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Effect of Dose-Response of Zinc and Manganese on Siderophores Production

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Abstract: Problem statement: This study was conducted to find and determine whether the siderophores of the four environmental *Pseudomonas* spp. isolates possess a sequestering activity towards essential transition metals (Zn and Mn) other than iron. **Approach:** Four fluorescent Pseudomonads isolated from various environments, were characterized analytically (Isoelectric focusing), biologically (pyoverdine-mediated uptake) and genetically (16S rDNA sequencing). By means of spectrophotometric measurements, it was possible to establish and compare the levels of pyoverdine production, in two different nutrient-poor media. **Results:** The strains were assigned, by sequencing, to *P. fluorescens*, *P. aeruginosa*, *P. putida* and *P. mosselii* isolated, respectively from soil, compost, sea water and waste water treatment plant. These bacterial strains were recognized as producing diver's yellow-green siderophores types, when grown under conditions of iron starvation. The highest metabolite concentration was obtained with PsC132 and PsTp171 strains isolated respectively from compost and waste water treatment plant, in CAA medium. Strains grown in CAA medium exhibit a higher PVD level compared to SM medium. Mn (II) was found to promote pyoverdine biosynthesis, but rather, Zn (II) had no significant effect on siderophore production when compared to control medium. For both strains PsS29 and PsC132, the increase of iron concentration quenched siderophore production especially above 20 µM. Pyoverdine level declined with the high concentration of zinc but increased with Manganese concentration ranging up to 70 µM (in case of PsC132) and 300 µM (in case of PsS29). **Conclusion/Recommendations:** The ability of fluorescent *Pseudomonas*, isolated from wastewater treatment plant and from compost, to sequester zinc, point to a unique advantage of these species for divers bioremediation applications.

Keywords: Fluorescent pseudomonads, heavy metals, iron-poor media, siderophore production, bacterial strains, control medium, bacterial strains, non-proliferating conditions

INTRODUCTION

A number of transition metals are needed by bacteria as vital constituents, but their availability in the environment may not suffice to support microbial growth state (Ambrosi *et al*., 2002; Adarsh *et al*., 2007). Some authors found that production of pyoverdines contributes to the bio-control capacity of the fluorescent *Pseudomonas*. Conversely, other studies failed to establish a link between production of pyoverdine and antagonism against phytopathogenic fungi Kumar *et al*.,2008; Saidi *et al*., 2009; Yang *et al*., 2009.

Pseudomonas spp. has been shown to produce siderophores able to chelate any available iron (Henry *et al*., 1991). Due to the critical need for iron in aerobic metabolism, bacteria living in neutral environments are normally faced to the nutritional iron deficit resulting from the low solubility of iron in its oxidized state (Winkelmann *et al*., 1987; Ambrosi *et al*., 2002). In order to satisfy their need to iron, microorganisms start to excrete large amounts of specific Fe^{3+} scavenging molecules (siderophores), when cells are grown under iron deficiency (Braun and Braun, 2002). The Fe (III)-siderophore complex is then transported into bacterial cell via cognate-specific receptor to enzymatic

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reduction (Meyer *et al*., 2000; Cornelis and Matthijs, 2002). Pyoverdine (PVD), the fluorescent siderophore produced by the rRNA groupI species of genus *Pseudomonas*, constitutes a large family of iron chelators (Wahyudi *et al*., 2011). This Yellow-green fluorescent pigment is composed of three structural parts: dihydroxyquinoline chromophore responsible of the fluorescence, a variable peptide part comprising 6 to 12 amino acids and a side chain, generally a dicarboxylic acid or a dicarboxylic amid (Meyer *et al*., 2000; Cornelis and Matthijs, 2002). As the peptide part interacts with specific cell surface receptors, pyoverdine type recognition allows for *Pseudomonas* strain classification (Meyer *et al*., 2002).

Some siderophores can bind, besides iron, other essential metals (K, Mg,) that serve as micronutrients, used for redox processes and regulation of osmotic pressure (Visca *et al*., 1992; Bruins *et al*., 2000; Parker *et al*., 2004; Shinozaki-Tajiri *et al*., 2004). Moreover, microorganisms able to produce siderophores can protect themselves by binding toxic metals (Al, Pb, Cd,) (Mureseanu *et al*., 2003; Olmo *et al*., 2003).

Although essential metals have important biological role, at high levels they can damage cell membranes, alter enzyme specificity, disrupt cellular functions, damage the DNA structure (Bruins *et al*., 2000; Canovas *et al*., 2003; Teitzel *et al*., 2006) and can reduce crop yields and soil fertility (Stuczynski *et al*., 2003).

The objectif of the research was to evaluate environmental *Pseudomonas* spp in siderophores sequestering activity towards essential transition metals (Zn and Mn) other than iron. The effect of pyoverdin production was studied in two metal poor mediums (succinate and casamino Acid). The dose-response effect of Fe (III), Zn (II) and Mn (II) was then tested on siderophore production.

MATERIALS AND METHODS

Isolation of *Pseudomonas* fluorescent strains: In this study the bacterial strains were isolated from various media: soil (PsS29), compost (PsC132), sea water (PsWs140) and waste water treatment plant (PsTp171).

Fluorescent pseudomonad colonies were isolated on King's B medium (Scharlau) and identified under UV light at 366 nm. Purified single colonies were further spread onto KB agar plates to obtain pure cultures. Stock cultures were made in Luria Bertani broth containing 50% (w/v) glycerol and stored at-80°C.

IEF analysis and PVD-mediated iron uptake: The iron-poor liquid Casamino Acid (CAA) growth medium used for this study was composed as follows (per liter): 5 g of low-iron Bacto Casamino Acid (Difco), 1.54 g of $K_2HPO_4 \cdot 3H_2O$ and 0.25 g of $MgSO_4 \cdot 7H_2O$. CAA medium was mainly used for PVD-IEF analysis and PVD purification through the Amberlite XAD-4 (XAD) procedure as previously described by Meyer *et al*. (2002). The cultures were incubated on a rotary shaker (200 rpm) at 25°C. The model 111 mini-IEF cell from Bio-Rad was used. Casting of the gels (5% polyacrylamide containing 2% Bio-Lyte 3/10 ampholytes) and electric focusing were performed according to the manufacturer's recommendations. One-microliter samples of PVDs (aqueous XAD-purified solutions (6.5 mg mL^{-1})), or of culture supernatants (40-h CAA-grown culture supernatant concentrated 20-fold by lyophilisation) were used in this experiment. PVD bands in the gel were visualized under UV light at 365 nm and photographed immediately after focusing. Their respective isoelectric pH values (pHi values) were determined with "Easy win 32" program as described by Fuchs *et al*. (2001). This allowed assigning each band to the corresponding pHi value. A mixture of seven known pyoverdines bands (3.95, 4.6, 5.2, 7.25, 7.75, 8.8 and 9.2) was used as internal pHi standard (Meyer *et al*., 2002).

The PVD-mediated iron uptake analysis was conducted as previously described by Meyer *et al*. (2002). Iron-starved cells were then incubated in succinate medium under non-proliferating conditions in the presence of a label mix containing ^{59}Fe-PVD complex. Aliquots of the bacterial suspension were withdrawn at different time intervals and rapidly filtered on 0.45 μm porosity membrane. Cells remaining on the filters were thoroughly washed and their radioactivity, measuring the amount of label iron incorporated during the incubation time, was determined using a Gamma 4000 Beckman radioactivity counter. Control assays without bacteria were performed simultaneously to verify the complete solubility of labelled iron through PVD complexation.

Partial sequencing of the 16S rDNA gene: PCR amplifications were performed using the following primers: forward primer Ps-for (5'-GGTCTGAGAGGATGATCAGT-3') and reverse primer Ps-rev (5'-TTAGCTCCACCTCGCGGC-3') for 16S rRNA gene.

The 16S rRNA gene PCR products were purified using the QIAquick Wizard PCR purification Kit (Promega, USA), according to manufacturer's instructions. Sequences of the PCR products obtained with Ps-for/Ps-rev primers were aligned and corrected manually with Chromas Pro (version 1.34). Similarity matrix of 16S rRNA gene sequences with closest neighbours and identification were achieved using RDP utilities (Ribosomal Database Project II: http://rdp.cme.msu.edu/html).

Pyoverdine assay: The media used in this investigation were the King's B, the succinate medium (SM, g L^{-1}: K_2HPO_4, 6.0; KH_2PO_4, 3.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 1.0; Succinic acid, 4.0; pH 7.0) and Casamino Acid media (CAA, g L^{-1}: $K_2HPO_4 \cdot 3H_2O$, 1.54; $MgSO_4 \cdot 7H_2O$, 0.25; Bacto Casamino Acid (Difco), 5.0; pH 7.0).

All these media were prepared with deionized water. To prevent siderophore interaction with other elements, glassware was cleaned in 6 M HCl and repeatedly rinsed with ultrapure water.

The culture broth was inoculated with actively grown culture (16 h in King's B medium) and grown in 60 mL of iron deficient succinate broth and CAA media at 28°C for 48 h under constant shaking of 150 rpm using an incubator shaker (ZHWY-2102 P). Samples of 1 mL were taken at 5, 10, 24, 36 and 48 h interval times respectively. Bacterial growth was estimated by spectrophotometry at 600 nm. The culture broth were then withdrawn and centrifuged at 10,000 rpm for 15 min at 4°C. Decimal dilutions of supernatants were done in deionised water. The amount of siderophores excreted into the culture medium was determined by spectrophotometry at 405 nm (Spectro UVS-2700 Dual BEAM LABOMED, INC) in 1 cm cells against media blank. Pyoverdin levels were expressed as the ratio of A405/A600 (Stintzi *et al*., 2000). Three repeated experiments were envisaged.

Effect of metal concentration on growth and pyoverdine production: Metal Salts were used in the following forms: $ZnSO_4 \cdot 7 H_2O$ (iron content, <10 ppm); $MnSO_4 \cdot H_2O$ (iron content, <0.001%) and $FeSO4 \cdot 7 H_2O$ (iron content, >99.9%). Stocks of 10mM $ZnSo_4$ and $MnSo_4$ salts were prepared and sterilized with 0.22 µm filters under aseptic condition. These stock solutions were incorporated in autoclaved CAA media (CAA + Zn and CAA + Mn) and SM media (SM + Zn and SM + Mn) at a final concentration of 60 µM for each metal.

In order to determine the threshold level of metals at which growth and/or siderophore biosynthesis are stimulated or repressed, PsS29 and PsC132 strains in CAA medium were monitored as a function of increasing amounts of Fe (III), Zn (II) and Mn (II) from 0.1-475 µM in 5 mL CAA medium.

RESULTS

Strain characterisation: In an attempt to assign isolates to bacterial species, the strains referenced as PsS29, PsC132, PsWs140 and PsTp171 and selected in this investigation, showed a 16S rDNA sequence related to four *Pseudomonas* species, namely: *P. fluorescens*, *P. aeruginosa*, *P. putida* and *P. mosselii*, respectively.

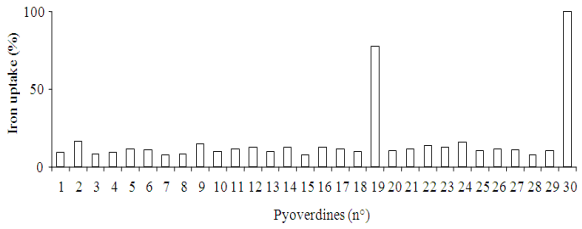
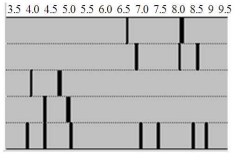


Fig. 1: Homologous and heterologous PVD-mediated ^{59}Fe incorporation by PsWs.140 strain. Ordinate values correspond to ^{59}Fe-radioactivity incorporated into the cells expressed in percentage, with 100% representing the incorporation obtained when using the homologous pyoverdine as ^{59}Fe-iron chelator (pyoverdine number 30 in abscissa). The other pyoverdines tested in abscissa (numbers 1 to 29 as alkaline pyoverdines correspond to the structurally different pyoverdines synthesized by the following bacterial strains: 1: *Pseudomonas* sp. G24, 2: *Pseudomonas* sp. G83, 3: *Pseudomonas* sp. G84, 4: *Pseudomonas* sp. G169, 5: *Pseudomonas* sp. CFML 96-312, 6: *Pseudomonas* sp. CFML 96-318, 7: *Pseudomonas* sp. CFML 95-275, 8: Lille25, 9: *Pseudomonas sp.* strain E8, 10: *P.fluorescens* SB8.3, 11: *Pseudomonas* sp. A6, 12: *P.fluorescens* PL8, 13: *P.Kilonensis*, 14: *P.fluorescens* ATCC 13525, 15: *P.fluorescens* 18.1, 16: *P.aeruginosa* PAO1, 17: *P.putida* CFML 90-136, 18: D46, 19: *P. putida* G168, 20: 96-192, 21: *P. libanensis* CFML 96-195, 22: *Pseudomonas* sp. PS6-10, 23: *P.putida* (Gwose), 24: Lille 40 (9AW), 25: *P.fluorescens* ATCC 17400, 26: *P.fluorescens* 1.3, 27: *Pseudomonas* sp. 96-319, 28: *Pseudomonas* sp. CHO59, 29: *Pseudomonas* sp. CIP 75.23

The NCBI Accession Numbers for the 16S rRNA gene sequences of the four isolates (PsS29, PsC132, PsWs140 and PsTp171) determined in this present study are respectively HM 627585, HM 627574, HM 627619 and HM 627603.

In order to investigate whether the four *Pseudomonas* species produce different pyoverdins, the siderotyping method was used. Table 1 illustrates the different PVD-IEF patterns upon analyzing the culture supernatants of the four strains grown under iron-deficient conditions (CAA medium). PsS29 and PsTp171 were characterized by acidic PVD-IEF profiles, with bands ranging between pHi 4.0 and 5.1. PVD-IEF profiles from strain PsWs140 produced two main bands (pHi 8.9 and 7.3) and a minor band with pHi value of 8.5.

Table 1: Pyoverdine isoelectrophoretic patterns, ^{59}Fe incorporation and 16S rDNA affiliation

Strains	PVD-IEF Patterns pHi	^{59}Fe uptake				Closest 16S rDNA Sequence
		PAO1	G168	PL9	LBSA1	
PsC. 132		+				*P. aeruginosa*
PsWs. 140			+			*P. putida*
Pss. 29				+		*P. fluorescens*
PsTp.171					+	*P. mosselii*
Internal pHi standard						

+: homologous incorporation. PAO1: *P. aeruginosa*, G168: *P. putida*, PL9: *Pseudomonas* spp. and LBSA1: *Pseudomonas* spp

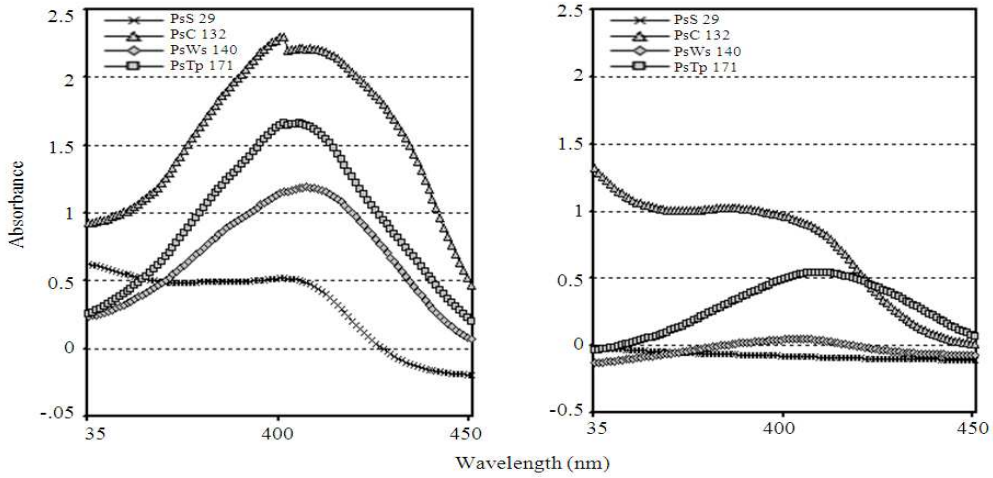


Fig. 2: Absorption spectra (350-450 nm) of the un-diluted supernatants from strains: *P. fluorescens* (PsS29), *P. aeruginosa* (PsC132), *P. putida* (PsWs140) and *P. mosselii* (PsTp171) CAA (a) and SM growth media (b)

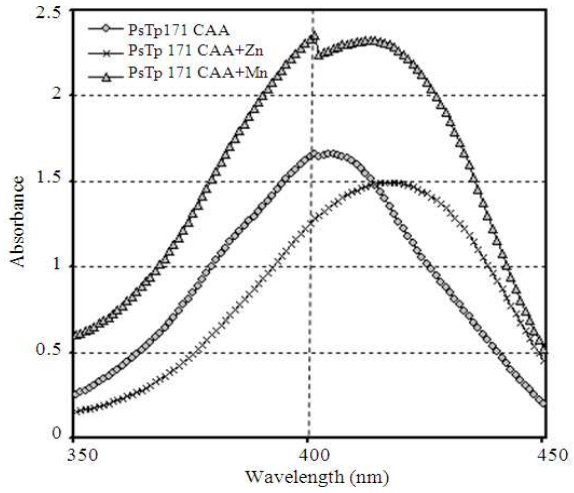


Fig. 3: Absorption spectra (350-450 nm) of the un-diluted clear supernatants from strain *P. mosselii* (PsTp171) grown in indicated media: CAA, CAA+Zn and CAA+Mn

The fourth strain (PsC132) showed two bands with pHi values of 8.5 and 6.9. (Table 1 demonstrate a schematic PVD pattern of the four tested strains).

For the purpose to confirm the classification reached by PVD-IEF characterization, the four strains were analysed for their capacity to incorporate iron under the form of a PVD-iron complex. The strains PsS29, PsTp171, PsWs140 and PsC132 cross-reacted with their own PVDs and with the type strains PL9, LBSA1, G168 and PAO1, respectively (Table 1). As example of homologous and heterologous ^{59}Fe incorporation, the strain PsWs140 showed primordially, a strict specificity of recognition toward its own pyoverdine. Figure 1 showed that the strain PsWs140 exclusively incorporated iron bound to its pyoverdine with a 100% efficiency (PVD number 30) indeed to the pyoverdine of *P. putida* strain G168 with a 78% efficiency (PVD number 19) (Data not shown for other strains).

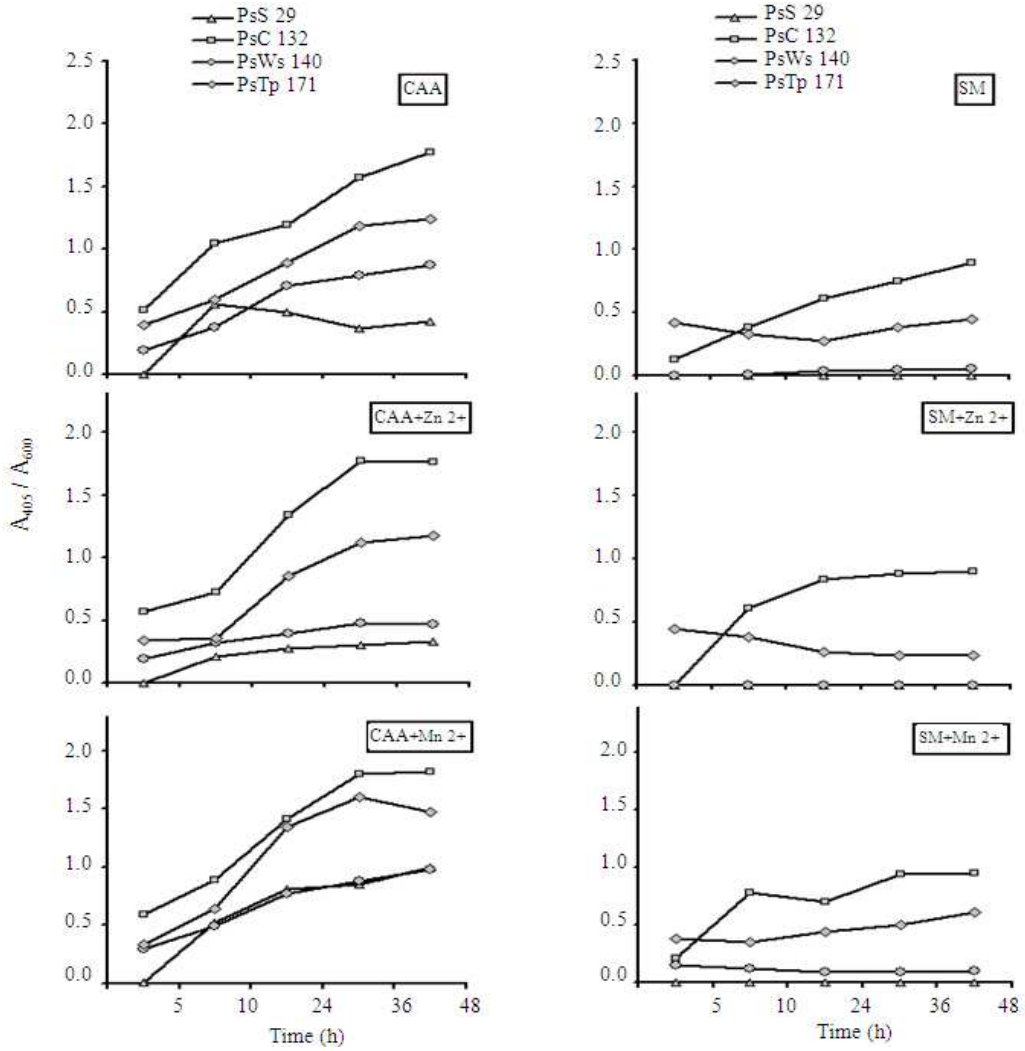


Fig. 4: Influence of supplementation of two trace elements (Zn and Mn) in CAA (a) and SM (b) media on pyoverdine levels of four different fluorescent *Pseudomonas* strains *P. fluorescens* (PsS29), *P. aeruginosa* (PsC132), *P. putida* (PsWs140) and *P. mosselii* (PsTp171) at different time intervals. The supernatants were diluted 1:10 in deionised water. Samples were collected in triplicate. A_{405}/A_{600} ratio expressed the pyoverdine level (Stintzi *et al*., 2000)

Strains growth related to pigment synthesis: Spectrophotometric analysis of the un-diluted bacteria supernatant showed an absorption area between 350 and 450 nm with a sharp peak at about 400 nm (Fig. 2), characterizing the PVD siderophore type. The maximum absorbance obtained for the strain PsS29 was at 400 nm. The other strains saved the maximum absorbance between 405 and 410 nm.

The determination of siderophore production by the strains used in this study allowed their separation in three types: PsC132 produced the highest siderophore concentration; followed by strains PsTp171 and PsWs140. The least siderophore production was obtained by PsS29 (Fig. 2a). The cells grown on the CAA medium presented siderophore content nearly 2.5-fold higher as compared to cells developed in SM medium. No PVD production was detectable for strain the PsS29 in SM medium (Fig. 2b).

Strains grown in CAA medium (Fig. 4a) exhibit a higher PVD level ranging from 1.98-fold (PsC132) to 15.26-fold (PsWs140) compared to SM medium (Fig. 4b). So, this is due to a significantly higher purity of the CAA medium (lower iron contamination).

Considering the PVD levels related to growth cycle (Fig. 4) the siderophore released by the studied strains in CAA and SM media started after 5h of incubation

and increased up to 36-48h then declined afterward. In SM medium, apart PsS29 enable to produce PVD, PsWs140 and PsTp171 strains showed no significant increase in the level of PVD during incubation time (Fig. 4b). The strains were found to produce maximum siderophore quantity during the stationary phase of culture growth (Sharma and Johri, 2003).

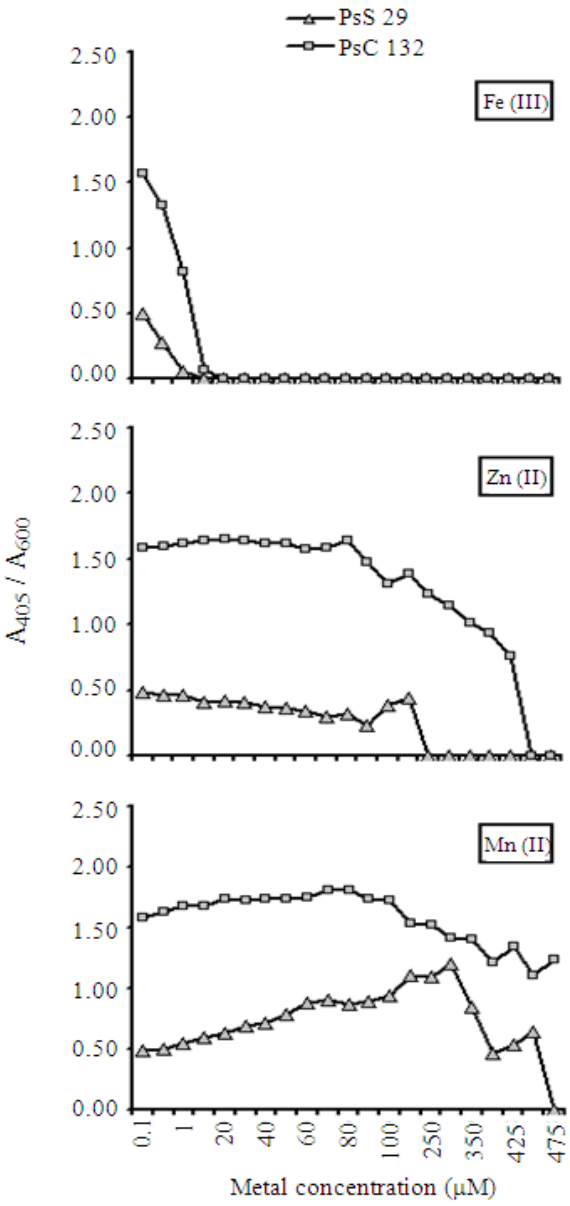


Fig. 5: Dose-response effect of various metals on pyoverdine levels of *P. fluorescens* (PsS29) and *P. aeruginosa* (PsC132) in CAA medium. A_{405}/A_{600} ratio expressed the pyoverdine level (Stintzi *et al*., 2000)

While studying the influence of heavy metals, it was observed that the presence of Mn^{2+} in the extracellular medium (CAA and SM) promoted significantly PVD production (Braud *et al*., 2009; Sharma and Johri, 2003). After 48h of growth, PsC132 present a slightly increase in siderophore level in CAA and SM media enriched with Mn supplementation (respectively 1.03-fold and 1.06-fold increase). The presence of 60 µM Mn^{2+} increased PVD production of PsTp171 strain by nearly 1.2-fold in CAA medium (Fig. 3 and 4a) and nearly 1.37-fold in SM medium (Fig. 4b). However, supplementing CAA and SM media with exogenous Zn2+, in case of PsTp171 strain, caused a decrease in siderophore levels (respectively 1.05-fold and 1.91-fold decrease) as compared to the control (CAA and SM without metal supplementation) (Fig. 3-5).

DISCUSSION

The identification based on the 16S rDNA sequencing was reconfirmed by using the siderotyping, an easy and powerful method, giving a rapid discrimination between fluorescent pseudomonads producing a particular pyoverdin (Meyer *et al*., 2002). Therefore, the four studied strains produced compounds belonging to four different siderovars indicating the presence of various pyoverdin structures. The 59Fe incorporation technique confirms this data. The detection of different siderovars confirms the diversity of the four strains used.

The determination of the absorbance of the clear supernatants obtained from all cultures developed for 48 h in CAA and SM medium was performed to determine whether the maximum absorbance was at 400 nm. As shown in Fig. 2, the maximum absorbance varied from 400 to 410 nm which may indicate the diversity of compounds produced by these strains. This multiplicity may be due to the nature and the number of the aminoacyl residues in the peptide moiety (Carrillo-Castaneda *et al*., 2005).

Moreover, the level of siderophore production was compared in the two medium. The pyoverdine production was greater in CAA media. Numerous investigations have shown that the synthesis of PVDs by fluorescent pseudomonads was affected by different environmental factors, notably the chemical nature of the organic carbon and energy source, the degree of aeration of the growth medium, pH, light and trace elements (Gouda and Greppin, 1965; Meyer *et al*., 1978). Although the different media usually have varied levels of iron contamination, Sharma and Johri (2003) suggested that synthetic media are in all cases better than the complex medium for siderophore production.

Moreover, Carrillo-Castaneda *et al*. (2005) demonstrated that iron concentration in the growth medium is an important nutritional factor which determines siderophore biosynthesis.

Strains PsTp171 and PsC132, producing the largest amounts of pyoverdine are isolated respectively from wastewater treatment plant and from compost. Several authors noted the important role of strains isolated from these origins in bioaccumulation of heavy metals. Isolates from complex sources were faced frequently to compete towards micronutrients such as heavy metals via siderophore production (Lovley *et al*., 1997; Hassen *et al*., 2001). Hussein *et al*. (2005) noted that a group of *Pseudomonas* sp. isolated from the effluent of wastewater treatment plant in western Alexandria, possess the ability to tolerate and to uptake different heavy metals (Cu (II), Ni (II)).

Metal ions have definite influence on siderophore production. While Mn^{2+} increased the siderophore production, Zn^{2+} decreased this production. This result is consistent with the work of Sayyed *et al*. (2005), who carried out a notably decrease in the amount of siderophore produced in the case of SM media supplemented with Zn^{2+}. Authors reported that the metal ion can substitute Fe^{2+} in the intracellular control of siderophoregenesis. In addition, Braud *et al*. (2010) indicated that besides pyochelin, PVD is able to sequester metals from the extracellular medium of the bacteria, decreasing metal diffusion into the bacteria. In their study, PVD was able to sequester Al^{3+}, Co^{2+}, Cu^{2+}, Eu^{3+}, Ni^{2+}, Pb^{2+}, Tb^{3+} and Zn^{2+} from the extracellular medium. Baysse *et al*. (2000) noted that the repression of pyoverdin production by vanadium and explained that uptake of several metals by siderophores was possible.

The dose-response effect of heavy metals on PVD level by the highest (PsC132) and the lowest (PsS29) siderophore producing strains was investigated using the CAA medium growth. Results show that pyoverdine production was inversely related to Fe^{3+} concentration, whereas growth was directly proportional to iron concentration (Meyer *et al*., 1978; Visca *et al*., 1992; Villegas *et al*., 2002; Manwar *et al*., 2004). Djibaoui and Bensoltane (2005) observed a complete decline of siderophore production with 200 µg/L of iron as threshold level. This fact reflects the iron requirement for microbiological cellular processes (Sayyed *et al*., 2005).

On the other hand, concentrations higher then 250 µM of zinc declined the siderophore production. Visca *et al*. (1992) demonstrated that the pyoverdine production was not affected by metals (Zn(II), Mo(VI), Co(II), Ni(II) and Cu(II)) at concentrations up to 10 µM but was repressed at higher concentrations. Therefore, PsC.132 and PsS.29 were highly tolerant to Mn since their pyoverdine production was completely repressed only by high Mn concentrations (> 475 µM).

CONCLUSION

This study revealed by means of the spectrophotometry measurement, classification of environmental strains by levels of siderophore production. Both fluorescent Pseudomonads, *P. aeruginosa* and *P. mosselii* were able to provide higher yields of PVDs especially in CAA medium and in media supplemented with Mn^{2+}. Moreover, at concentration >20 mM of iron, siderophore biosynthesis is completely quenched.

In culture medium supplemented with zinc, the amount of PVD is lower than that excreted in control medium (no added metal). This result indicates that pyoverdins of studied strains might be able to complex zinc instead of iron. These strains would have the ability to chelae one of essential component and make it inaccessible to other bacteria (competition phenomenon). In addition, this property could be used in the case of decontamination area contaminated with an excess of zinc. Bioremediation using bioaccumulation and chelating of heavy metals contaminating industrial waste may be an alternative processes and/or additives to conventional methods (physical and chemical). Therefore, the ability of fluorescent *Pseudomonas*, isolated from wastewater treatment plant and from compost, to sequester zinc, point to a unique advantage of these species for divers bioremediation applications.

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