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Research Paper

OPTIMIZATION AND PARTIAL CHARACTERIZATION OF SIDEROPHORE PRODUCED BY *Pseudomonas* SPECIES ISOLATED FROM AGRICULTURAL SOIL

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Abstract

In the present study a siderophore producing bacteria was isolated from agricultural soil and characterized as *Pseudomonas* species. It was optimized for siderophore production maximum sideophore production was observed at 6.5 pH, 30^0C and when glucose was used as a carbon source. The type of siderophore was determined and was found to be hydroxamate type. Siderophore was partially extracted by ethyl acetate and functional groups were studied by infrared spectroscopy. The isolate was susceptible to all the tested antibiotics.

Key words: PGPR, hydroxamate siderophore, biofertilizer.

INTRODUCTION

Rhizobacteria are essential components of soil. Rhizobacteria are root colonizing bacteria that forms symbiotic association with many plants. They are involved in a variety of biotic activities of the soil ecosystem to make it dynamic for nutrient turn over and sustainable for crop production [6]. They stimulate plant growth through mobilizing nutrients in soils, producing numerous plant growth regulators, protecting plants from phytopathogens by controlling or inhibiting them, improving soil structure and bioremediating the polluted soils. Rhizobacteria promotes plant growth in two ways; Direct and indirect plant growth promotion. They promote plant growth directly by producing siderophore, IAA, ammonia, etc. Rhizobacteria act as a bio control agent in indirect plant growth promotion. Many rhizobacteria have been reported to produce antifungal metabolites like, HCN, phenazines, pyrrol-nitrin, 2, 4-diacetylphloroglucinol, pyoluteorin, viscosinamide.[8]. Worked on 55 *Pseudomonas aeruginosa, 22 P. Putida, 26 P. Cepacia and 37 P.* Fluorescens strains and they screened these bacteria for their plant growth promoting activity. Most *P. aeruginosa* strains showed positive PGPR activity as compared to other species of *Pseudomonas*. The study showed that *Pseudomonas* as an effective plant growth promoting bacterium.

Both microorganisms and plants require a high level of iron, and obtaining sufficient iron is even more problematic in the rhizosphere where plant, bacteria and fungi compete for iron [9]. Iron is a vital nutrient for almost all forms of life. To survive with such a limited supply of iron, Bacteria acquire irons by secreting siderophores. Siderophore (from Greek iron carriers) are defined as relatively low molecular weight. Ferric ion specific chelating agent elaborated by bacteria and fungi growing under low iron stress. The role of these compounds is to scavenge iron from the environment and to make them mineral which are almost always essential to microbial cell. Generally, rhizobacteria differs regarding the siderophore cross-utilizing ability; some are proficient in using siderophores of the same genus (homologous siderophores) while others could utilize those produced by other rhizobacteria of different genera (heterologous siderophores) [1]. Siderophores act as solubilizing agents for iron from minerals or organic compounds under conditions of iron limitation. The role of these compounds is to scavenge iron from the environment and to make the mineral, which is almost always essential, available to the microbial cell. [9,10] Siderophores have been related to virulence mechanisms in microorganisms pathogenic to both animals and plants. In addition, they have clinical applications and are possibly important in agriculture. Microorganisms growing under aerobic conditions need iron for a variety of functions including reduction of oxygen for synthesis of ATP, reduction of ribotide precursors of DNA, for formation of heme, and for other essential purposes. A level of at least one micromolar iron is needed for optimum growth. Most siderophores are either Hydroxamate or catechols type. For detection of siderophore, the chrome azurol sulfonate assay has become widely used. It has been demonstrated that plants are able to use the hydroxamate-type siderophores ferrichrome, rodotorulic acid and ferrioxamine B; the catechol-type siderophores, agrobactin; and the mixed ligand catechol-hydroxamate-hydroxy acid siderophores biosynthesized by saprophytic root-colonizing bacteria. All of these compounds are produced by rhizospheric bacterial strains, which have simple nutritional requirements, and are found in nature in soils, foliage, fresh water, sediments, and seawater. [8]

Tailor A. J., *et al*., (2012) carried out extensive screening for the siderophore producing bacteria from the sugarcane rhizosphere. They found *Pseudomonas fluorescens* strain as a promiscus candidate for crop improvement and protection due to its PGPR activities [12].

The present study has been undertaken for production, detection and estimation of siderophores produced by plant growth promoting rhizobacteria.

MATERIAL AND METHODS

Isolation of siderophore producing bacteria

Sugarcane rhizoshere soil sample collected in a sterile vial from a field near Ahmednagar, Maharashtra, India and stored at 4^0C until use. 0.1g of soil sample was then inoculated in 100ml of succinate broth**.** The flask was incubated at 37^0C under shaking conditions for 48 hours. After incubation enriched sample was inoculated on CAS agar plate by spread plate technique and incubated for 48 hours. After incubation the plate was observed for yellow – orange colour zone around the colony which indicates siderophore production by the bacteria.

Identification of the bacteria

Bacteria was identified by various morphological and biochemical test according to Bergye's manual of determinative bacteriology.

Optimization of siderophore production

Effect of temperature: Sterile succinate broth tubes were inoculated by isolate. Tubes were kept for incubation for 48h at different temperatures (20°C, 28°C, 30°C, 32°C and at 40°C). Control tubes were kept for each temperature. After incubation, 2ml culture from each tube was centrifuged and absorbance of supernatant was taken at 400nm and siderophore concentration was detected using Meyer and Abdallah method as follows [11]

$A = \varepsilon cl$

Where A= absorbance at 400nm, ε = 20,000 (molar extinction coefficient), C= concentration, L= length in cm

Effect of pH: Sterile succinate broth tubes with pH from 3, 3.5, 4, 4.5, 5, 5.5 up to 9 were prepared. Isolate was then inoculated in each tube and kept for incubation for 48 hours at37^0C on shaking incubator. Siderophore concentration was determined by method suggested by Meyer and Abdallah, 1978 [11].

Effect of different carbon source: Succinate media was prepared by replacing its carbon source. 7 different carbon sources were used; they are glucose, fructose, sucrose, succinic acid, maltose, lactose and starch. Isolate was inoculated in each tube and tubes were incubated at 37^0C for 48 hours on shaking incubator. Uninoculated control tubes were kept for each carbon source. After incubation, siderophore concentration was determined by method suggested by Meyer and Abdallah, 1978 [11].

Siderophore production with respect to the growth

1m sample was taken from 24 hours old culture of isolate and centrifuged at 1000 rpm for 10 min. Cell pellet was washed with sterile saline and resuspended in 1 ml sterile succinate broth and mixed. Then this sample was inoculated in 500 mL sterile succinate broth. Immediately, 2 ml sample from broth was taken and then flak was incubated on shaker at 37^0C. Then absorbance of sample was taken at 600 nm to check the growth and siderophore concentration was determined by method suggested by Meyer and Abdallah, 1978 [11]. Same procedure was repeated after 2 hours interval to determine siderophore concentration and growth.

Siderophore type determination: Hydroxamate type of siderophore was determined by Csaky Assay [7]. 1 ml cell free supernatant was mixed with 1m of 6 N H_2SO_4 and boiled for 6h or at130°C for 30 min. then 3 ml of Sodium Acetate Solution was added. This was followed by addition of 1 ml of 1% sulphanilic acid solution and then 0.5 mL iodine solution. After 3-5 min excess iodine was destroyed by adding 1 mL of 2% aq. solution of sodium arsenate solution. 1 mL of α-napthylamine solution was then added to the mixture. Finally 10 mL of distilled water was added to it and mixture was then allowed to stand for 30 min. After incubation of 30 min mixture was then observed for formation of pink color. A negative control was kept which includes all the components except culture sample.

Catecholate type of siderophore was determined by Arnow's Assay (2). The assay was performed by mixing the 1 ml of cell free supernatant, 1ml 0.5 M HCL, 1 ml Nitrite-Molybdate reagent (10 %sodium nitrate (W/V) and 10 % sodium molybdate(W/V) in double distilled water), and 1ml of 1 M NaOH. The mixture was then incubated for 5 minutes. Formation of yellow color indicates presence of catecholate type of siderophores. A negative control was kept which includes all the components except culture sample.

Extraction of siderophore

siderophore was extracted using ethyl acetate [12]. The pH of the 200 ml of cell free supernatant was adjusted to 2 with the help of concentrated HCl and then equal volume of ethyl acetate was added in it. The solution was mixed properly for 15 min. and then allowed to settle. The organic fractions were collected and subjected to drying by evaporation. The dried crystals of siderophores were then dissolved in sterile distilled water.

Infrared spectroscopic analysis of extracted siderophore

Extracted siderophore sample was subjected to infrared spectroscopic analysis and probable function groups present in siderophore were predicted.

Antibiotic susceptibility pattern

Antibiotic susceptibility pattern of isolate was studied by disc diffusion method [4]. The isolate was grown in nutrient broth medium for 24h, at 37^0C. A 0.1 ml of the overnight grown culture of isolate was spread on nutrient agar plate. The antibiotic disc of known potency was then placed on the agar surface and the plate was incubated at 37^0C for 24 hours and the zones of inhibition around the antibiotic disc was observed.

RESULTS AND DISCUSSION

Isolation and Identification

A single siderophore producing colony was observed on CAS agar plate after the incubation. This colony showed a yellow-orange coloured zone around the colony. After 24h incubation on nutrient agar plate following colony characters were observed for the isolate.

Size	Shape	Color	Margin	Consistency	Elevation	Opacity	Gram character	Motility
<1mm	Circular	Green	Entire	Soft	Convex	Opaque	Gram negative	Motile

Isolate was a Gram negative motile short rod. It produced a green diffusible pigment on nutrient agar and it followed a strict respiratory metabolism i.e. catalase and oxidase negative as well as negative for sugar fermentation test. Hence according to Bergey's manual, the isolate was identified as a *Pseudomonas* species.

Optimization

Concentration of siderophore increased with the temperature. The maximum siderophore production was found at 30^0C. But when the temperature was further increased, concentration of siderophore was reduced. Tailor A.J., *et al*., (2012) found optimum temperature for siderophore production as 29ºC for *Pseudomonas fluroscences.* The maximum siderophore production was observed at pH 6.5 [12]. But when the pH was further increased, concentration of siderophore was reduced. This indicated that the optimum pH for siderophore production by the isolate is 6.5. Bholay A. D., *et al*., (2012) worked on fluorescent *Pseudomonas* as plant growth promoting rhizobacteria and their siderophoregenesis [5]. The optimum pH for siderophoregenesis for their bacteria was 7. The effect of different carbon sources on siderophore production was studied. The Maximum siderophore production was obtained when glucose was used as a carbon source. The lowest siderophore production by isolate was obtained when carbon source was sucrose. Tailor A.J., (2012)

studied characterization and optimization of siderophore production from *Pseudomonas fluroscences* [12]. They also obtained maximum siderophore production when glucose was the carbon source.

Siderophore production with respect to the growth

Siderophore production with respect to growth of isolate was studied. During first 4h, bacterial growth was slow but after 6h growth was increased significantly and it remained nearly constant till 34. Siderophore was not detected during first 4h. But there is a constant increase in siderophore production after 6h. The rate of siderophore production was further increased after 30h and this rate remained approximately constant till 34h.

Type determination of siderophore

After performing Csaky assay pink color was developed in test which indicated presense of hydroxamate group in the siderophore. Cell free supernatant gave negative test with Arnow's assay which means absence of Catecholate group.

Infrared spectroscopic analysis

Infrared spectroscopic analysis of extracted siderophore was carried out. From the spectra generated probable functional groups were detected. Extracted siderophore contains amine group, benzene ring, amide group. All these functional groups are commonly found in hydroxamate type of siderophores like enterobactin, pyroverdin etc.

Table no.1: Functional groups of siderophore

Sr. No.	Wave number (cm^{-1})	Functional group
1	1117.80	Amine CH stretch
2	2800-3000	Aromatic C-H stretch
3	1614.49	Amide NH bending / amine bending
4	3200-3400	NH_2streach and phenolic O-H stretch
5	1575-1600	Aromatic C=C stretch

Antibiotic susceptibility

The isolate was susceptible for all tested antibiotics. As isolate having plant growth promoting abilities, it can be used as biofertilizer. As a biofertilizers, it is expected that the bacteria should not be multi drug resistant since biofertilizers are used in the field and farmers comes in direct contact of these bacteria. Bakthavatchalu S., *et al.*, (2012), worked on identification of multi-trait PGPR isolate and evaluation of their potential as biocontrol agent.[3] In their study, fluorescent *Pseudomonad* showed resistance to tetracycline, erythromycin and cotrimazole, whereas it exhibited sensitive to rifampicin, kanamycin and gentamicin.

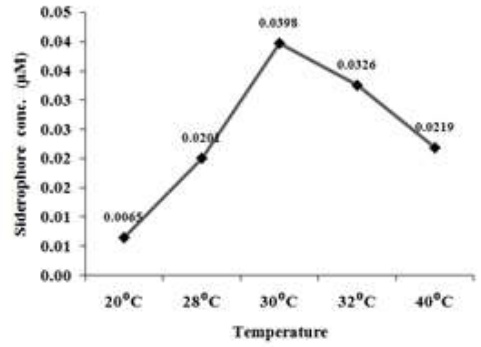

Fig. 1: Effect of Temperature

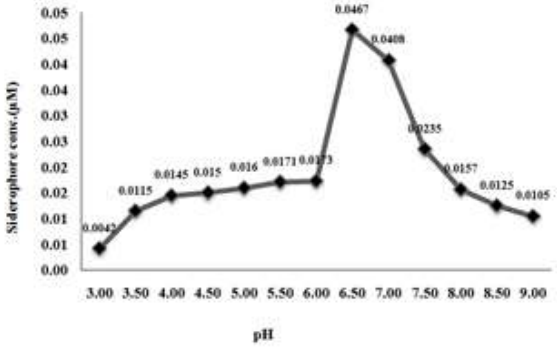

Fig.2 : Effect of pH

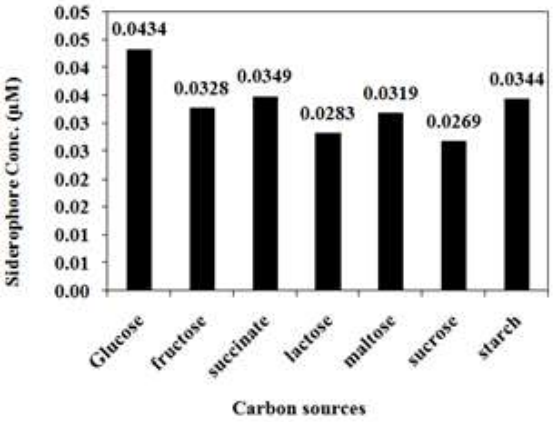

Fig. 3: Effect of Carbon source

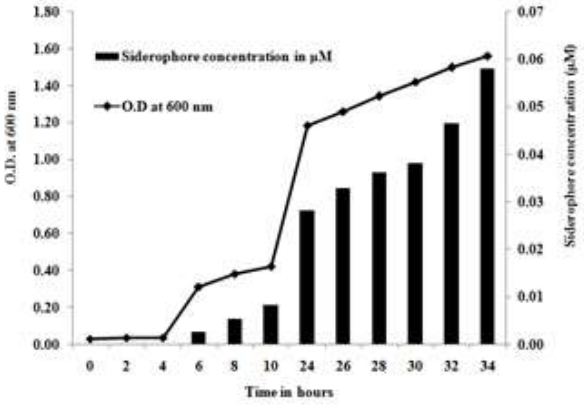

Fig. 4: Growth verses siderophore production

Fig.5: Siderophore production

Fig.:6 Antibiotic susceptibility

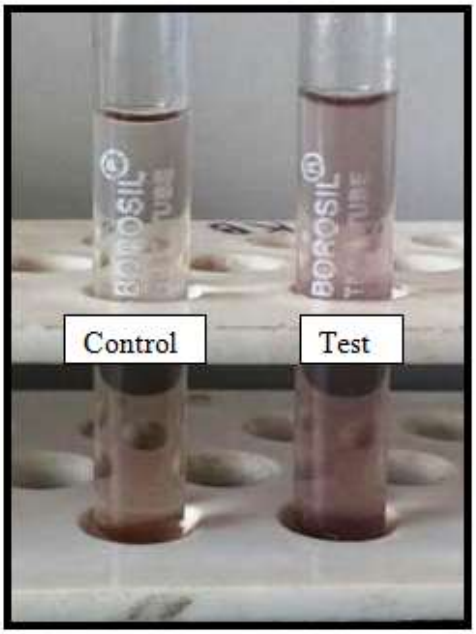

Fig. 7: Hydroxamate type of siderophore

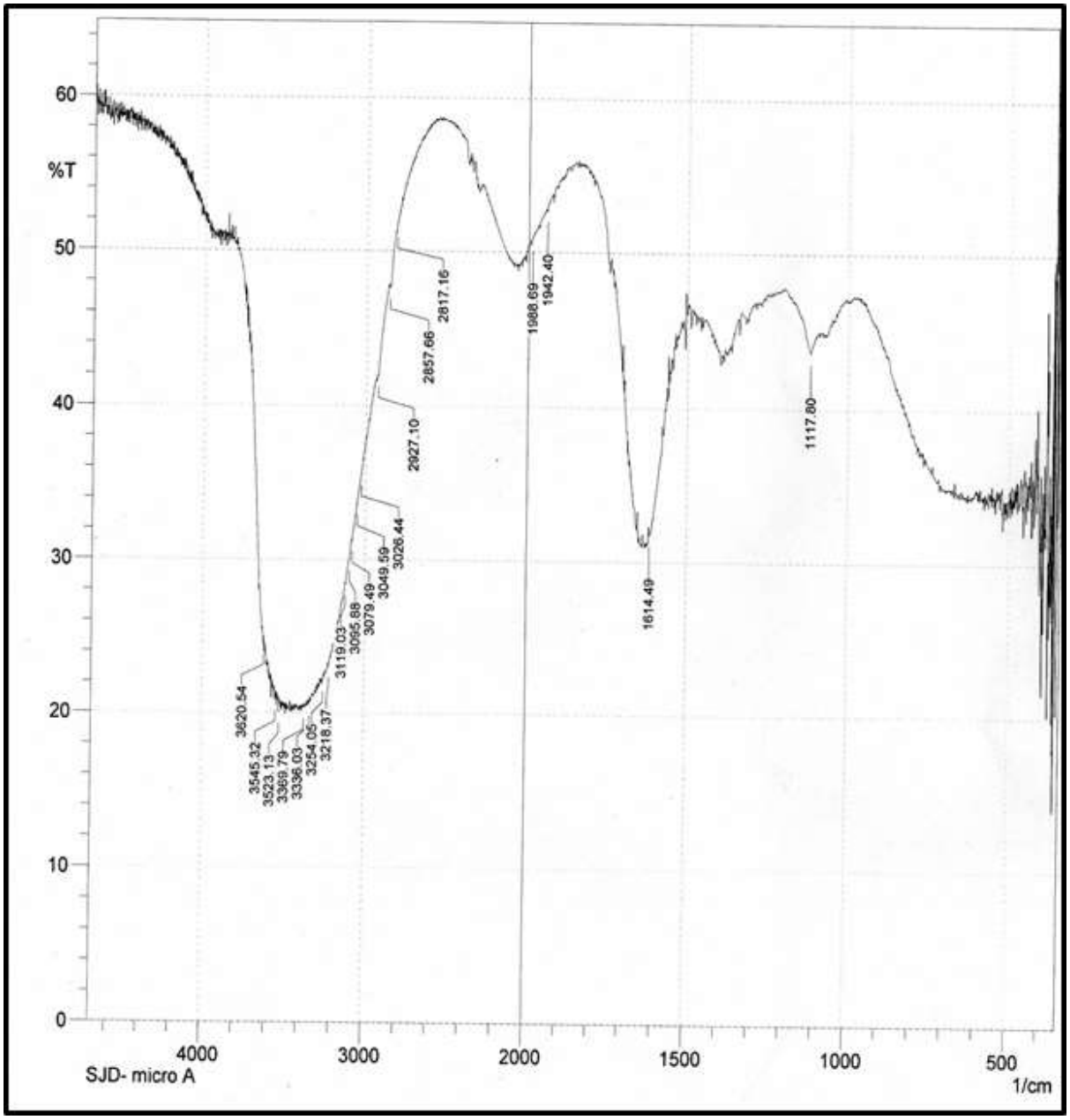

Fig. 8: Infrared spectra of siderophore

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