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L-asparaginase and L-glutaminase Producing *Actinomycetales* from Bhitarkanika Mangrove Sediment- A First Report

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The under explored mangrove sediment of Bhitarkanika, a potent source for the isolation of new actinobacteria and were recognized as a source of novel antibiotics and anticancer agents having unusual structure and properties. In the present study twenty actinobacterial isolates were obtained and screened for L-asparaginase and L-glutaminase production. Only four isolates were found to exhibit the above activity. 16S rDNA analysis revealed one isolate i.e. DSOIIA as a new strain of *Actinomycetales bacterium* BkSoiiA bearing Genebank Accession number: KC573069 and positive for L-asparaginase and L-glutaminase production.

Key words: *Actinomycetales bacterium,* actinobacteria, Bhitarkanika, L-asparaginase, L-glutaminase**.**

Actinobacteria play a quite important role in natural ecological system. They are also prolific producers of antibiotics, antitumor enzymes, enzyme inhibitors and immune modifiers, widely applied in agriculture, forestry, pharmaceutical and other industries. The actinobacterial population density is less common in marine sediments relative to terrestrial soils[1]. Actinobacterial resources in extreme environments (i.e. high and low temperature, high or low pH, high salt concentration etc.) so far have received comparatively little attention from microbiologists. Traditionally, actinomycetes have been isolated from the terrestrial sources only and the first report of mycelium forming actinobacteria being recovered from marine sediments appeared several decades ago[2]. Recently, the marine derived actinobacteria are recognized as a source of novel antibiotics and anticancer agents with unusual structure and properties[3].

Mangrove forest of Bhitarkanika, Odisha, occupy a thalassic ecosystem and impart immense ecological importance for maintaining marine life, their high productivity and in supplying organic material to coastal marine ecosystems[4-6]. The under explored mangrove sediment of Bhitarkanika is a potent source of new actinobacteria. The present investigation was aimed to identify the actinobacterial isolates having ability to produce L-asparaginase and L-glutaminase.

MATERIALS AND METHODS

Study area

Bhitarkanika being the second largest mangrove ecosystem in India covers an area of 672 sq. km extending between 20° 4' to 20° 8' North latitude 86° 45' to 87° 50' East longitude along the east coast of Bay of Bengal, in Kendrapara district of Odisha, India. The estuarine rivers of

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Bhitarkanika i.e. Brahmani, Baitarani, Kharasrota, Dhamra, Pathasala, Maipura, Hansua, and Hansina are criss-crossed by numerous creeks and channels during their course flow into the Bay of Bengal, thus providing a peculiar ecological niche for the growth and development of rich and varied mangrove life forms[7-10].

Sample Collection

Sediment samples were collected from different areas i.e. Khola, Balizore, Dangamal, Suazore of Bhitarkanika , from a depth of 5cm to 15 cm with a sterile spatula in sterile polythene bags from above locations and transported to the laboratory for microbiological analysis.

Enumeration and Isolation

All the media used were procured from Hi-Media, Mumbai and prepared as per manufacturer 's instructions. All the experiments were carried out in triplicate.

Sediment samples were dried and processed for selective isolation of actinobacteria[11-14]. Then 10 fold serial dilutions of sediment samples were made using sterile 50% sea water[15]. About 0.1ml of the serially diluted sample was taken and spreaded over both Actinomycetes Isolation Agar and Starch Casein Agar medium[16] supplemented with 50 µg/ml of nystatin to inhibit fungal growth and 80µg/ml cycloheximide to minimize other bacterial growth respectively.

Screening of the isolates for L-asparaginase and L-glutaminase production

Preliminary screening of the isolates was done for L-asparaginase and L-glutaminase production by plate assay technique[17] on Glucose Asparagine Agar and Minimal Glutamine Agar respectively supplemented with glycerol, antibacterial and antifungal substance. Phenol red was used as an indicator. The pH was adjusted to 7.0. Plates were spot inoculated with different actinbacterial isolates. The zone diameter was studied after 5 days of incubation.

Biochemical and Physiological Tests

The isolates showing maximum zone diameter during preliminary screening were selected and biochemical tests of the selected ones were done for identification following Bergey's manual of Determinative Bacteriology[18]. All the isolates were also screened for different carbon sources and amino acids utilization, extracellular enzyme production and antibiotic sensitivity. The effect of physiological parameters like pH, salt and temperature tolerance on the growth was also studied.

DNA extraction and PCR amplification from actinobacteria

Actinobacterial DNA extraction was done followed by PCR amplification[19,20]. A fragment of genomic DNA of the isolate DSOIIA was used for amplification of 16S rDNA gene. The universal 16S rDNA primer, forward primer 8F, and reverse primer 1492R were used for amplification. The PCR amplification was carried out in a reaction mixture containing 2µl of genomic DNA as template, 1 μl dNTP mix (2.5mM each), 100 ng/μl each of forward and reverse primer, 1X *Taq* DNA polymerase assay buffer (10X), 3U *Taq* DNA polymerase enzyme (Bangalore Genei Ltd., Bangalore, India), and distilled water enough to make up the volume of reaction mixture to 50 μl. Amplification conditions were 2min initial denaturation at 96^{o}C, 30cycles of denaturation at 96^{o}C for 45sec., 30 sec. of primer annealing at 56^{o}C, 2 min elongation at 72^{o}C, and a final extension of 8 min at 72^{o}C. The amplification was carried out in a Thermal Cycler (Bio rad, Thermocycler).

Electrophoresis and Sequencing of 16S rRNA Gene

15 μl of PCR amplified product was electrophoresed on 1.2% (w/v) low-EEO agarose gel in 1X TBE buffer (45mMTris-borate, pH 8.3, and 1mM Na 2 EDTA) at 100 V for 2 h. The gel was stained with ethidium bromide in a final concentration of 0.5 μg/ml. A single discrete PCR amplicon band of 1500 bp was observed and photographed under UV light (Gel Image-1). The amplified 16S rRNA gene from the isolate was eluted and purified from the gel slice using the GeneiPure TM Gel Extraction Kit (QIAGEN). Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1332bp of 16S rDNA gene was generated from forward and reverse sequence data using aligner software.

Sequence Alignment of 16S rRNA Gene

The 16S rDNA gene sequence of the isolate DSOIIA was used to carry out BLAST[21] with the nr database of NCBI GenBank database. Based on maximum identity score, first ten

sequences were selected and aligned using multiple alignment software program ClustalW version 1.6[22]. Distance matrix was generated using RDP database, and the phylogenetic tree was constructed using MEGA 4.0[23].

RESULTS AND DISCUSSION

Twenty actinobacterial isolates obtained from Bhitarakanika mangrove forest sediment were screened for L-asparaginase and L-glutaminase production. Only 4 isolates named as DSOIIA, DSedIC, DSedIIA, and DSedIIC were found positive for the above two enzymes. DSOIIA and DSedIIA showed L-asparaginase activity. DSoII A, DSedI C and DSedII C all the three isolates were positive for L Glutaminase (Table: 1).

Morphological characteristics, biochemical tests, extracellular enzyme production, different growth parameters were studied for DSOIIA . Analyzing all the above parameters, DSOII A was selected for 16S rDNA sequencing for identification. Figure 1 shows the 1500bp region of the amplicon band.

After forward sequencing of DSOIIA using 8F universal forward primer 565 bp sequence was obtained (ACGAGCG……. CAGGCTAGA GTCTTGT) and a reverse sequence of 942bp (TTGCAACCC……. TGCTTCTTCTGCGAG) was obtained by using 1492 universal reverse primer.

The evolutionary history was inferred using the Neighbor-Joining method[24]. The bootstrap consensus tree inferred from 500 replicates[25] is taken to represent the evolutionary history of the taxa analyzed[25]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches[25]. The evolutionary distances were computed using the Kimura 2-parameter method[26] and are in the units of the number of base

Table 1. Plate Assay for L-asparaginase and L-glutaminase

Enzyme	Isolates zone diameter (mm)[a]							
	DSOIIA		DSedIC		DSedIIA		DSedIIC	
	Colony	Pink zone	Colony	Pink zone	Colony	Pink zone	Colony	Pink zone
Asparaginase	29.67± 1.54	19.67± 1.15	-	-	12.33± 1.52	0.67± 0.58	-	-
Glutaminase	34.67± 1.53	33.67± 0.58	38.67± 1.15	11.33± 1.53	-	-	13.33± 1.5	23± 1.73

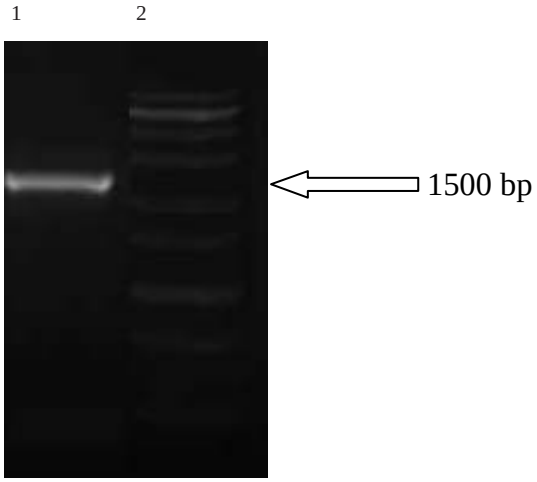
[a] numbers in the table indicate mean of triplicate plate reading ± standard deviation ; - for negative results

Table 2. Sequence match (BLASTN) Producing Significant Alignments

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JQ924072.1	Actinomycetales bacterium AA1502	2449	2449	100%	0.0	99%
JQ924075.1	Actinomycetales bacterium AA1507	2438	2438	100%	0.0	99%
JQ924077.1	Actinomycetales bacterium AA1519	2422	2422	100%	0.0	99%
JQ660195.1	Cellulomonas hominis strain S6-233-1	2303	2303	100%	0.0	98%
JQ659324.1	Micrococcus endophyticus strain L3-101	2170	2170	100%	0.0	96%
	Curtobacterium plantarum strain CIP 108988	2145	2145	100%	0.0	96%
EU556503.1	Uncultured actinomycete clone Z851020	1971	1971	100%	0.0	93%
DQ521381.1	Dietzia sp. ice-oil-124	1607	1607	100%	0.0	89%
JF833747.1	Uncultured Streptomyces sp. clone D1	2309	2309	99%	0.0	98%
HQ696525.1	Actinobacterium YH66	1358	1358	99%	0.0	85%

substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1247 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4[23]. A phylogenetic tree was constructed using 11 strains including isolates DSOIIA the type strains of *Actinomycetales bacterium* AA1502 ,*Actinomycetales bacterium* AA1507 , *Actinomycetales bacterium* AA1519 , *Cellulomonas hominis* strain S6-233-1 , *Micrococcus endophyticus* strain L3-101, *Curtobacterium plantarum* strain CIP 108988, Uncultured actinomycete clone Z851020, *Dietzia* sp. ice-oil-124, Uncultured *Streptomyces* sp. clone D1, *Actinobacterium* YH66 (fig 2)

Bhitarakanika being one of the largest



Lane 1: 16S rDNA amplicon band
Lane 2: DNA marker

Fig. 1. 16S rDNA amplicon band

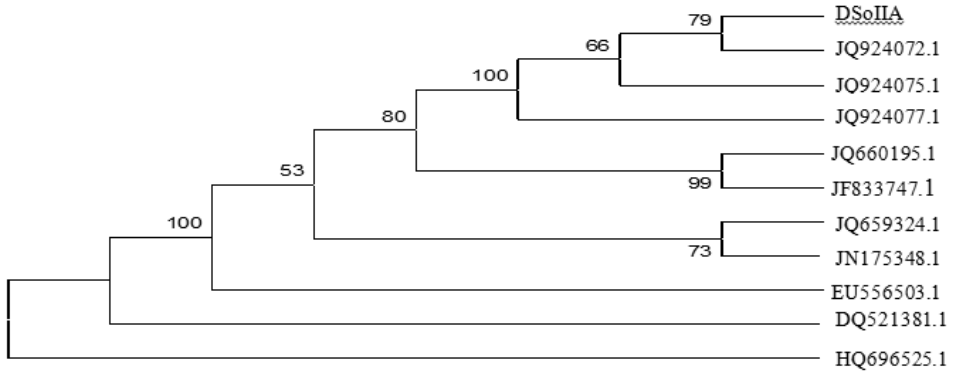


Fig. 2. Phylogenetic relationship between isolates DSOIIA and other 10 taxa determined form 16S rRNA gene sequence similarities

mangrove forests in India remains scarcely explored as far as the potential actinobacteria producing anti-tumor enzymes is concerned. only one report on *Streptomyces* isolated from phyllospheric region of Bhitarakanika mangrove was reported by Gupta *et al*. 2009[7,8]. *Actinomycetales bacterium* having L glutaminase activity has not yet been reported from Bhitarakanika mangrove forest. The present findings on occurrence of *Actinomycetales bacterium* producing both L-asparaginase and L –glutaminase is not exactly corroborating with others. However *Streptomyces olivochromogenes* from sediment of mangrove rhizosphere of Parangipettai costal area was reported[27]. *Streptomyces* SBUI has also been reported from marine sediment of Cape Camorin coast producing L glutaminase[28]. Dhevangi and Purani (2006)[29] had isolated *Streptomycetes* PDK 7 from Parangipettai and Cochin mangrove sediments samples. Narayana *et al.*, 2008 isolated *Streptomyces albidoflavus* exhibiting L asparaginase activity[30]. A lot of research work on actinobacteria producing anti-tumor enzyme has been reported from southern coastal mangrove sediment of India[27-30].

Clustering on the basis of the neighbour-joining algorithm showed that isolates DSOIIA formed a sublineage with type genus *Actinomycetales bacterium* at a similarity 99% which are isolated from marine sediment (NCBI site). The strain next best matches with *Cellulomonas hominis* strain S6-233-1 and

Micrococcus endophyticus strain L3-101 at a similarity of 98% and 96% respectively. *Curtobacterium plantarum* strain CIP 108988, Uncultured actinobacteria clone Z851020, *Dietzia* sp. ice-oil-124, Uncultured *Streptomyces* sp. clone D1 and *Actinobacterium* YH66 were 93, 89, 98 and 85%, respectively. Isolate DSOIIA have high G+C content. Microscopic, morphological and biochemical characters of the isolate identified it as *Actinobacteria*. However 16S rDNA gene sequences of DSOIIA deposited in NCBI GenBank revealed it as *Actinomycetales bacterium* (Accession number: KC573069). This was a first report of *Actinomycetales bacterium* producing L-asparaginase and L-glutaminase from Bhitarkanika mangrove sediment.

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