CHAP: 3
SIDEROPHORE PRODUCTION BY RHIZOBACTERIA UNDER METAL STRESS.
CHAPTER 3:

ABSTRACT

In iron depleted standard succinic acid medium, 1000 µgml⁻¹ of siderophore was produced by NT1. MM9 and FMM media were found to optimize siderophore production ability while no siderophore production was observed in citrate and nutrient agar medium. Increasing concentration of iron showed an inverse relationship between growth and siderophore production. Amount of fluorescence produced increased up to 2 µM concentration of FeCl₃. Siderophore production was enhanced by Zn²⁺ (12 µM) whereas it reduced in presence of Ni²⁺ (12 µM) in SSM medium. However, only NT1 showed development of fluorescence, which could be correlated with siderophore producing ability of the strain and was maximum (35.294 rfu) at 6 µM Zn²⁺. Presence of Ni at 6 and 12 µM decreased the growth of all isolates except T15. Siderophore production doubled in both T5 and T15, no change was reported in siderophore production in C4 whereas it inhibited the growth of NT1 and C5 proving to be inhibitory at higher concentration of 12 µM. Biocontrol efficiency of NT1 was found to be related to siderophore production potential. The antagonistic agent present in siderophore was found to be resistant to high temperature pressure. Crystallization of fine needle shaped crystals and their FTIR followed by UV spectral analysis revealed the presence of pyoverdin which was also influenced by pH. Dominant peaks were also observed at 14.906 min, 16.957 min and 18.444 min during HPLC analysis shows presence of atypical pyoverdin. Studies were also conducted on mass spectra.
Chapter 3: Siderophore Production By Rhizobacteria Under Metal Stress.

3.1 INTRODUCTION

Iron is the most abundant element found in the earth crust. It is also the most vital micronutrient for bacterial growth being a co-factor in about 140 different enzymatic processes and iron containing proteins. Even then, plants grown on calcareous or alkaline soils suffer from iron deficiency. Absence or deficiency of Fe$^{3+}$ in plants may hamper plant growth and development of chlorophyll, thylakoid synthesis and chloroplast formation. (Miller et al, 1995). Iron forms insoluble hydroxides at neutral and basic pH in soil and therefore it is not promptly available (Jurkevitch, 1992). In most environments the amount of free iron (approx $10^{-9}$ M) is below the concentration required by most microorganisms for growth. To fulfill their requirements for iron, bacteria have developed several strategies including 1) reduction of ferric to ferrous ions 2) direct acquisition of iron from host protein 3) secretion of high affinity iron chelating compounds called siderophores and uptake of heterologus siderophores (Llamas et al, 2006).

Under such iron starvation conditions, bacteria, fungi and plants secrete small, specialized efficient iron (III) chelator molecules commonly known as siderophores (Drechsel & Jung, 1998). After the iron-siderophore complexes have formed, these now soluble complexes are internalized via active transport into the cells by specific membrane receptors (Glick et al, 1999). Following either cleavage or reduction to the ferrous state, the iron is released from the siderophore and used by a cell (Glick et al, 1999).

Lankford (1973) coined the term siderophore to describe low molecular weight (approximately 600 to 1500 daltons) molecules that bind ferric iron with an extremely high affinity. Siderophore was derived from a Greek term meaning iron carrier (Chincholkar et al, 2003). The dominant iron-binding ligands of siderophores are hydroxamates and catecholates (phenolates). Bacterial siderophores are structurally diverse where as fungal siderophores are dominated by hydroxamate siderophores (Chincholkar et al, 2003). On the other hand, plant siderophores are linear hydroxy- and amino-substituted iminocarboxylic acids, such as mugineic and Siderophore production by rhizobacteria under metal stress.
aventic acids (Sugiura et al, 1981). Many bacteria are capable of producing more than one type of siderophore or have more than one iron-uptake system to take up multiple siderophores (Neilands, 1981). Wide arrays of beneficial plant-associated bacterial genera, e.g. Pseudomonas, Azotobacter, Bacillus, Enterobacter, Serratia, Azospirillum and Rhizobium secrete various types of siderophores (Lopez & Henkels, 1997; Glick et al, 1999).

The uptake of iron under limiting conditions is particularly well studied in bacteria where bacterial siderophore bind to fix iron and transport it to an iron siderophore receptor protein present on the cell wall of microbe. These siderophores play a vital role in extracellular solubilization of iron from minerals and organic substances (Hu and Boyer, 1996).

Under iron limiting conditions, most aerobic microorganism produces at least one siderophore and in some cases, a single bacterial strain can produce two or more. Each siderophore probably has a specific role in metal acquisition. One molecule may be important for the acquisition of iron, while another may be responsible for transport of some other metals. P. aeruginosa PAO1 secretes two different types of siderophores; pyoverdine, which demonstrates binding affinity for iron and pyochelin, which shows affinity for other metals as well as biocontrol efficiency. Siderophores of different bacteria are designated and named on the basis of the name of organism as well as their iron- free or iron bound form. For eg. Enterobactin, Pseudobactin, Azotobactin, Rhizobactin, Rhizoferrin, Pyoverdin etc. There are a variety of biochelators produced by microorganism which do not function as siderophores and some rather well known siderophores appear to have additional activities apart from transport of metals such as antioxidant and antibiotic action.

Interest in PGPR has recently gained interest because of the possible use of siderophore as biopesticides, which act as antibiotics also and thus play its role in plant growth promotion in double mode (1) supply of plant nutrients (2) Control of phytopathogens through direct competition. It has been clearly studied that yellow green siderophore producing Pseudomonas species exert biocontrol effect on crops. The antimicrobial activity of siderophores can have significant ecological effects. For

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example the siderophores of fluorescent pseudomonads are responsible for antagonism towards various strains of fungi and some *Pseudomonas* spp that are pathogenic to plants. Siderophore production by PGPR as a constituent of biological products is of great interest because of its possibilities in the substitution of chemical pesticides (De Villegas *et al.*, 2002). Many PGPR have been employed efficiently as biocontrol agents and in present time certain commercial products in markets as bacteriostatic and fungistatic agents in combination with other antibacterial factors will certainly raise a great interest.

Among the large diversity of bacteria, *Pseudomonads* are characterized due to their ability to produce a special yellow-green water-soluble pigment, which acts as ferrous chelator. These pigments or siderophores are known as pyoverdin or pseudobactins. These pyoverdins are strong iron specific chelator with high affinity for Fe(III) where as low affinity chelators produced by pseudomonads are known as pyochelin which act primarily for biocontrol of phytopathogen. Pyoverdins when placed under UV light give fluorescence. Today more than 40 pyoverdin peptide chain compositions have been identified in the group containing arginine dehydrolase positive, saprophythic or opportunistic animal pathogenic fluorescent *Pseudomonas* species. Pyoverdins are now used as a major tool in systematics, identification as well as characterization/classification of closely related pseudomonad species. Siderovar regroup strains that produce pyoverdins with same peptide chain. There are different siderovars in certain species such as *P. fluorescens* has 19 siderovars where as *P. putida* has 13 siderovars (Bultreys *et al.*, 2003).

The work was therefore undertaken to study siderophore produced by selected plant growth promoting rhizobacteria in presence of different metals and thus act as metal cheletors. Study was also undertaken to find out biocontrol efficiency of siderophores produced and then to further characterize them.
3.2 MATERIALS AND METHODS

3.2.1 Growth study of selected isolates in MM9 medium.

Generation time of best five selected isolates (NT1, T15, T5, C4 and C5) was determined using MM9 medium. Actively growing 1 mL inoculum of all isolates was added to 100 mL MM9 medium and incubated on rotary shaker with 200 rpm at room temperature. Enhancement of growth was checked every 2 h at 540 nm on UV-Vis spectrophotometer and generation time was calculated until the culture achieved stationary phase.

3.2.2 Induction of siderophores in MM9 medium.

Simultaneous induction of siderophore production was also checked every time where 1 mL of cell free supernatant was added with 1 mL of CAS shuttle solution. Decolorisation of blue colour from the CAS shuttle solution shows the production of siderophores by the organism. Amount of siderophore produced can be checked as siderophore units by taking OD at 630 nm and can be calculated as follows (Sayyed et al, 2005).

\[% \text{ Siderophore Unit} = \frac{(Ar-As)}{Ar} \times 100\]

where

\(Ar = \text{absorption of Reference}; As = \text{Absorption of Sample.}\)

Induction was also confirmed by agar cup method where 0.8 mL of culture supernatant was added into wells bored in CAS blue agar plate. Presence of siderophore can be checked as zone of discoloration of CAS dye around the well as well as by Csaky’s method as mentioned in chapter 2.

3.2.4 Screening for suitable media for siderophore production.

Siderophore production was checked in different media using different sugars and amino acids to screen the most suitable media for the isolates for siderophore production. These media were MM9 (Schwyn & Neilands, 1987). Fiss’s Modified Minimal media (FMM) (Clark et al, 2004), Standard Succinate Medium (SSM), Standard Citrate Medium (SCM), Barbhaiya and Rao Medium (BRM) and Nutrient Broth (Sayyed et al, 2005).
3.2.5 **Siderophore production by wild type and rifampicin tagged mutants.**

Because siderophore production is an important mechanism of plant growth promotion, a comparative study of siderophore production by the selected isolates before and after tagging with rifampicin was carried out. A set of active cultures of selected wild type and rifampicin resistant isolates were spot inoculated on CAS blue agar plates and then incubated at 30 °C and checked for yellow-orange halo around the colonies after 24 h. The difference in the ratio of zone and colony was calculated to find the difference in siderophore production by wild type and rifampicin tagged mutants.

3.2.6 **Effect of different ferrous concentration on siderophore production.**

In order to determine the threshold level of ferrous at which siderophore biosynthesis is repressed in PGPR, liquid succinic acid medium was initially deferrated and then externally added with different ferrous concentration ranging between 0-20 μM. This was then inoculated with selected isolates and incubated for 30 h at 30 °C and checked for production of siderophores (Sayyyed et al., 2005).

3.2.7 **Estimation of Fluorescence.**

Fluorescence produced by isolates at different FeCl₃ concentrations was estimated by using Quinine Bisulphate method (Totter and Moseley, 1952). A relative fluorescence produced was estimated from the intensity of the fluorescence using Fluorispectrometermeter (Hitachi, Model F-2000).

3.2.8 **Heavy metal remediation.**

Different heavy metals viz. ZnCl₂, NiCl₂, CdSO₄ and K₂Cr₂O₇ were selected because of their toxic effects on plants. A modified CAS dye was developed in which 1 mM Zn, Ni, Cd and Cr replaced Fe. This dye was then added into the deferrated medium. PGPR were then streaked on the modified CAS agar plate and incubated for 3 d at 30 °C temperature. A clearing zone around the colony was recorded every 24 h which showed chelation of heavy metals due to the production of siderophore (Renshaw et al., 2003).

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3.2.9 Influence of heavy metals on siderophore production.

Effect of heavy metals like Zn and Ni at 2 different concentrations was also studied to check the tolerance and siderophore production potentials of selected PGPR at these two concentrations. ZnCl₂ and NiCl₂ from filter sterilized 0.1 mM was added to sterilized deferrated SSM so as to achieve 6 and 12 μM concentration individually. Influence of these metals was checked on siderophore production along with growth, pH and fluorescence after 24 h and 30 h (Sayyed et al, 2005).

3.2.10 In-vitro antagonistic potential of siderophore extract against phytopathogens.

Biocontrol of cell free supernatant of PGPR activated in ferrous free SSM was checked for activity against *Macrophomina phaseolina* and *Aspergillus niger*. A 5 mm diameter mycelia disc was taken from an actively growing colony of *M. phaseolina* and *A. niger* grown on PDA. The disc was then placed in the center of the PDA plate. Agar cups were bored in the PDA plates at a distance of 2 cm from the mycelia disc. The cups were filled with 0.02 mL, 0.05 mL and 0.1 mL of autoclaved and nonautoclaved filtered supernatant. Plates were then incubated at 37 °C. After every 24 h growth inhibition of mycelia disc was checked and noted down till no further change was observed. Control well contained sterile distilled water (De Villegas et al, 2002).

3.2.11 Crystallization and FTIR studies of siderophores.

Siderophore produced were crystallized to study their chemical structure. The 30 h culture grown at 30 °C was centrifuged and cell-free supernatant was added with saturated FeSO₄ solution to get maximum orange colour of ferrated siderophores. The pH was adjusted to 3.0 with H₂SO₄ and 50 % ammonium sulphate solution was added to deproteinize. The orange colour was extracted in benzyl alcohol and filtered through double filter paper, diluted with several volumes of diethyl ether and extracted with small volumes of water. The aqueous phase was concentrated in a rotary vacuum evaporator and set aside in cold to crystallize. The

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filtrate was neutralized, reduced to dryness and extracted in dry hot methanol. The methanol extract was reduced in volume over water bath in 60 °C, till the first precipitate appeared and allowed to stand overnight at room temperature to crystallize. The crystals were then separated out on whatman filter paper no. 44. Crystals obtained after extraction of the siderophores from the supernatant were then used for their FTIR analysis and compared with the FTIR of standard hydroxamic acid crystals (Dave and Dube, 1999).

3.2.12 Partial purification of siderophore for spectral analysis.

Culture was grown in deferrated SSM and incubated at 30 °C for 30 h. Culture was then centrifuged and 100 mL of cell free supernatant was collected and its pH was adjusted to 6.0 with 6 N HCL. This supernatant was then passed through XAD-4 column (25 x 2.5 cm) with a flow rate of 60 mL in 1 h. After complete removal of the fluorescence from the supernatant, the column turns green proving the adsorption of fluorescence pigment on the resin. This was then washed twice with deionised water and then eluted using 50 % methanol (Carson et al, 2000). Six different fractions of 1.5 mL each were collected and studied for the presence of fluorescence under U.V. light. The peak of absorbance was checked using UV visual spectrophotometer. Influence of pH on the shift of absorption peak was checked on partially purified pyoverdins at various pH (3.0, 5.0, 7.0 and 10.0) and compared with standard P. fluorescens at pH 7.0 and 3.0 (Bultreys et al, 2001).

3.2.13 Detection of NT1 siderophores by HPLC.

After incubation for 30 h, active culture of NT1 was collected in 2 mL micro-centrifuge tubes and mixed with 40 μL of 1 M FeCl₃ and centrifuged for 20 min. This was then filtered through 0.2 mm membrane filter and pH adjusted to 5.0-5.5. Pyoverdin production was estimated by measuring the absorbance at 403 nm. HPLC was performed as discussed (Bultreys et al, 2003).
3.2.14 MS studies of partially purified pyoverdin.

The molecular mass of the pyoverdin was determined by mass spectrometry using Electron spray Ionization Solvent H$_2$O, CH$_3$OH, CF$_3$COOH 50:50:1, capillary temperature 230 °C, spray voltage 3.4 - 3.6 kV (Barelmann et al., 2002). The study was conducted at Institute of organic chemistry, Koeln, Germany by Dr. H. Budzekiewicz.

3.3 RESULTS
3.3.1 Growth of selected PGPR in MM9.

Maximum growth of each selected isolate was observed after 26 h of incubation which was delayed by 2 h than that in nutrient broth. Stationary phase continued up till 30 h after which decline phase started. Isolate NT1 entered in to log phase after 2 h, C4 showed log phase after 4 h, T15 showed log phase after 6 h and C5 showed log phase after 8 h of incubation. Thus overall it can be said that each isolate showed a delayed growth ranging from 2 h to 8 h except NT1 although the growth was comparatively lower than that observed in nutrient medium (Fig. 3.1).

3.3.2 Induction of siderophore production by PGPR.

When checked for induction of siderophore production, results show that siderophore production was induced only after 8 h of incubation. The presence of siderophore was observed only after 8 h in supernatant of isolates when checked by CAS shuttle solution. NT1 showed presence of 85 % of siderophores in 1 mL of supernatant after 24 h. When 1 mL of NT1 supernatant collected after 8, 24, 26, 28 and 30 h was added in to CAS blue agar cups, zones of ferrous chelation from CAS dye was observed after 24 h of incubation where maximum zone of deferration was reported to be 7 mm after 26 h of incubation (Pic. 3.1). The results thus show that as the growth increased in the deferrated medium, the amount of siderophore produced also increased in the supernatant revealing that siderophore production is an important mechanism of survival during the growth of culture in iron deficient medium.
3.3.3 Screening of most efficient medium for siderophore production.

When different media were tried to screen out the most suitable medium, FMM, SSM and MM9 were found to be the best solid media for siderophore production. All the isolates showed highest zone of ferrous chelation on FMM medium followed by SSM and MM9. Maximum zone of ferrous chelation on FMM agar was observed in T15 (18 mm) followed by NT1, T5 and C4 (15 mm). NT1 also showed highest zone of ferrous chelation on SSM (12 mm) and MM9 (11 mm), C4 and C5 when grown on MM9 agar showed a zone of 10 mm and 8 mm respectively where as T5 and T15 showed zones of 7 mm on MM9 agar. On SSM agar, C4 and C5 showed a zone of 9 mm and 11 mm where as T5 showed a zone of 10 mm followed by T15 (7 mm). No zone of ferrous chelation was observed on SCM, BR medium or nutrient agar plate (Tab. 3.1).

When the same liquid media were tried for the quantitative estimation of siderophore production, SSM was found to be the most efficient medium. All isolates produced higher amount of siderophores when grown in SSM medium followed by MM9. About five fold increase in siderophore production by NT1 was observed as compared to that in MM9 medium (Fig.3.2). Production of fluorescence was reported in NT1 and T15 only along with siderophore production in SSM (Pic.3.2). No fluorescence was observed when grown in SCM revealing a complete absence of siderophores in the medium especially by NT1 and T15. Quantitative estimation of hydroxamates in SCM further supported that there is a complete absence of siderophores in the medium.

All the isolates showed increase in pH from 6.8 - 7.0 to 9.5 to 10.0. Results also show that all isolates continued to grow up to 30 h and then started declining. A simultaneous decline in siderophore production was also reported in all isolates during decline phase.

3.3.4 Comparative siderophore production by wild type and rifampicin tagged mutants.

Tagging of all the isolates with 100 μg mL⁻¹ rifampicin reduced the colony size of each isolate on the CAS blue agar plate resulting in to reduced size of ferrous

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Fig. 3.1 Growth of isolates in MM9 medium.

Fig. 3.2. Comparative study of quantitative siderophore production by selected isolates in SSM, MM9 and FMM media.

Siderophore production by rhizobacteria under metal stress.
Pic. 3.1 Siderophore induction in NT1 isolate after different time (h) of incubation.

Pic. 3.2 Fluorescence production by NT1 in SSM medium.

Pic. 3.2 Fluorescence production by NT1 in SSM medium.

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chelation zone as compared to that of wild type isolates. But when the ratio of zone size and colony size was calculated results show that both, wild type and mutants, show equal ratio revealing that although the size of the zone of iron chelation reduced in tagged mutants their siderophore production efficiency has not been effected. This is an important achievement for these selected isolates to work as PGPR because siderophore production is an important trait working at different levels of plant growth. If this property is lost or is reduced after developing the selected PGPR, its potential to perform as biofertilizers may be lost (Tab.3.2).

3.3.5 Effect of different FeCl₃ concentrations on growth and siderophore production pattern.

All the isolates showed increase in growth along with the increase in FeCl₃ concentration revealing that ferrous is an important element for their growth. Maximum growth enhancement was reported for different isolates was reported at different concentrations of Fe. NT1 and C4 showed maximum growth at 5 μM FeCl₃ concentration where as C5 showed maximum growth at 10 μM FeCl₃ concentration. T5 and T15 showed highest growth at 2 μM FeCl₃ concentration (Fig.3.3). This shows that although ferrous is vital for growth of all these PGPR, the threshold level of each isolate for optimum growth was found to be different. On the other hand results also show that presence of higher amount of ferrous beyond certain concentrations can be inhibitory to the organisms resulting in to their reduced growth.

Maximum siderophore production by different isolates was found to be at different FeCl₃ concentrations. NT1, T5 and T15 showed maximum siderophore production at 1 μM FeCl₃ concentration where as C4 and C5 showed highest siderophore production at 5 μM FeCl₃ concentration (Fig.3.4). A continuous decrease in siderophore production was reported in all isolates after reaching the threshold level of Fe for optimum siderophore production along with the increase in FeCl₃ concentration. This also proves that siderophore production is a response to the presence or absence of ferrous in the medium. The results here shows that although an increase in growth of all isolate was reported when the concentration of FeCl₃ was increased, this increase was at the cost of siderophores produced by the isolate.

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Tab.3.1 Comparative siderophore production by selected isolates on different media showing zone size (mm).

<table>
<thead>
<tr>
<th>Media</th>
<th>NT1</th>
<th>T5</th>
<th>T15</th>
<th>C4</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM9</td>
<td>11</td>
<td>7</td>
<td>7</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>SSM</td>
<td>12</td>
<td>10</td>
<td>7</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>FMM</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>SCM</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>BR</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>N. agar</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
</tr>
</tbody>
</table>

Tab.3.2 Comparative siderophore production by wild type and rifampicin tagged mutants.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Colony size (C) (mm)</th>
<th>Zone size (Z) (mm)</th>
<th>Z:C Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT1. WT</td>
<td>6</td>
<td>15</td>
<td>2.5</td>
</tr>
<tr>
<td>NT1 Rif+</td>
<td>1.5</td>
<td>4</td>
<td>2.6</td>
</tr>
<tr>
<td>T5 WT</td>
<td>4</td>
<td>10</td>
<td>2.5</td>
</tr>
<tr>
<td>T5 Rif+</td>
<td>1.5</td>
<td>3.4</td>
<td>2.26</td>
</tr>
<tr>
<td>T15 WT</td>
<td>5</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>T15 Rif+</td>
<td>1.3</td>
<td>2.5</td>
<td>2</td>
</tr>
<tr>
<td>C4 WT</td>
<td>4</td>
<td>11</td>
<td>2.7</td>
</tr>
<tr>
<td>C4 Rif+</td>
<td>1.5</td>
<td>3.4</td>
<td>2.26</td>
</tr>
<tr>
<td>C5 WT</td>
<td>4</td>
<td>10</td>
<td>2.5</td>
</tr>
<tr>
<td>C5 Rif+</td>
<td>1.5</td>
<td>4</td>
<td>2.6</td>
</tr>
</tbody>
</table>

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Fig. 3.3 Influence of different FeCl₃ concentrations on growth of selected PGPR.

Fig. 3.4 Influence of different FeCl₃ concentrations on siderophore production by selected PGPR.

Siderophore production by rhizobacteria under metal stress.
Production of fluorescence was reported in NT1 and T15 only. Highest fluorescence was reported in NT1 followed by T15. The fluorescence of NT1 changed from dark green (+4) in absence of FeCl₃ to brilliant green (+3) and finally to fluorescence yellowish green (+2) as the FeCl₃ concentration increased from 0 μM to 20 μM. T15 also showed fluorescence in absence of ferrous but never showed brilliant green pigmentation. Its fluorescence ranged from fluorescent yellowish green (+2) to yellowish green to canary yellow (+1) along with the increase in ferrous concentration. NT1 showed fluorescence up to 20 μM FeCl₃ concentration whereas T15 showed fluorescence only up to 15 μM FeCl₃ concentration. When relative intensity of fluorescence was measured it was observed that maximum fluorescence was obtained at 2 μM FeCl₃ concentration in both NT1 and T15 after which a continuous decline in fluorescence intensity was observed (Fig. 3.5). Thus the production of fluorescent pigment accompanied production of siderophores in the medium by NT1 and T15 proving that it is a response to siderophore production in presence and absence of FeCl₃.

3.3.5 Heavy metal chelation by selected isolates through siderophore production.

Results of heavy metal chelation by selected isolates through siderophore production shows that all isolates could chelate out zinc and cadmium whereas nickel was chelated out by only NT1 and T15. None of the isolate could grow in presence of chromium revealing its toxicity to all selected PGPR. Maximum zone of zinc chelation was observed in NT1 and T15 (21 mm) after 48 h of incubation. Maximum cadmium chelation was reported by C4 (25 mm) whereas maximum nickel chelation was reported by T15 (29 mm) after 48 h of incubation (Tab. 3.3). NT1 and T15 showed chelation of all the three metals except chromium.
Fig. 3.5 Relative fluorescence of NT1 and T15 at different FeCl₃ concentrations.

<table>
<thead>
<tr>
<th></th>
<th>Zone size</th>
<th>Zone size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h (mm)</td>
<td>48 h (mm)</td>
</tr>
<tr>
<td></td>
<td>Cr  Ni</td>
<td>Zn   Cd</td>
</tr>
<tr>
<td>NT1 WT</td>
<td>ng  14</td>
<td>13   7</td>
</tr>
<tr>
<td>T5 WT</td>
<td>ng  ng</td>
<td>7    10</td>
</tr>
<tr>
<td>T15 WT</td>
<td>ng  18</td>
<td>16   7</td>
</tr>
<tr>
<td>C4 WT</td>
<td>ng  ng</td>
<td>11   16</td>
</tr>
<tr>
<td>C5 WT</td>
<td>ng  ng</td>
<td>12   14</td>
</tr>
</tbody>
</table>

(Cr; Chromium, Ni; Nickel, Zn; Zinc, Cd; Cadmium, ng: no growth).

Tab. 3.3 Heavy Metal remediation through siderophore production by selected isolates.
3.3.6 Influence of different heavy metals on growth and siderophore production by selected PGPR.

When Zn was added to deferrated SSM at 6 and 12 μM concentrations, isolates showed an increase in growth as Zn concentration increased from 6 to 12 μM (Fig3.6) but this increase in growth was lesser than that in deferrated medium without Zn. A simultaneous decrease in siderophore production was also reported by all isolates along with increase in growth and Zn concentration (Fig3.7). Presence of Zn also influenced the production of fluorescence by NT1 and T15. Maximum siderophore production at 6 μM was reported in C5 (3000 μg mL\(^{-1}\)) after 24 h which then decreased to 800 μg mL\(^{-1}\) after 30 h. At 12 μM Zn, both C5 and T15 showed highest siderophore production (2500 μg mL\(^{-1}\)) which then reduced after 30 h of incubation and almost to half in T15. On the other hand siderophore production increased in NT1 from 800 μg mL\(^{-1}\) to 1350 μg mL\(^{-1}\) after 30 h of incubation where as it remained the same in C4. Lowest siderophore was reported in T5 after 24 as well as 30 h of incubation. Here it was surprising to note that siderophore production was highest after 24 h and not after 30 h as observed in absence of zinc (Fig3.7).

When Ni was added to deferrated SSM at 6 and 12 μM concentrations, the growth of cells increased at 12 μM concentration as compared to 6 μM NiCl\(_2\) concentrations. Maximum increase in growth was reported in NT1 after 24 h and in T15 after 30 h of incubation in presence of 6 μM NiCl\(_2\) (Fig3.8). Siderophore production increased as incubation increased at 6 μM NiCl\(_2\) concentration except in T5 where maximum siderophore production was reported in C4 (1124 μg mL\(^{-1}\)). In presence of 12 μM NiCl\(_2\) siderophore production was highest in NT1 after 24 h which then decreased after 30 h. On the other hand in T15 and C4 siderophore production increased as incubation time increased from 24 to 30 h where as in T5, it remained constant up to 30 h. Unlike others, C5 did not show any growth in presence of 12 μM Ni (Fig.3.9).

A similar increase and decrease in fluorescence along with siderophore production was observed in NT1 and T15 in presence of Zn and Ni at both concentrations.
Fig. 3.6 Growth of selected isolates at different Zn concentrations and time intervals.

Fig. 3.7 Influence of Zn and time on siderophore production by selected isolates.

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Fig. 3.8 Growth of selected isolates at different Ni concentrations and time intervals.

Fig. 3.9 Siderophore production at different Ni concentrations and time intervals.

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3.3.7 In-vitro antagonistic potential of siderophore extract against phytopathogens.

A clear zone of mycelial growth inhibition was observed for *M. phaseolina* and *A. niger* when tested against NT1 where as T5 and T15 showed zone of inhibition only against *A. niger*. Maximum zone of growth inhibition (20 mm) was observed with NT1 extract. Both autoclaved and nonautoclaved extracts gave equal zones of mycelial growth inhibition at all concentrations (Pic. 3.3). Mycelial inhibition with T5 and T15 siderophore extract was observed only against *A. niger* and not against *M. phaseolina* that too with non autoclaved filtered extract. This also shows that biocontrol potential of T5 and T15 was not siderophores based. When checked for the mycelial inhibition of *M. phaseolina* by siderophores extract of selected PGPR, results show that only NT1 siderophore extract was able to inhibit the growth of *M. phaseolina*. Both autoclaved and non autoclaved extracts gave zone of inhibition of *M. phaseolina* mycelium revealing that the biocontrol efficiency of NT1 siderophore was not affected or destroyed even after heating it at higher temperature and pressure (Fig. 3.4). Figure also shows that sterilized extract of NT1 siderophore is able to inhibit growth of *M. phaseolina* at all concentrations ie. 0.02 mL, 0.05 mL and 0.1 mL.

3.3.8. Extraction of siderophore crystals from the supernatant.

Extraction of ferrichrome type of hydroxamates by Benzyl alcohol method yielded fine needle shaped crystals after entire process. These crystals were then collected and studied for FTIR analysis on KBr pellets range between the ranges of 2.5 to 14 (4000-400 cm\(^{-1}\)). FTIR results show that the crystals obtained were of ferrichrome type with hydroxamate functional group, which correlated with the peaks obtained from the FTIR analysis of PBHA crystals. Peaks were observed at 3190, 2360, 1602, 1566, 780 and 530 cm\(^{-1}\), which were identical to those obtained from PBHA crystals FTIR analysis. But along with these peaks two more peaks were also observed at 1405 cm\(^{-1}\) and 1105 cm\(^{-1}\) revealing the presence of one -C-H bending with functional group -CH\(_2\) and one -N-O structure due to functional group N-O bending. A peak at 1566 cm\(^{-1}\) resembled antisymmetric stretching of COO\(^{-}\) reported by Krishnan *et al*, (2007) in succinic acid single crystals (Fig. 3.10).
CHAPTER 3

Pic. 3.3 Biocontrol of *Asp. niger* by siderophore extract of NT1.

Pic. 3.4 Biocontrol of *M. phaseolina* by siderophore extract of NT1.

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3.3.9. Spectral Analysis.

When siderohore containing supernatant of NT1 was passed through the XAD-4 column, most of the pigment present in the supernatant was adsorbed on the resin (Pic. 3.5). After complete loading of the siderophore on the column, six different fractions were eluted. When all the six fractions were checked for UV spectral analysis, reports show that there was just one major peak at 408 nm with absorption of more than 3.0 in fraction 1, which corresponds to the PfB type of pyoverdin, but simultaneously there were other peaks too. Fraction (II) showed 2 major peaks at 300 nm and 410 nm with absorption of 2.5 and 3.4 respectively. Fraction (III) showed a clear major peak between 408-410 nm with absorption at about 3. Fraction 4, 5 and 6 showed no peaks. This shows a clear absence of purified pyoverdin in these two fractions (Fig.3.11). The absorption spectra of the pyoverdin extracted were found to be pH sensitive. At lower pH (3.0-5.0) the peaks were found at 385 nm where as at pH 7.0, the major peak was found to be at 410 nm (Fig.3.12).

3.4.7 Detection of Pyoverdins of NT1 siderophore by HPLC.

Dominant peak for this pyoverdin appeared at 14.906 min, 16.975 min and 18.444 min. The results obtained with HPLC program reveals the ΔRT of 0.070 with distinguished HPLC of P. syringae LMG13190 and ΔRT of 0.341 with standard HPLC of P. cichorii LMG showing presence of Pa A type of atypical pyoverdine (Fig. 3.13).

3.4.8 ESIMS spectral analysis.

As the pyoverdin profiles obtained were complex, the dominant pyoverdins of the reference strains as reported by Fuch and Budzekiewicz, (2001) were used for comparisons. ESIMS when performed using purified supernatant of NT1, yielded several peaks as mentioned in the Tab 3.4. The peaks were obtained in a wide range of m/z starting from 399 m/z to 1239 m/z. The peak at 399 m/z represents presence of A1 fragment with a loss of one H2O molecule. Next visible peak was at 417 m/z which represent A1 fragment showing presence of succinic acid side chain. Peak 445 m/z shows presence of serine where as 504 shows B55-H2O fragment representing Thr-AhO-Ala-Ly-Ser side chain. Peak at 575 m/z shows present of Lys-Ser fragment.
The highest peak observed was at 717 m/z showing presence of Y_6'' fragment of pyoverdin. This side chain contains amino acids like Ala-Thre-Oho-Ala-Ly-Ser in sequence. Peak 1067 shows loss of succinic acid from pyoverdin of *P. fluorescens* showing molecular mass of 1167 as reported by Fuch and Budzekiewicz, (2001).

The result also shows that the siderophore of NT1 contains C terminal with OHOOrn side chain. Peaks at 1105 m/z represent side chain with Asp-Lys-OHasp-Ser-Ala-Ser-cOHOOrn. Peak at 1123 m/z shows presence of CH_3CHO+Thr and a peak at 1187 m/z represents a side chain of Ser-Dab-Gly-Ser_OHasp-Ala-Gly-Ala_Gly-cOHOrn. Peak at 1149 m/z represents a loss of H_2O molecule from the previous peak 1167 m/z visible in the spectra. Certain peaks i.e 1149 m/z and 1167 m/z showed a gradual loss of H_2O molecule from the actual molecule 1187 which resembles the presence of amino acid side chain Ser-Dab-Gly-Ser-OHasp-Ala-Gly-Ala-Gly-cOHOrn.

3.4 DISCUSSION

The isolates entered into log phase after 8 h of incubation on shaker in deferrated MM9 medium which was delayed by 4 h than that in nutrient medium and achieved its stationary phase after 30 h which was found to be 6 h late. Here glucose served as sole C source and glutamic acid served as sole N source. The growth cycle was extended by 6 h when compared to its growth in nutrient rich medium. Similar results were also obtained by Carson *et al.*, (2000) when *S. meliloti*. was grown in MSM-YE medium, which showed an increase MGT in absence of iron in the medium. A delay in growth was also reported by Sayyed *et al.*, (2005) in deferrated medium. They reported that siderophore production started only after 12 h and continued up to 24 to 30 h after which a decrease in growth along with siderophore production. All the isolates showed production of siderophore up till 30 h of incubation. A simultaneous increase in growth was also reported till 30 h after which a decline in both siderophore and growth was observed. This has also been reported that in *Pseudomonas aeruginosa* pyoverdin production was optimum at

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Fig. 3.10 FTIR analysis of NT1 siderophore crystals

Pic. 3.5 Fluorescence absorbed on the XAD-4.

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Fig. 3.11 UV spectra of the NT1 siderophore extract.

Fig. 3.12 Influence of pH on UV spectra of NT1.

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Fig. 3.13 HPLC analysis of siderophore produced by NT1.
### Tab. 3.4 ESI MS results of NT1 siderophore.

<table>
<thead>
<tr>
<th>No.</th>
<th>m/z</th>
<th>Fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>399</td>
<td>$A_1{-}H_2O$</td>
</tr>
<tr>
<td>2</td>
<td>417</td>
<td>$A_1$ showing presence of succinic acid side chain</td>
</tr>
<tr>
<td>3</td>
<td>445</td>
<td>Presence of Serine</td>
</tr>
<tr>
<td>4</td>
<td>504</td>
<td>$B_5S{-}H_2O$: Thr-Aho-Ala-Ly-Ser</td>
</tr>
<tr>
<td>5</td>
<td>575</td>
<td>$B_2$ Lys-Ser</td>
</tr>
<tr>
<td>6</td>
<td>717</td>
<td>$Y_6''$</td>
</tr>
<tr>
<td>7</td>
<td>774.16</td>
<td>$[M+2H]^+$</td>
</tr>
<tr>
<td>8</td>
<td>1067</td>
<td>Loss of succinic acid from previous molecule with 1167 m/z</td>
</tr>
<tr>
<td>9</td>
<td>1123</td>
<td>$CH_3CHO{+}$Thre</td>
</tr>
<tr>
<td>10</td>
<td>1149</td>
<td>Loss of one $H_2O$ from 1167 m/z</td>
</tr>
<tr>
<td>11</td>
<td>1167</td>
<td>Loss of one $H_2O$ from 1187 m/z</td>
</tr>
<tr>
<td>12</td>
<td>1105</td>
<td>$Asp$-Lys-Ohasp-Ser-Ala-Ser-cOHOrn</td>
</tr>
<tr>
<td>13</td>
<td>1187</td>
<td>$Ser$-Dab-Gly-$Ser$-OHasp-Ala-Gly-Ala-Gly-cOHOrn.</td>
</tr>
</tbody>
</table>

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40 h when the culture just entered in to the stationary phase (Barbhaiya and Rao, 1988) where as Jadhav and Desai, (1992) have reported a maximum siderophore production after 22 h of incubation in *Rhizobium* which proves that production of siderophore is maximum when the culture has just entered into the stationary phase and was parallel with growth.

Hu and Boyer, (1996) reported that during log phase a significant amount of siderophore is produced intracellularly which diffuses out in to the surrounding iron deficient medium. This diffusion stops when intracellular and extracellular levels of siderophore reach equilibrium. This usually occurs when an iron deficient cell reaches stationary phase. Incubation of cultures till achievement of stationary phase was also observed to be optimum in our growth phase study for siderophore production and was therefore selected for checking maximum siderophore production.

Increase in pH was accompanied with increase in siderophore concentration. The pH increased from 6.8 to a maximum of 10 along with siderophore production. This change in pH in the medium during siderophore production was also reported by Budzikiewicz, (1993) who reported that alkalinity is important to avoid siderophore destruction showing that pyoverdins are labile in presence of acids or O₂. On the contrary Sharma and Johri, (2003) showed that higher pH is rather destructive to siderophores as reported in siderophores of *Pseudomonas* strain GRP3A, a variant of GRP3 isolated from *Glycine max* rhizoplane. This may be due to the fact that alkaline pH helps in excess solubilization of iron which increases the iron content of the medium (Sayyed et al, 2005).

When different media were tried to screen the most suitable media for siderophore production, MM9 and FMM were the best medium whereas no siderophore production was observed with citrate and nutrient broth where as very little siderophore was obtain with succinate on solid medium. This increase in siderophore production on solid MM9 and FMM media as compared to SSM may be due to the presence of glucose which can be an easy carbon source for all the PGPR. Glucose can be easily utilized into any biochemical process thereby enhancing the

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growth of the bacteria which finally results into increased demand of ferrous by cells resulting into increased production of siderophores (De Villegas et al, 2002)

Better quantitative production of siderophores was reported in both liquid MM9 as well as liquid SSM medium revealing that aeration and agitating conditions can influence the production of siderophores by the microorganisms. Increase in siderophore obtained with SSM medium shows that presence of succinic acid enhances the siderophore in all these isolates. Sharma and Johri, (2003) also obtained similar results. They have reported that addition of succinic acid in the medium increase the production up to 3 folds. De Villegas et al, (2002) have reported that succinate can be easily utilized in siderophore synthesis as compared to growth which presumably may be due to the vital role of succinate in the synthesis of siderophore, especially pyoverdins where 3-amino moiety of the chromophore is substituted with various acetyl groups derived from succinate, malate or α-ketoglutarate. A complete absence of siderophore in citric acid added medium can be attributed to the lower iron requirement of the isolates in the presence of citric acid (Sharma and Johri, 2003). Nutrient broth being a rich medium for growth of isolates did not create any iron deficiency in the medium and hence siderophore production was inhibited in all the isolates.

All the isolates showed increase in growth with increase in FeCl₃ concentration revealing that presence of FeCl₃ is vital for their growth but at the cost of simultaneous decrease in siderophore production. Increase in FeCl₃ concentration had a negative influence on siderophore production. This was also reported by De Villegas et al, (2002) who stated that concentration of FeCl₃ above 10 μM has a negative effect on siderophore production whereas Manninen and Sandholm, (1993) reports that highest siderophore production occurs only at iron concentration at and above 50 μgML⁻¹. Our results show maximum siderophore production occurs at 2 μM FeCl₃ which declines thereafter up to 20 μM of Fe.

Amount of fluorescence produced by the isolates NT1 and T15 increased along with the concentration of FeCl₃ up to 2 μM concentration after which a continuous decrease was reported. This increase was parallel to siderophore

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produced by the isolate which definitely shows a relation between siderophore production and fluorescence. Similar results were also found when Fe was replaced by Zn and Ni in the medium.

All the isolates showed chelation and remediation of zinc and cadmium followed by nickel. This shows that metals like zinc and cadmium can be easily chelated out by the siderophore produced by the isolates where as nickel could be removed in a little amount. This may be due to the fact that hydroxamate types of siderophores have equal affinity for metals like zinc and cadmium or manganese but may not be able to bind to other metals (Saxena et al, 1998). Here it needs to be remembered that all our isolates produced hydroxamate type of siderophores.

Presence of Zn at 12 μM concentration increased the growth of all isolates as compared to 6 μM concentration. This increase in growth was also accompanied with increase in siderophore production except by NT1. It can also be concluded that in absence of ferrous in the medium, Zn supports the growth of the organism but does not adversely affects the amount of siderophore produced even at higher concentration. Sharma et al, (2003) also report that Zn²⁺, Cu²⁺ and Mn²⁺ increase the siderophore production. Our studies also showed an increase of almost 50 % in siderophore production by C4 and C5 at 12 μM Zn²⁺. Gupta et al, (2001) also reported that P. aeruginosa can utilize metal ions for enhancement of growth and siderophores under deferrated medium. They also reported 29 % increase in siderophore production in presence of Zn whereas Mn and Fe also supported growth and siderophore production up to 12 μM concentration.

Presence of Ni at 6 and 12 μM decreased the growth of all isolates except T15. Siderophore production doubled in both T5 and T15 at 12 μM NiCl₂ as compared to 6, no change was reported in siderophore production by NT1 whereas C4 showed 40 % reduction in siderophore production at 12 μM NiCl₂ than at 6 μM NiCl₂. 12 μM NiCl₂ inhibited the growth of C5 proving to be inhibitory at higher concentration of 12 μM. Rachid and Ahmed, (2005) have reported that both growth and siderophore production were inhibited in presence of heavy metals like lead, mercury and
cadmium. Absence of any trace metal reduces the growth of the cells but with increased siderophore production showing an inverse relationship between growth and siderophore production as studied by Meyer and Abdallah, (1978).

The presence of metals other than Fe is known to stimulate siderophore formation in a number of bacteria and fungi. Hu et al, (1996) reported that presence of aluminium enhanced production of schizokinen by B. megaterium. Similar results were also reported by Hofte et al, (1993) suggesting that metals may be directly involved in siderophore biosynthesis. This increase in siderophore production may be related to some metabolic pathway leading to siderophore synthesis.

Inhibition of mycelial growth by siderophore extract of NT1 with and without autoclaving reveals that the antagonistic potential of NT1 does not need any physical contact and is not affected even when subjected to higher temperature. Thus it is resistant to high temperature and temperature. The results therefore suggest the role of siderophores in biocontrol. Similar results were also obtained by De Villegas et al, (2002) when sterile filtered supernatant of P. aeruginosa inhibited the growth of S. rolfsii within 7 d. Results say that siderophore produced in absence of Fe shows maximum inhibition of phytopathogens as compared to siderophore produced in presence of Fe. This also shows that P. aeruginosa produces maximum amount of siderophore in absence of Fe and as the concentration increased siderophore production decreased. Johri et al, (1997) also reported the role of fluorescent Pseudomonas strain RBT13 in tomato plant disease management in which the organism produced siderophores which exhibited in vitro antagonism against several fungal phytopathogens and simultaneously increase crop production. Sindhu et al, (1997) also reviewed the role of rhizobacteria in plant pathogen inhibition. Loper and Buyer, (1991) also suggested that fluorescent siderophores of Pseudomonas depleted ferrous from microenvironment of phytopathogens and thereby using Fe pool exclusively for itself rendering plant pathogens deficient of Fe and thus curbing their growth and infection potential. In vitro antagonism by cell free culture filtrate was also reported by Singh et al, (2008) where cell free culture filtrate of B. subtilis BN1 inhibited 60 % of growth of M. phaseolina. Because siderophore

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extract of only NT1 showed biocontrol potential, it was selected for further characterization studies.

FTIR results show that the crystals obtained were of ferrichrome type with hydroxamate functional group, which correlated with the peaks obtained from the FTIR analysis of PBHA crystals. Peaks were observed at 3189, 2360, 1602, 1566, 780 and 530 cm\(^{-1}\), which were similar to those obtained from PBHA crystals FTIR analysis. But along with these peaks two more peaks were also observed at 1495 and 1105 wave number revealing the presence of one -C-H bending with functional group -CH\(_2\) and one -N-O structure due to functional group N-O bonding, which shows that although these are hydroxamate crystals they still contain other functional groups, which were not observed in FTIR analysis of standard hydroxamic acid crystals. There are reports that hydroxamate crystals of fungal siderophore contained functional groups like methyle, amide, secondary amine, methylene, N-O bond and a ring structure (M-O) where M=Fe (Dave and Dube, 1999). The functional chemical group reported by them resembled ferrichrome siderophores as was observed in the present study.

The absorption spectra of the extracted pyoverdin were found to be pH sensitive. At lower pH (3.0-5.0) the peaks were found at 385 nm where as at pH 7.0, the major peak was found to be at 410 nm. Similar results were also quoted by Xiao and Kissalita (1995) who reported that atypical pyoverdins had double peaks at low pH values (i.e. 366 & 384 nm for Pf-A, 368 & 384 for Pf-B and 369 & 385 nm for Pf-C). However at higher pH values (pH 7), the pyoverdine spectra had single peaks (407 nm for Pf-A and Pf-B where as Pf-C has a peak at 408 nm on UV spectra. Bultreys et al, (2003) reports that as the pH of the pyoverdin containing supernatant is reduced, the absorption maxima moves towards the lower ultra-violet range. According to him, P. asplenii shows absorption maxima at pH 7.0 at about 407 nm, which became 406 nm at pH 4.0 and 405 nm at pH 3.5. Thus it behaves as typical pyoverdine at lower pH (3.0) and as atypical pyoverdine as the pH increased from 3.0 to 7.0 and above.
Dominant peak for this pyoverdin appeared at 14.906 min, 16.975 min and 18.444 min. RT data allowed discrimination between pyoverdin with different peptide chains produced by different species of *Pseudomonas* (Bultreys *et al*, 2003). In visual tests a change in colour of production medium that accompanied in the pH 7.0 to 4.0 indicates atypical pyoverdin production (Bultreys *et al*, 2001). The results obtained with HPLC program reveals the ΔRT of 0.070 with distinguished HPLC of *P. syringae* LMG13190 and ΔRT of 0.341 with standard HPLC of *P. cichorii* LMG showing presence of PaA type of atypical pyoverdin. Similar results were also reported by Bultreys *et al*, (2003) where they mentioned that their isolate *P. Cichorii* LMG 2162 differed from the reference strain *P. syringae pv syringae* B301D by about ΔRT 0.160 min. They also reported that HPLC method of analyzing pyoverdin production in the culture medium shows greater diagnostic potential than the visual, spectrophotometric and IEF-based methods but we found that UV spectral analysis is of preliminary importance to confirm the presence of pyoverdin in the extract.

Siderophores known as pyoverdins produced by the fluorescent members of the bacterial genus are very complex in structures. Their peptidic part- linear or partially cyclic-comprises unusual and partially modified amino acids which makes their interpretation difficult. Free pyoverdins as well as the ferri pyoverdins generally give abundant [M+H]+ ions occasionally accompanied by [M+2H]+2 with lower intensity as is observed over here at 774 m/z peak where as peak at 557 m/z represent [M+2H] with a loss of CO and H$_2$O molecules (Fuch and Budzikiewicz, 2001).

Most important is the formation of fragment A$_1$ which has been observed in all pyoverdins showing presence of succinic acid side chain with Ser as first amino acid. Present report also show a peak at 417 m/z which represent the presence of fragment A$_1$ containing Ser as first amino acid in the side chain which supports that the NT1 siderophore extract contains pyoverdin type of siderophores. The obtained ESIMS spectrum resembles the ESI spectrum of *P. fluorescens* P19 pyoverdin obtained by Fuch and Budzekiewicz, (2001) where they mention that ESI of [M+H]+ of

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by Fuch and Budzekiewicz, (2001) where they mention that ESI of [M+H]⁺ of pyoverdin shows a peak at 1169 reporting that OH transfer is possible from other amino acids (Asp, Fho, Ser etc). The present pyoverdin contains cOHOm C-terminal resembling the peptide chain of Py 2798. Here cyclic chain is if Ser/Dab which refers to the condensation product of the γ-NH₂ group of Dab with the amide carbonyl group of the preceding amino acid giving a tetrahydro pyrimide ring as mentioned by Fuchs et al, (2001).

3.5 CONCLUSION

The siderophore producing property in presence and absence of metals like Fe, Zn and Ni is an important achievement of any rhizobacteria to perform as PGPR. Medium composition as well as time of incubation also effects production of siderophore in different isolates. NT1 was found to be most promising amongst all selected isolates to act as PGPR as it showed the potential of large amount of siderophore production in presence and absence of heavy metals like Fe, Zn and Ni. Zn enhanced siderophore production. Fluorescence increased along with increase in the siderophore and growth. Siderophore extract of NT1 also exhibited biocontrol potential against fungal phytopathogens even after autoclaving revealing that antagonistic potential of NT1 was not lost even after subjecting it to higher temperature. It also revealed siderophore production as a major mechanism of biocontrol. C4 and C5 exhibited a totally different trait of siderophore production where the amount of siderophore released by them increased as FeCl₃ concentration increased. Spectral analysis of NT1 siderophore extract showed that it was apyoverdin type of siderophore. FTIR also confirmed it to have peaks resembling to peaks of standard PBHA. The purified compound obtained from the supernatant was considered as true pyoverdine because of its fluorescence and a single peak between 408 nm and 410 nm when analysed by UV spectrum. ESIMS results also showed that the type of NT1 siderophore is pyoverdin which resembles to that of P. fluorescens P19. The extract was found to contain many peaks that matched with the standard peaks of P. fluorescens P19. However the extract also contained few peaks which did not match with any of the standard peak and hence could not be

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identified. This is quite possible because there are about fifty identified and partially identified pyoverdin of *Pseudomonas* itself and many more are under study. It may be possible that the pyoverdin the present isolate NT1 may be slightly varying with the already identified pyoverdins due to the difference at species or strain level. All the isolates showed better performances in siderophore production in presence of different metals and hence will be studied for their effect on plant growth under different stresses.