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Characterization and phylogenetic analysis of novel polyene type antimicrobial metabolite producing actinomycetes from marine sediments: Bay of Bengal India

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ABSTRACT

Objective: To isolate and indentify the promising antimicrobial metabolite producing *Streptomyces* strains from marine sediment samples from Andrapradesh coast of India. **Methods:** Antagonistic actinomycetes were isolated by starch casein agar medium and modified nutrient agar medium with 1% glucose used as a base for primary screening. Significant antimicrobial metabolite producing strains were selected and identified by using biochemical and 16S rDNA level. Minimum inhibitory concentrations of the organic extracts were done by using broth micro dilution method. **Results:** Among the 210 actinomycetes, 64.3% exhibited activity against Gram positive bacteria, 48.5 % showed activity towards Gram negative bacteria, 38.8% exhibited both Gram positive and negative bacteria and 80.85 % isolates revealed significant antifungal activity. However, five isolates AP-5, AP-18, AP-41 and AP-70 showed significant antimicrobial activity. The analysis of cell wall hydrolysates showed the presence of LL-diaminopimelic acid and glycine in all the isolates. Sequencing analysis indicated that the isolates shared 98.5%-99.8% sequence identity to the 16S rDNA gene sequences of the *Streptomyces* taxons. The antimicrobial substances were extracted using hexane and ethyl acetate from spent medium in which strains were cultivated at 30℃for five days. The antimicrobial activity was assessed using broth micro dilution technique. Each of the culture extracts from these five strains showed a typical polyene-like property. The lowest minimum inhibitory concentrations of ethyl acetate extracts against *Escherichia coli* and *Curvularia lunata* were 67.5 and 125.0 μg/mL, respectively. **Conclusions:** It can be concluded that hexane and ethyl acetate soluble extracellular products of novel isolates are effective against pathogenic bacteria and fungi.

1. Introduction

Actinomycetes are Gram-positive bacteria showing a filamentous growth like fungi. They are aerobic and present in various ecological habitats such as soil, fresh water, back water, lake, compost, sewage and marine environment[1]. They are predominant in dry alkaline soil. Actinomycetes DNA are rich in G+C content with the GC% of 57-75. They are considered highly valuable as they can produce various antibiotics and other therapeutically useful compounds with diverse biological activities. Actinomycetes is one of the most attractive families of industrial bacteria on account of their superior potential for producing valuable secondary metabolites including antibiotics, anti-cancer drugs, immune suppressors and enzyme inhibitors. Actinomycetes have been well known for the production of secondary metabolite. Many of the presently used antibiotics such as streptomycin, gentamicin, rifamycin and erythromycin are the products of actinomycetes. The genus *Streptomyces* is represented in nature by the largest number of species and varieties, producing the majority of known antibiotics among the family Actinomycetaceae. *Streptomyces* are well known sources of antibiotics and other important novel metabolites, including antifungal agents[2], antitumor agents[3], antihelminthic agents[4] and herbicides. Discovery of new antibiotics produced by *Streptomyces* still continues, for example, mediomycins A[5], and clethramycin[6], were isolated from *Streptomyces* mediocidicus[7]. They grow extensively in soils with rich organic matter. Marine environment contains a wide range

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of distinct *Streptomyces* that are not present in the terrestrial environment. Though some reports are available on antibiotic and enzyme production by marine actinomycetes, the marine environment is still a potential source for new actinomycetes, which can yield novel bioactive compounds and industrially important enzymes[8].

The purpose of this study was isolation, identification and characterization of antimicrobial metabolite producing actinomycetes from coastal region of Andrapradesh, India. This study also involved the extraction of polyenic antifungal metabolites and its bio prospective study against various plant and animal pathogenic bacteria, fungi and dermatophytes.

2. Materials and methods

2.1. Chemicals and enzymes

The genomic DNA isolation kit and pGEM-T vector were purchased from Promega (Madison, WI, USA). The high fidelity pfx polymerase was purchased from Invitrogen. The mini-prep and DNA gel extraction kits were purchased from Qiagen (Mannheim, Germany). PCR master mix with a novel Top DNA polymerase was purchased from Bioneer. Glucose and all other chemicals were obtained from Himedia (India) and Sigma-Aldrich (St. Louis, MO, USA).

2.2. Sample collection

Soil samples were collected from different locations in Andra Pradesh coast of the Bay of Bengal, India. The sediment samples were collected at 2-3 m depth using grab sampler. The sediments and water samples were collected in sterile polypropylene bags and screw cap bottles, respectively. The collected samples were brought to the laboratory for isolation of marine actinomycetes and the locations, nature of sample were recorded.

2.3. Isolation of actinomycetes

Samples (2 g, wet weight) were diluted ten times in sterile saline water (NaCl 9 g/L), homogenized by vortexing 15 min. Isolation was carried out on starch casein agar medium supplemented with 40 μg/mL actidione which inhibits the development of antagonist fungi and other soil eukaryotic microorganisms. The plates were incubated at 30 ℃ for 7 days. Actinomycetes colonies were recognized on the basis of morphological characteristics by light microscopy. Most actinomycetes showed a vegetative mycelium and aerial hyphae, others showed only the substrate mycelium. Isolates were maintained on ISP-2 agar medium by storage at 4 ℃ for 2 months. Alternatively, cultures were re-suspended in 20% glycerol and stored at -80 ℃.

2.4. Antimicrobial activity

Antimicrobial activities of the isolates were checked by growing the cells on modified nutrient glucose agar (MNGA) plates by single streak in the center. The plates were incubated at 30 ℃ for five days. The test organisms were inoculated perpendicular to the antagonist on the agar medium. Bacteria were incubated at 37℃for 18 h and fungi were incubated at 28℃for 48 h. The microbial inhibitions were observed by determining the diameter of the inhibition zones.

2.5. Culture characteristics and biochemical identification

Actinomycetes colonies were characterized morphologically and physiologically following the directions given by the International *Streptomyces* Project (ISP) [9] and Bergey's Manual of Systematic Bacteriology[10]. Cultural characteristics of pure isolates in various media were recorded after incubation for 7, 14 and 21 days at 30. Morphological observations were made with a light microscope using the method of Shirling and Gottlieb[10]. Actinomycetes were identified to the species level by comparing the morphology of spore bearing hyphae with entire spore chain and structure of spore as described in Bergey's manual. Micro morphology was observed by slide culture method[11]. Biochemical identifications of the isolates were done by API 20E micro tests (Himedia, Mumbai). Cell wall composition was analyzed by the method of Lechevalier and Lechevalier[12], using thin layer chromatography plates as described by Staneck and Roberts[13].

2.6. Amplification of 16s rDNA and sequencing

Genomic DNA was extracted with the Gen Elute Bacterial Genomic DNA kit (Sigma). 27F and 1492R primers were used to amplify 16S rDNA fragments by PCR (Table 1) (Bio-Rad I cycler). The conditions for thermal cycling were as follows: initial denaturation of the target DNA at 95℃for 10 min followed by 30 cycles of amplification, denaturation at 95℃for 2 min, primer annealing at 58℃for 1 min and primer extension at 72℃for 2 min. At the end of the cycle, the reaction mixture was held at 72℃for 10 min and cooled to 4 oC. Amplified DNA was visualized at 100 V and 400 mA for 25 min on agarose gel [1% (w/v) in TAE buffer 1x, 0.1 μL ethidium bromide solution]. Concentration of DNA was determined by nanodrop spectrophotometer (Nanodrop™ 1000). The amplified PCR products were purified by QIAquick® PCR purification Kit (Qiagen Ltd., Crawley, UK). The PCR product was ligated into the pGEM-T cloning vector by following the instructions given by the manufacture (Promega, Madison, WI, USA). Plasmids were transformed into *Escherichia coli* DH5α competent cells. Recombinant transformants were selected by blue/white colony screening. Individual white colonies were grown at 37℃overnight with rotary shaking in 25 mL of LB medium containing ampicillin. After plasmid preparation, 2 μL (out of 50 μL) of each sample was amplified by PCR (Bio-Rad I cycler) using M13-F and M13-R primers to check for the presence of insert DNA. Only plasmids containing the expected 1 500 bp inserts were sequenced. The obtained sequences were subjected to BLAST at http://www.ncbinlm-nih.gov/ se arch in NCBI database for phylogenetic relationship. The evolutionary history was inferred using the Neighbor-Joining method. Evolutionary analyses were conducted in MEGA5[14].

Table 1
Oligonucleotide primers used for 16S rDNA amplification and sequencing.

Primer	Sequences
27 FP	AGA GTT TGA TCG TGG CTC AG
1492 RP	GGT TAC CTT GTT ACG ACT T
M13 FP	GTT TTC CCA GTC ACG AC
M13 RP	CAG GAA ACA GCT ATG AC

2.7. Nucleotide sequence accession numbers

The 16S rDNA gene sequences determined for AP-5, AP-18, AP-41 and AP-70 strains in this study were deposited in GenBank database, under the accession numbers: JQ283103, JQ283104, JQ283105 and JQ283106, respectively.

2.8. Cultivation and extraction of metabolites

Strains AP-5, AP-18, AP-41 and AP-70 were cultivated at 30℃for five days in a sterile 500 mL Erlen-mayer flask containing 100 mL MNGA medium. At the end of the fermentation cycle the culture was filtered and the supernatant was separated by centrifuging at 8 000 rpm for 15 min. The supernatant pH was adjusted to five by using 0.1 N HCl and then extracted with hexane and ethyl acetate (3*300 mL). The solvent phase was concentrated by using vacuum at 35℃to obtain the crude extract for antimicrobial screening.

2.9. Antibacterial activity

The minimum inhibitory concentrations (MICs) of crude extracts were tested against different bacteria by broth micro dilution method[15].

2.10. Antifungal activity

The antifungal activity and minimum inhibitory concentration (MIC) were performed according to the standard reference method[15]. Extracts were dissolved in water together with 2% dimethyl sulfoxide (DMSO). The initial test concentration was serially diluted two-fold. Each well was inoculated with 5 μL of suspension containing 104 spore/mL of fungi. The antifungal agent fluconazole was included in the assays as positive control. Plates were incubated 24, 48 or 72 h at 28℃up to seven days for dermatophyte strains. MIC was determined as the lowest concentration of the extracts inhibiting the visual growth of the test cultures. Three replications were maintained to confirm the antifungal activity.

2.11. Microorganisms

Bacteria: *Bacillus subtilis* (*B. subtilis*) MTCC 441, *Enterococcus faecalis* (*B. faecalis*) ATCC 29212, *Staphylococcus aureus* (*S. aureus*) ATCC 25923, *Staphylococcus epidermidis* (*S. epidermidis*) MTCC3615, *Escherichia coli* (*E. coli*) ATCC25922, *Klebsiella pneumoniae* (*K. pneumoniae*) ATCC 15380, *Proteus vulgaris* (*P. vulgaris*) MTCC 1771, Ps*eudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853 and Erwinia sp. MTCC 2760 and fungi: *Trichophyton rubrum* (*T. rubrum*) MTCC 296, *Trichophyton mentagrophytes* (*T. mentagrophytes*) 66/01, *Trichophyton simii* (*T. simii*) 110/02, *Epidermophyton floccosum* (*E. floccosum*) 73/01, *Scopulariopsis* sp. 101/01 *Aspergillus niger* (*A. niger*) MTCC 1344, *Botyritis cinerea* (*B. cinerea*), *Curvularia lunata* (C. lunata) 46/01 and *Candida albicans* (*C. albicans*) MTCC 227 were used for the experiment.

2.12. Detection of polyenic antibiotics

Polyene production of culture extracts was examined by disc diffusion method using *C. albicans* as the indicator[16]. Sterile agar medium was dispensed in a 90 mm petri dish which was composed of two separate layers. In the first layer, 15 mL of the YM medium (yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, glucose 1.0%) formed a base layer in the petri dish. After solidification, a 0.1 mL suspension of 0.7 OD600 nm *C. albicans* mixed with 15 mL of sterile YM medium at (40-50 ℃) was poured immediately onto the base layer to constitute the upper layer. The crude extracts (5 mg) obtained from hexane and ethyl acetate were dissolved in 1 μL of respective solvents and used for assay. Only hexane and ethyl acetate solvents containing discs were used as the negative control, the methanol extract from the *Streptomyces* noursei strain was used as the positive standard. In order to facilitate the diffusion of polyene metabolite containing extracts into the medium, the plates were incubated for 2 days at 37 ℃, and the inhibitory zones were measured and charted.

2.13. Determination of antibiotic sensitivity and resistance pattern

Antibiotic sensitivity and resistance of the isolated bacteria were assayed by disc diffusion method of Bauer *et al*[17]. Bacterial inoculum was prepared by growing cells in SCA medium for 48 h at 30 ℃. Petri plates were prepared with 25 mL of sterile SCA medium (Diffco). The test culture was swabbed on the top of the solidified media and allowed to dry for 10 min. Different antibiotics loaded discs were placed on the surface of the medium and left for 30 min at room temperature for diffusion of the antibiotics. The plates were incubated for 48 h at 28 ℃. After incubation, the organisms were classified as sensitive or resistant to an antibiotic according to the diameter of inhibition zone given in standard antibiotic disc chart.

3. Results

3.1. Isolation and screening of actinomycetes for antimicrobial metabolites

The present investigation involved in the isolation of antagonistic potential actinomycetes from four marine mud samples obtained from coastal area of Andra Pradesh, India. A total of 210 suspected actinomycetes were isolated and purified based on their capability to grow on SCA medium and ISP-2 medium, respectively. All the strains were screened for their antimicrobial activity against various bacteria (Gram positive and Gram negative) and fungi (filamentous fungi and dermatophtes). All the suspected actinomycetes showed activity against either bacteria or fungi in primary screening. Gram positive bacteria *B. subtilis*, *E. faecalis*, *S. aureus* and *S. epidermidis* exhibited good antibacterial activity. Approximately 50% (105 isolates) of the isolates exhibited activity against one or more bacteria. From the total (210), 78% of the isolates showed activity against one bacterium, while 54.8% revealed activity against two bacteria and 31% showed activity against all the tested bacteria. This indicates that actinomycetes are the potential candidates for broad range antimicrobial metabolites, which may be the results from natural selection of the microorganisms in order to survive in a competing environment. The results showed that 64.3% of the actinomycetes produced antibacterial substances towards only Gram positive bacteria, 48.5% exhibited the activity towards Gram negative bacteria and 38.8% against both Gram positive and negative bacteria. Out of the 210

isolates, only 80.85% isolates exhibited significant antifungal activity in primary screening. The highest antifungal activity of *Streptomyces* strains was recorded against *A. niger* (56%), followed by *C. albicans* (52%), *T. mentagrophytes* (47%), *T. rubrum* (45%), *Scopulariopsis* sp. (41%), *C. lunata* (38%), *T. simii* (38%), *B. cinerea* (29%) and lowest for *E. floccosum* (28%). Actinomycetes strains AP-5, AP-18, AP-41 and AP-70 showed better activity against all the tested bacteria and fungi. So these strains were studied further for production of bioactive metabolite and identification by biochemical and physiological characteristics, 16s rRNA amplification and sequencing.

3.2. Morphological characteristics of strains

The new bacterium designated as *Streptomyces* sp. AP-5, AP-18, AP-41 and AP-70 recovered from marine sample exhibited antimicrobial activity against Gram positive, Gram negative bacteria and fungi. Gram staining indicated that all the strains were Gram-positive filamentous bacteria. Culture characteristics of strains were derived on the basis of observations made after 7, 14 and 21 days of incubation on ISP-2 media. According to the cultural characteristics, AP-5 grew well on ISP-2, ISP-5 and ISP-6 but the colonies were spreading (Table 2). Growth of strains AP-18, AP-41 and AP-70 were found to be poor in ISP-2 and ISP-5. Strain AP-18 mycelia color was greyish and spore chain was light black in color. Strains AP-5, AP-41 and AP-70 displayed dark grey in color. The colonies of *Streptomyces* were elevated, convex and powdery in nature. The spore morphology of these strains produced aerial coiled mycelia and the spores arranged in chains. Spore chain arrangements were observed using research microscope at 1000X showing that of all the isolates bear spore chains of three or more and non-motile in nature. Isolates form a hook like structure which can only be found in the *Streptomyces* genus.

Table 2
Cultural characteristics of the strains in different medium.

Characteristics	Strains			
Growth	AP-5	AP-18	AP-41	AP-70
Czapek-dox agar (CA)	Moderate	Good	Good	Good
Glycerol tyrosine agar (GTA)	Good	Good	Moderate	Moderate
Peptone yeast agar (PYA)	Good	Good	Good	Good
Glycerol asparagine agar (GAA)	Good	Good	Good	Good
Tryptone yeast glucose agar (TYGA)	Good	Good	Good	Good
Starch casein agar (SCA)	Good	Good	Good	Good
Vegetative mycelia	Moderate, brown	Good, brown	Moderate, yellow brown	Good, light brown
Aerial mycelia	Abundant, light gray	Abundant, dark gray	Moderate, gray	Moderate, white
Spore	Poor, light gray	Brown, light grey	Brown	Grey
Soluble pigment	Colourless	Colourless	Colourless	Dark grey

Table 3
Biochemical and physiological characteristics of strains.

Character		AP-5	AP-18	AP-41	AP-70
Growth at	45℃	-	-	-	-
	37℃	+	+	+	+
	30℃	+	+	+	+
Growth at pH	5	-	-	-	-
	6	+	+	+	+
	7	+	+	+	+
	8	+	+	+	+
	9	-	-	-	-
Growth at NaCl (%)	2	+	+	+	+
	4	+	-	+	+
	6	-	-	-	-
	8	-	-	-	-
Extracellular Enzyme	Amylase	+	+	+	+
	Cellulase	+	+	-	-
	Protease	+	+	+	+
	Lipase	-	-	-	-
	Gelatinase	+	+	+	+
	Hydrolysis of				
	Casein	+	+	+	+
	Hypoxanthine	+	+	+	+
	Tyrosine	+	+	+	+
	Xanthine	+	+	+	+
	Esculin	+	+	+	+
	Urea	V	V	V	V
	Lysozyme resistance	-	-	-	-

These micro morphological and spore colors and mycelia properties strongly suggested that AP-5, AP-18, AP-41 and AP-70 strains belonged to the genus *Streptomyces*.

Table 4
Growth pattern of the strains in different carbon sources.

Carbon source	AP-5	AP-18	AP-41	AP-70
Glucose	+	+	+	+
Lactose	+	+	+	+
Rhamnose	+	+	+	+
Sucrose	+	+	+	+
L-Arabinose	+	+	+	+
m-Inositole	−	−	−	−
Salicin	+	−	−	−
D-Fructose	+	+	+	+
Galactose	+	+	+	+
D-Mannitol	+	+	+	+
D-Xylose	−	−	+	−
Maltose	+	+	+	+
Trehalose	−	−	−	−
Melibiose	−	−	−	−
Glycerol	−	−	−	−
Salicin	+	+	+	+
Dulcitol	+	+	+	+
Sorbitol	+	−	−	−
Adonitol	−	−	−	−
Arabitol	−	−	−	−
Erytritol	−	−	−	−
Melezitose	−	+	+	+
Xylitol	+	+	+	+
Sorbose	+	+	+	+

+: Positive (more than 90%); −: Negative (more than 90%).

Table 5
Growth pattern of the strains in different nitrogen sources.

Nitrogen source	AP-5	AP-18	AP-41	AP-70
L-Arginine	+	+	+	+
L-Histidine	+	+	+	+
L-Cystine	+	+	+	+
L-Valine	+	+	+	+
L-Phenylalanine	+	+	+	+
L-Theronine	+	+	+	+
L-Serine	+	+	+	+
L-Methionine	+	+	+	+
L-Apargine	+	+	+	+
L-Alanine	+	+	+	+
L-Glutamine	−	+	+	+

+: Positive (more than 90%); −: Negative (more than 90%).

3.3. Biochemical and physiological characterization of strains

Biochemical identifications presented in the API 20E micro tests (Himedia, Mumbai) were determined. Biochemical tests revealed that these isolates were mesophilic and unable to produce hydrogen sulphide and reduce nitrate. The optimal pH and temperature for AP-5, AP-18, AP-41 and AP-70 were 6.0-8.0 and (30-37 ℃), respectively (Table 3). All strains showed good growth on medium amended with sodium chloride up to 2%; poor growth was observed at 6% and no growth was seen at 8% NaCl. All the strains displayed positive results for amylase, protease and gelatinase enzyme production test. Table 4 showed that strains AP-5, AP-18 AP-41 and AP-70 could grow on glucose, lactose, rhamnose, sucrose, arabinose, fructose, galactose, mannitol, maltose, salicin, dulcitol, xylitol and sorbose. However, they could not grow on innocitol, xylose, trehalose, melibiose, glycerol adonitol, arabitol and erytritol, xylose, mannose, cellobiose, rhamnose, xylitol, arabinose, inositol, sorbitol and arabitol. Utilization of various carbon sources by new isolates indicated a wide pattern of carbon source assimilation. All the strains exhibited good growth on various nitrogen sources like leucine, histidine, tryptophan, serine, glutamic acid, lysine, arginine, methionine and tyrosine (Table 5). The analysis of cell wall hydrolysates showed the presence of LL-diaminopimelic acid and glycine without any characteristic sugar pattern. Taxonomic studies indicated that it belongs to the *Streptomyces* genus.

3.4. Antibiotic sensitivity

Antibiotic sensitivity test was conducted against most commonly used antibiotics for bacterial infections by means of disc diffusion method. All strains revealed similar sensitivity pattern (Table 6). Strains AP-5, AP-18, AP-41 and AP-70 were sensitive to most of the tested antibiotics. All the strains exhibited the highest sensitivity towards β-lactamase inhibitor imipenem followed by fluroquinolone antibiotics such as moxifloxacin, nalidixic acid, norfloxacin, ofloxacin and sparfloxacin, respectively. Aminoglycoside and cephalosporin antibiotics streptomycin and cefpodoxime did not inhibit the growth of the strains. Streptomycin resistance indicated its typical feature.

3.5. Comparative 16S rDNA analysis

PCR-amplified 16S rDNA of AP-5, AP-18, AP-41 and AP-70 were completely sequenced and analyzed for the similarities. The NCBI BLAST search proGram showed that the sequence data of AP-5 had high identity (99%) to those of *Streptomyces* roseoverticillatus with a bits score and E value of 2815 and 0, respectively. Strains AP-18 and AP-41 displayed high similarity towards *Streptomyces* roseorubens and *Streptomyces septatus*. The evolutionary tree was drawn using the Neighbor-Joining method (Figure 1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates)[18]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method[19] and are in the units of the number of base substitutions per site.

3.6. Antibacterial activity

Hexane and ethyl acetate extracts obtained from the isolates showed significant antimicrobial activity against Gram positive and Gram negative bacterial pathogens (Table 7). Hexane extracts of strains AP-5 and AP-41 exhibited MIC 125.0 μg/mL for Gram positive bacteria (*B. subtilis*, *E. faecalis*, *S. aureus* and *S. epidermidis*). MICs for Gram negative bacteria *E. coli*, *P. vulgaris* *P. aeruginosa*, *Erwinia* sp. and *K. pneumonia* were 250.0 and 500.0 μg/mL, respectively. Hexane extracts of AP-18 showed comparatively similar kind of activity for Gram positive and Gram negative bacteria. Hexane extracts obtained from strain AP-70 revealed MIC 1 000.0 μg/mL for *P. vulgaris*,

Table 6
Comparison of sensitivity of strains towards various antibiotics.

Antibiotic group	Antimicrobial agent	Disc potency (μg)	Diameter of inhibition zone (mm)*			
			AP-5	AP-18	AP-41	AP-70
Aminoglycoside	Amikacin	30	25	20	22	21
	Gentamicin	10	27	22	21	25
	Kanamycin	30	25	28	21	21
	Streptomycin	10	0	0	0	0
	Tobramycin	10	31	26	23	19
Carboxypenicillin	Carbenicillin	50	25	22	20	19
	Ampicillin	50	25	21	25	26
β-lactamase inhibitor	Augmentin	30	28	25	22	25
	Imipenem	10	30	27	30	31
	Ticarcillin	75	25	27	29	33
Fluroquinolone	Ciprofloxacin	5	39	35	31	21
	Gatifloxacin	5	32	28	24	28
	Levofloxacin	5	37	31	29	28
	Moxifloxacin	5	30	26	24	25
	Nalidixic acid	30	35	18	15	17
	Norfloxacin	10	38	29	21	26
	Ofloxacin	5	34	30	28	26
	Sparfloxacin	5	32	26	24	25
Cephalosporin	Cefpodoxime	10	0	0	0	0
	Cetriaxone	30	30	32	21	25
Polymixin	Colistin	10	11	9	0	0
Sulphonamide	Co-Trimoxazole	25	25	21	20	15

*Zone of inhibition was measured after incubating the strains at 48 h at 30℃ in SCA medium.

Table 7
MIC (μg/mL) solvent extracts of isolated strains against bacteria.

Microorganisms	Streptomycin	Hexane extract				Ethyl acetate extract			
		AP-5	AP-18	AP-41	AP-70	AP-5	AP-18	AP-41	AP-70
B. subtilis (MTCC 441)	2.5	125.0	500.0	125.0	500.0	125.0	250.0	_	_
E. faecalis (ATCC 29212)	25.0	125.0	250.0	125.0	500.0	125.0	250.0	_	_
S. aureus (ATCC 25923)	6.3	125.0	250.0	125.0	250.0	125.0	125.0	_	_
S. epidermidis (MTCC3615)	50.0	125.0	250.0	125.0	250.0	125.0	125.0	_	_
E. coli (ATCC25922)	50.0	250.0	500.0	250.0	250.0	_	500.0	67.5	125.0
K. pneumoniae (ATCC 15380)	50.0	500.0	500.0	500.0	500.0	_	500.0	125.0	125.0
P. vulgaris (MTCC 1771)	25.0	250.0	250.0	500.0	1 000.0	_	250.0	125.0	500.0
P. aeruginosa (ATCC 27853)	50.0	250.0	250.0	500.0	1 000.0	_	250.0	125.0	500.0
Erwinia sp. (MTCC 2760)	50.0	250.0	250.0	250.0	1 000.0	_	250.0	250.0	500.0

Table 8
MIC (μg/mL) of organic solvent extracts of strains against fungus.

Microorganisms	Ketoconazole	Hexane extract				Ethyl acetate extract			
		AP-5	AP-18	AP-41	AP-70	AP-5	AP-18	AP-41	AP-70
T. rubrum (MTCC 296)	50.0	125.0	250.0	250.0	250.0	500.0	1 000.0	500.0	1 000.0
T. mentagrophytes (66/01)	12.5	125.0	250.0	125.0	250.0	250.0	250.0	500.0	1 000.0
T. simii (110/02)	25.0	250.0	125.0	125.0	125.0	250.0	250.0	250.0	1 000.0
E. floccosum (73/01)	25.0	250.0	125.0	250.0	125.0	500.0	500.0	250.0	500.0
Scopulariopsis sp. (101/01)	12.5	67.5	250.0	250.0	250.0	125.0	500.0	250.0	125.0
A. niger (MTCC 1344)	12.5	67.5	250.0	125,0	250.0	125.0	500.0	125.0	125.0
B. cinerea	25.0	67.5	250.0	67.5	125.0	500.0	500.0	125.0	125.0
C. lunata (46/01)	12.5	125.0	125.0	67.5	125.0	125.0	500.0	125.0	125.0
C. albicans (MTCC 227)	12.5	67.5	125.0	67.5	125.0	125.0	250.0	125.0	125.0

Table 9
Antifungal bioassay for the detection of polyene antibiotics (mm).

Sample	Hexane extract		Ethyl acetate extract		Nystatin
	5.0 mg/mL	2.5 mg/mL	5.0 mg/mL	2.5 mg/mL	0.1 mg/mL
AP-5	15	–	–	–	25
AP-18	12	–	–	–	
AP-41	17	–	–	–	
AP-70	12	–	–	–	

Zone of inhibition was measured after incubating the strains at 48 h at 37 ℃.

P. aeruginosa, *Erwinia* sp., and *E. coli*, *S. epidermidis*, *S. aureus* and *B. subtilis* and *E. faecalis* showed 250.0 and 500.0 μg/mL. The antagonistic activity of the crude metabolite extracted from the isolates did not exhibit comparable activity to that of standard antibiotics. Ethyl acetate extracts of strains AP-41, AP-70 and AP-5 showed activity against Gram negative and Gram positive bacteria, respectively. Ethyl acetate extracts of AP-5 showed MIC 125.0 μg/mL for *B. subtilis*, *E. faecalis*, *S. aureus* and *S. epidermidis*.

3.7. Antifungal activity

Hexane and ethyl acetate extracts of AP-5, AP-18, AP-41 and AP-70 were screened against fungi. All the isolates exhibited a marked antagonistic activity against the fungal pathogens and ethyl acetate extract showed comparatively better activity than hexane extract (Table 8). Hexane extracts of strain AP-5 exhibited MIC 125.0 μg/mL for T. rubrum, T. mentagrophytes and C. lunata, B. cinerea, A. niger and Scopulariopsis sp. showed good activity 62.5 μg/mL. Hexane extracts of all the strains showed MIC 125.0 μg/mL for T. simii except strain AP-5. Hexane extracts obtained from strain AP-18 inhibited the growth of all the tested fungi at 250 μg/mL concentration. Strains AP-5, AP-41, AP-70 and AP-18 inhibited the growth of *C. albicans* at 67.5 and 125.0 μg/mL, respectively. Ethyl acetate extracts of all the strains showed significantly less activity than hexane extracts. MIC study of ethyl acetate extracts revealed that strains AP-5, AP-41 and AP-70 showed comparatively better activity than other strains. *C. albicans* showed MIC 125 μg/mL for all the strains except AP-18. Dermatophytic fungus (T. rubrum, T. mentagrophytes, *T. simii*, *E. floccosum* and *Scopulariopsis* sp.) did not show good activity at lesser concentrations when compared to other fungi (*A. niger*, *B.cinerea*, *C. lunata* and *C. albicans*).

3.8. Detection of poyene antibiotics in the crude extract

Each of the culture extract was analyzed for polyene specific characteristics antibiotics by an antifungal bioassay method using *C. albicans* in order to determine if the five identified *Streptomyces* strains produce the putative polyene compounds in the culture. Polyene antibiotic nystatin-producing *S. noursei* used as a positive control in this study revealed inhibition zone of 25 mm against *C. albicans* (Table 9). Hexane extracts of the strains showed zone of inhibition at 5 mg/mL concentration. Ethyl aceteate extracts of strains AP-5 and AP-70 revealed inhibition zone at concentration 5 mg/mL and 2.5 mg/mL, respectively. Ethyl acetate extracts of strain AP-18 did not exhibit activity; this indicated that the crude extract did not contain polyene antibiotic.

4. Discussion

The finding of new bioactive compounds is a never ending process to meet the everlasting demand for novel bio-molecules with antimicrobial properties in order to contest human and plant pathogens. It is more important to screen actinomycetes because they are the essential sources of potent molecules. Marine environment is the biggest reservoir of chemical and biological diversity. Therefore, research focus on marine environment has been gaining importance in recent years. However, still it has not been fully explored and there is tremendous potential to identify novel organisms with various biological properties. The present investigation showed that actinomycetes tentatively identified as *Streptomyces* species have strong antimicrobial activities against pathogenic bacteria, fungi and dermetophytes. Although soils are considered excellent sources for the isolation of actinomycetes with diverse potential[20-22], several actinomycetes have been isolated from marine samples[23-26]. The isolation of actinomycetes from marine sediments was well documented; yet the proportion of these filamentous bacteria which represents the indigenous marine micro flora remains unclear.

In our previous experience[27], most of the *Streptomyces* grow well on starch SCA medium. So SCA medium supplemented with antibacterial and antifungal agent was used for isolating *Streptomyces* strains. In the present study among the 210 actinomycetes isolated from marine environment, most of the isolates exhibited antimicrobial activity. Remarkably, the morphlogy of majority of the marine actinomycetes was similar to *Streptomyces* species. Sujatha *et al* reported that cell wall composition is an important criterion for the identification of *Streptomyces* and chemotaxonomic investigation using isomeric diaminopimelic acid (DAP) configuration was already established[28,29]. The spore morphology is considered as one of the important characteristics in the identification of *Streptomyces* and it greatly varies among the species. It has been found that the majority of the marine isolates produced aerial coiled mycelia and the spores arranged in chains as already reported by Mukherjee and Sen[30,31] *Streptomyces* strains AP-5, AP-18 AP-41 and AP-70 showed good antimicrobial activity in solid medium and also in fermented spent broth. Most of the secondary metabolites and antibiotics are extracellular in nature and extra cellular products of actinomycetes showed potent antimicrobial activities[32].

Actinomycetes are useful biological tools in the production of antimicrobials against bacteria and fungi[33]. In general, *Streptomyces* are primarily saprophytic and are best known from soils where they contribute significantly to the turnover of complex biopolymers and antibiotics. In the past two decades however, there has been a decline in the discovery of new lead compounds from common soil derived actinomycetes. For this reason, the cultivation of actinomycetes taxa has become a major focus in the search for the next generation of pharmaceutical agents[34]. The antimicrobial crude extracts from *Streptomyces* species

AP-5, AP-18 AP-41 and AP-70 were recovered using hexane and ethyl acetate solvent. However, most of the antimicrobial compounds are extracted using ethyl acetate.

From the present study, it is clear that a novel isolate produced hexane and ethyl acetate soluble extracellular product which is effective against pathogenic test bacteria and fungi. In view of the decline in the discovery of new lead compounds in recent years, further investigations on isolates would lead to some useful products.

Conflict of interest statement

We declare that we have no conflict of interest.

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