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Identification of Polyketide Gene from *Streptomyces roseoverticillatus* and Its Antimicrobial Properties against Pathogenic Bacteria and Fungi

Mariadhas Valan Arasu[1], Thankappan Sarasam Rejiniemon[2], V. Aldous J. Huxley[2], Soundarrajan Ilavenil[6], Veeramuthu Duraipandiyan[1], Naif Abdullah Al-Dhabi[1], Da Hye Kim[3,4], Kyung Dong Lee[5] and Ki Choon Choi[6]*

[1]Department of Botany and Microbiology, Addiriyah Chair for Environmental Studies, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia.
[2]Department of Zoology, Thiru Vika Government Arts College, Thiruvarur - 610 003, Tamil Nadu, India.
[3]The United Graduate School of Agricultural Sciences, Tottori University, Tottori-Shi, 680-8553, Japan.
[4]Faculty of Life and Environmental Science, Shimane University, Matsue-Shi, 690-8504, Japan.
[5]Department of Oriental Medicine Materials, Dongsin University, Naju, Republic of Korea.
[6]Grassland and forage division, National Institute of Animal Science, RDA, Seonghwan-Eup, Cheonan-Si, Chungnam, 330-801, Republic of Korea.

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The aim of this study was isolation and characterization of antimicrobial metabolite producing actinomycetes from forest soil. A new actinomycete designated ERIAS-10 was selected based on antibacterial and antifungal activity in the primary screening. Biochemical, physiological, morphological and carbohydrate fermentation pattern of the isolate was characterized and confirmed that the isolate belong to *Strepomyces* species. The 16S rRNA gene sequence shared 100% sequence similarity to *Streptomyces roseoverticillatus*. The antimicrobial substances were extracted with organic solvents from the spent medium in which ERIAS-10 was cultivated. Antimicrobial activity was assessed using the broth micro dilution technique. The minimum inhibitory concentration (MIC) of the extracts against *Enterococcus faecalis* was 7.81 µg/mL and *Aspergillus niger, A. flavus, A. clavatus* and *Fusarium oxysporum* was 15.63 and 31.25 µg /mL respectively, whereas, *Humicola grisea* showed MIC at 150 µg/mL level. Cloning and sequence analysis of ketoacyl synthase gene revealed similarity to the type II polyketide synthase gene of *Streptomyces* species. The results showed that the antimicrobial potential of ERIAS-10 isolate could be suitable for pharmaceutical applications.

Key words: *Streptomyces roseoverticillatus,* Antimicrobial activity, Minimum inhibitory concentration, Type II polyketide synthase.

Antimicrobial and bio-prospective agents are widely used in several fields, including human and animal therapy, protection of plants and crops from insects and pests, and preservation of foods from spoilages. Whereas, antimicrobial metabolites synthesized from chemical routes are the most prescribed drugs in the worldwide, but their effectiveness is facing serious clinical concerns especially due to the emergence of resistant bacteria and fungi (Metsa-Ketela *et al.* 2002). To overcome the difficulties related to the resistance of pathogens towards various commercial antimicrobial agents, novel natural products

* To whom all correspondence should be addressed.
Tel.: +82-41-580-6752; Fax: +82-41-580-6779;
E-mail: choiwh@korea.kr

derived from plants, invertebrates and microorganisms has been screened for their better bioactivities. By employing modern analytical techniques under various screening programs, the identification of natural compounds from medicinal plants and microbes exceeded one million (Pimentel-Elardo *et al*. 2010). Among that, the majority of the biologically active compounds were isolated and characterized from microorganisms (22500), 45% is produced by actinomycetes, 38% by fungi and 17% by unicellular bacteria (Demain and Sanchez 2009). Actinomycetes are capable of producing many types of secondary metabolites with commercial importance are a group of prokaryotic organisms, which are Gram positive and G-C rich bacteria. Actinomycetes, especially *Streptomyces,* are commonly present in soil, marine sediments and agriculture places are traditionally known to be rich sources of antibacterial, antifungal, anti-infective, antitumor, antihelminthic and herbicidal agents (Thakur *et al*. 2007; Yang *et al*. 2010). Recently, the number of isolation of new compounds from terrestrial actinomycetes has decreased, whereas the rate of re-isolation of known compounds has increased (Okoro *et al*. 2009). Therefore, the isolation of novel actinomycetes from inadequately explored habitats have promised a raise in the prospect of discovering new natural compounds that can be developed as a resource for applied microbiological research and drug discovery (Arasu *et al*. 2009). Relatively, the Western Ghats region of Kanyakumari district has rarely been explored for microbial diversity and microbial metabolites. Due to the large geographical variation, differences in soil type and their nutrient contents, it is quite likely that the distribution of antibiotic producing actinomycetes is also variable. Hence, there is an immense possibility to identify new actinomycetes in the Western Ghats to discover novel bioactive compounds. Therefore, this study aimed in isolation and characterization of antimicrobial metabolite producing actinomycetes from the Western Ghats region, India.

MATERIALS AND METHODS

Chemicals and enzymes

The DNA isolation kit and pGEM-T vector were purchased from Promega (Madison, WI, USA). DNA gel extraction kits were obtained from Qiagen (Mannheim, Germany). PCR master mix with a novel *Top* DNA polymerase was procured from Bioneer (Seoul, Korea). Glucose and all other chemicals were bought from Himedia (India) and Sigma-Aldrich (St. Louis, MO, USA).

Isolation of actinomycetes

Soil samples were collected from different locations at Western Ghats region of Kanyakumari district, India. One gram of the soil was suspended in 100 ml sterile saline water (NaCl 9 gL^{-1}), homogenized by vortexing for 15 min, and incubated in an orbital shaker incubator at 30°C with shaking at 200 rpm for 3 h. Isolation was carried out by following the method of Arasu *et al*. (2009). Isolates were maintained in the ISP-2 agar medium by storage at 4°C for 2 months. Alternatively, cultures were re-suspended in 20 % glycerol and stored at -80°C.

Screening of actinomycetes for antimicrobial activity

Antimicrobial activities of the isolates were checked by growing the cells on Modified Nutrient Glucose Agar (MNGA) plates by single a streak in the center. The test organisms, bacteria strains (*Bacillus subtilis* MTCC 441, *Staphylococcus aureus* ATCC 25923, *S. epidermidis* MTCC 3615 and *Enterococcus faecalis* ATCC 29212) and fungi strains (*Aspergillus niger* MTCC 1344, *A. flavus*, *A. clavatus* and *Curvularia lunata* 46/01) were studied. The test organisms were inoculated perpendicular to the antagonist on the agar medium. Bacteria were incubated at 37°C for 18 h and fungi were incubated at 30°C for 48 h. The microbial inhibitions were observed by determining the diameter of the inhibition zones (Arasu *et al*. 2013). An isolate with very good antagonistic activity was selected and named as ERIAS-10.

Biochemical and morphological characteristic of isolate ERIAS-10

The isolate ERIAS-10 was characterized morphologically and physiologically by following the method of International *Streptomyces* Project (ISP) and Bergey's Manual of Systematic Bacteriology (Locci 1989). Micro morphology was observed by slide culture method Williams, and Cross 1971) briefly, sterile square cover slips were inserted at an angle of 45^0 in sterile ISP-2, sabouraud dextrose agar; potato dextrose agar;

modified nutrient glucose agar; glycerol asparagine agar; peptone yeast agar and nutrient agar medium in petri dishes. Individual isolates were transferred to the intersection of the medium and cover slip. The cover slips were removed after seven days of incubation, air-dried and morphological characters were noted. Biochemical identifications were done by API 20E micro tests (Himedia, Mumbai).

Antibiotic sensitivity pattern of isolate ERIAS-10

Antibiotic sensitivity and resistance of isolate ERIAS-10 was assayed by the disc diffusion method of Bauer *et al*. (1996).

Molecular characterization by amplification and sequencing of 16S rRNA and Polyketide synthase (PKS)

The 16S ribosomal RNA gene was amplified from the genomic DNA of the strain ERIAS-10 by PCR with Taq DNA polymerase using the primers, 27 forward primer (5' AGA GTT TGA TCG TGG CTC AG 3') and 1492 reverse primer (3' GGT TAC CTT GTT ACG ACT T 5'). The amplified PCR products were purified by QIAquick® PCR purification Kit (Qiagen Ltd., Crawley, UK). The PCR product was ligated into the pGEM-T cloning vector by following the instructions given by the manufacturer (Promega, Madison, WI, USA). Plasmids were transformed into *Escherichia coli* DH5α competent cells. The plasmid was amplified by PCR using M13- forward primer (5' GTT TTC CCA GTC ACG AC 3') and M13- reverse primer (3' CAG GAA ACA GCT ATG AC 3') to check for the presence of insert DNA. Only plasmids containing the expected 1,500 bp inserts were sequenced by Solgent Co. Ltd. (Seoul, Korea) and deposited in Gene Bank database under the accession number JQ283103. The obtained sequences were subjected to BLAST at http://www.ncbinlm-nih.gov/ search in the NCBI database. Evolutionary history was inferred using the neighbor-joining method. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*. 2011).

In *Streptomyces* sp., the genes involved in the synthesis of polyketides are clustered in PKS (*Polyketide synthase*) operons (Arasu *et al*. 2013). Putative *ketoacyl synthase* gene (KS) fragment was amplified from the genomic DNA using degenerative primers 5' -TSG CST GCT TGG AYG CSA TC- 3' and 5' TGG AAN CCG CCG AAB CCT CT- 3'. The products were gel-purified and sequenced. The obtained sequences were subjected to BLAST at http://www.ncbinlm-nih.gov/ search in the NCBI database.

Fungal biomass inhibition effect of Streptomyces sp. ERIAS-10

The isolate ERIAS-10 cultivated in MNGA showed promising activity against fungal pathogens in cup plate assay. Therefore, the fungal biomass inhibition effect of cell-free supernatants was assayed. Aliquots (25 ml) of supernatant with 25 ml of PD broth were placed in 250 ml flasks and inoculated in triplicate with each test fungus. The culture flasks were incubated at 30°C for 5 days. Flasks without fermentation broth were the positive control. The growth performance of all fungal strains was checked separately in PD broth. After the incubation, fungal growth was measured by harvesting the cells, which were air-dried on pre-weighed Whatman #1 filter paper. Average fungal biomass was calculated for each test fungus and compared with the fungal biomass of positive controls.

Extraction of antimicrobial metabolites

The spore suspensions of the isolate ERIAS-10 were inoculated in 5L sterile flask containing 2.5 L MNG broth supplemented with 0.5% calcium carbonate and 0.5% corn steep liquor and incubated at 30^{0}C for six days. At the end of fermentation, the spent broth was separated by centrifugation at 8000 rpm, 10 min and extracted the antimicrobial metabolites using hexane, ethyl acetate and chloroform using 5L separating funnel. The solvent phase was concentrated by using vacuum at 40^{0}C to obtain the crude extract. The obtained extracts were evaluated for antimicrobial activities against bacteria and fungi.

Antimicrobial activity

The minimum inhibitory concentrations (MIC) of the methanol fractions were tested against different test organisms by the broth micro dilution method (NCCLS 1999).

RESULTS

Several suspected actinomycetes strains were isolated from Western Ghats forest soil samples according to their ability to inhibit the growth of pathogenic Gram positive, Gram negative bacteria, and filamentous fungi on MNGA medium (Fig.1). One isolate designated ERIAS-10 showed good antibacterial activity in cross streak method.

The isolate ERIAS-10 showed a high antibacterial activity against *S. aureus* to the extent of 17 mm inhibition area followed by *E. faecalis* (15 mm), *S. epidermidis* (14 mm), and *B. subtilis* (10 mm), in addition the fermentation spent medium exhibited significant antifungal activity was selected for further antimicrobial metabolite extraction (Fig.1). This isolate was identified as a Gram-positive, filamentous bacterium and produced white color spore while growing on different agar medium.

Table 1. Culture characteristics of *Streptomyces* sp. ERIAS-10 in different media after 7 days of incubation

Medium	Growth	Aerial mycelium	Substrate mycelium	Spores	Pigments
ISP-1	Moderate	Moderate, pale white	Present, soft white colour	white colour	No
ISP-2	Good	good, Pale white	Present, soft grey colour	white colour	No
SDA	Good	good, grey	Present, soft grey colour	white colour	No
PDA	Good	grey	Present, soft grey colour	white colour	No
MNGA	Good	grey	Present, soft white colour	white colour	No
GAA	Moderate	white	Light grey colour	white colour	No
PYA	Moderate	white	grey colour	white colour	No
NA	Moderate	white	white colour	white colour	No

ISP-1, ISP-2: International Streptomyces Project medium; SDA: Sabouroud Dextrose Agar; PDA: Potato Dextrose Agar; MNGA: Modified Nutrient Glucose Agar; GAA: Glycerol asparagines agar; PYA: Peptone yeast agar; NA: Nutrient Agar

Table 2. Comparative sensitivity pattern of *Streptomyces* sp. ERIAS-10 towards various antibiotics

Antibiotic group	Antimicrobial agent	Disc potency (µg)	Diameter of inhibition zone (mm)* ERIAS-10	Pattern
Aminoglycoside	Amikacin	30	0	R
	Gentamicin	10	15	S
	Kanamycin	30	17	S
	Streptomycin	10	0	R
	Tobramycin	10	19	S
Carboxypenicillin	Carbenicillin	50	22	S
	Ampicillin	50	19	S
²-lactamase inhibitor	Augmentin	30	31	S
	Imipenem	10	23	S
	Ticarcillin	75	11	S
Fluroquinolone	Ciprofloxacin	5	41	S
	Gatifloxacin	5	28	S
	Levofloxacin	5	32	S
	Moxifloxacin	5	31	S
	Nalidixic acid	30	0	S
	Norfloxacin	10	22	S
	Ofloxacin	5	21	S
	Sparfloxacin	5	16	S
Cephalosporin	Cefpodoxime	10	0	R
	Cetriaxone	30	29	S
Polymixin	Colistin	10	31	S
Sulphonamide	Co-Trimoxazole	25	27	S

Zones of inhibition were measured after incubating the strains for 72 h at 30°C in Starch casein agar medium (SCA).

According to the cultural characteristics, ERIAS-10 grew well on SDA, PDA, MNGA and the colonies were spreading and spore forming (Table 1). ERIAS-10 grew well in a wide range of carbon sources: glucose, fructose, sucrose, lactose and starch, whereas, it could not utilize xylose. The isolate was able to produce extracellular enzymes such as amylase, gelatinase and protease. Antibiotic sensitivity tests were conducted against the most commonly used antibiotics for bacterial infections. Streptomycin resistance indicated its typical feature (Table 2). The morphological, biochemical, physiological and antibiotic resistance patterns studies indicated that the isolate ERIAS-10 belongs to the *Streptomyces* genus.

The 16S rRNA gene (1476 bp) of ERIAS-10 was completely sequenced and analyzed for the similarities. The NCBI BLAST search program showed that the sequence data had a high identity and similarity (100%) to *Streptomyces roseoverticillatus* with a bits score and E value of 2726 and 0, respectively. The evolutionary tree was drawn using the Neighbor-Joining method (Fig 2). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). These results authenticated that ERIAS-10 isolate belongs to *Streptomyces roseoverticillatus.*

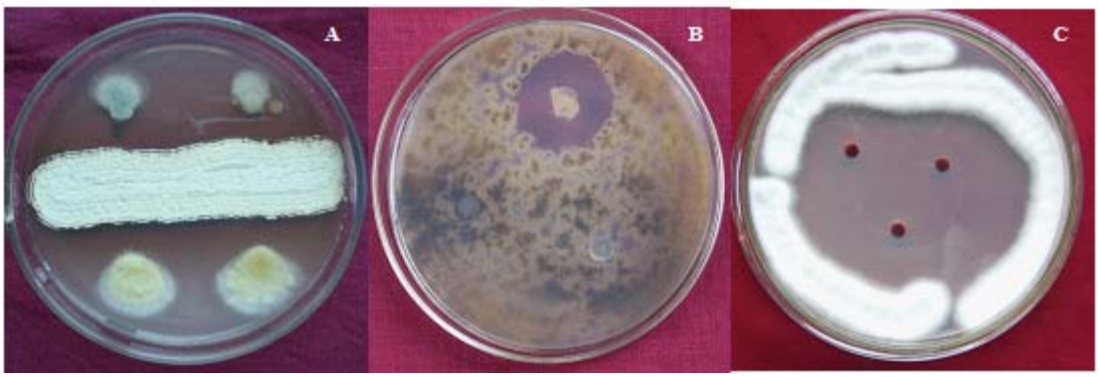
Additionally, the ERIAS-10 isolate was sequenced for KS genes which are essential for the synthesis of polyketide antibiotics in *Stretomyces* sp. PCR amplification yielded approximately 606 bp. It shared 100% nucleotide sequence identity to KS of *Streptomyces* sp. HB132 with a bits score and E value of 416 and 0, respectively. The KS gene sequence was comparable to those of *Streptomyces flavogriseus, S. nodosus* subsp. *Asukaensis, S. albulus* and *S. auratus* and many other *Streptomyces* species which are known to produce polyketide antibiotics.

Table 3. Antifungal activities of the extracts obtained from *Streptomyces* sp. ERIAS-10

Microorganisms	Minimum inhibitory concentration (MIC) (µg/mL)			
	HE	CL	EA	S
Gram positive				
Bacillus subtilis	125	>250	62.5	2.5
Staphylococcus aureus	125	>125	31.25	6.25
Staphylococcus epidermidis	250	250	15.63	25
Enterococcus faecalis	62.5	>250	7.81	25
Gram negative				
Escherichia coli	250	NA	125	25
Klebsiella pneumoniae	250	NA	125	25
Fungi				
Candida albicans	250	NA	62.50	12.50
Aspergillus niger	250	NA	15.63	12.50
Aspergillus flavus	>250	NA	31.25	12.50
Aspergillus clavatus	>250	NA	31.25	6.25
Aspergillus oryzae	125	NA	62.50	12.50
Curvularia lunata	125	NA	125.00	12.50
Fusarium oxysporum	62.5	NA	31.25	31.25
Gibberella moniliformis	31.25	NA	15.63	15.63
Humicola grisea	>250	NA	125.00	31.25
Penicillium chrysogenum	125	NA	62.50	12.50
Penicillium roqueforti	125	NA	62.50	12.50

S; control antibiotics, (Streptomycin sulfate for bacteria and Ketoconazole for fungal strains), HE; (Hexane extract); CL: (Chloroform extract); EA; (Ethyl acetate extract); NA: no activity at 500 µg/mL concentration.

The addition of fermentation spent broth together with fungal growth medium, significantly suppressed the growth of fungi, compared with that of controls based on dry weight measurements of fungal biomass (Fig. 3). The greatest antifungal growth inhibition was recorded against *A. clavatus* (83.33 %), followed by *P. roqueforti* (81.96 %), *A. flavus* (67.47 %) and *F. oxysporum* (62.56 %) respectively. These results confirmed that the fungal inhibition of the cell-free culture supernatant was due to the presence of the extracellular other inhibitory compounds.



(A) Antifungal activity of *Streptomyces* sp. ERIAS-10against fungal
(B) Activity of *Streptomyces* sp. ERIAS-10against fungal pathogen *Aspergillus fumigates* by overlaid agar method.
(C) Activity of *Streptomyces* sp. ERIAS-10against fungal pathogen *Aspergillus* by broth diffusion method.

Fig. 1. Antifungal activity of *Streptomyces* sp. ERIAS-10

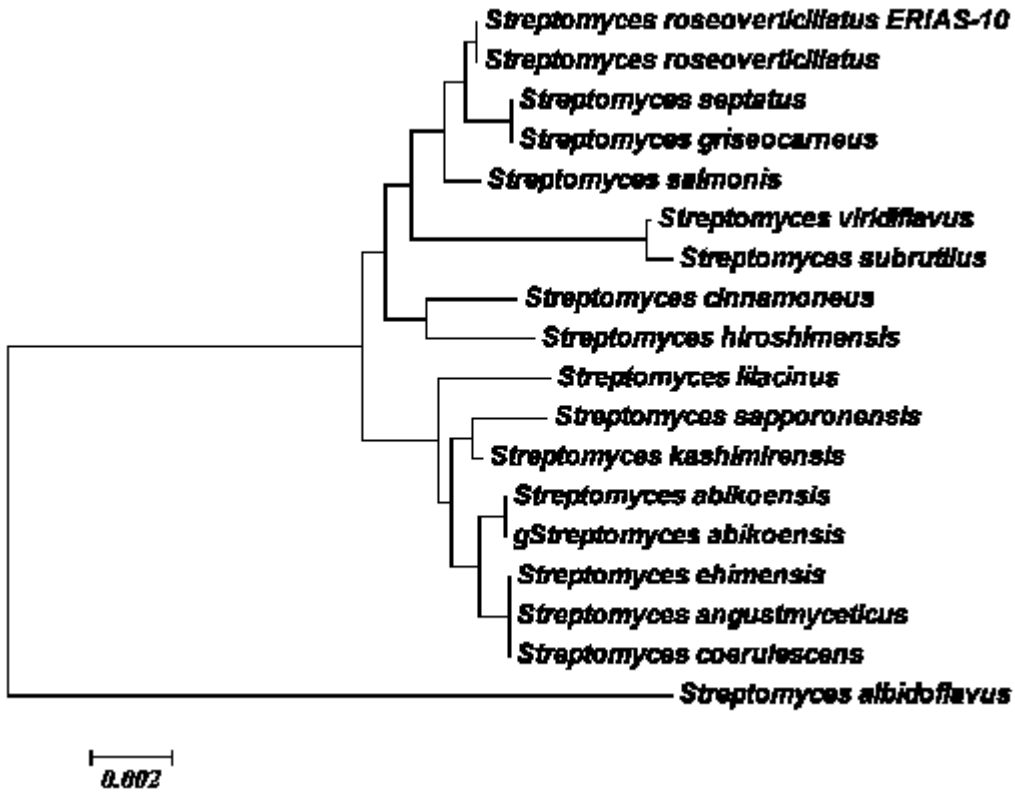


Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence showing the relationship between *Streptomyces* strains and species belonging to the genus *Streptomyces*. The tree was constructed using the neighbour-joining method

Ethyl acetate extracts obtained from the isolate showed comparatively good antimicrobial activity against Gram positive and Gram negative bacterial pathogens (Table 3). Hexane extracts exhibited MIC value 62.5 to 250 µg/ml for Gram positive bacteria and showed 250 µg/ml for both Gram negative bacteria, whereas, the MIC values of the ethyl acetate extracts were slightly similar or lesser than the standard broad spectrum antibiotic streptomycin.

Ethyl acetate extracts of ERIAS-10 revealed good antifungal activity against all the tested fungi compared to the other extracts (Table 3). Among the fungi, *A. niger* and *G. moniliformis* showed activity at 15.63 µg/ml, whereas, *A. flavus*, *A. clavatus* and *F. oxysporum* exhibited 31.25 µg/ml. Other fungal strains showed moderate activity. Chloroform extracts did not show activity at concentration 500 µg/ml. Standard antifungal drug ketaconazole showed MIC values ranging from 6.25 to 31.25 µg/ml.

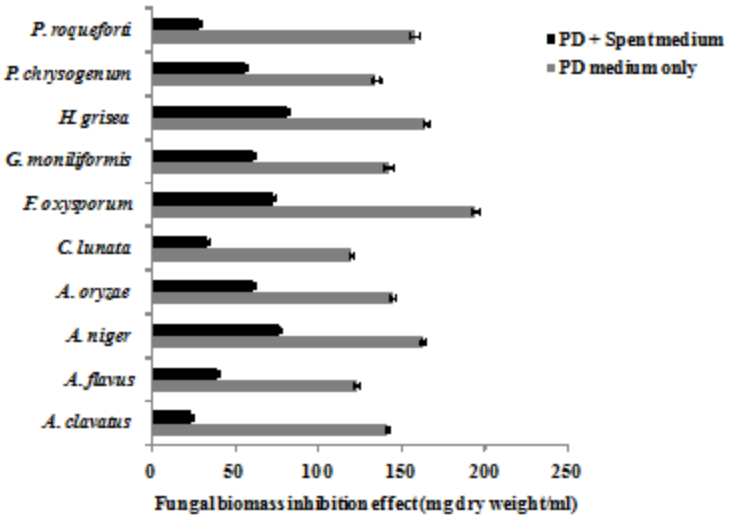


Fig. 3. Fungal biomass inhibition effect of *Streptomyces* sp. ERIAS-10

DISCUSSION

The Western Ghats forest soil samples are highly enriched in nutrients and leaf composts, therefore identification of valuable bacterial communities including fungi and actinobacteria with potential biological activities. Most of these areas have not been explored for bio-prospecting and/or microbial ecology studies. Many reports claimed that, actinomycetes have been evaluated as a source of bioactive compounds based on their distribution in various habits such as forest soil, sediments and marine environments (Crawford *et al*. 1993; Zheng *et al*. 2000). On the basis of this objective, novel *Streptomyces* sp. ERIAS-10 with promising antimicrobial activity was able to select for the bio-prospecting study. Morphological, biochemical, physiological and antimicrobial sensitivity properties described above confirmed that the strain ERIAS-10 was classified in the genus *Streptomyces* Taddei *et al*. 2006; Chater *et al*. 2010). ERIAS-10 was able to utilize a wide variety of sugars and secrete extracellular enzymes give promising clues for applied value for further research on these isolates. Further, this isolate is sensitive to most of the commonly used antibiotics was coincides with the results of Hamid, (2011). Beside the biochemical parameters, the phylogenetic analysis based on the sequence of 16S rRNA confirmed its placement in the genus *Streptomyces*.

The broad antimicrobial spectrum of this strain against *S. aureus*, *C. albicans*, *A. clavatus*, *A. flavus P. roqueforti* and *F. oxysporum* which cause serious mycotic infections and plant diseases, allowed us to consider this bacterium as a new strain of *S. roseoverticillatus*, and designated as *S. roseoverticillatus* strain ERIAS-10. *In vitro* studies conducted earlier also indicated

the antagonistic action of actinomycetes to the fungal pathogens causing plant diseases (Taechowisan *et al*. 2009). Reports claimed that *Streptomyces* sp. isolated from soil samples suppress the growth of plant pathogens such as *F. oxysporum* and *R. solani* under field conditions and exhibited activity against *C. gloesporioides* and *C. eragrostides* (Joo 2005; Soares *et al*. 2006; Zhao *et al*. 2012). It appeared that the antifungal activity of ethyl acetate extract was typically more pronounced on filamentous fungi. This result is consistent with previous screenings of *Streptomyces*, which showed good activity against *A.niger*, *Scopulariopsis* sp, *E. floccosum* and *T. simii* and a wide range of Gram positive and Gram negative pathogens (Arasu *et al*. 2013). The molecular characterization of the strain towards functional antibiotic group exhibited positive results for the presence of the polyketide synthesizing gene. Polyketides are synthesized by serialized reactions of a set of enzymes called polyketide synthase (Staunton and Weissman 2001). The presence of PKS genes in ERIAS-10 is a good indication of the polyketide molecules. The presence of type II PKS gene in ERIAS-10 seemed to have a direct correlation with ethyl acetate extract, which might be polyketide in nature (Maestro and Sanz, 2007). The present investigation also reveals that novel *S. roseoverticillatus* ERIAS-10 showed activity against a variety of opportunistic and phytopathogenic bacteria and fungi and also the presence of type II PKS gene in ERIAS-10 may lead to the discovery of a new lead compound and the expansion of diversity of pharmaceutical compounds.

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