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European Journal of Experimental Biology, 2014, 4(5): 46-52

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Effect of antimicrobial activity of UV mutated actinomycetes SP isolated from mangroves

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ABSTRACT

Totally seven actinomycetes were isolated from Mangrove soil region of Maravakkadu Reserve forest, Thanjavur district. Tamil Nadu. Antimicrobial activity was carried out for 3 dominant species of wild and UV mutated strains of these isolates against five human pathogenic bacteria and five fungal pathogens. The wild type strain Streptomyces albus showed maximum antimicrobial activity and it was subjected to Gas Chromatography with Mass Spectrometry analysis to find out the bioactive compounds.

Keywords: Biodiversity of actinomycetes ,antimicrobial activity,UV mutated and GC-MS analysis

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INTRODUCTION

Mangroves are great ecological, economic and social significance further, mangroves occurring along estuaries, back waters and the deltas function as the most important link between the land and sea. Such mangrove forests are estimated to cover an area of about 17 million hectares in World wide. The total area of mangroves in India is estimated to be 6,740 sq.km. In Tamilnadu, mangrove coverage is about 150 sq.km at Pichavaram and Muthupet (Ajith Kumar, 1998).The results of extensive screenings have led of the discovery of about 4,000 antibiotic substances from bacteria and fungi, many of which have been applied in human medicine, veterinary and agriculture. Most of them are produced from *Streptomyces*. Most *Streptomyces* and actinomycetes are used in the production of a diverse array of antibiotics including aminoglycosides, macrolides, β-Lactams, peptides, polyenes, polyether, tetracyclines, etc. In searching for new antibiotics, over 1,000 different bacteria, Actinomycetes, *Streptomycetes*, fungi and algae have been investigated. Recently, the rate of discovery of new compounds from terrestrial actinomycetes has decreased whereas the rate of isolation of known compounds has increased.

Marine actinomycetes remain an important source in the search for novel bioactive compounds. So far terrestrial substrates have been predominantly exploited as sources of actinomycetes; whereas the marine habitat has received much less attention. There are a few reports on bioactive compounds from marine actinomycetes in recent times. The marine environment an important source of novel anticancer, antiviral, antibacterial and antifungal as well as industrially important enzymes.

Actinomycetes are usually interest to scientist and industrialists because of their ability to produce useful products such as antibiotics, enzymes, pigments and vitamins (Goodfellow and Haynes, 1984). Few reports are available on antagonistic actinomycetes collected from marine soils.

MATERIALS AND METHODS

Sample Collection

Mangrove soil samples were collected at a depth of 5- 10 cm from two regions of Maravakadu, Thanjavur,TamilNadu, then it was transferred aseptically into sterile polythene bags for further study.

Physico-Chemical analysis of soil

The physico-chemical parameters of the soil samples were carried out by using Soil analysis kit (model 191E). The soil texture, soil pH, conductivity, turbidity, salinity, Total Dissolved Solids (TDS) and Dissolved oxygen (DO) were determined.

Isolation of Actinomycetes (Porter *et al*., 1960)

Isolation of actinomycetes was performed by plating technique using starch casein agar (Kuster and Williams, 1964) medium. The medium was prepared and sterilized at 121°C at 15 lbs pressure for 15 minutes. Then it was supplemented with Griseofulvin and streptomycin to prevent the bacterial and fungal growth. The medium was poured into the sterile petriplates. The collected soil samples were diluted upto 10^{-6} and 0.1 ml of the diluted samples was spread over the agar plates. The inoculated plates were incubated at 28 ± 2°C for 7 – 10 days. After incubation actinomycetes colonies were observed, and used for further investigation.

Purification of Actinomycetes

Streak plate method was used to purify the culture of actinomycetes. After inoculation, the plates were incubated at 28 ± 2°C for 7 – 10 days and slants were maintained in starch casein agar medium and stored at 4°C for further investigation

Coverslip Culture Technique (Pridham *et al.*, 1958)

Actinomycetes culture plates was prepared and 2-4 sterile coverslips were inserted at an angle of 45°. The actinomycetes culture was slowly released at the intersection of medium and coverslip. The plates were incubated at 28 ± 2°C for 4-8 days. The coverslips were removed and observed under the high power magnification. The photomicrography was taken using Nikon microscope. The morphological features of spores, sporangia and aerial and substrate mycelium were observed and recorded. Among the isolate, predominate organisms were selected for further studies.

Physical Mutation

The selected strains were cultured in test tubes containing 9 ml Starch Casein broth. The tubes were inoculated with one loopful of the strain and incubated in a rotatory shaker at 250 rpm at 30°C for 72-96 hrs. After incubation, the tubes were removed from the shaker and 3ml of each culture was exposed to UV radiation at a distance of 30 cm for 180 seconds. Then, 1ml of the exposed cultures was transferred to 9 ml of Glycerol-Starch broth medium and the tubes were incubated for 72-96 hours on a rotary shaker at 250 rpm for 30°C. After incubation, the tubes were removed from the shaker and the broth was centrifuged at 2000 rpm for 20 min and the supernatant was used to examine the post-mutation effect on the strain for antibacterial activity

Preparation of actinomycetes extract

The most intense antagonistic activity of the Actinomycetes was selected and its antimicrobial spectrum was tested against the pathogenic bacteria and fungi. The selected isolates were inoculated separately into 500 ml conical flask casein broth, and shaken at 28 ±2°C and 250 rpm for seven days, after incubation the staling substances were filtered through filter paper (What man No.1) and then through sietz filter (G5). The filtrates were transferred aseptically into the conical flasks and stored at 4°C for further assay an equal volume of ethyl acetate was added to the cell free culture filtrate

Antimicrobial activity of Actinomycetes

Antibacterial assay

The sterilized nutrient agar medium was poured into each sterile Petriplate and allowed to solidify. Using a sterile cotton swab, fresh bacterial cultures such as *Klebsiella pneumoniae, Staphylococcus aureus,S.pyogenes,Bacillus subtilis* and *Alcaligenes* sp with known population count was spread over the plates separately. Six mm diameter well was made over the media inoculated with appropriate bacteria, and then supernatant of these actinomycetes culture 100 µl was added into the each well. All the plates were incubated at 37º C for 24 – 48 hours. After the incubation period the results were observed and measured the zone of inhibition in diameter.

Antifungal Assay

Potato dextrose agar medium was prepared and sterilized. Then it was poured into each petriplate and allowed to solidify. After solidification, using sterile cotton swabs fresh fungal pathogens such as *Aspergillus oryzae, A.terreus, A.niger, A. sulphureus* and *Penicillium chrysogenum* was spread over the plates. Six mm diameter well was made over the media and inoculated with appropriate fungi. Then supernatant of these actinomycetes culture is added into the well. All the plates were incubated at 27º C for 48 - 72 hours. The diameter of inhibition zone was measured.

Gas Chromatography-Mass Spectrometry Analysis of Actinomycetes filtrates

The selected isolates were inoculated separately into 500 ml conical flask casein broth, and shaken at 28 ±2°C and 250 rpm for seven days, after incubation the staling substances were filtered through filter paper. The mycelial mat was collected and crushed by using methanol in the pestle and mortar. Then it was vortexed for 30 min. and centrifuged at 20,000 xg. The supernatant was collected and the compounds present in the filtrates were analysed by using GC-MS technique. GC-MS system equipped with Elite-1 column (100% dimethyl poly siloxane, 30 cm x 0.25 mm ID x 1 µm df) was used to analyze the compound present in the actinomycetes extracts. Column condition was programmed as column oven temperature 110°C (2min) – 5°C/min, temperature of inject port 250°C. Helium was used as a carrier gas (1 ml/min) (Roy, *et al.*, 2006). The peaks of components in gas chromatography were subjected to mass-spectral analysis. The spectra were analyzed from the available library data, NIST - MS Search (version 2.0).

RESULTS

Physico-chemical analysis of soil

The physico-chemical parameters of the soil samples from the two different regions of Maravakadu, Thanjavur district, Tamil Nadu, were analyzed (Table: 1).

Table:1Physico-chemical analysis of soil samples from two regions of Maravakadu

S.No.	Physico-chemical Parameters	Region 1	Region 2
1.	pH	8.0	8.1
2.	Soil texture	Loamy sand	Loamy sand
3.	Conductivity	7.0	7.5
4.	Salinity (ppt)	7.2	7.8
5.	Turbidity (Ntu)	3.30	3.43
6.	TDS (ppt)	3.90	6.7
7.	DO 9mg/litre)	1.6	1.9

Isolation and identification of actinomycetes

A total of seven different *Actinomycetes* isolates were screened from two soil samples of mangrove region.The cultural and microscopic characterization of actinomycetes were identified as *Actinomadura livida, Nocardiopsis* sp. *Thermomonospora* sp. *Saccharoplatyspora hirsute, Streptomyces albus, S cyaneus* and *S griseoflavus*, were frequently isolated from the soil samples (Table: 2.)

Table: 2 Isolation of Actinomycetes from mangrove soil

S.No.	Isolation of actinomycetes	Region 1	Region 2
1.	*Streptomyces griseoflavus*	+	-
2.	*S. albus*	+	-
3.	*S cyaneus*	-	+
4.	*Actinomadura livida*	+	-
5.	*Nocardiopsis sp.*	-	+
6.	*Thermonospora sp.*	-	+
7.	*Saccharopolyspora hirsute*	-	+

(+) - present, (-) -Absent

Biochemical characteristics of actinomycetes

All the actinomycete isolate were Gram positive organisms. Among the various biochemical characteristics studied indole, methyl red, voges proskaur and catalase test observed as negative result for all the tested actinomycetes. Citrate and urease test indicated positive result for all the tested isolates. Nitrate reduction test positive for *Streptomyces cyaneus, S. griseoflavus, S. albus* and *Nocardiopsis* sp (Table: 3)

Table: 3 Biochemical characteristics of Actinomycetes

Isolation of actinomycetes	Gram's staining	Indole	Methyl red test	Voges proskauer	Citrate	Urease	Catalase	Nitrate
Streptomyces griseoflavus	+ve	-	-	-	+	+	-	+
S. albus	+ve	-	-	-	+	+	-	+
S. cyaneus	+ve	-	-	-	+	+	-	+
Actinomadura livida	+ve	-	-	-	+	+	-	-
Nocardiopsis sp	+ve	-	-	-	+	+	-	+
Thermonospora sp.	+ve	-	-	-	+	+	-	-
Saccharopolyspora hirsute	+ve	-	-	-	+	+	-	-

+ denotes Positive result: - denotes Negative result

Anti-microbial activity of Actinomycetess

Only three actinomycetes were tested against ten pathogenic organisms. Five bacteriaspecies *Klebsiella pneumonia, Streptococcus pyogenus, Staphylococcus aureus, Alcaligens sp* and *Bacillus subtilis* and five fungal species namely *Aspergillus oryzae A terreus, A niger, A sulphureus* and *Penicillium chrysogenum* were tested against three actinomycetes

Antimicrobial activity of wild and mutated actinomycetes

Antibacterial Activity

The actinomycetes isolates *Streptomyces albus, Streptomyces cyaneus* and *Actinomadura livida* which showed antibacterial activity against tested human bacterial pathogens were treated with physical (UV) mutation and to study the effect of mutation on their antibacterial activity. Both wild and mutated strains were checked for their antibacterial activity against human bacterial pathogens viz. *K. pneumoniae, S. aureus, S. pyogens, Alcaligenes* sp and *Bacillus subtilis* (Table: 4)

Antibacterial activities of wild and UV-mutated actinomycetes

Isolation of actinomycetes	*K.pneumoniae*			*S.aureus*			*S.pyogenes*			*B.subtilis*			*Alcaligenes sp.*		
	PM	NM	VA	PM	NM	VA	PM	NM	VA	PM	NM	VA	PM	NM	VA
	Zone of Inhibition (mm)														
Actinomadura livida	14	16	-2	10	10	0	-	-	-	-	-	-	-	-	-
Streptomyces albus	17	16	+1	18	17	+1	-	-	-	-	-	-	27	19	+8
Streptomyces cyaneus	15	10	+5	11	10	+1	-	-	-	-	-	-	-	-	-

PM – Physical mutation NM – Non mutated VA – Variation

Non-mutated

Non-mutated *Streptomyces albus* showed inhibition activity against *K. pneumoniae* (16mm), *S.aureus* (17mm) and *Alcaligenes* sp (19mm) *S. pyogenes and B. subtilis* showed no inhibition activity.

Physical mutation

UV- mutated *Streptomyces albus* showed inhibition activity against *K. pneumoniae* (17mm), *S.aureus* (18mm), and *Alcaligenes* sp. (27mm). No inhibition was observed against the *S. pyogenes* and *B.subtilis*.

Non-mutated
Non-mutated *Streptomyces cyaneus* showed inhibition activity against *K. pneumoniae* (10mm) and *S. aureus* (10mm). No inhibitory activity was observed in *S. pyogenes, B. subtilis* and *Alcaligenes* sp

Physical mutation
UV mutated strain *Streptomyces cyaneus* showed inhibition zone against*K. pneumoniae* (10mm), *S. aureus* (11mm), *S. pyogens, B. subtilis*, and *Alcaligenes* sp. Showed no inhibition activity.

Non mutated
Non mutated *Actinomadura livida* showed inhibition zone against *K. pneumoniae* (16mm), and *S. aureus* (10mm), *S. pyogenes, B. subtilis* and *Alcaligenes* sp showed no inhibition activity.

Physical mutation
UV mutated *Actinomadura livida* showed inhibition zone against K. *pneumoniae* (14mm), *S. aureus, S. pyogenes, B. subtilis* and *Alcaligenes* sp showed no inhibition activity and antifungal activity. Both wild and mutated strains were checked for their antifungal activity against human fungal pathogens viz. *Aspergillus oryzae, A.terreus, A. niger, A. sulphureus* and *P. chrysogenum*

Antifungal activities of wild and UV-mutated actinomycetes

Isolation of actinomycetes	*Aspergillus oryzae*			*A.terreus*			*A.niger*			*A.sulphureus*			*Penicillium chrysogenum*		
	PM	NM	VA	PM	NM	VA	PM	NM	VA	PM	NM	VA	PM	NM	VA
	Zone of Inhibition (mm)														
Actinomadura livida	3	0	+3	-	-	-	18	12	+6	-	-	-	-	-	-
Streptomyces albus	4	0	+4	12	4	+8	21	18	+3	-	-	-	22	20	+2
S. cyaneus	-	-	-	-	-	-	18	12	+6	-	-	-	-	-	-

PM – Physical mutation NM – Non mutated VA – Variation

Non mutated
Non strain *Streptomyces albus* showed inhibition zone against *A. terreus* (4mm), *A. niger* (18mm), *P. chrysogenum* (20mm). No inhibition was observed against *A. oryzae* and A. *sulphureus*.

Physical mutation
UV mutated strain *Streptomyces albus* showed inhibition zone against *A. oryzae* (3mm), *A. niger* (12mm). No inhibitory activity observed in *A. terreus, A. sulphureus* and *P. chrysogenum.*

Non mutated
Non mutated strain *Streptomyces cyaneus* showed inhibition zone against *A. niger* (12mm), No inhibition activity was observed against *A. oryzae, A. terreus, A. sulphureus* and *P. chrysogenum*.

Physical mutation
UV mutated strain *Streptomyces cyaneus* showed inhibition zone against *A. niger* (18mm). No inhibition activity was observed against *A. oryzae, A. terreus, A. sulphureus* and *P. chrysogenum*

Non mutated
Non mutated strain *Actinomadura livida* showed inhibition zone against *Aspergillus niger* (12mm). No inhibition zone was observed against *A oryzae, A terreus, A sulphureus* and *P chrysogenum.*

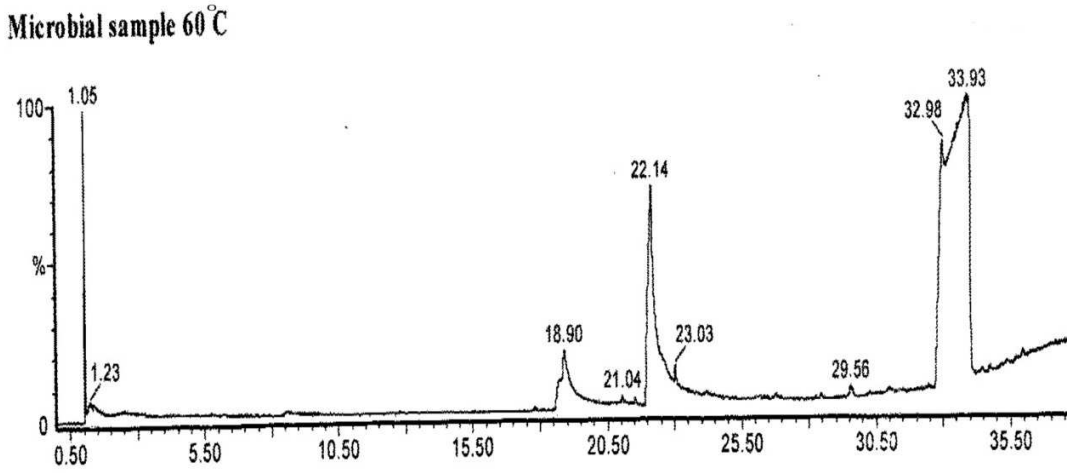
Physical mutation
UV mutated strain *Actinomadura livida* showed inhibition zone against *A oryzae* (3mm) and *A. niger* (18mm *)*. Inhibition activity was not observed against *A terreus, A sulphures* and *P chrysogenum*

GC-MS analysis of *S.albus*
Totally 7 compounds were observed in the methanolic extracts of *S. albus*. The peaks indicates that the compounds were determined as 1-Octanol, 2,7-dimethyl (8.56R_t, Oxalic acid, allylnonyl ester (12.79R_t), Pentanoic acid, 4-methyl (17.81R_t), n-Hexadecanoic acid (18.90R_t) Oleic Acid (22.14R_t), 1,10-Hexadecanediol (29.56R_t), Griseofulvin (33.93R_t) (Table 6, fig 1).

Table: 6 Compounds in the culture filtrate of *Streptomyces albus* identified based on the GC-MS analysis

No.	RT	Name of the compound	Molecular Formula	Molecular Weight	Peak Area
1.	8.56	1-Octanol, 2,7-dimethyl-	$C_{10}H_{22}O$	158	0.04
2.	12.79	Oxalic acid, allylnonyl ester	$C_{14}H_{24}O_4$	256	0.02
3.	17.81	Pentanoic acid, 4-methyl-	$C_6H_{12}O_2$	116	0.06
4	18.90	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	8.18
5.	22.14	Oleic Acid	$C_{18}H_{34}O_2$	282	23.38
6.	29.56	1,10-Hexadecanediol	$C_{16}H_{34}O_2$	258	0.51
7.	33.93	Griseofulvin	$C_{17}H_{17}ClO_6$	352	67.82

Gas Chromatography – Mass Spectrometry Analysis of *Streptomyces albus*



DISCUSSION

Actinomycetes are usually of interest to Scientists and Industrialists because of their ability to produce useful products such as antibiotics, enzymes, pigments and vitamins (Goodfellow and Haynes, 1984). A few reports are available on antagonistic actinomycetes from marine soils. The physico-chemical analysis of the Mangrove soil samples from two different regions of Maravakadu showed variation in salinity, conductivity, Total Dissolved Solids (TDS) and Dissolved Oxygen (DO) except the pH and Turbidity. Thus it is obvious that *Streptomyces* is ubiquitous and adapted to diverse habitats, which vary widely in space and time, and also to diverse habitats, and also to diverse environmental condition. In the present study totally seven different actinomycetes isolates were screened from two soil samples of mangrove region viz; *Actinomadura livida*, *Nocardiopsis* sp, *Thermomonospora* sp, *Saccharoplatyspora hirsute*, *Streptomyces albus*, *S cyaneus* and *S griseoflavus*, were frequently isolated from the soil samples. Dhanasekaran *et al.,* (2005) isolated 65 actinomycetes from 32 soil samples collected from Cuddalore, East Coastal region of Tamilnadu. Biochemical characteristics of the actinomycetes were also used as the characters for identification (Nikolova, *et al*., 2004; Gottieb, 1961; Kim and Goodfellow, 2002). In the present study totally 7 strain of actinomycetes were identified by morphological, cultural, biochemical and physiological Characteristics Colony formation, vegetative a nd aerial mycelium, structure of sporophores and spores are the most important features of identification of Strepto myces (Krasilnikor,1960;Wasksman,1961;kuster,1963

In the present investigation antimicrobial activities was tested in using three actinomycetes such as S*treptomyces albus*, *S cyaneus* and *Actinomadura livida*. Vijaykumar *et al.*,(2005a; 2005b) reported that among

the 68 isolates of actinomycetes only 25 (37%) isolates were found to possess antimicrobial potentiallity. Phillips (1960) reported that UV-mutated actinomycetes increase antibiotics production than that of non-mutated actinomycetes Mutated *Penicillium chrysogenum* produced antibiotics 10 times higher than that of the non-mutated strain. In the present investigation mutated actinomycetes showed high antimicrobial activity when compared with non-mutated actinomycetes after mutation. The strain *Streptomyces albus* (UV treated) increased the antimicrobial activity against all the tested human bacterial and fungal pathogens. Due to the mutation, partial active gene of these strains which were responsible for the production of antibiotics could have been activated. But the strain *Streptomyces cyaneus and Actinomadura livida (*UV treated) showed high level of antimicrobial activity depending on the tested human bacterial and fungal pathogen. Secondary metabolites in *Streptomyces albus* were analysed by using Gas Chromatography-Mass spectrometry. The compounds were indicated as 1-Octanol, 2,7-dimethyl (8.56Rt, Oxalic acid, allylnonyl ester (12.79Rt), Pentanoic acid, 4-methyl (17.81Rt), n-Hexadecanoic acid (18.90Rt), Oleic Acid (22.14Rt), 1,10-Hexadecanediol (29.56Rt), Griseofulvin (33.93Rt). Kenawy (2001) found that the polymers containing 8-hydroxyguinoline moiety were inhibitory to *E.coli*, *B.subtilis* and *Trichophyton rubrum*

CONCLUSION

The physico-chemical analysis of the Mangrove soil samples from two different regions of Maravakadu Totally 7 marine *Actinomycetes* such as *Actinomadura livida*, *Nocardiopsis* sp. *Thermomonospora* sp. *Saccharoplatyspera hirsute, Streptomyces albus*, *S. cyaneus* and *S. griseoflavus*, belonged to five genera were isolated from Mangrove soil sample of Maravakadu, Thanjavur District, Tamil Nadu.Among the seven *Actinomycetes* species isolated, three isolates *Streptomyces albus*, *Streptomyces cyaneus* and *Actinomadura livida* were tested for antimicrobial activity against five bacterial and five fungal pathogens. Among them *Streptomyces albus* was showed the maximum antimicrobial activity against bacterial and fungal pathogens.GC-MS analyses revealed that totally 7 compounds were found in *Streptomyces albus.* Among them the maximum percentage of peak was occupied by griseofulvin (67.82%). Thus the mangroves provide niches for dense and diversified function groups of *Actinomycetes* involving in the antimicrobial activity. They have great potential as antimicrobial agents and may also provide pharmaceutically important novel days. This study also gives basic information for those who are interested in studying the role of actinomycetes and antimicrobial activity in mangrove habitat, with many opening to further the studies in this aspect.

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