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Antifungal Metabolites from Sponge Associated Marine *Streptomyces* sp. Strain (ERIMA-01)

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***Streptomyces* sp. strain ERIMA-01 was isolated from marine sponge. The strain was grown in ISP 2 medium to study the morphology and biochemical characteristics. The strain was subjected to 16S rRNA and identified as *Steptomyces* sp. The nucleotide sequence of the 16S rRNA gene of *Streptomyces* sp. strain ERIMA-01 exhibited close similarity with other *Streptomyces*. The accession number was FJ865352. Antifungal metabolite production of ERIMA-01 was evaluated using five different fermentation media. Most active broth (MNGA) was extracted with ethyl acetate. The ethyl acetate extract inhibited the growth of all the tested fungi (*Trichophyton mentagrophytes* at 62.5µg/ml, *Trichophyton rubrum* at 31.25 µg/ml, *Trichophyton simii* at 125 µg/ml, *Scopulariopsis* sp at 125 µg/ml, *Aspergillus flavus* at 250 µg/ml, *Aspergillus niger* at 62.5 µg/ml, *Botrytis cinerea* at 125 µg/ml, *Candida albicans* at 15.2 µg/ml, *Candida krusae* at 31.25 µg/ml, *Candida tropicalis* at 125 µg/ml and *Candida parapsilosis* at 125 µg/ml. Most active ethyl acetate extract was subjected to GC-MS analysis which showed 38 components. The major compound was 1- tetradecanol. The present paper reports the isolated *Streptomyces* sp. Showed good antifungal activity.**

***Keywords*:** *Streptomyces* sp, ethyl acetate, methanol, ISP, 16S rRNA, GC-MS, Antifungal, ERIMA-01(Entomology Research Institute Marine Actinomycetes).

Actinomycetes are one of the most attractive sources of new bioactive metabolites. Rare actinomycetes have been shown to be an important source of novel and useful antibiotics (Lazzarini *et al*., 2001). Many actinomycetes isolates from deep-ocean source contain NRPS and PKS pathways, the hallmarks of secondary metabolite production. Despite their abundance, however, marine sediments and marine invertebrates are relatively untapped sources for new secondary metabolites (Baltz, 2007). Marine actinomycetes have been found in symbiosis with different marine invertebrates, especially sponges (Piel, 2004; Kim and Fuerst, 2006).

Marine sponges are sessile invertebrates that have developed effective strategies to protect themselves against viruses, bacteria, and eukaryotic predators. One of these defense mechanisms is the production of secondary metabolites (Proksch, 1994).

Sponges are known as prolific sources of bioactive compounds that could be used to treat various diseases (Faulkner *et al*., 1999). The primary producers of bioactive compounds are symbiotic microorganisms hosted by marine sponges (Faulkner *et al*., 2000; Kobayashi, 2000). Sponge - microbe associations involve a diverse range of heterotrophic bacteria, cyanobacteria,

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facultative anaerobes, unicellular algae, and archaea (Webster and Hill, 2001; Hentschel *et al*., 2002, 2006; Thacker, 2005).

Streptomyces is the largest antibiotic-producing genus in the microbial world discovered so far. Majority of the antibiotics so far reported are obtained from *Streptomyces* sp. (Ravel *et al*., 2000). There is an urgent need for the development of novel antifungal agents with superior therapeutic effects and higher safety levels as fungal infections are quite prevalent, especially in immunocompromised patients. Among natural sources, several antifungal compounds are reported from the genus *Streptomyces* (Castillo *et al*., 2002; Fukuda *et al*., 2005; Fushimi *et al*., 1989; Qureshi *et al*., 2001; Ueki *et al*., 1996).

The present work deals with the isolation of a *Streptomyces* sp. strain ERIMA-01 from sponge collected from Tsunami affected area in south east coast of India and its antifungal activity. The identification of the strain, based on the cultural, morphological, physiological and biochemical characteristics, as well as 16s rRNA is reported. Production of antifungal metabolites was optimized using different media.

MATERIALS AND METHODS

Isolation of actinomycetes from marine sponge

Streptomyces sp. strain ERIMA-01 was isolated from the marine sponge (*Dendrilla* sp.) from south east coast of Tamil Nadu, India. Isolation and enumeration of actinomycetes were performed by the sample dilution plate technique (Ellaiah *et al*., 1996) using starch casein agar medium (g/l: starch 10, casein 0.3, KNO_3 2, $NaCl_2$, K_2HPO_4 2, $MgSO_4$-$7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4$-$7H_2O$ 0.01 and agar 18). Plates containing ERIMA-01 were stored at 4^0C. For long storage, the strain was grown in International S*treptomyces* Project-2 (ISP-2) broth for 7 days. Glycerol was added to make the final concentration 15% and stored at -20 °C (Maniatis *et al*., 1989).

Physiological, biochemical and cultural Characteristics

Biochemical, morphological, and physiological characteristics of the potential antimicrobial producer (ERIMA-01) was determined by adopting standard methods. Media used were those recommended by Shirling and Gottlieb (1966) in the International Streptomyces Project (ISP) and by Waksman (1961). Mycelium was observed after incubation at 28° C for 2 weeks. Colors were determined according to Prauser (1964). The pigmentation of aerial mycelium and structure of sporophores (highly characteristic and useful in the classification of Streptomycetes) were observed by cultivating the strains in ISP-4. The utilization of carbon and nitrogen sources by the strain was carried out according to the method of Gottlieb (1961).

Test organisms

The following pathogenic bacteria were used for experiments: *Bacillus subtilis* MTCC 44, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiela pneumonia* ATCC 15380, *Proteus vulgaris*, *Erwinia amylowora*, *Salmonella paratyphi –B* and *Xanthomonas campestris.* The bacterial cultures were obtained from the Institute of Microbial Technology, 39- (Sector 39-A), Chandigarh- 160036, India. The following fungi were used for experiments: *Trichophyton mentagrophytes* (66), *T. rubrum* (57), *T. simii* (110), *Scopulariopsis* sp. (101), *Aspergillus niger* MTCC 1344, *Aspergillus flavus*, *Botyritis cinerea* and yeast *Candida albicans* MTCC 227, *Candida krusie*, *Candida tropicalis*, *Candida parapsilosis.* The fungal and yeast cultures were obtained from Department of Microbiology, 92 Christian Medical College, Vellore, Tamil Nadu, India.

Preparation of fungal spore

The filamentous fungi were grown on Sabouraud Dextrose Agar (SDA) slants at 28°C for 10 days and the spores were collected using sterile doubled distilled water and homogenized. Yeast was grown on Sabouraud Dextrose Broth (SDB) at 28°C for 48 h.

Preliminary screening

The *Streptomyces* sp. strain ERIMA-01 was inoculated on Modified Nutrient Glucose Agar (MNGA) plates by single streak in the center. The plates were incubated at 28°C for 4 days. The test pathogenic bacteria (inoculum size was 10^8 CFU/ml)were inoculated perpendicular to the antagonist on the agar medium and incubated at 37°C for 24 h. Filamentous fungi (inoculums size was 1×10^5 CFU/ml) were spotted and incubated at 28°C for 96 h. The microbial inhibitions were observed by

determining the zone of inhibition from the antagonist.

Secondary screening

Antimicrobial activity of ERIMA-01 was determined by standard cup plate method using Gram positive and Gram negative bacteria and fungi. Assay plates were prepared by inoculating 20 ml of Mueller–Hinton agar medium with test organism. Agar-cups (6mm diameter) were filled with 50 µl of mycelia-free culture filtrate in triplicate and the plates were incubated at 37°C for 24 h. The diameter of zone of inhibition was measured.

Antibiotic production in different media and screening

Antibiotic production was studied in a shake flask with designated media. The compositions were as follows (per litre of distilled water): Medium1, Sabouraud Dextrose Agar (SDA-Himedia): Dextorose 20g, Peptone 10g, Agar 17g; Medium 2 (Yeast extract Dextrose Agar- YEDA): 10 g of Dextrose, 5 g of yeast extract, 18 g of agar, pH 5.8 to 6.2.; Medium 3 (Antibiotic production media for Actinomycetes-SS):Starch 25g, Glucose 10g, Yeast 2g, $CaCO_3$, Trace salts 1µl; Medium 4 (International Streptomyces Project- ISP-2): 4 g of glucose, 4 g of yeast extract and 10 g of malt extract (Shirling and Gottlieb 1966). Medium 5 (Modified Nutrient Glucose Agar- MNGA) : 10 g of glucose, 5 g of peptone, 3 g of beef extract, 3 g of dry yeast, 3 g of NaCl, 3 g of $CaCO_3$ (Omura *et al*., 1989). The pH of each medium was adjusted to 7.0 prior to autoclaving.

Seed culture was prepared in a 500 mL Erlenmeyer flask containing 100 ml X-medium by inoculating a loop full of the slant culture and incubating at 28°C on shaker (200 rpm) for 48 h. Antibiotic production was observed in all the media by inoculating a 500ml flask (100 ml medium) with seed culture and growing under the same conditions for 168 h. Fermented broth of the cultures was centrifuged and the supernatant was tested against pathogenic fungi. The antifungal activity in the culture broth was monitored for 7 days by the conventional well diffusion assay (Egorov, 1995). The fungal spore suspension (inoculum size was 1×10^5 CFU/ml) was seeded over the Mueller Hinton Agar (MHA- Himedia). Each well of 10 mm in diameter was filled with 50 µl of the supernatant. The diameters of zones of inhibition were measured after 72 h and recorded in mm.

Extraction of antimicrobial metabolites

The spore suspensions of the culture were inoculated on MNGA Broth media and incubated at 28°C at 200 rpm under dark for eight days. Fermented broth of the cultures was centrifuged and the supernatant was collected. The pH of the supernatant was reduced using HCl up to 2 pH and then extracted thrice with ethyl acetate in the ratio of 1:1 (v/v). This was kept at 4°C for 24 h. Two layers appeared. The organic phase was separated using separating funnel**.** Then the aliquot was decanted and concentrated using vacuum rotary evaporator. The crude extract was obtained and stored at 4°C. The same method was used for separating methanol extract. The extract was dissolved in Dimethyl Sulfoxide (DMSO). This was screened for antifungal activity.

Minimum inhibitory concentrations (MIC)

The crude extract (10 mg) was dissolved in 1ml of Dimethyl sulfoxide (DMSO): water (1:9) and used for antifungal study using standard broth microdilution method [Duraipandiyan *et al*., 2009] and the MIC was calculated. Mueller Hinton broth (Himedia, Mumbai) was prepared and sterilized by autoclaving at 121°C, 15 lbs for 15 minutes. The required concentration of the extract (0.5mg/ml, 0.25mg/ml, 0.125mg/ml, 0.0625mg/ml, 0.03125 mg/ml, 0.01562 mg/ml and 0.00781 mg/ml) was added to the 96 well micro titer plate containing 0.1ml broth. The 10¼l of fungal spore suspension was introduced into the respective wells and the final inoculum size was 1×10^5 CFU/ml. The plates were incubated at 27°C for 3days. Positive control and solvent control (DMSO) were also included. MIC was determined as the lowest concentration of the compound which inhibited complete growth.

Gas chromatography-mass spectrometry (GC-MS) analysis

The active ethyl acetate extract was quantified using gas chromatograph (GCMS-Shimadzu) equipped with a CPB-capillary column (mm inner diameter X 50 m length) mass spectrometer (ion source 200ºC, RI 70 eV) programmed at 40-280ºC with a rate of 4ºC/min. Injector temperature was 280ºC; carrier gas was He (20 psi). Sample volumes of 1 ¼l were injected with a split ratio of 25:1 using a hot-needle. GC-MS analysis carried out at Sargam Laboratory Service, Private Ltd, Chennai-600 089, India.

Molecular identification of active ERIMA-01 using 16s rRNA

The strain ERIMA-01 was grown for 5 days at 28°C with agitation in 500 ml flasks containing 100 ml of ISP2 medium. Biomass were harvested by centrifugation at 8,000 *g* for 10 min and washed twice with double-distilled water. About 200 mg of mycelia was used for DNA extraction as follows: the sample was dispersed in 800 µl of the aqueous lysis solution (100 mM Tris–HCl, pH 7; 20 mM EDTA; 250 mM NaCl; 2% SDS; 1 mg ml^{-L} lysozyme). About 5 µl of a 50 mg ml^{-L} RNase solution was added and the suspension was incubated at 37°C for 60 min. About 10 µl of a proteinase K solution (20 mg ml^{-L}) was added and the lysis solution was reincubated at 65°C for 30 min. The lysate was extracted with an equal volume of phenol and centrifuged at 7,000 *g* for 10 min. The aqueous layer was re-extracted with phenol (50-50%, v/v) and then by chloroform (50–50%, v/v). DNA was recovered from the aqueous phase by the addition of NaCl (150 mM final concentration) and two volumes of 95% (v/v) cold ethanol prior to centrifugation. The precipitated DNA was cleaned with 50 µl of 70% (v/v) ethanol, centrifuged at 7,000 *g* for 10 min, resuspended in 50 µl of TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8) and stored at -20°C. The purity of DNA solutions was checked spectrophotometrically at 260 and 280 nm, and the quantities of DNA were measured at 260 nm.

The 16S ribosomal RNA was amplified using the PCR method with *Taq* DNA polymerase and primers 27f (5^{1} AGT TTG ATC CTG GCT CAG 3^{1}) and 1492b (5^{1}ACG GCT ACC TTG TTA CGA CTT 3^{1}). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. PCR amplification was detected by agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining.

The PCR product obtained was sequenced by an automated sequencer (Genetic Analyser 3130, Applied Biosystem, USA). The same primers as above were used for this purpose. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks using the NCBI BLAST available at http:// www.ncbi-nlm-nih.gov/. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987).The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007).

RESULTS AND DISCUSSION

Streptomyces sp. strain ERIMA-01 was isolated from the marine sponge samples. The isolated actinomycete was tested for antibacterial and antifungal activities against both Gram positive and Gram negative bacteria and fungi using streak method (Fig 1, Table 1). Preliminary screening revealed that ERIMA-01 showed activity against fungi but not against bacteria.

Table 1. Antimicrobial activity of ERI MA-01 against bacteria and fungi using solid media (Streak method) of MNGA

Tested microbes	Inhibition
Bacteria	
Staphylococcus aureus	-
Bacillus subtilis	-
Enterococcus faecalis	-
Escherichia coli	-
Pseudomonas aeruginosa	-
Klebsiella pneumoniae	-
Salmonella paratyphi –B	-
Erwinia amylowora	-
Proteus vulgaris	+
Xanthomonas campestris	-
Fungi	
Trichophyton mentagrophytes (66)	+
Trichophyton rubrum (57)	+
Trichophyton simii (110)	+
Scopulariopsis sp (101)	+
Aspergillus flavus	+
Aspergillus niger	+
Botrytis cinerea	+
Candida albicans	+
Candida krusie	+
Candida tropicalis	+
Candida parapsilosis	+

-; no activity, +; Activity

Biochemical and culture characteristics

Gram staining indicated that ERIMA-01 was Gram-positive filamentous bacterium. The morphological studies proved that the colonies were white, opaque, rough, leathery and hard to remove due to branching filaments that had grown into the media. Biochemical and morphological characterization indicated that the antibiotic-producing strain was actinomycete. It exhibited high sensitivity towards ampicillin, streptomycin, ciprofloxacin, cephaloridine and gentamycin. The strain grew on a range of agar media showing morphology typical to *Streptomyces* (Anderson and Wellington, 2001). The colour of the mycelium was white. Aerial mycelium with white and gray colour series was the most abundant. It did not produce diffusible pigments on several agar media. The strain exhibited salt tolerance (up to 0.8%); it may be placed in the intermediate salt tolerance group. The strain also showed various biochemical activities such as H_2S production. It had the capability to produce different enzymes such as amylase, protease and catalase. The utilization of carbohydrates and nitrogen sources, the growth characteristics on different temperatures and pH and other characteristics are summarized in Tables 2 and 3. The cultural characteristics (Pigment production), morphological characteristics and antimicrobial activities of different *Streptomyces* isolates have been reported by several investigators (Oskay *et al*., 2004; Sultan *et al*., 2002).

Table 2. Morphological and biochemical tests for identification of ERI MA-01 on ISP2

Characters	ERIMA-01
Gram reaction	Positive
Mycelium	Aerial mycelium
Color of the mycelium	White
Production of diffusible pigment	-
Range of temperature for growth	27°C-45°C
Optimum temperature for growth	30°C
Range of Ph for growth	5.5- 7.5
Optimum Ph for growth	7.0
Hydrolysis of	
Protease	+
Catalase	+
Amylase	+
Lipase	-
Gelatinase	+
H_2S production	+
Utilization of	
L-Arabinose	-
Fructose	+
Galactose	+
Inositol	-
D-Mannitol	+
Rhamnose	-
Soluble starch	+
Sucrose	+
Xylose	-
Cellulose	-
Glucose	+
Growth in the presence of antibiotics	
Streptomycin 10 µg	S
Ampicillin 10µg	S
Ciprofloxacin5µg	S
Cephaloridine 30µ	S
Gentamicin 10µg	S

+: presence; -: absence; S: Sensitive

Antibiotics production in different media and screening against fungi

Strain ERIMA-01 exhibited broad spectrum of antifungal activity in MNGA liquid broth medium. Hence it was chosen as the production medium for the antibacterial compounds. The antibacterial activity started at

Table 3. Physiological activities of ERIMA-01 on different media

Growth at pH	ISP-2	ISP-3	ISP-4
4	-	-	-
5	-	-	-
6	-	+	-
7	+	+	+
8	-	-	-
9	-	-	-
Growth at temperature			
50	-	-	-
37	+	+	+
30	+	+	+
27	+	+	+
20	+	-	-
10	-	-	-
Growth at NaCl (%)			
0.1	+	+	+
0.2	+	+	+
0.4	+	+	+
0.8	+	+	+
1.6	-	-	-

-: no growth; +: growth present

Table 4. Antimicrobial activity of ERI MA-01 against bacteria and fungi using different fermented media broth (Well diffusion method)

Tested microbes	Zone of inhibition in dia (mm)				
	SDA	YEDA	SS	ISP2	MNGA
Bacteria		-	-	-	-
Staphylococcus aureus	-	-	-	-	-
Bacillus subtilis	-	-	-	-	-
Enterococcus faecalis	-	-	-	-	-
Escherichia coli	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-
Klebsiella pneumoniae	-	-	-	-	-
Salmonella paratyphi –B	-	-	-	-	-
Erwinia amylowora	-	-	-	-	-
Proteus vulgaris	16	18	16	25	17
Xanthomonas campestris	-	-	-	-	-
Fungi					
Trichophyton mentagrophytes (66)	13	17	16	11	15
Trichophyton rubrum (57)	20	22	19	16	24
Trichophyton simii (110)	20	18	22	21	18
Scopulariopsis sp (101)	14	20	20	10	16
Aspergillus flavus	16	20	21	19	19
Aspergillus niger	17	24	23	20	24
Botrytis cinerea	18	25	25	20	24
Candida albicans	17	21	18	15	21
Candida krusie	18	23	21	21	25
Candida tropicalis	16	21	22	14	24
Candida parapsilosis	14	20	24	19	24

-: no activity
SDA (Sabourad Dextrosre Agar), YEDA (Yeast Extract Dextrose Agar)
SS- (Antibiotic production Media), ISP-2 (International *Streptomyces* Project-2)
MNGA (Modified Nutrient Gulucose Agar)

Table 5. Minimum Inhibitory concentration of Ethyl acetate and Methanol extract of *Streptomyces* sp (ERIMA-01) against fungi

Tested organisms	µg/ml		
	Ea	Me	Ketoconazole
Trichophyton mentagrophytes (66)	62.5	125	<12.5
Trichophyton rubrum (57)	31.25	125	<12.5
Trichophyton simii (110)	125	250	<12.5
Scopulariopsis sp (101)	125	125	<12.5
Aspergillus flavus	250	500	<12.5
Aspergillus niger	62.5	125	<12.5
Botrytis cinerea	125	500	nt
Candida albicans	15.62	>500	25
Candida krusae	31.25	125	25
Candida tropicalis	125	500	25
Candida parapsilosis	125	500	25

Ea- Ethyl acetate extract; Me- Methanol extract; nt: not test

the beginning of the exponential phase of growth (2 days) and reached maximum on sixth day. The pH between 7.0 and 7.5 was good for antifungal metabolite production at the optimum temperature of 30^{0}C. Five different media were used for antibiotic production; it was tested against bacteria and fungi. There was no activity against bacteria but all the fermented media inhibited the growth of tested fungi at 50µl/well **(**Fig 2). MNGA medium inhibited the growth of filamentous fungi and yeast such as *T. mentagrophytes* (15 mm), *T. rubrum* (24 mm), *T. simii* (18 mm), *Scopulariopsis sp* (16 mm), *A. flavus* (19mm), *A. niger* (24 mm), *B. cinerea* (24 mm), *C. albicans* (21mm), *C. krusae* (25 mm), *C. tropicalis* (24 mm) and *C.parapsilosis* (24 mm). Based on the zones of inhibition, the broth medium 5 (MNGA) was found to be good for antibiotic production. Different carbon sources were added in the media, to produce various concentrations of antibiotics. The results are summarized in Table 4.

Table 6. GC-MS analysis of active ethyl acetate extract of *Streptomyces* sp. strain ERIMA-01

RT	Name of the compound	Molecular Formula	Molecular Weight	Peak Area %
3.797	1,4-Butanediol	$C_4H_{10}O_2$	90	6.80
5.844	1,4-Butanediol,diacetate	$C_8H_{14}O_4$	174	1.04
6.474	3,5,5-Trimethyl-Cyclohex-2-Enone	$C_9H_{14}O$	138	0.61
7.635	n-Dodecane	$C_{12}H_{26}$	170	0.68
7.746	1-dioxidetetrahydrothiophene	$C_4H_8O_2S$	120	3.73
10.450	Tetradecane	$C_{14}H_{30}$	198	1.14
11.434	1-Dodecanol	$C_{12}H_{26}O$	186	0.83
11.910	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206	1.21
12.865	1-Pentadecene	$C_{15}H_{30}$	210	1.53
12.954	Hexadecane	$C_{14}H_{34}$	226	3.27
14.032	1-Tetradecanol,Acrylate	$C_{17}H_{32}O_2$	268	11.59
14.333	4-N-Nonylphenol	$C_{15}H_{24}O$	220	0.70
14.658	3-Deuterio-4-T-Butylcyclohexanone	$C_{10}H_{17}DO$	155	1.86
15.125	1-Nonadecane	$C_{19}H_{38}$	266	3.67
15.200	Octadecane	$C_{18}H_{38}$	254	4.72
15.443	3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	210	0.88
15.569	(4E)-2,2-Dimethyl-4-Decene	$C_{12}H_{24}$	168	0.69
15.739	(1-Propylheptadecyl)Benzene	$C_{26}H_{46}$	358	0.55
16.388	(1-Methyldodecyl)Benzene	$C_{19}H_{32}$	260	1.26
16.516	7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276	1.40
16.876	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	2.74
16.907	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	1.24
17.235	n-Eicosane	$C_{20}H_{42}$	282	3.44
17.388	Lauryl-beta-mercaptopropionate	$C_{15}H_{30}O_2S$	274	2.35
18.022	Succinic acid, di(2,4-dimethylpent-3-yl)ester	$C_{18}H_{34}O_4$	314	0.92
18.562	(6Z)-6-Octadecenoic acid	$C_{18}H_{34}O_2$	282	1.69
18.768	Stearic acid $$ n-octadecanoic acid	$C_{18}H_{36}O_2$	284	1.52
18.841	n-Hentriacontane	$C_{31}H_{64}$	436	0.61
18.903	3,6-diisobutyl-2,5-piperazinedione	$C_{12}H_{22}N_2O_2$	226	1.32
19.093	n-Docosane	C22H46	310	2.00
20.803	n-Tetracosane	C24H50	338	1.51
22.038	1,2-benzenedicarboxylic acid	C24H38O4	390	3.64
22.355	(9E)-9-Hexacosene	C26H52	364	1.12
22.389	n-Heneicosane	C21H44	296	1.97
23.139	Nonacosane	C29H60	408	2.10
23.863	n-Hexatriacontane	C36H74	506	3.63
25.249	Tetracontane	C40H82	562	3.20
25.998	Tetratetracontane	C44H90	618	2.10

Carbon and nitrogen sources that were slowly assimilated were also preferred for antibiotic synthesis as they did not favor high specific growth rates. Limitation of nutrients is responsible for the onset of antibiotic biosynthesis (Doull and Vining, 1990; Sanchez and Demain, 2002). The best C-source for the production of antifungal metabolites was MNGA medium.

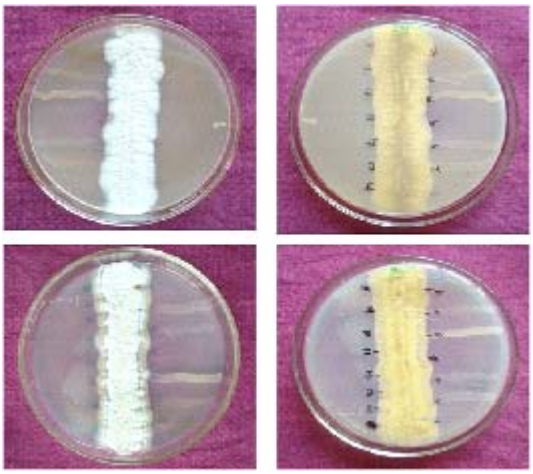
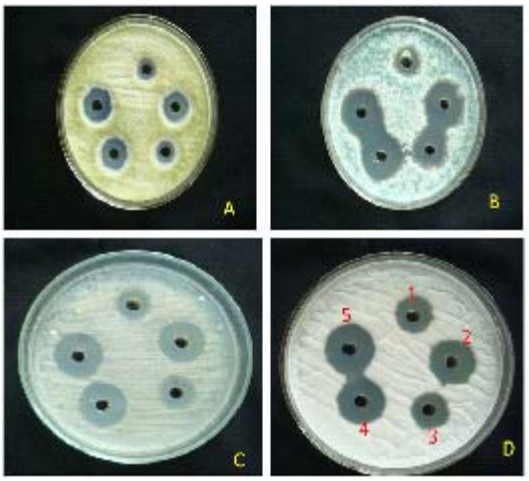


Fig. 1. A 7 days old culture of Streptomyces sp. ERIMA-01. inhibited the growth of bacteria and yeast (preliminary screening- streak method



A- *T. mentagrophytes*, B- *B. cinerea*, C- *Scopulariopsis* sp, D- *A. niger*
1- SDA (Sabourad Dextrosre Agar), 2- YEDA (Yeast Extract Dextrose Agar)
3- SS- (Antibiotic production Media), 4- ISP-2 (International *Streptomyces* Project-2)
5- MNGA (Modified Nutrient Gulucose Agar)

Fig. 2 . Antifungal screening of different fermented media of Streptomyces sp. ERIMA-01 against filamentous fungi

Extraction of antimicrobial metabolites

The spore suspensions of the culture were inoculated on MNGA (2 litre) medium and incubated at 28°C for 10 days. Ethyl acetate was used for extraction. The organic phase was concentrated using rotary evaporator at 40°C. Similarly methanol was used for extraction. The yield of extracts was: ethyl acetate (800mg) and methanol (600 mg).

Antifungal activity of crude metabolites of ERIMA-01

Ethyl acetate and methanol extracts of ERIMA-01 were tested for their antifungal activity against pathogenic fungi using microdilution method. The ethyl acetate extract inhibited the growth of all the tested fungi; *T. mentagrophytes* at 62.5µg/ml, *T. rubrum* at 31.25 µg/ml, *T. simii* at 125 µg/ml, *Scopulariopsis sp* at 125 µg/ml, *A. flavus* at 250 µg/ml, *A. niger* at 62.5 µg/ml, *B. cinerea* at 125 µg/ml, *C. albicans* at 15.2 µg/ml, *C. krusae* at 31.25 µg/ml, *C. tropicalis* at 125 µg/ml and *C. parapsilosis* at 125 µg/ml. Methanol extract inhibited the growth of *T. mentagrophytes* at 125µg/ml, *T. rubrum* at 125 µg/ml, *T. simii* at 250 µg/ml, *Scopulariopsis sp* at 125 µg/ml, *A. flavus* at 500 µg/ml, *A. niger* at 125 µg/ml, *B. cinerea* at 500 µg/ml, *C. krusae* at 125 µg/ml, *C. tropicalis* at 500 µg/ml and *C. parapsilosis* at 500 µg/ml (Table 5). The above results indicated that the ethyl acetate extract of ERIMA-01 significantly inhibited the growth of *C. albicans*, *T. rubrum* and *C. krusae*. Shahin (2003) reported the antimicrobial activity of *Streptomyces* sp. MU106. He observed more than 31 mm diameter inhibition zone against *C. albicans* and *C. tropicalis*.

Kavitha and Vijayalakshmi (2010) have reported the antimicrobial activity of *S. rochei* against bacteria and fungi. They found that the ethyl acetate extract of *S. rochei* inhibited the growth of *A. niger, A. flavus* and *C. albicans* at 1000 µg/ml. Our findings showed that *Streptomyces* sp. strain ERIMA-01 inhibited the growth of *A. niger, A. flavus* and *C. albicans* at very low concentrations of 250, 62.5 and 15.62 µg/ml respectively. Sultan *et al*. (2002) studied the antimicrobial activity of *Streptomyces* sp. They isolated active metabolites from ethyl acetate extract of *Streptomyces* sp. Various researchers have reported the antimicrobial activities of

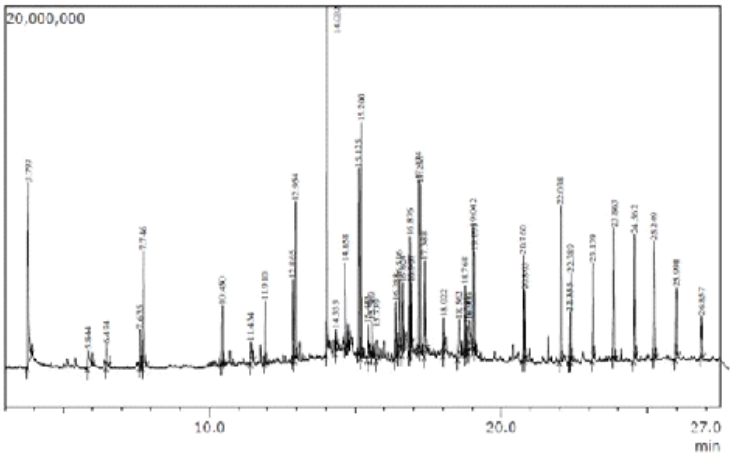


Fig. 3. GC-MS spectrum of Streptomyces sp (ERIMA-01) active ethyl acetate extract

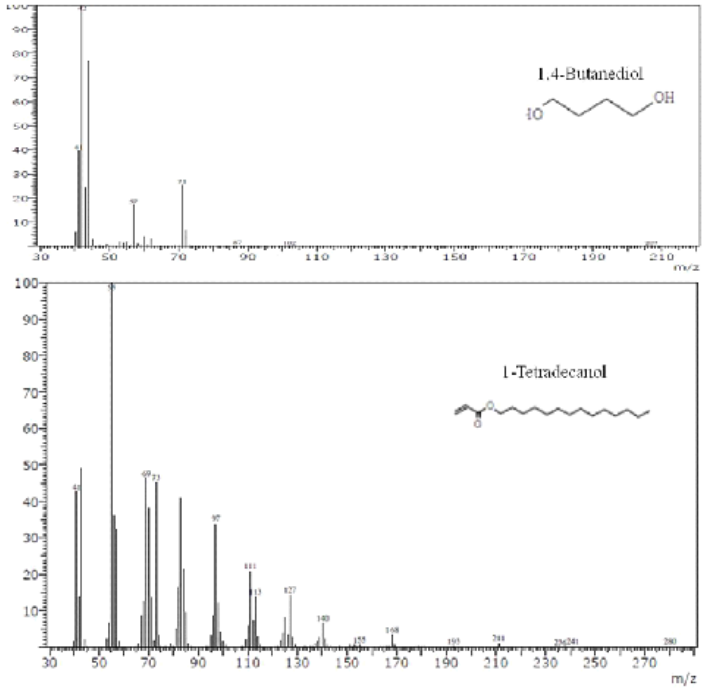


Fig. 4. Mass spectrum of major compounds

actinomycetes (Boruwa *et al*., 2004; IMPTEC, 1998; Lemriss *et al*., 2003). Kavitha *et al*. (2010) reported the antifungal activity of *Streptomyces* TK-VL333 isolated from laterite soils. They found that MIC value for *C. albicans* was 65 µg/ml; for *A.niger* it was 85 µg/ml and for *A. flavus* it was 90 µg/ml. Our results showed MIC value for *C. albicans* was 15.62 µg/ml and for *A.niger* it was 62.5 µg/ml. These values were very low compared to previous reports.

Many species of actinomycetes, particularly those belonging to the genus *Streptomyces,* are well known for the production of antifungal agents that inhibit several plant pathogenic fungi (El-Tarabily *et al*., 2000; Xiao *et al*., 2002; Errakhi *et al*., 2007; Joo, 2005). The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of antifungal compounds (Ouhdouch *et al*., 2001; Getha and Vikineswary, 2002; Fguira *et al*., 2005;

Taechowisan *et al*., 2005). Many researchers have reported the antifungal activity of *Streptomyces* sp. (Mukhergee and Sen, 2006; Jain and Jain, 2007; Kumar and Kannabiran, 2010; Duraipandiyan *et al*., 2010; Valan Arasu *et al*., 2008, 2009).

GC-MS analysis of the active extract

The active ethyl acetate extract of ERIMA-01 was quantified using GC-MS chromatograph (Fig 3). The GC-MS spectrum revealed the presence of many compounds (Table 6). The major compounds were: 1,4-Butanediol (6.80%), 1-Dioxidetetrahydrothiophene (3.73%), Hexadecene (3.27%), 1-Tetradecanol (11.59), 3-Deuterio-4-T-Butylcyclohexanone (1.86%), 1-Nonadecene (3.67%), Octadecene (4.72%), 7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione (1.40%), 1,4-Diaza-2,5-Dioxo-3-Isobutyl Bicyclo [4.3.0] Nonane (2.01%), n-Hexadecanoic acid (2.74%), Dibutyl phthalate (1.24%), n-Eicosane (3.44%), Stearic acid (1.52%), 1,2-Benzenedicarboxylic Acid (3.64%), n-Hexatriacontane (3.63%) succinic acid (0.92%) and 3,6-Diisobutyl-2,5-Piperazinedione (1.32%). The molecular weight and molecular formula were: 1,4-Butanediol (m/z 90; $C_4H_{10}O_2$) **(**Fig. 4), 1-Tetradecanol (m/z: 268 $C_{17}H_{32}O_2$) (Fig 4). These compounds were responsible for antifungal activity. Previously the above compounds have been reported as antifungal and antibacterial agents. 1,4- Butanediol isolated from the *Lactobacillus plantarum* was used as antifungal agent in agriculture to control fungus infection of plants (Barer and Clark, 1977). 1- Tetradecanol showed antibacterial activity against *S. aureus* (Togashi *et al*., 2007). Uquncu *et al*. (2010) reported that tetradecanol, Hexadecane, pentadecane and n-Hexadecanoic acid from essential oils of mosses (*Tortula muralis)* had shown activity against bacteria and fungi. The compound 3,6-Diisobutyl-2,5-Piperazinedione was found in ethyl acetate extract of ERIMA-01. The derivative of 3-benzyl-2, 5-piperazinedione was isolated from the ethyl acetate extract of the fermentation liquid of the marine fungus *Trichoderma atroviride* G20-12 (Sun *et al*., 2009). 1,2-Benzenedicarboxylic Acid in ethyl acetate extract of ERIMA-01 may have brought about the inhibitory effect on tested fungi. The same compound was reported from *Nocardia levis* MK-VL113 and showed antibacterial and antifungal activities (Kavitha *et al*., 2010). Succinic acid, stearic acid n-hexadecanoic acid, 1-nonodecene, n-eicosane stearic acid, pentadecene, tetradecene, hexadecene and benzenecarboxylic acid were identified as antifungal agents in the crude extracts and fat-soluble constituents of *Holotrichia diomphalia* larvae (Dong *et al*., 2008).

Phylogenetic studies and species identification

The phylogenetic position of ERIMA-01 was established by amplifying the 16S rRNA region and the sequence was examined by BLAST analysis **(**Fig. 5). The 16S rRNA genome sequence of the strain showed 87% similarities with that of *Streptomyces* sp; hence, strain was assigned to the *Streptomyces* sp. cluster and the 16S rRNA sequence was submitted to the NCBI GenBank with accession number FJ865352. Clustering analysis obtained by the NJ method showed that the strain ERIMA-01 was taxonomically very close to

TTTGTTGGGTTAACGTCGCGACTTATCGTGCAGTCGACGATGACCGGTTTCGCCGGG
GATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCTGCACTCTGGGACAA
GCCCTGGAAACGGGGTCTAATACCGGATACGACCTTCGAGCGCATGCTTGAGGGTG
GAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTTATCAGCTTGTTGGTGGGGTG
ATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGCGACCGGCCACACT
GGGGACTGAGACACGGCCCAGACTCCTACCGGGAGGCAGCAGTGGGGGAATATTG
CACAATGGGGGCGAAAGCCTGATGCAGCGACCGCCGCGTGAAGGGATGACGGCCCT
TCGGGTTGGAAACCTTCTTTCACCAGGGAAGAAGCAGAGTGACGTTCCTGCCAGG
AAGAAGCGCCGGTTACTTACGTGCCAGCGGCGGGGGAAATCCGTAGGGGGCAGGT
TTGTCCGGAATTTTTGGGCGAAAAGAGCTTGTAGGCGGCTTTGTCGTGCCGAAATGT
AAAAGCCCGGGGTTTAACCCCGGGCTCTGTATTCGTATACGGGCTGGTCAATATTCG
GTAGGGAAATCGGAATTCCTTGGTGTAGCGGGAAATGCGCAATTTCGGGGAAAACC
CGGTGGCGAAGGGGGCTTTTGGCCGATTCTACGCTGAGGAAGCAAAGCTGGGGGC
CACAGATTAGAACCCCTGGTAGCCCACGCGGAAACGGTGGGACATTGGGTGTGGCC
AATTTCCCAAGTTGTCGTGCAGGAAACTGAAGTATTAGTGTCCCCGCGATGGCGATA
CCGGCAAGATAGACTGAACCCTTAATGTGTAGTTGTGAGGGGCGTTGCT

Fig. 5. 16 S ribosomal RNA gene sequence of the *Streptomyces* sp. starin ERIMA-01

Streptomyces sp. Similar findings have been reported by Anansiriwattana *et al*. (2006) and Kavitha *et al*. (2010). Singh *et al*. (2008) partially purified antifungal metabolites from *Streptomyces capoamus* from agricultural soil and identified it based on physiological and biochemical characteristics and 16S ribosomal RNA sequence homology studies.

Actinobacteria were reported to be the major producers of biologically active compounds. Two-thirds of microbial derived antibiotics were obtained from this source, especially *Streptomyces* sp. The production of microbial metabolites can be substantially increased by manipulating the nutritional conditions, physical parameters, and genetic makeup of the producing organisms. The nature and concentration of some components of the fermentation medium also have a marked effect on antibiotic production (Aharonowitz, 1980; Martin and Demain, 1980; Omura and Tanaka, 1986; Parekh *et al*., 2000; Sanchez and Demain, 2002). The present study investigated the production of antifungal metabolites from *Streptomyces* sp. ERIMA-01 using different media. The crude ethyl acetate extract showed significant antifungal activity. However, most of the antimicrobial compounds have been extracted using ethyl acetate (Franco and Countinho, 1991).

Our results indicated that the antifungal compounds were produced extracellularly in fermented medium. Most of the secondary metabolites and antibiotics are extracellular in nature. Extra cellular products of actinomycetes showed potent antimicrobial activities (Bernan *et al*., 1994; Hacene *et al*., 2000). The results indicated the dependence of the production of antimicrobial compound(s) on medium constituents. Similar findings have been reported by Vilches et al. [1990] and Holmalahti et al. [1998] who showed that the nature of medium composition strongly affected antimicrobial compound production in different organisms.

CONCLUSION

The study on the influence of different nutritional media and culture conditions on antimicrobial compound production of *Streptomyces* sp. strain ERIMA-01 indicated that the highest biological activities were obtained when MNGA medium was used as a base. Extracellular metabolites in the culture filtrates of ERIMA-01 inhibited the growth of pathogenic fungi.

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