Research Paper

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Catechol Siderophore Produced by *Klebsiella Pneumoniae* Isolated from Rhizosphere of *Saccharum Officinarum L.*

Medical Science

KEYWORDS : *Klebsiella pneumoniae*; Siderophore; CAS agar; FTIR; XAD2

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ABSTRACT

In present study, 27 different isolates were obtained from 22 sugarcane fields in Sangli district area, based on soil textures and irrigation status of the area. 18 isolates produced siderophores under iron limiting conditions on chrome azurol – S agar medium. The isolate SUP IV showed maximum siderophore production in Burk's medium with maximum 43% decolorization of CAS reagent in liquid CAS assay. Optimum yield of siderophore was obtained at pH 7.2. The isolate failed to grow at temperature 370C, but flourished well at 300C. The culture was identified as Klebsiella pneumoniae based on 16S rRNA gene sequencing and phylogenetic studies using MEGA 4. The siderophore extraction and purification was achieved using XAD2 column. Colorimetric reactions prove that purified siderophore is of catecholate type. Fourier – transform infrared (FTIR) analysis showed prominent peaks at 3351 cm-1, 1654 cm-1, 1033 cm-1, which supported the colorimetric results. The study represents first report for production of siderophore by rhizospehric Klebsiella in the area.

Introduction

Iron is fourth most abundant element on Earth's crust which is involved in variety of crucial biological processes such as photosynthesis, nitrogen fixation, methanogenesis, H_2 production, oxygen transport, gene regulation, DNA biosynthesis/repair, and detoxification of free radicals and thus is essential to almost all forms of life (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 and Neilands, 1982).

The name *siderophore* is derived from the Greek word – 'iron carrier'. 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 known to scavenge iron from the environment with high specificity and make the iron available to the microbial cells (Neilands, 1995 and Leong, 1986).

Though some of the known siderophores have been shown to chelate other metal ions, their specificity towards the iron remained as most consistent feature (Chincholkar, et al., 2007a). 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., 2007b). 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, et al., 2006). Though *Klebsiella* have been extensively studied for nitrogen fixing mechanisms (Iniguez, et al., 2004), plant growth promoting *Klebsiella* are still to be focused in detail (Sachdev, et al., 2009). Present study highlights siderophore producing *Klebsiella pneumoniae* from sugarcane rhizosphere.

Materials and Methods

Collection of Soil Samples

Twenty seven soil samples from various sugarcane fields in Sangli district area were collected by composite sampling method. The samples were kept in sterile polyethylene bags and immediately brought to the laboratory and subjected for enrichment.

Enrichment and isolation of nitrogen fixers

Enrichment of nitrogen fixing organisms was done in Ashby's Nitrogen free Mannitol broth at 30^{0}C for 72 h. Upon proper enrichment, isolation was done on Ashby's Nitrogen free Mannitol agar medium at 30^{0}C for 48 h. Colonies showing distinct morphology were selected and maintained on agar slopes of the same medium in test tubes. Total 27 isolates were obtained from soil samples.

Screening of Siderophore Positive Strains

The siderophore positive isolates were screened using Chrome Azurol - S Agar (CAS agar) (Schwyn and Neilands, 1987). In order to obtain actively growing cultures, the isolates were grown in respective broth media for 24 hours. Fresh cultures were adjusted to O.D. 0.1 (~10^6 CFU ml^{-1}) (Shakhawat, 2007) and were inoculated in 5 µl quantities on CAS agar plates and grown at 30^{0}C for 7 days. The orange halo surrounding the colony indicated siderophore production. 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 the following formula:

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

Where,
Ar = Absorbance of reference

As = Absorbance of sample at 630 nm.

The most potent siderophore producing isolate SUP IV identified as *Klebsiella pneumoniae* was selected for further study.

Phylogenetic studies of siderophore producing *Klebsiella sp.*

The potent siderophore producing culture was identified by 16S rRNA sequencing from the Molecular Biology Unit, NCCS, Pune using universal Eubacteria-specific primers (Pidiyar et al., 2004). Sequenced 16S rRNA gene, was used for sequence analysis at the Ribosomal Database Project (RDP-II) and NCBI-BLAST (http//:www.ncbi.nlm.nih.gov/BLAST). The phylogenetic tree was constructed using 1000 base pair aligned sequences by the neighborhood joining method using MEGA 4.1.2 software (Tamura et al., 2007).

Production of siderophores

The siderophore production for selected isolate of *Klebsiella sp.* was achieved in Burk's medium (Antony and William, 1998) with slight modification, where the original Burk's medium was supplemented with 0.5% casamino acids. The culture was incubated in shaking condition (150 rpm) at 30^{0}C for 48 h.

Extraction and purification of siderophores

The culture from 350 ml Burk's medium (Antony and William, 1998) was centrifuged at 7000 × *g* for 10 min. to remove cell pellet. The supernatant was then acidified to pH 2 using 6 M HCl to make siderophores less soluble in the medium. The acidified supernatant was then 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, 2011) was performed. The detection limit of the method is 1x10^{-5} M.

All reagents except sodium arsenite were used as described by Csaky, sodium arsenite was replaced by sodium thiosulfate solution in order to avoid the environmental hazards caused due to sodium arsenite.

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). One ml sample was acidified using equal volume of 0.5 M HCl, to which 1.0 ml of nitrite-molybdate solution (mixture of NaMoO4 and NaNO2 10% each) were added. On thorough mixing, the solution turned yellow. Then equal volume of 1.0 M sodium hydroxide was added and the solution was thoroughly mixed. 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.

Results and Discussion

Members of genus *Klebsiella* are well known for their plant growth promoting activity by production of various beneficial components such as IAA (Govindarajan et al., 2007), since a long time due to their significant plant growth promoting activity. This paper highlights the study of potent *Klebsiella pneumoniae* isolate obtained from rhizosphere of sugarcane and its ability to produce siderophores. Variance was found in isolate numbers with respect to different soil textures i.e. eleven isolates from black soil, six isolates form yellow, five isolates form brown and rocky soils respectively were obtained. The isolates when cultivated at 37^{0}C, a remarkable distinction was observed for isolate SUP IV, which unlike other members of the genus (*Klebsiella*), flourished well at 30^{0}C and failed to grow at 37^{0}C.

Phylogenetic analysis

Figure 5 depicts the phylogenetic tree for the sequence of isolate SUP IV which shows significant similarity to *Klebsiella pneumoniae* drawn by bootstrap method using MEGA 4. Sequence of the same bacteria has been deposited under Accession number AB744027 in DDBJ/EMBL/GenBank database (Figure 1).

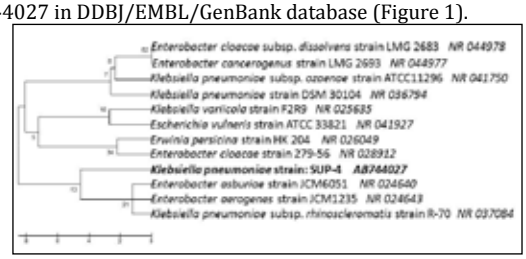

Figure 1: The phylogenetic position of the isolate obtained using MEGA 4. The sequence of the Klebsiella strain used in this study is indicated in bold letters.

While screening for siderophore positive strains, it was noted that out of 27, only 18 isolates were CAS positive. Also, the number of CAS positive isolates greatly varied with soil texture and irrigation status of the soil (Figure 2).

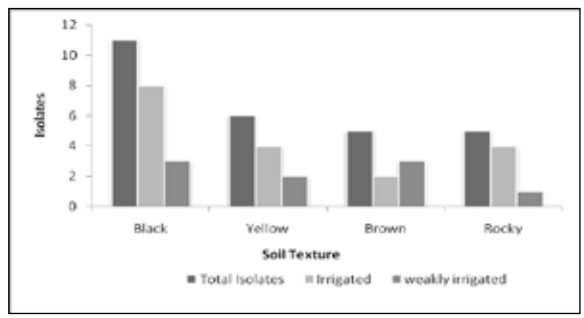

Figure 2: Total number of CAS positive isolates with respect to soil texture and their distribution with respect to irrigated and dry (weakly irrigated) soils.

pH optimization:

During screening for potent siderophore producer, it was observed that pH had a great influence on siderophore production. When the isolates were grown in media with different pH ranging from pH 6.0 to 7.5, it was monitered that, isolates produced more siderophores at pH 7.1 and decreased below and above 7.1, thus the potent isolate – SUP IV was further studied for its siderophoregenic behavior in a pH range 6.5 to 7.5 (Figure 3).

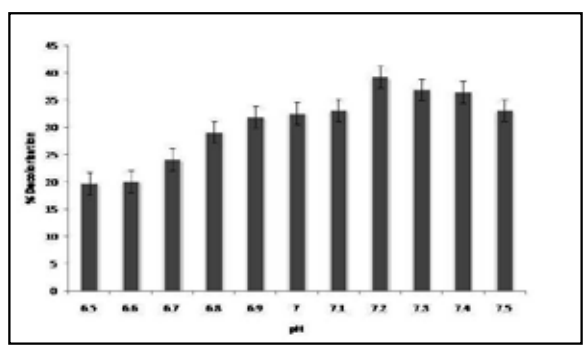

Figure 3: Variation in siderophore production detected in terms of percent decolorization of CAS reagent by isolate SUP IV seen in Burk's medium with pH ranging from 6.5 to 7.5.

During primary screening experiment, nine isolates were selected for secondary screening where, consistent results of the isolate SUP IV favored its selection for further study. All the screening experiments were run in triplicate sets (Figure 4).

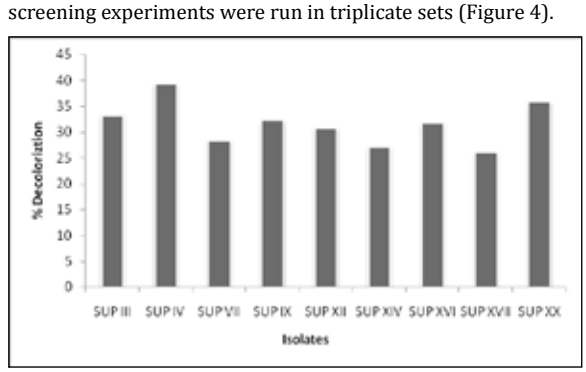

Figure 4 : Percent decolorization of CAS reagent by isolates showing higher selection ratio on CAS agar.

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Though the pH of CAS agar is 6.8 according to the standard references, most isolates including *Klebsiella sp.* showed a little and almost same amount of siderophore production even after incubation for 168 hours (7 days) at 30^0C. When pH of the medium was raised up to 7.2, optimum siderophore production was observed. Thus pH below 7.2 was assumed to be sub-optimal for siderophore production.

During the study of development of orange halo surrounding the colony, it was observed that distinct halo started to develop surrounding the isolates after 24 hours of incubation. Some isolates showed zones with definite margin, while some showed diffused margin zones indicating difference in ability of siderophores to remove iron from CAS – Fe^{+++} complex.

Purified siderophore showed CAS and Arnow's test positive, indicating presence of catecholate nature of the siderophore. The Csakey's test for hydroxamate was found to be negative suggesting no any reactive hydroxamate ligand.

FTIR analysis of purified samples of siderophore from *Klebsiella pneumoniae* showed broad peak at 3351 cm^{-1}, indicating presence of Ar–OH moiety. Appearance of peak at 2963 cm^{-1} showed presence of saturated alkanes. The spectrum also provides evidence for presence of amide linkage in the structure. The intense peak at 1654 cm^{-1} typically indicated an amide C=O stretch suggesting a secondary amide. The N-H stretch was not distinctly observed as the sample was analyzed in methanol (Murugappan et al., 2011). The presence of very intense and broad peak at 3351 cm^{-1} indicated intermolecular hydrogen bonds showing slight dispersion and thus the peak which appeared in the region of 3200 cm^{-1} in case of KBr analysis (Actis et al., 1986), was not observed in the spectra. Further, peaks at 1033 cm^{-1} indicated –C-O-C for ether linkages (Figure 5).

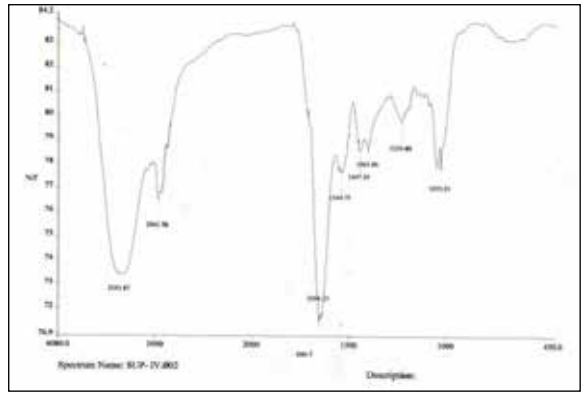

Figure 5: Fourier – Transform Infrared (FTIR) spectrum of purified catechol siderophore of isolate SUP-IV identified as Klebsiella pneumoniae.

According to the results obtained in the present study, the potent isolate identified as *Klebsiella pneumoniae* could be tried for bioremediation of iron deficient agricultural land to meet the iron requirements of the cash crops through siderophores.

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