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Antimicrobial activity of a mangrove *Streptomyces sp*. M16

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Abstract : An actinomycete isolate M16 was isolated by dry heat (70°C) pre-treatment method on starch casein agar media, from the soil sample that was collected nearer to the root region of the mangrove *Avicennia marina* from the back water area, Ariyankuppam, Puducherry (UT). Antimicrobial activity of isolate M16 was tested against twelve bacteria, eight multicellular fungi and a unicellular *candida albicans*. Broad spectrum antimicrobial activity was confirmed by cross streak method. The isolate M16 was identified at genus level through the scanning electron microscopy and it was named tentatively as *Streptomyces sp* M16. *Streptomyces sp*.M16 was found to be active in having antibacterial, antifungal and anticandida properties.

Key words : Dry heat treatment, Antimicrobial activity, Agar plug, Cross Streak, Well Diffusion method, *Avicennia marina*, Mangrove Back water area.

Introduction

Microbiologists have extracted many active secondary metabolites from different types of microbes that are useful to the pharmaceutical world. The group of microbes that have high G+C content, are actinomycetes. They have been exploited in pharmaceutical industries for finding many novel antibiotics, especially the genus streptomyces has contributed its best than compared to other genus belong to actinomycetes. The antibiotic substances produced by them display antibacterial[1,2], antifungal[3,4], anticancer, antiprotozoic, antiviral, anticandida[5,6] and insecticidal properties[7]. The antibiotics produced by the Streptomycetes are safer than the antibiotics naturally synthesized by the fungi and bacteria. The search of new and novel antibiotics is important for the fight against new emerging drug resistant pathogens. Neglected habitats like mangroves are proving to be a good source of novel actinomycetes and bio active compounds[8]. The present investigation aims at finding better antimicrobial compound for controlling the bacterial and fungal human diseases.

Materials and Methods

Isolation of mangrove actinomycetes

The actinomycete isolateM16 was isolated from soil sample of *Avicennia marina*, from the Ariyankuppam back water area, Puducherry, India by dryheat pretreatment (70°C for 15 min)[9,10], pour plate method[11] using Starch casein agar[12] supplemented with Fluconazole 80µg/ml and Nalidixlic acid 75µg/ml. The actinomycete isolate M16 was subcultured in Yeast malt extract agar slants.

Screening of isolate M16 for antimicrobial activity

Test organisms used in this study

The following test bacteria were procured from Microbial Type Culture Collection-Chandigarh. The gram negative bacteria were *Pseudomonas aeruginosa* (MTCC-424), *Shigella flexneri* (MTCC-1457), *Bordetella bronchiseptica* (MTCC-6837), *Salmonella typhi* (MTCC-3220), *Vibrio cholera* (MTCC-3906), *Proteus vulgaris* (MTCC-744), *E.coli* (MTCC-1687), *Klebsiella pneumonia* (MTCC-4031), *Pseudomonas fluorescens*, *Enterococcusfaecalis* (MTCC-439) and gram positive bacteria are *Staphylococcus aureus* (MTCC-96), *Bacillus subtilis* (MTCC-441) andOne unicellular fungi-*Candida albicans* (MTCC-183).

The fungi used were *Microsporum gypseum* (MTCC-4494), *Trichophyton mentagrophytes* (MTCC-8476), *Epidermophyton floccosum* (MTCC-7880), *Fusarium oxysporum* (MTCC-1755) and *Rhizoctonia solani* (MTCC-1236), procured from MTCC, Chandigarh. The following fungi- *Alternaria alternata, Curvularia lunata* and *Colletotrichum gloeosporioides* were obtained from the laboratory collection.

Preparation of test organisms

Test bacteria were maintained in nutrient agar broth, pH-7. Test fungi were maintained in potato dextrose broth and in PDA slants, pH-6.5. These were stored in refrigerator at 4^{o}C for future use. 12-24 hours old bacterial liquid cultures, candida culture and 3-5 days old fungal plate cultures were used for antimicrobial study.

Invitro screening for antimicrobial activity

Primary screening by agar plug method was studied [13], secondary screening by agar well diffusion method was done [14]and confirmatory test was done by cross streak method [15].

Morphological characterization

Cover slip culture technique

Cover slip culture technique[16] was employed to study the micro-morphology of isolate M16. A loop of inoculam of the isolate M16 was streaked on nutrient agar plates. Then the sterilized glass coverslips were inserted at an angle of 45^{o} in the medium intersecting the streak lines. The plates were incubated at 28^{o}C. The isolate M16 grew both on medium and also on the inserted cover glass. The cover glasses were removed after 7 days from petriplates and observed under the microscope (Labomed, USA, Lx300, ivu 3100 at magnification 40 x) and photographed.

Gram staining

The mycelium of the isolate M16 (7 days) was analyzed to check whether it is gram (+) ve or (-) ve by following the Gram's standard procedure.

Scanning Electron Microscopy (SEM)

The structure, arrangement of spores on the mycelium of the active isolate M16 was examined with the help of scanning electron microscope. The seven days growth on the coverslip was used for scanning electron microscopy. The coverslip with the growth of isolate M16 was carefully removed without disturbing the surface. The coverslips with culture were air dried, mounted on the metal stub, sputter coated with carbon (5-10 nm) and viewed under SEM - Hitachi, Model: S-3400N at Central instrumentation facility (CIF), Pondicherry University, Puducherry, at an accelerate voltage of 15000 voltage and photographed.

Results and Discussion

The wet pH of mangrovesoil sample collected from *Avicennia marina* was 7.7. Drying and heating enhanced the isolation of rare actinomycetes. Dry heat method supported to get biologically active actinomycete for antimicrobial activity[17, 18]. The actinomycete isolate M16 was subcultured in yeast malt extract agar-ISP2. The great majority of antibiotics that have been isolated in numerous screening programs concerned with the

search for new therapeutic agents have been tested primarily for their activity against different bacteria[19]. Accordingly, ten gram negative bacteria and 2 gram positive bacteria, 8 multicellular fungal pathogens and a unicellular *Candida albicans* were procured from MTCC; Chandigarh was used for antimicrobial study.

Antibacterial activity of the isolate M16

The isolate M16was subjected for antibacterial activity in primary screening by agar plug method. It was concluded that the mangrove actinomycete isolate M16 was strong in inhibiting the growth of *Pseudomonas aeruginosa* followed by *Bacillus subtilis.*

Table 1: Antibacterial activity of isolate M16 in primary screening by agar plug method

S. no	Isolate code	Measurement of zone of inhibition in millimeter										
		E.c	k.p	p.v	p.a	s.t	s.f	v.c	B.b	p.f	E.f	B.s	S.a
16	M16	-	-	-	20±0.3	-	-	-	-	-	-	6±0.2	-

E.c-*E.coli,* **K.p**-*Klebsiella pneumoniae*,**P.v**-*Proteus vulgaris,* **P.a**-*Pseudomonas aeruginosa,* **S.t**-*Salmonella typhi*,**S.f** -*Shigella flexneri,* **V.c**-*Vibrio cholera,* **B.b**-*Bordetella bronchiseptica*,**P.f**-*Pseudomonas fluorescens,* **E.f**-*Enterococcus faecalis,* **B.s**-*Bacillus subtilis,***S.a**-*Staphylococcus aureus.*

Antibacterial activity of isolate M16 in secondary screening by agar well diffusion method

The isolate M16was subjected for secondary screening by agar well diffusion method. The isolate grew very well and produced antibiotic compound large quantity in liquid media Well diffusion method supported to study about the antibiosis from liquid media easily. It was noted that the antibiotic production and antibacterial potency of the actinomycete isolate M16 in liquid media was varying from the antibiotic production and antibacterial potency in solid agar medium.

Table 2: Antibacterial activity of isolate M16 by agar well diffusion method

Isolate code	Zone of inhibition in mm											
	E.c	k.p	p.v	p.a	s.t	s.fl	v.c	B.b	p.f	E.f	B.s	S.a
M16	-	-	6±0.2	16±0.3	-	13±0.1	10±0.05	-	-	-	12±0.2	-

The isolate M16 showed antibacterial activity towards *Pseudomonas aeruginosa* followed by *Bacillus subtilis*both in agar plug method (Solid media) and in agar well diffusion method (Liquid media). So, it was subjected further for confirmatory test.

Cross streak method to confirm the antimicrobial activity of isolate M16

It was noted that the isolate M16 grew better in nutrient agar plate and this abilityof isolate M16 made easy to perform cross streak of tested bacteria and candida against it, for confirmatory test in the plate.

Plate 1: Antibacterial activity of isolate M16by cross streak method

Gram negative bacterial pathogen like *Pseudomonas aeruginosa* pose serious threat to public health and resistance to multiple antibiotics is also being increasingly reported. Results of our study revealed that the *Streptomyces sp* M16 actively inhibited the growth of *Pseudomonas aeruginosa*. It is supported from the study that dealt that actinomycetes from the mangrove rhizosphere sediment will be a good source for the isolation metabolite effective against Gram negative bacterial pathogens[2].

Antifungal activity of isolate M16 by dual culture method

Table 3: Antifungal activity of isolate M16 by dual culture method

Isolate code	**Fungi used in antifungal activity, Inhibition in mm**							
	M.g	***T.m***	***E.f***	***C.l***	***A.a***	***R.s***	***C.g***	***F.o***
M16	No growth	No growth	No growth	10±0.5	20±0.3	No growth	3±0.2	18±0.3

M.g*- Microsporum gypseum,* ***T.M****- Trichophyton mentagrophytes,* ***E.f****- Epidermophyton floccossum,* ***C.l****-Curvularia lunata,* ***A.a****- Alternaria alternata,* ***R.s****-Rhizoctonia solani,* ***C.g****-Colletotrichum gleosporioides,* ***F.o****-Fusarium oxysporum*

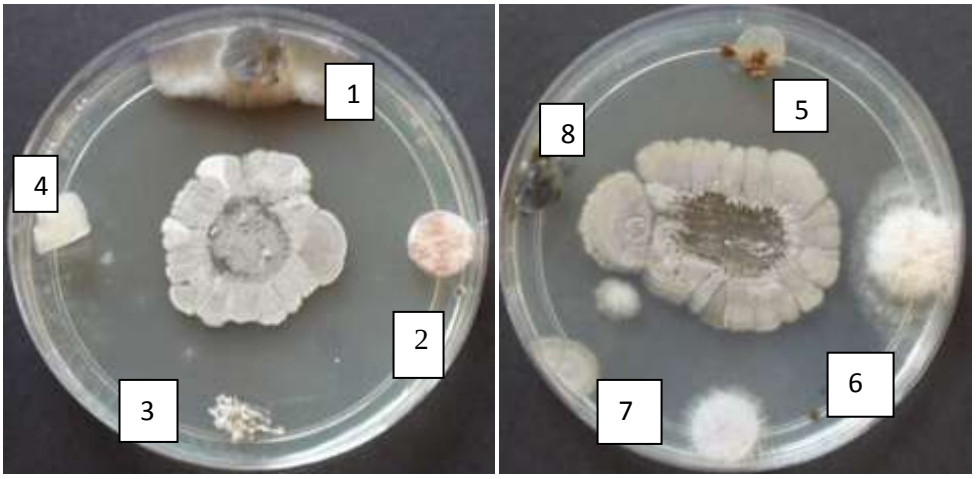

Plate 2: Antifungal activity of isolate M16

1.*Alternaria alternata* **2.***Microsporum gypseum***3.***Trichophyton mentagrophytes***4.** *Epidermophyton floccossum***5.***Rhizoctonia solani***6.***Colletotrichum gleosporioides* **7.***Fusarium oxysporum***8.***Curvularia lunata*

The isolate M16 was active for one gram +ve and one gram negative bacteria. The antifungal potential of isolate M16 was also been observed in confirmatory test by dual culture plate method.

The isolate M16 inhibited the growth of all the 8 fungi tested and it was also been observed that the isolate M16 was most active for candida (100% inhibition), no growth of *Candida albicans* was observed in the cross streak plate for confirmatory test for bacteria and candida. The studies [5, 6] stated that the marine and mangrove actinomycetes have very good anticandida properties. The isolate M16 was very active against *Candida albicans* that cause severe thrush infections in mouth, nail and genital systems in human beings. It controlled the growth of bacteria- *Pseudomonas aeruginosa* that cause noscomial infections.

Table 4: Antimicrobial spectrum of isolate M16

S.no	Test organisms used for antimicrobial activity	Inhibition in mm
Bacteria (+) ve		
1	*Bacillus subtilis*	22
2	*Staphylococcus aureus*	-
Bacteria (-)ve		
3	*Bordetella bronchiseptica*	-
4	*Enterococcus faecalis*	-
5	*Pseudomonas aeruginosa*	32
6	*Shigella flexneri*	No biofilm
7	*Pseudomonas fluorescens*	-
8	*Salmonella typhi*	-
9	*Vibrio cholera*	No biofilm
10	*Proteus vulgaris*	thin
11	*E. coli*	No biofilm
12	*Klebsiella pneumoniae*	-
Unicellular human fungi		
13	*Candida albicans*	100% inhibition
Multicellular human fungi		
14	*Microsporum gypseum*	100% inhibition
15	*Trichophyton mentagrophytes*	100% inhibition
16	*Epidermophytonfloccosum*	100% inhibition
17	*Curvularia lunata*	10±0.5
18	*Alternaria alternata*	20±0.3
Multicellular plant fungi		
19	*Rhizoctonia solani*	100% inhibition
20	*Colletotrichum gloeosporioides*	3±0.2
21	*Fusarium oxysporum*	18±0.3

The other test fungal pathogens showed decreasing sensitivity towards the isolate M16 were *Alternaria alternata* (20±0.3mm)>*Fusarium oxysporum* (18±0.3mm)> *Curvularia lunata* (10±0.5mm)>*Colletotrichum gloeosporioides* (3±0.2mm).The isolate M16 was most active against dermatophytic fungi- *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Epidermophytonfloccosum*. 100% growth arrest was seen in thosedermatophytes. The isolate M16 suppressed the growth of the phyto fungal pathogen *Rhizoctonia solani*(100%).

The isolate M16 was active for respiratory fungal pathogens -*Alternaria alternata*. Most of the fungal phytopathogens were controlled by the isolate M16. The broard spectrum activity of the isolate M16 against the

Candida albicans (unicellular) and fungi (multicellular) was unique and better.Our research results are supported by the research reports that dealt about antifungal activities of different species of actinomycetes. Actinomycete-fungusantagonism has been demonstrated for a wide variety of plant pathogens suchas *Alternaria sp.*[20]. *Rhizoctonia sp.*[21], *Colletotrichum sp.* and *Cuvvularia sp.* [22].Actinomycete-fungusantagonism is important in the biocontrol studies. Soil and seed borne fungal diseases are controlled with the help of the antagonistic actinomycetes, especially streptomyces sp[23]. Our research work is highly supported by the statement that states that *Streptomyces* strains isolated from mangrove sediment produce potential antibacterial, antifungal and broad spectrum antibiotic compounds[1].

Morphological characterization of the isolate M16

The colony morphology of isolate M16 was ridged at the centre, more or less round to ovoid in shape and annulated, initially cream white, produced grey aerial mycelium and later it looked like grey strain, produced yellowish brown substrate mycelium. It produced non diffusible yellowish brown pigment.

Plate 3: Colony morphology of isolate M16

The isolate M16 grew well on potato dextrose, yeast malt extract, nutrient agar. The growth and development of mycelium from inoculam appeared from next day onwards from the media. The microscopic study revealed that the mycelium was branched with long chains of sporophores.

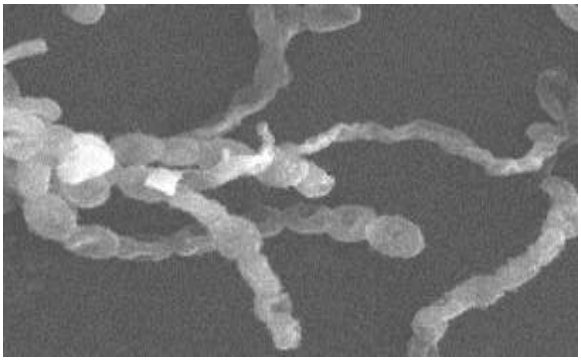

Plate 4: SEM photographs of isolate M16
A. Well developed mycelium and attached sporesin chains

The strain of the isolate M16 was identified as gram positive with the help of standard gram strain procedure. SEM analysis revealed that the spore surface is not smooth, biconcave in shape and isolate had aerial mycelium with long chains of flexous sporophores and has given initial idea that the isolate M16 belongs to the genus Streptomyces.It is a preliminary work, further studies are needed to evaluate thenature of compound present for antagonistic potentiality of *Streptomyces sp*.M16.

Conclusions

The genus Streptomyces is a noteworthy microbes having biopotential for better antagonism. *Streptomyces sp. M16*is active against dermatophytes, candida, respiratory pathogens and plant pathogens. Biologically active compounds with variety of application in the different fields of biological area are highly motivated and targeted study in the competitive pharmaceutical world. Isolation and bioprospecting of Streptomycetes group from the unexplored areas like mangroves got more importance, because mangrove Streptomycetes play vital role in producing novel bioactive compounds with antibacterial, antifungal, antiparasitic, anticancer, insecticidal properties etc.,. Since, the mangrove Streptomyces sp. M16 showed broad spectrum antifungal activity, it would be effectively used to cure human, animal and plant fungal diseases in future.

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