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SIDEROPHORE MEDIATED METAL UPTAKE BY *PSEUDOMONAS FLUORESCENS* AND ITS COMPARISON TO IRON (III) CHELATION

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ABSTRACT

Siderophores are iron chelating substances that are also known to bind metals such as Molybdenum and Lead. The pigment produced by *P. fluorescens* is a siderophore whose ability to bind to different metal ions was investigated in this study. Pigment production was increased in the presence of Sodium, Potassium, Lead, Molybdenum and Cadmium. Siderophores were detected using CAS assay and catechol - siderophores by Arnow assay. Peak changes and changes in extinction coefficient values are indicators of complex formation**.** Maximum difference was observed in siderophore free Iron and Iron-siderophore complexes. Some changes were also noted for siderophore- metal complexes for all metals included in this study. Thus siderophore of *P. fluorescens* shows a tendency to form complexes with almost all metals tested.

Key words: pigment, CAS assay, metal ions, complex formation, peak changes, molar extinction coefficient.

INTRODUCTION

Iron, one of the most essential microelements for virtually all living cells, is usually abundant in the environment, particularly in soils and natural aquifers. However, its bioavailability is relatively low, which is connected with a dramatically decreased solubility of ferric species under physiological pH values owing to their complete hydrolysis. This has resulted in the development of special biologically regulated mechanisms of Fe(III) solubilization, e.g., involving specific natural low-molecular-weight chelating agents (siderophores) which transport iron(III) to the cell surface in the form of a complex, with further Fe(III) release from the latter in the course of its reductive assimilation (Kamnev *et al.,* 2000). Many microorganisms possess high affinity iron uptake system mediated by the action of low molecular weight iron chellators termed as siderophores (Lankford, 1973; Neilands, 1981). The fluorescent *Pseudomonas* are characterized by yellow green pigments that fluoresce under UV irradiation and function as siderophores termed pyoverdins, and pseudobactins (Meyer and Abdallah, 1978). The following study investigates the ability of siderophores to chelate other metal ions as has been reported for iron.

MATERIALS AND METHODS

Ioslation of *Pseudomonas spp* was done on Kings A and B medium (Deshmukh, 1997) from rhizopheric soil as well as phyllospheres of ornamental plants. Colonies with characteristic pigments were tested biochemically and identified using the Bergey's manual of systematic Bacteriology. Pigment production was tested in SM medium (Meyer and Abdallah, 1978), Barabhaiyya and Rao medium (BR) (Barabhaiyya and Rao, 1985), Pseudomonas F broth, Cetrimide broth, Nutrient broth, LB medium (Deshmukh, 1997), Low iron Fiss minimal media (Vellore, 2001) and M63 succinate minimal broth (Khare *et al.,*1997) respectively.

Metal tolerance and siderophore production.

The ability to tolerate metals was determined by incorporating 16 different metal salts individually at 200 µg/ml (Sayyed *et al*., 2005) in SM medium. Absorbance at 600 nm along with total cell proteins (Lowry *et al.,* 1951) were determined and intensity of pigment formation was noted on a visual scale of 1 to 4 after 48h of growth at 30 ^{0}C.

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CAS assay

CAS assay (Schwyn and Neilands, 1987) was carried out for detection of siderophores. *Pseudomonas fluorescens* was grown in Fiss's iron deficient medium (at iron concentration of 135µg/ml) as well as in iron modified Fiss minimal media (5.56 mg/l; Vellore, 2001). CAS agar plates were used for detection of siderophores. Culture supernatant (20µl) was applied to wells of CAS agar plates and incubated at 30^0C for 5 days. Siderophore-producing (Sid^+) culture supernatant develops an orange halo.

Arnow assay

The presence of catechol-phenolic type siderophores was detected using Arnow assay in cell free supernatants grown in low as well as high iron Fiss's minimal media (Arnow, 1937).

Extraction and TLC

Pseudomonas fluorescens was grown for 3 days in BR medium and Nutrient broth which were suitable for intense pigment production. The culture free broth was extracted with equal volumes of isopropanol and chloroform (Tobie, 1945) and the resultant solvent fractions was allowed to evaporate under refrigeration. The extract was redissolved in 5ml of distilled water and used as a crude siderophore preparation for TLC a**s** well as for metal complexation studies. TLC was done using a tertiary mixture of n-butanol, acetic acid and distilled deionised H_2O in a ratio of 3:1:1. (Johnson, 1977). Plates were dried and observed under UV light for detection of fluorescence.

Detection and estimation of siderophores

Culture supernatants from King's B medium was used for recording absorbance over a wavelength of 200-700nm. *Pseudomonas fluorescens* was grown in Fiss's iron deficient medium for 48h.Culture supernatants were used for determining the amount of siderophore in culture medium at λ_{max} of 400 nm (Rachid and Bensoltane, 2005) and calculated as follows:
Concentration = O.D/ ε x1 (where molar coefficient ε = 2000 moles/litre) (Jayaraman, 1996).

Metal complexation studies

Metal-siderophore complexation was allowed at metal concentration of 50µg/ml for 3 h. at room temperature. A spectral scan (200 to 700 nm) was conducted to determine the λ_{max} of siderophore free metal and metal – siderophore complexes. Sampling was carried out in triplicate and average values have been reported. The molar extinction coefficients and 'ε' (% moles/litre) were calculated (Jayaraman, 1996) as follows:
A or E = log 100/T = 2-logT (where A is the absorbance and E, the extinction)
ε = E / C x l (where C or concentration =0.05 g/litre and l or length of light path = 1cm)

RESULTS AND DISCUSSION

The isolate obtained on King's B medium was biochemically confirmed as *P. fluorescens* using Bergey's manual of systematic Bacteriology (Table 1). Typical yellowish blue UV fluorescence was detected on TLC plates. When such spots were eluted and redissolved in isopropanol or chloroform, the λ_{max} was found to be 363 nm (Fig. 1), characteristic of pyoverdins (360 to 420nm). However the elute could not be used in subsequent metal chelation studies as the quantity of siderophores containing elute was too little for metal chelation. Metal chelation studies were thus carried out after solvent extraction of the growth medium. The solvent phase was allowed to evaporate and the residue was resuspended in deionised water. Since the pigment is itself a siderophore and an iron chelator (Meyer, 2000) therefore fluorescence and peak at 363 nm confirms the production of siderophores. The siderophore color could mainly be attributed to the 402 nm peak (Bultreys *et al.*, 2001).

Table 1. Biochemical characteristics of *P.fluorescens*.

Biochemical tests	Results
Catalase	+
Oxidase	-
Lecithinase	+
Gelatin hydrolysis	+
Starch hydrolysis	-
Argenine dihydrolase	+
Urease	+
Nitrate reductase	-
Growth on King's B medium	+
Cetrimide agar	+
Growth at 41^0C	-
Growth at 4^0C	+
Gram's nature	Gram negative
UV Fluorescence	+

The peaks for partially purified siderophore was in the range of 290 to 300 (Table 4 computed from Figs. 2A to 2O). Since the pigment was extracted from the growth medium,

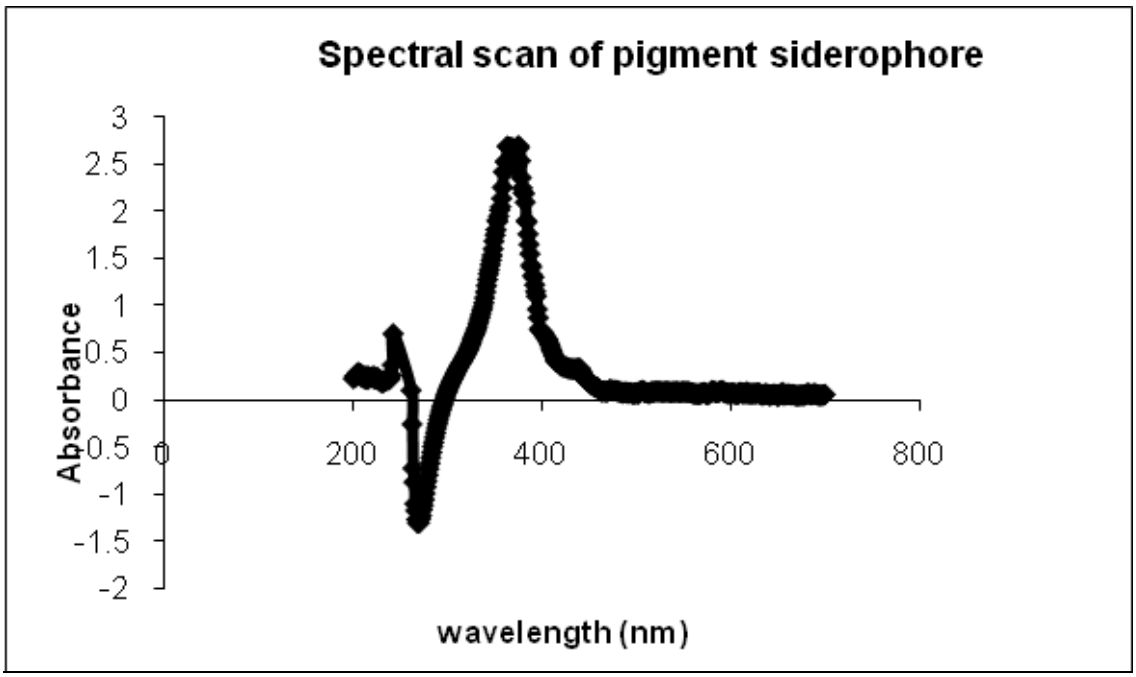


Figure 1. Spectral scan of the eluted siderophore-pigment after TLC.

Table 2. Differences in λmax and molar extinction coefficient with reference to siderophore free and metal incorporated siderophore complexes.

Metal added	Siderophore free –metal		Siderophore-metal complexes	
	λmax	ε (% moles/litre)	λmax	ε (% moles/ litre)
Sodium	293	37.7	290	34.9
Potassium	292	37.9	286	35.4
Calcium	292	39.6	305	31.9
Iron	295	31.9	300	43.4
Zinc	294	37.1	287	34.4
Lead	291	40.8	299	29.3
Silver	300	31.9	300	31.9
Mercury	297	33.8	302	31.2
Cadmium	291	39.7	299	31.9
Chromium	292	39.6	302	31.9
Nickel	291	41.2	299	29.3
Molybdenum	288	41.9	297	29.3
Copper	295	36.6	300	31.9
Magnesium	290	40.9	299	29.4
Manganese	292	41.0	301	31.9
None	293	37.7	290	33.9

interference in absorption with respect to unused media constituents, degraded products and metabolites produced cannot be ruled out.

Several researchers reported the presence of siderophore, a polar substance with bands of absorption at different wavelengths such as 260 nm and 402 nm, 448 nm, absorbance maxima of 365 and 410 nm for pyoverdins pss and its ferric chelate, respectively (Cody and Gross, 1987), 350 – 600 nm in absence and presence of iron (Parker *et al.*, 2004). The reports indicate a shift on the longer wavelength side after iron chelation and the same has also been observed in this study.(Fig. 2B). Peak shift on the longer wavelength side has also been observed with

respect to Ca (Fig. 2D), Pb (Fig. 2F), Hg (Fig. 2H), Cd (Fig. 2C), Cr (Fig. 2A), Ni (Fig. 2K), Mo (Fig. 2J), Cu (Fig. 2E, Mg (Fig. 2I), Mn (Fig. 2G; Table 2).

Excellent growth and pigment production was detected in BR medium. BR medium has succinate which favors pigment production and is used as a carbon source by *Pseudomonas*. It was reported by Meyer and Abdallah (1978) that pyoverdin excretion in *P. fluorescens* increased in standard succinate medium under iron depleted conditions. Barbhaiya and Rao (1985) also reported that succinate and ammonium sulphate were the best sources of carbon and nitrogen for pyoverdin production. Chodat and Gouda (1961) have attributed an important role in pyoverdin synthesis to the nature of the carbon source for growth and succinate is classified as a chromogenic substrate increasing sidorophore yields. Total cell proteins decreased considerably in presence of Silver, Chromium and Copper as heavy metals exert oligodynamic action. Many metallic elements have been observed to inhibit the growth of bacteria and to inactivate enzymes (Charles *et al*., 1948). Metal free media (control) showed higher total proteins and Manganese, Magnesium or Potassium did not inhibit growth. Mn, Mg or K are physiological ions and hence did not inhibit the growth of the organism. It was reported by Abd Rahman *et al*. (2004), that metal ions such as K^+, Mg^{2+} and Ca^{2+} maximized enzyme production in *Pseudomonas aeruginosa*.

Intensity of the pigment was highest in presence of Sodium, Potassium, lead, Molybdenum and Cadmium (Table 3). Sayyed *et al*. (2005) reported that lead triggered growth as well as siderophore synthesis. Siderophores have been shown to have a high affinity for a variety of metal ions concerned such as Cd, Cu, Pb, Zn, Al etc (Chamongkolpradit *et al*., 2008). Thus their chelation probably reduced their toxicity and hence allowed pigment production.

Siderophore indicating orange halos were observed after 5 days of incubation on CAS medium. Pinkish–red colour was noted in Fiss's low iron medium in presence of excess of NaOH (catechol siderophores) but could not be detected in modified Fiss's medium (high iron). Highest concentration of siderophore was detected in metal free low iron Fiss minimal media. The production of siderophores depends on iron concentration in the medium and was thus in agreement with the findings of Kloepper *et al.* (1980). Low or no iron stimulated the production of siderophores (Tables 2 & 3).

CAS assay is the universal assay for detection of siderophores. The principle of this assay is based on a color change of CAS from blue to orange resulting from siderophoral removal of Fe from the chrome azurol dye (Guan *et al*., 2001). Arnow assay was also done to detect catechol – phenolic siderophores. Catechol combines with nitrous acid giving a yellow colour, which becomes intense orange red in presence of excess NaOH.

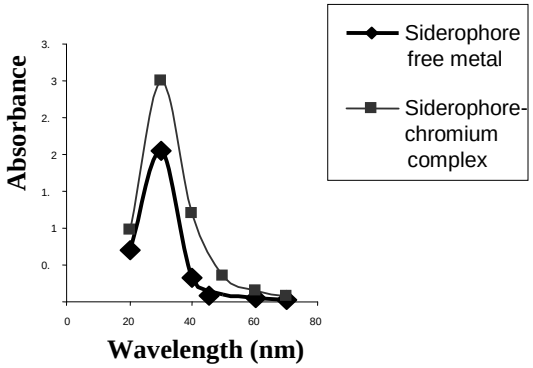
Table 3. Growth and pigment production in SM medium.

Metal	A_{600}	Total proteins (µg/ml)	Intensity of pigment
Sodium	1.423	14.75	++++
Potassium	2.106	22.95	++++
Calcium	1.317	19.67	+++
Iron	1.332	13.11	-
Zinc	1.076	14.75	+
Lead	1.673	16.39	++++
Silver	0.648	6.55	-
Mercury	0.929	11.47	-
Cadmium	1.248	11.47	++++
Chromium	0.462	6.55	-
Nickel	0.545	11.47	-
Molybdenum	1.251	19.67	++++
Copper	0.662	8.19	+
Magnesium	1.553	22.95	+++
Manganese	2.708	27.86	+++
None	2.674	32.78	++++

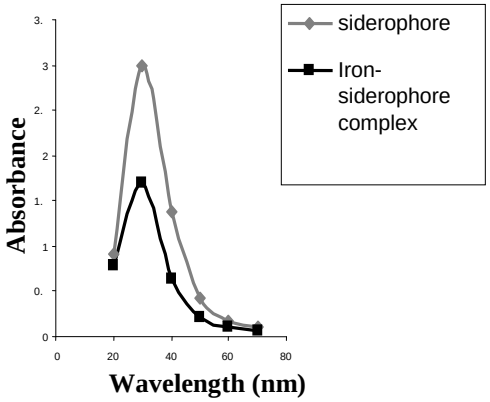
Table 4. Estimation of siderophore produced in Fiss's minimal medium.

Metal added	A_{400}	Siderophores (µg/ml)
Sodium	1.264	0.0632
Potassium	1.583	0.0791
Calcium	1.397	0.0698
Iron	0.629	0.0314
Zinc	1.273	0.0636
Lead	1.218	0.0609
Silver	1.183	0.0591
Mercury	1.166	0.0583
Cadmium	1.232	0.0500
Chromium	1.207	0.0603
Nickel	1.174	0.0587
Molybdenum	1.232	0.0616
Copper	1.182	0.0591
Magnesium	1.410	0.0705
Manganese	1.252	0.0626
None	1.716	0.0858

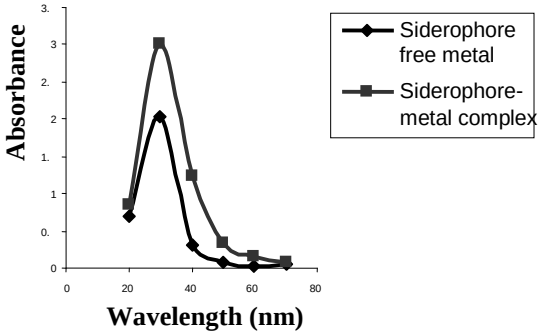
2A. Scan of chromium and Siderophore-Chromium complex



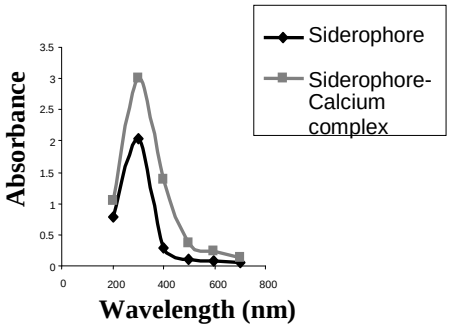
2B. Scan of Iron and Iron- Siderophore complex



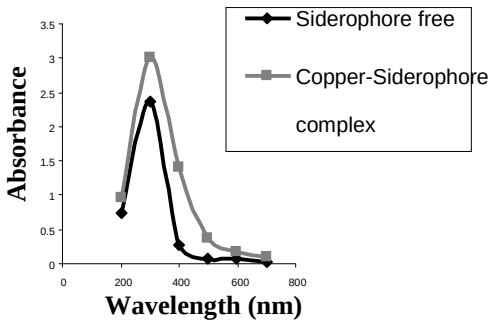
2C. Scan with Cadmium and Cadmium-Siderohore complex



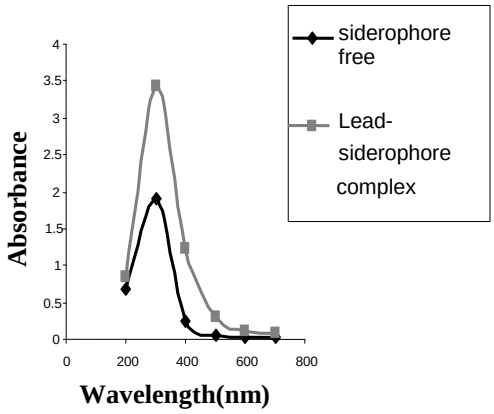
2D. Scan with Calcium and Calcium-Siderophore complex



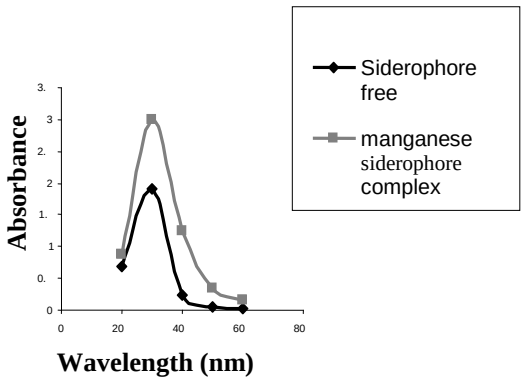
2E. Scan with copper and siderophore-copper complex



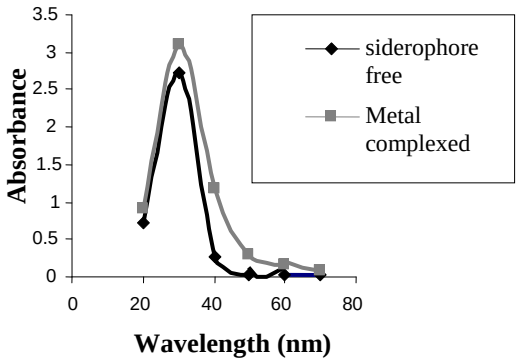
2F. Scan with lead and lead-siderophore complex



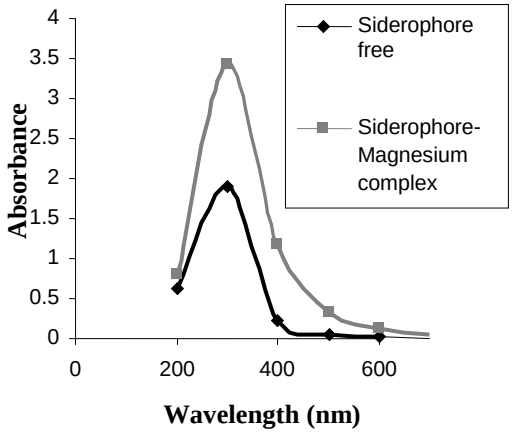
2G. Scan of Manganese and siderophore-Mn complex manganese complex



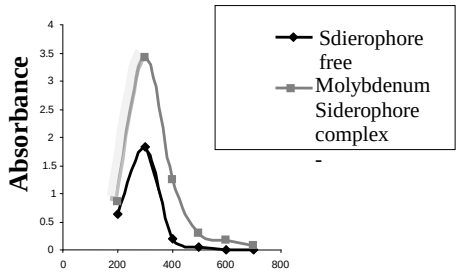
2H. Scan of Mercury and Mercury-siderophore complex



2I. Scan of Magnesium and Siderophore-Magnesium complex



J. Scan of Molybdenum and siderophores-Mo complex



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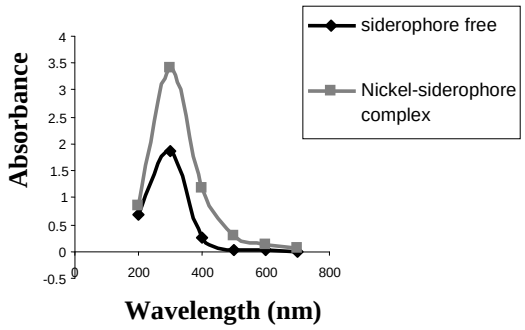
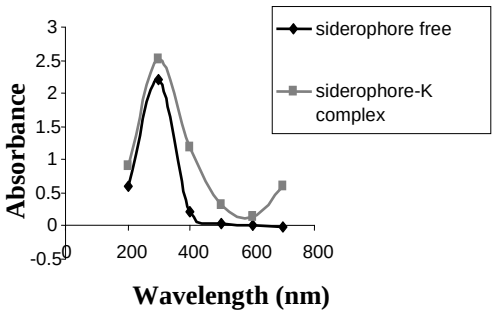
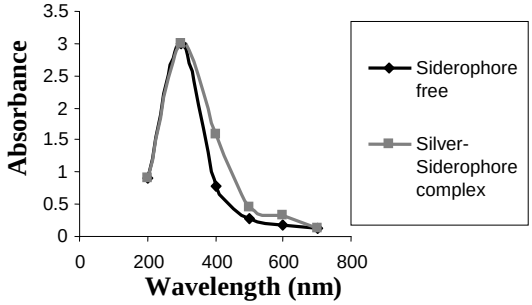
2K. Scan of Nickel and Nickel- siderophore complex



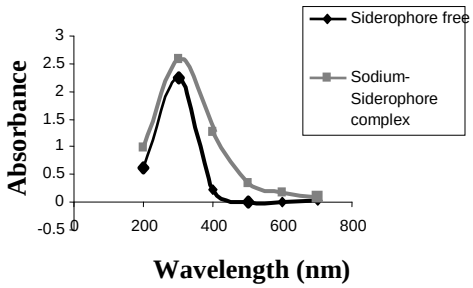
2L. Scan of Potassium and Siderophore – Potassium complex



2M. Scan of Silver and Silver- Siderophore complex



2N. Scan of Sodium and Siderophore-Sodium complex



2O. Scan of Zinc and Siderophore-Zinc complex

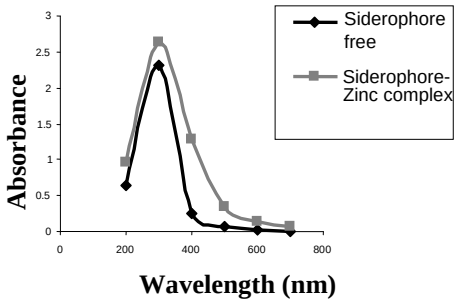


Figure 2. A – O. Spectral scan with respective metals in the presence and absence of the siderophore.

Presence of Potassium, Magnesium and Calcium had little inhibitory effect on siderophore production compared to controls (Table 4). It was reported by Nies (1999) that some of the metal cations play an important role as trace elements in biochemical reactions. Therefore pigment production and growth was not affected in presence of these metal ions.

Widening of FWHM on the longer wavelength side has been noted for most metals except iron (Fig. 2B). Fall in graph also indicates the ability of the siderophore to show absorption over longer wavelengths i.e. 500-700 nm (Table 2 and Figs. 2A to 2O). Full width at half maximum (FWHM) is a criterion that indicates complex formation (Griem, 1968). Widening of FWHM on the longer wavelength side indicates some sort of complex formation with the siderophore. It also indicates that the atomic, ionic and molecular densities have increased. Density, a measure of mass (Griem, 1968) had increased because of possible incorporation of the metal in the siderophore. Such an increase in FWHM has also been reported with respect to Iron citrate-Lotibactin, a siderophore synthesized by *Mesorhizobium loti* (Morton *et al..,* 2007). The fall is graph has been found to be exceptionally sharp in siderophore free metal solution.(Figs. 2A to 2O). Thus siderophore allowed the measurement of absorbance, which was not possible in the absence of the siderophore.

Molar extinction coefficients have been determined in partially purified extracts of siderophores after chloroform extraction (Demange *et al.,* 1988; Meyer and Abdallah, 1978). The extinction coefficient for Silver and Silver – siderophore complex remained unchanged indicating no complex formation as silver is a noble metal (Table 2 & Fig. 2M). Complex formation also changes the transmission coefficient of light. The extinction coefficient was reduced in presence of siderophore for most of metals except iron.

The role of alkalophilic bacterial siderophores in removal of iron, gallium and aluminium from culture media has been reported by Gascoyne *et al*. (1991). Chamongkolpradit *et al*. (2008) had reported that siderophores have

high affinity for Cd, Cu, Pb, Zn, Al. It was reported by several researchers (Khare *et al*., 1997; Sayyed *et al*., 2005; Rachid and Bensoltane, 2005) that siderophores are able to bind with metals such as Pb and Mo along with Fe. Parker *et al*. (2004) reported the scavenging of Manganese by pyoverdins.

It can be deduced that siderophore production is reduced at higher concentrations of iron (Table 3) and fluorescent siderophores are produced in the presence of some metal ions. It was also observed that it forms transient complexes with these metal ions as concluded from changes in λmax, FWHM and extinction coefficients.

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