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Extraction and Characterization of Catecholic Siderophores in *Bacillus* Sp. Isolated from the Mangrove Sediments

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Abstract: *Iron is a requisite nutrient for the growth and proliferation of bacteria. The paucity of iron in the harsh environments such as mangrove has driven many bacteria to evolve sophisticated means to acquire iron from their surroundings, including the use of high-affinity iron chelates termed siderophores. These siderophores have important ecological and commercial applications. In view of this, an attempt was made to detect the presence of siderophores from some bacteria isolated from sediments collected from mangrove area. After detection, these siderophores were chemically characterized and the bacterial strain was phylogenetically identified.*

Key words: *Siderophores, Bacillus spp., Mangroves, Iron, Phylogenetic study*

1. INTRODUCTION

The transition metal iron is an essential micronutrient for almost all organisms, including bacteria, fungi and plants. The bioavailability of iron is very less in the mangrove environment because of the reduced solubility of various inorganic iron salts in seawater. Various organisms have evolved active strategies to overcome this lack of iron and acquire this precious essential mineral element [1].

Siderophores are low molecular weight organic compounds produced by a variety of microorganisms, fungi and plants growing under iron limiting conditions [2]. These compounds primarily chelate the ferric iron [Fe(III)] from different terrestrial and aquatic habitats and thereby make it available for the cells. They are also reported to form complexes with other essential elements such as Mo, Mn, Co and Ni in the environment making them available to the microbial cells [3].

Oweing to their important role in weathering soil minerals and soil formation, enhancing plant growth, biocontrol of pathogens, nuclear fuel processing, bioremediation of pollutants, biogeochemical recycling of Fe in the ocean, bio-bleaching of pulps and as biosensors; siderophores have attracted considerable attention in last few years [4].

Siderophores are divided into three main families depending on the functional group, i.e. hydroxamates, catecholates and carboxylates. More than 500 different types of siderophores are known [5, 6].

Even though siderophores have been reported from a variety of organisms inhabiting diverse environments; the study of marine siderophores is in its infancy as compared to their terrestrial counterparts. Therefore, the present investigation was carried out to detect and characterize catecholic siderophores of unique *Bacillus* spp. isolated from sediments collected from mangrove environment.

2. MATERIALS AND METHODS

2.1. Isolation of Bacteria

The sediment sample was collected from mangrove areas of Ratnagiri, Maharashtra state, West cost of India. The sample was collected in sterile falcon tube and brought to the laboratory. The sample was serially diluted in sterile sea water and an aliquot (100µl) was spread plated on Zobell Marine Agar. The plates were incubated at 30^0C for 48-72 h. Single colonies were picked and purity was confirmed

by repeated streaking. The purified bacterial cultures were maintained on Zobell marine agar slants and these bacteria were further used for the detection of siderophores.

2.2. Detection of Siderophore Positive Cultures

The universal chrome azurol sulfonate (CAS) assay was used to detect siderophores, produced by the bacteria [7]. The sediment derived bacterial cultures (SB) were spotted on CAS agar plates and incubated for 48 h. After incubation period, spots were observed for colour change, if any.

2.3. Quantification of siderophores

The Spectrophotometric method using CAS reagent described by Alexander and Zuberer [8] was used to quantify siderophore production by SB+ strain.

2.4. Mass Culture of Siderophore Positive MS2 Culture

The siderophore positive MS2 culture was selected further for mass culture. Seed culture was prepared in 150ml of deferrated minimal media (sucrose 2g, KH2PO4 0.03g, CaCl2.2H2O 1.15g, MgSO4.7H2O 0.49g, tryptone 3g per litre). After 3 days of incubation, the inoculums were transferred to 2.5 liters of deferrated minimal media. The culture flasks were incubated on shaker (30ºC, 100 rpm) for 7 days. After incubation, siderophores were extracted by centrifuging the culture broth of MS2 and then by adsorbing on a polymeric resin XAD-2 and washing with methanol.

2.5. Chemical Characterization of Siderophores

Thin-layer chromatography (TLC) was performed on 0.25-mm-thick silica gel 60 F254 in butanol-acetic acid-water (12:3:5). Infrared (IR) spectra of active fraction were recorded using FTIR spectrometer in range of 500-4000 cm^{-1} in a KBr pellet.

2.6. Phylogenetic Analysis of MS2 Bacterial Isolate

Identification of siderophore producing bacterial culture was carried out by using 16S rRNA gene sequences. DNA was extracted from stationary phase culture using the standard phenol-chloroform extraction procedure.

The DNA isolated from MS2 was subjected to polymerase chain reaction (PCR) amplification using Biometra thermal cycler (T-Personal 48). The universal primers 27f (5'-GAGTTTGATCCTGGCTCA-3') and 1385r (5'-CGGTGTGT(A/G)CAAGGCCC-3'), were used for PCR amplification. The PCR reaction mix contained 2.5µl of 10X buffer, 1µl of each primer, 2.5µl of 2.5mM of each dNTP, 2.5 Units of Taq DNA polymerase and 1µl Template DNA and 8.5µl nuclease free water. The PCR amplification cycle consist of, a cycle of 5 min at 94°C; 35 cycles of 1min at 94°C, 1 min at 60˚C, 2 min at 72°C; and additionally 1 cycle of 7 min at 72°C. The PCR reagents used were procured from GeNei and the primers were synthesised by Bioserve Biotechnologies (INDIA) Pvt. Ltd.

The PCR amplification product was purified using the geneclean kit (Bio 101). The recovered fragment was sequenced on a Li-Cor 4200 automated sequencer using sequencing primers. The obtained sequences were aligned using ABI prism Auto assembler v. 2.1 software (Perkin Elmer) and entered into the BLAST.

3. RESULTS

3.1. Sediment Derived Bacteria (SB)

Total twelve bacterial isolates (MS1 - MS12) were obtained from the mangrove sediment samples. The morphological characteristics are given in Table 1.

Table1. *Morphological characteristics of bacteria isolated from mangrove sediments*

Isolate No.	Colony Size (mm)	Elevation	Translucency	Colour
MS1	0.5	Flat	Translucent	Cream
MS2	1	Umbonate	Translucent	Cream
MS3	0.5	Raised	Transparent	Cream
MS4	0.5	Raised	Transparent	Cream
MS5	0.5	Raised	Opaque	Yellow
MS6	0.5	Raised	Transparent	Cream
MS7	1	Flat	Translucent	Cream
MS8	1	Raised	Transparent	Cream
MS9	2	Raised	Opaque	Cream
MS10	3	Flat	Translucent	Cream
MS11	3	Flat	Translucent	Cream
MS12	0.5	Raised	Transparent	Cream

3.2. Screening of Bacterial Isolates for Siderophore Production

Out of the twelve bacterial isolates, eight isolates showed the production of siderophores. In CAS plate assay, cultures formed an orange halo around the culture indicating siderophore production by the culture. The change in colour from blue to orange was due to chelation of iron bound to CAS by the siderophore resulting in free CAS which turns into characteristic orange/red. Thus, cultures showing colour change were considered positive for siderophore. The details of screening results are given in Table 2.

In CAS plate assay, cultures form an orange halo around the culture indicating siderophore production by the culture. This diameter of this halo is given in mm.

Table2. *Screening of sediment derived bacterial for the production of siderophores.*

SAB strains	Siderophore production	Zone diameter (mm)	Abs readings (630nm)	Abs Reference (630nm)	% Siderophore units
MS1	-	-	-	-	-
MS2	+++	28	0.511	0.668	23.5
MS3	+	3	0.653	0.668	2.2
MS4	+	6	0.638	0.668	4.5
MS5	++	19	0.585	0.668	12.4
MS6	-	-	-	-	-
MS7	-	-	-	-	-
MS8	++	7	0.633	0.668	5.2
MS9	+++	28	0.511	0.668	23.5
MS10	++	8	0.628	0.668	6.0
MS11	++	7	0.633	0.668	5.2
MS12	-	-	-	-	4.5

3.3. Quantification of Siderophore Production

Out of the eight Sid+ MS cultures, one prominent culture (MS2) was chosen further for the quantification of siderophores. In this assay, the production of siderophore by MS2 was found to be 23.5%. siderophore unit (Table 2)

3.4. Chemical Characterization of Siderophores

3.4.1. Thin Layer Chromatography

The TLC plate was examined under UV light or sprayed with ninhydrine to detect peptide/amino acid compounds. To detect catechol-type compounds, plate was sprayed with the reagents of the 1% ferric ammonium citrate and then with 1% potassium ferricyanide. TLC analyses demonstrated only the presence of the catechol of Rf 0.45 cm found in MS2 extract.

3.4.2. FTIR analysis

The infrared spectrum analyses of MS2 extract in a KBr pellet showed peak at 3000, 3100 cm-1 for aromatic C-H stretching, 916 and 648 cm-1 for aromatic C-H bending and broad peak at 3300 cm-1 indicating the presence of aromatic OH moiety of siderophores. Appearance of peak at 2958 cm-1 showed presence of saturated alkanes. The spectrum also provides evidence for the presence of amide linkage in the structure. The intense peak at 1643 cm-1 typically indicated an amide C=O stretching suggesting a secondary amide functionality. The peak at 3172 cm-1 is attributed to NH stretching. Furthermore, a peak at 1118 cm-1 indicated -C-O-C- bond of ether linkage. Thus spectrum indicated the presence of catechol siderophores (Fig. 1).

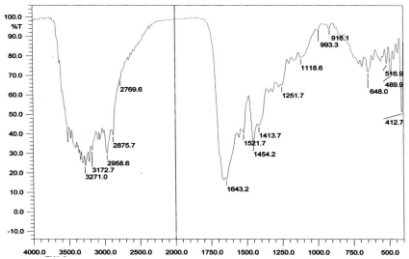

Figure1. *FTIR spectrum of MS2 (Siderophore positive) bacterial extract*

3.5. Phylogenetic Study of the Siderophore Positive Bacterial Isolate

Phylogenetic analysis of the sequence of the amplified 16S rRNA gene of the siderophore producing bacterium produced a 520 bp product. Results of BLAST analysis after alignment with the database showed that prominent Sid+ strain was Bacillus sp. (Table 3).

Table3. *Results for BLAST analysis of the gene sequence of the unknown bacterium*

Description	Max score	Query cover	E value	Ident	Accession
Bacillus licheniformis strain SB9 16S ribosomal RNA gene, partial sequence	961	100%	0.0	100%	KF443077.1
Bacillus sp. JBP-9 16S ribosomal RNA gene, partial sequence	948	100%	0.0	99%	KM675938.1
Bacillus sp. mrinalini4 16S ribosomal RNA gene, partial sequence	948	100%	0.0	99%	KF909132.1
Bacillus sp. H-95 16S ribosomal RNA gene, partial sequence	948	100%	0.0	99%	KF021777.1
Bacillus sp. H-61 16S ribosomal RNA gene, partial sequence	948	100%	0.0	99%	KF021745.1
Bacillus licheniformis strain 1351 16S ribosomal RNA gene, partial sequence	948	100%	0.0	99%	JN645997.1
Bacillus licheniformis strain IARI-AB-14 16S ribosomal RNA gene, partial sequence	948	100%	0.0	99%	JN411306.1
Bacillus licheniformis strain SAT2-11 16S ribosomal RNA gene, partial sequence	948	100%	0.0	99%	HQ236037.1
Bacillus licheniformis strain WMA-BD2 16S ribosomal RNA gene, partial sequence	942	100%	0.0	99%	KT008385.1
Bacillus sp. DP-7 16S ribosomal RNA gene, partial sequence	942	100%	0.0	99%	KP238218.1

4. DISCUSSION

Siderophores and their substituted derivatives have a large number of applications in agricultural, environmental and medical sciences. Microorganisms produce a wide range of siderophores and different bacteria and fungi are known to use different siderophore-mediated Fe transport systems [4].

Most of the bacterial siderophores especially those from *Bacillus* spp. are known to be catecholates [9, 10, 11] as also indicated in the present investigation. However, isolation of hydroxamate siderophores of *Bacillus* spp. have also been reported recently [6].

TLC and FTIR spectrum indicated the presence of catecholic siderophores. It was further confirmed by comparing IR data of MS2 siderophore samples with the reported IR spectrum of catecholic siderophores. The IR spectra of MS2 metabolites were matching with spectrum of catecholate type siderophore reported by Actis *et al*., [12].

Phylogenetic study carried out using 16S rRNA gene sequences clearly indicated that the bacterium was from the Genus *Bacillus* highlighting the usefulness of such genetic markers for accurate species identification.

5. CONCLUSION

This investigation highlights the importance of mangrove sediment derived bacteria for the production of siderophores. Further purification and characterization of siderophores from strain MS2 are ongoing in order to explore this source in the field of medicine, agriculture and biotechnology.

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