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Chemical Characterization and Antifungal Activity of Siderophores of *Pseudomonas stutzeri* EGB_3 Isolated from the GUT of Earthworm (*Eisenia foetida)*

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***Pseudomonas stutzeri, an* Earthworm (*Eisenia foetida)* gut bacteria produces extracelluar siderophores when grown in succinic acid medium under iron deficiency. The siderophores were found to be pyochelin type. These siderophores were antagonistic to fungal pathogens like *Alternaria alternata*, *Fusarium oxysporum*, *F.solani*, *F.moniliformae*, *F.udum*, *Macrophomena phaseolina*, *Rhizoctonia solani*, *Colletotrichum capsicii*, *Aspergillus flavus* and *A.niger*.**

Key words: Siderophores, *Pseudomonas stutzeri,* phytopathogens, pyochelin, antagonistic activity.

Living organisms require iron as a component of proteins involved in important life processes such as respiration, photosynthesis and nitrogen fixation. Iron is one of the major elements in the earth's crust but soil organisms such as plants and microbes have difficulty in obtaining sufficient iron to support their growth because of the formation of ferric oxides under aerobic conditions, which cannot be readily transported into cells. Under such iron starvation bacteria, fungi and plants secrete small, specialized efficient iron (III) chelator molecules commonly known as siderophores[1]. Lankford[2] coined the term siderophore to describe low molecular weight (approximately 600 to 1500 daltons) molecules that bind ferric iron with an extremely high affinity. Siderophore was derived from a Greek term meaning "iron carrier"[3]. The dominant iron-binding ligands of siderophores are hydroxamates and catecholates (phenolates), but carboxylate, oxazoline, α-hydroxy carboxylate and keto hydroxyl bidentate siderophores have also been found[4]. In addition, hybrid siderophores with more than one type of ligand group exist. Many bacteria are capable of producing more than one type of siderophore or have more than one iron-uptake system to take up multiple siderophores[5]. Wide arrays of beneficial plant-associated bacterial genera, *e.g*. *Pseudomonas, Azotobacter, Bacillus*, *Enterobacter*, *Serratia*, *Azospirillum* and *Rhizobium* secrete various types of siderophores[6,7].

Siderophores produced by certain strains of fluorescent *Pseudomonas* spp. have been linked to suppression of soil-borne plant diseases. It has been suggested that siderophores act antagonistically by sequestering iron from the environment, restricting growth of the pathogen. Convincing evidence for the involvement of siderophores in disease suppression is readily available[8]. Thus, disease suppression under controlled laboratory conditions is only an indication of the efficacy of the biocontrol agent in the field. *Pseudomonas* siderophores have also been implicated in inducing systemic resistance (ISR) in plants[9], *i.e*. enhancement of the defence

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capacity of the plant against a broad spectrum of pathogens. Siderophores produced by biocontrol agents are pyoverdin, salicylic acid and pyochelin. Some siderophores are also good chelators of some elements other than iron. For example, pyochelin is a good Cu^{2+} and Zn^{2+} chelator[10] and when these elements are increasingly made available to the bacteria[11]. Siderophores may directly stimulate the production of other anti-microbial compounds. Under certain conditions, siderophores can function as a diffusible bacteriostatic or fungistatic antibiotic. The species of *Pseudomonas* present in the suppressive soils reduce the pathogenic population by producing siderophores[12]. We therefore attempted to characterize the siderophores of *Pseudomonas stutzeri,* an isolate from the earthworm gut and its antifungal activity was determined.

MATERIAL AND METHODS

Isolation of *Pseudomonas stutzeri*

P.stutzeri was isolated from gut of earthworm (*Eisenia foetida)* by serial dilution and plating techniques[13]. Isolates were characterized as *P. stutzeri* based on biochemical tests[14,15], Bergey's Manual of Systematic Bacteriology[16] and 16S rRNA analysis.

Characterization of siderophores

The isolates were grown in 10 ml of iron depleted King's B medium (KB)[17] and incubated in an orbital shaker at 28^0C,180 rpm for 18-24h. Cultures of all the isolates grown for 18-24h, were inoculated separately into100ml of succinic acid medium at 1% inoculum level ($15x10^6$ cfu mL^{-1}) and grown with shaking at 28^0C in an orbital shaker for production of siderophores. Three replications were maintained. After 48h incubation, the cultures were centrifuged at 10,000rpm for 15 min and supernatant was examined for siderophores by qualitative $FeCl_3$ test[18] and UV/Vis spectrophotometer[19].

Chemical nature of siderophores

Hydroxamate, catecholate and carboxylate nature of siderophores was ascertained by examining absorption maxima (λmax) of ferric-siderophore complex in UV/Vis spectrophotometer. The λmax for ferri hydroxamates is in between 420-450nm, catecholate siderphores at 320, 250 & 210nm, ferric-catecholate at 495nm and copper-carboxylates at 190-280 nm[20].

Quantification of siderophores

The amount of siderophores present in the supernatants of six production media was determined and calculated by using absorption maxima and molar absorption coefficient (λmax 403 and e 20000 cm^{-1}). Percent of siderophore units were calculated by growing the organisms in six production media (pH 7.2) as per the modified method of Payne [21].

Test organisms

Fusarium oxysporum, *Fusarium solani*, *Fusarium moniliformae*, *Fusarium udum*, *Macrophomena phaseolina*, *Rhizoctonia solani*, *Colletotrichum capsicii*, *Aspergillus flavus* and *Aspergillus niger.*

Antagonistic activity of siderophores

Antagonistic activity of siderophores against fungal isolates was examined by Agar Plate Assay Technique. 1 ml of suspension containing about 10^5 spores of each test fungi was mixed with 20 ml of melted, cooled Potato Dextrose Agar (PDA) and poured into separate sterile petri plates. 20 µl of siderophore extract from *Pseudomonas stutzeri* was placed in 5mm diameter wells made in the solidified agar plates and 3 replicates were maintained. In control wells 10 µl of siderophore extract mixed with 10 µl of $FeCl_3$ (2%) was added. All the plates were incubated at 28^0C for 3-4 days.

RESULTS AND DISCUSSION

20 bacterial isolates were isolated from the earthworm gut. Among them one promising isolate was identified as *P. stutzeri* on the basis of morphological, biochemical tests as per Bergey's Manual of Systemic Bacteriology by Palleroni (Table 1) and 16S rRNA sequence analysis.

Production of siderophore was confirmed by positive $FeCl_3$ test. Cell free supernatants showed maximum absorption between 403-405 nm which conferred the presence of siderophores. Ferric sederophore complexes showed maxima at 210 nm which is the characteristic feature for catecholate nature of siderophores. Ethylacetate extraction of culture supernatants were applied on silica gel plates. Compound was observed in the ethylacetate extraction with an Rf of 0.4. TLC

Table 1. Morphological and biochemical tests for identification of EGB_3

Test	EGB_3
Colony morphology	
Configuration	Wrinkled, cream, round, concentric
Margins	Smooth
Surface	Butyraceous
Pigmentation	-
Turbidity	+
Opacity	Translucent
Gram's reaction	Negative
Cell shape	Rods
Size(¼m)	1 -3 µm in length, 0.5 µm in width
Arrangement	Singles
Spores	-
Motility	+
Physiological tests	
Growth at temperature (^{0}C)	
4^{0}C	+
37^{0}C	+
41^{0}C	+/w
45^{0}C	-
Growth in NaCl (%)	
2	+
5	+
7	w
10	w
Growth at pH	
4	-
5	-
6	W
7	+
8	W
Growth under anaerobic condition	+
Biochemical tests	
Indole test	-
Methyl red test	+
Voges proskauer test	-
Citrate utilization test	+
H_2S production	+
Gelatin hydrolysis	w
Urea hydrolysis	+
Starch hydrolysis	+
Lectinase	_
Lipase (Tween 80 hydrolysis)	+
Catalase test	+
Oxidase test	+
Denitrification	+
Levan formation from sucrose	-
Arginine dihydrolase	-
Nutritional characteristics	+
Starch	+
Maltose	+
Glucose	+
Glycerol	+
Succinate	+
β-Alanine	-
L-Histidine	-
L-Arginine	-
L-Lucine	-
D-Alanine	+

analysis of siderophore revealed the production of pyochelin type of siderophores by *P.stutzeri*. Quantitative analysis of siderophores (Table 2) showed that EGB_3 isolate is producing high quantity of siderophores in succinic acid medium and very low amounts in TSA medium. From the above results the best medium for siderophore production is the succinic acid medium. The siderophores produced by *P.stutzeri* was found effective against all the fungal pathogens in agar plate assay in comparison with control wells. These studies revealed that siderophores exerted maximum impact on *Fusarium sps* than compared to other *sps* (Table 3).

Table 2. Quantitative estimation of siderophores produced by EGB_3 isolate in six production media

S. No	Production media	Siderophores produced
1	TSA	16.8×10^{-6}mg/ml
2	KB medium	17.2×10^{-6} mg/ml
3	GNSA	17×10^{-6} mg/ml
4	CAA	60.6×10^{-6} mg/ml
5	T-medium	45×10^{-6} mg/ml
6	SA	72.5×10^{-6} mg/ml

Table 3. Antimicrobial activity of *Pseudomonas stutzeri*

Test organism	EGB_3
F. oxysporum	+++
F. udum	+++
F. solani	+++
F. moniliformae	+++
Macrophomena phaseolina	++
Rhizoctonia solani	++
Colletotrichum capsicii	++
Aspergillus flavus	++
Aspergillus niger	++

Note: 5-9 mm (+) weak inhibition, 10-19 mm, (++) (moderate inhibition) and >20 mm, (+++) strong inhibition.

CONCLUSION

P. stutzeri were found to produce very stable pyochelins type siderophores which exerted maximum antifungal activity aganist all the tested plant pathogenic fungi.

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