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Antimicrobial and Antiproliferative Activities of *Streptomyces carpaticus* (MTCC-11062) Isolated from Marine Sediment of Visakhapatnam Sea Coast

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A new potent antagonistic actinomycetes strain was isolated from marine sediment samples of Visakhapatnam sea coast at Bay of Bengal, India. The isolate grows on starch casein agar medium with creamy-white substrate mycelium and blackish-gray aerial mycelium. On the basis of cultural characteristics, biochemical, chemotaxonomic tests and phylogenetic studies based on 16S rRNA gene sequence it was identified as *Streptomyces carpaticus* sp and deposited in microbial type culture collection IMTECH Chandigarh India with an accession number of MTTC-11062. The isolate showed broad spectrum antimicrobial activity against the pathogenic bacterial and fungal species studied and also inhibited the proliferation of HeLa and HepG2 cell lines with an IC_{50} values of 15.04 ¼g/ml and 18.36¼g/ml respectively. The survival rate of $HepG_2$ cell lines was higher than of HeLa cell lines at the same concentration of antibiotic.

Key words: Marine sediment, Actinomycetes, *Streptomyces carpaticus*, Antimicrobial activity, antiproliferative activity,16SrRNA

Actinobacteria are filamentous gram positive bacteria considered as intermediate organisms between the bacteria and fungi with 67-78% G + C content (Ventura *et al*. 2007). Actinobacteria are the best sources of secondary metabolites and industrially useful enzymes. Majority of these compounds, such as antibacterial, antifungal, antiproliferative and immunosuppressive agents are derived from the genus *Streptomyces* (Berdy, 2005). These actinobacteria are distributed widely in the marine and terrestrial habitats (Pathom-Aree *et al*. 2006). Traditionally, these bacteria have been isolated from terrestrial sources. Since the biological diversity of marine ecosystem is larger than that of the terrestrial and the difference in extreme environmental conditions with that of the terrestrial ones, it is assumed that marine *Streptomyces* have different characteristics from those of terrestrial counterparts and might produce novel bioactive secondary metabolites (Jensen *et al*. 2005). Over the past ten years the marine actinobacteria have become recognized as a source of novel antibiotics and anticancer agents with unusual structures and properties (Bull &Stach, 2007).

The marine ecosystem of south east coast of Bay of Bengal is mostly unexplored and may provide a rich source of actinobacteria producing novel and efficient bioactive compounds (Suthindhiran&Kannabiran, 2010). Only limited research on characterization of marine *Streptomyces* producing bioactive compounds

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from the sediments of Bay of Bengal has been reported (Siva kumar&Ramana, 2013). The present study aims to isolate and functionally characterize the indigenous marine Streptomyces producing bioactive compounds, and to screen their antimicrobial and antiproliferative activities.

MATERIALS AND METHODS

Organism

Strain MB-162 was isolated from marine sediment sample collected near Visakhapatnam sea coast, Andhra Pradesh, India, using starch casein agar medium (ISP medium 4; International Streptomycete project, Shirling and Gottlieb; 1966) incubated at 28ÚC for 2 weeks. The isolate was maintained on ISP medium - 4 slants with pH of 8.0, 50% (v/v) sterilized sea water was used for media preparation. The strain was deposited in Microbial Type Culture Collection IMTECH, Chandigarh, India as strain MTTC-11062.

Morphological, physiological and biochemical characteristics

The morphology of the strain was studied by inoculating on various ISP media (Shirling and Gottlieb, 1966) and incubating at 28ÚC for 2 weeks. Morphology of the strain was observed by light microscope (Olympus) and scanning electron microscope (JEOL; JSM-6610LV).The physiological and biochemical characteristics of the strain MB-162 was studied by growing on a number of culture media described by Shirling and Gottlieb, 1966; Williams *et al*., 1983. The utilization of carbon source and acid production were studied by using culture media suggested by Gordon *et al*., 1974. The colors were determined by comparing the color chips from the ISCC-NBS color charts standard samples No. 2106 (Kelly 1964).

Chemotaxonomy

The fatty acid analysis was performed by obtaining the cell mass grown on TSB agar plates (trypticase soy broth) that had been incubated for 10 days at 28ÚC.The fatty acids were extracted, methylated and analysed using the standard MIDI (Microbial Identification) system (Sasser, 1990; Kämpfer and Kroppenstedt, 1996). Whole cell sugars were determined by the method of Lechevalier and Lechevalier, 1980. Phospholipid analysis was carried out as described by Lechevalier *et al*., 1981.

Molecular characterization

The chromosomal DNA of the strain MB-162 was isolated by the method described by Sambrook *et al*., (1989). The genomic DNA G+C content of the strain was determined by thermal denaturation method of Marmur and Doty (1962). The 16S rDNA was amplified by PCR using primer A f (5 –AGTTGATCCTGGCTCAG -3¹) and a primer B r (5¹ -GGCT/TACCTTACGACTT -3¹). The amplified 16S rDNA fragment was separated by agarose gel electrophoresis and the sequence (1466nt) was determined. The almost complete 16S rDNA sequence of the strain MB-162 was compared with the sequences of known *Streptomyces* species retrieved from databases.Sequence data was aligned with CLUSTAL_X (Thompson *et al*., 1997). The evolution tree was rooted with *Streptomyces sp.* MTCC7779 (accession no. GU563884.1) as out group, was inferred by using maximum likelihood method by Felsenstein (1981) with PHYLIP package. The topology of the resultant tree was evaluated based on 1000 resamplings (Felsenstein, 1985). Dendroscope program was used to display, edit and print phylogenetic trees (Huson *et al*., 2007).

The almost complete 16S rDNA sequence (1466nt) of the strain MB-162 was deposited in EMBL with an accession number of HF586482.

Production of secondary metabolites

Aseptical inoculation of these pores from a mature slant culture of the streptomyces strain was done in 50ml of production medium (SSmedium) composition (g/L) soluble starch- 25, glucose -25 , yeast extract – 2 , caco3 and trace elements – 1ml by submerged fermentation condition in 250 ml Erlenmeyer flask by incubating at 28^{0}C for 4 days. After incubation the broth was centrifugated at 5000 rpm for 15 minutes and the clear supernatant was used for further analysis.

Antimicrobial activity

The clear supernatant was assayed for antagonistic activity using agar well diffusion method. The assay plates were seeded with *B.subtilis* (MTCC 441), *S.aureus* (MTCC3160), *B.cereus* (MTCC 430), *E.coli* (MTCC 443), *P.aeruginosa* (MTCC 424), *P. vulgaris* (MTCC 426) using muller hinton agar and *C.albicans* (MTCC 227), *C.cerevisiae* (MTCC 170),

A.flavis (MTCC 3396) and *A.niger* (MTCC 961) using yeast extract malt extract agar. Wells of 6mm diameter were prepared and 50¼l of clear supernatant was added into each well. The plates were incubated for 24hrs at 37^{0}C for bacterial species and 48hrs at 28^{0}C for fungal species after incubation inhibition zones were measured.

Extraction of metabolites

The clear supernatant obtained from the production medium was extracted with ethyl acetate. Equal volumes of the supernatant and ethyl acetate were mixed and shaken vigorously. The ethyl acetate phase containing antibiotic was separated from aqueous phase, it was then allowed to evaporate in water bath at 80^{0}C and the residue obtained was weighed.

Anti-proliferative studies

The cervix carcinoma (HeLa) cells and liver carcinoma ($HepG_2$) cells were obtained from the national center for cell (NCCS) Pune, India. The cells were grown in RPMI 1640 medium supplemented with 4.5g/L glucose , 2mM L-glutamine and 10% FBS , incubated in 5% CO_2 at 37^{0}C. Cells at a density of 1000 cells/well were seeded into 1ml of medium in a 96 well culture plates and allowed to grow in a CO_2 incubated with 5% CO_2 for 48hrs at 37^{0}C. After incubation antibiotic at various concentrations (1, 2, 4, 8, 16, 32, 64, 128 and 256¼g/ml) to the plates and incubated at 37^{0}C in 5% CO_2 incubated. Triplicates were maintained and the medium containing with out antibiotic was taken as control. After 24 hrs 5¼l of MTT [3-(4, 5-Dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (0.5mg/ml) in PBS was added to each well and was incubated for 2hrs at 37^{0}C. The medium with MTT was removed from the wells and equal volumes of DMSO were added to dissolve the formazan crystal, the absorbance was measured at 570nm using ELISA reader. The IC50 values were calculated by non-linear regression analysis using Masterplex ReaderFit software. The % cell inhibition was determined using following formula (Freshney *et al*., 2006).

% of inhibition = mean absorbance of sample/ mean absorbance of control X 100

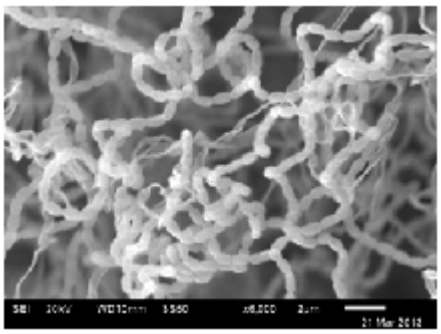


Fig. 1. Scanning electron micrograph indicating the spore chain morphology of the strain MB-162

Fig. 2. phylogenetic tree of *Streptomyces carpaticus* obtained by maximum likelihood Method

Table 1. Cultural characteristics of the strain MB-162

Media	Growth	Diffusible pigment	Aerial mycelia	Substrate mycelia
Yeast malt extract agar (ISP 2 medium)	Good	Brown	Pale black	Light grey
Oat meal agar (ISP 3 medium)	Poor	Absent	Light grey	White
Inorganic salt starch agar (ISP 4 medium)	Good	Reddish Brown	Grey	White
Glycerol asparagine agar (ISP 5 medium)	Good	Reddish Brown	White	Light brown
Peptone yeast extract iron agar (ISP 6 medium)	Good	Black	Light grey	White
Nutrient agar	Poor	Absent	Pale black	Dark Brown

Note: colours taken from ISCC-NBS COLOUR CHARTS standard sample No. 2106 (Kelly 1964)

Table 2. Biochemical characteristics of the strain MB-162

Strain	MB-162
Gram's reaction	+
Cell shape	Rods
Spore(s)	+
Motility	-
Growth at temperatures	
4ÚC	-
10ÚC	+
25ÚC	+
30ÚC	+
37ÚC	+
42ÚC	+
55ÚC	-
Growth at pH	
pH 5.0	-
pH 6.0	+
pH 7.0	+
pH 8.0	+
pH 9.0	+
pH 10.0	+
pH 110	+
pH 12.0	+
Growth on NaCl (%)	
0.5	+
2.0	+
4.0	+
6.0	+
8.0	-
10.0	-
11.0	-
12.0	-
Biochemical Tests	
Catalase test	+
Oxidase test	+
Methyl Red	+
VogesProskauer test	-
Casein hydrolysis	-
Arganinedihydrolase	+
Indole	-
Citrate	+
Nitrate Reduction	+
Urease	+
Gelatin hydrolysis	+
Starch hydrolysis	-
Esculin hydrolysis	+
Tween 40	-
Tween 60	+
Tween 80	+
Acid Production from	
Adonitol	-
Sorbitol	-
Dextrose	+
Fructose	+
Inositol	-
Lactose	+
Maltose	+
Mannitol	+
Raffinose	-
Rhamnose	+

(+) -utilization; (—) - non utilization

Table 3. Fatty acid composition percentage of the strain MB-162

Fatty acid	%
iso-$C_{14:0}$	6.04
iso-$C_{15:0}$	3.30
anteiso-$C_{15:0}$	3.38
iso-$C_{16:0}$	48.04
iso-$C_{16:0}$ H	4.40
$C_{16:0}$	15.75
anteiso-$C_{17:0}$	2.08
cyclo-$C_{17:0}$	8.9
$C_{18:0}$	3.70
Summed feature 3	6.49

Summed feature 3 contains iso-$C_{15:0}$ 2OH and/ or $C_{16:1}$ *w7c*;

RESULTS AND DISCUSSION

Morphological observation of the 14 days old culture of the strain MB-162 grown on ISP-4 medium revealed that the strain had typical characteristic features of genus *Streptomyces*.The strain has abundant, well developed substrate and aerial mycelia without fragmentation. Long spore chains in spiral were born on the aerial mycelium; the spores were rod shaped with smooth surface and non motile (Fig.1).The strain grows well on different tested media. The colour of the aerial mycelia varied from white to light grey and the substrate mycelia varied from grey to pale black. Cultural characteristics of the strain were shown in Table-1. Diffusible pigments were produced on most of the tested media, except nutrient agar. melanin was produced on peptone yeast extract iron agar. The strain grew well at between pH 6.0 to 12.0, with an optimum pH value of 8.0 to 10.0. The strain grows at a temperature between 10ÚC to 42ÚC, with an optimum at 30ÚC. Strain MB-162 exhibited tolerance to NaCl (w/v) upto 6.0 % (Table-2).

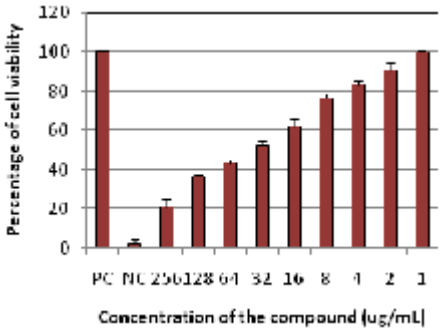


Fig. MTT assay with HeLa cell lines

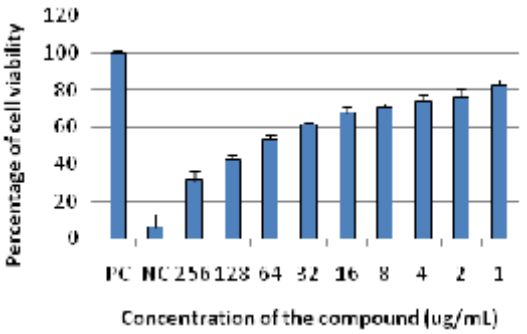
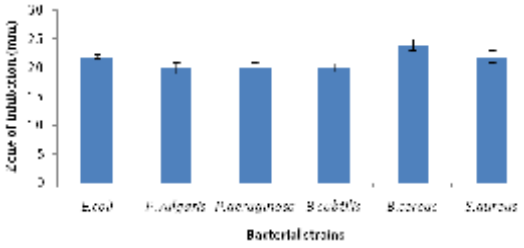
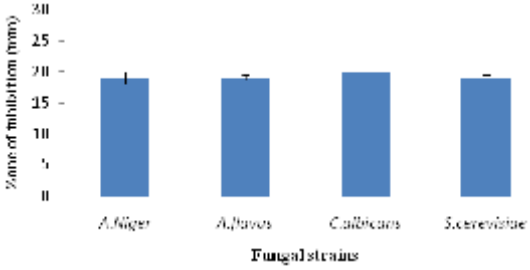


Fig . MTT assay with $HepG_2$ cell lines





The cell wall of the strain MB-162 contained LL-diaminopimelic acid and glycine, representing the cell wall characteristic of chemotype I (LechevalierandLechevalier 1970 a,b). The cell wall hydrolysates have no characteristic sugars. The diagnostic phospholipids identified were phosphatidyl ethanolamine and diphosphatidyl glycerol. The major fatty acids identified were iso-$C_{16:0}$ (48%), $C_{16:0}$ (15%) and cyclo-$C_{17:0}$ (8%) (Table 3). Thus phenotypic characters and chemotaxonomic data revealed that the strain MB-162 belongs to the genus *Streptomyces*.

The G+C content of the DNA of the strain MB-162 was determined as 86.3%. The almost complete 16S rDNA sequence of the strain MB-162 has been determined and was deposited in EMBL database with an accession number of HF586482. The almost complete 16S rDNA sequence of the strain MB-162 was aligned manually with corresponding partial 16S rDNAsequences of genus *Streptomyces* retrieved from GenBank, EMBL, and DDBJ databases by using BLAST (Altschuletal, 1997). Phylogenetic analyses based on 16S rDNA revealed that the 16s rRNA gene sequence of strain MB-162 contain 1466 base pairs. The comparative analysis of 16s rRNA gene sequence and the estimation of phylogenetic relationship showed that the strain formed a distinct lineage in the tree and showed the closest level of sequence similarity of 99% with *Streptomyces carpaticus* FGP40 (genBank accession number KF991640.1)

The antimicrobial principles secreted by the strain *Streptomyces carpaticus* (MTCC-11062) exhibited broad antimicrobial spectrum. Among all the bacterial strains tested *B.cereus* showed highest zone of inhibition followed by *S.aureus* and *E.coli* of 24mm, 22mm and 22mm respectively. Among the fungal strains tested *C.albicans* showed highest zone of inhibition of 20mm.

Streptomyces carpaticus (MTCC-11062) was also effective in inhibiting the proliferation of HeLa cell lines and $HepG_2$ cell lines in a dose dependent manner. The IC_{50} values were 15.04 ¼g/ml and 18.36¼g/ml respectively. The survival rate of $HepG_2$ cell lines was higher than of HeLa cell lines at the same concentration of antibiotic.

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