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STUDY OF CATECHOL SIDEROPHORE FROM A NEWLY ISOLATED *Azotobacter sp.* SUP-III FOR ITS ANTIMICROBIAL PROPERTY

Shirishkumar Supanekar[1] Ajay Sorty[2] Avinash Raut[3]*

Address(es): Shirishkumar Supanekar
[1]Department of Microbiology, Willingdon College, Sangli. Maharashtra. India. Pin: 416416. Phone: +919422592993.
[2]Department of Biotechnology, Willingdon College, Sangli. Maharashtra. India. Pin: 416416.
[3]Department of Microbiology, Yashwantrao Chavan College of Science, Karad. Maharashtra. India. Pin: 416410.

*Corresponding author: shirishkumarsupanekar@gmail.com

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ABSTRACT

In present study, the isolate SUP III 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 culture was identified as *Azotobacter sp.* 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 peaks at 3402 cm-1, 1652 cm-1, 1032 cm-1, and 1112 cm-1 which supported the colorimetric results. Antimicrobial activity of the purified siderophore showed significant zones of inhibition for some pathogens. This type of study has not been previously reported in this area.

Keywords: *Azotobacter* sp., Siderophore, CAS agar, FTIR, XAD2

INTRODUCTION

Iron is fourth most abundant element on Earth's crust involved in variety of essential biological processes. Many life processes use iron as one of the important elements, e. g. photosynthesis, respiration, and nitrogen fixation **(Vraspir and Butler, 2009)**. Iron is also included in vital cellular reactions such as photosynthesis, nitrogen fixation, methanogenesis, H_2 production, the TCA cycle, oxygen transport, gene regulation, DNA biosynthesis/repair, and detoxification of free radicals **(Andrews *et.al.*, 2003; Crichton and Ward, 1998)**. The ability of organisms to produce siderophores and siderophore mediated specific iron uptake by elaborating cells is a strategy adapted by microorganisms to overcome the low Fe^{+++} stress in variety of environments **(Cox, 1989; Neilands, 1982)**. Many siderophores are known to chelate other metal ions but their affinity towards Fe^{+++} seems consistent **(Arnow, 1937)**. Siderophores are known to enhance the rhizosphere colonization of ligand producing cells and also the bioavailability of soluble iron to plants **(Vansuyt, *et al.*, 2007).** Additionally, siderophores have been shown to impart inhibitory effect on phytopathogens by limiting the Fe^{+++} supply in surrounding environment, thus acting as indirect plant growth promoters **(Chincholkar, *et al.*, 2007)**. Considering the occurrence of chelating groups, siderophores are categorized into two main classes – Hydroxamate - type siderophores and phenolate or 2,3-dihydroxybenzoate (DHB) i.e. catechol – type siderophores **(Actis, *et al.*, 1986; Neilands, 1995).** The siderophores with carboxylate and hydroxyl donor groups have also been demonstrated. Many of the siderophores belong to the mixed ligand – types (**Neilands, 1995)**. Additionally, siderophores are of significant medicinal importance. Siderophores are thought to act as potential drug delivery agents for treatment of infections caused by antibiotic resistant microbes **(Nagoba and Vedpathak, 2011)**. Desferrioxamine B is a siderophore elaborated by *Streptomyces pliosus* is being used in treatment of iron overload in patients suffering from beta – thalassemia **(Aessopos, *et al.* 2007).**

Bacteria belonging to genus *Azotobacter* are a diverse group of free-living diazotrophic microorganisms commonly occurring in soil which are non-symbiotic nitrogen fixers. These have also been well characterized as plant growth promoting organisms and are being exclusively used as biofertilizer inoculants in many countries. The siderophore production along with nitrogen fixation will serve for the fulfillment of both nitrogen as well as iron (Fe^{+++}) demand in agriculture particularly in iron deficient soils. Also, considering the medicinal importance of siderophores, it is needful to detect involvement of the action of the same against human pathogens. Present study highlights the potent siderophoregenic *Azotobacter sp.* SUP III and possible use of the catecholate ligand against common human pathogens.

MATERIAL AND METHODS

Collection of Soil Samples

Twenty-two soil samples were collected by composite sampling method from the rhizosphere of *Apios americana* from various crop fields of Sangli district (M.S.). The samples were kept in sterile polyethylene bags and immediately brought to the laboratory and subjected for enrichment.

Enrichment and isolation of *Azotobacter* sp.

Enrichment of *Azotobacter sp.* was done in Ashby's Nitrogen free Mannitol broth at 30^0C for 72 h. Isolation was done on Ashby's Nitrogen free Mannitol agar medium at 30^0C for 48 h. Total 26 different isolates were obtained and maintained on agar slants having same medium composition.

Screening of Siderophore producing Strains

The siderophoregenic isolates were screened using Chrome Azurol - S Agar (CAS agar) **(Schwyn and Neilands, 1987)**. Fresh cultures were adjusted to O.D. 0.1 and were inoculated in 5µl quantities on CAS agar plates and grown at 30^0C 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)**.

Phylogenetic studies

The most potent siderophore producing isolate SUP III identified as *Azotobacter sp.* was selected for further study.
The potent siderophoregenic culture was identified by 16s rRNA sequencing from the Molecular Biology Unit, NCCS, Pune. Sequenced 16S rRNA gene, was used for phylogenetic 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 (p-distance model) was constructed using MEGA 4 by neighbor joining method keeping bootstrap value of 1000 replicates **(Tamura *et al.* 2007)**.

Production, extraction and purification of siderophores

The siderophore production for selected isolate of *Azotobacter sp.* was achieved using Burk's medium **(Subba Rao, 1977)** with slight modification, where the original Burk's medium was supplemented with 0.5% casamino acids. The 350 mL batch of culture was incubated in shaking condition (150 rpm) at 30^0C for 48 h. Cells were pelleted at 7000 ×*g* for 10 min. The supernatant was acidified to pH 2 using 6 M HCl and mixed with conditioned XAD2 resin and stirred thoroughly for 3 hours. The slurry was 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 40mL of 5% methanol. 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 -20^0C till further use.

Determination of Hydroxamate and Catecholate type siderophores

Hydroxamate group was detected using Csaky's test **(Csaky, 1948)**, with slight amendment **(Velasquez, 2011)**. The original method described by Csaky uses sodium arsenate solution, which was replaced, by sodium thiosulfate solution in order to avoid the environmental hazards caused due to sodium arsenate.
Catecholate-type siderophores were detected using Arnow's test **(Arnow, 1937)**. One mL sample was acidified using equal volume of 0.5 M HCl, then 1.0 mL of nitrite-molybdate solution (mixture of $NaMoO_4$ and $NaNO_2$ 10% each) was added. This was added with equal volume of 1.0 M sodium hydroxide and mixed. The intense red color indicated presence of catechol compounds. The intensity of red color was measured at 510 nm using a single beam UV-Vis spectrophotometer of Systronics make.

FTIR analysis

Infrared (IR) spectra of the active fraction of siderophore was done in courtesy of USIC, Shivaji University, Kolhapur using a Magna 550 model of FTIR spectrometer, Nicolet Instruments Corporation, USA in the range 50-4000 cm^{-1} as described earlier. **(Tank *et al.* 2012)**.

Antimicrobial activity of purified siderophore

In-vitro antimicrobial activity of purified siderophores was detected using agar cup method in Luria agar medium **(Shah *et al.* 1992)**. Twenty ml sterilized, cooled Luria agar was seeded with 10^7 CFU ml^{-1} of fresh 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. Sterile double distilled water was used as control. The plates were kept at 15^0C for 30 min and then incubated for 60 hrs at 37^0C for human pathogens and 30^0C for plant pathogen.

RESULTS AND DISCUSSION

Isolation and phylogenetic analysis of *Azotobacter sp*.

Variance was found in isolate numbers with respect to different soil textures i.e. 12 isolates from black soil, 4 isolates from yellow, 6 isolates from brown and 4 isolates from rocky soils were obtained. This can be co-related to various factors like pH, cultivated crop, carbon, nitrogen and phosphorous content of soil **(Line and Margaret, 1969)**, soil texture **(Hegazi, 1979)**, influencing occurrence of diazotrophic population in soils. The potent siderophoregenic isolate SUP III was isolated from black soil. Figure 1 depicts the phylogenetic tree for the sequence of isolate SUP III which shows significant similarity to *Azotobacter sp.* drawn by bootstrap method (p-distance model) using MEGA 4. Sequence of the 16S rRNA fragment of same isolate has been deposited under Accession number AB742373 in DDBJ/EMBL/GenBank database.

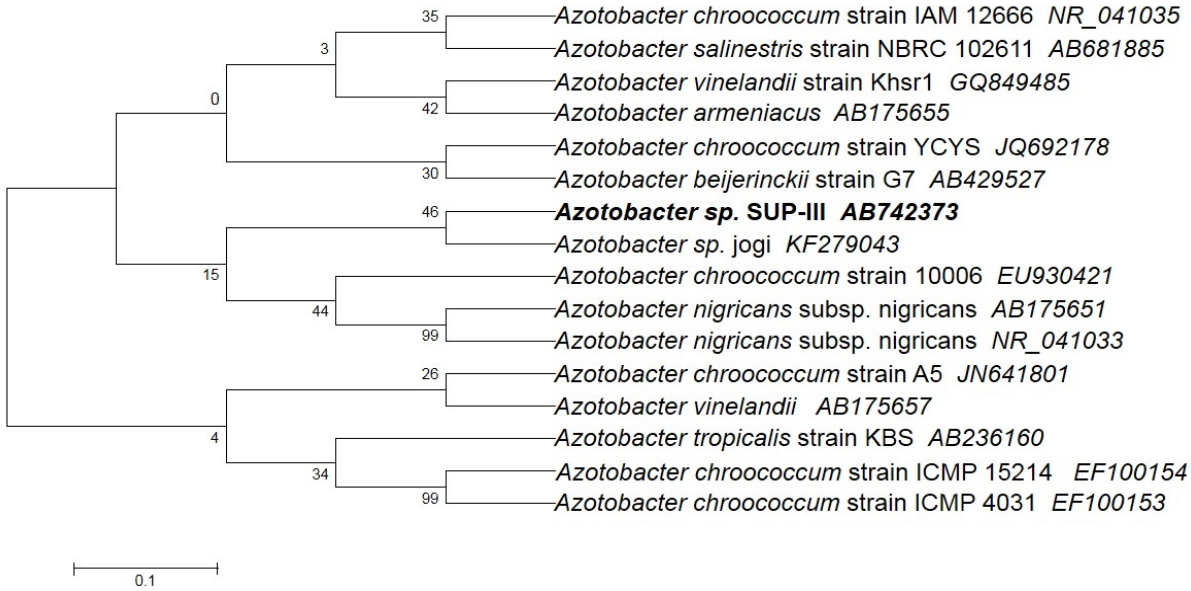

Figure 1 The phylogenetic position of the isolate obtained using MEGA 4. The *Azotobacter sp*. used in this study is indicated in bold letters.

Out of 26, only 15 isolates were CAS positive with variable siderophoregenic ability. Also, the number of CAS positive isolates greatly varied with soil texture and irrigation status of the soil (Figure 2).

Maximum number of CAS positive *Azotobacter* isolates was obtained from irrigated black soil samples as, appropriate moisture and dissolved nutrients are readily available in such conditions, while the same non irrigated soil texture showed presence of little number of CAS positive isolates showing reduced siderophoregenesis in reduced moisture conditions.

Screening of siderophore producing strains

During screening for potent siderophore producer, pH was seen to have great influence on siderophore production by the isolates. It was found that, isolates produced more siderophores at pH 7.1 on CAS agar than that of pH 6.8, which is the original pH recommended for CAS agar **(Schwyn and Neilands, 1987)**. Thus the potent isolate SUP III was further studied for its siderophoregenic behavior in a pH range 6.5 to 7.5 (Figure 3), where the isolate was found more siderophoregenic at pH 7.2.

Determination of Hydroxamate and Catecholate type siderophores

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 negative suggesting absence of reactive hydroxamate ligand, which was in accordance with FTIR results.

FTIR Analysis

FTIR analysis of purified siderophore showed broad peak at 3402 cm^{-1}, indicating presence of Ar–OH moiety. Appearance of peak at 2949 and 2842 cm^{-1} showed presence of saturated alkanes. The intense peak at 1652 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, 2011)**. The presence of very intense and broad peak at 3402 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 1032 and 1112 cm^{-1} indicated –C-O-C for ether linkages (Figure 4).

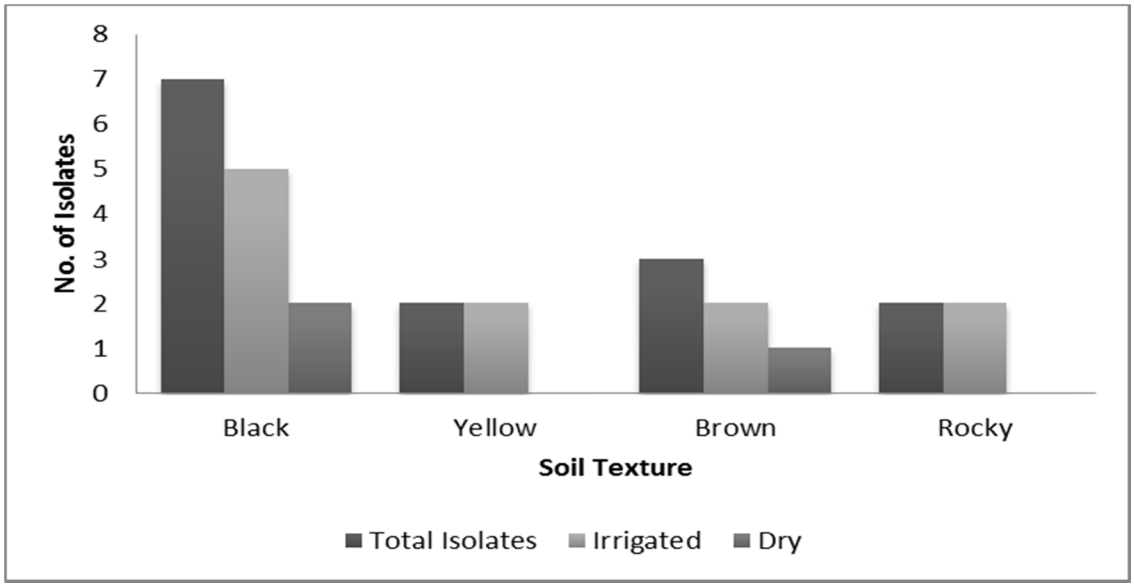

Figure 2 Total number of CAS positive isolates with respect to soil texture and their distribution with respect to irrigated and dry (non irrigated) soils. *Azotobacter sp.* SUP-III presented here was obtained from black soil.

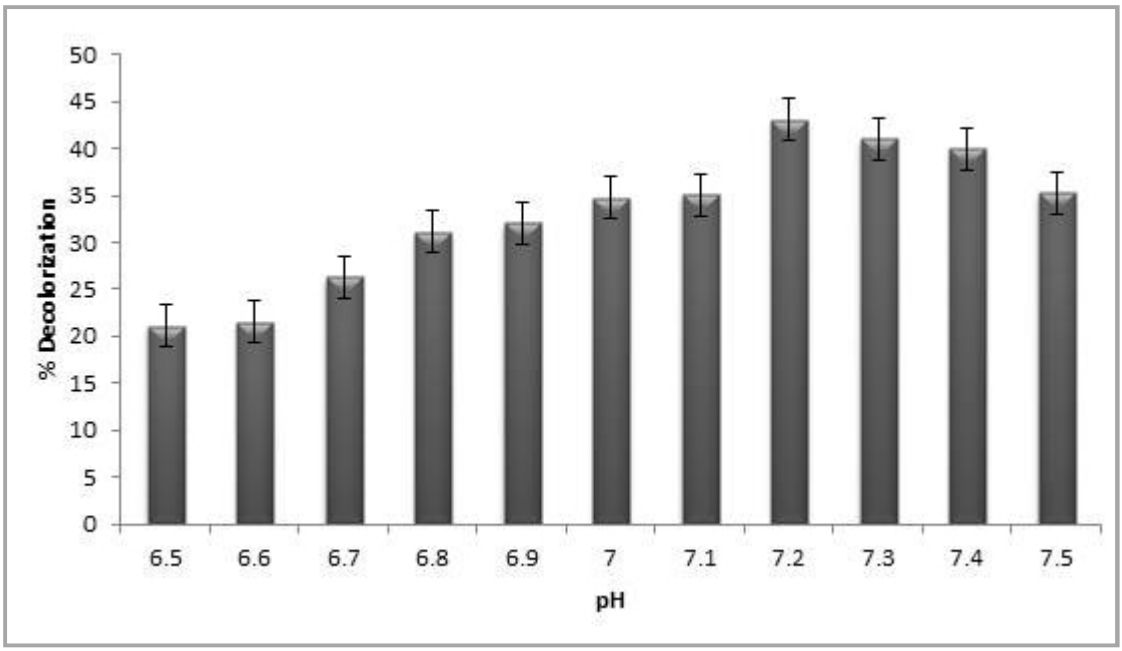

Figure 3 Variation in siderophore production detected in terms of percent decolorization of CAS reagent by isolate SUP III seen in Burk's medium with pH ranging from 6.5 to 7.5.

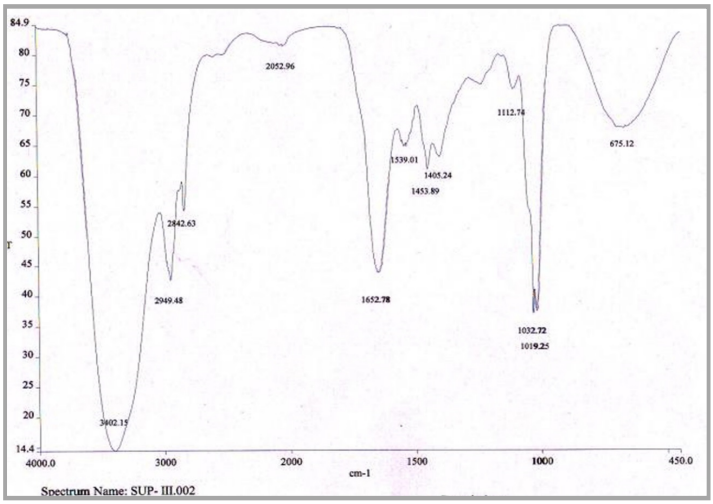

Figure 4 Fourier – transform infrared (FTIR) spectrum of purified siderophore.

Antimicrobial activity of purified siderophore

The plant rhizosphere inhabitants have been known to exert growth promoting effects on plants directly by enhancement of nutrient uptake, growth hormone production, or indirectly by antibiotics, bacteriocins, or siderophores production that adds inhibitory effect on the growth of phytopathogenic bacteria **(Strutz and Christie, 2003)** and fungi **(Manwar, 2004).** The Staphylococci are known to possess a range of receptors for hydroxamate siderophores **(Sebulsky, *et al.* 2000)**, this highlights the use of siderophores to conjugate antibiotics for treatment of multidrug resistant strains of staphylococci. The involvement of siderophore elaborated by *Azotobacter sp.* SUP III in the same was detected against *Pseudomonas aeruginosa, Proteus vulgaris, Candida albicans, Bacillus subtilis, Serratia marcescens, Salmonella typhimurium* and *Xanthomonas* sp. The inhibition zones with a diameter greater than 10 mm were considered inhibitory, thus growth inhibition was obtained in case of *Proteus vulgaris* (12 mm), *Bacillus subtilis* (15 mm), *Salmonella typhimurium* (13 mm) and *Candida albicans* (16 mm), while rest of the above pathogens except *Xanthomonas* sp. (no inhibition); showed inhibitory zones of diameter less than 10 mm. When the effect was cross checked using deferreted Luria agar and siderophore – Fe^{+++} complex as iron source in the wells, all the organisms except *Xanthomonas* sp. were unable to flourish. *Xanthomonas* sp. remained unaffected probably because of presence of complementary receptors for the siderophore. Though the inhibition of pathogens could have occurred because of inability to utilize the

siderophore produced by *Azotobacter sp.*, the extent of growth inhibition is still a part of further investigation.

CONCLUSION

The overall results of present study suggest that, the potent siderophoregenic *Azotobacter sp.* SUP III could be employed in bioremediation of iron deficient soils in agriculture and for biocontrol of pathogens as well. Also, the antimicrobial action of the purified siderophore highlights its use in medicine.

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