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Antibacterial activity of soil actinomycetes from the mangrove *Avicennia marina*

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Abstract
Totally 25 actinomycetes were isolated by dry heat (70 ºC) 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). All the 25 actinomycetes were subjected for primary screening against the 10 gram negative bacteria, 2 gram positive bacteria by Agar plug method. The total percentage of inhibition by actinomycetes against bacteria in primary screening was noted as *E.coli*-8%, *K. pneumoniae*-4%, *P.vulgaris*-4%, *P.aeruginosa*-24%, *S.typhi*-4%, *S.flexneri*-16%, *V.cholera*-4%, *B.bronchiseptica*-52%, *P.fluorescens*-0%, *E.faecalis*-8%, *B.subtilis*-40%, *S.aureus*-8%. Totally 20 (80%) of actinomycetes showed antibacterial activity towards any one of the tested bacteria, 5 (20%) actinomycetes showed no antagonistic activity. Only 2 actinomycetes were selected from *A.marina* and that were subjected for secondary screening. Broad spectrum antibacterial activity was confirmed by cross streak method for selected antagonistic actinomycetes.

Keywords: Dry heat treatment, Anti-bacterial activity, Agar plug, Cross Streak, Well Diffusion method, *Avicennia marina*, Mangrove Back water area.

1. Introduction
Actinomycetes are the strongest antagonists among microbes. The antibiotic substances elaborated by them display antibacterial, antifungal, anticancer, antiprotozoal and antiviral properties. Of the ten thousand known antibiotics produced by microbes over a decade ago, about 70% are of actinomycete origin: of them, representatives of the genus *Streptomyces* account for two thirds (Miyadoh, 1993) [15]. Actinomycetes are potent source of antibiotics, besides vitamins and enzymes, and such antagonistic actinomycetes of marine origin are being regularly reported (Krasilnikov, 1962; Okami *et al*., 1976; Pisano *et al*., 1986: Weyland and Helmke, 1988; Do *et al*., 1991; Farooq Biabani *et al*., 1997: Pusecker *et al*., 1997: Romero *et al*., 1997; Williams *et al*., 1999) [11, 18, 19, 28, 5, 6, 20, 23, 29]. Few reports that soil is a major source of actinomycetes (Sivakumar *et al*., 2005; Vijayakumar *et al*., 2007; Dhanasekaran *et al*., 2008) [24, 25, 4]. Members of actinomycetes which live in marine environment are poorly understood and only few reports are available pertaining to actinomycetes from mangroves (Sivakumar, 2001; Vikineswari *et al*., 1997; Rathna kala & Chandrika, 1993; Lakshmanaperumalsamy, 1978) [26, 21, 13]. Mangrove ecosystem is the most productive ecosystem diversified with variety of microbes (Kathiresan and Bingham, 2001) [10]. The search of new and novel antibiotics and other bioactive microbial metabolites is important for the fight against new emerging pathogens (Good fellow *et al.*, 1989, Berdy, 2005, Busti *et al*., 2006) [7, 2, 3]. Isolation of actinomycetes from unique unexplored natural habitats is of interest to avoid re-isolation of strains that produce known bioactive metabolites. Neglected habitats are proving to be a good source of novel actinomycetes and bio active compounds.
The present investigation aims at finding better antibacterial compound for controlling the bacterial human diseases, with the help of mangrove actinomycetes that are selectively isolated from the soil near the root region of *Avicennia marina (*Forsk*)*. Vierh - Avicenniaceae*,* from the Ariyankuppam back water area, Puducherry, India.

2. Materials and methods
Collection of soil sample
Soil sample near the mangrove plant*,* Avicennia marina (Forsk). Vierh – (Avicenniaceae*)* in Ariyankuppam back water estuary, Puducherry (Lat 11 º46´03'' to 11 º53´40'' North and Longi 79 º49´45" to 79 º48´00" East) was collected, packed in sterile plastic containers and transported immediately to the laboratory. The pH of the fresh soil sample was determined (Reed and cummmings, 1945) [22]. Then the soil sample was air dried for 7-10 days at 40 ºC,

Crushed and sieved to remove the shells and debris and stored.

Soil analysis
Physio-chemical nature of soil sample was analysed in soil testing laboratory, Department of Agriculture, Puducherry, India.

Isolation of mangrove actinomycetes
The soil sample was subjected to dryheat (70 ºC for 15 min) (Hayakawa *et al*., 1991) [8] pretreatment to enhance the chances of isolating rare and novel actinomycetes. After pretreatment, one gram soil was mixed and serially diluted in sterile water blanks. 0.1 ml of last two dilutions ($10^{-5}$and 10^{-6}) was inoculated by pour plate method (Zheng *et al*., 2000) [30] using Starch casein agar (Kuster and Williams, 1964) [12] supplemented with Fluconazole 80µg/ml and Nalidixic acid 75µg/ml. Plates were incubated at 30 ± ºC for up to 30 days. Plates were periodically examined for actinomycetes colonies. Selected colonies were transferred to Yeast Malt extract agar slants and maintained in the same medium.

Screening of actinomycetes for antibacterial activity
The following test bacteria were procured from Microbial Type Culture Collection-Chandigarh. The gram negative bacteria are *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, Enterococcus faecalis* (MTCC-439) and gram positive bacteria are *Staphylococcus aureus* (MTCC-96), *Bacillus subtilis* (MTCC-441).

Preparation of test bacteria
Test bacteria were maintained in nutrient agar broth, pH-7. These were stored in refrigerator at 4ºC for future use. 12-24 hours old bacterial liquid cultures were used for antibacterial study

Invitro screening for antibacterial activity
Primary screening by agar plug method (Mohan raj *et al*., 2011)
All the 25 isolates were primarily screened for anti-bacterial activity by agar plug method. All isolates were grown in four different media- ie- Starch casein agar, potato dextrose agar, nutrient agar and yeast malt extract agar in petri plates by close streak and allowed to grow for 10 days under laboratory conditions for better growth and antibiotic production. 8 mm radial agar plugs were cut from the culture plates. Placed on the test bacteria seeded nutrient agar plates. The plates were kept in refrigerator overnight for diffusion of antibiotic compound from agar discs of actinomycetes. Then the plates were kept in incubator at 30 ˚C for 5 minutes for evaporation. Inhibition zones of the pathogenic strains around the plugs can be measured within 24-78 hours and inhibition zones were measured in millimeter.

Secondary screening by agar well diffusion method (Murrey *et al*., 1995)
Nutrient broth was prepared in test tubes, pH-7.5 was maintained. Tubes were inoculated with 2 active isolates, incubated at room temperature for 10-12 days. Nutrient agar plates were prepared, seeded with test bacteria, 8 mm wells were aseptically bored in test bacteria seeded nutrient agar plates. The wells were filled with *100µl* of culture filtrate. The antibacterial activity of metabolites was determined based on the diameter of zones of inhibition after 24-30 hrs of incubation.

Cross streak method (Lemos *et al*., 1985)
Antibacterial activity of the isolates was tested and confirmed further by cross streak method. A loop full of spores and mycelium of the isolates M20 was streaked across nutrient plates and incubated at 28 ˚C for 10 days. The 12 test bacteria, 24 hrs growths were streaked perpendicular to the line of growth of the actinomycetes. Plates were prepared in triplicate and incubated at 30 ˚C for 24 hrs and the zone of inhibition was recorded in mm.

3. Results and Discussion
The wet pH of mangrove sediment sample collected from *Avicennia marina* was 7.7. The following are the physico-chemical analysis of soil sample.

Table-1: Physico-Chemical analysis of mangrove soil sample from Avicennia marina from Ariyankuppam backwater estuary

Parameter	Soil status
pH	7.7
E.C status	0.1
Lime status	Normal
Soil Texture	Clay-loamy
Macro-Nutrients	
Nitrogen	55.00 (VL)
P_2O_5	1.23 (VL)
K_2O	101 (M)
Micro-Nutrients	
Cu	0.827(L)
Zn	2.508(H)
Mn	3.788 (M)
Fe	16.126 (H)

The soil analysis results showed that there were very low available Nitrogen, P_2O_5 and Cu. Micro-Nutrients like Zn and Fe were high in their available form, Mn was medium.

Isolation and maintenance of actinomycetes
Totally 25 actinomycetes were isolated from soil sample that was collected near *Avicennia marina* by after dry heat (70 ºC for 15 min) pretreatment method. Dry heat method yielded bioactive actinomycetes for antimicrobial activity (Janaki *et al*., 2015, Baskaran *et al*., 2011) [1]. The isolated actinomycetes were sub cultured in yeast malt extract agar-ISP2. Rare actinomycetes are considered as the strains whose isolation frequency by conventional methods is much lower than that of commonly occurring actinomycete strains. Subsequently, employing pretreatments of soil ie. Drying and heating enhanced the isolation of rare actinomycetes. 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 (Waksman *et al*., 1952) [27]. Accordingly, ten gram negative bacteria and 2 gram positive bacteria, procured from MTCC, Chandigarh was used for antibacterial study.

Antibacterial activity of actinomycetes from *Avicennia* marina
The 25 isolated actinomycetes from *Avicennia marina* were Subjected for antibacterial activity in primary screening by Agar plug method.

Table 2: Antibacterial activity of actinomycetes from *Avicennia marina* 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
1	M1	-	-	-	-	-	-	-	39	-	-	12	-
2	M2	-	-	-	-	-	-	-	8	-	-	-	-
3	M3	-	-	-	-	-	4	4	-	-	-	-	-
4	M4	-	-	-	-	-	-	-	-	-	-	-	-
5	M5	-	-	-	-	-	-	-	28	-	-	16	-
6	M6	-	-	-	-	-	-	-	-	-	4	-	-
7	M7	-	-	-	-	-	-	-	28	-	-	-	-
8	M8	-	-	-	-	-	-	-	-	-	-	12	-
9	M9	-	-	-	4	-	-	-	20	-	-	-	-
10	M10	10			25	12	12	-	10			4	8
11	M11	-	-	-	-	-	-	-	-	-	-	-	-
12	M12	-	-	-	-	-	-	-	-	--	-	6	-
13	M13		-	-	-	-	-	-	-	-	-	-	-
14	M14	-	-	-	-	-	-	-	14	-	-	-	-
15	M15	-	-	-	-	-	-	-	20	-	-	-	-
16	M16	-	-	-	10	-	-	-	-	-	-	6	-
17	M17	-	-	-	-	-	-	-	-	-	-	-	-
18	M18			12	6				20			20	
19	M19	-	-	-	-	-	-	-	-	-	-	-	-
20	M20	12	14	-	26	-	12	-	20	-	12US	20	12
21	M21	-	-	-	-	-	8	-	-	-	-	-	-
22	M22	-	-	-	-	-	-	-	-	-	-	-	-
23	M23	-	-	-	-	-	-	-	10	-	-	6	-
24	M24	-	-	-	-	-	-	-	16	-	-	10	-
25	M25	-	-	-	12	-	-	-	5	-	-	-	-

The total percentage of inhibition by actinomycetes against bacteria in primary screening was noted as *E.coli*-8%, *K. pneumoniae*-4%, *P.vulgaris*-4%, *P.aeruginosa*-24%, *S.typhi*-4%, *S.flexneri*-16%, *V.cholera*-4%, *B.bronchiseptica*-52%, *P.fluorescens*-0%, *E.faecalis*-8%, *B.subtilis*-40%, *S.aureus*-8%. Totally 20 (80%) of actinomycetes showed antibacterial activity towards any one of the tested bacteria, 5 (20%) actinomycetes showed no antagonistic activity (Figure 1). Only 2 actinomycetes were selected from *A. marina* and that were subjected for secondary screening.

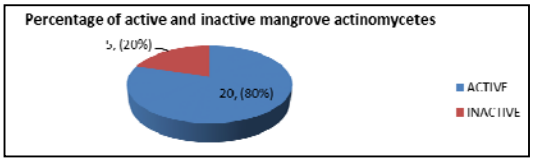


Fig 1: Percentage of active and inactive actinomycetes from A.marina

It was concluded that the mangrove actinomycetes were strong in their antibacterial activity in primary screening by agar plug method.

Antibacterial activity of actinomycetes in secondary screening by agar well diffusion method

The active isolates selected from primary screening were subjected for secondary screening by agar well diffusion method. The isolates which grew very well and produced antibiotic compound large quantity in liquid media were selected as most potent isolates. 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 actinomycetes in liquid media was varying from the antibiotic production and antibacterial potency in solid agar medium.

Table 3: Antibacterial activity of active isolates in secondary screening by agar well diffusion method

S.no	Mangrove plants	Isolates 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
1	Avicennia	M10	10	-	6	20	20	10	-	8	-	--	-	8
2	marina	M20	-	-	-	16	12	18	10	14	-	-	20	10

Out of 25 actinomycetes from *A. marina*, two active isolates were selected from primary screening of antibacterial activity and subjected for secondary screening by agar well diffusion method. The inhibition percentage for bacteria by actinomycetes were noted as *E.coli*-50%, *K. pneumoniae*-0%, *P.vulgaris*-50%, *P.aeruginosa*-100%, *S.typhi*-100%, *S.flexneri*-100%, *V.cholera*-50%, *B.bronchiseptica*-100%, *P.fluorescens*-0%, *E.faecalis*-0%, *B.subtilis*-50%, *S.aureus*-100%.

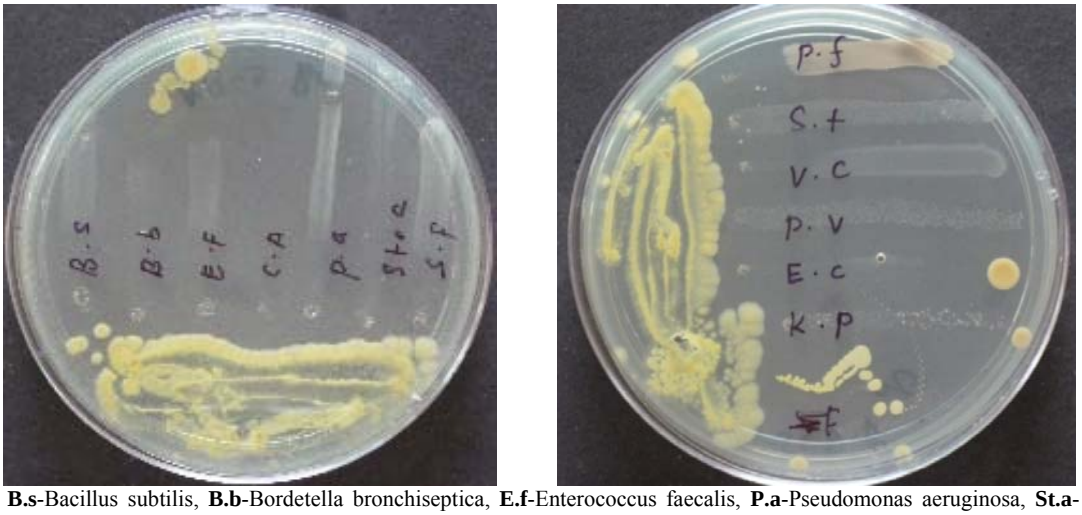
It was observed that the inhibition of number of tested bacteria by the actinomycetes by agar plug method in primary screening was not coincided hundred percentage with the agar well diffusion method in secondary screening, because some actinomycetes grew well in solid medium and had shown antagonistic activity effectively towards the tested bacteria by agar plug method, but same isolates in liquid medium did not show any antagonistic activity towards the tested bacteria by agar well diffusion method in secondary screening. In few cases of actinomycetes, number of inhibition of tested bacteria by the actinomycetes by agar plug method in primary screening coincided with agar well diffusion method in secondary screening. The active isolates, strong in their

antibacterial activity both in agar plug method (Solid media) and in agar well diffusion method (Liquid media) were selected for confirmatory test for antibacterial activity.

Cross streak method to confirm the activity of selected isolates

Cross streak method was used for confirmation of antibacterial activity for most active isolates. The active isolates which grew better in nutrient agar plates were subjected to cross streak method. It was noted that the active isolates inoculated in nutrient agar plates showed very good antibacterial activity than the isolates inoculated in starch casein agar, potato dextrose agar, yeast malt extract agar. Presence of peptone in nutrient agar may be a one of the reason for exhibiting very good antibacterial activity.

The isolate M20 was active for both gram +ve and gram negative bacteria. The bacterial test species in the order of decreasing sensitivity were *Pseudomonas aeruginosa* (32mm) = *Bordetella bronchiseptica* (32mm) = *Bacillus subtilis* (32mm)> *Shigella flexneri* (30mm) = *Staphylococcus aureus* (30mm)>*Enterococcus faecalis* (28mm)> *E. coli* (22mm)> *Pseudomonas fluorescens* (12mm) = *Vibrio cholera* (12mm)> *Salmonella typhi* (8mm) = *Klebsiella pneumonia* (8mm). Very thin detached colonies without the biofilm formation of *Proteus vulgaris* and *Klebsiella pneumonia* were also observed.



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

Plate 1: Antibacterial activity of isolate M20 by cross streak method

4. Conclusion

Antibiotic resistance is evident both in human and phyto pathogens. The wide spread antibiotic resistance in microbes initiated the search and find new antibiotics to control the resistant microbes. In nature, antagonistic microbes play a vital role by suppressing the activity and spread of plant and human pathogens. Actinomycetes are the strongest antagonists among microbes, producing effective and safe antibiotics. Since the mangrove actinomycetes are potential source for producing antibiotics for bacteria, these can be used in the pharmaceutical field to find novel drugs for bacterial infections.

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