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Research Article

GAS CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS OF BIOACTIVE CONSTITUENTS FROM THE MARINE *STREPTOMYCES*

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ABSTRACT

Objective: The objective was to study the antimicrobial activity of active eluent of thin layer chromatography (TLC) from *Streptomyces* isolated from marine sample.

Methods: To determine the antimicrobial activity by agar well diffusion method. Active compounds were extracted by solvent extraction method and purified using TLC. The presence of active compounds was confirmed by gas chromatography-mass spectrometry (GC-MS) analysis. Identification of active *Streptomyces* by 16S rRNA partial gene sequencing method.

Results: The active strain was identified as *Streptomyces cacaoi* strain SU2 (JF730119). The GC-MS analysis revealed that the presence of dodecane, eicosane, cetene, diethyl phthalate, phthalic acid isobutyl nonyl ester, thieno[3,2-e] benzofuran and bis (2-ethylhexyl) phthalate.

Conclusion: The methods adopted and active metabolites extracted and purified can be fruitfully employed for obtaining novel antibiotic compounds to treat human pathogenic bacterial and fungal infections.

Keywords: *Streptomyces*, GC-MS, Antimicrobial, Thin layer chromatography, Marine soil.

INTRODUCTION

Marine derived *Streptomyces* have become a focus in the research for novel secondary metabolites. However, streptomycetes from marine samples have rarely undergone screening for novel metabolites, and there is evidence that streptomycetes usually make up only a small proportion of the bacterial flora of marine habitats with absolute numbers of streptomycetes much lesser than in terrestrial habitats [1]. The marine streptomycetes are unique for antibiotic production compared to other sources [2], due to variations in physical, chemical and biological factors.

Actinomycetes population has been identified as one of the major group of soil population [3], which may vary with the soil type. Among Actinomycetes, *Streptomyces* group is the dominant. In particular, the genus *Streptomyces* is an important group of actinomycetes because of its ability to produce many types of secondary metabolites. *Streptomyces* are Gram-positive bacteria, which comprise a group of branching unicellular microorganisms. They produce branching mycelium which may be of two kinds (substrate mycelium and aerial mycelium) [4].

Members of the genus Actinomycetes especially *Streptomyces* sp. have been recognized as a prolific producer of useful bioactive metabolite with a broad spectrum of activities. *Streptomyces* have many vital bioactive compounds with high commercial values and are able to produce a wide variety of antibiotics and extra-cellular enzymes [5].

This study was carried out to screen and identify novel *Streptomyces* from marine soil sample for their active constituents acting on clinically resistant strains of infectious organisms.

METHODS

Isolation of *Streptomyces*

Marine soil samples were collected from a different region of Chennai coastal areas. The collected soil samples were air dried for 4 days to prevent bacterial and fungal contamination. Isolation of *Streptomyces* were carried out by spread plate method using 50% seawater in starch - casein agar medium [6]. The different colonies were identified and subcultured in ISP-2 agar medium [7].

Extraction of bioactive compounds

All the strains were processed for extraction of crude compounds by ethyl-acetate extraction method [8]. The strains were inoculated in ISP-2 broth containing 50% sea water and incubated for 7 days in a shaker incubator at 24°C. After incubation culture broth was centrifuged at 8000 rpm for 15 minutes and the supernatant was collected and mixed with an equal volume of ethyl acetate. The extracted crude compounds were dried at 40°C. The crude compounds were processed for secondary screening by agar well diffusion method [9] to confirm the presence of both antibacterial and antifungal bioactive metabolites.

Purification of active crude compounds

The purification of active crude compounds were separated by thin layer chromatography (TLC) method [10]. The readymade pre-coated TLC plates were used for separation of active crude compounds. Using the capillary tube, a row of spots of the active eluent was applied a line 1.5 cm above from the bottom of TLC plates. The spots were left to dry. The TLC plate was placed vertically in a trough containing the solvent (hexane-ethyl acetate) 1:9. When the solvent moved up to 80% of TLC plate, the plate was taken out and dried. The Rf values were calculated. Then the plates were viewed under ultraviolet-light. Each band were scrapped out separately and collected in different vials. Then the each band compounds were checked again for bioactive metabolites by agar well diffusion method.

Identification of bioactive metabolites

The active eluent compounds from TLC plates were identified using gas chromatography and mass spectrometry (GC-MS) method [6]. The mass spectrum was recorded using AGILENT GC-MS 5975C. MS under the temperature at 70°C.

Identification of active strain

The most active strain was process to identified based on cultural characteristics, microscopic spore morphology and 16S rRNA partial

gene sequencing. The 16S rRNA partial gene sequencing was performed for the active strain by Rana and Salam [11].

RESULTS AND DISSCUSSION

Isolation of *Streptomyces*

A total of 45 isolates were isolated from the marine soil samples based on their different colony morphology and color variations using starch casein agar medium. Five fast growing strains were further selected and tested for antimicrobial activity by secondary screening. A total of 68 Actinomycetes were isolated from near sea shore marine environment locations of Bigeum Island, South West coast of South Korea. The majority of these isolates were assigned to the genus *Streptomyces* of which one isolate is showing broad spectrum of antimicrobial was on the basis of their morphological, physiological and biochemical properties [6].

Extraction of bioactive compounds

The effective antimicrobial compounds were obtained from ethyl acetate extraction method. The 10 µl of bioactive crude compounds were showed most active against *Escherichia coli* and *Aspergillus niger*. Zone of inhibition of active strain showed (Table 1). Ethyl acetate crude extract were checked for their antibacterial activity by agar well diffusion method such as *Pseudomonas aeruginosa, Salmonella typhi, E. coli, Staphylococcus aureus, Bacillus cereus* where two pathogens were found to be highly susceptible *P. aeruginosa* (20 mm), *S. typhi* (19 mm) in the concentration of 20 mg/ml [12].

Purification of active crude compounds

The antimicrobial crude compounds were extracted from ethyl acetate extraction method and then purified and separated by TLC. Only one band was observed, and the active compounds were confirmed by agar well diffusion method for the same test organisms. The Rf value of the active band was measured as 0.40 in TLC and showed both antibacterial and antifungal activity (Fig. 1). In yet another report, *Streptomyces* isolates obtained from marine sponges produced antimicrobial compounds that showed Rf values ranging from 0.40 to 0.78 in TLC analysis, which confirmed the production of polyene substances [13].

Identification of bioactive metabolites

The active band from TLC was subjected to GC-MS analysis. The identification of the compounds were based on the peak area, molecular weight and molecular formula. This area is directly proportional to the quantity of the compound present in the active band. GC-MS analysis showed the presence of 16 compounds showed in Table 2 (Fig. 2). The ethyl acetate extract of *Streptomyces cavouresis* KUV39 was subjected to GC-MS analysis and the compound present in the extract showed the presence of 19 compounds [5].

Identification of active strain

The active strain was identified by cultural characteristics; microscopic spore morphology and 16S rRNA partial gene sequencing. The aerial color of the strain was white in color and reverse side pigment yellow in color. The scanning electron microscopic morphology of spores were appeared as spiral (Fig. 3). The morphological, micro-morphological, physiological and biochemical characteristics were obtained for the

Table 1. Secondary screening: Zone of inhibition in mm

	Zone of inhibition in mm
Pathogenic bacteria	
Staphlococcus aureus	18
Bacillus subtilis	16
Escherichia coli	20
Klebsiella pneumonia	14
Psedomonas aeruginosa	12
Pathogenic fungus	
Aspergillus niger	24
Aspergillus flavus	18
Candida albicans	14
Candida glabrata	10
Alternaria alternata	14
Trichoderma viridae	16

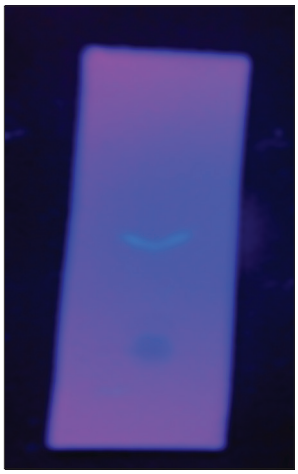

Fig. 1: Thin layer chromatography

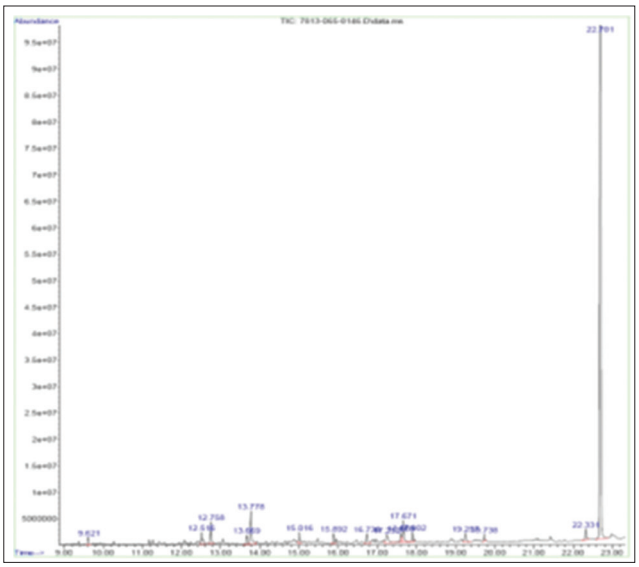

Fig. 2: Gas chromatography-mass spectrometry analysis of active strain

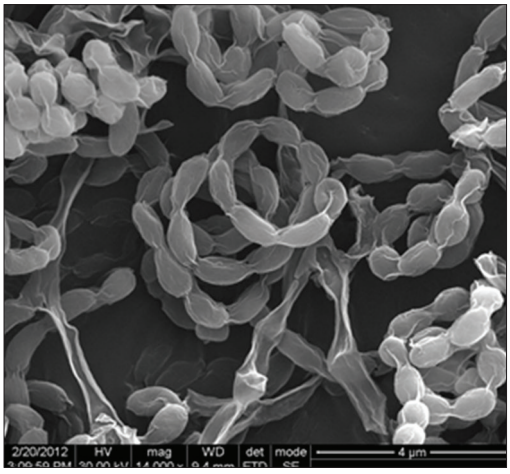

Fig. 3: Scanning electron micrograph of *Streptomyces cacaoi*

Table 2: Antimicrobial compounds identified in the ethyl acetate extract by GC-MS

Peak no	R.Time	Name of the compound	Molecular formula	Molecular weight	Area %	Activity
1	9.621	Dodecane	$C_{12}H_{26}$	170	0.66	Antioxidants, antimicrobial
2	12.516	Eicosane	$C_{20}H_{42}$	283	1.38	Antibacterial, antifungal
3	12.758	Phenol, 2,5-bis (1,1-dimethyl ethyl)	$C_{14}H_{22}O$	206	2.24	Antimicrobial
4	13.669	Cetene	$C_{16}H_{32}$	29	0.82	Antioxidants
5	13.778	Diethylphthalate	$C_{12}H_{14}O_4$	222	3.92	Antimicrobial
6	15.010	2-methyloctacosane	$C_{29}H_{60}$	409	0.81	Antifungal
7	15.896	1-Octadecane	$C_{18}H_{36}$	252	0.86	Antifungal
8	16.738	Phthalic acid, isobutyl nonyl ester	$C_{21}H_{32}O_4$	348	1.11	Antimicrobial, antioxidants
9	17.247	Heneicosane	$C_{21}H_{44}$	297	1.86	Antibacterial
10	17.610	n-Hexa decanoic acid	$C_{16}H_{32}O_2$	256	1.02	Cosmetics, antioxidants
11	17.671	Di-butyl phthalate	$C_{16}H_{22}O_4$	278	2.64	Antifungal
12	17.900	1-nonadecene	$C_{19}H_{38}$	267	0.85	Antioxidants, antimicrobial
13	19.251	Thieno[3,2-e] benzofuran	$C_{10}H_6OS$	174	1.20	Antibacterial
14	19.745	1-Decosene	$C_{10}H_{20}$	140	0.46	Antifungal
15	22.331	Diiso octyl phthalate	$C_{24}H_{38}O_4$	391	1.11	Anticancer, antibacterial
16	22.701	Bis (2-ethyl hexyl) phthalate	$C_{24}H_{38}O_4$	391.56	79.06	Antimicrobial

GC-MS: Gas chromatography-mass spectrometry

active strains s1d, s5a, s5e, s5k, s6b, s7c and s8e were tested [14]. The 16S rRNA sequence analysis of the active isolates yielded 1449 base pairs, and NCBI BLAST search analysis showed that the sequence was 98% similar to the sequence of *Streptomyces cacaoi* strain SU2. The 16S rRNA sequence was submitted in the GenBank under the accession number (JF730119).

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