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Polyene antibiotics from *Streptomyces* sp.S177.

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ABSTRACT

An antagonistic Streptomyces strain was isolated from the shallow water sediment in Vizhinjam, Thiruvananthapuram coast. Based on the cultural and morphological characteristic studies, the strain was identified as new Streptomyces sp. Screening of secondary metabolites obtained from the selected strain led to the isolation of active fraction by using preparative TLC.The fraction was employed for the minimum inhibition (MIC) assay against seven clinical pathogens. Among these, Bacillus subtilis was found to be highly sensitive to the compound. Further the UV spectral analysis of active fraction revealed two absorption peaks, denoting the presence of active compounds of Polyene nature.

Key words: Streptomyces, Antagonistic property, Polyene.

INTRODUCTION

Marine microbes are particularly attractive because they have not been as extensively exploited as their terrestrial counterparts, and because of the high potency required for bioactive compounds to be effective in the marine environment. Among the antibiotic producing microbes, the class Actinobacteria represents a broad range of valuable and prominent sources of pharmaceutically active metabolites in which, the members of the genus *Streptomyces* alone contributes more than half of the naturally occurring metabolites discovered up to date [1]. *Streptomyces* are Gram-positive bacteria that grow in soil, marshes and coastal marine environments and forms filamentous mycelium like eukaryotic fungi.

Secondary metabolites are synthesized by pathways, which are often connected and influenced by primary metabolism. In fact the intermediate metabolites from primary metabolism, serve as precursors for biosynthesis of secondary metabolites and the composition of the culture medium, closely connected with the metabolic capacities of the producing organism, greatly influence the biosynthesis of the bioactive molecules Fourati *et al*. [2]. The goal of this study was to search for microbial strains with potent antimicrobial activity against emerging human pathogens.

MATERIALS AND METHODS

Sampling station

Sediment samples for the present study was collected off Vizhinjam (8°22'N latitude and 76°57'E), Southwest coast of India from a depth of 10 m using SCUBA. Sediment samples were collected in sterile polythene bags and transported to the laboratory within minimum possible time to avoid the external microbial contamination and excessive proliferation.

Isolation of Streptomyces

One gram of sediment was serially diluted and plated it on starch casein agar medium (Soluble starch:10g; Casein:1g; Agar:18g; Aged seawater:500ml; Distilled water:500ml; pH 7.2±0.2; autoclaved at 15 lbs for 15 min; Nalidixic acid:20µg/ml; Nystatin: 25µg/ml; Cycloheximide: 100µg/ml) [3] using spread plate method. The plates were incubated in an inverted position for 7-15 days at 28±2°C [4]. Based on the colour and morphological differences, powdery colonies were counted and restreaked thrice in a yeast extract malt agar (ISP2) (Glucose: 4g; Yeast extract: 4g; Malt extract: 10g; Agar: 18; Aged seawater: 500ml; Distilled water: 500ml; pH 7.2±0.2; autoclaved at 15lbs for 15 min) medium to get an axenic culture. The spore stocks were prepared from the culture grown on ISP2 medium and stored in refrigerator for further identification and antagonistic studies [5].

Morphological and cultural characterizationof the bioactive compound producing Strain

The morphology of aerial hyphae, substrate mycelium and spore chains of a 14 day culture sample of S177 were examined by light and scanning electron microscopy. Spore morphology was studied by examining gold-coated dehydrated specimens using the JEOL (JSM-5610LV), scanning electron microscope. The presence of soluble pigments and the melanoid pigment was investigated on International Streptomycetes Project medium 2 (ISP medium 2) and peptone yeast extract ion agar (ISP medium 6).Physiological tests (Modified from Pridham and Gottlieb, [6]: Utilization of various carbon and nitrogen sources was examined. Each source was added at a final concentration of 1% (w/v) and 0.1% (w/v) respectively.

Fermentation

The S177 spores were inoculated in 200 ml of a seed medium composed of starch 1% (w/v), glucose 0.5% (w/v), yeast extract 0.2% (w/v), tryptone 0.5% (w/v), K_2HPO_4 1% (w/v), $MgSO_4.7H_2O$ 0.05% (w/v). The pH was adjusted to 7.0 and incubated at 28ºC for 3 days. The inoculums were used to seed an 8 litre fermenter containing 6 litre of the production medium composed of starch 1% (w/v), glucose 0.5% (w/v), yeast extract 0.2% (w/v), peptone 0.5% (w/v), soyabean oil 0.15% (v/v) K_2HPO_4 0.1% (w/v), $MgSO_4.7H_2O$ 0.05% (w/v), $CaCO_3$ 0.3% (w/v).

Product recovery

The fermented broth was centrifuged at 8000 rpm for 10 min. The culture filtrate was extracted twice with ethyl acetate. The extracts from the culture filtrate and mycelium were mixed and concentrated in vaccuo to dryness. After dehydration with anhydrous Na_2SO_4, the solution was further concentrated in vaccuo.

Thin layer chromatography (TLC)

Thin layer chromatographic technique was used for the analysis of antibacterial compounds. The extracts were spotted on the baseline of the silica gel plates at 1.0 cm and then allowed to dry at room temperature. Then the plates were placed in pre-saturated TLC chamber which contains the mobile phase chloroform:methanol (3.4:0.6) for active fraction. Then the chromatogram was

developed and dried for few minutes. It was visualized under ultraviolet (UV) light and spots were marked. The $R_f$values for each band were measured. Further the antibacterial compounds were separated in preparative plates (Preparative-TLC) for further use with the same procedure as used in TLC. The resulting bioactive fraction was subjected to UV-visible scanning spectrophotometer.

Minimum inhibition concentration

MIC of the active fraction (strain S177) were evaluated and compared with the antibacterial antibiotic (Tetracycline). The MICs were between 20 and 200 µg/ml. Active fraction and Tetracycline (µg/ml) were prepared in DMSO solvent and 50 µg/ml sample were loaded onto sterile discs. The procedure, then followed was exactly the same as described above.

RESULTS AND DISCUSSION

Totally 108 *Streptomyces* spp. were isolated from the sediment samples. Glycerol asparagine medium showed better than the Actinomycetes agar for primary isolation (figure 1). *Streptomyces* spp. developed various pigments (cream to yellow and orange, pink, green, black, red or gray), glabrous, wrinkled, or granular colonies of acid-fast bacteria. Then the higher antagonistic activity of strain S177 was further selected on the basis of antibiotic activity against clinical pathogens. The selected strain has grey coloured aerial mycelium, yellow coloured substrate mycelium with no melanoid pigment production (table 1). The spore surface was hairy with recti flexible spore chain. It utilized all the selected carbon and nitrogen sources (table 3). Morphological differentiation in *Streptomyces* involved the formation of a lawn of aerial hyphae on the colony surface that stands up into the air and differentiates into chains of spores (figure2). Earlier, the screening of *Streptomyces* sp. from Veli Lake of Thiruvananthapuram coast was done by Suja Devan, [7]. In addition, the strain S177 showed some differences with other *Streptomyces* species respect to morphological, physiological and biochemical properties. These Phenetic results support the classification of the isolate S177 as a new strain. Actinomycetes especially *Streptomycetes* are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites and notably antibiotics [8], immunosuppressive agents [9], and enzymes [10]. Among the different types of drugs prevailing in the market, antibacterial drugs are very significant and have an important role in the control of bacterial diseases [11]. In this study, fermentation was carried out for 72 h, after which the yield of antibiotic had reached its maximum and the culture broth was subsequently harvested. Ethyl acetate and acetone was used for extraction of selected *Streptomyces* culture. When the crude extract was subjected to preparative TLC methods[12], 4 major and 3 minor compounds were detected. Each of the fractions was individually tested with seven clinical pathogens. Finally the compound was extracted using the solvents Chloroform:Methanol (3.4:0.6) which showed higher antagonistic activity against the pathogens. The history of new drug discovery processes shows that novel skeletons have, in the majority of cases, comes from natural sources [13]. The MIC activities of the bioactive fraction was tested by agar plate diffusion assay. The active fraction of the strain *Streptomyces* sp. S177 were found to be active against a wide variety of test organisms for which the MIC values ranged from 10 to 250 µg/ml (Table 4). Among the bacteria tested, *Bacillus subtilis* was found to be highly sensitive to the compound and it exhibited better activity than commercial antibiotic tetracycline. The UV spectral data for the ethyl acetate extract of the selected strains from fermented broth are shown in figure 3. The major absorption peaks at 239.6nm and 279.2nm peaks indicate the production of active compounds of polyene nature with a broad-spectrum of antibacterial activity. The spectral data were consistent with those obtained previously [14]. Studies reported during the past many years have led to the widely accepted

conclusion that polyene antibiotics function by binding with the sterol molecules in susceptible cells to cause permeability change and eventual death of the cell.

Table.1. Aerial and substrate mycelial colours and soluble pigment of *Streptomyces*S177

Media	Aerial Mycelium	Substrate mycelium	Soluble Pigment
Glycerol/calcium malate agar	Peach	Cream	Pink
Actinomyces agar	Light pink	Yellow	Brownish red
Glycerol asparagine agar	Yellow	Cream	Pink
Inorganic salt-starch agar	Peach	Cream	Brown
Kuster's agar	Yellow	Yellow	Red
Nutrient agar	Yellow	Yellow	Brown
Potato dextrose agar	Peach	Light pink	Brown

Table-2. Observation of Streptomyces sp. S177 under Scanning electron microscope

Spore chain morphology	Retinaculiaperti
Spore chain	10-40 spores per chain
Spore surface	Smooth.

Table-3. Influence of Carbons and amino acids on the growth of *Streptomyces* sp.S177

Influence of Carbon on the growth of *Streptomyces spp*											Influence of amino acids on the growth of *Streptomyces sp.* S177				
Dex	Fru	Glu	Raf	Ino	Lac	Mal	Man	Sta	Suc	Xyl	L-His	Ala	Asp	Gly	Van
0.039	0.086	0.067	0.38	0.095	0.064	0.200	0.081	0.087	0.171	0.073	0.007	0.028	0.018	0.010	0.019

Dex-Dextrose, Fru-Fructose, Glu-Glucose, Raf –Raffinose, Ino-Inositol, Lac-Lactose, Mal-Maltose, Man-Mannitol, Sta-Starch, Suc-Sucrose, Xyl- Xylose, L-His- L-Histidine, Ala- Alanine, Asp- Asparagine, Gly- Glycine, Van- Vanilin

Table 4. Antibacterial activity of active fractions

Clinical Pathogens	Minimum inhibition concentration of the active fractions (µg/ml)	
	Active fraction	Tetracycline
Escherichia coli	150	50
Klebsiella pneumonia	100	22
Salmonella typhi	50	25
Bacillus subtilis	40	50
Staphylococcus aureus	50	50
Proteus vulgaris	100	50
Pseudomonas aeruginosa	120	50

Figure 1.Total *Streptomycetes* population isolated from the sediment samples

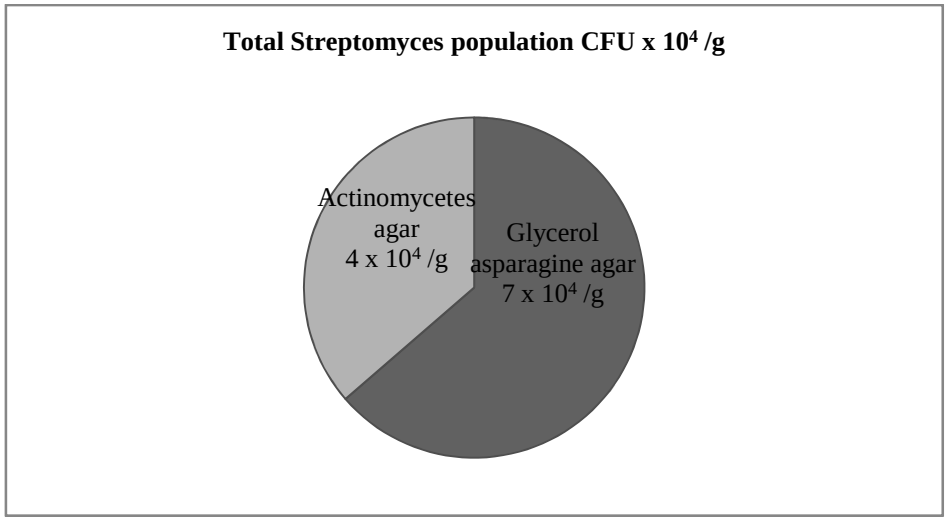


Figure 2.Light and Scanning electron micrographs of selected *Streptomyces sp*. S177

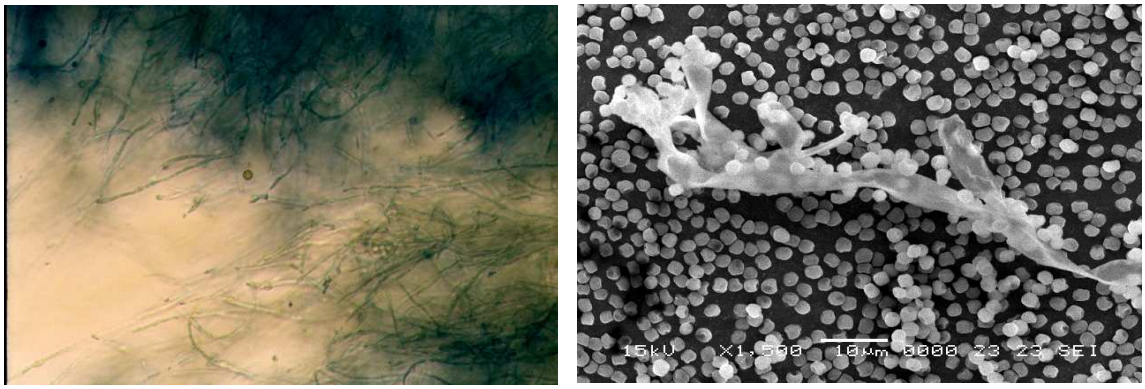
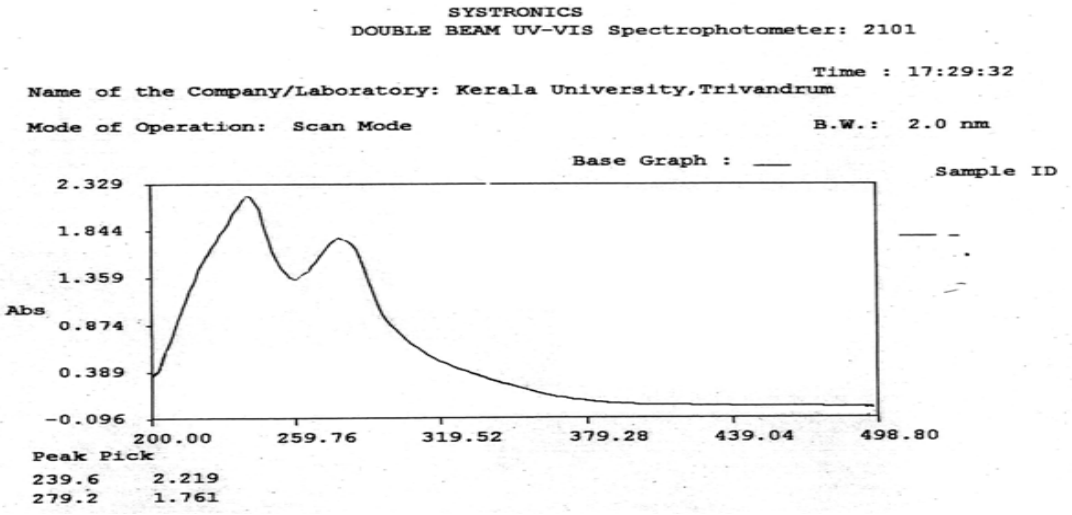


Figure3. UV visible analysis of active fractions



CONCLUSION

The present study demonstrated that, the antimicrobial activity of the selected *Streptomycetesspp*S77 was attributed towards the presence of polyene natured compounds. It appeared to be the dominant potential strain and its broad antagonistic properties against gram positive, gram negative pathogenic bacteria is also a boon to the pharmaceutical application and their validity towards the developing field of biotechnology.

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