hours, and growth and siderophore production was determined as mentioned in section 3.2.4. A control was maintained without added iron.

3.2.8 Binding of siderophores produced by *Pseudomonas aeruginosa* TMR2.13 and *Bacillus amyloliquefaciens* NAR38.1 to Fe$^{+2}$ and Fe$^{+3}$

TMR2.13 and NAR38.1 were inoculated in MSM with 0.2% glucose, and the flasks were incubated at 28 ºC at 150 rpm for 24 hours. The culture broths were centrifuged at 10,000 rpm for 10 minutes to obtain the cell free supernatant. For studies with the siderophore produced by TMR2.13, fluorimetric analysis was carried out with RF-5301 PC Shimadzu spectrofluorometer at excitation and emission wavelengths of 400 and 467 nm, respectively. Fluorescence quenching was studied by adding 10 µl of Fe$^{+2}$/Fe$^{+3}$ solutions to 3 ml of crude dilute culture supernatant to achieve a final concentration of 3.3 µM (Xiao and Kisaalita 1998). To determine binding of zerovalent Fe nanoparticles to the siderophore, Fe nanoparticles were added to 3 ml of diluted culture supernatant to achieve a final concentration of 0.0125, 0.025 and 0.05 M.

To determine binding of siderophore produced by NAR38.1 to Fe$^{+2}$ and Fe$^{+3}$, the siderophore was dissolved in deionised water at a concentration of 10 µg/ml. To 0.5 ml of siderophore, 0.5 ml of Fe$^{+2}$ (0.0001, 0.0005, 0.001, 0.005, 0.01 and 0.05 M) or Fe$^{+3}$ (0.0001, 0.0005, 0.001, 0.005, 0.01 and 0.05 M) was added and the solution was centrifuged to remove the iron-bound siderophore. To determine binding of Fe nanoparticles, 3.25 µg of siderophore
was dissolved per ml of deionised water. To 0.5 ml of siderophore, 0.5 ml of 0.025 M and 0.05M Fe nanoparticles was added separately and siderophore bound to Fe nanoparticle was removed by centrifugation. Unbound siderophore in the solution was estimated using Arnow’s test.

3.3 Results and Discussion

3.3.1 Plant growth promoting properties

In recent years, siderophore producing rhizobacteria have gained increased attention. Siderophores are beneficial to both, bacteria that produce them and the plant colonized by the siderophore producers. Siderophores have a dual mechanism of enhancing plant growth: either by enhancing iron availability to the host and the plant or by making iron unavailable to plant pathogens by chelating it and thus inhibiting their growth. In the previous chapter it was observed that both the isolates produced CAS detectable siderophores, therefore, these organisms were also screened for their other plant growth promoting properties such as phosphate solubilization, HCN production and ammonia production.

A large database is available to support the role of fluorescent pseudomonads in plant growth promotion (Jagadeesh et al. 2001). Further, pseudomonads have also been recognized as biocontrol agents against certain soil-borne plant pathogens. They are characterized by the production of yellow-green pigments termed pyoverdines, which fluoresce under UV light and function as ‘siderophores’ (Demange et al. 1987). The role of Gram negative bacteria as PGPR has been well documented in contrast to that of Gram positive bacteria especially Bacillus spp. Plant growth promoting effects of B. amyloliquefaciens have been reported only recently and PGPR activity is attributed to the production of metabolites like indole acetic acid and siderophores (Mishra and Kumar 2012).
On screening for plant growth promoting properties, both the isolates showed ammonia production. *P. aeruginosa* TMR2.13 scored positive for other plant growth promoting properties like phosphate solubilization and HCN production as opposed to *B. amyloliquefacies* NAR38.1 (Fig. 3.1).

Phosphate solubilizers bring about favourable changes in the soil microenvironment which leads to solubilization of inorganic phosphate (Mehta *et al.* 2010). Indole acetic acid (IAA) is a phytohormone and induces root elongation (Fallik *et al.* 1989, Kende and Zeevaart 1997), growth of roots and shoots in response to light and gravity (Kaufman *et al.* 1995), cell division in tissues, cell differentiation, and the formation of adventitious roots (Bashan *et al.* 2008). Production of HCN by PGPR protects plants from diseases (Marques *et al.* 2010). Plant growth promoting bacteria are usually *Bacillus* or *Pseudomonas* spp. and have been applied to a large range of plant spp. (Haas and Defago 2005).

### 3.3.2 Determination of the functional group of siderophore

To determine the functional group of the siderophore, the supernatant of the culture grown in iron-free MSM was subjected to various tests. The supernatants containing siderophores of *P. aeruginosa* TMR2.13 showed presence of carboxamate functional group. The cell-free supernatant of TMR2.13 showed a sharp peak at 400 nm (Fig. 3.2) and emitted fluorescence at 467 nm when excited at 400 nm, characteristic of the yellow pigment, pyoverdine, frequently produced by *Ps. aeruginosa*. The pigment shows a strong affinity for iron and therefore acts as a siderophore in iron limiting media (Braud *et al.* 2009, Cornelis *et al.* 2009).
Fig. 3.1: Plant growth promoting properties of *P. aeruginosa* TMR2.13 a) Phosphate solubilization b) HCN production c) Ammonia production d) Ammonia production by *B. amyloliquefaciens* NAR38.1

Fig. 3.2: Spectrophotometric scan of pyoverdine produced by TMR2.13
Pyoverdines are composed of three parts: i) a fluorescent chromophore based on a quinolin cycle responsible for the yellow-green color and bright fluorescence of pyoverdine; ii) a side-chain containing a carboxylic acid residue branched to an amino group of the chromophore; iii) a peptidic chain attached to a carboxyl group on the chromophore. The differences in the side chain reflects the diversity in pyoverdine structures (Meyer 2000).

*B. amyloliquefaciens* NAR38.1 was found to produce catechol type of siderophore. Previous studies have shown that catecholate siderophores predominate in certain Gram-negative genera, namely enterobacteria, *Vibrio*, but are also seen in the nitrogen-fixing *Azotobacteria* and the plant-associated agrobacteria. Catecholates may be common in this group because of their lipophilic nature, complex stability, high environmental pH and a weak nitrogen metabolism (Das *et al.* 2007). Catecholate siderophores are known to be produced by *Magnetospirillum magneticum* (Calugay *et al.* 2006), *Bacillus subtilis* (Corynebactin), *Corynebacterium glutamicum* (Corynebactin), *Azotobacter vinelandii* (protochelin, azotochelin), *Aeromonas hydrophila* (Amonabactins), *Chryseomonas luteola* (Cheyseomonin) and *Rhodococcus erythropolis* (heterobactins) (Winkelmann 2004).

Production of hydroxamate type of siderophores is important in an environment where a large amount of plant litter is present and is degraded by acid - producing bacteria such as *Enterobacter, Listeria* or *Lactobacillus* spp. In such ecosystems, iron may be present in large amounts in humic matter; however, it is not easily available to microorganisms and necessitates the production of siderophore. Ferrioxime is one such siderophore, which can extract iron from soils containing large amounts of humic acid, and is produced by streptomycetes (Winkelmann 2004). However, in the present study, although the culture NAR38.1 was isolated from a similar ecosystem, (mangrove ecosystem, where the plant litter
is being continuously degraded following a detrital cycle) the isolate produced the catecholate type of siderophore. Plant litter in mangrove ecosystems comprises of major macromolecules such as tannins, waxes, pectins etc. *Bacillus* are known to breakdown such unusual molecules thereby resulting in their proliferation (Ilori *et al.* 2007, Qureshi *et al.* 2012). This study has also shown the occurrence of *B. amyloliquefaciens* in the mangrove ecosystem at Ribander with production of the catecholate type of siderophore by the same to overcome iron stress.

### 3.3.3 Effect of pH on growth and siderophore production

The isolates TMR2.13 and NAR38.1 were isolated from the rhizosphere of a sand dune creeper *Ipomoea pes-caprae* and from mangroves. The salinity in such ecosystem varies due to tidal fluxes and also results in microniches which have an alkaline/acidic pH which is a result of breakdown of plant litter (Agate *et al.* 1988). In order to understand the effect of such changes in pH and salinity on siderophore production, the isolates were exposed to varying pH and salt concentrations. For *P. aeruginosa* TMR2.13, optimum pH for growth and siderophore production was found to be pH 7 (Fig. 3.3a). Sayyed *et al.* (2004) have also reported pH 7 to be optimum for siderophore production in *P. fluorescens*. While changes in pH did not considerably affect growth, the siderophore production was decreased to a large extent at pH 5 in this isolate. In case of NAR38.1, the changes in pH also did not show any significant effect on the growth, however, the siderophore production was found to decrease under acidic and alkaline conditions (Fig. 3.3b). Optimum siderophore production as determined by Arnow’s test was observed at pH 7. The decrease in siderophore production under acidic pH could be attributed to solubilization of iron at lower pH resulting in iron availability (Agate *et al.* 1988). Ferric complexes of catecholates are unstable at acidic pH, therefore, in nature, biosynthesis of the hydroxamate type of siderophore is preferred over synthesis of the catecholates (Winklemann 2004). It has also been reported that at low pH,
destruction of siderophores is noted specially in *Pseudomonas aeruginosa* and therefore an increase in pH is reported during growth (Villegas *et al.* 2002) which protects the siderophore.

3.3.4 Determination of the effect of NaCl on growth and siderophore production in the selected isolates

During our study it was noted that the increase in NaCl concentrations increased growth of *P. aeruginosa* TMR 2.13, however, siderophore production was found to decrease (Fig. 3.4a). Siderophore production also decreased considerably with increase in salt concentrations from 0 to 5% in *B. amyloliquefaciens* NAR38.1, however, growth was not affected upto 2% of NaCl (Fig. 3.4b). Further, increase in NaCl (≥3%) showed reduction in growth which resulted in decrease in siderophore production. Such effects of NaCl on siderophore production have been noted in *Rhizobium* strains nodulating *Macrotyloma uniflorum*. These strains showed increased siderophore production upto 8-9% of salt concentration, but siderophore production ceased when salt concentration was increased further (Prabhavati and Mallaiah 2008).
Fig. 3.3: Effect of varying pH on growth and siderophore production in a) *P. aeruginosa* TMR2.13 b) *B. amyloliquefaciens* NAR38.1
Fig. 3.4: Effect of varying NaCl concentrations on growth and siderophore production in a) *P. aeruginosa* TMR2.13 b) *B. amyloliquefaciens* NAR38.1
3.3.5 Effect of iron on growth and siderophore production in *Pseudomonas aeruginosa* TMR2.13 and *B. amyloliquefaciens* NAR38.1

Iron is one of the most important micronutrients required by bacteria for their metabolism as a cofactor for a large number of enzymes and iron containing proteins (Harrington and Crumbliss 2009). However, its availability in the environment may not be sufficient to support microbial growth. The effect of iron on growth of the selected isolates was determined in presence and absence of added iron in MSM with 0.2% glucose as the carbon source. It was observed that presence of iron did not affect growth profiles of the cultures but completely suppressed siderophore production (Fig. 3.5 and 3.6).

Further studies with NAR38.1 showed production of siderophore upto 1 µM of Fe$^{2+}$ and upto 30 µM of Fe$^{3+}$ (Fig. 3.7), suggesting that higher levels of Fe$^{3+}$ are required to suppress siderophore production as compared to the Fe$^{2+}$. This could be possibly because of the well known fact that Fe$^{3+}$ form is not the highly soluble form of Fe as opposed to Fe$^{2+}$ ion which has a much better solubility (Chu 2010).

However, the effect of iron with *P. aeruginosa* TMR2.13 showed a significant siderophore production upto 54 µM which was suppressed at 108 µM irrespective of it being divalent or trivalent (Fig. 3.8). The fact that pyoverdine binds to both the forms of Fe, as has been reported earlier (Xiao and Kisaalita 1998), results in the suppression of siderophore production with the same concentration of Fe$^{2+}$ and Fe$^{3+}$. 
Fig. 3.5: Growth profile and siderophore production by *P. aeruginosa* TMR2.13 in presence and absence of added iron

Fig. 3.6: Growth profile and siderophore production by *B. amyloliquefaciens* NAR38.1 in presence and absence of added iron

Fig. 3.7: Effect of Fe$^{2+}$ and Fe$^{3+}$ concentrations on siderophore production by *B. amyloliquefaciens* NAR38.1
Earlier studies on the effect of iron concentration on siderophore production by *Ps. aeruginosa* have shown siderophore production even with 248 µM of Fe$^{3+}$ (Villegas et al. 2002). Rachid and Ahmed (2005) have reported Fe$^{3+}$ levels of 200 µg/ml being inhibitory to siderophore production by *P. fluorescens*, while Sayyed et al. (2004) have reported suppression of siderophore production in *P. fluorescens* and *P. putida* at concentration of 20 µM. These reports and the observations made in the present study implicates that the iron requirement varies with the strains.

3.3.6 Binding of siderophores produced by *Pseudomonas aeruginosa* TMR2.13 and *Bacillus amyloliquefaciens* NAR38.1 to different forms of iron.

Amongst the different forms of iron, Fe$^{3+}$ iron is known to have higher affinity for siderophores in contrast to Fe$^{2+}$. This extreme difference in the affinity is important for release of Fe$^{3+}$ from siderophore by reducing it to Fe$^{2+}$ (Xiao and Kisaalita 1998). The ability of siderophores produced by the selected isolates to bind to Fe$^{2+}$/Fe$^{3+}$ and zerovalent iron nanoparticles was determined. It was interesting to note that the fluorescence of pyoverdine produced by the isolate TMR2.13 was quenched by 45.58% with Fe$^{2+}$ immediately, while Fe$^{3+}$ quenched the fluorescence by 67.08% within 30 seconds (Fig. 3.9a). Xiao and Kisaalita (1998) have demonstrated that pyoverdines also bind and oxidize Fe$^{2+}$, with the binding of pyoverdines to Fe$^{2+}$ being faster than to Fe$^{3+}$ and suggested that the phenomenon could be due to the precipitation of Fe$^{3+}$ as Fe(OH)$_3$. The most interesting observation was the binding of pyoverdine to zerovalent iron nanoparticles. Nanoparticles quenched fluorescence by 22.25, 36.56 and 62.32 % when added at concentrations of 0.0125, 0.025 and 0.05 M respectively (Fig. 3.9b).

In the present study, very low concentrations of Fe$^{3+}$ (0.001M) resulted in complete binding of the siderophore produced by *B. amyloliquefaciens* NAR38.1 than those of Fe$^{2+}$ (0.05M).
The intensity of the peak at 510 nm is directly proportional to the amount of siderophore which is not bound to Fe. Studies with zerovalent iron nanoparticles did not show any binding with siderophore confirming its affinity for trivalent form of iron.

This chapter illustrates the various aspects about the selected siderophore producing isolates. Both the isolates showed ammonia production with *P. aeruginosa* TMR2.13 being positive for other plant growth promoting properties like phosphate solubilization and HCN production. Functional group tests proved that the siderophores produced by TMR2.13 and NAR38.1 were of carboxamate and catecholate type respectively. Optimum siderophore production for both the isolates was obtained at pH 7, 0% NaCl and with no added iron. Furthermore, addition of iron to the growth medium of both the isolates suppressed siderophore production without affecting growth profiles. Studies with NAR38.1 showed production of siderophore upto 1 µM of Fe\(^{+2}\) and upto 30 µM of Fe\(^{+3}\). However, interestingly, siderophore production was seen upto 54 µM and suppressed at 108 µM of Fe\(^{+2}\) as well as Fe\(^{+3}\) in *P. aeruginosa* TMR2.13. Siderophore produced by *P. aeruginosa* TMR2.13 showed binding to Fe\(^{+2}\), Fe\(^{+3}\) as well as to Fe\(^{0}\). While, siderophore produced by NAR38.1 had much higher affinity for Fe\(^{+3}\) as compared to Fe\(^{+2}\).

Biosynthesis and secretion of siderophores is known to be related to the requirement of iron for metabolism of specific growth substrates. The presence of aromatic compounds along with easily metabolisable co-substrates supports the production of siderophores. Iron concentration is one of the many factors influencing efficient clean-up of pollutants. In natural environments, the availability of iron is usually low, more so, in marine and coastal ecosystems as well as in arid environments where the concentration of iron can be
Fig. 3.8: Effect of Fe$^{2+}$ and Fe$^{3+}$ concentrations on siderophore production by *P. aeruginosa* TMR2.13

Fig. 3.9: Fluorescence spectra of pyoverdine illustrating fluorescence quenching with a) Fe$^{2+}$ and Fe$^{3+}$ b) Fe$^{0}$ nanoparticles.

Fig. 3.10: Binding of Fe$^{2+}$ and Fe$^{3+}$ to siderophore produced by *B. amyloliquefaciens* NAR38.1.
as low as $10^{-18}$ M. Such iron limiting conditions may have a direct implication on the siderophore production during utilization of aromatic compounds. Isolate TMR2.13 which produced siderophore and also showed growth on sodium benzoate was selected further to study the effect of sodium benzoate on siderophore production. Sand dune samples were also enriched to isolate siderophore producing organisms which could also utilize sodium benzoate as the sole carbon source. These results have been compiled in the next chapter.