Research Paper Microbiology

Siderophoregenic Klebsiella pneumoniae SUP II from Wheat (Triticum aestivum) Rhizoplane

* Supanekar Shirishkumar Vishnu ** Sorty Ajay Madhusudan

* Department of Microbiology, Willingdon College, Sangli-416415

** Department of Biotechnology, Willingdon College, Sangli.

ABSTRACT

In present study, 43 different isolates were obtained from 22 wheat fields in Sangli district area. Twenty seven isolates found siderophoregenic under iron limiting conditions on chrome azurol – S agar medium. The isolate SUP II showed maximum siderophore production in Fiss Glucose Mineral medium with maximum 69.81% decolorization of CAS reagent in liquid CAS assay. Optimum yield of siderophore was obtained at pH 7.1. 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 proved that purified siderophore is of hydroxamate type. Fourier – transform infrared (FTIR) analysis showed prominent peaks at 3401 cm^{-1}, 1548 cm^{-1}, 1644 cm^{-1}, 1718 cm^{-1}, supporting the colorimetric results. Purified siderophore inhibited Bacillus subtilis, Salmonella typhimurium and Serratia marcenscen in vitro. The potent siderophoregenic behavior of the isolate Klebsiella pneumoniae SUP II highlights its crucial role in iron recycling in wheat rhizosplane.

Keywords : *Klebsiella pneumoniae* • Siderophore • CAS agar • FTIR • XAD2

Introduction

Iron is one of the essential elements involved in variety of vital biological processes and thus is essential to almost all forms of life. The siderophoregenesis and specific transport of iron into the ligand-producing cell is an important adaptation for survival and success in various iron limited environments (Cox 1989, Neilands 1982). The bioavailability of iron in the rhizospheric soils at neutral pH is very low; thus siderophores play an important role in iron recycling in the rhizosphere.

Siderophores are well known for their ability sequester iron from the environment with high specificity and make the iron available to the microbial cells (Neilands 1995, Leong 1986). Thus, siderophores play an important role in iron recycling as bioavailability of iron is suboptimal at neutral pH.

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., 2007). 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). 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 pneumonia* SUP II from wheat rhizosplane.

Materials and Methods

Collection of Samples

The sampling spots were located in various 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 ^{0}C - 42.0 ^{0}C. Healthy appearing wheat plants in close proximity of diseased plant were selected for sampling. The root portion was cut and packed in sterile polythene bags and brought to laboratory, washed thrice with distilled water and then suspended in sterile saline (0.85% NaCl) solution and kept shaking at 150 rpm for 3 hours. The suspension was streaked on nitrogen free mannitol agar and incubated at 30^{0}C for 72 h. Morphologically distinct colonies were preserved on nitrogen free mannitol agar slopes after further purification.**

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 and were inoculated in 5µl quantities on Chrome – Azurol Ssulfonate (CAS) agar plates and grown at 30^{0}C for 7 days. More potent strains were further subjected for liquid CAS assay in which the comparison of siderophore production was done in terms of percent decolorization (Pyane 1994), calculated using formula:

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

Where,

Ar = Absorbance of reference

As = Absorbance of sample at 630 nm.

Phylogenetic studies

The potent siderophore producing culture was identified by 16S rRNA sequencing from the Molecular Biology Unit, NCCS, Pune using universal Eubacteria-specific primers 16F27 (5'-CCA GAG TTT GAT CMT GGC TCA G-3') and 16R1525XP (5'-TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC-3') (Pidiyar VJ et.al 2004). Sequenced 16S rRNA gene, was used for sequence analysis at the Ribosomal Database Project (RDP II; Michigan State University, East Lansing, MI) and the National Centre for Biotechnology Information (Bethesda, MD) (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 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 acidified to pH 2 using 6 M HCl and mixed with preconditioned XAD2 resin and stirred thoroughly for 3 hours. Then slurry was poured into glass column and liquid was drained at a flow rate of 30 mL/h. Siderophores 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.

Csaky's assay
Hydroxamate type of siderophores were detected using Csaky's test (Csaky 1948), with slight amendment (Velasquez et al., 2011); where sodium arsenite was replaced by sodium thiosulfate solution in order to avoid the environmental hazards caused due to sodium arsenite.

Arnow's assay:
The selective determination of catecholate-type siderophores was carried out by Arnow's colorimetric test for catechol (Arnow 1937).

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 *Klebsiella pneumonia* 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.

Siderophoregenesis and cell mass:
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).

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). Wells of 10 mm diameter were made in the seeded 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.

Results and Discussion:
Klebsiella members 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 rhizoplanic *Klebsiella pneumoniae* isolate from wheat and its ability to produce siderophores.

Phylogenetic analysis:
The phylogenetic tree for the sequence of isolate SUP II 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 AB766060 in DDBJ/EMBL/GenBank database (Figure 1).

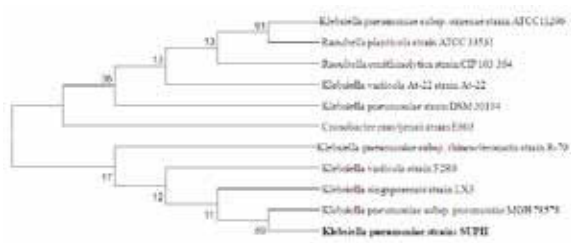

Figure 1: The phylogenetic position of the isolate obtained using MEGA 4. The sequence of the *Klebsiella pneumoniae* strain SUP II used in this study is indicated in bold letters.

While screening for siderophore positive strains, it was noted that out of 43, only 27 isolates were CAS positive.

The CAS agar with pH 6.8 yielded poor results during primary screening, thus CAS agar with pH 7.2 was used for primary screening. During screening for potent siderophore producer, it was observed that pH was having great influence on siderophore production thus, the isolate SUP II was studied for its siderophoregenic behavior in a pH range of 6.5 to 7.5, where the isolate showed more siderophoregenesis at pH 7.1 with marked decrease below and above the value. Thus, pH below and above 7.1 proved suboptimal for siderophoregenesis by *Klebsiella pneumoniae* SUP II.

Purified siderophore showed CAS and Arnow's test negative, indicating absence of catechol moiety in the structure. The Csakey's test for hydroxamate found positive suggesting hydroxamate nature of the siderophore.

In order to correlate siderophore production with increase in cell mass, Fiss - glucose mineral medium with pH 7.1 was used. Maximum siderophore production was achieved at 96 hours of incubation when the cell mass was also at its peak (Figure 2); indicating maximum siderophore production in late log phase and thus proving it a secondary metabolite.

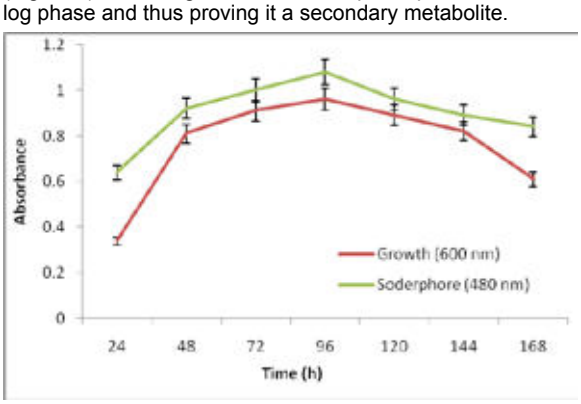

Figure 2: Siderophoregenesis by *Klebsiella pneumoniae* SUP II with rise in cell mass.

FTIR analysis of purified samples of siderophore from *Klebsiella pneumoniae* SUP II showed broad peak at 3401 cm^{-1}, indicating presence of Ar–OH moiety with hydrogen – bonded alcoholic –OH groups. Appearance of peak at 2945 cm^{-1} was due to –CH stretches of saturated alkanes. The spectrum also provides evidence for presence of amide linkage in the structure. The intense peak at 1644 and 1548 cm^{-1} typically indicated a secondary amide with CO-NH linkage. The N-H stretch was not distinctly observed as the sample was analyz-

ed in methanol (Murugappan et al., 2011). The C=O stretch due to ester group was seen at 1718 cm^{-1}. The presence of very intense and broad peak at 3401 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 AL et al., 1986), was not observed in the spectra. Further, peaks at 1032 and 1019 cm^{-1} indicated –C-O-C for ether linkages (Figure 3).

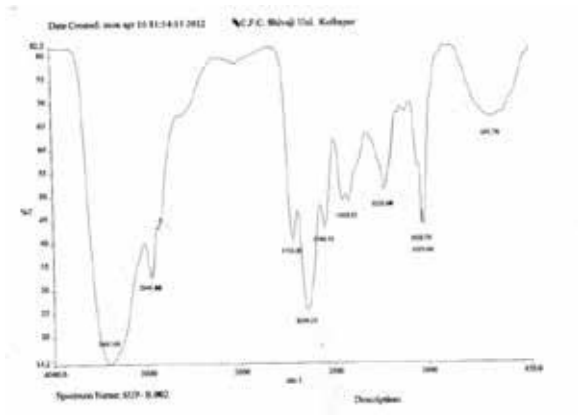

Figure 3: Fourier – transform infrared (FTIR) spectrum of purified siderophore.

The siderophores are well known for biocontrol of phytopathogens, also, considering medicinal importnace of microbial siderophores, such as their use in deferratization of patients with transfusion induced siderosis and beta thalasemia, (Daar and Pathare 2006). Involvement of siderophore produced by *Klebsiella pneumoniae* SUP II in the same was detected with purified siderophore, by the agar cup method. Distinct zones of inhibitions were obtained for human pathogens, although some showed ability to extract chelated iron from the siderophore.

The zones of inhibition with a diameter ≥ 10 mm was considered as inhibitory, thus inhibition of growth was obtained in case of *Bacillus subtilis* (11 mm), *Salmonella typhimurium* (10 mm), *Serratia marcenscen* (12 mm) while the *Xanthomonas* sp. and *Proteus vulgaris* showed inhibitory zones of dimeter less than 10 mm. When the effect was cross checked using deferreted Luria agar with siderophore – Fe^{+++} complex as iron source, all the organisms except *Pseudomonas aeruginosa* and *Candida albicans* were unable to flourish. The *Pseudomonas aeruginosa* and *Candida albicans* showed stimulation which probably because of presence of complementory receptors (OMP) expressed for the siderophore uptake. Though the inhibition of pathogens could have occurred because of inability to utilize the siderophore produced by *Klebsiella pneumoniae* SUP II, the extent of growth inhibition is still a part of further investigation.

From findings in the present study, the siderophore obtained from the isolate *Klebsiella pneumoniae* SUP II can be used to control the animal pathogens and the isolate can also be used in the agriculture to meet the iron requirements of cash crops.

Acknowledgements
This work was supported by University Grants Commission (11th plan). Authors are indebted to Dr. Yogesh Shouche, Scientist F, NCCS Pune, for conducting 16s rRNA sequencing. Authors are also indebted to Dr. Chaitanya Kulkarni for his kind help in FTIR analysis.

REFERENCES

1. Cox, C. D. (1989). Importance of iron in bacterial virulence. In Metal Ions and Bacteria. Beveridge, T. J. & Doyle, R. J. (eds.) John Wiley, New York, pp.207-246 | 2. Neilands, J. B. (1982). Microbial envelope proteins related to iron. Annu Rev Microbiol. 36: 285-309 | 3. Neilands, J. B. (1995). Siderophores: Structure and Function of Microbial Iron Transport Compounds, J biol chem. 270:45:10 pp.26723–26726 | 4. Leong, J. (1986). Siderophores: Their biochemistry and possible role in the biocontrol of plant pathogens. Annu Rev Phytopathol. 24:187-209 | 5. Vansuyt, G., Robin, A., Briat, J. F., Curie, C. and Lemanceau, P. (2007). Iron acquisition from Fe-pyoverdine by Arabdopsis thaliana. Mol Plant-microbe Interact. 20: 441-447 | 6. Chincholkar, S. B., Chaudhari, B. L. and Rane, M. R. (2007). Microbial Siderophores: State of art. In: Microbial Siderophores. Chincholkar SB and Varma A (eds) Springer Verlag, Germany, pp.233-242 | 7. Actis, A. L., Fish, W., Crosa, J. H., Kellerman, K., Ellenberger, S. R., Hauser F. M. and Loehr J. S. (1986). Characterization of Anguibactin. A novel Siderophore from Vibrio anguillarum 775(pJM1). J of Bact. 167:57-65 | 8. Xie, X. J., Wang, and Yuan, H. (2006). High-resolution analysis of catechol-type siderophores using polyamide thin layer chromatography. J Microbial Methods. 67:390-393 | 9. Iniguez, A. L., Dong, Y. and Triplett, E. W. (2004). Nitrogen fixation in wheat provided by Klebsiella pneumonie 342, Mol Plant Microbe Interact. 17:1078 | 10. Sachdev, D. P., Chaudhari, H. G., Kasture, V. M., Dhavale, D. D. and Chopade, B. A. (2009). Isolation and characterization of indole acetic acid (IAA) producing Klebsiella pneumonie strains from rhizosphere of wheat (Tritium aestivum) and their effect on plant growth. Ind J Exp Biol. 47:993-1000 | 11. Schwyn, B. and Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. Anal Biochem. 160:47-56 | 12. Pyane, S. M., (1994). Detection, isolation and characterization of siderophores. Methods in Enzymol. 235:329-344 | 13. Pidiyar, V. J., Jangid, K., Patole, M. S. and Shouche, Y. S. (2004). Studies on cultured and uncultured microbiota of wild Culex Quinquefasciatus mosquito midgut based on 16s ribosomal RNA gene analysis. Am J Tropic Med Hyg. 70:597-603 | 14. Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 Mol Biol and Evol. 24:1596-1599 | 15. Vellore, J. (2001). Iron acquisition in Rhodococcis erythropolis strain IGTS8: Isolation of non-siderophore producing mutant. M.S. Thesis. East Tennssee State University, Johnson City, TN. | 16. Csaky, T. (1948). On estimation of bound hydroxylamine in biological materials. Acta Chemica Scandinavica. 2:450-454 | 17. Velasquez, I. B. (2011). Characterization of Siderophores in the Southern Ocean, Ph.D. thesis. University of Otago, Dunedin, New Zealand | 18. Arnow, E. L. (1937). Colorimetric determination of the components of 3,4 - dihydroxyphenylalanine – tyrosine mixtures. J Biol Chem. 118:531-537 | 19. Shah, S., Karkhanis, V., and Desai, A. (1992). Isolation and Characterization of Siderophore, with Antimicrobial Activity, from Azospirillum lipoferum M. Curr. Microbiol. 25: 347-351 | 20. Govindarajan, M., Soon-Wo, K., and Hang-Yeon, W. (2007). Isolation, molecular characterization and growth-promoting activities of endophytic sugarcane diazotroph Klebsiella Sp. GR9. World J Microbiol Biotechnol. 23:997 | 21. Murugappan, R. M., Aravinth, A., Karthikeyan, M. (2011). Chemical and structural characterization of hydroxamate siderophore produced by marine Vibrio harveyi. J Ind Microbiol Biotechnol. 38:265–273 | 22. Daar, S., and Pathare, A. V. (2006). Combined therapy with desferrioxamine and deferiprone in beta thalassemia major patients with transfusional iron overload. Ann. Hematol., 85: 309-315 |