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EVALUATION AND BIOCHEMICAL CHARACTERIZATION OF A DISTINCTIVE PYOVERDIN FROM A PSEUDOMONAS ISOLATED FROM CHICKPEA RHIZOSPHERE

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ABSTRACT

Microbial siderophores confiscate the available ferric ions around the roots and trigger a reaction resulting in plant growth promotion. In our study, a high level of siderophore production was observed from a newly isolated *Pseudomonas sp.* from the rhizosphere of Chickpea plants. Under an iron depleted condition in Standard Succinic acid medium a 1000 μgmL^{-1} of siderophore production was achieved. Increasing the concentration of iron showed an inverse relationship between growth and siderophore production. Fourier Transform Infrared Spectroscopy (FTIR) analysis of the purified crystals, its UV spectral analysis and High Pressure Liquid Chromatography (HPLC) revealed the identity of the siderophore as similar to that of pyoverdin with distinctive characters. Electron spray ionization mass spectroscopy (ESIMS) shows presence of abundance of A_1 ions (419 m/z) and branching of amino acids from B_1-B_5. This pyoverdin contains a cyclic tetra peptide but Serine and Arginine are missing. Based on our analysis and deviations from the reported structure of pyoverdin it is suggested that this pseudomonas produces distinctly characterized pyoverdin siderophore.

Key words: Fluorescense, hydroxamates, collision activation, pyoverdin, *Pseudomonas*

INTRODUCTION

Iron is essential for processes such as respiration, photosynthesis and nitrogen fixation but microbes have difficulty obtaining enough iron to support their growth because iron is in immobilized form of insoluble ferric hydroxide in soil and cannot be transported in the cells (1). Ability to produce siderophores by an organism under iron limiting conditions can promote plant growth by directly supplying iron for plant utilization and by removing iron from the environment for the growth of phytopathogens thereby reducing their competitiveness (2). It has been studied that yellow green siderophore producing *Pseudomonas* species exert biocontrol effect on phytopathogens there by enhancing plant growth. *Pseudomonas fluorescens* and *P. putida* produce siderophores of two general types, Pyochelin and Pyoverdin.

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Each siderophore has a specific role in metal acquisition. Pyochelins are phenolate siderophores derived from salicylic acid and cysteine. Pyoverdins are water soluble pigments that fluoresce yellow-green under ultraviolet light. All pyoverdins are structurally similar containing of a chromophore moiety, which gives bacteria its fluorescent nature, and a dicarboxylic acid (4). More than 40 pyoverdin peptide chain compositions have been identified in the group containing arginine dehydrolase positive, saprophytic or opportunistic animal pathogenic fluorescent *Pseudomonas* species. The chemistry and biology of different siderophores have been discussed by Hider and Kong (3).

Pyoverdins are now used as a major tool in identification, systematics, and characterization of closely related pseudomonad species. There are different siderovars, regroup strains that produce pyoverdins with same peptide chain, in certain species. *P. fluorescens* has 19 siderovars where as *P. putida* has 13 siderovars (5).Each siderophore has a specific role in metal acquisition. *Puesdomonas PAO1* secretes both types of siderophores, Pyoverdin , which exhibit binding affinity for iron and Pyochelin which shows affinity for other metals and has a biocontrol potential. Siderophores from different bacteria are designated and named on the basis of name of organism for eg. Enterobactin, Agrobactin , Rhizobactin etc. In this study, we isolated a distinctively characterized siderophore produced by a *Pseudomonas sp.* isolated earlier from rhizosphere of *Cicer arietinum* and biochemically characterized its type and variety in order to reveal the identity of the type of siderophore.

MATERIALS AND METHODS

Bacterial strain, culture conditions and growth study

Bacterial strain of *P. fluorescens* was isolated and identified by 16sRNA from the rhizosphere of chickpea crop. This isolate was screened for Plant Growth Promoting potential after studying its various PGP (Plant Growth Promoting) traits as reported by us earlier (6). The isolate was maintained on nutrient agar at 5 ^{o}C until used. The sequence is deposited in Gene Bank Accession No. MSC2 HQ179576.

Induction and estimation of siderophores in MM9 medium

Actively growing inoculum of *Pseudomonas* culture was added to MM9 medium (8) and incubated on rotary shaker (200 rpm) and generation time calculated. Simultaneous induction of siderophore production was also checked by taking 1 mL of cell free supernatant was added with 1 mL of Chromeazurol S (CAS) shuttle solution. Amount of siderophore units was calculated as Percentage of Siderophore Unit = (Ar-As)/Ar x 100 (where Ar = absorption of Reference and As = Absorption of Sample) as reported earlier(7). Induction was also confirmed by Agar Well method where the culture supernatant was added into bored-wells in agar plates of MM9 medium supplemented with Casamino acids and CAS dye . Presence of siderophore can be checked as zone of discoloration of CAS dye around the well after 24 h of incubation at 30^{0}C.

Deferrated Standard Succinic Medium (SSM) was inoculated with 24 h old culture and incubated at 30 ^{o}C. Siderophore production was checked after 24 h and 30 h when culture attained its stationary phase. Detection was done using CAS shuttle solution as described by (8). A simultaneous change in pH was also checked. Type of siderophore was checked by performing various methods like Arnows (9) for detecting catecholate type of siderophore and Csaky's (10) method for detecting hydroxamate type of siderophore.

Effect of different $FeCl_3$ concentration on siderophore production and Fluorescence

In order to determine the threshold level of ferrous at which siderophore biosynthesis is repressed in fluorescent *Pseudomonas*, the deferrated liquid succinic acid medium and then externally added with different ferrous concentration ranging between 0-20 µM. This was then inoculated and

incubated for 30 hours at 30°C and checked for production of siderophores. Fluorescence produced by isolates at different $FeCl_3$ concentrations was estimated by using Quinine Bisulphate method (11) using Spectrofluorimeter (Hitachi, Model F-2000) at an excitation of 295 nm and emission at 485 nm.

Crystallization and FTIR studies of siderophores

The produced siderophore was crystallized to study the chemical structure. The 30h old culture was centrifuged and cell-free supernatant was added with saturated $FeSO_4$ solution to get maximum ferreted siderophores. The pH was adjusted to 3.0 with H_2SO_4 and 50 % ammonium sulphate solution was added to deproteinize. The aqueous phase was concentrated in a rotary vacuum evaporator and set aside in cold to crystallize. The filtrate was neutralized, reduced to dryness and extracted in dry hot methanol. The crystals were then separated out on Whatman filter paper no. 44. Crystals obtained were then used for their FTIR analysis and compared with the FTIR of standard hydroxamic acid crystals(12).

Partial purification of siderophore for spectral analysis

Culture was grown in deferrated SSM , the cell free supernatant was collected and its pH was adjusted to 6.0 with 6 M HCL. This supernatant was then passed through XAD-4 column (25x2.5 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. (13). 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 according to Bultreys et al,(13) who has linked the siderophore production to pH.

Detection and Comparison of Pyoverdins by HPLC and MS

After incubation for 30h, the culture was centrifuged for 20 min. This was then filtered through 0.2 µm membrane filter and pH adjusted to 5.0 - 5.5. Pyoverdin production was estimated by measuring the absorbance at 403 nm. By using HPLC, the retention times (RT) of peaks with comparable heights were analyzed. The HPLC analyses were performed with Nucleosyl C18 columns and a Waters 2190 system.

The molecular mass of the pyoverdin was determined by mass spectrometry using Electron spray Ionization Solvent H_20, CH_3OH, CF_3COOH 50:50:1 capillary temperature 230°C spray voltage 3.4 - 3.6 kV (15).

RESULTS AND DISCUSSION

Induction and Estimation of siderophore in the medium

The isolate entered into log phase after 8h of incubation on shaker in deferrated MM9 medium which was 4 h later than that in nutrient medium and achieved its stationary phase after 30h (Figure 1). Here glucose served as sole C source and glutamic acid served as sole N source. Similar results were also obtained by Carson *et al.* (13) when *S. meliloti* was grown in MSM-YE medium, which showed an increase MGT in absence of iron in the medium. The result shows that siderophore production was induced along with the growth of the isolate (Figure 2), and was observed just after 8h when the culture entered into logarithmic phase and continued to increase until the culture attained stationary phase i.e. 30h. Results show that hydroxamate types of siderophores, with high ferrous affinity, are present in the supernatant. Siderophore units (%) in the culture broth were found to be 85 % (Table 1). Sayyed *et al.* (6) also found presence of 87 % of siderophore units in the medium.

Siderophore production by the isolate carried out on solid CAS blue agar showed a 22 mm clear zone of orange colour representing iron chelation. Supernatent from culture was tested with Arnow reagent (Hydroxmate) and Csaky method

(Catecholate). The supernatant turned pink upon addition of ferric perchlorate indicating the hydroxmate group of siderophore. Quantitative estimation showed that the organism produced a maximum of about 1000 µg mL^{-1} of hydroxamate type of siderophore in culture medium (Table 1) after 24h of incubation which remained constant even after 30 h of incubation. The pH of the media changes as the culture ages and uses up the available nutrients. Siderophore production also increases as iron is depleted from the media. Therefore increase in pH may be coincidental to increase in siderophore concentration. The pH increased from 6.8 to a maximum of 10 along with siderophore production. It is reported that alkaluine pH helps in solubilization of iron which results into more iron content in medium and hence a decline in siderophore production (7). This change in pH of the medium during siderophore production was also reported by Budzikiewicz (16) mentioning that alkalinity is important to avoid siderophore destruction. On the contrary Sharma and Johri (17) showed that higher pH is rather destructive to siderophores.

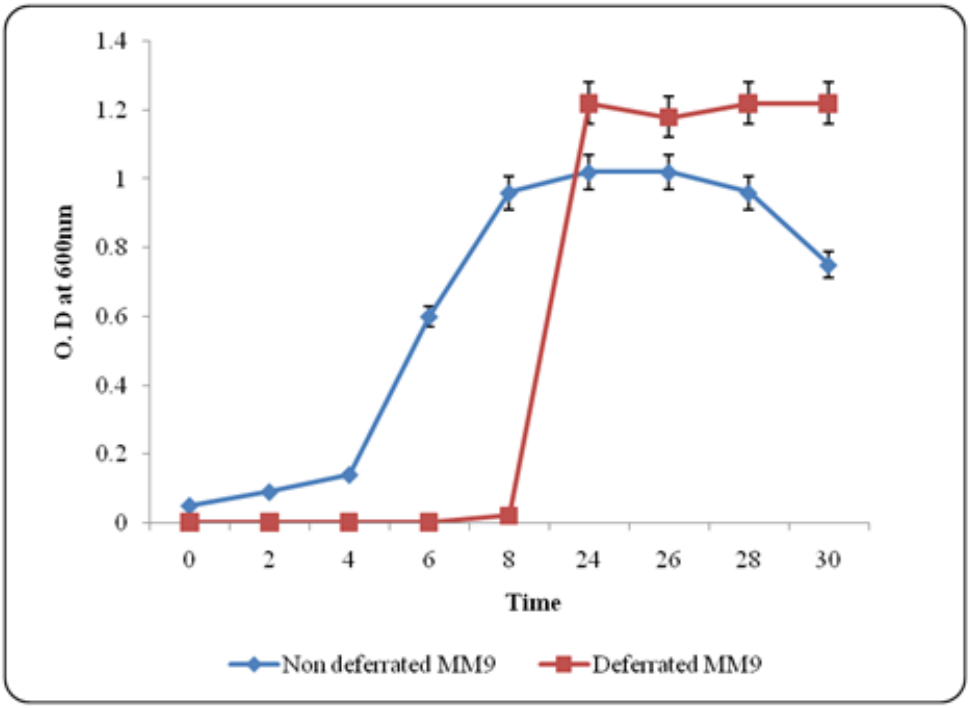

Figure 1. Growth rate of *P. fluorescence* under deferreted and non deferrated MM9 medium. The growth was measured in terms of optical density at 600 nm.

Table 1. Detection characterization and quantification of Pyoverdins in SSM. Quantitative estimation showed that the organism produced a maximum of about 1000 µg mL^{-1} of hydroxamate type of siderophore in culture medium after 24 hours of incubation which remained constant even after 30 hours incubation.

Time	Sid. Unit	Hydroxamates	Zones around agar cup (mm)	pH
24 h	85%	1000 µg mL^{-1}	22	8.0
30 h	86%	1000 µg mL^{-1}	22	10.0

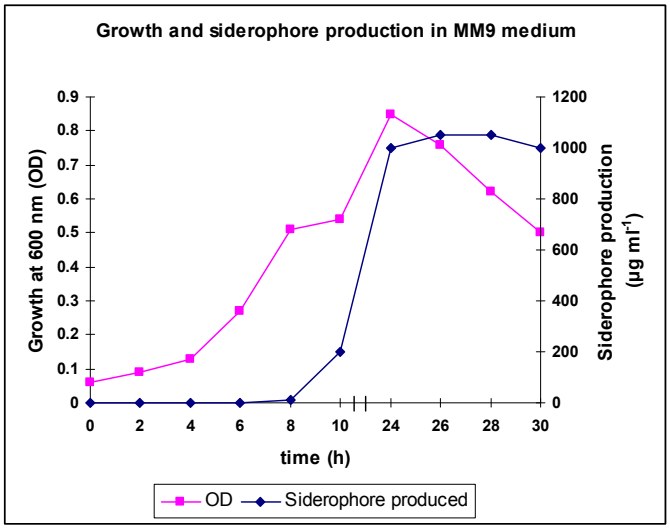

Figure 2. Growth Phase and siderophore induction study of *P. fluoresence* in MM9 Medium.

The growth of the isolate was expressed in optical density (■) at 600 nm and its siderophore production (♦) was given concurrently.

which shows that pyoverdin biosynthetic genes and thereby fluorescence are under the control of iron regulated promoters.

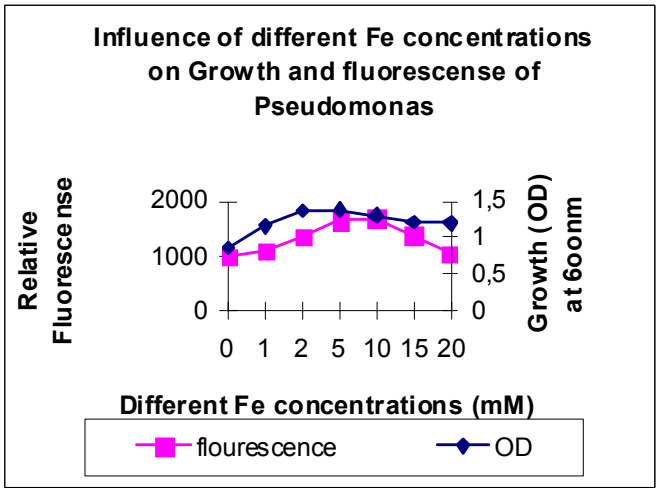

Figure 3. Influence of different concentrations of $FeCl_3$ on growth and Fluorescence of *P.fluorescence.*

Effect of different $FeCl_3$ concentration on siderophore production

Pseudomonas showed increase in growth with increase in $FeCl_3$ concentration revealing that presence of $FeCl_3$ is vital for its growth (Figure 3) .This was also reported by De Villegas (17) who stated that concentration of $FeCl_3$ above 10 µM has a negative effect on siderophore production whereas Manninen and Sandholm (19) 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 $FeCl_3$ which declines thereafter up to 20 µM of Fe. An increase in fluorescence was reported along with increase in $FeCl_3$ concentration up to 2 mM $FeCl_3$ concentration. It can be reported that production of fluorescence is a response to presence or absence of $FeCl_3$ in the medium. Amount of fluorescence produced by the isolate *Pseudomonas* increased along with the concentration of $FeCl_3$ but up to just 2 mM concentration after which a continuous decrease was reported (Figure 3). This increase was parallel to growth of the isolate,

Extraction and purification 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}). This scale was selected according to PBHA crystals used as standard. FTIR results (Figure 4) show that the crystals obtained had 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 wave number, which are same as 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 (12). As observed in the present study, the certain functional chemical group resembled ferrichrome siderophores.

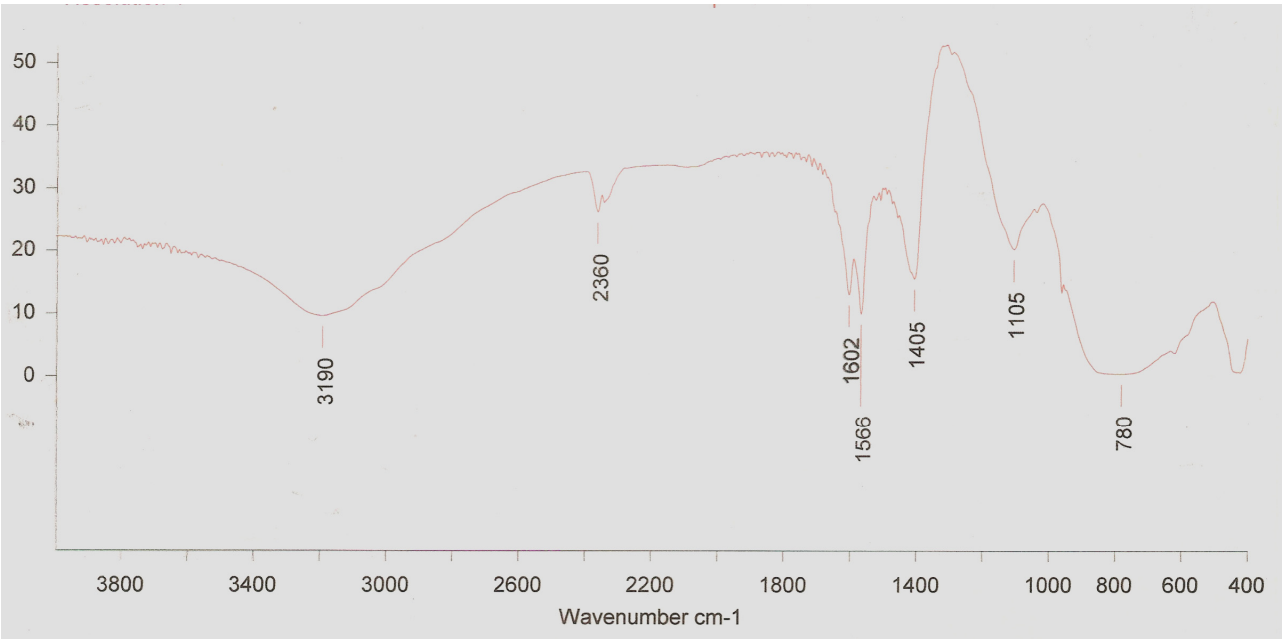

Figure 4. FTIR analysis of siderophore crystals. FTIR analysis of the crystals obtained after extraction and purification of the siderophore showing peaks similar to that of standard PBHA crystals alongwith two more peaks at 1105 and 1495.

Spectral Analysis

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 (Figure 5). Similar results were also quoted by Xiao (18) 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 and 385 nm for Pf-C). However at higher pH values (pH 7), the pyoverdin 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 (5) report that as the pH of the supernatant containing pyoverdin 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 putative pyoverdin at lower pH (3.0) and as distinctive pyoverdin as the pH increased from 3.0 to 7.0 and above. All pyoverdins have a common feature in its molecular structure i.e. a constant quinoline chromophore group bound to a peptide chain and to a dicarboxylic acid or a dicarboxylic amide (21). and the presence of three iron-binding ligands. One ligand is located in a catechol chain and one on hydroxamic acid chain derived from ornithin or b-hydroxyaspartic acid. The atypical feature of the pyoverdin of *P. syringae* and *P. viridflavia* is the presence of two OH-Asp residues of Ornithin in the chelation of iron. This feature influences the spectral characteristics of Fe(III) chelated atypical pyoverdin (14). The main difference observed between siderophores produced by different *Pseudomonas* is the arrangement of L and D amino acids. Pyoverdins can be further segregated as typical and atypical pyoverdins.

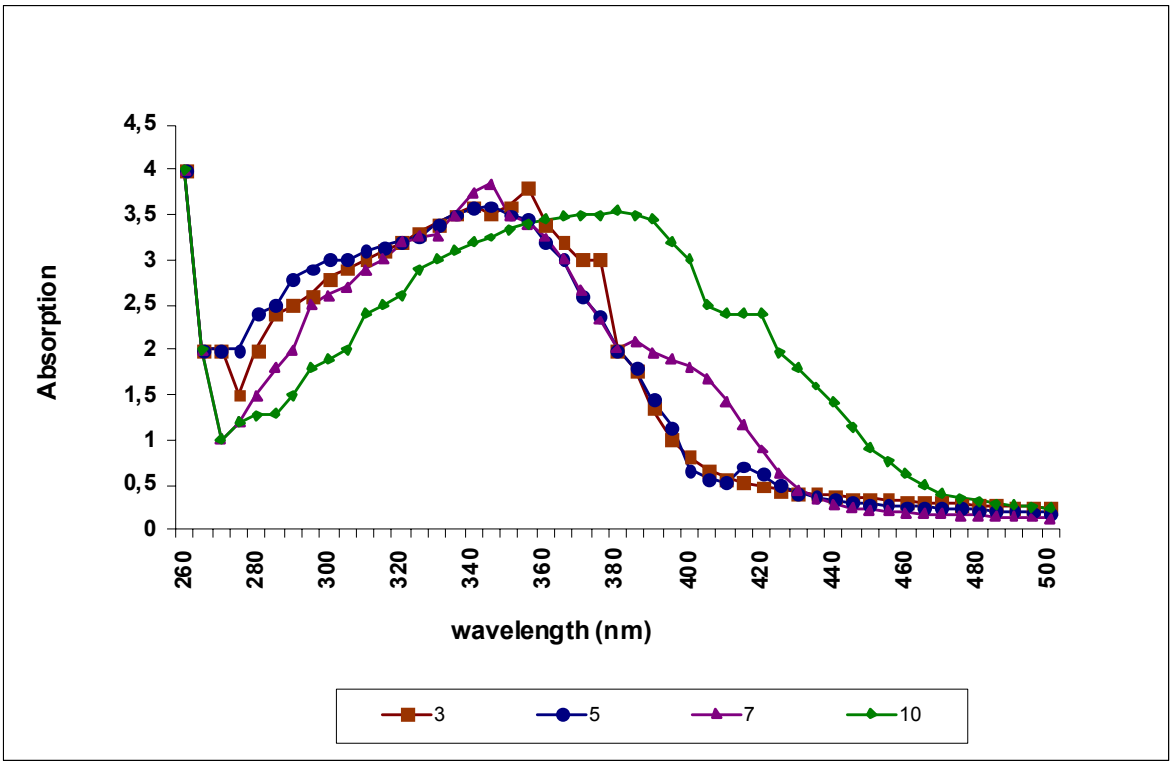

Figure 5. Influence of changes of pH on absorption spectra of pyoverdin. UV-absorption spectra of siderophore at different pH. (3.0, 5.0, 7.0, 10.0).

Detection and Comparision of Pyoverdins by HPLC and ESIMS

Dominant peak for this pyoverdin appeared at 14.906 min, 16.975 min and 18.444 min (Fig: 6). RT data allowed discrimination between pyoverdin with different peptide chains produced by different species of *Pseudomonas* (5). In visual tests a change in color of production medium that accompanied in the pH 7.0 to 4.0 indicated atypical pyoverdin production(14). Reference strain *P. syringae* B301D ΔRT of 18.783 min and *P. chicorii* LMG 2162 RT of 19.075 min, *P. syringae pv. syringae* PSP1 with RT of 18.758 min. Two smaller peaks at 17.922 min and 18.444 min were obtained in our sample. These other peaks could be correlated to minor peaks found in *P. fuscovaginae* (14.906 and 16.975 min). The result obtained with HPLC chromatogram 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.

As the pyoverdin profiles obtained were complex, the dominant pyoverdins of the reference strains as reported by Fuch and Budzekiewicz (22) were used for comparisons. ESIMS when performed using purified supernatant of the culture, yielded several peaks as mentioned in the Table 2. The peaks were obtained in a wide range of m/z starting from 399 m/z to1239 m/z. The peak at 399 m/z represents presence of A_1 fragment with a loss of one H_2O molecule. Next visible peak was at 417 m/z which represent A_1 fragment showing presence of succinic acid side chain. Peak 445 m/z shows presence of serine where as 504 shows B_{5S}-H_2O 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 at 1067 m/z shows loss of succinic acid from

pyoverdin of *P. fluorescens* showing molecular mass of 1167 m/z as reported (23). The result also shows that the siderophore contains C terminal with OHOrn side chain. Peaks at 1105 m/z represent side chain with Asp-Lys-OHasp-Ser-Ala-Ser-cOHOrn. 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 reperesents a loss of H_2O molecule from the previous peak 1167 m/z visible in the spectra. Certain peaks i.e 1149 m/z and1167 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 (Table 2). Siderophores like 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_2O molecules(22). 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 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, (22) where they mention that ESI of $[M+H]^+$ of pyoverdin shows a peak at 1169 m/z reporting that OH transfer is possible from other amino acids (Asp, Fho, Ser etc). The present pyoverdin contains cOHOrn 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 and Budzekiewicz (22).

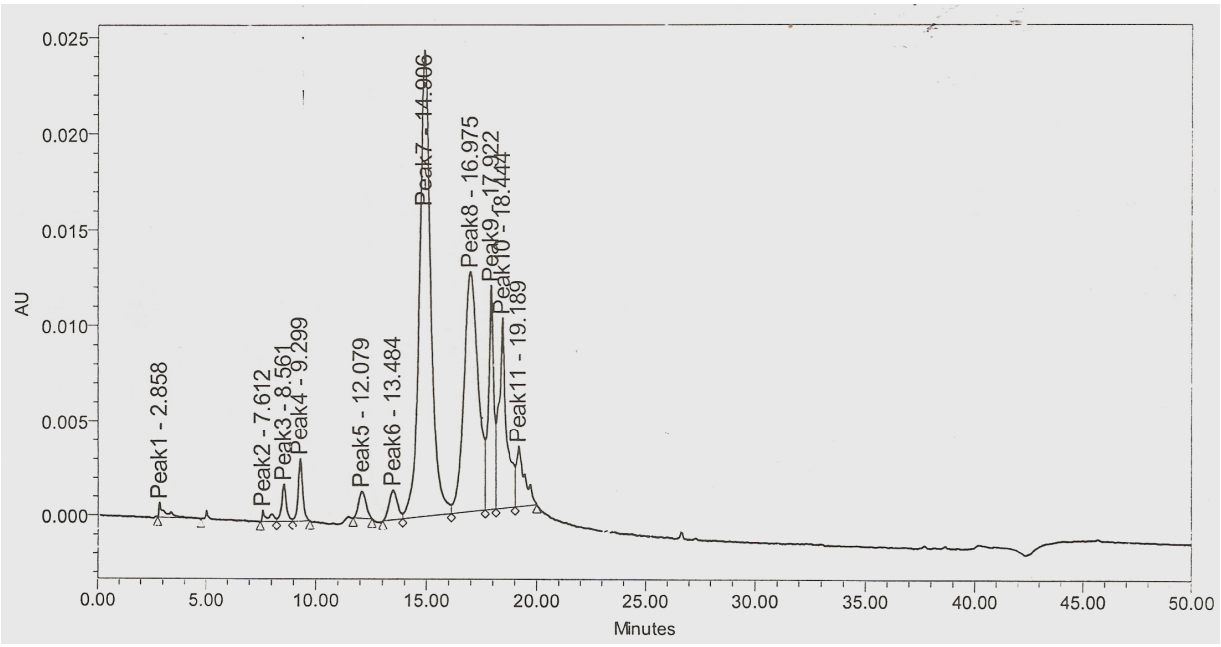

Figure 6. HPLC analysis of the pyoverdin. HPLC chromatogram of the siderophore produced in SSM medium by *P. fluorescence*.

Table 2. ESIMS analysis of the siderophore produced by the isolate.

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

CONCLUSION

Pseudomonas have been studied widely as siderophore producers and siderotyping is an important step in identification of *Pseudomonas* as it is species specific. The novel isolate *Pseudomonas fluorescens* shows some distinct features which have not been observed so far in and suggests a new type of Pyoverdin.

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