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Cytotoxicity of Streptomyces Sp. Isolated from Mangrove Sediments

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ABSTRACT

An attempt was made to isolate and culture a strain of actinomycetes from mangrove sediment samples. The isolate was identified as a member of Streptomyces genus using 16S rRNA gene sequence analysis. A crude extract of the cultured isolate was prepared in n-butanol and studied for its cytotoxic activity using MTT assay. Results were promising against PC12 and HeLa cell lines under study.

KEYWORDS | Actinomycetes, Streptomyces, Mangroves, Cytotoxicity, 16S rRNA

INTRODUCTION

Actinomycetes are primarily aerobic and spore forming Gram positive bacteria with high G+C content. As their name reflects (in Greek, "atkis" means ray and "mykes" means fungus), they share some morphological features with fungi. They are ubiquitous in nature, found both in terrestrial and aquatic habitats, including mangroves and sea sediments (Saini et al., 2015). Actinomycetes are known to be responsible for the production of about half of the discovered bioactive secondary metabolites, notably antibiotics, antitumor agents, immunosuppressive agents and enzymes (Lam, 2006).

Mangrove forests are among one of the world's most productive tropical ecosystems that protect coastal zones from erosion, and provide food and shelter for a large number of commercially valuable fin and shell-fishes. They are harboring enormous microbial diversity and are now exploited for the production of many bioactive compounds (Azman et al., 2015). A perusal of literature indicated that although there are numerous reports available on isolation of actinomycetes from mangrove sediments, majority of these have been focused on studying their antimicrobial and enzymatic potential and literature on their cytotoxic activity is scanty.

In view of this, the present investigation was undertaken to isolate a strain of actinomycetes from sediment samples collected from mangrove region and then to study the cytotoxic potential of the same. The isolate was identified using the sequence of amplified 16S rRNA gene.

MATERIALS AND METHODS

Mud sample for isolation of actinomycetes was obtained from mangrove area of Ratnagiri, Maharashtra state. The sediment samples were collected in sterile zip lock bags, brought to the laboratory and processed within 24 hours.

Isolation of the actinomycetes

For isolation, 1 g of sediment sample was crushed using a sterile mortar and pestle and suspended in 10 ml of sterile distilled water by vortexing for 2 min on maximum speed. A 10-3 serial dilution of the suspension was prepared. This dilution of the sample was spread onto actinomycetes Isolation agar (with 50% seawater) using the standard spread plate technique. The media was inoculated with two kinds of antibiotics. Rifampicin was used for the inhibition of bacterial growth while cycloheximide was used for the inhibition of fungal growth. The plates were incubated at 40°C for a minimum period of 2-3 weeks to allow growth of slower growing microorganisms and also for the purpose of sporulation. Seven colonies were selected based on their morphology, size and colour appearance on the agar plates. The selected colonies were then sub-cultured onto separate agar plates to ensure their purity. The purified cultures were maintained on actinomycetes agar slants. Out of the seven cultures, one unique culture RMS6 was further selected for phylogenetic analysis and preparation of extracts.

Phylogenetic analysis

DNA was extracted from the selected isolate using HiPurA Bacterial Genomic DNA Purification Kit (Himedia, MB505) with appropriate treatment with lysozyme, RNase A and Proteinsae K solutions. After purification, the concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation A11454806498). The DNA isolated from actinomycetes isolate was subjected to polymerase chain reaction (PCR) amplification using Biometra thermal cycler (T-Personal 48). Gel electrophoresis was performed using 1.0% agarose (Seakem, 50004L) to analyze the size of amplified PCR product. The size obtained was approx. 643bp for 16S rRNA region. Molecular phylogeny of actinomycetes isolate was determined by amplifying genomic 16S rRNA region which was later sequenced using Applied Biosystems 3730xl DNA Analyzer, USA. Two primers specific to 16S rRNA region used in this study were 235F and 878R. The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI) to find out the phylogenetic position of the isolate.

Preparation of the extract

The isolate RMS6 was cultured on modified FM 17 medium and incubated for eight days at 30°C and 100 rpm. After incubation, actinomycetes culture (1 L) was mixed with 150 ml of n-butanol. The mixture was kept at 40°C for 24 hrs stirred for 20 mins, centrifuged, and the butanol layer was separated and then evaporated using a rotary evaporator. Dry residue (100–150 mg) was stored below 5°C until further use.

Cytotoxicity study

Initially, 5 cell lines viz. PC3 (prostate), T47D (breast), HCT-116 (colon), PC12 and HeLa were taken for cytotoxicity assay at 100 µg / ml. In the initial screening, this extract was found to be active against only PC12 and HeLa cell line. Therefore, only these two cell lines were used for further cytotoxicity assays at various concentrations of the extract.

The extract obtained from actinomycetes RMS6 was tested for cytotoxicity against PC12 cells and human cervical HeLa cells. The PC12 cells were grown in medium DMEM (supplemented with 10% horse serum and 5% foetal calf serum) and HeLa cells in RPMI (with 10% foetal calf serum). These cells were

seeded into 96-well plates at a concentration of 1.4 × 104 cells/cm2 and incubated at 37°C for 24 hours. The extract was reconstituted in distilled water at a concentration of 1 mg/ml and tested at 4 different concentrations- 5, 25, 50, and 100 µg/ml (n = 4). After adding the desired concentrations of the extract to seeded cells in 96-well plates in a volume of 200 µl, the plates were incubated at 37°C for 72 hours. Cell viability was determined using the methylthiazolyldiphenyl-tetrazolium (MTT) bromide colorimetric assay (Scudiero et al., 1988). The plates were read at 595 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio Rad 3550) after overnight incubation at 37°C. Percentage inhibition of the cell growth was calculated with respect to the control.

RESULTS

Phylogenetic analysis

The partial gene sequence of the amplified 16S rRNA gene of the extracted DNA from the actinomycetes isolate RMS6 produced a 556 bp product (Fig.1). This sequence when analysed using BLASTn tool indicated that the isolate is a Streptomyces sp. as it showed 99% sequence similarity with many Streptomyces species which were already deposited in the nucleotide database (Table 1).

Figure 1: Partial gene sequence of 556 bp of the actinomycetes isolate RMS6

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ACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGA
GGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGC
AGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCG
GAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGCCCGGGGCTTAA
CCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTA
GCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTG
ACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GGTGGGCACTAGGTGTGGGCGACATTCCACGTCGTCC
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Table 1: Phylogenetic neighbours of the isolate RMS6 based on partial 16S rRNA gene sequence

Description	Ident.	Accession
Streptomyces pseudogriseolus strain NRRL B-3288 16S ribosomal RNA gene, partial sequence	99%	NR_043835.1
Streptomyces cellulosae strain NRRL B-2889 16S ribosomal RNA gene, partial sequence	99%	NR_043815.1
Streptomyces thermocarboxydus strain NBRC 16323 16S ribosomal RNA gene, partial sequence	99%	NR_112585.1
Streptomyces flavoviridis strain NBRC 12772 16S ribosomal RNA gene, partial sequence	99%	NR_041218.1
Streptomyces gancidicus strain NBRC 15412 16S ribosomal RNA gene, partial sequence	99%	NR_041179.1
Streptomyces lusitanus strain NBRC 13464 16S ribosomal RNA gene, partial sequence	99%	NR_041143.1

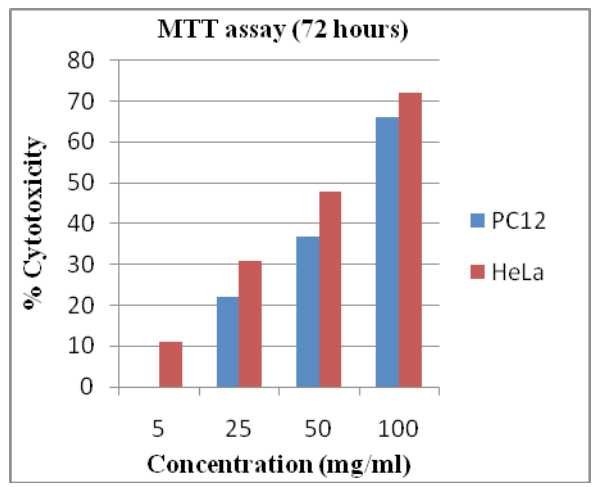
Cytotoxicity study

Crude extract prepared from the isolate RMS6 showed promising cytotoxic activity against PC12 and HeLa cells in a dose dependent manner. 100 µg/ml of the extract was found to have 66% cytotoxicity on PC12 cells and 72% cytotoxicity on HeLa cells (Table 2 and Fig. 2)

Table 2: Cytotoxicity of RMS6 on PC12 and HeLa

Concentrations of the extract	PC12 cells (% inhibition)	HeLa cells (% inhibition)
5 µg/ml	-	11%
25 µg/ml	22%	31%
50 µg/ml	37%	48%
100 µg/ml	66%	72%

Figure 1: Results for MTT assay showing % inhibition of growth of PC12 and HeLa cells



DISCUSSION

The present study indicates that the crude butanol extract prepared from the actinomycetes isolate Streptomyces sp. has a promising cytotoxic activity on human cervical cell line HeLa and rat neuronal cell line PC12 suggesting that the extract could have a broad spectrum activity.

Recently, mangrove ecosystem is becoming a hot spot for studies of bioactivities and the discovery of natural products.

Huang et al., (2008) and Hu et al., (2009) have reported the cytotoxic, antitumor activities from the actinomycetes isolated from mangroves sediments from China.

Promising cytotoxicity on HEP 2 (laryngeal carcinoma cells) and Hep G2 (Hepatocellular carcinoma) cell lines by MTT cell proliferation assay was also reported from a bioactive compound isolated from a unique Streptomyces species isolated sediments of Marakkanam coast of Bay of Bengal, India by Saurav and Kannabiran, (2012).

Ser et al., 2015 isolated Streptomyces pluripotens MUSC 137 strain from the mangrove soil of Tanjung Lumpur, Pahang, Malaysia which showed potent cytotoxic activity against breast cancer cell (MCF-7), colon cancer cells (HCT-116) and lung cancer cell (A549).

In the present study, the bioactive strain was isolated from the mangrove soil. So based on the results of the present study and earlier reports, it is suggested that more such screening studies should be undertaken in mangroves and coastal areas to explore the huge unexplored source of actinomycetes that can have a high potential to produce active secondary metabolites.

The use of phylogenetic and molecular evolutionary approaches has been of great importance to the classification methods. Some organisms that are in advertently placed in an ineligible group are now classified appropriately thanks to the advance-

ment in molecular techniques. Recently, the identification of the species and phylogenies are commonly derived from 16S rDNA and the use of polymerase chain reaction (PCR) for sequence analyses and many researchers have reported the use of gene sequences of 16S rRNA to identify actinomycetes as done in the present investigation (Patil et al., 2011; Isik et al., 2014; Sharma, 2014; Ser et al., 2015).

CONCLUSION

The present investigation highlights the cytotoxic activity of the actinomycetes isolated from the mangroves sediments on PC12 and HeLa cells. We can also conclude that molecular marker like 16S rRNA gene sequence can be effectively used for the identification of the unknown microbes up to genus level.

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