TAXONOMY, FERMENTATION, BIOLOGICAL ACTIVITY, ISOLATION AND CHARACTERIZATION OF COMPOUND FROM A NEW STRAIN *Streptomyces coelicolor* STRAIN SU6

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Abstract

An Actinomycetes isolate SU 4 active against clinical fungal pathogen was isolated from the coastal soil sample of Veerampattinam, Pondicherry. Based on morphological and biochemical characteristics it was identified as *Streptomyces coelicolor* also submitted to GENBANK with the accession number JQ828940 as *Streptomyces coelicolor strain SU 6*. The active compound isolated from Streptomyces coelicolor strain SU 6 by submerged fermentation using ISP-2 medium was characterized based on spectral data. The following compounds diisobutyl phthalate (16.82%) and 1,2-Benzenedicaarboxylic acid, Bis(2-ehtylehexyl) ester (65.26%) were found abundantly with retention time 15.645, 21.620 respectively. Crude extract of the active isolate exhibited LC50 in 62.5 µg against Hep-2 cell line, < 250µg in VERO cell line. This study clearly proves that the marine sediment derived actinomycetes with bioactive metabolites can be expected to provide high quality biological material for high throughout biochemical and anticancer screening programs.

Keywords: Actinobacteria, Biocombatability, Anti-tumor activity, antifungal activity.

I. INTRODUCTION

Cancer still represents one of the most serious human health problems despite the great progress in understanding its biology and pharmacology. The usual therapeutic methods for cancer treatment are surgery, radiotherapy, immunotherapy and chemotherapy [1]. These techniques are individually useful in particular situations and when combined, they offer a more efficient treatment for tumors. Actinomycetes are prolific producers of antibiotics and majority of the antibiotics in clinical use today are produced by them. Apart from antibiotics, actinomycetes also produce other bioactive secondary metabolites, anticancer (mitomycin and daunomycin)[2] and immunosuppressive agents (rapamycin and FK506)[3].

Although these organisms are continually studied extensively, it is clear that the rate of discovery of novel metabolites from terrestrial actinomycetes is decreasing and that new source of bioactive substances must be explored [4]. 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[5]. Antitumor antibiotics produced by actinomycetes are among the most important cancer chemotherapeutic agents including members of the anthracycline, bleomycin, actinomycin, mitomycin and aureolic acid families[6,7].

Their study is expected to become an important component in the production of new natural bioactive products. The current study was undertaken to investigate this biodiversity and to isolate and screen marine sediment derived actinomycetes with biological activity.

II. MATERIALS AND METHODS

Microorganism

The strain SU 4 was isolated from marine sediment samples were collected from Veerampattinam, Pondicherry. India, using starch casein agar supplemented with cycloheximide and nalidixic acid. The strain was deposited at GENBANK with the accession number JQ828940.

Primary Screening

The bioactivity of the isolate SU 4 was determined by cross streak method [8] using clinical fungal pathogens viz *Candida albicans* NCIM 3471, Candida glabrata. NCIM 3236, *Trichoderma viride* NCIM 1053 and *Aspergillus niger* NCIM 563.

Taxonomic Studies

The morphological, cultural, physiological, and biochemical characterization of the isolate was carried out as described in International *Streptomyces* Project (ISP)(9). The morphological characters of the isolate SU 4 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 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), temperatures (10, 20, 30, 40 and 50°C) was also recorded. The biochemical characterizations of the isolate was also studied by the procedures of[10] using medium (B) of the following composition: glucose, 5.0 g.; KNO_3, 1.0 g.; K_2HPO_4, 0.5 g.; $MgSO_4$, 0.2 g.; $FeS0_4$, 0.01 g.; agar 15.0 g.; distilled water, 1000 ml, adjusted to pH 6.8 with 0.1N NaOH. The filter sterilized sugars such as D-Glucose, sucrose, lactose, Fractose, D-Maltose, D-Galactose, D-Mannitol, and Starch were given at 1% level.

Molecular sequencing:

Genomic DNA was isolated from cells as described by[11]. The 16S rRNA gene of strain SU 4 was amplified by polymerase chain reaction, using two universal bacterial primers, 1492R (5'-GGTTACCTTGTTAC GACTT-3') and Eubac27F (5'-AGAGTTTGATCCTGGCTC AG-3');[12]. 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. The phylogenetic tree was constructed based on neighbor joining method.

Fermentation

Fermentation process was studied using shake flasks. The fermentation medium used was 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).

Preparation of Extracts

After the ten days fermentation, 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.

Cytotoxic activity (MTT assay)

The human laryngeal cancer cell line (Hep-2) and VERO cell line were obtained from Cancer institute, Chennai, India. Cells were grown as monolayer culture in MEM medium and incubated at 37°C in a 5% of CO2 atmosphere. Hep-2 and VERO cells (100 µ l) were seeded in 96 well plates at a concentration of 5 $X10^3$ cells/ml for 24 hrs. After the incubation the culture medium was replaced with 100ml serum free medium containing various concentrations (3.9, 7.8, 25,15.6,31.2, 62.5, 125, 250, 500, 1000 and 2000 µ g/ml) of actinomycete extracts at 24 hrs and 48 hrs. After that, the medium was refreshed with 100 µ l of serum free medium (MEM) and 20 µ l of MTT (5 mg/ml of (3, 4,5 - dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide) was added. The micro-titer plates were incubated for three hours in dark. The developed colour was measured with ELISA reader at 570 nm. Triplicates were maintained for each treatment. IC_{50} values were determined by calculating the % of viability:

$$\% \text{ of viability} = \frac{\text{Mean Test OD}}{\text{Mean OD of Control}} \times 100$$

Characterization

The isolated active compound was subjected to spectral analysis for characterization. GC-MS [SHIMADZU QP2010] instrument at GC column oven Temperature 70°C, Injector Temperature 200°C at spilt mode ratio 40 with a flow rate of 1.51 ml/min was used. The MS with Ion source temp 200°C, Interface temp: 240°C, Scan range : 40 – 1000 m/z, Event time 0.5 sec, Solvent cut time: 5mins, MS start time : 5 (min), MS end time : 35 (min), Ionization : EI (-70ev) was used.

III. RESULTS AND DISCUSSION

The isolate SU 4 exhibited extensively branched grey colored aerial mycelium and white colored substrate mycelium.

Fig. 1: The Isolate SU4

(Fig: 1) The aerial mycelium is straight segmented, with Retinaculum –apertum (RA) type spores. The spores are open loops, hooks, or extended spirals of wide diameter (Fig: 2). It utilizes starch, galactose, mannitol, Fractose and sucrose grow well at 20 – 40°C in pH 7-11.

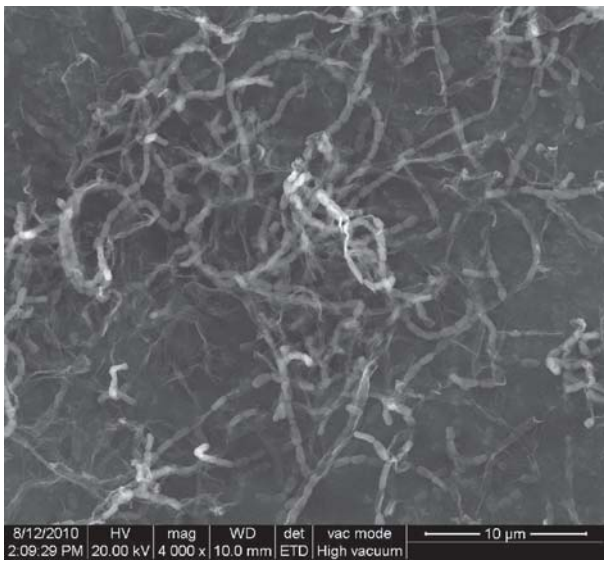

Fig. 2 Scanning Electron Micrograph Of *Streptomyces coelicolor* strain SU-6

The phylogenetic tree was constructed based on neighbor joining method shows that the isolate is most closely related to *Streptomyces* which is also supported by the high boot strap value. Based on the molecular taxonomy and phylogeny the isolate was identified as *Streptomyces coelicolor* and designated as *Streptomyces coelicolor strain SU6* (Fig: 3).

Biological Activities

The isolate SU 4 exhibited antifungal activity against all the tested clinical fungal pathogens with the presence of zone of growth inhibition (Fig: 4).

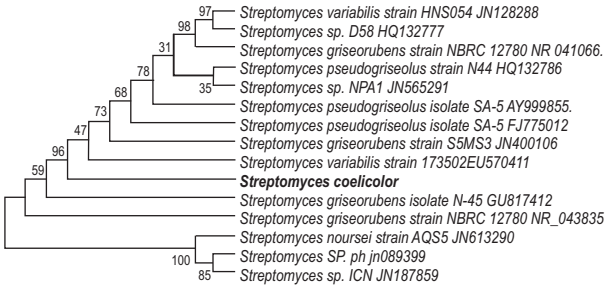

Fig. 3 Phylogenetic tree of *Streptomyces coelicolor* strain SU-6 based on 16S rRNA gene sequence

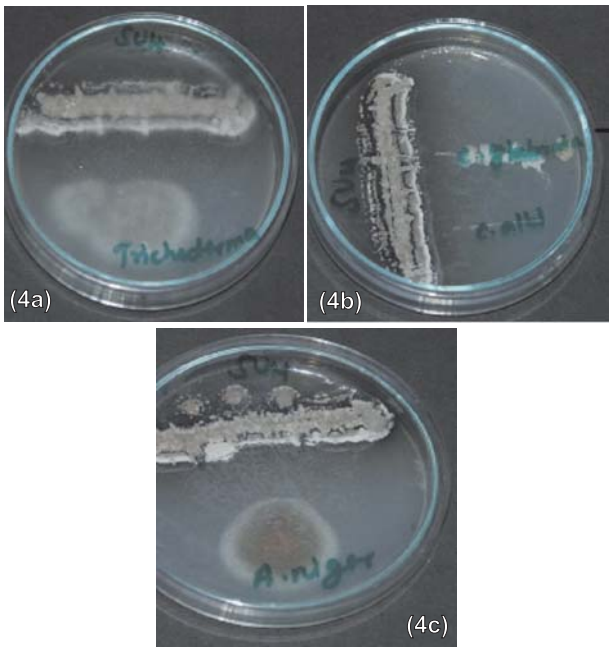

Fig. 4 Antifungal efficacy of *Streptomyces coelicolor* strain SU-6

4a: Zone of Inhibition of Isolate SU 4 Against *Trichoderma viride*

4b: Zone of Inhibition of Isolate SU 4 Against Candida albicans and Candida glabrata

4c: Zone of Inhibition of Isolate SU 4 Against Aspergillus niger

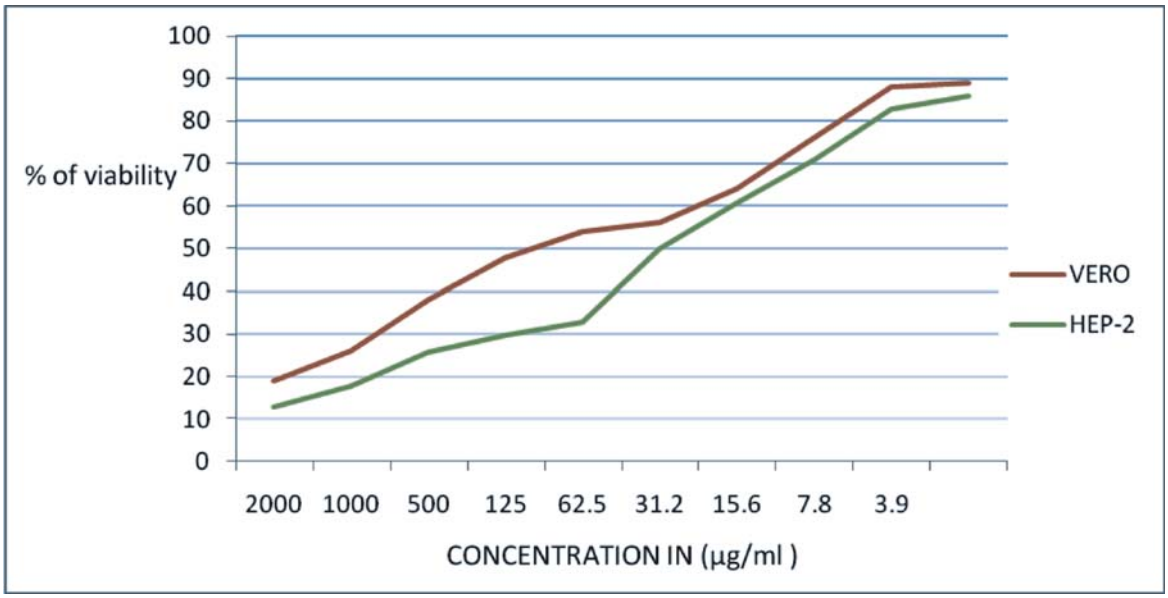

Fig. 5 MTT Cytotoxicity for the Crude Extract on Hep-2 Cell Line and VERO Cell Lines

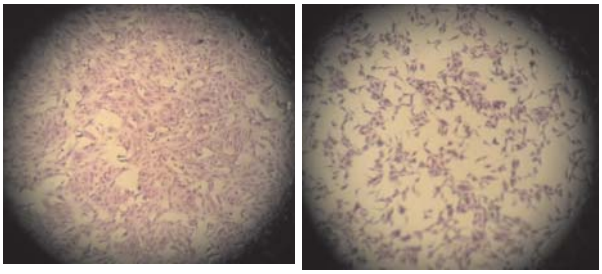

Fig. 5a Light Microscopic Images of Hep-2 Control And SU-4 Isolate Crude Extract Treated Hep-2 Cells After 48 hr Incubation

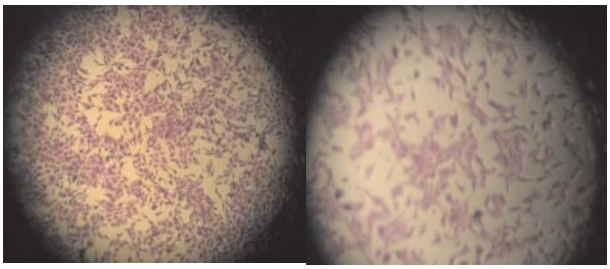

Fig. 5b Light Microscope Images of VERO Control And SU-4 Isolate Crude Extract Treated VERO Cells After 48 hr Incubation

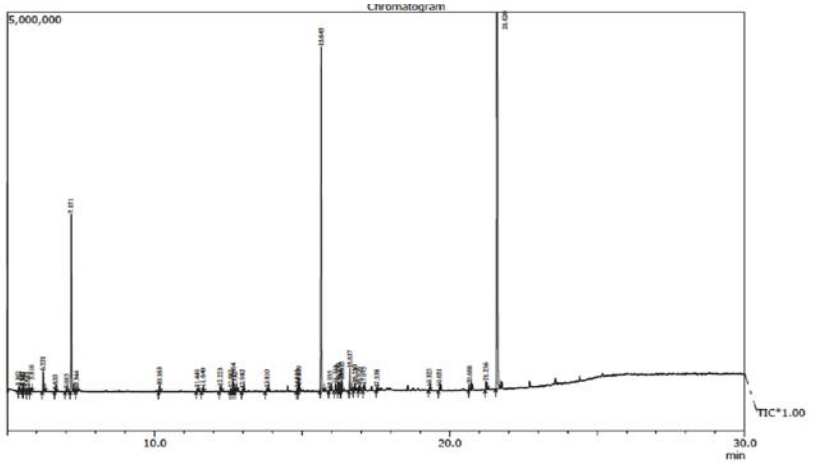

Fig. 6 GC MS Mass Spectrum

The crude extracts from culture filtrate exhibited anticancer activity. The extracts showed activity (IC50 of 62.5 μ g/ml against Hep-2 cell line and < 250 μ g/ml in VERO cell line) g/ml) (Fig:5). Previously we have reported a *Streptomyces* strain with antitumor activity against cancer cell line and less cytotoxicity activity against normal cell line[13].These values are very close to the criteria of cytotoxicity activity for the crude extracts, as established by the American National Cancer Institute (NCI) is in IC50 < 30 μ g /ml.

The GC MS analysis shows that the active principle might be 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (12.17%), Isooctyl phthalate (15.29%) with the retention time 7.169, 15.642 and 21.612 respectively (Fig: 6).

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