CHAPTER 5

Characterization and Purification of Siderophores

CHARACTERIZATION AND PURIFICATION OF SIDEROPHORES

5.1. Introduction

Pyoverdine, the siderophores secreted by fluorescent pseudomonads, is composed of 3 distinct parts, a dihydroxyquinoline chromophore, a variable peptide arm consisting of 6 to 12 amino acids and a dicarboxylic acid (or its cognate monoamide) which is attached to the chromophore. Pyoverdine contains both catechol and hydroxamate functional groups to coordinate iron binding (Meyer, 2000; Cornelis and Matthijs, 2002; Ballouche et al., 2009). Pyoverdines are secreted from the bacterial cell and chelates ferric iron in the environment mainly by the hydroxamate and hydroxyacid groups present within the peptide moiety of the molecule (Loper and Henkels, 1999; Ravel and Cornelis, 2003; Visca et al., 2007).

Detection of siderophores is most readily achieved in iron limited media, which generally means either a synthetic (minimal) recipe or introduction of a complexing agent that will render the iron selectively unavailable (Neilands, 1995). Although most siderophores are either hydroxamates or catecholates, earlier tests based on such functional groups proved unreliable since they are absent from a few siderophores. The chrome azurol sulfonate (CAS) assay has become widely used since it is comprehensive, exceptionally responsive, and convenient (Schwyn and Neilands, 1987). The CAS assay may be applied on agar surfaces or in solution. It is based on the colour change that accompanies transfer of the ferric ion from its intense (extinction coefficient of at least 100,000) blue complex to the siderophore. Since siderophores differ substantially in structure, no uniform procedure is available for their isolation. Most are water-soluble, and it is thus usually expedient to drive the siderophore into an organic solvent, such as benzyl alcohol or phenol-chloroform, in order to eliminate salt (Buyer et al., 1993; Neilands, 1995).

The antimicrobial activity of siderophores can have significant ecological effects. For example, the siderophores of fluorescent pseudomonads are

responsible for antagonism toward various strains of fungi and some *Pseudomonas* spp. that are pathogenic to plants (Buyer and Leong, 1986). In addition, microbial siderophores can serve as iron sources for plants (Bar-Ness et al., 1991; Robin et al., 2007), and the production of a siderophore by *Pseudomonas putida* has been shown to enhance the yield of potato tubers (Bakker et al., 1986). Sometimes bacteria secrete multiple siderophores where 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 metal. Pyochelin, one of two siderophores produced by *Pseudomonas aeruginosa* PAO1, has a relatively low affinity for iron. However, pyochelin binds a variety of metals (Cuppels et al., 1987), and regulation of pyochelin synthesis correlates with its relative affinity for Mo(VI), Co(II), and Fe(III) (Visca et al., 1992). Pyoverdine, the other siderophore produced by PAO1, demonstrates a binding affinity and a regulatory response typical of a transport molecule specific for iron (Wendenbaum et al., 1983).

A number of pseudobactins/pyoverdines have been isolated and characterized from several fluorescent *Pseudomonas* species (Budzikiewicz, 1993; Khalil-Rizvi et al., 1997). Purification of the hydroxamate-type siderophore generally includes passing acidified culture supernatant through an amberlite column (XAD-2 or XAD-16), followed by passing the concentrated siderophore through a Sephadex LH-20 hydrophobic column and finally through HPLC on a C18 hydrophobic column (Buyer et al., 1993; Storey, 2005). In the present study, the siderophores produced by all the 20 selected antagonistic isolates were characterized by the standard assays for hydroxamate, catecholate and carboxylate types. Then the hydroxamate siderophore produced by the *Pseudomonas putida* strain AS04 was separated by first passing through amberlite coloumn and then through Sephadex LH-20 hydrophobic column. Before undertaking the purification procedure, the siderophore production by the strain was optimized under different media supplements, temperatures and incubation periods. The most suitable condition for siderophore production was maintained for the

purification process. The partially purified siderophore was scanned in a UV-VIS spectrophotometer.

5.2. MATERIALS AND METHODS

5.2.1. Media for siderophore production

Since siderophores are mainly produced under iron-limiting conditions, the medium used for siderophore production was prepared with restricted amount of iron. Fiss-glucose minimal media (Vellore, 2001) was used as an iron-restricted media. Media was prepared by dissolving 5.04 g KH_2PO_4 and 5.04 g L-asparagine in 960 ml deionized distilled water (DDW) and pH was adjusted to 6.8. After autoclaving, 10 ml of each of the following solutions (each autoclaved separately) was added to the 960 ml sterile media: 50% glucose, 0.005% $ZnCl_2$, 0.001% $MnSO_4$ and 0.4% $MgSO_4.7H_2O$. To minimize the trace amount of iron, all media components were prepared with DDW and all glassware used for media storage and for growth of the culture were treated with concentrated HNO_3 and rinsed with DDW.

5.2.2. Detection of hydroxamate siderophores

The iron-restricted Fiss-glucose minimal media was inoculated with a 24 h culture (NA) of each of the selected 20 bacterial isolates and incubated at 30°C for 30 h. Following incubation, the culture was centrifuged at 10,000 rpm for 15 min to obtain culture supernatant. This supernatant was used for siderophore characterization.

5.2.2.1. Tetrazolium test

Detection of hydroxamate siderophore was carried out following the method of Snow (1954). This test is based on the capacity of hydroxamic acids to reduce tetrazolium salt by hydrolysis of hydroxamate groups using a strong alkali. The reduction and the release of alkali shows red color. To a pinch of Triphenyl tetrazolium chloride salt, 1–2 drops of 2N NaOH and 1 ml of the test culture supernatant were added. Instant appearance of a deep red colour indicated hydroxamate siderophores.

5.2.2.2. Ferric chloride test

Following the method described by Neilands (1981) hydroxamate siderophore production was confirmed by $FeCl_3$ test. To 1 ml of culture supernatant, 1ml of 2% ferric chloride solution was added. The formation of red or purple colour indicated the presence of siderophore. A peak between 420 and 450 nm of ferrated siderophores indicated its hydroxamate nature.

5.2.3. Detection of catecholate siderophores

For detection of catecholate siderophore, Fiss-glucose minimal media was inoculated with all bacterial isolates and incubated at 30°C for 30 h. Tests were conducted with the culture supernatant obtained from each isolate and used for siderophore characterization.

5.2.3.1. Arnow's test

Arnow's method (Arnow, 1937) was used to determine whether the siderophore produced by the isolated bacterial strains was of catecholate type. This test is performed by mixing the following in order: 1 ml culture supernatant, 0.1 ml 5M HCl, 0.5 ml Nitrite-Molybdate reagent and 0.1 ml 10N NaOH. These were allowed to incubate for 5 minutes for the reaction to occur completely. Catechol gives a yellow color when reacted with nitrous acid and changes to an intense orange-red when made strongly basic (pH 10) (Holzberg and Artis, 1983). Absorbance was measured at 515 nm.

5.2.3.2. Ferric chloride test

The ferric chloride test for detection of catecholate siderophores was performed following the method of Neilands (1981). One ml of culture supernatant was added to 1ml of 2% $FeCl_3$ solution and the absorption maxima at 495 nm indicated the presence of catecholate siderophore.

5.2.4. Detection of Carboxylate siderophores

Spectrophotometric test was conducted following the methodology of Shenker et al., (1995). To 1 ml of culture supernatant, 1 ml of 250 µM $CuSO_4$ and 2 ml of acetate buffer (pH 4) were added. The solution was

scanned in the wavelength range of 190–280 nm and was observed for the peak of absorption of siderophores. An uninoculated medium was kept as blank. The copper complex shows absorption maximum between 190 and 280 nm.

5.2.5. Optimization of siderophore production

Conditions can be optimized to achieve the maximum amount of siderophore production. Hence, before attempting to purify the siderophore compound, optimization is necessary in order to acquire a higher yield of the compound. Of the 20 siderophore producing isolates used in the present study, the *Pseudomonas putida* strain AS04 produced maximum siderophore units in the quantitative test. Therefore, AS04 was selected for optimization and purification experiments.

5.2.5.1. Siderophore production in different media suppliments

Different media combinations were tried in order to optimize siderophore production; Fiss-glucose minimal medium was used as the base which was supplemented with four different carbon and nitrogen sources either individually or in combination. The alternate carbon sources included sucrose (1%) and mannitol (1%). The additional nitrogen sources were $(NH_4)_2SO_4$ and NH_4Cl (1% each). Fiss-glucose medium was supplemented separately with 1% maltose, 1% mannitol, 0.1% $(NH_4)_2SO_4$, 0.1% NH_4Cl, 1% sucrose + 0.1% $(NH_4)_2SO_4$, 1% sucrose + 0.1% NH_4Cl, 1% mannitol + 0.1% $(NH_4)_2SO_4$ and 1% mannitol + 0.1% NH_4Cl. Supplemented media (500 ml) were distributed equally into five 250ml Ehrlenmeyer flasks and sterilized. The flasks were inoculated with a 24 h NA culture of AS04 and incubated for 36 hours at 30°C on a rotary shaker. Following incubation, the cultures were centrifuged at 10,000 rpm for 15 minutes and the culture supernatant was used to measure the percent siderophore units produced (described in section 4.2.1).

5.2.5.2. Siderophore production after different periods of incubation

Fiss-glucose minimal medium (200 ml) taken in two 250 ml flasks (100 ml each) was inoculated with 1.5 ml of a 24 h NB culture of AS04. The culture was grown for a maximum of 40 hours, approximately 5 ml aliquots were removed at each 2-hour interval ranging from 0-40 hours and growth was measured at OD 600 nm. Next, the culture aliquot was centrifunged and culture supernatant was collected for estimation of siderophore.

5.2.5.3. Siderophore production at different incubation temperatures

Siderophore production was assessed after different periods of incubation. Fiss-glucose minimal medium was prepared in six 250 ml flasks holding 50 ml each, sterilized and inoculated with 24 h culture of AS04 in NA. The flasks were incubated at different temperatures (4°, 20°C, 30°C, 37°C, 43°C and 55°C). After 30 hours, growth was measured and the siderophore produced was estimated for each culture and values were recorded.

5.2.6. Purification of siderophore

For partial purification of siderophore, large volumes of culture were grown in the optimized Fiss-glucose medium. Typically it was done by preparing 5 litres of medium and dispensing 150 ml of it in each 500 ml conical flask. A seed culture was grown by inoculating AS04 strain in the same medium and incubated overnight. This fresh culture was used as the seed inoculum to inoculate each flask with 1.5 ml medium. All of the conical flasks were incubated for 30 hours at 30°C on a rotary shaker. After incubation, the culture supernatant was collected by centrifuging at 7,000 rpm for 15 minutes. The supernatant was then acidified to pH 2.0 with 6M HCl in order to make the siderophore less soluble in water.

5.2.6.1. Amberlite XAD-2 chromatography

The acidified supernatant of AS04 bacterial culture was passed through a 30X5 cm column packed with Amberlite XAD-2, which binds cyclic compounds. Prior to this, the column was prepared by suspending approximately 60 g of XAD-2 in DDW and the mixture was kept at room

temperature overnight so that the material can completely absorb water. The column was then packed (approximately 20 cm) with the prepared XAD-2 and it was equilibrated with four bed volumes of DDW. Next, the acidified supernatant was passed through the column and the flow-through was collected. When all supernatant had been run, the column was washed with two bed volumes of DDW. This DDW-wash was also collected in a separate container. The column was then eluted with approximately 250 ml of methanol. When the fraction coming out of the column appeared yellow and the flow rate increased, it indicated that only methanol was present in the fraction. This fraction was also collected separately as fraction No. 2. Fractions were collected until the flow-through became colourless. Altogether five 50 ml fractions were collected. The column was then washed with four bed volumes of methanol, followed by four bed volumes of DDW to re-equilibrate the column.

The flow-through, DDW-wash, and all fractions were collected were evaluated for their siderophore content by Tetrazolium salt test. Fractions showing positive result for the siderophore were combined in a 250 ml round-bottom flask and dried on a rotary vacuum evaporator (Eyela, A-1000S, Japan), keeping the temperature at 25°C. The concentrated sample was re-dissolved in 5 ml methanol and stored at -20°C till further purification.

5.2.6.2. Sephadex LH-20 chromatography

Sephadex LH-20 is a material that separates compounds based on their hydrophobicity. It was prepared by suspending 5.0 g LH-20 in methanol and stirring for around 20 minutes. The material was then packed into a 50 x 1.5 cm column (almost upto the top of the column) and was equilibrated with four bed volumes of methanol. The 5ml concentrated sample was loaded on the column and eluted with methanol. Approximately 20 fractions (3ml each) were collected and tested for presence of siderophore by performing thin layer chromatography (TLC). Fractions positive for siderophore were combined in a 100 ml round bottom flask and evaporated to dryness using a rotary vacuum evaporator. The dried sample was then

re-dissolved in 2-3 ml methanol. The sample was stored at –20°C until further studied.

5.2.6.3. Thin layer chromatography

Different fractions of Sephadex LH-20 column were tested for the presence of siderophore by TLC. Concentrated samples of siderophore were spotted on 7X4 cm^2 precoated TLC silica gel 60 F_{254} plates (Merck, India) and were allowed to dry. The plates were developed in n-butanol:acetic acid:DW (12:3:5) until the solvent front reached the top of the plate. Plates were then dried and sprayed with 0.1 M $FeCl_3$ in 0.1 N HCl. The formation of a brown spot indicated a hydroxamate-type siderophore (Storey, 2005).

5.2.6.4. Spectral scan analysis

Spectral scan analysis was done following the method described by (Jalal and van der Helm, 1991). A spectral scan in 300-700 nm was done on the partially purified siderophore sample to characterize the type of hydroxamate. A dihydroxamate shows absorption maxima in the range of 500-520 nm and that of trihydroxamate is in the range of 420-440 nm.

5.3. RESULTS

5.3.1. Detection of siderophores

All the isolates were tested for the type of siderophore produced by each strain. Results, summarized in Table 15, showed that only one type of siderophore was produced by each of the strains.

5.3.1.1. Hydroxamate

Altogether 15 strains out of the 20 tested showed positive result in the tetrazolium test. A red colour appeared immediately indicating the presence of siderophore in the culture supernatants (Fig. 25a). The strains that tested positive were MD01, CB02, AS01, AS04, BB05, BB07, CR07, CR10, CR12, CR13, MB01, MB05, NG04, NG05 and NG07. In the $FeCl_3$ test, a spectral scan showed peaks at 420-440 nm wavelength which confirmed

hydroxamate production by all the 15 strains tested positive in the Tetrazolium test.

5.3.1.2. Catecholate

Arnow's test detected the presence of catecholate siderophore in the culture supernatants of 5 strains out of the 20 tested (Fig. 25b). The strains that tested positive were CR04, CR14, JL11, KT05 and MB02. A spectral scan showed peaks at 495 nm which confirmed catecholate production by all these strains tested positive in the Arnow's test.

Table 15: Types of siderophore produced by bacterial isolates[a]

Bacterial strains	Type of siderophore		
	Catecholate	Hydroxamate	Carboxylate
BB05	-	+	-
BB07	-	+	-
JL11	+	-	-
MD01	-	+	-
CB02	-	+	-
AS01	-	+	-
AS04	-	+	-
CR04	+	-	-
CR07	-	+	-
CR10	-	+	-
CR12	-	+	-
CR13	-	+	-
CR14	+	-	-
MB01	-	+	-
MB02	+	-	-
MB05	-	+	-
NG04	-	+	-
NG05	-	+	-
NG07	-	+	-
KT05	+	-	-

[a] : '+': positive; '-': negative.

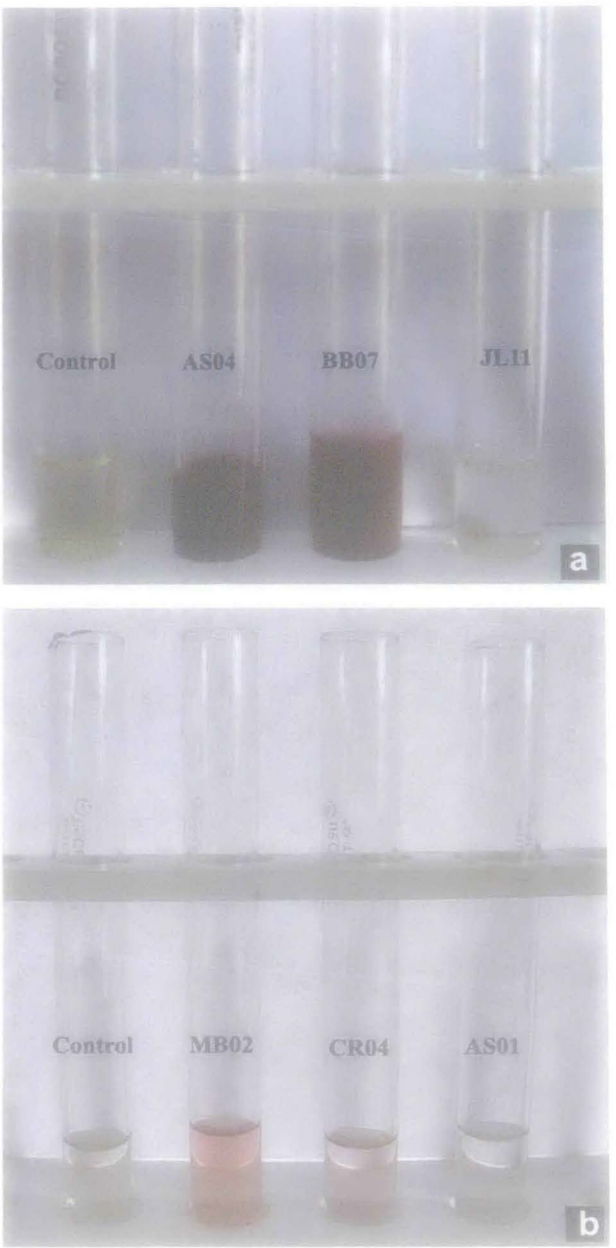

Fig.25: Characterization of siderophore:

(a)Tetrazolium salt test showing immediate red colouration indicating presence of hydroxamate type siderophore produced by bacterial isolates AS04 and BB07. Absence of red colour indicating negative result by isolate JL11 when compared to control;

(b) Arnow's test showing pink colouration indicating the presence of catecholate type of siderophore produced by the isolates MB02 and CR04. Colourless medium indicating negative test by the isolate AS01 when compared to control.

5.3.1.3. Carboxylate

A spectral scan in the 190-280 nm wavelength region did not show characteristic peak indicating that none of the isolated bacterial strains produced carboxylate type of siderophore.

5.3.4. Optimization of siderophores production

5.3.4.1. Media components

In order to achieve maximum siderophore production, the Fiss-glucose minimal medium which was used in the preliminary characterization was required to be optimized. A variety of media combinations were tried to optimize siderophore production. Fig. 26a depicts a graphical representation of the results, which summarizes the effect on siderophore production of each media type tried based on the CAS assay for estimation. The addition of all the media supplements showed some increase in the amount of siderophore production (Table 16). Addition of 1% sucrose and 0.1% $(NH_4)_2SO_4$ to the original Fiss-glucose minimal media showed maximum increase in the amount of siderophore produced (from 59.70 to 89.04% units). Next to it, the combination of 1% mannitol and 0.1% NH_4Cl increased the amount of siderophore produced to 81.86% units. Sucrose was better carbon source than mannitol and $(NH_4)_2SO_4$ was better than NH_4Cl as a nitrogen source when tested individually.

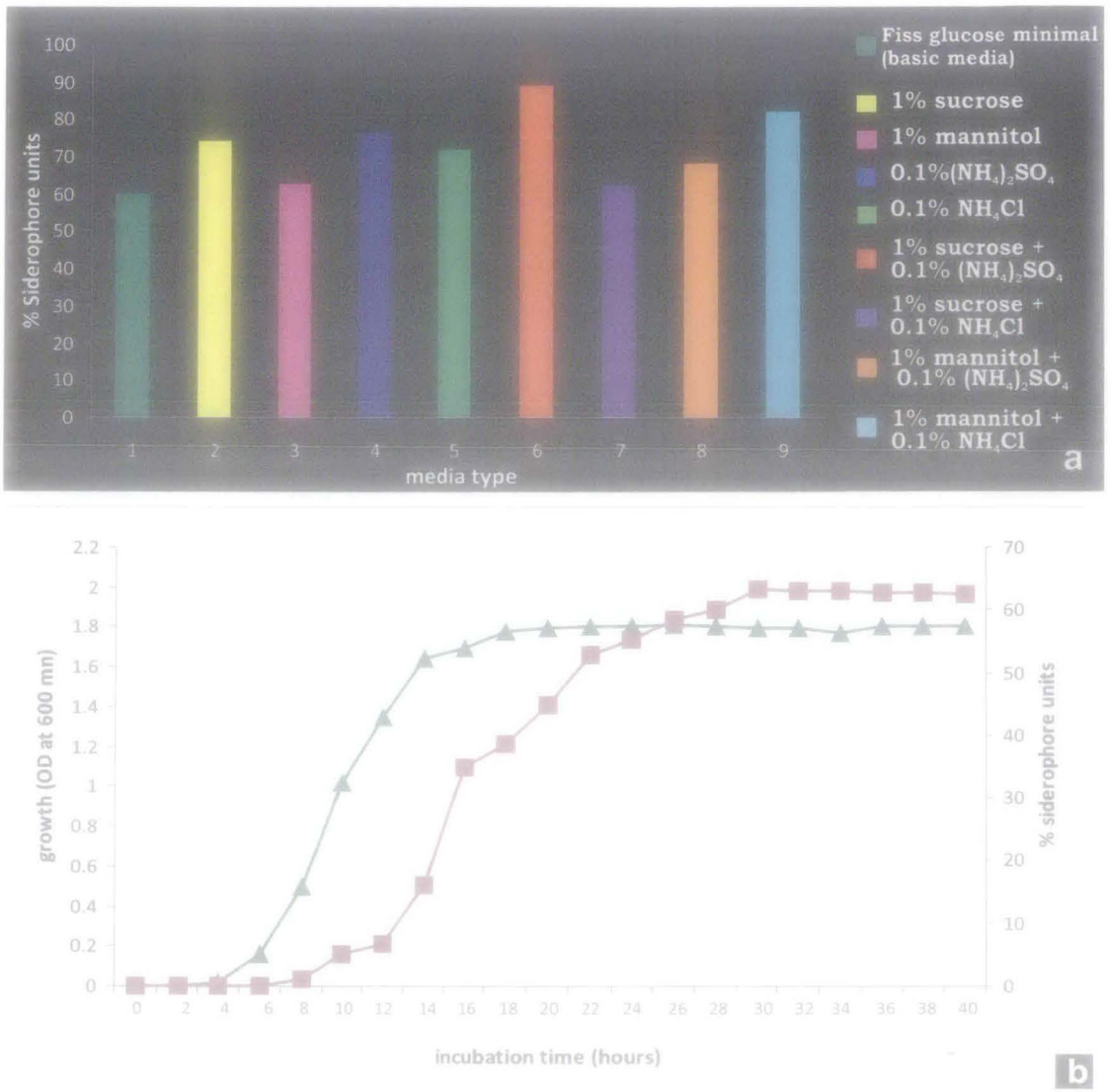

Fig 26: (a) Siderophore production by the bacterial isolate, *Pseudomonas putida* strain AS04 in presence of various medium supplements;

(b) The effects of incubation time on growth (-▲-) and siderophore production (-■-) by *Pseudomonas putida* strain AS04.

Table 16: Siderophore production in various media supplements

Medium Supplements	% Siderophore units
Fiss glucose minimal (basic media)	59.70±0.47
1% sucrose	74.21±0.67
1% mannitol	62.89±0.73
0.1% $(NH_4)_2SO_4$	76.49±0.84
0.1% NH_4Cl	71.92±0.82
1% sucrose + 0.1% $(NH_4)_2SO_4$	89.04±0.62
1% sucrose + 0.1% NH_4Cl	62.00±0.56
1% mannitol + 0.1% $(NH_4)_2SO_4$	68.24±0.67
1% mannitol + 0.1% NH_4Cl	81.86±0.72

5.3.4.2. Incubation time

In order to determine the optimum incubation time for maximum siderophore production by the isolate AS04, the culture was grown for a maximum of 40 hours, and both growth and siderophore production were measured at each 2-hour intervals, starting from zero hour. Fig. 26b shows the growth curve for strain AS04 along with the amount of siderophore produced at the various incubation times measured. Results indicated that AS04 begins producing siderophore after 8 hours post-inoculation, with maximum production occurring at 30 hours (Table 17).

5.3.4.3. Incubation temperature

As described previously, *Pseudomonas putida* strain AS04 was grown at varying temperatures (4°C, 20°C, 30°C, 37°C, 43°C and 55°C) to assess its effects on siderophore production. Figure 27 and Table 18 shows that both growth and siderophore production were highest at 30°C.

Table 17: Siderophore production at different incubation time

Incubation time (h)	Growth (OD at 600 nm)	% Siderophore units
0	0.000	0.00
2	0.002	0.00
4	0.019	0.00
6	0.160	0.00
8	0.498	01.06
10	1.023	05.03
12	1.345	06.78
14	1.664	16.01
16	1.698	34.76
18	1.780	34.64
20	1.782	44.78
22	1.800	52.87
24	1.802	55.34
26	1.811	58.34
28	1.803	60.12
30	1.799	63.23
32	1.797	62.97
34	1.768	62.78
36	1.807	62.79
38	1.806	62.79
40	1.806	62.45

Table 18: Optimization of incubation temperature for highest siderophore production

Incubation temperature (°C)	Growth (OD at 600 nm)	% Siderophore units
4	0.006	0.00
20	1.528	22.62
30	1.842	61.34
37	1.383	37.81
43	0.294	07.43
55	0.011	02.09

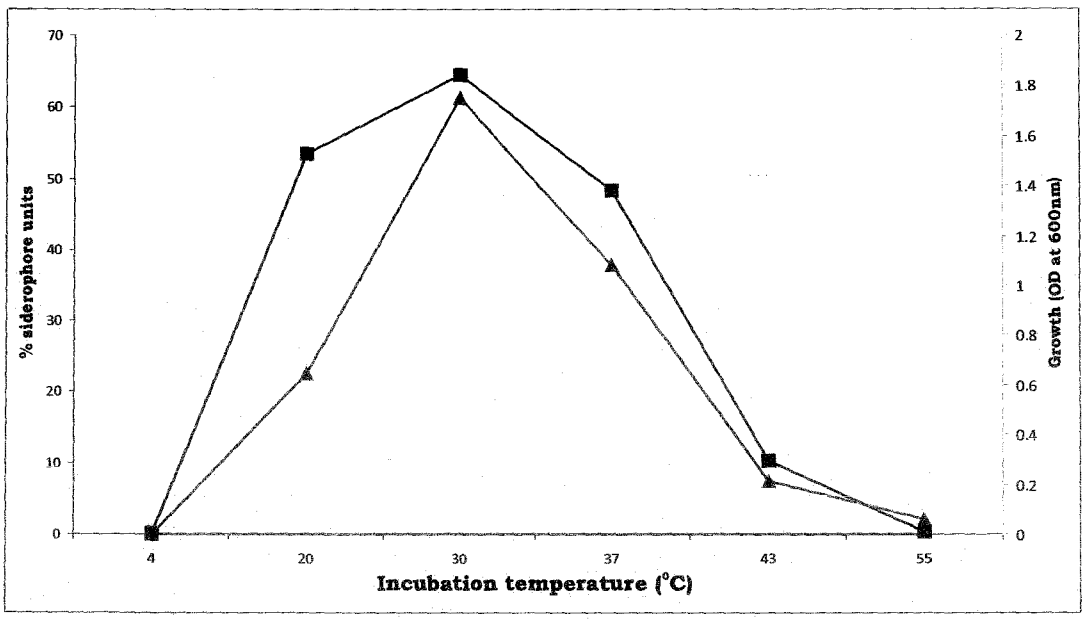

Fig.27: Optimization of siderophore production by *Pseudomonas putida* strain AS04 at various incubation temperatures.

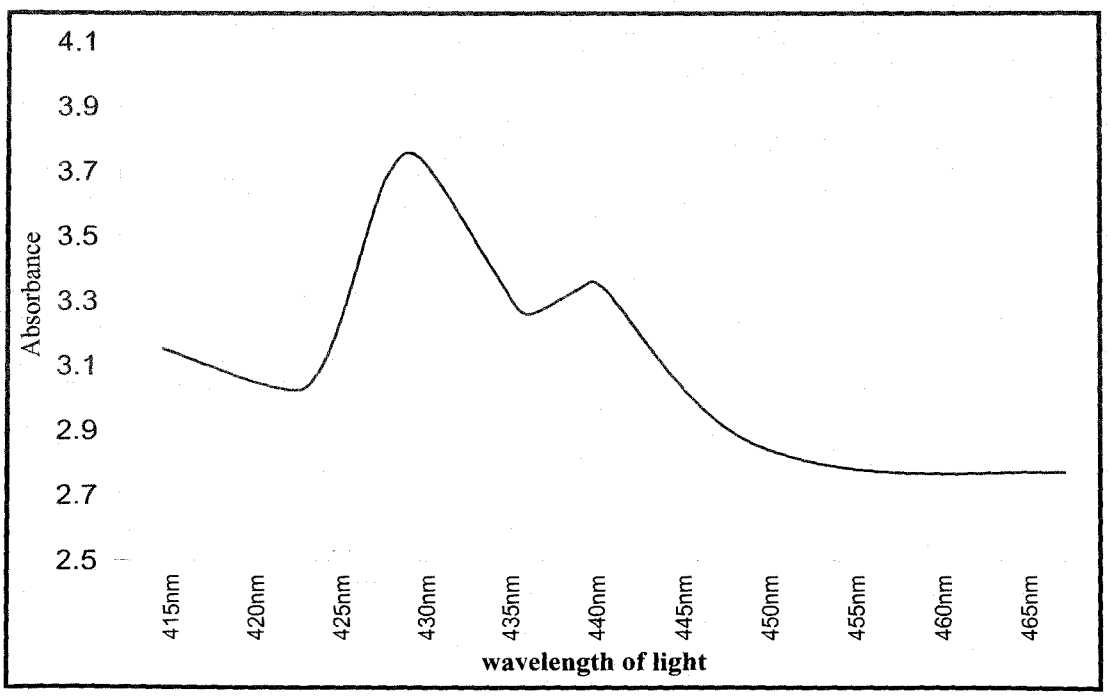

Fig. 28: Spectral scan of partially purified siderophore of *Pseudomonas putida* strain AS04 showing a peak at 430 nm.

5.3.6. Purification of siderophore

5.3.6.1. Amberlite XAD2 chromatography

On Amberlite XAD-2 chromatography several fractions were collected. The first fraction was mostly water and colourless. Fraction 1 was colourless, fractions 2 and 3 were dark yellowish brown in colour and had a putrid odour, and fractions 4 and 5 were light yellow in colour (Fig. 29). The flow-through, DDW wash, and all fractions collected were tested for their siderophore content using Tetrazolium salt test. Fractions 2 and 3 showed the highest amount of siderophore content based on intensity of the colour.

5.3.6.2. Sephadex LH20 chromatography

The fractions tested positive for sideophore content were pooled and concentrated and were further purified by Sepladex LH20 chromatography. Altogether 20 fractions were collected and each fraction was monitored for siderophore content by TLC. Initial fractions were colourless. Fractions 8-11 were pale yellow, the colour intensity increased gradually and the later fractions (fractions 14-19) were brown.

5.3.6.3. TLC

The formation of brown spot by spraying with $FeCl_3$.HCl indicated a hydroxamate-type siderophore. It was observed that fractions 17, 18 and 19 produced intense brown spots (Fig. 30) thus confirming presence of hydroxamate siderophore.

5.3.6.4. Spectral scan analysis

A spectral scan (300-700 nm) with the partially purified siderophore showed a major peak at 430nm (Fig. 28) that confirmed that AS04 produced trihydroxamate type of siderophore.

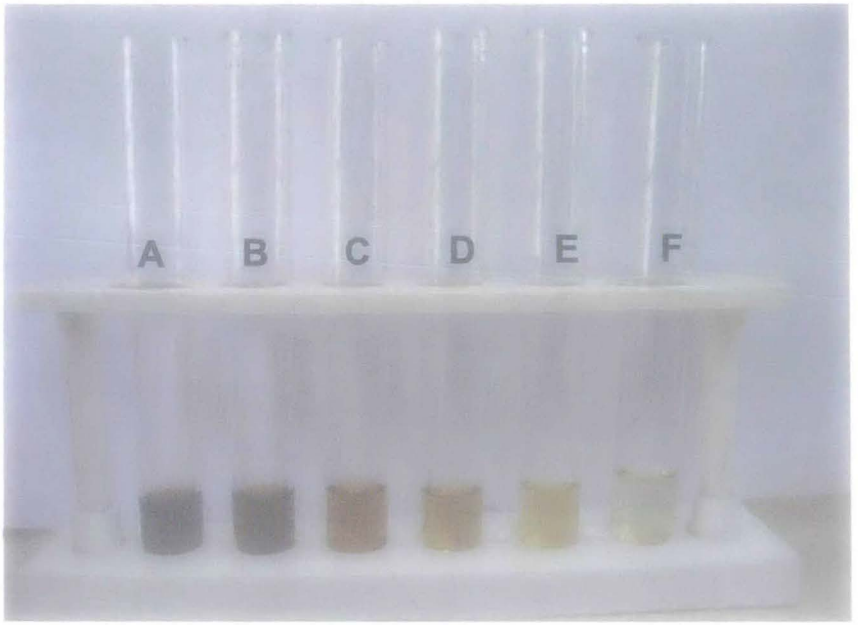

Fig. 29: Tubes with increasing colour intensity, containing Amberlite XAD-2 fractions collected and tested for their siderophore content; A: fraction 2, B: Fraction 3, C: Fraction 4, D: Fraction 5, E: flow-through, F: DDW wash.

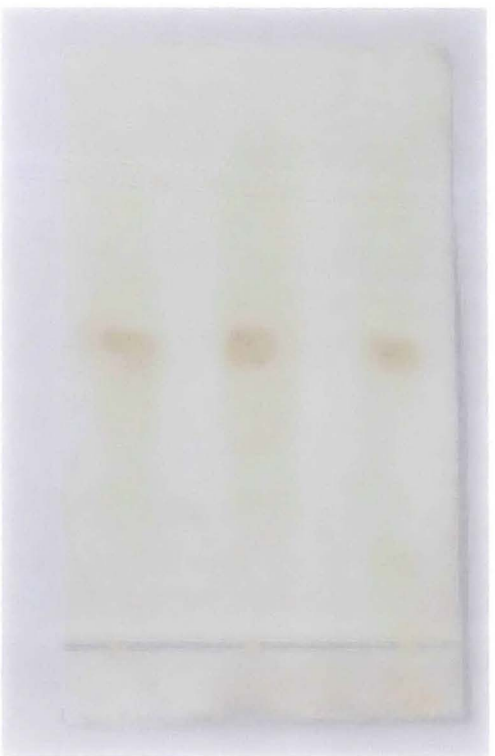

Fig.30: Detection of hydroxamate siderophore as intense brown spots using Thin Layer Chromatography.

5.4. DISCUSSION

The screening of antagonistic bacteria from rhizosphere soil during the present study was based on siderophore production in CAS agar medium. The siderophores produced by each strain was quantified by the CAS shuttle assay and *Pseudomonas putida* strain AS04 was found to be the highest producer of siderophore. In this chapter the siderophore produced by each of the selected antagonistic isolates were chemically characterized and the siderophore produced by strain AS04 was partially purified and analysed by UV spectrophotometry.

Studies on siderophore characterization form a wide array of microorganisms are well documented. The universal chrome azurol S (CAS)-agar plate assay (Schwyn and Neilands, 1987) is the most well-known and widely used method for detection of siderophore production by microorganisms in solid medium (Machuca and Milagres, 2003). This assay is based on a competition for iron between the ferric complex of an indicator dye, chrome azurol Sulphonate (CAS), and a chelator or siderophore produced by microorganisms. The iron is removed from CAS by the siderophore, which apparently has a higher affinity for iron (III). The most positive reaction results in a colour change of CAS-reagent (usually from blue to orange). The blue dye can be applied as a solution test, or alternatively, it can be incorporated in the solid growth medium and used for screening several microorganism siderophore producers by direct plating mode. The authors concluded that the direct plating mode for screening microorganisms is probably applicable to all Gram-negative bacteria. Thus in our study we used siderophore screening which resulted in the isolation of most of the culturable gram negative bacteria. However, Schwyn and Neilands (1987), observed that the detergent hexadecyltrimethyl-ammonium bromide (HDTMA) used in preparation of the CAS medium may be toxic to some microorganisms, especially gram positive organisms and fungi. Thus it was not surprising that in our study, most of the isolates, post-screening were gram negative. We found that only six bacteria out of the twenty selected were gram positive and all of them belonged to the genus *Bacillus*.

Of the twenty antagonistic isolates, 15 strains have been found to produce hydroxamate type of siderophore while the rest 5 produced catecholate type. None of the strains produced carboxylate siderophore. The methodologies followed for the characterization tests; Arnow's test, Neiland's $FeCl_3$ test and the tetrazolium salt test are universally accepted. Several researches have been conducted on characterizing siderophores from fluorescent pseudomonads. Bano and Musarrat (2003) characterized the siderophore produced by *Pseudomonas aeruginosa* NJ-15 and identified it as hydroxamate-type. Chaiharn et al. (2009) isolated siderophore producing antagonistic strains against rice pathogens *Fusarium oxysporum* and *Pyricularia oryzae*. Characterization of siderophores showed that among 18 isolates, all except three were hydroxamate type. *Pseudomonas aureofaciens* AR 1 was the best siderophore producer overall and secreted hydroxamate type siderophore. Gupta et al. (2002) characterized the siderophore produced by antagonistic isolate *Pseudomonas* GRC2. The 48-h-old culture filtrate of the strain showed a major peak at 400 nm when tested by the Neiland's $FeCl_3$ method which revealed that the siderophore was of hydroxamate type. Arora et al. (2001) screened 12 rhizobial isolates for siderophore production of which two strains, RMP3 and RMP5 showed hydroxamate type of siderophore. Santos-Villalobos et al. (2012) isolated *Burkholderia cepacia* XXVI from mango rhizosphere which was found to be effective against the mango pathogen, *C. gloeosporioides*. The bacterium was found to produce siderophore that was involved in the antagonistic activity. The class of siderophore produced by this strain was identified using colorimetric test (Tetrazolium test) and spectrophotometric assays ($FeCl_3$), showing that this metabolite belongs to hydroxamate class, due to the appearance of a deep red colour and the presence of an only peak at 420 nm, respectively.

In the present study, *Pseudomonas putida* strain AS04 was selected for further studies on siderophore characterization and purification. Siderophore production was optimized in order to select the most suitable carbon and nitrogen source that would be best for siderophore production. The amount of siderophore produced increased from 59.70 to 89.04% units

on addition of 1% sucrose and 0.1% $(NH_4)_2SO_4$ to the original Fiss-glucose minimal media. When tested individually, sucrose and $(NH_4)_2SO_4$ was better than mannitol and NH_4Cl as carbon and nitrogen sources respectively. The optimum incubation time was evaluated for determining the appropriate time for siderophore extraction and to determine when siderophore production begins in this organism. Results revealed that AS04 begins producing siderophore after 8 hours post-inoculation, with maximum production occurring at 30 hours. Similarly, the optimum incubation temperature recorded for growth and siderophore production were 30°C. Siderophores are secondary metabolites and a culture may begin producing siderophore at the later phases of growth, with production increasing as cultures are grown for longer periods (Storey, 2005). In the stationary phase, the amount of siderophore was increased due to a state of iron starvation attributed to the consumption of this element in the culture medium as a result of bacterial multiplication (Crosa, 1997; Lim et al., 1998; Cowart, 2002; Trivedi et al., 2008).

Siderophore production by the biological control strain *Pseudomonas aeruginosa* was optimised by Villegas et al. (2002). The highest metabolite concentration was obtained in glucose and glutamic medium. Kanimozhi and Perinbam (2011) investigated siderophore mediated antagonistic activity of *Pseudomonas fluorescens* Lp1 isolated from soil against common fungal pathogens of plant. The authors optimized the siderophore production under varied physiochemical conditions and also partially purified the siderophore. The siderophore production was optimum during the incubation time of 24 to 30 h at neutral pH. The yield of 73.0% was obtained in a medium amended with malic acid among organic acids tested, the organic nitrogen source peptone yielded 72.5% and inorganic source urea yielded 75.5%.

Once the growth conditions had been optimized in the present study, it was then possible to produce large amounts of siderophore by growing *P. putida* AS04 in batch cultures. Accordingly, about 5 litres of culture was grown under the optimized conditions and the siderophore was partially purified

by passing acidified supernatant through an XAD-2 column, followed by passing the concentrated siderophore through a Sephadex LH-20 hydrophobic column. At each stage of purification, the tetrazolium salt test was done to track the siderophore. During and after purification through the Sephadex LH-20 column, the concentrated fractions were tested for presence of siderophore in TLC plates. A single brown spot was obtained at each stage indicating the presence of hydroxamate siderophore.

Spectral scan of the purified siderophore showed a peak at 430 nm. Spectral scans (300-700 nm) can indicate whether a dihydroxamate or a trihydroxamate- type siderophore is present (Jalal and van der Helm, 1991). Spectral scans of the purified siderophore isolated from *P. putida* AS04 indicated that it was of trihydroxamate type.

Purification of siderophores from rhizobacteria has been well documented in literature. Kanimozhi and Perinbam (2011) partially purified the siderophore produced by the antagonistic strain *Pseudomonas fluorescens* Lp1 by passing the acidified culture supernatant through an ion exchange chromatographic column containing Amberlite IR120 (Na+) and eluting the siderophore with 50% methanol. Sokol et al. (1992) isolated a novel iron-binding compound named azurechelin from the ethyl acetate extracts of the supernatants from *Pseudornonas cepacia* cultures by Sephadex G-10 column with aqueous methanol 10% v/v as the mobile phase. The structure was determined by UV-VIS spectroscopic analysis. The authors concluded that it was a novel compound with neither the typical characteristics of catechol nor of hydroxamate compounds. Buyer et al. (1993) purified the siderophore of plant-growth-promoting *Pseudomonas* strain B10 by passing the culture supernatant through a column of Amberlite XAD-16 and the red-brown material was eluted with 1:1 water-methanol. The resultant was purified further by gel filtration and identified by a specific ELISA protocol. This methodology was extended to detecting siderophores in the rhizosphere of barley. Fekete et al. (1989) purified hydroxamate siderophore from *Azotobacter chroococcum* B-8 by Sephadex G-10-120 size exclusion column using a 50% methanol-water mobile phase. The orange fractions were

pooled and evaporated under vacuum to about 1 ml before injection of 250-pLI portions onto a reverse-phase high-performance liquid chromatography. Storey (2005) purified hydroxamate siderophore from *R. leguminosarum* IARI 917 by using XAD-2 column followed by Sephadex LH-20 chromatography and HPLC. The siderophore thus purified was analysed by mass spectrometry and the structure was determined as the dihydroxamate siderophore schizokinen.

In the present study characterization of the siderophores from all twenty isolates showed that most were hydroxamate type. The siderophore production from the strain *P. putida* AS04 which recorded highest production during preliminary studies was optimized for three parameters, media composition, temperature and incubation time. The optimized conditions were used for bulk culture of the strain for partial purification of the siderophore. Purification was done by affinity chromatography and LH-20 chromatography that separates compounds based on hydrophobicity, and the resultant compound produced a peak at 430 nm at ferrated state. The compound was identified to be of trihydroxamate type.