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ISOLATION, CHARACTERIZATION AND SCREENING FOR ANTICANCER PROPERTY OF SEDIMENT DERIVED ACTINOMYCETES FROM SOUTH EAST COAST OF INDIA

S. SUDHA SRI KESAVAN*

Assistant Professor, Department of Biotechnology, Sathyabama University, Jeppiaar Nagar, Rajiv Gandhi Salai, Chennai-600 119, India.

ABSTRACT

In the screening for actinomycetes with anticancer property, fifty two isolates were isolated using a starch casein agar medium from twenty marine sediment samples collected from the southeast coastal regions of India. Ten morphologically different isolates were selected and screened for cytotoxic active isolate by Brine shrimp lethality assay. One of the most potent isolate was selected for identification up to species level based on chemotaxonomic, cultural, physiological, biochemical analysis, and 16S rRNA gene sequencing. The ethyl acetate crude extracts of the identified isolate was analyzed by FT-IR and was subjected to MTT assay. The crude extract exhibited anticancer activity towards MCF-7 and HeLa cell lines with the IC_{50} of 19.95 and 25.1 µg/ml respectively and cytotoxic towards the VERO cell lines with the IC_{50} of >199.5µg/ml. The results of the present study revealed that the tentatively identified isolate *Streptomyces globisporous* strain SU7 could be pursued as a promising agent for the development of therapeutic anticancer drugs.

KEYWORDS: **Actinomycetes, cytotoxic, *Streptomyces globisporous,* MCF-7, HeLa.**

***Corresponding author**

S. SUDHA SRI KESAVAN
Assistant Professor, Department of Biotechnology, Sathyabama University, Jeppiaar Nagar, Rajiv Gandhi Salai, Chennai-600 119, India.

INTRODUCTION

Marine microorganisms are of extensive interest as a new and promising source of biologically active compounds [1]. They produce a mixture of metabolites, some of which can be used for drug development [2]. Among the microorganisms, actinomycetes gained special importance due to their capacity to produce bioactive secondary metabolites and enzymes. The discovery of actinomycetes from marine environments has been a productive area of research in the past decade. However, little is known about the diversity of actinomycetes from marine samples compared to the diverse range of actinomycetes isolated from terrestrial environments [3]. The isolated compounds from marine actinomycetes has a broad spectrum of biological activities such as antibiotic, antifungal, toxic, cytotoxic, neurotoxic, antimitotic, antiviral and antineoplastic activities[4] . Members of this group are producers in addition of clinically useful antitumor drugs such as anthracyclines (aclarubicin, daunomycin and doxorubicin), peptides (bleomycin and actinomycin D), aureolic acids (mithramycin), enediynes (neocarzinostatin), antimetabolites (pentostatin), carzinophilin, mitomycins and others [5,6]. The aim of the present study is to give an insight into the steps which are involved in screen and identify a potential actinomycete strain with anti-cancer activity.

MATERIALS AND METHODS

Collection of sample

Twenty marine sediment samples were collected from four different locations of south coastal regions of India (Ennore, Muttukadu, Verampattinum and Pulikat) into sterile polythene bags. From each sample site five sediment samples were collected with 1m distance in the intertidal region at 10cm depths from the soil surface. The soil samples were processed by drying at 50^{o}C in order to prevent bacterial and fungal contaminations [7].

Isolation of Actinomycetes

The collected marine sediment samples were immediately transferred to the laboratory condition. Approximately one gram of soil sample was aseptically transferred into 99ml of pre sterilized 50% seawater and serially diluted. 100 µl of diluted samples were transferred to molten starch casein agar medium (10g.1^{-1} soluble starch, 1 g.1^{-1} of casein and 18 g.1^{-1} of agar made up with 50% of sea water) and incubated at 27±2° C for 7- 8 days. After incubation, colonies appeared on the agar medium were re-streaked in the same agar medium.

Extraction of cell free crude extracts

A loopful of selected actinomycetes strain was inoculated into 150ml of ISP2 medium (4.0g.1^{-1} of glucose, 10.0 g.1^{-1} of malt extract and 4.0 g.1^{-1} of yeast extract, pH 7.2±2°C made up with 50% sea water) for 10 days under continuous shaking (100 rpm). After that, cell free broth was adjusted to pH 5.0 with 1N hydrochloric acid and equal volume (1:1) of ethyl acetate was added and mixed by vigorous shaking and kept without disturbance. The organic phase was collected and evaporated in incubator at 60-70°C and the residue was stored at-20°C for further use.

Brine shrimp lethality assay

Dried cysts of *Artemia salina* were incubated in natural seawater (1g.1-1) at 28-30°C under constant aeration for 48hrs. After hatching, active naupli free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Different concentrations of stock solutions were prepared by dilution with dimethyl sulphoxide (DMSO) so as to obtain 31.25, 62.5, 125, 250, 500 and 1000 µg.ml^{1} of ten isolated actinomycete cell free extracts. Ten artemia naupli were added into each concentration of extract in 96 microtitration well plate. Control was maintained with 0.2% of DMSO [8] instead of extract. After 24 hrs, dead shrimp was counted using microscope. Larvae did not

exhibit any internal or external movement during several seconds of observation was calculated as dead and the percentage of mortality was calculated and the data were transformed to the probit analysis for the determination of IC_{50} of the crude extract.

Identification of Actinomycetes

The isolates were identified based on morphological characters followed by the methods of Bergey's manual of determinative bacteriology and confirmed by 16S rRNA sequencing.The morphological, cultural, physiological and biochemical characterization of the isolate was carried out as described in International Steptomyces Project (ISP) [9]. The morphological characters of the selected isolate was examined by using light microscope as well as scanning electron microscope. The cultural characters of the isolate was studied by cultivating it on different media namely ISP1, ISP2, ISP4, ISP5 and ISP7 and incubated for 7- 10 days at 28 °C. colony morphology including color of the aerial mycelium, substrate mycelium, reverse side color, melanin pigment production and production of diffusible pigments were recorded.The physiological characters such as growth at different pH (5, 7,9,10 and 11), temperature (10C, 20C, 20C, 40C, and 50C) was also recorded.

Molecular sequencing

Genomic DNA was isolated from cells as described by[10]. The 16S rRNA gene of strain SU 5 was amplified by polymerase chain reaction, using two universal bacterial primers, 1492R (5′-GGTTACCTTGTTAC GACTT-3′) and Eubac27F (5′-AGAGTTTGATCCTGGCTC AG-3′)[11]. The amplified products were purified using TIANgel mini purification kit, ligated to MD18-T simple vector (TaKaRa), and transformed into competent cells of Escherichia coli DH5α.16S rRNA gene fragment was sequenced using forward primer M13F (−47) and reverse primer M13R (−48). The derived 16S rRNA gene sequence was compared to the GENBANK database (NCBI), to search for similar sequences using the basic local alignment search tool algorithm.

FT-IR Spectrum Analysis

The crude extract was analyzed for its chemical nature by the Fourier Transform Infrared spectrum of. Electromagnetic radiation ranging between 2500nm to 20,000nm is passed through a sample and is absorbed by the bands of the molecules in the sample causing them to stretching or bending. The wavelength of the radiation absorbed is characteristic of the bond absorbing it. IR spectrum was recorded on a Bruker FT-IR instrument equipped with AT-XT Golden gate accessories. The spectra were scanned in the 400 to 4000cm^{-1} range. The spectra were plotted as percentage transmittance versus wave number. The spectra obtained through those samples were compared and interpreted for the shifting of functional peaks.

Cytotoxicity assay

The human laryngeal cancer cell line MCF-7, HeLa and VERO cell line were obtained from Veterinary College, Chennai, India. Cells were grown as monolayer culture in MEM medium and incubated at 37°C in a 5% of CO_2 atmosphere. For the preliminary screening MCF-7 and HeLa and VERO cells (100µl) were seeded in 96 well plates at a concentration of $5X10^3$ cells/ml for 24 hrs. After the incubation the culture medium was replaced with 100ml serum free medium containing various concentrations (3.87, 7.75, 15.5, 31.25, 62.5, 125, 250, 500, 1000 and 2000µg/ml) of actinomycete extracts at 24 hrs and 48 hrs. After that, the medium was refreshed with 100µl of serum free medium (MEM) and 20µl of MTT (5mg/ml of (3, 4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide) was added. The micro-titer plates were incubated for three hours in dark. Triplicates were maintained for each treatment. The developed colour was measured with ELISA reader at 570 nm. The IC_{50} was determined by Linear regression analysis using Microsoft office excel work sheet. By plotting a graph of Log (concentration of compound) vs % cell inhibition, the concentration of compound required to inhibit 50 % cell growth (IC_{50}) was determined. A line drawn from 50 % value on the Y axis meets the curve and interpolate to

the X axis. The X axis value gives the Log (concentration of compound). The antilog of that value gives the IC_{50} value [12].

RESULTS AND DISCUSSION

Twenty marine sediment samples were collected from four different coastal regions of South India (Ennore, Muttukadu, Verampattinum and Pulikat). From each site 5 sediment samples were collected and pooled together in the laboratory to made one sample. Fifty two isolates were isolated using starch casein agar medium from the 5 costal sediment samples (Figure 1).

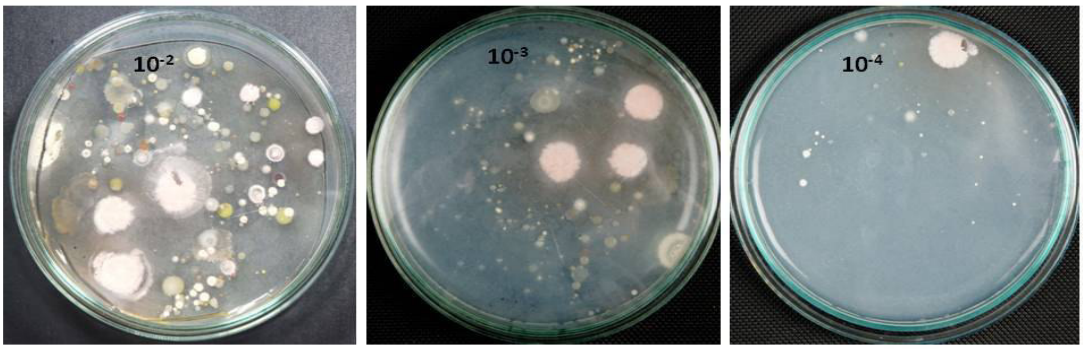

Figure 1
Isolated actinomycetes on Starch Casein Agar

Brine shrimp lethality assay was conducted on each of the ten selected isolates extracts at six different concentrations 31.25, 62.5, 125, 250, 500 and 1000µg/ml (Solis et al 1993). The extract of the actinomycete isolates PCL1, SU1, SU2, SU3, SU4, SU5 and SU13, shows LC_{50} in < 1000µg/ml. The isolates SU2, SS12 and SU3 showed LC_{50} in > 500µg/ml and the isolates SS1, SS4, and SU19 showed the LC_{50} in > 1000µg/ml [13, 14]. Among the fifty two isolates based on cytotoxic activity against brine shrimp larvae the isolates were selected for identification. A comparison of the characteristics of the present selected strains with the descriptions of the reference cultures revealed that the selected strains SU1 shares the properties of *Streptomyces globisporous* (Table 1). Figure 2 shows the spore morphology of the isolate SU1. The partial 16S rRNA sequence of strain SU1was aligned manually with available *Streptomyces* (16S rRNA) sequences retrieved from Gen bank using cluster X version. Phylogenic trees were inferred by using tree making algorithms, the neighbor joining and maxim likelihood algorithms from the phylip package. The isolates was closely related to the type strains of *Streptomyces globisporous*, The nearest homolog species for the isolate SU1, the homolog species are *S. globisporous* sub sp*. Globisporous* strain 13669A EU741221. The nucleotide sequence of the tentatively identified isolate SU1 was submitted as *Streptomyces globisporous* strain SU7 with the accession number KF551875.

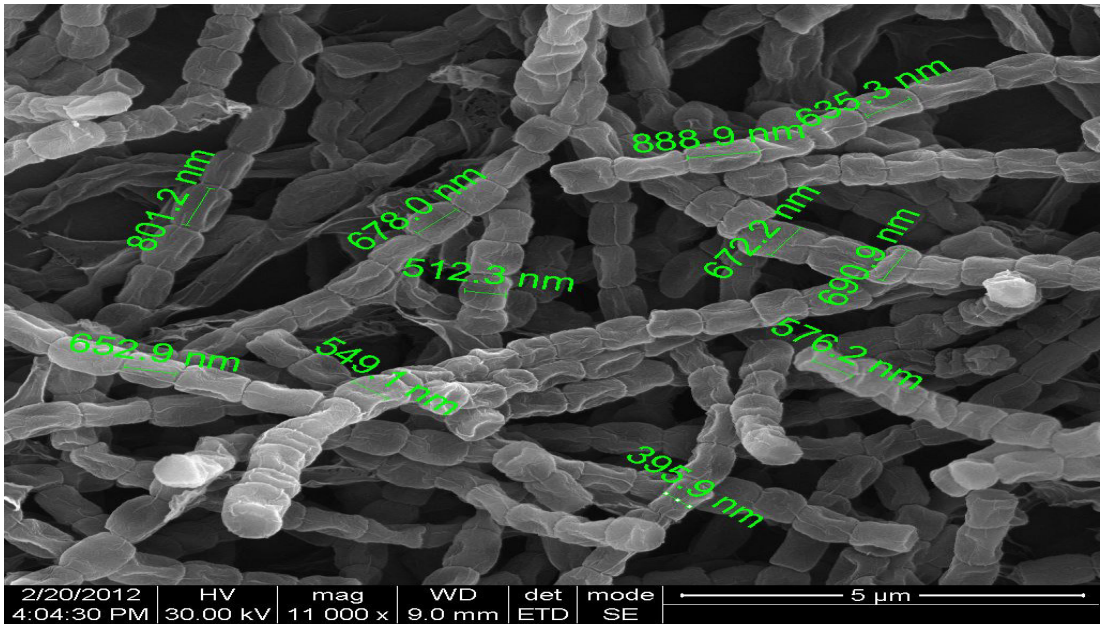

Figure 2
Scanning electron micrography of Streptomyces globisporous strain SU7

Table 1
Comparison between SU1 and Streptomyces globisporous C1027 (Waksman 1953)

S.No	Characters studied (as per Nonomura key)	SU1	*S.globisporous* C1027(Waksman 1953)[15]
1.	Colour of aerial mycelium	White	Ivory yellow to yellowish
2.	Melanoid pigment	-	-
3.	Reverse pigment	Yellowish brown	colorless-cream
4.	Soluble pigment	-	-
5.	Spore chain	R – rectus or straight	RA
6.	Spore surface	Sm	Sm
7.	Gelatin liquefaction	+	+
8.	Nitrate reduction	+	+
9.	Starch hydrolysis	+	+
10.	Milk coagulation	-	+
11.	H_2S production	+	+
12.	Cellulose degradation	+	-
	Carbon source assimilation		
13.	Arabinose	-	+
14.	Xylose	+	+
15.	Inositol	+	-
16.	Mannitol	+	+
17.	Fructose	+	+
18.	Rhamnose	+	+
19.	Sucrose	+	-
20.	D-glucose	+	+

R-Rectus or Straight; Sm-smooth, + positive, - negative, +/- slow growth. ND- No data

FT-IR spectrum of the crude extract showed the values at 3435cm^{-1}, 2959cm^{-}1, 2926cm^{-1}, 2857 and 2153cm^{-1} corresponding to hydroxyl groups; while the ester carbonyl group (C=O) bands seen at 1727cm^{-1} and 1653cm^{-1}. The bands observed at 1448, 1375, 1273, 1230, 1216, 1117 were assigned

to the C-N stretching vibrations of aromatic and aliphatic amines respectively; 1075 corresponds to ethers (C-O-C), 742 corresponds to aromatic (C-H) band (Figure 3).

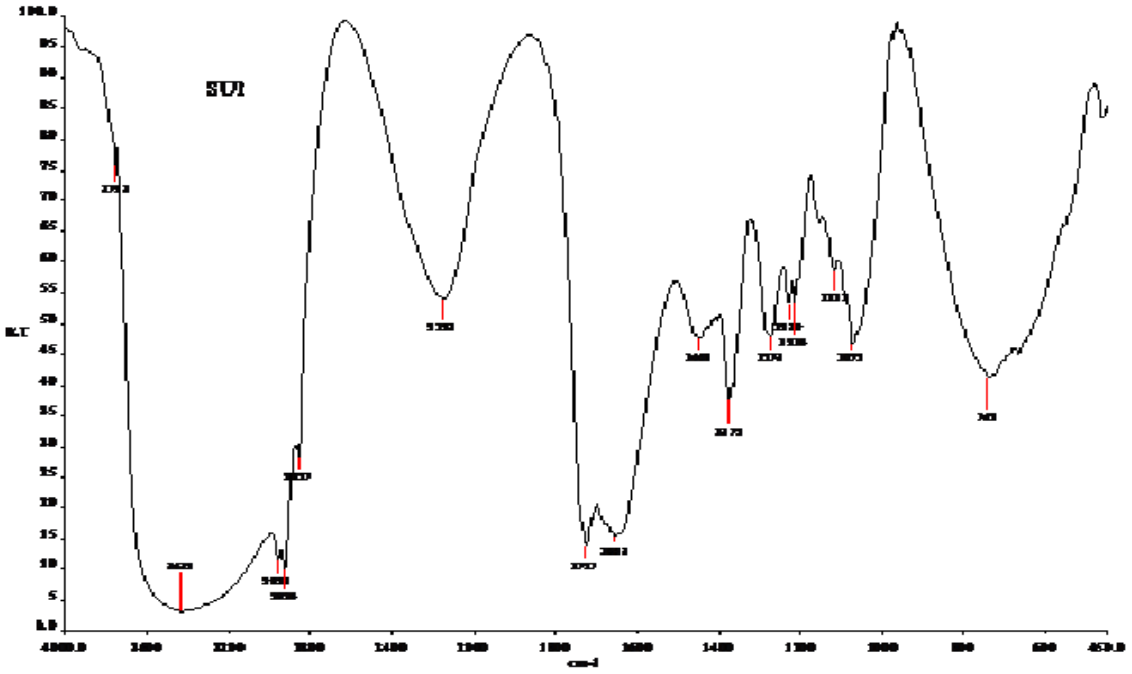

Figure 3
FT-IR spectrum of crude extract of Streptomyces globisporous strain SU7

The anticancer activity of the crude extract was screened against MCF-7 and HeLa cell lines by MTT assay. The crude extract was highly toxic toMCF-7 and HeLa cell lines with the minimum IC_{50} of 19.95µg /ml and 25.1 µg /ml and relatively high IC_{50} of 199.526µg /ml against VERO cell lines (Table 2 and Figure 4). In similar studies Balachandran and his team (2012)[16] reported the cytotoxic effect of actinomycete isolates ERIA-31 and ERIA-33 against human adeno carcinoma cancer cell line (A549) and the recorded IC_{50} for ERIA-31 was 57.04 g/ml and IC_{50} for ERIA-33 was 55.07g/ml.

Table 2
IC_{50} and R^2 for the crude extract of Streptomyces globisporous strain SU7 against two human cancer cell lines and VERO normal cell lines

Cell lines	IC_{50} (µg/ml) for crude extract of isolate SU 1	R^2
VERO	199.526	0.974
MCF-7	19.95	0.977
HeLa	25.1	0.986

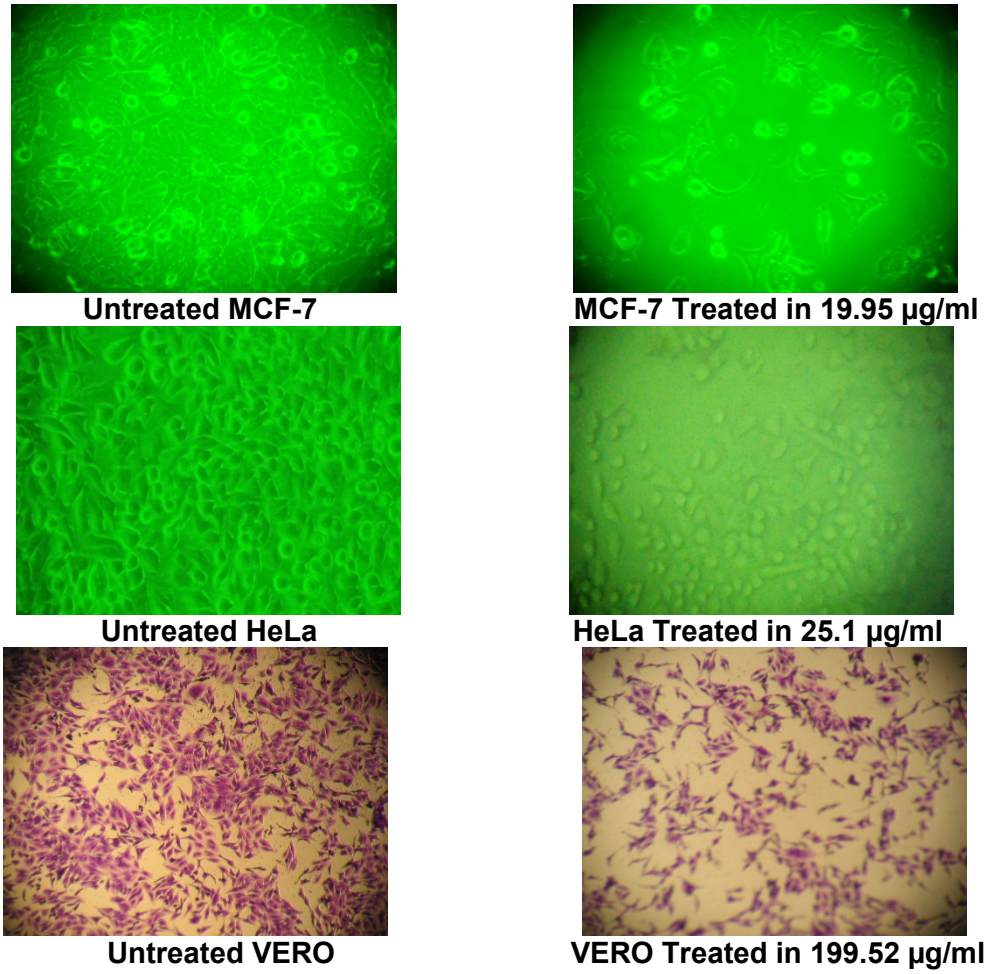

Figure 4
Shows the light microscope images of control and crude extract of Streptomyces globisporous strain SU7 treated VERO, HeLa, MCF-7 cell lines (40X)

CONCLUSION

This study results were evidence for that the South East costal habitats might be a rich source of actinomycetes with active principles. The isolate SU 1 was screened for its cytotoxic activity against cancer cell line and was identified as *Streptomyces globisporous* strain SU 7 by 16S rRNA sequencing. This study confirms that an ethyl acetate extract of *Streptomyces globisporous* strain SU 7 possesses anticancer activity against cancer cell lines, however, further purification of the crude extract is needed to know more about the efficacy against cancer cell lines as well as in vivo systems.

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