Bioassay, characterization and estimation of siderophores from some important antagonistic fungi

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ABSTRACT

The primary mechanisms of plant disease suppression by biological control agents are by production of antimicrobial secondary metabolites like siderophores, antibiotics, volatile substances etc. Siderophores are low molecular iron chelating compounds produced by fungi and bacteria under iron stress condition. The present study is focused on the detection, estimation and characterisation of siderophores from different soil borne fungal biocontrol agents like *Trichoderma* spp., *Beauveria* spp. and *Metarhizium* spp. Isolates of *Trichoderma* have shown positive results in CAS agar plate whereas *Beauveria* and *Metarhizium* have shown negative results. The isolates with positive results were opted for characterization and quantitative estimation of siderophore. *T. harzianum* produced a maximum percentage of siderophore (85.00%), followed by *T. viride* (65.50%), *T. asperellum* (60.27%) and *T. longibrachiatum* (45.50%). While studying the typification of siderophores, it was found that *T. harzianum* recorded with maximum hydroxymate and carboxylate production whereas *T. viride*, *T. asperellum* and *T. longibrachiatum* recorded with lesser production of hydroxymate and carboxylate as confirmed by color intensity. But none of the isolates was recorded with catecholate production. Through spectrophotometric analysis, it has been found that slightly alkaline pH was more favorable for the siderophore production for different spp. of *Trichoderma*. The productions of siderophore from *Trichoderma* spp. not only scavenge iron which lead to inactivation of those enzymes of the pathogen where iron plays the critical role as cofactor which supplies that iron to the host plant resulting in plant growth promotion. Siderophore also has a high degree of influence on medical science in MRI imaging. So mapping of different strains of *Trichoderma* with high antagonistic efficacy along with profound potency of siderophore production will encourage eco friendly management of fungal diseases on crops, crop improvement and enrichment of medical science.

Key words: antagonistic fungi, siderophore, pH, crop improvement.

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INTRODUCTION

Siderophores are generally produced by microorganisms, both aerobic and facultative anaerobic and monocotyledonous plants under low-iron stress conditions (Ratledge and Dover, 2000). They have less than 1000 daltons molecular weight and are extracellular in nature, which selectively bind iron (Fe^{3+}). The production of siderophores by plant growth promoting microbes (PGPM) and biocontrol agents (BGA) are the important mechanisms for plant growth promotion (Kloepper *et al.,* 1980, Sayyed *et al.,* 2005) and disease suppression (Eland and Baker, 1985; Seuk *et al.,* 1988; Buysense *et al.,* 1996; Husen, 2003). To combat plant pathogens, siderophore producing bacteria are often used as biocontrol agents (Gram, 1996). A pivotal role in the energy metabolism in aerobic and semi-aerobic microorganisms has been regulated by siderophores (Schippers, 1988). With increasing pH (> 6), the amount of siderophore attenuates gradually in soil for microorganisms and plants. The first report of a siderophore production came into limelight from *Ustilago sphaerogena* (Neilands, 1952). After that several fungi and bacteria were reported with siderophore production. Competition for iron among microorganisms is carried out by releasing siderophores (Leong and Rev, 1986). Typically, microbial sideophores are classified into three major groups namely catecholares, hydroxamates and carboxylates, depending on the chemical nature of their coordination sites with iron (Winkelmann, 2002). Some sideophores are reported as phenolates (Hagg *et al.*, 1993) while others as mixed (both hydroxamate and catecholate functional groups) (Meyer and Abdallah, 1978).

Recently, in addition to agrifields (Kleopper *et al.*, 1980), microbial siderophores are utilized in medical science for antibiotic preparation (Trojan horse antibiotics) (Vertesy *et al.*, 1995; Benz *et al.*, 1982), in MRI (Magnetic Resonance Imaging) technique (Doble *et al.*, 2003) in cancer therapy (Miethke and Marahiel, 2007), as antimalarial therapeutics (Gysin and Crenn, 1991), antisleeping sickness (Breidbach and Scory, 2002)

The main objectives of this study were to screen some microbes for their ability to produce siderophore, characterization and quantitative assay of it.

MATERIALS AND METHODS

In our laboratory, previously three species of *Trichoderma* were isolated and characterized growing on PDA (Potato Dextrose Agar) at 28±1ºC for 7-days in BOD and identified phenol typically following published keys of Domsch *et al.*, 1980 and Nagamoni *et al.*, 2006 and through nucleotide sequencing (PCR based ITS1-5.8s-ITS2 of rDNA) followed by NCBI BLAST. They were published in NCBI Genbank as *Trichoderma asperellum* (GenBank accession no. KY966022), *Trichoderma viride* (GenBank accession no. KY966032), *Trichoderma harzianum* (GenBank accession no. KY966020). *Trichoderma longibrachiatum* was Phenotypically characterized, identified and deposited into IMTECH, Chandigarh, India under Accession no. MTCC11582. All three strains of *Beauveria bassiana* were molecularly identified earlier in this laboratory, deposited and published in NCBI database as SKG BB 2012 / SILKWORM (GenBank: accession no. KM604668.1) (BB1); SKG/BB/2014/LC1(GenBank: accession no. KM491553.1) (BB5). *Beauveria bassiana* 13, *Metarhizium anisopliae* 3 and *Metarhizium anisopliae* 6 were deposited to the Microbial Culture Centre of Ramakrishna Mission Vivekananda Centenary College as BB13, MAT3 and MAT6 respectively. Although they are different in their morphological and cultural characteristics their molecular characterization has not yet been established.

In vitro detection of siderophores

Chrome Azurol S (CAS) agar method is generally used to detect the mobilization of iron. For detection of siderophore production antagonistic fungi were cultured on PDA plates. After growing on plates, 5mm fungal mats from each isolate were scooped out from PDA plate and placed on CAS agar plate following the protocol of Schwyn and Neiland (1987) with a few modifications and incubated in BOD at $28±1^0$C for seven days. Fe-CAS indicator gave the medium a blue color. When the iron ligand complex was formed the release of the free dye was accompanied with a color change. Iron mobilization was done via the production of complex acids or siderophores. The Fe (III) gave the agar a rich blue color and concentration of siderophores excreted by iron starved organisms gave a color change to orange. The orange hallow surrounding the colony indicated the excretion of siderophore and its dimension evaluated the amount of siderophore excreted.

Characterization of siderophores

For characterization of siderophores, fresh cultures of the antagonistic fungi with siderophore producing property were inoculated into Grimm-Allen broth media (K_2So_4 1gm, Ammonium acetate 3 gms, K_2HPo_4 3gms, Citric acid 1gm, Sucrose 20gms, Water 1000ml, pH 6.8, Medium supplemented with thiamine, Cu++,Mn++,Zn++ and Mg++) and incubated at

30°C for 15 days in order to classify ther subsequent siderophore.

Glassware preparation

To avoid iron contamination all glass wares were kept in 6M HCl overnight and rinsed with double distilled water several times to remove any iron trace. Further removal of iron trace was achieved by adding 8 hydroxyproline dissolved in chloroform to the media. Repeated washing of the media with chloroform was done to ensure complete removal of iron and residual of 8 hydroxyquinoline which could inhibit fungal growth (Messenger and Ratledge, 1985).

Fifteen day old culture incubated at 28±1C was centrifuged and cell free supernatants were used for assay of hydroxymate, catecholate and carboxylate nature of siderophore by following the test.

Detection of hydroxymate nature of siderophore

Tetrazolium Test (Xichung and Boyer 1996)

The property of hydroxymic acid to reduce tetrazolium by hydrolysis of hydroxymate groups using a strong alkali was adopted. The reduction and release of alkali were confirmed with the formation of red colour. To a pinch of tetrazolium salt, 1-2 drops of 2N NaOH and 0.5 ml of the test sample were added. Presence of hydroxymate siderophore was confirmed with instant appearance of a deep red coloration.

Detection of catecholate nature of siderophores

One mL of freshly prepared 2% $FeCl_3$ was added to 1 mL of the culture supernatant. Development of wine color indicated catecholate nature of siderophores.

Detection of Carboxylate nature of siderophores

Disappearance of pink colour after addition of 1mL of fungal culture filtrate to pink coloured solution of 3 drops of NaOH and 1 drop of phenolpthalein indicates presence of carboxylate siderophores (Vogel, 1992).

Detection of mono di and trihydroxymate nature of hydroxymate siderophores

pH dependent absorption maxima was analyzed for the fungi producing hydroxymate siderophores to distinguish mono, di and tri hydroxymates. Spectrophotometrical analysis of ferric complexes for a shift in λ max (nm) at different pH was done (Jalal *et al.,* 1990). Trihydroxymates showed little or no shift when pH ranges from 4-7. Dihydroxymates dissociated at pH 4-5 and showed wide shift. Monohydroxymates showed a shift when pH dropped to 4 (500-520 nm).

Estimation of siderophore

To estimate the production of siderophore cell free supernatant of the fungal cultures were subjected to CAS-Shuttle assay (Pyne, 1993; Payne, 1994). The culture supernatant of 0.5mL was mixed with 0.5 ml of CAS reagent and absorbance was measured at 630 nm against a reference of 0.5 mL of uninoculated broth and 0.5 mL of CAS reagent. Siderophore content in the supernatant was calculated by using following the formula:

% of siderophore units= $A_r - A_S / A_r \times 100$

where Ar = absorbance of reference at 630 nm and As = Absorbance of sample at 630 nm.

RESULTS AND DISCUSSION

The data presented in Table 1 revealed that all the four potent antagonistic spp. of *Trichoderma* namely *T. viride, T. harzianum, T.longibrachiatum* and *T. asperellum* produced considerable amount of siderophore production but all three isolates of *Beauveria bassiana* and two isolates (MAT3 and MAT6) of *M. anisoplae* didn't produce siderophore.

Table 1. In vitro detection of siderophore production on CAS agar plate by antagonistic fungi

Sample	Siderophore production
Trichoderma viride	+++
Trichoderma harzianum	++
Trichoderma longibrachiatum	++
Trichoderma asperellum	++
Beauveria bassiana BB 13	-
Beauveria bassiana BB5	-
Beauveria bassiana BB 1	-
Metarhizium anisopliae MAT6	-
Metarhizium anisopliae MAT3	-

*For each set of experiment, 3 replicas were done. +++ indicates more amount of production of siderophore whereas ++ indicates mediocre amount of siderophore production and – indicates no production of siderophore.

Table 2. Quantitative production of siderophores by different microorganisms:

Antagonist	Siderophore % ±SD
Trichoderma viride	65.50 ±0.18 (54.03)
Trichoderma harzianum	85.00 ±0.37 (67.21)
Trichoderma longibrachiatum	45.50±0.630 (42.42)
Trichoderma asperellum	60.27±0.875 (50.89)

*For each set of experiment, 3 replicas were done. Value within the parenthesis denotes the angular transformational value

The results presented in Table 2 showed that *T. harzianum* produced maximum percentage of siderophores than by *T.viride*, *T.asperellum* and *T. longibrachiatum*. Different organisms produced different percentage of siderophores in their culture as reported by many authors (Breidbach *et al.*, 2002; Hussain *et al.*, 2012). Hussein and Joo (2012) reported that *T. harzianum* produced 92.33% of siderophores in MEB medium. In our study *T. harzianum* also exhibited comparable result. Moreover, other mineral factors such as Zn^{2+} and Cu^{2+} also influence fluorescent siderophore production in medium (Dimpka, 2009). Promotion of siderophore production was also induced by Cu^{2+} and Ni^{2+} in *P. fluorescence putida* (Chakraborty and Roy, 1964). Hussain and Joo (2012) also observed that *Aspergillus niger* produced 87.99%, 87.99%of siderophores. All the four isolated species of *Trichoderma* produced hydroxymate and carboxylate types of siderophores, whereas none of them was able to produce catecholate type as wine colour was not developed after performing the confirmatory test for catecholate type (Table 3). It was also recorded that *T. harzianum* produced maximum amount of hydroxymate in comparison to *T. viride*, *T. longibrachiatum* and *Trichoderma asperellum* as the pink color intensity was noted to be the highest after experimentation with culture filtrate of *T. harzianum*. In case of carboxylate, *T. harzianum* was again recorded with maximum production as the pink colour of the phenolpthalin reagent disappeared in maximum amount in comparison to the culture filtrates of *T. viride*, *Trichoderma asperellum* and *T. longibrachiatum*). In the previous work of Ghosh *et.al.* (2015), the effect of different media on siderophore production by *T. viride*, *T. harzianum*, *Bacillus megatericus*, *B. subtilis*, *Pseudomonas aeruginosa* and *Candida famata* was recorded. On the basis of shift in λ max as a result of pH change of growth medium the results indicated that dihydroxymates were most common with shifts up to 24 in λ max (*T. viride*, *T. harzianum* and *T. asperellum* recorded with a λ max shift of 24, 21 and 24 respectively) whereas trihydroxymates showed little change in λ max (*T. longibrachiatum* recorded with λ max shift of 8) (Table 4).

Table 3. Characterization of different kinds of siderophores produced by antagonistic fungi:

Sample	Hydroxymate	Catecholate	Carboxylate
Trichoderma viride	++	-	+
Trichoderma harzianum	+++	-	+++
Trichoderma longibrachiatum	+	-	+
Trichoderma asperellum	++	-	++

*For each set of experiment, 3 replicas were done; + indicates low production of hydroxymate, catecholate and carboxylate type of siderophores.++ sign denotes mediocre amount of subsequent siderophore production, +++ signifies high production of siderophores. whereas – indicates no production of subsequent siderophores.

In conclusion, among the tested fungi all spp. of Trichoderma namely *Trichoderma viride, T. harzianum, T. asperellum* and *T. longibrachiatum* produced siderophores in qualitative test on CAS agar medium and quantitative test in Grim-Allen broth media. These three fungi produced 45.50-85.00% of siderophore in Grim-Allen medium. *T.harzianum* produced maximum percentage

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Table 4. Detection of mono, di and tri hydroxamates

Test Fungi	λ max (nm) of ferrate siderophores		λ max shift	Inference
	pH	**λmax (nm)**		
Trichoderma viride	4	-	24	Dihydroxymate
	5	410		
	6	425		
	7	428		
	8	430		
	9	434		
Trichoderma harzianum	4	-	21	Dihydroxymate
	5	424		
	6	435		
	7	429		
	8	414		
	9	426		
Trichoderma longibrachiatum	4	-	8	Trihydroxymate
	5	414		
	6	417		
	7	422		
	8	421		
	9	-		
Trichoderma asperellum	4	-	24	Dihydroxymate
	5	412		
	6	422		
	7	428		
	8	436		
	9	429		

of siderophores than *T.viride, T. asperellum* and *T. longibrachiatum*. While studying the charaterisation of siderophores it was observed that *T. harzianum* produced maximum hydroxymate and carboxylate whereas *T. viride*, *T. asperellum* and *T. longibrachiatum* gave lesser yield of hydroxymate and carboxylate as confirmed by color intensity.

While going into further analysis among the hydroxymate types, *T. longibrachiatum* was only analyzed with the production of trihydroxymate type of siderophores, whereas *T. viride, T. harzianum* and *T. asperellum* were characterized with the dihydroxymates, as confirmed through spectrophotometritc analysis. Therefore, this study indicates that the ability of siderophore production by these microbes, which are universally recognized biocontrol and plant growth promoting agents, is in appreciable amount;. Modern application of siderophores in agriculture, medical science and environment science is increasing. This study may have a huge impact in international perspective as it may encourage more production of siderophore commercially and more utilization of it in modern science.

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REFERRENCES

Arnow, L.E.1937. Colorimetric determination of the components of 3, 4 –dihydroxyphenylalanine tyrosine mixtures. *The Journal of Biological Chemistry,* **118**: 531–537.

Benz, G., Schroder, T., Kurz, J., Wunsche, C., Karl, W., Steffens, G., Pfitzner, J. and Schmidt, D., 1982. Konstitution der desferriform der albomycine d1, d2 and c. Angewandte Chemae International Edition, 94: 552-553 Suppl., 1322-1335.

Breidbach, T., Scory, S., Krauth-Siegel, R.L. and Steverding, D. 2002. Growth inhibition of bloodstream forms of *Trypanosoma brucei* by the iron chelator deferoxamine. *International Journal of Parasitology*, **32**(4): 473-479.

Buysens, K., Heungens, J. and Poppe Hofte, M.1996. Involvement of pyochelin and Pyoverdin in suppression of *pythium*-induced Damping-Off of Tomato by Pseudomonas aeruginosa 7NSK2. *Applied and Environmental Microbiology,* **62**(3): 865-871.

Chakrabarty, A.M. and Roy, S.C. 1964. Nature of endogenous reserve material in a strain of *Pseudomonas fluorescens-putida. International Journal of Biochemistry in jaban*,**92**: 105-112.

Dimpka, C.O., Merten, D., Svatos, A., Buchel, G. and Kothe, E. 2009. Metal-induced oxidative stress impacting plant growth in contaminated soil is alleviated by microbial siderophores. *Soil Biology and Biochemistry*, **41**(1): 154-162.

Doble, D.M. J., Melchior, M., Osullivan, B., Siering, C., Xu, J., Pierre, V. C. and Raymond, K. N. 2003. Toward optimized high-relaxivity MRI agents: the effect of ligand basicity on the thermodynamic stability of hexadentate hydroxypyridonate/catecholate gadolinium (III) complexes. *Inorganic Chemistry*, **42**: 4930-4937.

Domsch KH, Gams W and Anderson TH. Compendium of soil Fungi. Vol –I and II, *Academic Press, London*, 1980.

Eland, Y., and Baker, R. 1985. Influence of trace amounts of cations and iderophore-producing pseudomonads on chlamydospore germination of Fusarium oxysporum. *Infection Ecology and Epidemiology,* **75**: 1047-1052.

Ghosh, S.K., Pal, S. and Chakraborty, N. 2015. The qualitative and quantitative assay of siderophore production by some microorganisms and effect of different media on its production. *International Journal of Chemical Science*, **13**(4): 2015, 1621-1629.

Gram, L.J. 1996. The influence of substrate on siderophore production by fish spoilage bacteria. *Journal of Microbiological Methods,* **25**(3): 199-205.

Gysin, J., Crenn, Y., Da Silva, P. and Luiz, B. 1991. Siderophores as anti parasitic agents. Catherine, *United States Patent,* **5**:192-807.

Haag, H., Hankte, K., Drechsel, H., Stojiljkovic, I., Jung, G. and Zahner, H. 1993. Purification of yersiniabactin: a siderophore and possible virulence factor of *Yersinia enterocolitica*. *Journal of General Microbiology*, **139**: 2159-2165.

Husen, E. 2003. Screening of soil bacteria for plant growth promotion activities in vitro, *The Indian Journal of Agricultural Science,* **4**(1): 27-31.

Hussein, K.A. and Joo, J. H. 2012. Potential of siderophore production by bacteria isolated from heavy metal: polluted and rhizosphere soils. *Korean Journal of Soil Science and Fertiliser,* **45**(5): 798-804.

Jalal, M.A.F. and Dick, V.D.H.1990. In handbook of microbial iron chelates, edited by G.G. Winkelmann, CRC Press, pp15.

Kloepper, J.W., Leong, J., Teinize, M. and Schroth, M.N. 1980. Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. *Nature,* **286**:885-886.

Leong, A.J. 1986. Siderophores: their biochemistry and possible role in the biocontrol of plant pathogens. *Annual Review of Phytopathology,* **24**: 187-209.

Messenger, A.J.M. and Ratledge, C. 1985. Comprehensive Biotechnology. Vol 3, edited by M.Moo-Young (*Pergamon Press*). pp 275.

Meyer, J.M. and Abdallah, M.A. 1978. The fluorescent pigment of pseudomonas fluorescens: biosynthesis, purification and physicochemical properties. *Journal of General Microbiology***,** **107**: 319.

Miethke, M. and Marahiel, M.A. 2007. Siderophore-based iron acquisition and pathogen control. *Microbiology and Molecular Biology Review,* **71**(3): 413-461.

Milagres, A.M., Machuca, A. andNapoleao, D. 1999. Detection of siderophore production from several fungi and bacteria by a modification of chrome azurol S (CAS) agar plate assay. *Journal of Microbiological Methods,* **37**: 1-6.

Nagamani A, Kunwar IK, Manoharachary C. Hand book of Soil Fungi. *I.K. International, New Delhi,* 2006, 477.

Neiland, J.B.1981. Microbial iron transport compounds (siderophores) as chelating agents, In development of iron chelators for clinical use. Edited by A. E. Martwell, W.J.Anderson and J.D. Madman (*Elsevier Press, North Holland. Amsterdam*).

Neilands, J.B. 1952. A Crystalline Organo-iron pigment from a rust fungus (Ustilago sphaerogena). *Journal of The American Chemical Society,* **74**: 4846.

Payne, S.M. 1994. Detection, isolation, and characterization of siderophores. *Methods in Enzymology***,** **235**:329-344.

Pyne, S.M. 1993. Iron acquisition in microbial pathogenesis. *Trends in Microbiology,* **1**: 66-69.

Ratledge, C. and Dover L G. 2000. Iron metabolism in pathogenic bacteria. *Annual Review of Microbiology,* **54**: 881-941.

Sayyed, R. Z., Badgujar, M, D,, Sonawane, H,M,, Mhaske, M.M. and Chincholkar, S.B. 2005. Production of microbial iron chelators (siderophores) by fluorescent Pseudomonads. *Indian Journal of Biotechnology,* **4**, 484-490.

Schippers, B. 1988. Philosophical Transactions of the Royal London. B, 318, 283-293.

Schwyn, B. and Neiland. J.B. 1987. Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry,***160**:47–56.

Seuk, C., Paulita, T. and Baker, R. 1988. Attributes associate with increased bio-control activity of fluorescent Pseudomonads. Journal of Plant Pathology,4(3): 218-225.

Vertesy, L., Aretz, W., Fehlhaber, H.W., Kogler, H. 1995. Salmycin A–D, *Antibiotika aus Streptomyces violaceus,* DSM 8286, mit Siderophor-Aminoglycosid-Struktur. *Helvetica Chimica Acta,* **78**: 46-60.

Vogel, A.L. 1992. In elementary practical organic chemistry (*CBS Publishers*). 2190

Winkelmann, G. 2002.Microbial siderophore mediated transport. *Biochemical Society Transactions*, **30**: 691-695.

Xychung, H. and Boyer, G.L. 1996. Siderophore-Mediated Aluminum Uptake by *Bacillus megaterium* ATCC 19213. *Annalitycal Chemistry*, **68**: 1812.

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