Isolation, Discovery of Bioactive Compounds, Phylogenetic Analysis of *Streptomyces* sp. Hb084 and its Cytotoxic Studies against MCF-7

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ABSTRACT

Infectious disease is the number one cause of death in tropical countries accounting for approximately half of all fatalities. The chemotherapeutic treatments of infectious diseases are also getting limited due to the emergence of antibiotic resistant pathogens. Actinomycetes play a quite important role in natural ecological system and they are also profile producers of antibiotics, antitumor agents, enzymes, enzyme inhibitors and immune modifiers which have been widely applied in industry, agriculture, forestry and pharmaceutical industry. In the present study, Actinomycete isolates were screened from the mangrove soil samples, totally 5 Actinomycetes strains were isolated. The isolates were grown in various medium to illustrate the cultural and morphological characteristics. Secondary screening was done to identify the isolates which shown broad spectrum activity against selected bacterial pathogens. Extracted compound was tested for antibacterial activity against 12 Bacterial pathogens including MRSA (Methicillin resistant *Staphylococcus aureus*) by Agar well diffusion method. The compounds were studied for short term *in vitro* cytotoxicity using Dalton's lymphoma ascites cells (DLA). MTT Assay results showed the IC_{50} value against MCF-7 cells were 19.84µg/ml. The results suggest that the crude extract is less toxic to normal cells and toxic to MCF-7 cells. Various biochemical tests were performed to describe the biochemical characterization of the sample MS5. 16s rRNA sequence of the effective MS5 was sequenced and analyzed by PCR amplification method. The microbe was found to be most similar to *Streptomyces* sp. HB084 (GenBank entry (GU213489). Biologically active compounds were identified from the extract compound by TLC, GC-MS and FT IR analysis.

Key words: Streptomycetes, Cytotoxicity, Actinomycetes, 16SrRNA, DLA, MTT.

INTRODUCTION

The demand for new antibiotics continues to grow due to the rapid emerging of multiple antibiotic resistant pathogens causing life threatening infection. Although, considerable progress is being made within the fields of chemical synthesis and engineered biosynthesis of antibacterial compounds, nature still remains the richest and the most versatile source for new antibiotics.

Actinomycetes have been proven as efficient producer of new secondary metabolites and have wide range activity such as antibacterial, antifungal, antitumor, immunosuppressive and larvicidal. Bioactive compounds like macrocyclic lactum, macrolide, quinine and peptide are present in actinomycetes. These compounds have range of activity like antibacterial, antifungal, antitumor etc [1].

MATERIALS AND METHODS

Sample Collection

Sediment samples were collected from different areas of the Thiruthipuram.mangrove ecosystem (Lat 10°11'56"N, Long 76°12'47"E) situated along the west coast of Kerala, India. The central portion of the 6-10 cm sediment sample was taken and transferred to a sterile bag and transported immediately to the laboratory.

Isolation of Actinomycetes

Soil sediments (1g) were serially diluted up to 10^{-8} dilution using sterile water. The dilutions such as 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were taken and inoculated in nutrient agar by spread plate method and the plates were incubated at 30°C and observed from 5th day onwards for 25 days. After incubation, actinomycetes isolates

were distinguished from other microbial colonies. The isolated colonies were inoculated into actinomycetes isolation agar (AIA) supplemented with Ketoconazole (0.01 mg/ml). The plates were incubated at 30°C for a period of 15 days until the growth was observed. The grown colonies were purified by sub culturing on Starch Casein Agar (SCA) supplemented with Ketoconazole (0.01 mg/ml) to inhibit the fungal growth**.**

Morphological Characterization

Gram staining was performed to see the morphology and examined under the light microscope.

Fermentation, antibiotic extraction and purification

ISP4 (International Streptomycetes project-4) was prepared and sterilized. A loopful of isolate was inoculated into a 500 ml flask containing 300 ml of ISP4 medium and incubated on a rotary shaker at 200 rpm at 35°C for 7 days. After 15 days, the culture was taken and centrifuged at 3000-5000 rpm for 10 minutes [2]. After centrifugation, the cell free supernatant was collected and filtered with Whatmann No.1 filter paper. Antibacterial compound was recovered from the filtrate by the solvent extraction method [3]. Equal volume of ethyl acetate was added to supernatant (1:1), shakedvigorously and allowed to settle for4 hours. The aqueous layer was collected in watch glass and kept in water bath at 70°C-80°C until the solvent evaporates and the product was grabbed [2].

In vitro screening of isolates for antimicrobial activity

Primary screening:

Morphologically distinct actinomycetes were subjected to antibacterial primary screening by cross streak plate technique, and the antagonistic activity was noted for further studies [3].

Secondary screening:

The antagonistic properties of the 5 isolates were determined by agar well diffusion method[4]. The partially purified extract obtained by the evaporation of the ethyl acetate extract was dissolved in 1ml distilled water. The test human pathogens were swabbed on the Muller Hinton agar and kept aside for 20 minutes. Then the extracted bioactive compound mixture was loaded into the wells bored on the medium at different concentration (50 µl, 100 µl & 200 µl). The plates were incubated at 30°C for 24 hours and the diameters of inhibition zones were measured.

In vitro Cytotoxicity study-1

The compounds from 5 isolates were studied for short term *in vitro* cytotoxicity using Dalton's lymphoma ascites cells (DLA).

$$\% \text{ Cytotoxicity} = \frac{\text{Number of dead cells}}{\text{No. of living cells + No. of dead cells}} \times 100$$

In vitro cytotoxicity assay -2

The human breast cancer cell line (MCF 7) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). For screening experiment, cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37^{0}C, 5% CO_2, 95% air and 100% relative humidity. After 48h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^{0}C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

$$\% \text{ cell Inhibition} = 100 - \text{Abs (sample)/Abs (control) x100.}$$

Isolation of genomic DNA

Isolation of genomic DNA is carried out by Sequencing Machine, ABI 3500 XL Genetic Analyzer with an Analysis protocolBDTv3-KB-Denovo Ver. 5.2

Phylogenetic analysis

Sequences were analyzed with Seq Scape Ver. 5.2 software, and thereby trees were generated. The assemblage of 16S rDNA gene sequences in each library was analyzed by rarefaction analysis. The number of

species in each clone library was determined by comparing closely related sequences using Weighbor. 16S rDNA sequences exhibiting a percentage of similarity of 98% or lower were considered for species authentication [5].

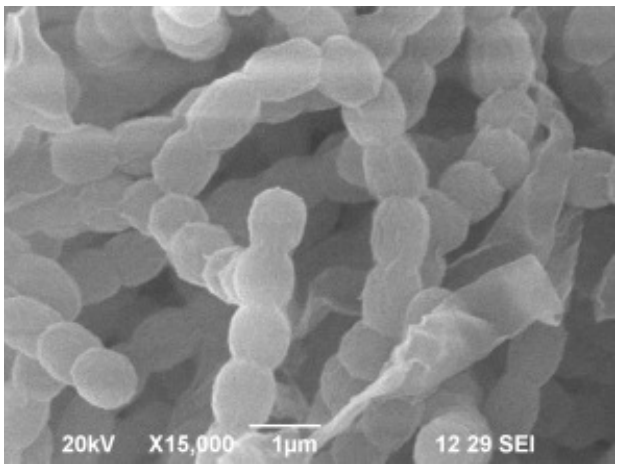

Fig. 1 Electron microscopic view of *Actinomycete* isolates

Partial purification by thin layer chromatography

The active compounds were separated on silica G 60 grade absorbent by mixture of chloroform and methanol (4:1) solvent.

Gas Liquid Chromatography-Mass Spectrometry

The partially purified active compound was analyzed by gas liquid chromatography-mass spectrometry (GC-MS).

Fourier Transform Infrared Spectrophotometer (FTIR)

The structure elucidation of the partially purified bioactive compound was further determined by FTIR [6].

RESULTS AND DISCUSSION

Screening of acetinomycetes

The colonies were characterized as tough, leathery, mucoid colonies which are partially submerged into the agar [7]. A total number of 5 *actinomycete* isolates were screened from mangrove soil. The colonies were small to medium sized, grayish white to pure white, pink and sandal in color, and powdery in nature. SEM analysis results indicate that the spores are arranged in chains, almost spherical in shape with a smooth surface (Fig.1).

Screening of antibacterial *Actinomycetes*

All the actinomycete isolates showed good antibacterial effect against the selected pathogens (Table 1). Among the five isolates, the isolate (MS5) was selected for further study based on their broad spectrum antibacterial activity and zone of inhibition against the test organisms.

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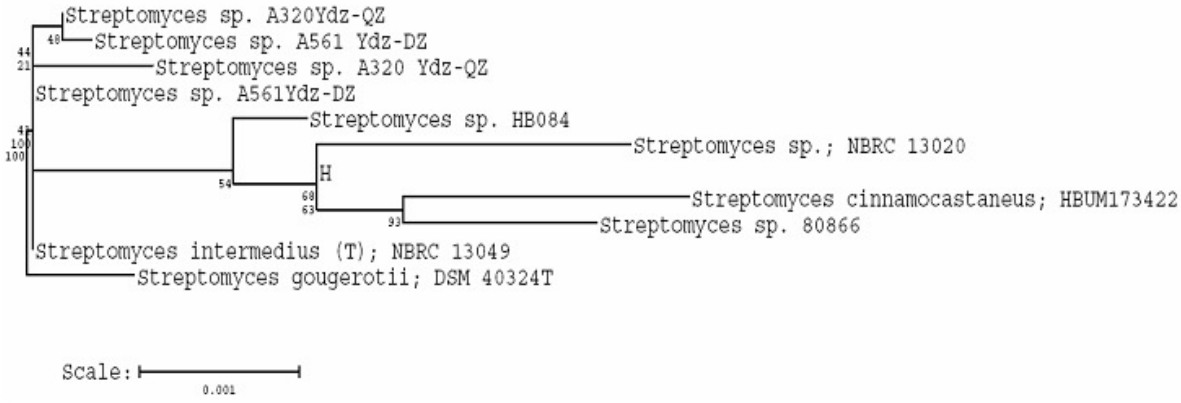

Fig 2 Phylogenetic tree of the sample (MS5)

Table 1: Antagonistic activity of Actinomycetes against human pathogens

Human Pathogens	Zone of inhibition (mm)														
	MS1			MS2			MS3			MS4			MS5		
	50 µl.	100 µl.	200 µl.	50 µl.	100 µl.	200 µl.	50 µl.	100 µl.	200 µl.	50 µl.	100 µl.	200 µl.	50 µl.	100 µl.	200 µl.
P.aeruginosa	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
B.cereus	_	16	19	17	20	23	_	13	15	_	15	17	10	13	15
K.pneumoniae	14	16	20	13	16	20	17	18	20	13	16	19	14	15	17
P.vulgaris	_	_	_	_	_	_	_	_	_	_	_	_	15	17	20
S.flexneri	17	23	25	20	25	30	_	12	13	12	17	21	16	18	20
S.mutants	3	19	20	18	20	23	_	14	17	13	15	20	15	16	20
E.coli	15	17	21	15	17	20	17	19	22	_	17	20	17	18	20
S.aureus	10	13	22	15	21	25	10	13	19	13	18	20	11	14	16
S.marsecens	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
S.typhi	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Enterococcus Sp.	13	16	20	14	17	20	16	18	21	_	_	_	14	16	20
MRSA	13	16	19	5	10	20	13	16	13	_	12	16	15	16	20

DLA Assay

The five isolates MS1, MS2, MS3, MS4 and MS5 were tested for DLA (Dalton's lymphoma ascites) assay. The isolate (MS5) showed activity with 90% of cell death in 200µg of sample, whereas MS2 showed 75% of cell death, MS1 with 70% of cell death and MS3 with 62% of cell death. MS4 showed less activity with only 12% of cell death. Thus MS5 was taken for further carcinogenicity analysis and molecular characterization (Table 2). The results obtained showed similar features as studied by Jensen et al 2005.

Table 2: Percentage of cell death in different drug concentration. (DLA Assay)

Drug concentration µg/ml	Percentage cell death(DLA)				
	MS1	MS2	MS3	MS4	MS5
200µg	70%	75%	62%	12%	90%
100µg	58%	40%	40%	06%	75%
50µg	40%	15%	30%	_	55%
20µg	20%	06%	19%	_	22%
10µg	08%	_	10%	_	11%

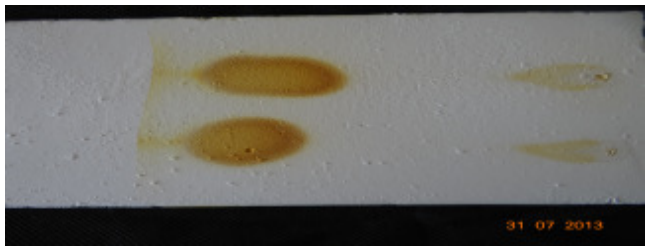

Fig 3: Thin layer Chromatography to separate the compounds

The cytotoxic effect of crude extract of MS5 on MCF-7 cells showed a concentration and time dependent activity. The IC_{50} value against MCF-7 cells was found to be 19.84µg/ml. At 37.5µg concentration, more than 70% cell death was observed. With 70µg, more than 90% of cell death occurred, and with 150µg

> 97% cell death observed. The results suggest that the crude extract is less toxic to normal cells and toxic to MCF-7 cells.

16S rRNA sequence analysis of the sample MS5

Based on the phylogenetic analysis, the strain was found to be highly related to *Streptomyces* sp. HB084 (GenBank entry: GU213489) exhibiting high similarity (~99 %) hence it designated as *Streptomyces*sp. HB084. The 16S rRNA gene sequence of *Streptomyces* sp.HB084 was already deposited in the GenBank (NCBI, USA) under the accession number GU213489.The next closest homologue was found to be *Streptomyces cinnamocastaneus*; HBUM173422 (Genbank entry : EU841658) Based on 16S rRNA gene sequencing, Phylogenetic position of effective Isolate 5 is assigned in to the genus *Streptomyces sp*. HBO84.

Phylogenetic analysis of the sample MS5%

Phylogenetic tree of the sample (MS5) is exhibited in Fig. 2.

Thin Layer Chromatography

The purification of secondary metabolites was performed using Thin layer chromatography technique. The biological active compound was extracted from fermentation broth. The active compounds were loaded in silica gel with chloroform and methanol solvent up to ¾ of the plate. The plates were exposed to iodine vapor and dark brown single spot was observed. The retention factor (R_f) of moved spot was 0.9cm (Fig.3).

GC-MS analysis

26 compounds were identified from the extract. Among the 26 compounds, Cyclododecane, n-Pentadeanol, 2-Undecene, 3 methyl- (Z), Undecyl trifluoro acetate, n-Nonadecanol-1, (2E) 3, 7, 11, 15-tetramethy l-2-hexadecene, 2, 3, 4, trimethyl-1-pentanol, Chrysene,4B, 5,6,12-Tetrahydro-2, 8-Dimethoxy-4B-methyl,n-Tetracosanol-1, 1-Heptacosanol, are the compounds showed high percentage of peak. In these, some compounds are used as volatile binding medium, flavoring ingredient in foods, medication for the treatment of eczema, bone cancer, osteoporosis, also used as anti-viral agent and pesticides (Fig.4).

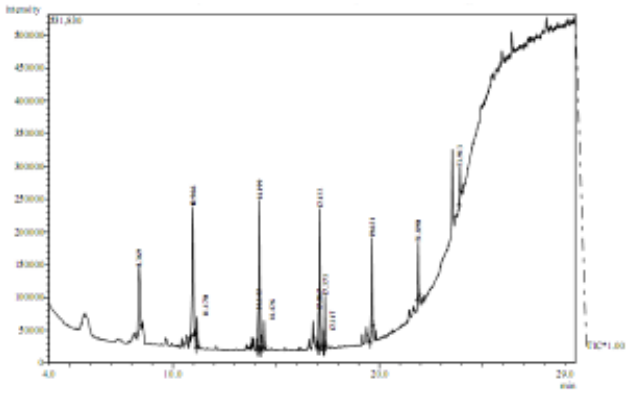

Fig 4: GC-MS analysis spectrum

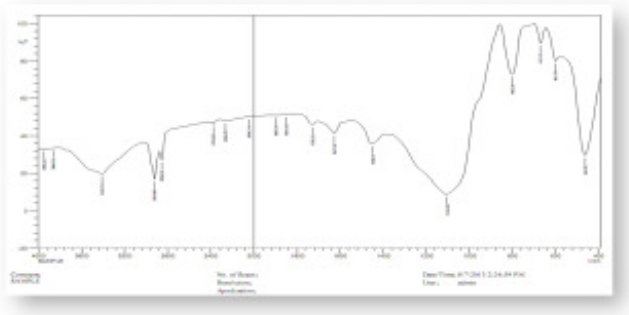

Fig 5: FTIR spectra of Antibacterial compound

With this method (FTIR), the functional groups present in the anti-microbial compounds were identified (Fig.5). The frequency at 3410 shows alcohols and phenols group present with O-H stretching. The frequency at 2854 shows alkanes with C-H Stretch. The frequency at 1728 shows aldehydes with C=O stretch. The frequency at 1103 shows carboxylic acids with C-O stretch. The frequency at 802 shows alkyl halides with C-Cl stretch. The frequency at 671 and 601 shows alkyl halides with C-Br stretch.

CONCLUSION

In the present study, the synthesis of Biologically Active compounds by Actinomycetes against bacterial pathogens and identification of antibacterial and antitumor compounds producing Actinomycetes in to genus level has been taken as the field of interest. The isolated actinomycetes, *Streptomyces* sp.HB084 was found to be a potent strain against various bacterial pathogens and tumor cell lines like DLA (Dalton's lymphoma ascites) and MCF-7.

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