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Screening and fractional purification of antimicrobial compound of *Streptomyces* sp. MAB 18 isolated from coastal sediment of Nagapattinam, south-east coast of India

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A total of 42 strains of actinobacteria were isolated from five different stations. Out of these 42 isolates, the suspected 10 actinobacterial strains were screened and inoculated for purification, and based on the preliminary screening results, MAB18 was taken to investigate the extraction evaluation of its antimicrobial property. Then it was tested against bacterial and fungal pathogens. The highest zone of inhibition was observed against *E. coli* (8.2±0.8mm), *P. aeroginosa* (10.4±0.6 mm), *Vibrio harveyi* (7.2±0.8mm), *S. typhi* (10.6±-0.4 mm) for bacteria and *A. niger* (11.2±0.8 mm), and *A. flavus* (9.8±0.2 mm) *C. albicans* (10.4±0.6mm), and *Penicillium* sp. (7.2±0.8mm), for fungi, whereas it was 21.4±0.6 and 20.4±0.6 mm for the standard ampicillin and nystatin. The antibiotic producing actinomycetes may be tapped as one of the India's potential source of novel antibiotics to be used against both bacterial and fungal pathogenic organisms.

[Keywords: Actinobacteria; Pathogens; *Streptomyces*; Antimicrobial activity; Antibiotics]

Introduction

Marine microorganisms have become an important source of study for novel drug discovery. Recently, both researchers and industrialists have concentrated on marine microorganisms for novel products. Due to the climatic conditions of the marine environment, almost each group of marine organism exhibits diversity of molecules with unique structural features, which are not found in terrestrial natural products. Although, the marine environment contains much greater biodiversity than the terrestrial, the efforts to exploit the marine biodiversity by identifying new chemical compounds have hardly begun[1].

Actinobacteria are the most economical and biotechnologically valuable class of prokaryotes producing bioactive secondary metabolites notably antibiotics[2] anti-tumor agents and immunosuppressive agents[3] and enzymes[4,5]. Actinomycetes are the main source of clinically important antibiotics, besides vitamins and enzymes. The *Streptomyces* are especially prolific, producing around 80% of total antibiotic products[6,7]. The marine actinomycetes account for a major fraction of microbial metabolites and, among them, *Streptomyces* is the distant and most abundant genus. Antagonistic actinomycetes of marine origin are being regularly reported[8,16]. However, it soon became apparent during screening programmes that some microbial metabolites were discovered more frequently than others. Rare or novel actinomycete taxa have become a major focus in the search for pharmaceutical agents[17].

In pharmaceutical industry, over several decades, isolated and screened millions of *Streptomyces* strains. Consequently, the chances of isolating a novel *Streptomyces* strain have substantially diminished, and so has the probability of discovering a novel compound also less. Therefore, it has been estimated that only a fraction of the antibiotics produced by *Streptomyces* strains has been discovered[18], and identifying the undiscovered portion will require a substantial effort. Consequently, a well-defined taxonomic study of actinobacteria is important to ascertain the correct species that produces novel secondary metabolites. Further, the discovery of novel antibiotic through screening of secondary metabolite is progressively more important.

Hence, the present study was carried out to screen antagonistic marine actinobacteria isolated from near-sea shore sediments along south-east coast of India. The potential antibiotic producing actinobacteria

strain was screened, identified and used for the investigation of antibacterial activity against different human clinical pathogens.

Materials and Methods

Isolation of actinobacteria

The sediment samples were serially diluted and plated on Actinomycetes Isolation Agar medium supplemented with 20 mg/l of cycloheximide to eliminate fungal and bacterial contaminations[19]. The plates were incubated at 28±2 °C for 7-28 days. The actinobacteria colonies that appear on the petriplates were counted. All the experiments were carried out in triplicate. Further, the strains were sub-cultured and incubated at 28 °C for 2-4 weeks to isolate good sporulating actinobacteria and then the strains were stored in the deep freezer at -20 °C. Further, the actinobacterial strains were identified using conventional methods, such as morphological characteristics (Aerial mass colour, melanoid pigments, reverse side pigments, and soluble pigments), physiological characteristics (Assimilation of carbon sources and assimilation of nitrogen sources),

chemotaxonomical analysis (Hydrolysis, thin layer chromatography, amino acids, whole-cell sugars), and micromorphological characteristics (Spore chain morphology). The strains were sub-cultured onto Actinomycetes Isolation Agar (AIA) medium and preserved in 20% glycerol stored at 4 ^{o}C until use.

In vitro screening of isolates for anti-bacterial activity

Morphologically distinct actinomycetes isolates were chosen for antibacterial and antifungal activity. The preliminary screening was made against the pathogenic test organisms using single line streak method[22] on agar medium. Pure actinomycetes isolates were inoculated in a single streak and incubated at 28 ^{o}C for four days for the production of any antibiotics. A single streak of each test organism was added perpendicular to the actinomycetes streak. Test organisms were placed perpendicular to culture streak and the plates were incubated for 24 h at 37 ^{o}C. Further, confirmation of the selected strain was subjected to molecular taxonomic studies.

Molecular identification

The genomic DNA was isolated from the selected strain using the method of Hopwood[21]. The DNA samples of the strain were amplified using a PCR kit (Synergy Scientific Services, Chennai, India) using consensus primers, the ~0.25 kb 16SrDNA fragment was amplified using high-fidelity PCR polymerase. The purified fragments were directly sequenced using Ampli Tag FS DNA sequencing kit (Applied Dio system). Sequence of 1478bp was perforated with a model 377 PRISM automatic sequencer (Applied Biosystems). The data were analyzed using applied biosystem DNA editing and assembly software and sequence comparisons were obtained using the Micro Seq Software. Sequence similarity search was made for the 16SrDNA sequence of the strain by applying its sequence to BLAST search of the NCBI (National Centre for Biotechnological Information, USA). The 16SrDNA gene sequence of the strain was manually aligned[21] with the sequences of species members of the same genus/family obtained from the GenBank database. Evolutionary tree was inferred by using the neighbour-joining method[22].

Fermentation

The fermentation conditions were optimized for the strain and fermentation was carried in 500 ml Erlenmeyer flask. Initially, this strain was grown at 28 ^{o}C in Starch Casein Agar (SCA) with the pH of 7.5 for one week. Spores were collected and cultured with 10 ml of the same medium broth. Cultivation of the strain was made by transferring 1 ml (ca. 10^8 cells/ml) of the spore suspension and incubated at 28 °C and 250 rpm for seven days in 500 ml Erlenmeyer flasks containing 100 ml of antibiotic production medium with 2% sucrose, 0.25% yeast extract, 0.1% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.001% $FeSO_4.7H_2O$, 0.002% NaCl, pH 7.5 and made up to 100% by using seawater.

Extraction

The crude culture broth of isolated strain was centrifuged at 10,000 rpm for 30 min at 4 °C by maintaining all the physico-chemical factors at optimum levels. Growth and antibiotic production were estimated at 30 ^{o}C, pH 7.5 and sodium chloride concentration of 2.0%. The total culture filtrate of the metabolites was used for solvent extraction using ethyl acetate in triplicate. The organic layer was collected and the solvent ethyl acetate was evaporated using vacuum rotary evaporator and lyophilized.

Purification

The crude antimicrobial compound (5mg) was chromatographed on silica gel (Merck, particle size

0.100 - 0.200 mm) column (22X5 cm) and eluted with chloroform: ethyl acetate (70:30) to give 10 major fractions. The dried residues of all the 10 major fractions were dissolved separately in specified volume of ethyl acetate (1 mg/ml) concentration and tested for their antimicrobial activities against the pathogenic organisms by disc-diffusion method. All the extracts and fractions were subjected to thin layer chromatography on silica gel (60 F 254 MERCK; 25 TLC aluminum sheets 20x20cm MERCK) and run in chloroform: ethyl acetate (70:30) to visualize the efficiency of separation of UV fluorescent substances. Each batch was repeated several times to confirm the results.

Determination of antibiotic compound

The purified antimicrobial compound with an Rf value of 0.43 was identified from ethyl acetate extract of the metabolites of isolated strain as reddish crystals with a melting point of 110-120 ^{o}C.

Test organism

The following test pathogenic organisms were used to test Minimum Inhibitory Concentration (MIC) of the extracts: bacterial strains such as *Escherichia coli, Pseudomonas aeruginosa, Vibrio harveyi, Salmonella typhii* and fungal such as *Aspergillus niger, A. flavus, Candida albicans* and *Penicillium* sp. were obtained from Raja Muthiah Medical College Hospital (RMMCH), Annamalai University, Tamil Nadu.

Preparation of inoculums

Inoculums of each bacterial strain was inoculated in 3 ml of Mueller Hinton Broth (MHB) and incubated at 37 °C for 24 h. After incubation period, the culture was diluted with sterile MHB to provide initial cell count of about 10^{-4} CFU/ml. The four pathogenic fungi were grown on Sabouraud Dextrose Agar (SDA) slants at 28 ^{o}C for 10 days and the spores were collected using sterile Milli Q water and homogenized.

Antimicrobial activity

Antimicrobial activity of MAB18 was assayed by disc diffusion method[23]. The pathogenic organisms were grown individually in the sterile nutrient broth for 24 hr at 37 °C in the case of bacteria and 28 °C for 72 h in the case of fungi. For antimicrobial assay, Mueller Hinton Agar (MHA) for bacteria and Sabouraud Agar for fungi were prepared poured aseptically into petriplates and allowed for solidification. Then the test organisms grown in the nutrient broth were swabbed into the sterile MH agar plates using sterile cotton buds. Agar wells were prepared using sterile cork borer (diameter 4 mm). Subsequently, 100 µl of MAB18 of extracted supernatant was added into each well. Negative control was prepared using the respective solvent. To compare the antibacterial and antifungal activities, ampicillin (30 µg/disc) and nystatin (20 µg/disc) were used as a positive control. For each strain five replicates were made. The petridishes were incubated at 8-10 °C for 16 h for the diffusion of the bioactive compound. For each strain five replicates were made. The plates were incubated for 24 hours at 37 °C for bacterial growth. After incubation, the plates were examined for the antimicrobial activity and zones of inhibition were recorded in millimeters and all the data were statistically analyzed and mean and standard deviation were calculated.

Determination of MIC

The MIC was determined by using the method of National Committee for Clinical Laboratory Standards[24]. The required concentrations (1000 mg/ml, 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml and 31.25 mg/ml) of extract MAB18 was dissolved in dimethyl sulfoxide (DMSO) (2%). These tests were made to find out the least concentration of MAB18 extracts that inhibit the growth of human pathogens. The test was carried out in 96 well microlititre plates, so that replicates of individual sample can be run. An inoculum of 5 ml from each well was inoculated. The antibacterial agent ampicillin and antifungal nystatin (20 µg/disc) were included in the assays as positive controls. The plates were incubated for 72 h for fungi and 24 h for bacteria at 37 ^{o}C. The MIC for bacteria was determined as the lowest concentration of the compound inhibiting the visual growth of the test cultures on the agar plate. Three replications were maintained.

Results

A total of 42 strains of actinobacteria were isolated from five different marine stations. Of these 42 isolates, the suspected 10 actinobacterial strains were isolated and inoculated on the Actinomycetes Isolation Agar medium for purification and pure colonies were maintained in the same agar slant at 4 °C and were named MAB5, MAB8, MAB14, MAB18, MAB20, MAB25, MAB29, MAB33, MAB36 and MAB41. All the 10 culture strains were screened against bacterial and fungal pathogens as

shown in Table 1. In antimicrobial screening test, the strain MAB18 inhibits the growth of all the bacteria and fungi which was selected for further study.

Molecular taxonomy (16S rDNA analysis)

Identification of actinomycetes on the basis of morphological characteristics is not reliable for all groups of organisms, including actinomycetes which possess limited morphological differentiation[25] and can be relied on the basis of biochemical tests and assimilation assays[26]. When compared to morphological and biochemical characterization methods, 16S rDNA analysis is found to be the novel and accurate method for identifying unknown species; 16S rRNA sequencing appears to have the potential ability to differentiate strains at the species level.

The 16S rDNA of the strain MAB18 was amplified through PCR which shows the molecular weight of 0.5 kb corresponding to that of the 1000 bp DNA ladder in 1% agarose gel. The amplified product was purified to remove the excess primer for sequencing and it was sequenced using the automated DNA sequencer. Sequence of the strain MAB18 showed partial 16S rDNA sequences, consisting of 1478 nucleotides (Fig. 1), which was submitted to the GenBank (National Center for Biotechnology

Table 1 —The antimicrobial activity of actinobacterial isolates against pathogenic bacteria and fungi (single steak method)

Strain Name	*E. coli*	*P. aeruginosa*	*V. harveyi*	*S. typhi*	*A. niger*	*A. flavus*	*C. albicans*	*Penicillium* sp.
MAB 1	+	+	+	+	-	-	-	-
MAB5	+	+	+	-	-	-	-	-
MAB8	+	+	+	+	-	-	-	-
MAB14	+	-	+	-	+	-	+	-
MAB18	+	+	+	+	+	+	+	+
MAB20	+	-	-	-	-	+	-	-
MAB25	+	-	-	-	+	-	-	-
MAB29	+	+	+	-	+	-	-	+
MAB33	+	+	-	+	-	-	+	-
MAB36	+	+	+	+	+	-	+	-
MAB41	+	+	+	+	+	-	-	-
MAB5	+	+	+	+	-	-	+	-

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   1 gagtttgatc ctggctcagg acgaacgctg gcggcgtgct taacacatgc aagtcgaacg
  61 atgaaccacc ttcgggtggg gattagtggc gaacgggtga gtaacacgtg ggcaatctgc
 121 cctgcactct gggacaagcc ctggaaacgg ggtctaatac cggatactga cctgccaagg
 181 catcttggcg ggtcgaaagc tccggcggtg caggatgagc ccgcggccta tcagcttgtt
 241 ggtgaggtaa tggctcacca aggcgacgac gggtagccgg cctgagaggg cgaccggcca
 301 cactgggact gagacacggc ccagactcct acgggaggca gcagtgggga atattgcaca
 361 atgggcgcaa gcctgatgca gcgacgccgc gtgagggatg acggccttcg ggttgtaaac
 421 ctctttcagc agggaagaag cgaaagtgac ggtacctgca gaagaagcgc cggctaacta
 481 cgtgccagca gccgcggtaa tacgtagggc gcaagcgttg tccggaatta ttgggcgtaa
 541 agagctcgta ggcggcttgt cgcgtcggtt gtgaaagccc ggggcttaac cccgggtctg
 601 cagtcgatac gggcaggcta gagttcggta ggggagatcg gaattcctgg tgtagcggtg
 661 aaatgcgcag atatcaggag gaacaccggt ggcgaaggcg gatctctggg ccgatactga
 721 cgctgaggag cgaaagcgtg gggagcgaac aggattagat accctggtag tccacgccgt
 781 aaacggtggg cactaggtgt gggcaacatt ccacgttgtc cgtgccgcag ctaacgcatt
 841 aagtgccccg cctggggagt acggccgcaa ggctaaaact caaaggaatt gacgggggcc
 901 cgcacaagcg gcggagcatg tggcttaatt cgacgcaacg cgaagaacct taccaaggct
 961 tgacatacac cggaaagcat cagagatggt gccccccttg tggtcggtgt acaggtggtg
1021 catggctgtc gtcagctcgt gtcgtgagat gttgggttaa gtcccgcaac gagcgcaacc
1081 cttgtcccgt gttgccagca actcttcgga ggttggggac tcacgggaga ccgccggggt
1141 caactcggag gaaggtgggg acgacgtcaa gtcatcatgc cccttatgtc ttgggctgca
1201 cacgtgctac aatggccggt acaatgagct gcgataccgc aaggtggagc gaatctcaaa
1261 aagccggtct cagttcggat tggggtctgc aactcgaccc catgaagtcg gagtcgctag
1321 taatcgcaga tcagcattgc tgcggtgaat acgttcccgg gccttgtaca caccgcccgt
1381 cacgtcacga aagtcggtaa cacccgaagc cggtggccca acccttgtg ggagggagct
1441 gtcgaaggtg ggactggcga ttgggacgaa gtcgtaac
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Fig. 1 — 16S rDNA sequence of the strain MAB18

Information, USA) and an Accession Number (JQ068140) was obtained for MAB18 strain.

Antimicrobial activity

Based on the preliminary screening results, MAB18 was taken to investigate the extraction evaluation of its antimicrobial property (Figs 1 and 2). Ethyl acetate was used as an extract for the recovery of antibiotics from strain MAB18 and purified by using chromatographic technique. Then it was tested against bacterial and fungal pathogens. The mean and standard deviation values are shown in Table 2. The highest zone of inhibition was observed against *E. coli* (8.2±0.8mm), *P. aeroginosa* (10.4±0.6 mm), *Vibrio harveyi* (7.2±0.8mm), *S. typhi* (10.6±-0.4 mm) for bacteria and *A. niger* (11.2±0.8 mm), and *A. flavus* (9.8±0.2 mm) *C. albicans* (10.4±0.6mm), and *Penicillium* sp (7.2±0.8mm), for fungi, whereas it was 21.4±0.6 and 20.4±0.6 mm for the standard ampicillin and nystatin Fig.3.

Minimum inhibitory concentration (MIC)

The MIC values of ethyl acetate extract of MAB18 are: *E. coli* (125 mg/ml) and *P. aeruginosa* (250 mg/ml), *Vibrio harveyi* (62.5 mg/ml), *Salmonella typhi* (250 mg/ml), *A. Niger* (250 mg/ml), *A. flavus* (500 mg/ml), *C. albicans* (500 mg/ ml) and *Penicillium* sp. (1000 mg/ml) as shown in Table 3.

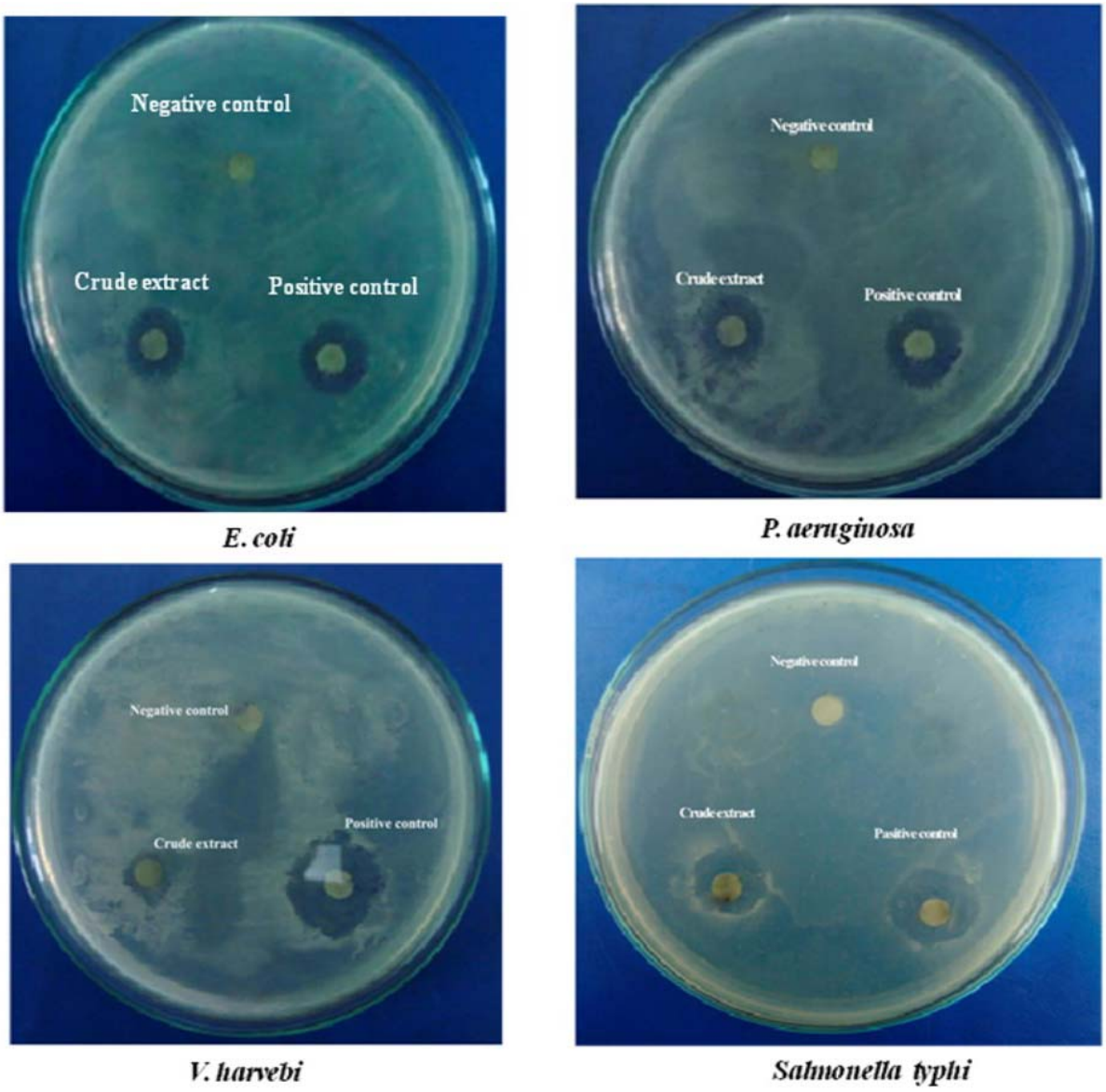

Fig. 2 —The antimicrobial activity of MAB18 against bacterial pathogen

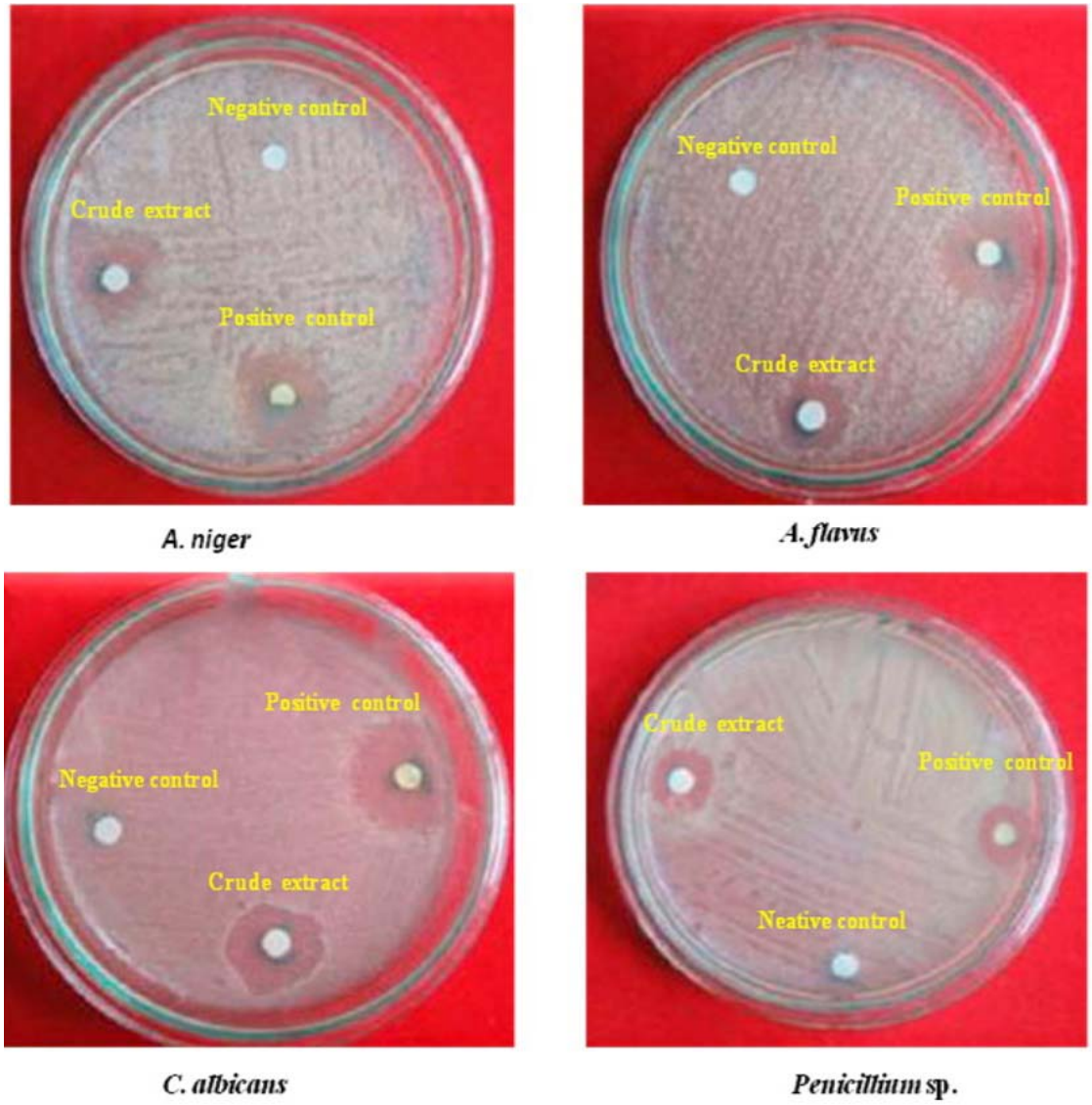

Fig. 3 —The antimicrobial activity of MAB18 against fungal pathogen

Table 2 —The antimicrobial activity (zone of inhibition) of MAB18 against pathogenic bacteria and fungi

Pathogens	Diameter of zone of inhibition (mm)
Bacteria	
Escherichia coli	8.2±0.8
Pseudomonas aeruginosa	10.4±0.6
Vibrio harveyi	7.2±0.8
Salmonella typhi	10.6±-0.4
Ampicillin (30µg/disc)	21.4±0.6
Fungi	
A. niger	11.2±0.8
A. flavus	9.8±0.2
C. albicans	10.4±0.6
Penicillium sp.	7.2±0.8
Nystatin (20µg/disc)	20.4±0.6

Discussion

The appearance of pathogenic microbes with increased resistance to established antibiotics provides a major incentive for the discovery of new antimicrobial agents[24]. However, investigation on isolation and identification of new bioactive compounds from marine microorganisms will be more effective in controlling diseases related to bacterial and fungal pathogens. Ability to produce a large number of chemically different secondary metabolites is associated mostly with the actinomycetes. In particular, the genus *Streptomyces* sp is widely reported for the production of various antibiotics which are used therapeutically[25]. Yedir et al.[26] showed that 21.88% of actinobacterial colonies

Table 3 —The minimum Inhibition concentration (MIC) of *Streptomyces* sp. MAB18 extract against bacterial and fungi pathogens

S. No.	Nature of substance	*E. coli*	*P. aeruginosa*	*V. harveyi*	*S. typhi*	*A. niger*	*A. flavus*	*C. albicans*	*Penicillium* sp.
1.	Extract of MAB 18	125	250	62.5	250	250	500	500	1000
2.	Control	12.5	25	50	50	6.25	12.5	6.25	12.5

isolated from terrestrial environment had significant growth inhibitory activity against pathogenic fungal strains. Abo-Shadi et al.[27] reported that the Streptomyces sp. strain A 24S4 isolated from plant rhizosphere region at Al-Madinah Al-Munawwarah, Saudi Arabia, exhibited 62.5% growth inhibitory activity against the clinically important fungal pathogens.

In the present study, the suspected actinobacterial strains were isolated and inoculated in the AIA medium and preliminary screening test was made to identify the potential strain. The strain MAB18 inhibits the growth of all the bacteria and fungi. The strain MAB18 was taken for further investigation for evaluation of its antimicrobial property. Ethyl acetate extracts of MAB18 was tested against bacteria and fungi pathogens. The strain *Streptomyces* sp. (MAB18) exhibits good antimicrobial activity against all bacterial as well as fungal pathogens. Sahin and Ugur[30] reported *in vitro* antimicrobial activity of three isolates as *Streptomyces antibioticus* (MU106, MU107) and *S. rimosus* (MU114) against Gram-positive and Gram-negative bacteria and yeasts. Dehnad *et al*.[31] reported 12 *Streptomyces* sp. isolates with different ability to produce antibiotics. Heng *et al*.[32] recorded *Streptomyces* sp. strain PM2 and PM4 as potential antifungal candidate to inhibit the growth of pathogenic fungi, *Colletotrichum capsisi* and *Colletotrichum gloeosporioides* isolated from chilli fruit. Annamalai *et al.*[33], working on *Alcaligens faecalis* (AU01) isolated from seafood industry effluent produced an alkaline protease and reported that the enzyme inhibited the growth of fish pathogens, such as *Flavobacterium* sp., *Pseudomonas fluorescens*, *Vibrio harveyi*, *Proteus* sp. and *Vibrio parahaemolyticus*. Naikpatil and Rathod[34] reported the antimicrobial and cytotoxic activity of *Streptomyces* sp. (KR-5) and proved its medical applications. They observed that the extract of *Streptomyces tanashiensis* strain (A2D) showed antibiotic activity against *Bacillus subtilis* (15 mm), *Staphylococcus aureus* (25 mm), *Escherichia coli* (21 mm) and *Klebsiella pneumonia* (23 mm). Manivasagan *et al.*[35] reported that the extract of *Streptomyces* sp. (PM-32) showed antibiotic activity against *Bacillus subtilis* (20 mm) and *E.coli* (16 mm). Ethyl acetate extract of MAB18 also showed inhibition against all the pathogenic fungi with the highest zone of inhibition against *A. niger* (11.2±0.8) and the least inhibition observed against *Penicillium* sp. (7.2±0.8 mm). In an earlier study, AL-Bari *et al.*[36] reported 13 mm (20 µg/disc) inhibition zone against *A. flavus* and Kavitha *et al.*[37] reported the antifungal effect of *Nocardia levis* MK-VL_113 against *Fusarium oxysporum.* Manivasagan *et al.*[35] reported that the extract of *Streptomyces* sp. PM-32 showed antibiotic activity against *A. flavus*.

Conclusion

The present investigation about the antagonistic activity of marine *Streptomyces* sp. exhibits very good inhibition activity against human pathogenic organisms. Hence, it has been concluded that sediments of Nagapattinam coast, Southeast coast of India possess antibiotic producing actinomycetes and may be tapped as one of the India's potential source of novel antibiotics. They have every possibility to be used against both bacterial and fungal pathogenic organisms.

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