Research Article

Facinating Actinomycetes from Unexplored Regions of Pichavaram Mangrove Ecosystem

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ABSTRACT

The present study focused on the isolation of fascinating microbes from mangrove region. Actinomycetes are one of the most attractive sources of all types of bioactive metabolites that have important applications in human medicine. Antibiotics are major secondary metabolites produced by Actinomycetes. One hundred and ten actinomycetes were isolated from mangrove soil sediments from Pichavaram in our study. Due to the broad spectral activity the present study was emphasized on the fifty four strains which possess good activity. The antagonistic activity of chosen actinomycetes were tested. The isolates were streaked on agar plate followed by the test pathogens (*Staphylococcus aureus*, *Klebsiella pneumonia*, *Proteus vulgaris*, *Escherichia coli*, *Enterococcus faecalis* and *Serratia marcences)* were cross streaked. *Nocardiopsis prasina* ACT 24 showed very good activity against the selected human pathogen Vancomycin resistant enterococcus. The screening reveal that antibiotic from ACT24 has potential to inhibit the vancomycin resistant enterococci and this can be exploited for further treatment in future by analysing the structure of the compound.

Keywords: Mangrove, Nocardiopsis, Isolation, Antimicrobial agent.

INTRODUCTION

Marine ecosystems are a rich source of novel actinomycetes which have the capacity to produce interesting new bioactive compounds including antibiotics[1]. The marine sediments were found to contain a wide range of unique microorganisms which were not found in terrestrial environment provided many interesting, unique and novel secondary metabolites. Mangroves are highly productive ecosystems. There is evidence that mangrove sediments contain high populations of actinomycetes. In recent years there has been a growing awareness of the potential value of marine water habitat as source of actinomycetes that produce useful metabolic products. Actinomycetes are the most economically and biotechnologically valuable prokaryotes. The few actinomycetes which have been isolated from mangrove habitats are a potentially rich source for discovery of anti-infection and antitumor compounds. Reports from other parts of the world describe the potential role of marine actinomycetes in production of bioactive compounds[2].

Actinomycetes are a kind of bacterial group that is intensively studied because of its antimicrobial activity. Actinomycetes are one of the most attractive sources of all types of bioactive metabolites that have important applications in human medicine. Antibiotics are major secondary metabolites produced by Actinomycetes. On the other hand, infectious diseases and resistant strains are increasing progressively due to antibiotics treatments. Therefore, efforts to find new antibiotics are crucial[3]. Antibiotic resistance continues to pose a significant threat to the clinical and pharmaceutical world. Many common hospital pathogens are becoming multi-drug resistant and are unable to treat with many/any of the commercially available antibiotics. There is a need for novel antimicrobial compound is greater now than ever before. In particular, actinomycetes from less-explored habitats show exceptional promise to produce novel antimicrobial compounds[4]. The present study was to investigate the antimicrobial activities for a potentially wider and more specific therapeutic use of actinomycete isolates from aquatic habitats.

MATERIALS AND METHODS

Sample collection

Marine sediments were collected from Mangrove Pichavaram from cuddalore, tamilnadu. Samples were collected random in polythene bags and transferred to laboratory for further investigation.

Isolation of actinomycetes

For each collected sample, 1g of the marine samples were suspended in 100 ml of distilled water then incubated in an orbital shaker incubator at 28 C with shaking at 200 rpm for 30 min. Mixtures were allowed to settle. Isolation and enumeration of actinomycetes were performed by serial dilution and spread plate technique. One gram of soil was suspended in 9 mL of sterile double distilled water. The dilution was carried out up to 10^{-5} dilutions. Aliquots (0.1 mL) of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were spread on the starch casein agar (Himedia). To minimize the fungal and bacterial growth, actidione 20 mg/L and nalidixic acid 100 mg/L were added. The plates were incubated at 30 °C for 10 d. Based on the colony

morphology, the actinomycete cultures were selected and purified on SCA (Himedia) medium[5].

Morphological characterization

Morphological and cultural characteristics of the selected actinomycetes strain was studied by inoculating in to sterile SCA (Himedia) media. The media were sterilized and poured in to sterile petridishes. After solidification of the media, the selected culture was streaked on the surface of the media and incubated at 28^{0}C for 7 days. Morphological characters such as colony characteristics, type of aerial hyphae, growth of vegetative hyphae, fragmentation pattern and spore formation were observed[6].

Screening of antimicrobial activity

Screening allow the discarding of many valueless microbes and helps in the isolation of the organism of interest in a large microbial population. The anti microbial activity of the isolates were tested by cross streak method employing nutrient agar medium for bacterial cultures (*Staphylococcus aureus*, *Bacillus subtillus*, *Klebsiella pneumonia*, *Escherichia coli*, *Enterococcus faecalis* and *Serratia marcenses*). The media were sterilized by autoclaving at 121˚C for 15 min and the molten sterile medium poured in to petridishes and allowed to solidify. Each plates were streaked with one isolate at the centre and incubated at 28˚C for 7 days. After 7 days test organisms were streaked perpendicular to the growth of isolate and incubated at 37˚C for 24hr[7].

Liquid culture of active substance

The active isolates resulted from primary screening, were tested for their extra cellular antibiotic production capabilities under submerged fermentation conditions. A loopful inoculum of potential actinomycetes strain (*Nocardiopsis prasina* ACT 24) was inoculated in to 500 ml. Erlenmeyer flask containing 100ml of Actinomycetes isolation broth and kept at 28˚C for 72hrs with continuous shaking. After incubation 20ml of aliquots transferred to 1000ml of SC broth and incubated at 28˚C for 7 to 10 days[8].

Extraction of active substance

After incubation the crude antibiotic extract was recovered from the broth culture filtrate by solvent extraction method using different solvents like Ethyl acetate, methanol, ethanol, dichloromethane and butanol in accordance with the description of author[9]. Ethylacetate were added to the filtrate in the ratio 1:1 (V/V) and shaken vigorously for 1hr to complete extraction. The ethylacetate phase that contained the antibiotic was separated from the aqueous phase and concentrated in vacuum at 60˚C using rotary evaporator. The residue obtained were weighed and reconstituted in 50% methanol to make a working concentration of 10 mg/ ml for antibacterial assay[10].

Antimicrobial activity assay

Antibacterial activities of the extracts were tested by using well diffusion method as described by Kirby-Bauer (1979) with modification. The inoculums was prepared by mixing a few bacterial colonies from exponential phase with 9ml of sterile nutrient broth and incubated at 37˚C for 24hrs. After incubation the 24hrs culture were used to swab on Muller hinton agar (MHA) surface. The well was prepared in the plate by using sterile cork borer (6mm in diameter). A volume of 100µl of 10mg/ml of extracts was carefully dispensed in to each well and allowed to diffuse for 2h and incubated at 37˚C for 24hrs. The sterilized methanol was used as negative control. After 24h of incubation, zone of inhibition around each well was recorded[11].

RESULT AND DISCUSSION

Isolation of actinomycetes

One hundred and ten isolates of actinomycetes were isolated from mangrove soil sediments from Pichavaram in our study. The isolates were isolated based on different colony morphology and similar colonies were not taken. They were purified and maintained on Starch Casein Agar medium in refrigerated condition.

The soil samples were dried and sieved to remove gravels and debris. They were processed and preheated at 55˚C to minimize the bacterial and fungal growth. The presence of large population of actinomycetes (Table-1) indicating that Pichavaram was suitable ecosystem for isolation of novel antibiotic producers.

Table 1: Number of actinomycetes colony from pichavaram soil sample

S.No	Sample site	Dilutions	No of actinomycetes colony		
			SCA	MNGA	AIA
1	P1	10^{-2}-10^{-6}	12	8	15
2	P2	10^{-2}-10^{-6}	8	2	15
3	P3	10^{-2}-10^{-6}	8	12	14
4	P4	10^{-2}-10^{-6}	15	5	6

SCA- Starch casein agar; MNGA- Modified nutrient glucose agar; AIA- Actinomycetes isolation agar.

Similarly, Valli *et al*., (2012) reported that the frequency of novel bioactive compounds discovered from terrestrial actinomycetes decreases with time, much attention has been focused on screening of actinomycetes from diverse environments for their ability to produce new secondary metabolites[12]. As marine environmental conditions are extremely different from terrestrial ones, it is summarized that marine actinomycetes have different characteristics and have adapted to life in the sea. Therefore they might produce different types of bioactive compound. Sirisha *et al*., (2010)[13] reported that among the 90 isolates 63 showed highest activity against test pathogens. out of 290 actinomycetes isolates 180 isolates were active against test pathogens[14].

Morphological characterization

The color of the substrate and aerial mycelium were varied. ACT-25 and ACT-36 produced diffusible pigment produced on production medium (Table-2). The author reported that two isolates was produced diffusible brownish pigment on peptone yeast extract agar[6].

Table 2: Colony characters of the selected isolates

Isolate	Aerial mycelium	Substrate mycelium	Soluble pigment
ACT-1	Greyish white	Greyish white	No pigmentation
ACT-23	Grey	Grey	No pigmentation
ACT-24	White	4352342342D	Z
ACT-35	White	White	No pigmentation
ACT-36	Sandal	Cream	Yellow

Among the 110 isolates isolated from marine sediments, 5 isolates showed antibacterial activities against at least one of the tested bacteria. In perpendicular streak plate method, results revealed that that the isolates ACT-34 and ACT-35 exhibited broad spectrum activities against tested bacteria. The isolate ACT-35 showed potential activity against more than four pathogenic organisms which mentioned earlier. Gebreselema Gebreyohannes *et.al* .,(2013) revealed that the isolate LT002 showed potential activity against *E.Coli* and *Pseudomonas aeroginosa*[11].

Screening of antimicrobial activity

Fifty four out of one hundred and ten actinomycetes isolates showed remarkable activity against gram positive and gram negative bacteria by cross streak and agar method. The antibacterial activity was measured by zone of inhibition (mm) (Table-3) against tested pathogens. Most of them were highly inhibits *Staphylococcus aureus* and *Enterococcus faecalis*. It could be desired that the antibacterial substance produced by isolates had a broad spectrum activity.

Due to the broad spectral activity the present study was emphasized on the fifty four strains which possess good activity. The isolates were cultivated in starch casein broth incubated in shaking condition for 5 days. The culture filtrate were collected and subjected to antibacterial activity by using well diffusion method. Based on zone of inhibition, five isolates were selected for further study namely ACT-1, ACT-24, ACT-25, ACT-35 and ACT-36.

The majority of antibiotics that have been isolated in the numerous screening program concerned with the search of new chemotherapeutic agents have been tested primarily for their activity against different bacteria[15]. The actinomycetes are prolific producers of antibiotics and other industrially useful secondary metabolites[16]. They have provided more than half of the naturally occurring antibiotics discovered to data and continue to be screened for useful compounds. The pretreatment was introduced to construct an efficient method with which to isolate the members of the *Streptomycetes species* cluster from soil[17]. In this present study the soil samples were pretreated at 50ºC.

Among the 31 actinomycetes isolated from water and sediments of lake tana, 13 isolates showed antibacterial activities against at least one of the tested bacteria[11].

Table 3: Sensitivity of pathogenic microorganisms against actinomycetes isolates

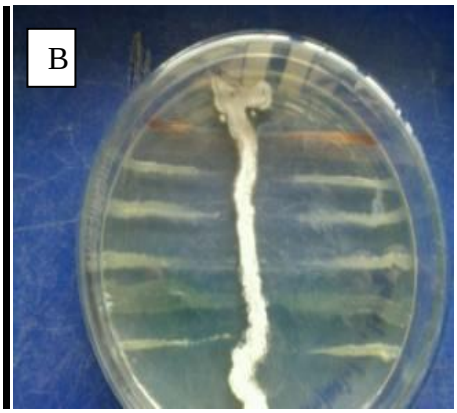
Isolates	Zone of inhibition (mm)					
	S.aureus	*B.subtilus*	*K.pneumonia*	*E.coli*	*S.marcences*	*E.faecalis*
ACT-1	23	22	11	13	19	21
ACT-21	-15	14	19	22	23	20
ACT-22	8	21	20	15	17	12
ACT-24	11	13	12	12	9	9
ACT-25	15	13	18	18	16	14
ACT-26	10	11	12	11	11	8
ACT-28	12	15	12	16	12	16
ACT-33	20	12	8	7	6	4
ACT-34	22	13	12	10	14	12
ACT-35	14	14	14	14	10	15
ACT-38	13	12	19	20	22	24
ACT-39	15	16	18	18	16	14
ACT-44	24	22	13	12	11	11

Table showing the antibacterial profile of thirteen isolates the zone of inhibition ranges from 4mm-24mm in diameter

Singh *et al.,* (2014) isolated 37 actinomycetes from phoomdi (floating putrefying vegetation) in Loktak Lake in Manipur, India. Twenty one (56.7%) isolates showed antimicrobial activities against test microorganisms in

primary screening[18]. Of these, 12 (32.4%) were found to have broad spectrum (antibacterial and antifungal) activities. Srisha *et.al*., (2013) reported that among the 90 isolates 63 showed highest activity against test pathogens[13]. Similarly, the author isolates 35 actinomycete isolates from 3 different soil samples collected in Coimbatore. By performing agar disc method, 12 actinomycetes produced antibiotic against test pathogens[19].

Plate 1: Antagonistic activity of actinomycetes against Gram positive and Gram negative pathogens



Nocardiopsis prasina ACT 24 showing activity against *S.marcences (11mm), E.faecalis (12mm), S.aureus (13mm), B.subtilus (13mm), K.pneumonia (8mm), E.coli (9mm)*

The antagonistic activity of chosen actinomycetes were tested. The isolates were streaked on agar plate followed by the test pathogens (*Staphylococcus aureus*, *Klebsiella pneumonia*, *Proteus vulgaris*, *Escherichia coli*, *Enterococcus faecalis* and *Serracia marcences)* were cross streaked. The strains produced different level of activity against six pathogens. If growth of the test organisms occurred in the entire streak line, then the antimicrobial activity of the isolate was recorded as negative. The result of primary and secondary screening revels that most of the active isolates were active against gram positive bacteria (*S.aureus*) than gram negative bacteria (*E. coli and P. aeruginosa*). The reason for different sensitivity between gram positive and gram negative bacteria could be explain to the morphological differences between these microorganisms, gram negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, The gram positive should more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier[14].

Agar well diffusion method was used to determine the efficacy of ACT1, ACT24, ACT25, ACT35 and ACT36. It was observed that all the isolates inhibit one or more pathogens, the zone of inhibition ranges from 2mm-34mm. The extracts were more effective in inhibiting gram positive bacteria than gram negative bacteria was revealed by wider zone of inhibition around the well (Table-4).

The particulate matter in the sample being tested is much less likely to interfere with the diffusion of the antimicrobial substance into the agar than in the filter paper disc. A polar compound would not be influenced by the hydroxyls on the surface of the paper and would diffuse easily. Thus, in this case, well agar diffusion method is more convenient than the disc variant. This theory explains at least in part, the higher sensitivity detected by well agar diffusion method[20].

The filtrate were bioassayed against *Staphylococcus aureus*, *Proteus vulgaris*, *Escherichia coli*, *Klebsiella pneumonia* and Vancomycin resistant *Enterococcus faecalis*. Five isolates exhibited high activity against test pathogens. The highest activity was seen in the strain ACT 24 which showed 34 mm of zone of inhibition against *Staphylococcus aureus.* ACT 36 showed (2mm) least activity. Since the strain ACT 24 showed highest activity was selected for further study.

Table 4: Antibacterial activity of the culture filtrates of the five selected isolates of actinomycetes against test pathogens.

Test organisms	Zone of inhibition (mm)				
	S.aureus	*P.vulcaris*	*VRE*	*E.coli*	*K.pneumonia*
ACT1	10	24	24	12	2
ACT24	34	23	28	18	3
ACT25	7	22	21	10	8
ACT35	4	7	10	11	13
ACT36	23	9	2	10	14
Streptomycin	2	26	24	4	5
Erythromycin	15	14	NG	17	15

VRE: Vancomycin Resistant Enterococcus, Streptomycin- Positive control, Erythromycin- Negative control, NG- No growth.

Nocardiopsis prasina ACT 24 showed very good activity against the selected human pathogen Vancomycin resistant enterococcus. ACT 35 showed lesser activity compared to other selected strains. ACT 1 and ACT 35 also showed similar results. Among the five strains ACT 24 selected for further antibiotic studies. Tahahashi *et al*,.

(1985) reported that the isolate *Nocardiopsis* was active to Gram positive and Gram-negative bacteria except *Serratia* and *Pseudomonas*[21].

The antimicrobial activity of DRQ 10 and DRQ 72 test isolates against *S.typhi* and *S.aureus* were similar. This may be due to the biogeographical attributes and habitat conditions from where the isolates obtained[22].

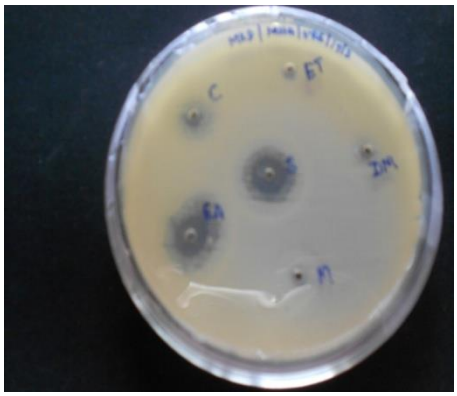
Well diffusion method is more sensitive than disc diffusion method. The previous study findings correlated with the existing report where the strain SRB25 was more similar to *Streptomyces* with antimicrobial property against multidrug resistance *S. aureus*[23].The active compound of Streptomyces KUAP106 was effective against Gram-positive, Gram-negative and unicellular filamentous fungi[24].

The selected isolates were subjected to secondary antimicrobial activity screening against clinical bacterial isolates VRE (Vancomycin resistant *Enterococcus*). Antimicrobial activity of ACT24 was more or less similar to that of standard antibiotics used as positive control. Yucel and Yamac (2010) reported that antimicrobial activity of isolate 1492 was higher than all of the isolates and the standard antibiotics[14].

Antimicrobial activity

The secondary metabolites of actinomycetes strains which were selected from primary screening were undergone for antimicrobial activity of well diffusion method. These strains produced different level of activity against test pathogens. However the maximum zone of inhibition 30mm in diameter (Plate-2) was noticed by the actinomycetes strain Nocardiopsis prasina ACT 24 .

Plate 2: showing the zone of inhibition of ethyl acetate solvents extract of *NOCARDIOPSIS PRASINA ACT 24 against Vancomycin Resistant Enterococcus*



. S- Sample; EA- Ethyl acetate; M- Methanol; DM- Dichloro methane; ET- Ethanol; C- Chloroform.

CONCLUSION

The need for new, safe and more effective antibacterial is a major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in drug resistant pathogens. The present study shown that marine sediments posses various antibiotic producing actinomycetes *Nocardiopsis prasina* ACT24 demonstrated the obvious inhibitory effect against *Vancomycin resistant enterococcus*. The potential of the compound in the control of *Vancomycin resistant enterococcus* should also be further evaluated.

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