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Isolation of actinomycetes from mangrove and estuarine sediments of Cochin and screening for antimicrobial activity

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ABSTRACT

Objective: To isolate and screen actinomycetes for antimicrobial activity from mangroves and estuarine soil samples of Cochin.

Methods: In the present study, sediment samples collected from mangroves and various stations of Cochin estuary were pretreated and actinomycetes were isolated on different selective media. The isolates were screened for antibiotic activity by following disc diffusion assay (Kirby-Bauer method) against human pathogens, fish pathogens and Gram-positive bacteria. The isolates were identified based on their morphology.

Results: Only 2 actinomycete isolates (ER7 and ER10) of the 50 isolates screened had antimicrobial activities against one or more pathogens tested. ER7 isolate showed higher antimicrobial activity as compared to that of ER10 isolate. The maximum inhibition zone of crude extract from ER7 was 16.7 mm. The methanol extract of ER7 showed antimicrobial activity against all the pathogens tested with a maximum zone of 21.0 mm. The isolates with antimicrobial activity were found to belong to the genus *Streptomyces*.

Conclusions: There is no significant report on bioactive actinomycetes from the present study areas. Potent antibiotics from the selected isolates could contribute to fight against several human and fish diseases. Further purification, structural elucidation and characterization are recommended to know the quality, novelty and commercial value of these antibiotics. Hence, the mangroves and estuary of Kochi show great promise for the discovery of bioactive actinomycetes.

1. Introduction

Actinomycetes are a group of microorganisms which morphologically resemble fungi and physiologically resemble bacteria[1]. They are Gram-positive, free living and saprophytic bacteria having high guanine-cytosine content (> 55%) in their DNA. They are the most economical and biotechnologically important class of prokaryotes producing secondary metabolites notably antibiotics, anti-tumor agents, immunosuppressive agents, enzymes and enzyme inhibitors[2].

Marine environment is a huge treasure accretion of marine actinomycetes resources[3]. As marine environmental conditions are different from terrestrial ones, it is observed that marine actinomycetes differ in physiological, biochemical and molecular characteristics from those of terrestrial counterparts and therefore might produce different types of bioactive compounds[4]. With an increase in the multi-drug resistance of the pathogens and collapse of usual terrestrial sources, marine drugs are in demand. Marine-derived antibiotics are more efficient in battling microbial infections because the terrestrial bacteria have not developed any resistance against them[5].

India has a wide sea area which is highly rich in biodiversity. A little of them is known about the actinomycete diversity of marine sediment, which is a bountiful resource. Many reports describe that the East Coast area is a major source of actinomycetes in India[6]. However, only few reports are available on actinomycetes diversity in the west coast of India and mangrove soil of India[7]. Mangroves are specialized marine environment which widely distributed along the coastlines and are rich in bacterial flora. Estuaries act as transition zone between two aquatic ecosystems, namely freshwater and marine. These are highly productive ecosystems and provide big support to the inhabitants of many coastal communities through their role in seafood production and nurturing of many valuable marine organisms. These areas are also rich in biodiversity and act as breeding and nursery grounds for fin/shell fishes.

The present work is aimed to study the antimicrobial activity of the actinomycetes isolated from the mangrove and estuarine sediments of Cochin estuary.

2. Materials and methods

2.1. Study area

The Cochin Estuary is the second largest wetland ecosystem in India sustaining rich bio-resources. The estuary is connected to the

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Arabian Sea at two locations, Cochin (latitude 9°58' N) and Azhikode (latitude 10°10' N) and is divisible into two parts: the southern arm extending from Cochin to the south and the northern arm extending from Cochin to Azhikode (Figure 1).

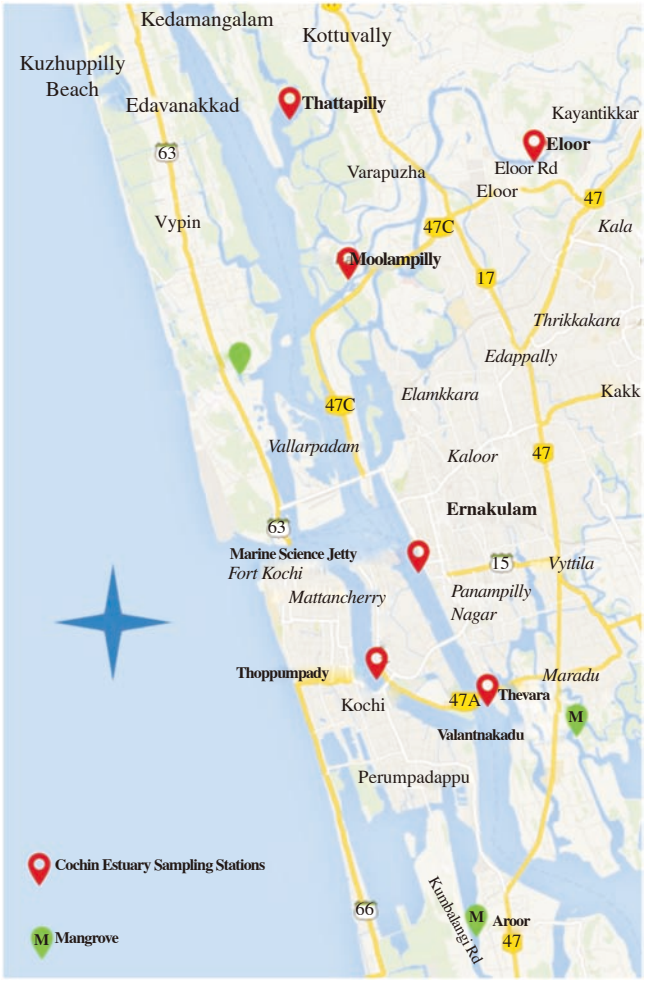

Figure 1. Map showing the sampling stations in Cochin Estuary and mangroves.

Sediment samples were collected from six different stations of Cochin estuary, Thevara, Thopumpady, Marine Science Jetty, Moolampilly, Thattapilly and Eloor and from three mangrove regions of Kochi-Puthuvyppu, Aroor and Valanthakadu.

Puthuvyppu is a mangrove nursery of fisheries station, inside the campus of Kerala Agricultural University and is free from sewage inputs. Aroor is a dwindling mangrove site with low plant density. Mangroves of Valanthakadu constitute a highly complex mangrove ecosystem with quite rich biodiversity.

2.2. Sampling and isolation of actinomycetes

A total of 22 marine sediment samples were collected from different stations using a van veen grab and were placed in small pre-labeled plastic bags using a sterile spatula and tightly sealed. The samples were preserved in an ice box and brought to the lab.

2.2.1. Pre-treatment of the sediment samples

Ten grams of the sediment from each location were weighed and suspended in 90 mL sterile saline water. It was then kept in an orbital shaker at 170 r/min for 30 min. The samples were then incubated at 65 °C for 5 min.

2.2.2. Isolation of actinomycetes from sediment samples

Actinomycetes were isolated by pour plate and spread plate technique by using starch casein agar, Kusters agar, glycerol-arginine agar and actinomycete isolation agar and incubated at 28 °C for 2 to 3 weeks. The media were supplemented with gentamicin sulphate (25 μg/mL) and nystatin (50 μg/mL) to minimize bacterial and fungal contamination, respectively. The actinomycete colonies that appeared on the petri plates were counted from the 7th day of incubation, up to the 28th day. Morphologically, diversed actinomycete colonies were sub-cultured, purified and maintained in nutrient agar (50% sea water) slants.

2.3. Primary screening

Agar slant cultures of actinomycete strains were inoculated into 250 mL Erlenmeyer flasks, each containing 50 mL of the production medium having the composition (g/L: 20 starch, 4 peptone and 8 yeast extract). The cultures were incubated on a rotary shaker (180 r/min) at ambient temperature for 14 days and the culture supernatant was tested for the antimicrobial activities by disc diffusion method by using the Kirby-Bauer technique[8].

2.4. Tested microorganisms

The antimicrobial activity of the isolates was tested against bacterial and yeast pathogens associated with human diseases, *viz. Escherichia coli* (*E. coli*), *Klebsiella* sp., *Proteus* sp., *Enterobacter* sp. and *Candida* sp. (yeast pathogen), Gram-positive bacteria, *viz. Bacillus* sp., *Planococcus* sp. and those associated with fish diseases, *viz. Vibrio parahemolyticus* MCCB 141, *Vibrio harveyi* MCCB 151 (*V. harveyi*), *Vibrio alginolyticus* MCCB 142 (*V. alginolyticus*) and *Aeromonas* sp. MCCB 152. All these isolates were obtained from the National Centre for Aquatic Animal Health, CUSAT, Kerala.

2.5. Secondary screening

The actinomycete isolates which showed antimicrobial activity against one or more pathogens were incubated in 50 mL production medium on a rotary shaker (180 r/min) at ambient temperature for 14 days. After the incubation period was finished, the extracellular metabolites were extracted with different solvents like *n*-hexane, ethyl acetate, chloroform, butanol and methanol. The extracts were concentrated in a rotary vacuum evaporator and tested for antimicrobial activity by disc diffusion method by using the Kirby-Bauer technique[8].

2.6. Identification of bioactive actinomycetes

The bioactive actinomycetes were identified up to the genera level based on morphological, cultural, physiological, biochemical, colour and carbon utilization tests as per the methods of the International *Streptomyces* Project (ISP)[9].

2.7. Statistical analysis

The statistical difference between mean values of the antimicrobial activity of the crude extract and its corresponding solvent extract of the positive isolate was determined by the student's *t*-test. Differences were considered significantly when the probability was less than 0.05.

3. Result

3.1. Sampling and isolation of actinomycetes

In the course of screening for novel antibiotics, 42 actinomycete strains were isolated from estuarine and mangrove sediments and collected from Cochin estuary and mangroves of Aroor,

Valanthakadu and Puthuvyppu. The isolates were designated as ER1 to ER42 based on their colony morphology observed on the master plate. The isolates were small to medium size, grayish white to pure white in colour, round and powdery. The majority of the strains were isolated from the Kusters agar as compared to Starch casein agar, Actinomycete isolation agar and glycerol-asparagine agar. The soil of Puthuvyppu mangroves gave a higher number of actinomycete isolates (13 isolates) as compared to other soil samples.

3.2. Screening of isolated actinomycetes for their antimicrobial activities

3.2.1. Primary screening

Out of 42 isolates, only 2 isolates (ER7 and ER10) showed antibacterial activity against human and fish pathogens. The ER7 isolate showed antimicrobial activity against all human and fish pathogens and the activity was comparatively higher than that of the ER10 isolate (Table 1). Both the isolates were not active against *Candida* sp. The antimicrobial activity of ER7 against the pathogens tested is given in Figure 2.

Table 1

Antimicrobial activity of the crude extract (cell free supernatant) of the isolates ER7 and ER10.

Tested pathogen	Diameter of zone of inhibition (mm)	
	ER7	ER10
Bacillus sp.	16.7	11.0
Planococcus sp.	14.0	10.0
Proteus sp.	11.0	-
Klebsiella sp.	12.0	9.0
Aeromonas sp.	9.7	-
E. coli	10.0	8.0
V. alginolyticus	15.0	-
Vibrio parahemolyticus	-	-
V. harveyi	10.0	
Candida sp.	-	-

3.2.2. Secondary screening

The antimicrobial activity of all the solvent extracts of the isolate ER7 was tested and it was observed that the methanol extract showed antimicrobial activity against all human and fish pathogens but not against the yeast pathogen *Candida* sp. (Table 2).

Table 2

Antimicrobial activity of the methanol extract (cell free supernatant) of the isolate ER7.

Tested microorganisms	Diameter of zone of inhibition (mm)
Bacillus sp.	21.0
Planococcus sp.	20.0
Proteus sp.	12.0
Klebsiella sp.	13.3
Aeromonas sp.	12.0
E. coli	13.0
V. alginolyticus	18.0
V. harveyi	12.0

3.3. Morphological and biochemical characterization of ER7 and ER10 isolates

The ER10 and ER7 actinomycete isolates showed excellent growth and abundant aerial mycelial formation on international streptomyces projects (ISP) medium No. 4 (inorganic salts-starch agar). The ER10 isolate showed good growth on ISP medium No. 2 (yeast extract-malt extract agar) and ISP medium No. 3 (oat meal agar) and moderate growth on ISP medium No. 5 (glycerol asparagine agar base). The ER7 isolate showed moderate growth on ISP medium No. 2, 3 and 5. The aerial mycelium of ER7 is simple and straight, flesh coloured with sparse substrate mycelium. The aerial mycelium of ER10 is simple and spiral, flesh coloured with sparse substrate mycelium. The aerial and substrate mycelium are media dependent. By comparing the morphology of spore-bearing hyphae of both the ER7 and ER10 isolates with the entire spore chain and structure of the spore chain with the actinomycetes morphologies, they found to belong to the genus *Streptomyces*. The morphological and biochemical characteristics of ER7 and ER10 are presented in Tables 3 and 4.

Table 3

Morphological and biochemical characteristics of ER7 and ER10 on different cultural mediums.

Medium	Isolate No.	Day of observation	Colour of aerial mycelium	Colour of substrate mycelium
ISP 2	ER7	7, 10, 14	-	Yellow
	ER10	7	White	Yellow
	ER10	10, 14	Flesh	Yellow
ISP 3	ER7	7, 10, 14	-	Yellow
	ER10	7, 10, 14	Flesh	Yellow
ISP 4	ER7	7, 10	-	Yellow
	ER7	14	Flesh	Yellow
	ER10	7, 10, 14	Flesh	Yellow
ISP 5	ER7	7, 10, 14	-	Yellow
	ER10	7, 10, 14	-	White

Table 4

Biochemical characteristics of ER7 and ER10.

Biochemical tests	ER7	ER10
Grams stain	+	+
Aerial mycelium	Flesh	Flesh
Colony colour	Flesh	Flesh
Melanin	-	+
Carbon sources (1% w/v)		
D-glucose	+	+
Sucrose	+	+
D-fructose	+	+
Raffinose	+	+
Cellulose		+
L-rhamnose	+	+
Mannitol	+	+
Inositol	+	+
L-arabinose	+	+
Casein	+	+
Xanthine	-	+
Hypoxanthine	-	-
Tyrosine	-	+
Urea	+	+

Figure 2. Antimicrobial activity against *Bacillus* sp., *Aeromonas* sp, *Klebsiella* sp. and *Proteus* sp. shown by ER7 isolate (Kirby-Bauer method).

3.4. Statistical data analysis

After performing the student *t*-test, it was found that there was no significance difference between the mean values of antimicrobial activities of the crude and methanol extracts of ER7.

4. Discussion

This study was undertaken with an aim of isolating novel antibiotic producers from unexplored ecosystem and selecting the potent strains. It seems timely to extend this approach to another poorly studied environment, such as the mangroves of Kochi and Cochin estuaries.

In this study, maximum morphologically different actinomycetes were obtained from mangroves than from estuarine sediment. Puthuvyppu mangrove is an ecosystem which is rich in traditional, medicinal and ornamental plants and where conditions are good for microbial growth. In the present study, the maximum number of actinomycetes was also isolated from Puthuvyppu mangroves.

Kusters medium was reported as the most suitable medium for the isolation of actinomycetes from the water, sediment, seaweed and molluscs samples since it contains glycerol that most actinomycetes are used as a carbon source[10,11]. In the present study, also, maximum number of actinomycetes were isolated from Kusters medium.

The bioactive strains, ER7 and ER10 were characterized and were found to belong to the genus *Streptomyces*. Several previous reports from different geographical locations around the world have described the occurrences of *Streptomyces* in different mangrove habitats. In the Indian context, 150 *Streptomyces* strains were isolated from Muthupet mangroves in Tamil Nadu[12]. Several Streptomycetes, *viz*., *Streptomyces alboniger*, *Streptomyces violaceus, Streptomyces moderatus and Streptomyces aureofasciculus* were also reported from the Vellar estuary on the southeast coast of India[11].

In the present study, the selected *Streptomyces* sp. were active against Gram-positive bacteria (*Bacillus* and *Planococcus* sp.) and Gram-negative human and fish pathogens tested. Previous studies also show that *Streptomyces* sp. isolated from mangrove soil in the eastern coast of Surabaya, Indonesia were capable of producing a series of antibiotics against Gram-positive and Gram-negative bacteria[13]. Two *Streptomyces* strains were isolated from the sediment samples of Thai mangroves which were active against Gram-positive and Gram-negative bacterial pathogens[14].

In the secondary screening, the methanol extract of ER7 was found to contain the antimicrobial compound as it was active against all the pathogens tested. The methanol extract of *Streptomyces* isolated from Parangipettai mangrove rhizosphere sediment also showed similar antibacterial activity against Gram-negative bacterial pathogens[10].

Many actinomycetes do not show antifungal activity[14,15]. In the present study, also the isolates did not show antifungal activity when tested against the yeast human pathogen *Candida* spp. The zone of inhibition shown by ER7 was ranging from 9.7 mm to 16.7 mm which was comparatively higher than that reported by Suguna and Rajendran[16].

The results of the present study were interesting and encouraging because the extracts showed antibacterial activity against a wide range of microbes, both Gram-positives and Gram-negatives. However, further purification of the bioactive compound, identification and checking the stability of the isolates are important before considering the isolates for antibiotic production.

Actinomycetes from marine habitat remain under explored. The present study on marine actinomycetes from Cochin estuary and mangroves of Kochi provides useful information on morphologically different actinomycetes and clearly indicates that they are potent sources of bioactive compounds.

Conflict of interest statement

We declare that we have no conflict of interest.

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