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Isolation and Identification of Actinomycetes from Mangrove Soil and Extraction of Secondary Metabolites for Antibacterial Activity

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AP and SKS designed the study, performed the statistical analysis and wrote the first draft of the manuscript. Author YKM helped in collection of sample and antibacterial studies. Authors SKT and BKS managed the literature searches and helped in editing of the manuscript. Author JKP did the statistical analysis, managed the literature searches and helped in editing of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

The mangrove ecosystem of India is an extensively unexplored source for actinomycetes with the potential to produce secondary metabolites of biological importance. In this study, twenty two actinomycetes were isolated from different soil samples collected from the Bhitarkanika mangrove forest along Odisha coast, India. These isolates were identified as *Streptomyces* sp. based on their morphological, physiological and biochemical characteristics as described in the *International Streptomyces Project*. Out of twenty two actinomycetes (designated as BSA-1 to BSA-22) isolates, only four isolates (BSA-5, BSA-10, BSA-11 and BSA-15) displayed significant antimicrobial

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properties in term of antagonistic activity against six human pathogenic bacterial strains (*Staphylococcus aureus*, *Shigella flexneri*, *Bacillus licheniformis*, *Bacillus brevis*, *Pseudomonas aeruginosa* and *Escherichia coli*). All these isolates exhibited excellent antimicrobial activity in a range of 14.0-22.0 mm as inhibition zone against the above studied human pathogens with highest activity displayed by the isolate BSA-11. The isolate, *Streptomyces* sp. BSA-11 was further identified up to the species level by 16S rRNA gene sequence analysis. The BLAST analysis confirmed that *Streptomyces* sp. BSA-11 was homologous to *Streptomyces himastatinicus* of order Actinomycetles and class *Actinobacteria*. The novel actinomycete, *Streptomyces himastatinicus* BSA-11 from Bhitarkanika has the ability to produce extracellular potent bioactive compounds which can be a potential source of many antimicrobials.

Keywords: Actinomycetes; antimicrobial activity; phylogenetic analysis; Streptomyces sp.

1. INTRODUCTION

Actinomycetes are aerobic, spore forming, filamentous and Gram positive bacteria with high G+C content which are excellent producers of novel antimicrobial agents [1]. Actinomycetes have the ability to produce exuberant secondary metabolites with biological significance, such as, antibiotic, antifungal, antiviral, anticancer, enzyme, immunosuppressant and other industrially useful compounds [2,3]. Aquatic actinomycetes have the great evidences of the discovery of several novel bioactive compounds, for example, rifamycin from *Micromonospora* [4] salinosporamide-A, an anticancer metabolite from a *Salinispora* strain [5], marinomycins from *Marinophilus* sp. [6], abyssomicin-C from *Verrucosispora* sp. and marinopyrroles from *Streptomyces* sp. [7,8]. Out of 22,500 well characterized biologically active compounds reported from different sources till now, above 45% are produced only by actinomycetes [9]. The genus, *Streptomyces* dominates the pharmaceutical industries as a potential producer of antibiotics, which accounts for more than 70% of the global antibiotic production and trading. *In lieu* of this, *Micromonospora* is less than one-tenth as many as *Streptomyces* in the production of antimicrobials [10]. At contemporary, Actinomycetes have occupied the pivotal place in the-then-hot areas of pharmaceutical research owing to their numerous potentials for production antibiotics and other therapeutic products [11]. To reiterate, actinomycetes play a vital role in the soil mineralization of organic matters, immobilization of nutrients, antibiosis and production of plant growth promoters [12,13] which can establish as a potential candidate for agricultural application.

Many actinomycetes are free living organisms widespread in the nature and both aquatic and/or terrestrial in their origin. The incessant search of novel organisms and their characterizations is an important and continuous exercise *vis-à-vis* to study on their existence and function in the ecosystem [14]. Marine environments are largely untapped source for the isolation of new microorganisms with the potential to produce active secondary metabolites [15]. Among such microorganisms, actinomycetes are of exceptional interest, as they are well known hyper producers of chemically diverse compounds having immense biological activities [15,16]. The escalating demand for novel antibiotics continues to grow, owing to the rapid emergence of multiple drugs/antibiotic resistant pathogens causing life threatening ailments and death. At present, significant progress is continuing within the fields of chemical synthesis and engineered biosynthesis of antibacterial compounds. As a consequence, the nature still remains as the unexplored, richest and the most versatile source for new antibiotics and metabolites [17,18]. Among various ecosystems, mangrove ecosystem is a largely unexplored and neglected source for actinomycetes with the potential to produce biologically active secondary metabolites. Therefore, in the present study, attempts have been made to isolate, characterize and identify the potent bioactive compounds producing actinomycetes from the Bhitarkanika mangrove forest along Odisha coast, India.

2. MATERIALS AND METHODS

2.1 Study Area

The Bhitarkanika mangrove forest is located at the confluence of the rivers Brahmani and Baitarani in Odisha and the second largest mangrove formation in Indian sub-continent next to the Sunderbans of West Bengal, India. The Bhitarkanika lies between 20°30' N to 20°50' N latitude and 86° 30' E to 87° 6' E longitude, extends over 139.39 sq. Km in Kendrapara district of Odisha, India. This ecosystem represents a salt tolerant, complex and a very dynamic environment that occurs only in tropical and subtropical inter-tidal areas. Comprising mangrove forests, rivers, creeks, estuaries, back water, accreted land and mud flats, Bhitarkanika is significant for its unique ecological, geomorphological and biological profile that has evolved over centuries to its present status.

2.2 Collection of Soil Samples

The soil samples were collected from different location of Bhitarkanika mangrove forest. The samples were collected in the month of January from top 4 cm soil profile, where most of the microbial activity takes place. Soil samples (approx. 500 g) were collected by using clean, dry and sterile polythene bags. The site selection was done by taking care of the point where widely varying characteristics such as, the organic matter, moisture content, particle size and color of soil, are possible so as to avoid contamination as far as possible. Samples were stored in ice boxes and transported to the laboratory where they were kept in a refrigerator at 4℃ until analysis.

2.3 Isolation of Actinomycetes from Soil Sample

The sample (1 g each soil) was taken for the serial dilution up to the 10^3 dilution, 0.2 ml of each dilution were inoculated in duplicate plates of the ISP-2 media with Nystatin and nalidixic acid as antifungal and antimicrobial agent [19] for the isolation of actinomycetes by spread plate technique. After incubation, all plates incubated at 28℃ in the incubator for 7 days. After incubation, plates were examined for the appearance of actinomycetes colonies. Total number of colonies in each set of plates were scored and recorded as CFU/g dry soil. Many colonies with different morphological and cultural characteristics, generally colony appeared with a tough, leathery or chalky texture; dry or folded appearance and branching filamentous with or without aerial mycelia were picked [20] from the isolation plates and streaked for pure culture. The stock cultures were maintained and transferred to fresh ISP-2 slants once in four months and stored at 4℃.

2.4 Screening of Antibiotic Producing Actinomycetes

Screening of antibiotic producing actinomycetes is carried out by the antimicrobial activity. The preliminary study was done by cross streak method [21] against six pathogenic bacteria, namely *Staphylococcus aureus*, *Shigella flexneri*, *Bacillus licheniformis*, *Bacillus brevis*, *Pseudomonas aeruginosa*, and *Escherichia coli*. The bacteria were maintained in nutrient agar (Hi-Media, India) slopes at 4℃ and sub-cultured before use. The selected isolates were streaked as parallel line on nutrient agar plates and incubated at 28℃ for 5 days. After observing a good ribbon- like growth of the actinomycetes on the Petri plates, the pathogen was streaked at right angles to the original streak of actinomycetes and incubated at 28±2℃. The inhibition zone was measured after 24 and 48 hours. A control plate was also maintained without inoculating the actinomycetes, to assess the normal growth of the bacteria.

2.5 Production of Bioactive Compounds

The actinomycetes isolates were selected for bioactive compounds production by submerged fermentation. Well sporulated 7 to 10 days isolates were taken and 5 ml of sterile water was added to each slant and spore suspension was added to a 250 ml conical flask containing 50 ml of the inoculum medium (Potato Dextrose Broth) and incubated at 28℃ on a rotary shaker (210-220 rpm) for 48 hours. After incubation, 5 ml of the inoculum medium was transferred to a 250 ml shake flask containing 45 ml of the production medium as described by Janardhan et al. [22]. Further, flasks were incubated at 28℃ for 6 days on a rotary shaker. After 6 days, 10 ml of the production medium was collected in sterile centrifuge tubes and centrifuged at 4000 rpm for 15 minutes to separate the fermented broth and the mycelium. The clear supernatant was used for the antibacterial assay by agar-well method [23].

2.6 Determination of Antimicrobial Activity

Antimicrobial activity was determined by Agar-Well Diffusion method [23]. The molten sterile nutrient agar medium was poured into sterile petriplate and inoculated with the test organisms by spread plate techniques. Wells were made using sterile borer; 50 µl of clear broth supernatant was added to each well. The plates were kept in a refrigerator for about 2 h to allow the diffusion of the bioactive metabolite. After 2 h, plates were incubated at 37℃ in an incubator. The inhibition zones in mili meter (mm) were measured after 24 h using an antibiotic zone reader. Further, actinomycetes isolates were selected for morphological and molecular identification.

2.7 Identification of Actinomycetes

2.7.1 Phenotypic identification

The cultural and morphological characteristics such as aerial mass color, reverse side pigment, melanoid pigments, spore chain morphology and spore morphology were determined as described by Shirling and Gottlieb [24]. The purified isolates were cultivated at 30℃ for 14 days on ISP-2 agar plates and their cultural characteristics were observed. Morphological observation was done by using a light microscope and scanning electron microscope (JSM-5410LV, Japan) of the cultures grown on ISP-2 agar plates at 30℃ for 7, 14 or 21 days. The ability of different actinomycetes isolates to utilize various carbon compounds i.e. D-glucose, L-arabinose, Sucrose, D-fructose, D-xylose, Raffinose, D-mannitol, Cellulose, Rhamnose, Inositol, as a source of energy was studied by following the method recommended in *International Streptomyces Project* (ISP) on Carbon utilization medium [25]. Decomposition of various compounds (cellulose, gelatine, starch, Tween 20) was examined using the basal medium recommended by Gordon et al. [26]. Temperature, NaCl, and pH tolerance were determined on ISP-2 medium. Finally, after all these experiments results were matched with the keys given in ISP for taxonomic identification.

2.7.2 Molecular identification

2.7.2.1 Extraction of DNA

Genomic DNA was extracted from actinomycetes isolates according to the CTAB method described by Hamedo and Makhlouf [27] with minor modifications. 50 mg mycelia was collected from pure culture and homogenized in liquid nitrogen in a pre-cooled mortar, transferred to a 15 mL tube containing 5 mL of 2X CTAB Buffer (1.4 mol/L NaCl, 100 mmol/L Tris-HCl (pH 8.0), 20 mmol/L EDTA, 2% CTAB), and 0.2% β-mercaptoethanol and mixed gently. The mixture was then placed in a 65℃ water bath for 30 min. The homogenate was extracted with an equal volume of chloroform:isoamyl alcohol (24:1). The homogenate was pelleted by centrifugation at 10,000 rpm for 10 minutes at 4℃ and the aqueous phase was removed to a new tube. Then 2/3 volume of cold iso-propanol was added to the tubes. Then the samples were incubated overnight at 4℃. After incubation the pellet was washed with 70% ethanol, dried, and re-suspended in 50 µL TE buffer. The DNA was stored at -20℃ for further use.

2.7.2.2 PCR amplification and DNA sequencing

The DNA was amplified and sequenced at Xcelris Labs Ltd., Ahmedabad, India. Fragment of 16S rDNA gene was amplified by Eppendorf Thermal Cycler using 8F and 1492R primers. A single discrete PCR amplicon band of 1500 bp was observed. The PCR amplicon was purified using QIA quick PCR purification kit (QIAGEN, UK) according to the manufacturer’s protocol. Forward and Reverse DNA sequencing reaction of PCR amplicon was carried out with 704F and 907R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA). Further the sequence obtained was subjected to phylogenetic analysis.

2.7.2.3 Phylogenetic analysis

The obtained sequences of *Streptomyces* sp. BSA-11 was submitted in the European Nucleotide Achieve Database (http://www.ebi.ac.uk/ena/) published with accession number KT223108. The sequence homology search was conducted for *Streptomyces*sp. BSA-11 (KT223108) using NCBI BLAST algorithm. Phylogenetic analysis was performed using the neighbour-neighbour joining algorithm with MEGA software (version 5.05) and the resulting tree was displayed by Tree View software (version 1.6.6) [28,29].

2.8 Statistical Analysis

The experiments were carried out in triplicates and the data was expressed as mean value ± standard deviation. The means of all the parameters were examined for significance by two way analysis of variance (ANOVA) and the differences between samples were determined by Duncan’s Multiple Range test using GenStat discovery (edition 3) statistical software package. Differences were considered significant at a probability level of $P<0.05$.

3. RESULTS

3.1 Isolation and Purification of Actinomycetes

Isolation plates developed various types of bacterial, actinomycetes and fungal colonies. Forty to fifty colonies were found per plate.

Colonies selected from each plate were 5 to 10 based on colony appearance. Colonies having characteristic features such as powdery appearance with convex, concave or flat surface and color ranging from white, gray to pinkish and yellowish were selected. Colonies observed on 1st and 2nd day was eliminated because actinomycetes are considered as slow grower. Total 22 colonies were selected, isolated and purified by pure culture techniques. All 22 isolates were designated as BSA-1 to BSA-22 (Bhitarkanika Soil Actinomycetes-1 to 22).

3.2 Screening of Antibiotic Producing Actinomycetes

The study was carried out by the cross streak method against six pathogenic bacteria, namely *Staphylococcus aureus*, *Shigella flexneri*, *Bacillus licheni formis*, *Bacillus brevis*, *Pseudomonas aeruginosa* and *Escherichia coli* and the result shown in Table 1. Among the 22 isolates, four isolates (BSA-5, 10, 11 and 15) were showing significant activity against all these tested pathogenic strains. BSA-1 and BSA-3 showed positive activity against *S. aureus* and *P. aeruginosa*. BSA-4 showed positive result against *B. brevis* and *E. coli* whereas BSA-8 and BSA-12 showed positive activity against *P. aeruginosa*. BSA-18 showed growth inhibition of *B. licheniformis*. BSA-20 and BSA-21 showed predominant activity against the different pathogenic organisms as given in Table 1. Further, ten isolates (BSA-2, 6, 7, 9, 13, 14, 16, 17, 19 and 22) showed negative activity against the tested pathogenic strains.

3.3 Production of Bioactive Compounds and Antimicrobial Activity

The bioactive compounds were produced by selected isolates (BSA-5, 10, 11 and 15), extracted and purified for antimicrobial activity. The agar well method is suitable for determinations of antimicrobial activity. The results of antimicrobial activity (inhibition zone diameter in mm) of extracellular compounds with a standard are given in Table 2. Among the four tested isolates, BSA-11 was showed higher antimicrobial activity (14.0-22.0 mm) against the pathogenic organisms. Besides, other three isolates (BSA-5, 10 and 15) showed moderate activity (8.5-18.0 mm) against all these pathogens. Hence, BSA-11 was selected for phenotypic and molecular identification based on antimicrobial activity.

Table 1. General observation of actinomycetes for antimicrobial activity

Name of isolates	*S. aureus*	*S. flexneri*	*B. licheniformis*	*B. brevis*	*P. aeruginosa*	*E. coli*
BSA-1	+	-	-	-	+	-
BSA-2	-	-	-	-	-	-
BSA-3	+	-	-	-	+	-
BSA-4	-	-	-	+	-	+
BSA-5	+	+	+	+	+	+
BSA-6	-	-	-	-	-	-
BSA-7	-	-	-	-	-	-
BSA-8	-	-	-	-	+	-
BSA-9	-	-	-	-	-	-
BSA-10	+	+	+	+	+	+
BSA-11	+	+	+	+	+	+
BSA-12	-	-	-	-	+	-
BSA-13	-	-	-	-	-	-
BSA-14	-	-	-	-	-	-
BSA-15	+	+	+	+	+	+
BSA-16	-	-	-	-	-	-
BSA-17	-	-	-	-	-	-
BSA-18	-	-	+	-	-	-
BSA-19	-	-	-	-	-	-
BSA-20	+	+	-	-	+	-
BSA-21	-	-	+	+	+	+
BSA-22	-	-	-	-	-	-

'+' Positive, '-' Negative

Table 2. Antimicrobial activity (inhibition zone in mm) of extracellular compounds

Test organisms	Penicillin-G	BSA-5	BSA-10	BSA-11	BSA-15
S. aureus	$11.8^{ghij*}\pm0.72$	$18.0^{cd}\pm1.0$	$8.5^{k}\pm0.5$	$16.0^{de}\pm2.0$	$14.0^{efgh}\pm1.0$
S. flexneri	$12.66^{fghi}\pm1.15$	$12.0^{ghij}\pm2.0$	$14.0^{efgh}\pm2.0$	$15.0^{ef}\pm1.0$	$12.0^{ghij}\pm0.5$
B. licheniformis	$11.0^{ijk}\pm1.0$	$10.86^{ijk}\pm1.5$	$13.0^{fghi}\pm2.0$	$20.0^{c}\pm1.73$	$12.0^{ghij}\pm2.0$
B. brevis	$26.42^{a}\pm1.24$	$10.0^{jk}\pm2.0$	$10.0^{jk}\pm2.0$	$22.0^{b}\pm1.0$	$10.0^{jk}\pm1.0$
P. aeruginosa	$0.0^{l}\pm0.0$	$14.0^{efgh}\pm2.0$	$11.4^{ijk}\pm1.5$	$16.46^{cd}\pm0.5$	$14.46^{efg}\pm1.6$
E. coli	$12.0^{ghij}\pm2.0$	$12.0^{ghij}\pm1.0$	$12.0^{ghij}\pm1.0$	$14.0^{efgh}\pm2.0$	$10.0^{jk}\pm2.0$

**Difference in the superscript letters indicate significant difference at probability P<0.05*

3.4 Identification of Actinomycetes

3.4.1 Phenotypic identification

The phenotypic characteristics of isolate BSA-11 was given in Table 3. The morphology of isolates BSA-11 grew on ISP2 agar medium was showed white color substrate mycelia whereas grey color found on starch casein agar medium. The color of reverse side pigments were found to be brown whereas soluble pigments were absent. The morphology of the spore was found as Spira-Spirales. The extra cellular enzyme (cellulose, amylase, lipase and catalase) production ability was shown in BSA-11. Further, after comparing the growth with negative and positive control, it was observed that glucose, fructose, rhamanose, raffinose, xylose, arabinose, starch, mannitol were the most assimilated carbon source by the isolate BSA-11. The optimum growth parameters such as temperature, pH and NaCl were determined. The growth was found to take place in the temperature range of 25-35℃ and 28℃ was found to be optimum for growth. The isolate BSA-11 was grown on ISP 2 at different pH values such as 5, 6, 7, 8 and 9 for 8 to 10 days, and pH 8.0 was found to be optimum for growth. Salt tolerance test was very important for to understand the native nature of the marine actinomycetes isolates. The BSA-11 was found to have the ability to tolerate up to 10% NaCl. After obtaining all the results from the experiment done were matched with the keys given for 458 species of actinomycetes included in ISP (*International Streptomyces Project*) and isolate BSA-11 was identified as *Streptomyces* sp. based on their characteristics. The match was done on the basis of the maximum percentage of resemblance of characteristics. Further, molecular study was carried out to identify up to the species level.

3.4.2 Molecular identification

3.4.2.1 DNA extraction and PCR amplification

The total genomic DNA was isolated from one actinomycete isolate (BSA-11) by adopting cTAB DNA extraction method. The quantity of extracted genomic DNA was found satisfactory and determined by taking the absorbance at 260 nm and 280 nm. The OD value was found to be in range of 140-180 µg/µl. The quality of extracted genomic DNA was determined by performing Agarose gel electrophoresis and the genomic DNA band at ~350 bp was observed (Fig. 1). The genomic DNA obtained was very good quality and yielded the expected PCR products using suitable primers (8F and 1492R). The PCR products sizes were approximately ~1500 bp (Fig. 2).

3.4.2.2 Phylogenetic analysis

From all the BLAST hits obtained for 16S rRNA genes of *Streptomyces* sp. BSA-11 (KT223108), fifteen homologous sequences of genus *Streptomyces* were retained on the basis of good sequence identity (98% to 99%), lowest e-value and query coverage (100%) as given in Table 4. A total of sixteen16S rRNA genes, including *Streptomyces* sp. BSA-11 were subjected to phylogenetic analysis. Maximum Parsimony (MP) tree was constructed using the close-neighbour interchange algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). Bootstrapping was performed for 10000 replicates. The bootstrap consensus MP tree was resulted using MEGA 5.05 for sixteen strains of genus *Streptomyces* clustered together in one group. The resultant bootstrap consensus parsimonious tree was shown in Fig. 3. The BLAST result obtained for *Streptomyces* sp. BSA-11 shown highest similarity with *Streptomyces himastatinicus* ATCC 53653, 16S rRNA gene (KT223108) with support of query coverage, 100%, identity 99% and e-value 0.0 which implicates *Streptomyces* sp. BSA-11 is one of the strong homologous of *Streptomyces himastatinicus*. Hence, the studied isolate (*Streptomyces* sp. BSA-11) was identified as *Streptomyces himastatinicus.*

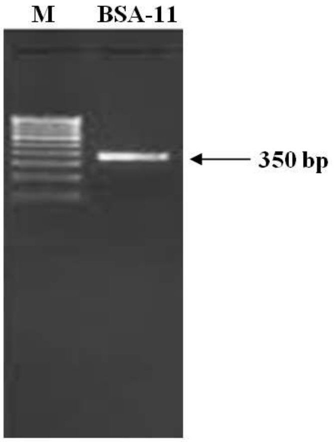

Fig. 1. Genomic DNA

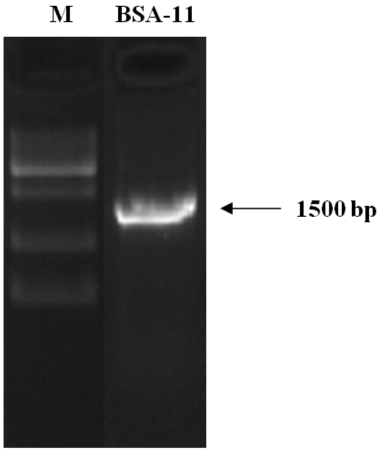

Fig. 2. PCR amplified product

4. DISCUSSION

Sediment samples collected from the Bhitarkanika mangrove forest were divided into two parts as wet sample and dry sample. Both samples were serially diluted and found that the no of isolates from the dry sample was more in number. Marine sediment samples are a valuable source for the isolation of actinomycetes [30]. All twenty two (22) isolates of actinomycetes was screened for their bioactive compound production ability. Among the 22 isolates, four isolates (BSA-5, 10, 11 and 15) showed positive activity against all tested pathogenic strains. Further, extracellular bioactive compounds produced by different isolates and evaluated their antimicrobial activity against the pathogenic strains. All the four isolates (BSA-5, 10, 11 and 15) showed significant results which are comparable with different reports [31,32]. Among the four tested isolates, BSA-11 was showed higher antimicrobial activity (14.0-22.0 mm) against the pathogenic organisms. It is observed that the new drugs, notably antibiotics, are urgently needed to halt and reverse the relentless spread of antibiotic resistant pathogens which use to cause life threatening infections and risk which are undetermined with the viability of healthcare systems [33]. Filamentous bacteria belonging to *Micromomospora* and *Streptomyces* sp. have a unique and proven capacity to produce novel antibiotics [34]. Hence the continued interest in screening such organisms for new bioactive and it is also becoming increasingly clear that un- and under-explored habitats, such as desert biomes and marine ecosystems, are a very rich source of novel actinomycetes which have the capacity to produce interesting new bioactive compounds, including antibiotics [35]. Another study done by Jeffrey et al. [36] shown the antagonistic activity of actinomycetes against four strains of pathogenic microbes (*Fusarium palmivora, Bacillus subtilis, Ralstonia solanacearum* and *Pantoae dispersa*). By all these discussions, it can be said as those actinomycetes produce some useful bioactive compounds. Therefore, the characterization was done by the phenotypic characterization and species affiliation by physiological and biochemical characteristics described by Das et al. [37]. The aerial mass color of almost all isolates was whitish and only one strain BSA-11 has shown gray color. Vanajakumar et al. [38] have also reported that white color series of actinomycetes they were the dominant forms. Color series were also recorded in soil, morphological observation of colonial characteristics such as amount and color of vegetative growth, and the presence and color of aerial mycelium and spores, and again the presence of diffusible pigments are recorded for each strain studied colonial growth on agar plate [39]. All isolates have shown the pigment formation. Some of the studies reported that the morphology of the spore bearing hyphae with entire spore chain along with substrate and aerial mycelium was examined under light microscope as well as scanning electron microscope [40,41]. The spore morphology was studied by considering different types of spore of actinomycetes under the microscope. Spore surface morphology was studied by microscope and found as Retinaculiaperti and Spira-Spirals. Microscopic analysis is very much specific as studies reported for spore surface morphology [42].

Table 3. Morphological and biochemical characterization of *Streptomyces* sp. BSA-11

Morphological and biochemical characteristics	Characters
Growth on media	
Pridhan Medium	Good growth
Starch casein Agar	Excellent growth
Glucose Asparagine Medium	Good growth
ISP2 Medium	Excellent growth
Cultural morphology	
Growth on ISP2	+++
Substrate mycelia	White
Aerial mycelia	-
Starch Casein gar (SCA)	
Substrate mycelia	Grey
Aerial mycelia	-
Pigmentation	
Reverse plate pigment	brown
Soluble pigment	-
Cellular and spore morphology	Spira-spirales
Vegetative cells	Branched and filamentous
Spore per chain	5-7 at the apex
Spore shape	Round to elliptical
Spore surface	Smooth
Gram response	Positive
Extra cellular enzymes	
Cellulase	+++
Amylase	+++
Lipase	++
Protease	-
Catalase	++
Carbon source utilization	
Glucose	Positive
Fructose	Positive
Rhamanose	Positive
Raffinose	Positive
Xylose	Positive
Arabinose	Positive
Maltose	Positive
Sucrose	Negative
Starch	Positive
Inositol	Negative
Mannitol	Positive
Growth parameters	
Temperature	25-35℃
pH	5.0-9.0 (optimum at pH 8.0)
NaCl	0-10% (optimum at 5%, w/v)

+++: Excellent; ++: Good; +: Moderate; -: Absent/Negative

Utilization of carbon sources like arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose were analysed for classification. Carbohydrate utilization was determined by growth on carbon utilization medium [25] supplemented with 1% carbon source at 28℃. Another major milestone in the identification of actinomycetes was the assimilation of carbon by actinomycetes. The tests include ten carbon sources which are sterilized by the membrane filtration method. Almost all the isolates have shown very luxuriant growth. But the studied isolates have shown less growth on sucrose and inositol. Pandey et al. [43] showed that for the optimum production of antibiotics, certain carbon sources are required. In that study the author and co-workers also suggested that pH might play an important factor in the production of antibiotic by actinomycetes. Ability to grow in different pH was carried out and all the isolate showed good growth on pH ranges from 5-9. As samples were collected from the mangroves, so it is quite expected that isolates can tolerate high diversification in salinity and isolates showed up to 10% NaCl tolerance. The hydrolysis of starch were evaluated by using the media of Gordon et al. [26] and liquefaction of gelatin was evaluated by the method of Waksman [44]. H_2S production test has been done by preparing the slant culture among all the strains nine strains have shown positive result. To know the overall activity of all the strains, various enzymatic screening has been done. These are the cellulose activity, caseinase activity, amylase activity, lipolytic activity and gelatinase activity. Gelatine hydrolysis was not shown by any isolate. But the amylase activity is shown by almost all the isolates. Cellulose and caseinase activity is shown almost in the same manner by all isolates. All these results have shown the same pattern of results obtained by previous works done by many researchers [45,46]. Finally, after all these experiments results have been matched with the keys given for 458 species of actinomycetes included in ISP and the species identification was done and it was found that all the isolates have been grouped under *Streptomyces* genus. Further molecular study is carried out for complete identification of studied isolates.

In molecular study, application of 16S rDNA gene is more simple, yet efficient, in identification of new *Streptomyces* strains [47]. It is worth noting that although 16S rDNA gene has less changes and transformation through evolution, it is deemed to be a superior candidate for taxonomic

Table 4. Homologous sequences resulted from BLAST search for 16S rRNA gene of *Streptomyces* sp. BSA-11 (KT223108)

Accession	Description	Max score	Total score	Query coverage	E value	Max identity
NR_044201.1	*Streptomyces himastatinicus* ATCC 53653	2566	2566	100%	0.0	99%
EU077189.1	*Streptomyces* sp. M1446	2481	2481	100%	0.0	98%
NR_041412.1	*Streptomyces sporocinereus* NBRC 100766	2462	2462	100%	0.0	98%
NR_041145.1	*Streptomyces hygroscopicus* NBRC 13472	2462	2462	100%	0.0	98%
NR_043379.1	*Streptomyces endus* NRRL 2339	2462	2462	100%	0.0	98%
AJ781368.1	*Streptomyces sporocinereus* LMG 20311	2462	2462	100%	0.0	98%
NR_114813.1	*Streptomyces hygroscopicus* NRRL 2387	2462	2462	100%	0.0	98%
AJ391819.1	*Streptomyces hygroscopicus* NRRL B-1477	2462	2462	100%	0.0	98%
FJ222814.1	*Streptomyces* sp. HV10	2459	2459	100%	0.0	98%
DQ445793.1	*Streptomyces javensis* NRRL B-24423	2457	2457	100%	0.0	98%
NR_041416.1	*Streptomyces yogyakartensis* NBRC 100779	2457	2457	100%	0.0	98%
NR_041141.1	*Streptomyces violaceusniger* NBRC 13459	2457	2457	100%	0.0	98%
AJ519937.1	*Streptomyces* sp. GE 90930	2457	2457	100%	0.0	98%
NR_043723.1	*Streptomyces demainii* NRRL B-1478	2457	2457	100%	0.0	98%
NR_114814.1	*Streptomyces violaceusniger* NRRL B-1476	2457	2457	100%	0.0	98%

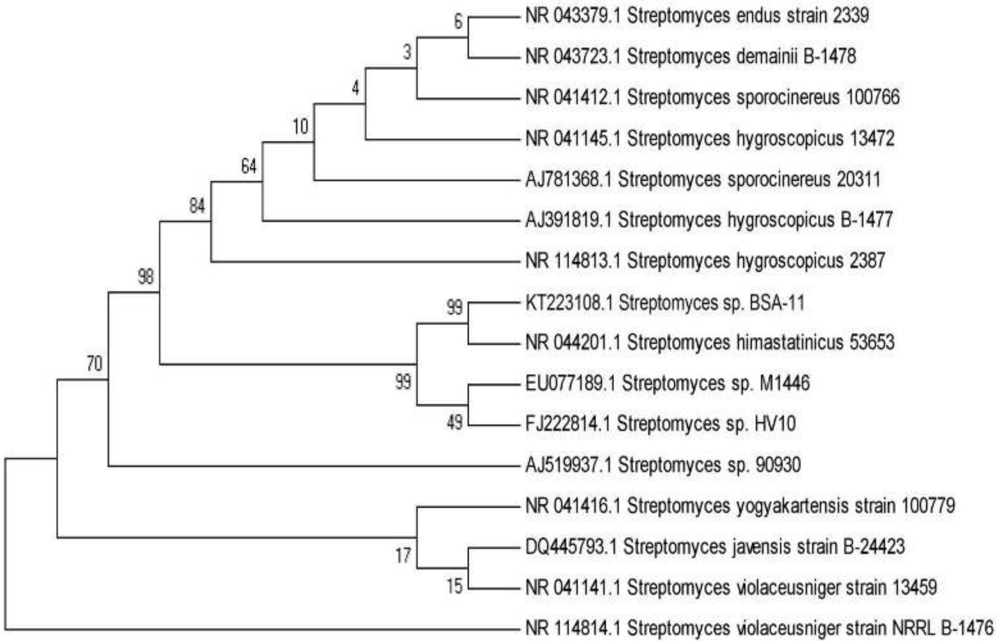

Fig. 3. Maximum parsimonious tree constructed using MEGA 5.05 based on homologous sequences of *Streptomyces* sp. BSA-11

studies because of 5' variable areas including α, β, δ, ε, and particularly variable γ part which shows relatively high polymorphism at the ′5 end of its structure [48-50] which could be exploited for studying the genetic diversity of various *Streptomyces* species. Among the four studied isolates, BSA-11 was chosen for molecular identification based on their good antimicrobial results. To confirm the identity of the isolate (BSA-11) as *Streptomyces* sp., molecular study was done based on 16S rDNA sequences, because nuclear 16S rRNA gene have been revealed to have some variable regions with sequence divergence [48-50]. The homologous were obtained from a sequence homology search using the BLAST algorithm shown a good similarity against *Streptomyces* species. The results obtained from the phylogenetic tree supported the fact that *Streptomyces* sp. BSA-11 (KT223108) are closely related to *Streptomyces himastatinicus* (ATCC 53653) which belongs to genus *Streptomyces* in order Actinomycetales of the class *Actinobacteria* which are evolutionary close on the basis of phenotypic and molecular characteristics. Hence, the *Streptomyces* sp. BSA-11 is identified as *Streptomyces himastatinicus*. Identification of new strains of *Streptomyces* have been frequently described in the literature using amplification of hyper variable regions that can provide strain specific signature [51,52]. Maleki et al. [53] have identified two new strains of *Streptomyces* spp. with high antibiotic production capacity and higher homology to *Streptomyces coelicolor* and *Sreptomyces albogriseolus* using the assessment of cultural, morphological and phylogenetic evaluation provided by 16S rDNA sequence analysis. In the similar work by the Higginbotham and Murphy [54], 16S rDNA sequence of the new strain exhibited higher homology with *Streptomyces lavendulae* and *Streptomyces globosus*.

Further, present study is comparable with study reported by Dezfully and Ramanayaka [55], in which strain ACTK2 was identified as *Streptomyces flavogriseus* from soil sample of Kodagu, Karnataka State (India) based on cultural, morphological, microscopic, biochemical and sequence analysis of 16S rRNA gene and showed antimicrobial activity against the Gram-positive bacteria *Staphylococcus aureus* (MTCC 96), *Bacillus subtilis* (MTCC 121), Gram-negative *Escherichia coli* (MTCC 729), *Enterococcus aerogenes* (MTCC 2829) and filamentous fungi (*Trichoderma harizianum* (MTCC6046), *Fusarium proliferatum* (MTCC 9375). Similarly, fifty four (54) bioactive actinomycetes strains capable of producing antimicrobial secondary metabolite from Sundarbans mangrove ecosystem were isolated by Sengupta et al. [56] and analyzed for antimicrobial activity against fifteen test organisms including three phytopathogens. Nine morphologically distinct and biologically active isolates were subjected to molecular identification study. 16S rDNA sequencing indicated eight isolates to reveal maximum similarity to the genus S*treptomyces*. Finally, the strain SMS_SU21, which showed antimicrobial activity with MIC value of 0.05 mg ml^{-1} and antioxidant activity with IC50 value of 0.242 ± 0.33 mg ml^{-1} was detected to be the most potential one [56]. Thus the studied actinomycete, *Streptomyces himastatinicus* BSA-11 from Bhitarkanika has also the ability to produce extracellular potent bioactive compounds which can be a potential source of many antimicrobials.

5. CONCLUSION

The number of drug-resistant pathogens are increasing now days, particularly the acquired multi-drug resistant strains, cause serious public health problem throughout the world. Therefore, the need for antimicrobial discovery and better treatments of these infections, particularly in hospitals where antibiotic resistance is immediately life threatening, is becoming a rapidly growing concern. The study of different environments throughout the world has yielded a lot of antimicrobial agents that are of great value for the treatment of many infectious diseases. Therefore isolation and purification of economically important secondary metabolites from actinomycetes of Bhitarkanika mangrove forest and characterization of the bioactive compounds is a challenging solution for exploring antimicrobial compounds from natural sources. *Streptomyces* sp. BSA-11 strain exhibited a broad spectrum of antimicrobial activity against the pathogenic bacteria. Further study is needed to characterize the bioactive compounds responsible for antimicrobial activity.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Rajan BM, Kannabiran K. Extraction and identification of antibacterial secondary metabolites from marine *Streptomyces* sp. VITBRK2. Int J Mol Cell Med. 2014;3(3): 130-137.
2. Xu D-B, Ye W-W, Han Y, Deng Z-X, Hong K. Natural products from mangrove actinomycetes. Mar Drugs. 2014;12(5): 2590-2613.
3. Abd-Elnaby H, Abo-Elala G, Abdel-Raouf U, Abd-elwahab A, Hamed M. Antibacterial and anticancer activity of marine *Streptomyces parvus*: optimization and application. Biotechnol Biotechnol Equip; 2015.
   DOI: 10.1080/13102818.2015.1086280.
4. Huang H, Lv J, Hu Y, Fang Z, Zhang K. Baos. *Micromonospora rifamycinia* sp. nov, a novel actinomycetes from mangrove sediments. Int J Syst Evol Microbiol. 2008;58(1):17-20.
5. Fehling RH, Buchanan GO, Minur TJ, Kauffman CA, Jensen PR, Fenical WR. Salinosporamide A: A highly cytotoxic proteasome inhibitor from a novel microbial source, a marine bacterium of the new genus *Salinospora*. Angew Chem Int Ed Engl. 2003;42(3):355-357.
6. Jensen PR, Gontang E, Minur TJ, Fenical W. Cultuerable marine actinomycete diversity from tropical Pacific Ocean sediments. Environ Microbial. 2005;7: 1039-1048.
7. Riedlinger J, Reicke A, Zahner H, Krishmer B, Bull AT, Maldonado LA. Abyssomicins, inhibitors of the para-aminobenzoic acid pathway produced by the marine Verrucosispora strain AB-18-032. J Antibiot. 2004;57(4):271-279.
8. Hughes CC, Prieto-Davo A, Jensen PR, Fenical W. The marinopyrroles, antibiotics of an unprecedented structure class from a marine *Streptomyces* sp. Org Lett. 2008;10:629-631.
9. Berdy J. Bioactive microbial metabolites; a personal view. J Antibiot. 2005;58(1):1-26.
10. Lam KS. Discovery of novel metabolites from marine actinomycetes. Curr Opin Microbiol. 2006;9:245-251.
11. Kumar SV, Sahu MK, Kathiresan K. Isolation and characterization of *Streptomycetes* producing antibiotics from a mangrove environment. Asian J Microbiol Biotechnol Env Sci. 2005;7(3): 457-464.
12. Anderson CR, Condron LM, Clough TJ, Fiers M, Stewart A, Hill RA, Sherlock PR. Biochar induced soil microbial community change: Implications for biogeochemical cycling of carbon, nitrogen and phosphorus. Pedobiologia. 2011;54(5): 309-320.
13. Sonia M-T, Naceur J, Abdennaceur H. Studies on the ecology of actinomycetes in an agricultural soil amended with organic residues: I. identification of the dominant groups of actinomycetales. World J Microbiol Biotechnol. 2011;27(10):2239-2249.
14. Subramani R, Aalbersberg W. Marine actinomycetes: An ongoing source of novel bioactive metabolites. Microbiol Res. 2012;167(10):571-580.
15. Solecka S, Zajko J, Postek M, Rajnisz A. Biologically active secondary metabolites from Actinomycetes. Cent Eur J Biol. 2012;7(3):373-390.
16. Manivasagan P, Venkatesan J, Sivakumar K, Kim S. Pharmaceutically active secondary metabolites of marine *actinobacteria*. Microbiol Res. 2014;169(4): 262-278.
17. Saravana PK, Duraipandiyan V, Ignacimuthu S. Isolation, screening and partial purification of antimicrobial antibiotics from soil *Streptomyces* sp. SCA 7. Kaohsiung J Med Sci. 2014;30(9):435-46.
18. Messaoudi O, Bendahou M, Benamar I, Abdelwouhid D-E. Identification and preliminary characterization of non-polyene antibiotics secreted by new strain of actinomycete isolated from sebkha of Kenadsa, Algeria. Asian Pac J Trop Biomed. 2015;5(6):438-445.
19. Mohanta YK, Behera S. Biosynthesis, characterization and antimicrobial activity of silver nanoparticles by *Streptomyces* sp. SS2. Bioproc Biosyst Eng. 2014;37(11): 2263-2269.
20. Mincer TJ, Jensen PR, Kauffman CA, Fenical W. Widespread and persistent populations of a major new marine actinomycetes taxon in ocean sediments. Appl Environ Microbiol. 2002;68:5005-5011.
21. Velho-Pereira S, Kamat NM. Antimicrobial screening of *actinobacteria* using modified cross-streak method. Indian J Pharm Sci. 2011;73(2):223-228.
22. Janardhan A, Kumar P, Viswanath B, Sai Gopal DVR, Narasimha G. Production of

bioactive compounds by actinomycetes and their antioxidant properties. Biotechnol Res Int. 2014;2014:1-8.

23. Murray PR, Baroon EJ, Pfaller MA, Tenover FC, Yolke RH, editors. Manual of clinical microbiology. 6th ed. Washington DC: American Society for Microbiology; 1995.
24. Shirling EB, Gottlieb D. Methods for characterization of *Streptomyces* species. Int J Syst Evol Microbiol. 1966;16:313-340.
25. Pridham TG, Gottlieb D. The utilization of carbon compounds by some actinomycetales as an aid for species determination. J Bacteriol. 1948;56:107-114.
26. Gordon RE, Barnett DA, Handerhan JE, Pang CHN. *Nocardia coeliaca, Nocardia autotrophica* and the *nocardin* strain. Int J Syst Evol Microbiol. 1974;24:54-63.
27. Hamedo HA, Makhlouf AH. Identification and characterization of actinomycetes for biological control of bacterial scab of *Streptomyces scabies* isolated from potato. J Biol Agric Healthc. 2013;3(13):142-153.
28. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4:406-425.
29. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731-2739.
30. Goodfellow M, Haynes. Actinomycetes in marine sediments in biological, biochemical and biomedical aspects of actinem. In: Ortiz-ortiz I, Bjalil LF, Yakoleff V, editors. London: Academic Press; 1984.
31. Edwards C. Isolation, properties and potential application of thermophilic actinomycetes. Appl Biochem Biotechnol. 1993;42:161-179.
32. Demain AL. Why do microorganisms produce antimicrobials? In: Hunter PA, Darby GK, Russel NJ, editors. Society of general microbiology, Fifty years of antimicrobials: prospective and future trend symposium 53, Cambridge: Cambridge University Press; 1995.
33. Talbot GH, Bradley J, Edwards JE Jr, Gilbert D, Scheld M, Bartlett JG. Bad bugs need drugs: an update on the development pipeline from the antimicrobial availability task force of the infectious diseases society of America. Clin Infect Dis. 2006;42(5):657-668.
34. Bentley SD, Chater KF, Cerdeno-Tarraga AM, Challis GL, Thompson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D. Complete genome sequence of the model actinomycetes *Streptomyces coelicolor* A3(2). Nature. 2002;417:141–147.
35. Hong K, Gao AH, Xie QY, Gao H, Zhuang L, Lin HP, et al. Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China. Mar Drugs. 2009;7(1):24-44.
36. Jeffrey LSH, Sahilah AM, Son R, Tosiah S. Isolation and screening of actinomycetes from Malaysian soil for their enzymatic and antimicrobial activities. J Trop Agric Food Sci. 2007;35:159-164.
37. Das A, Mazumder Y, Dutta BK, Shome BR, Bujarbaruah KM, Sharma G. *Clostridium perfringens* type A beta-2 toxin in elephant (*Elephas maximus indicus*) and pygmy hog (*Sus salvanius*) with haemorrhagic enteritis in Assam, India. Afr J Microbiol Res. 2008;2(8):196-201.
38. Vanajakumar SN, Natarajan R. Antagonistic properties of actinomycetes isolated from mollusks of the Porto Novo region, South India. In: Thompson M, Sorojini R, Nagabhushanam R, editors. Bioactive compounds from marine organisms. New Delhi: Oxford & IBH Publishing Co. Pvt. Ltd.; 1995.
39. Labeda DP, Testa RT, Lechevalier MP, Lechevalier HA. Glycomyces, a new genus of the actinomycetales. Int J Syst Evol Microbiol. 1985;35:417-421.
40. Vimal V, Mercy RB, Kannabiran K. Antimicrobial activity of marine actinomycetes, *Nocardiopsis* sp VITSVK5 (FJ973467). Asian J Med Sci. 2009;1:57-63.
41. Suneetha V, Zaved AK. Screening, characterisation and optimization of microbial pectinase. In: Shukla G, Varma A, editors. Soil Enzymology. Soil Biology-22. New York: Springer-Verlag Berlin Heidelberg; 2011.
42. Saurav K, Kannabiran K. Diversity and optimization of process parameters for the growth of *Streptomyces* VITSVK9. J Nat Environ Sci. 2010;1(2):56-65.
43. Pandey A, Shukla A, Majumdar SK. Utilization of carbon and nitrogen sources by *Streptomyces kanamyceticus* M27 for

the production of an antibacterial antibiotic. Afr J Biotechnol. 2005;4:909-910.

44. Waksman SA, editor. The actinomycetes classification identification and description of genera and species, vol 2. Baltimore, USA: Williams and Wilkins Company; 1961.
45. Kokare CR, Mahadik KR, Kadam SS, Chopade BA. Isolation of bioactive marine actinomycetes from sediments isolated from Goa and Maharashtra coastline (west coast of India). Indian J Mar Sci. 2004;33:248-256.
46. Sripreechasak P, Suwanborirux K, Tanasupawat S. Characterization and antimicrobial activity of *Streptomyces* strains from soils in southern Thailand. J Appl Pharm Sci. 2014;4(10):24-31.
47. Anderson AS, Wellington MHE. The taxonomy of *Streptomyces* and related genera. Int J Syst Evol Microbiol. 2001;51:797-814.
48. Shirling EB, Gottlieb D. Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. Int J Syst Evol Microbiol. 1968a;38:69-189.
49. Shirling EB, Gottlieb D. Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Int J Syst Evol Microbiol. 1968b;18:279-392.
50. Stach JEM, Maldonado LA, Ward AC, Goodfellow M, Bull AT. New primers for the class *Actinobacteria*: Application to marine and terrestrial environments. Environ Microbiol. 2003;5(10):828-841.
51. Kim SB, Falconer C, Williams E, Goodfellow M. *Streptomyces thermocarboxydovorans* sp. nov. and *Streptomyces thermocarboxydus* sp. nov., two moderately thermophilic carboxydotrophic species from soil. Int J Syst Evol Microbiol. 1998;48(1):59-68.
52. Roy S, Banerjee D. Bioactive endophytic actinomycetes of *Cinnamomum* sp.; isolation, identification, activity guided purification and process optimization of active metabolite. Am J Microbiol. 2015; 6(1):4-13.
53. Maleki H, Dehnad A, Hanifian S, Khani S. Isolation and molecular identification of *Streptomyces* spp. with antibacterial activity from northwest of Iran. Bioimpacts. 2013;3(3):129-134.
54. Higginbotham SJ, Murphy CD. Identification and characterisation of a *Streptomyces* sp. isolate exhibiting activity against methicillin-resistant *Staphylococcus aureus*. Microbiol Res. 2010;165(1):82-86.
55. Dezfully NK, Ramanayaka JG. Isolation, identification and evaluation of antimicrobial activity of *Streptomyces flavogriseus*, strain ACTK2 from Soil Sample of Kodagu, Karnataka State (India). Jundishapur J Microbiol. 2015;8(2): e15107.
DOI: 10.5812/jjm.15107
56. Sengupta S, Pramanik A, Ghosh A, Bhattacharyya M. Antimicrobial activities of actinomycetes isolated from unexplored regions of Sundarbans mangrove ecosystem. BMC Microbiol. 2015;15:170.
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