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Mixed Siderophoregenic Root Nodulating *Enterobacter cloacae shirishii SUP I* from Tamarindus indica L.

KEYWORDS: Enterobacter cloacae • Tamarindus indica • Mixed Siderophore • FTIR

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ABSTRACT *Nine different isolates were obtained from tamarind (Tamarindus Indica L.) root nodules samples collected from Sangli district (Maharashtra, India) area, out of which 5 isolates showed siderophoregenic behaviour under iron limiting conditions on chrome azurol – S (CAS) agar. The isolate SUP I seemed potent siderophoregenic on CAS agar, which was further confirmed in fiss glucose mineral medium where a maximum 72.16% decolorization of CAS reagent in liquid CAS assay was achieved. Maximum siderophore production was achieved at pH 7.0. In 16S rRNA gene sequencing and phylogenic analysis using MEGA4, the isolate showed affinity towards Enterobacter cloacae. From colorimetric reactions the purified siderophore was identified as hydroxamate – catacholate mixed type, with dominant hydroxamate moiety. Fourier transform infrared (FTIR) spectrum of the purified siderophore showed prominent peaks at 3303 cm-1, 3065 cm-1, 2934 cm-1, 1719 cm-1, 1648 cm-1 and 1545 cm-1, which supported the colorimetric analysis. The purified siderophore found inhibitory to Bacillus subtilis, Serratia marcenscens, and Salmonella typhimurium in vitro at 100 µg ml-1 concentration. The prominant siderophoregenic behavior of the isolate Enterobacter cloacae shirishii SUP I highlites its crucial role in biological recycling of Fe+++ in the ecosystem along with its use in bioremediation of iron deficient soils in agriculture. This study represents the first event to report siderophoregenic tamarind root nodulating Enterobacter cloacae.*

Introduction

Iron is fourth most abundant element on Earth's crust which is involved in variety of crucial biological processes (Neilands 1981). The siderophore production and specific transport of iron into the ligand-producing cell is an important adaptation for survival and success in a variety of iron limited environments (Cox 1989, Neilands 1982). The bioavailability of iron in the rhizospheric soils at neutral pH is very low, the siderophores play an important role in iron recycling in the rhizosphere.

Siderophores are relatively low molecular weight (400-1500 daltons) ferric ion chelators elaborated by many bacteria and fungi growing under iron starved conditions. Siderophores are involved in scavenging the iron from the environment with high specificity and make the iron available to the microbial cells (Neilands 1995, Leong 1986). Though some of the known siderophores exhibit the ability to chelate other metal ions, their specificity towards the iron remained as most consistent feature (Chincholkar et al. 2007). Siderophores play an important role in improving the rhizosphere colonization of the strain as well as in iron nutrition of plants (Vansuyt et al. 2007) and also indirectly promote plant growth by creating an antagonistic impact on phytopathogens (Chincholkar et al. 2007a). Basically, siderophores are mainly categorized in two types, viz., secondary hydroxamic acid types and catechol types (Actis et al. 1986). Also, most of catecholates are of 2, 3-Dihydroxybenzoic acid type and generally consists of 2, 3-dihydroxybenzoic acid and one or more amino acid residues (Xie 2006).

In order to detect the presence of siderophoregenic endophytic organisms and thus the role of siderophores in iron recycling in wild environment, the various wild legumes across the Sangli district area were studied. The potent microorganisms from such environments could serve in Fe^{+++} nutrition of crop plants as well. The medicinal importance of siderophores also highlights the need of elaborating the behavior of various organisms in presence of exogenous ferric- siderophore complex in iron deficient environment.

Materials and Methods

Collection of Samples

The sampling spots were located in various forest regions of Sangli district with geographical location -North Latitudes 16.4 to 17.1 **East Longitude 73.43 to 75.00 with total area of** 8601.5 Sq. Kilometer and the temperature range 14.0 0C - 42.0 0C**.** (The average rainfall per annum ranges from 400-450 mm. Agricultural status of the district shows Bajra, Jowar, Wheat, Rice Gram, Sugarcane, Groundnut, Turmeric, **Soybean,** Pomogranate, Grapes as major crops. Sugarcane is the annual crop cultivated in the district). The root regions of the samples were washed thoroughly first by tap water and then by distilled water for at least three times. The fresh, juvenile, pinkish nodules were selected from plant and surface sterilized using H_2O_2. The suspension of treated nodules was prepared in saline and was inoculated on Congo Red Yeast Extract Mannitol agar by streak plate method and grown at 30^0C for 36 h. The colonies were selected according to their distinct morphology. The isolates were maintained on the same medium without incorporation of the dye.

Screening of Siderophore Positive Strains

The siderophore positive isolates were screened using Chrome Azurol - S Agar (CAS agar) (Schwyn 1987). Fresh (24 h) cultures were adjusted to O.D. 0.1 (~10^6 CFU mL^{-1}) (Shakhawat 2007) and were inoculated in 5µl quantities on Chrome – Azurol Ssulfonate (CAS) agar plates and grown at 30^0C for 7 days. The strains showing higher selection ratio on CAS agar were further tested for their potency by using liquid CAS assay in which the comparison of siderophore production was done in terms of percent decolorization (Pyane 1994), calculated using formula:

$$\text{Percent decolorization} = \frac{Ar - As}{Ar} \times 100$$

Where,

Ar = Absorbance of reference

As = Absorbance of sample at 630 nm.

The most potent siderophore producing isolate SUP I identified as *Enterobacter cloacae* was selected for further study.

Phylogenetic studies

The potent siderophore producing culture was identified by 16S rRNA sequencing from the Molecular Biology Unit, NCCS, Pune. The phylogenetic tree was constructed using 1000 base pair aligned sequences by the neighborhood join-

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ing method using MEGA 4.1.2 software (Tamura et al. 2007).

Production of siderophores
The siderophore production for selected isolate of SUP I was achieved in Fiss glucose mineral medium (Vellore 2001). The culture was incubated in shaking condition (150 rpm) at 30^{0}C for 48 h.

Extraction and purification of siderophores
The culture was grown in 350 mL batch Fiss glucose mineral medium at 30^{0}C in shaking condition (150 rpm) for 48 hours. Cells were pelleted at 7000 × g for 10 min. The supernatant was then acidified to pH 2 using 6 M HCl and mixed with conditioned XAD2 resin and stirred thoroughly for 3 hours. The slurry was then poured into glass column and liquid was drained at a flow rate of 30 mL/h. The column was washed with 50 mL of double distilled water, followed by washing with 40 mL of 5% methanol to remove any non specific binding. Finally, siderophores and siderophore like compounds were eluted with three bed volumes of methanol. The extracts were evaporated to dryness and resuspended in double distilled water and stored at -20^{0}C till further use.

Chemical characterization of siderophores
Determination of Hydroxamate type siderophore using Csaky's assay
To detect presence of any active Hydroxamate group in the molecule, Csaky's test (Csaky 1948), with slight amendment (Velasquez et al. 2011) was performed in which all reagents except sodium arsenite were used as described by Csakey, sodium arsenite was replaced by sodium thiosulfate solution in order to avoid the environmental hazards caused due to sodium arsenite. The detection limit of the method is 1x10^{-5} M.

Determination of Catechol type siderophore using Arnow's assay:
The selective determination of catecholate-type siderophores was carried out by Arnow's colorimetric test for catechol (Arnow 1937). The intense red color indicated presence of catechol compounds in the sample. The intensity of red color was measured at 510 nm using a UV-Vis spectrophotometer.

FTIR analysis
Infrared (IR) spectra of the active fraction was recorded in USIC, Shivaji University, Kolhapur using a Magna 550 model of FTIR spectrometer, Nicolet Instruments Corporation, USA in the range 50–4000 cm^{-1} by methanol recording technique.

pH optimization:
Variation in siderophore production by the isolate SUP I was detected by growing the same in Fiss glucose mineral medium within a pH range 6.5 to 7.5 at 30^{0}C in shaking condition at 150 rpm.

Cell density and siderophore production
In order to study the siderophore production with increase in biomass, the cultures were grown in Fiss glucose mineral medium (with inoculums 1% v/v) at 30^{0}C for 168 hours in shaking conditions (150 rpm). The samples were withdrawn after every 24 hours and tested for cell density and siderophore production at 600 and 480 nm respectively using UV-VIS spectrophotometer (Sistronics 2000).

Determination of antimicrobial activity of purified siderophore
In-vitro antimicrobial activity of purified siderophore was detected using agar cup method in Luria agar medium (Shah et al. 1992). A Volume of 20 ml sterilized, cooled Luria agar was seeded with 10^7 CFU ml^{-1} of pathogen culture and poured in acid cleaned Petri plates. Wells of 10 mm diameter were made in the agar and purified siderophore solution of concentration 100 mg ml^{-1} in 100 µl quantity was added while, sterile double distilled water was used as control. The plates were kept at 15^{0}C for 30 min and then incubated for 60 hrs at 37^{0}C for human pathogens and 30^{0}C for plant pathogen.

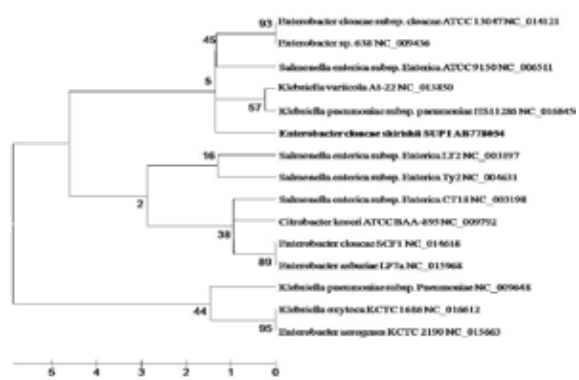
Candida albicans, Pseudomonas aeruginosa, Serratia marcescens, Proteus vulgaris, Salmonella typhimurium, Bacillus subtilis and Xanthomonas citri were studied for the assay. The active growth (18 hours culture) of microorganisms in Luria broth was used to seed the Luria agar.

Results and Discussion
In this study, wild legumes were sampled in order to isolate root nodulating bacteria, as routine consideration, we were expecting presence of Rhizobium spp. in the nodules; but surprisingly, we found most potent siderophoregenic isolate from tamrind root nodules which was identified as Enterobacter cloacae after 16S rRNA sequencing and the phylogenetic analysis, thus making this study the first event in the history to characterize the siderophoregenic root nodulating *Enterobacter cloacae*.

Since a long time, *Enterobacter cloacae* has been found to colonize roots nodules along with Rhizobium spp. but no attempts were made to characterize siderophoregenic abilities of such root nodulating *Enterobacter cloacae*. Very few events in the history record the isolation of *Enterobacter cloacae* from root nodules.

The *Enterobacter cloacae shirishii SUP I* was submitted to the DDBJ/EMBL/GenBank databases with the accession number AB778054 (Figure 1).

Figure: 1 The phylogenetic position of the isolate obtained using MEGA 4. The sequence of the *Enterobacter cloacae* **strain** *shirishii* **SUP I used in this study is indicated in bold letters.**



During collection of leguminous plants, little nodulation in watershed regions of the district was recorded. On the other hand, in well irrigated and regions with appropriate moisture, a good and consistent nodulation was observed.

During screening for potent siderophore producer, it was observed that pH seemed to have great influence on siderophore production thus, the isolate SUP I was studied for its siderophoregenic behavior in a pH range of 6.5 to 7.5. When the isolates were grown in media with different pH ranging from pH 6.5 to 7.5, it was found that, isolates produced more siderophore at pH 7.0 and decreased below and above pH 7.0 (Figure 2). In order to detect whether the siderophoregenic system or the activity of siderophore is affected at pH below or above 7.0, the isolate was grown in Fiss glucose mineral medium (pH 7.0) for 36 h and the supernatant was inoculated in the wells of 5 mm diameter in CAS agar with pH 6.5, 6.7,6.9,7.1,7.3 and 7.5 in 50 µl quantity. The results indicated that activity of siderophore itself at sub or hyper optimal pH is not affected, but its synthesis is affected resulting in decreased siderophore production.

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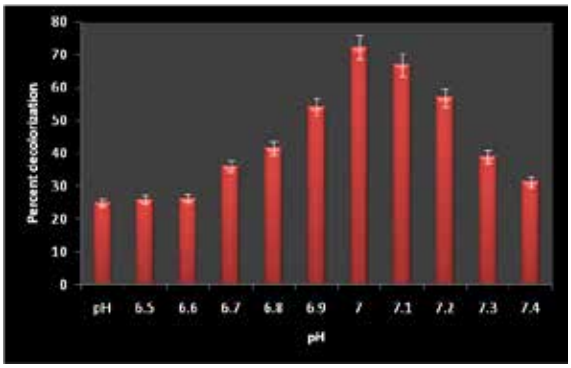

Figure: 2 Variation in siderophore production detected in terms of percent decolorization of CAS reagent by isolate SUP I seen in Fiss glucose mineral medium within a pH range of 6.5 to 7.5.

In order to correlate siderophore production with increase in cell mass, Fiss - glucose mineral medium with pH 7.0 was used. Maximum siderophore production was achieved at 96 hours of incubation when the cell mass was also at its peak (Figure 3); indicating maximum siderophore production in late log phase and thus proving it a secondary metabolite.

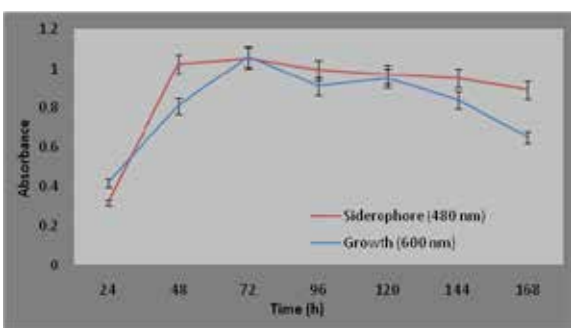

Figure: 3 Siderophoregenesis by the isolate SUP I with rise in cell mass.

In FTIR spectrum of the purified siderophore of *Enterobacter cloacae shirishii SUPI*, a broad peat at 3303 cm^{-1} was observed which indicated –OH stretch. The peak appearing at 3065 cm^{-1} confirmed the presence of unsaturation in the structure, probably the unsaturated ring usually seen in hydroxamic acid moiety present in the structure; further the peak at 2934 cm^{-1} also showed presence of alkenes, which was due to >C=H stretch. >C=O stretch was shown by the peak at 1719 cm^{-1}. The two peaks at 1648 and 1545 cm^{-1} respectively showed presence of secondary amide structure i. e. –C=O-NH, which confirmed the presence of conjugated amino acids. Two peaks at 1439 and 1407 were due to >C-H deformations (figure 4).

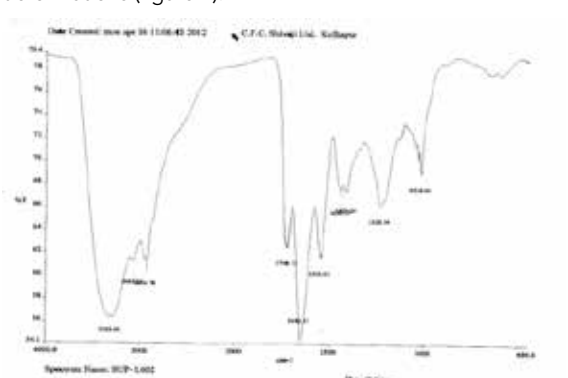

Figure: 4 Fourier – transform infrared (FTIR) spectrum of purified siderophore by the isolate SUP I.

Purified siderophore when subjected to chemical assay for determination of active functional moieties in the molecule, we found Arnow's test weakly positive, indicating presence of catechol moiety in the structure. The color developed after addition of excess NaOH was faint and was corresponding to standard tube with 10 µg ml^{-1}. The Csakey's test for hydroxamate was strongly positive which proved hydroxamic acid as dominant moiety in the molecule.

Thus from the results obtained from FTIR analysis and the chemical assays, the siderophore can be classified in "Mixed" category.

When the activity of purified siderophore produced by *Enterobacter cloacae shirishii SUP I* was detected, we found that the siderophore at a concentration of 100 µg ml^{-1} exhibited an inhibitory action on *Bacillus subtilis, Serratia marcescens,* and *Salmonella typhimurium* (Table 1).

Table: 1 Inhibitory action of catechol siderophore produced by the isolate SUP I at concentration of 100 mg ml^{-1} against common pathogens.

Pathogen	Zone of inhibition (mm)
Xanthomonas sp.	-
Proteus vulgaris	09
Candida albicans	05
Pseudomonas aeruginosa	-
Bacillus subtilis	11
Serratia marcescens	10
Salmonella typhimurium	10

The overall results from this study highlight the use of potent siderophoregenic *Enterobacter cloacae sirishii SUPI* in agriculture in bioremediation of iron deficient soils. Also, the inhibitory action of the purified siderophore against some of the studied pathogens indicates the role of the siderophore in medicine.

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