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ANTIMICROBIAL ACTIVITY OF MARINE ACTINOMYCETE *STREPTOMYCES DHINAKARAN* 2011 (JF751041)

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ABSTRACT

Fourteen actinomycetes were isolated from soil collected from five different coastal sites of Tamil Nadu. The actinomycete designated as "A" was selected as the most potent actinomycete. Among different solvents used, ethyl acetate was found to be more effective in extraction of bioactive compounds. Minimum Inhibitory Concentration of extract was found to be 35.6µg/ml for *Klebsiella pneumoniae*. The actinomycete "A" strain had the ability to produce enzymes and the stability of extract was ideal at 8^0C for 30 days. The stability of the extract was drastically reduced above 60^0C and below pH 6. 16S rRNA sequence of the strain A showed 99% similarity with *Streptomyces* sp. 2438. The accession number (JF751041) for the sequence of strain A was obtained from NCBI. Based on the phylogenetic evaluation, the actinomycete was designated as *Streptomyces dhinakaran* 2011, which was capable of producing extracellular bioactive metabolites, that inhibits the growth of *Klebsiella pneumoniae* and other bacterial and fungal pathogens.

KEY WORDS: ***Streptomyces dhinakaran* 2011, Antimicrobial, *Klebsiella pneumoniae*, 16S rRNA sequence.**

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INTRODUCTION

The marine sediments were found to contain a wide range of unique microorganisms which were not found in terrestrial environment [1,2] provided many interesting, unique and novel secondary metabolites [3]. Actinomycetes which were capable of producing antimicrobial compounds have been isolated not only from terrestrial habitats but also from marine environments [4,5]. Therefore actinomycetes from marine sediments are excellent promising source of natural bioactive compounds. In recent times, various new species and genera of marine actinomycetes were reported [6,7,8]. Most of the actinomycetes are known to synthesize bioactive secondary metabolites like enzymes, herbicides, pesticides, vitamins and antibiotics [9]. Almost 80% of the world's antibiotics are known to come from the genera *Streptomyces* [10]. Hence in the present study, the actinomycetes were isolated, purified, screened and evaluated for their antimicrobial activity from five different marine coastal soil of Tamilnadu, India.

MATERIALS AND METHODS

COLLECTION OF SOIL SAMPLES

The soil samples were collected from marine coastal areas of Tamilnadu, India like, Marina beach Chennai ($13^0$03'20.09''N and $80^0$17'01.32''E), Tuticorin beach ($8^0$44'37.95''N and $78^0$10'11.43''E), Silver beach Cuddalore ($11^0$44'24.08''N and $79^0$47'11.68''E), Muttom beach Kanyakumari ($8^0$08'19.44''N and $77^0$18'11.77''E) and from Rameshwaram beach ($9^0$17'16.77''N and $79^0$18'58.25''E) on January 2010. The soil samples were collected from 15cm depth using sterile spatula and brought to laboratory in sterile bottles.

ISOLATION AND SCREENING OF ACTINOMYCETES

Isolation of actinomycetes was carried out by serial dilution and spread plate technique in Starch Casein agar medium [11]. The actinomycetes isolated were purified by quadrant streaking method. Antagonistic activities of the pure isolates of actinomycetes were tested preliminarily by cross streak method [12] and secondary screening by agar well diffusion method [13].

TEST ORGANISMS

The test organisms were obtained from MTCC Chandigarh, India. *Streptococcus mutans*, *Lactobacillus acidophilus*, *Escherichia coli*, *Salmonella paratyphi*, *Shigella sonnei*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Aspergillus flavus*, *Candida albicans*, *Streptococcus* sp., *Bacillus subtilis* and *Pseudomonas aeruginosa* were used in the study.

EXTRACTION AND ANTIMICROBIAL ASSAY

From the selected antagonistic actinomycetes the antimicrobial compound was extracted by [14] method by using various solvents like ethanol, methanol, acetone, ethyl acetate and the antimicrobial assay was done by agar well diffusion method. Nitrofurantoin, Kanamycin and Clotrimazole were used as positive controls. Minimum Inhibitory Concentration was determined by tube dilution method [15].

STABILITY OF THE EXTRACT

The stability of culture filtrate was checked at different temperature viz 40^0C, 50^0C, 60^0C, 70^0C, 80^0C and 90^0C and at different pH viz 2, 4, 6, 8, 10 and 12. The shelve life of culture filtrate was checked at 8^0C and 30^0C for a period of 75 days. The antagonistic activity of the actinomycete culture filtrates was determined at regular intervals of fifteen days.

CHARACTERIZATON OF POTENT ACTINOMYCETE

Characteristics of the antagonistic isolate such as growth, colouration of aerial and substrate mycelia and formation of soluble pigment were tested in two different media including Starch Casein agar and Nutrient agar. Physiological characterization such as, the effect of temperature (4^0C to 50^0C), pH (6 to 12), sodium chloride concentration (1 to 4%) and antibiotic sensitivity against twelve antibiotics [16] were also studied. The potent actinomycete was also screened for enzymatic assays such as alkaline protease, amylase, lipase and urease using Casein agar, Starch agar and Tween 20 agar and Urea agar respectively [17].

The genomic DNA of strain A was isolated and amplified by PCR. The amplified DNA showed a molecular weight of 1.5 kilo base pairs when compared with the standard markers in agarose gel electrophoresis. 16S rRNA analysis was done using big dye terminator version 3.1 cycles sequencing kit and ABI 3130 Genetic Analyser (By Chromos biotech PVT. Ltd., India). The consensus 16S rRNA primers used in the reaction include 16S Forward primer 5'AGAGTRTGATCMTYGCTWAC3' and 16SReverseprimer5'CGYTAMCTTWTTACGR CT3'.The result of the 16S rRNA sequence of actinomycete "A" strain was obtained in the form of rough electrophoregrams in the automated sequencer (Genetic Analyzer ABI 3130). The sequences were converted into FASTA format. The sequence is blasted via BLAST software for comparison with the sequences contained in the data bank (GenBank). Alignment and similarity comparison were initially conducted by the Clustal W method [18]. The Phylogenetic tree was constructed with boot strap values using MEGA 5.05 software.

RESULTS AND DISCUSSION

Totally fourteen different types of actinomycetes were isolated from five marine coastal soil samples and were designated as W, W_1, W_2, W_3, WP, OG, CW_1, CW_2, CW_3, A, B, C, D and R. The growth of these strains was abundant in starch casein agar when compared to nutrient agar. Based on the results of primary (Table 1) and secondary screening (Table 2), actinomycetes strain A, B, D, W_3, R and CW_1 were selected as best strains among the fourteen actinomycetes.

Table 1
Primary screening of actinomycetes

Actinomycetes strain	Growth Inhibition			
	Pseudomonas aeruginosa	*Escherichia coli*	*Bacillus subtilis*	*Staphylococcus aureus*
A	+	+	+	+
B	+	+	+	+
C	-	+	-	+
D	+	+	+	+
R	+	+	+	+
W	+	+	+	+
W_1	+	+	+	+
W_2	+	-	-	+
W_3	+	+	+	+
CW_1	+	-	+	-
CW_2	+	+	-	+
CW_3	+	-	+	+
OG	+	+	-	+
WP	+	+	+	+

+ Present – Absent

Table 2
Secondary screening of actinomycetes by well diffusion method

Test organism	Zone of inhibition (mm)													
	A	B	C	D	R	CW_1	CW_2	CW_3	W	W_1	W_2	W_3	WP	OG
Aspergillus flavus	15	14	0	19	15	15	0	10	0	5	0	23	0	7
Shigella sonnei	7	13	0	6	10	13	15	0	0	0	0	25	6	0
Salmonella paratyphi	23	8	0	5	0	21	13	0	0	0	10	19	0	13
Klebsiella pneumoniae	9	7	0	6	11	10	11	0	0	0	0	17	0	0
Staphylococcus aureus	10	16	16	11	24	0	10	9	7	7	5	21	5	8
Lactobacillus acidophilus	0	0	0	9	0	0	16	0	15	18	0	19	14	14
Streptococcus sp.	16	17	25	23	18	0	11	0	12	12	0	0	11	15
Streptococcus mutans	16	14	22	15	6	21	0	7	6	0	18	18	6	14
Candida albicans	19	14	0	22	7	16	12	0	0	0	10	16	7	0
Pseudomonas aeruginosa	12	23	0	14	10	25	16	14	10	5	15	16	10	16
Bacillus subtilis	20	16	0	16	13	22	0	10	14	12	0	25	21	0
Escherichia coli	20	17	10	15	12	0	6	0	6	7	0	20	7	7

The ethyl acetate extract of actinomycete strain A exhibited better activity over the test organisms than other solvent extracts (Table 3). The antagonistic activity of ethyl acetate

extract of actinomycete strain "A" was better than commercial antibiotic, clotrimazole (9 mm), kanamycin (11 mm), nitrofurantoin (11 mm) and norfloxacin (19 mm). Actinomycete "A" strain was challenged against twelve commercially available antibiotics. Among those twelve antibiotics, actinomycete "A" strain was resistant to nine antibiotics including amoxyclav, erythromycin, nalidixic acid, chloramphenicol, nitrofurantoin, tetracycline, ciprofloxacin, kanamycin and penicillin G. "A" strain was sensitive to imipenem, norfloxacin and amikacin with 25 mm, 19 mm and 4 mm of zone of inhibition respectively (Table 4). Since A strain had good inhibition zone on *Klebsiella pneumoniae*, it was used as test organisms for finding the MIC and the stability detection tests. The MIC of strain "A" on *Klebsiella pneumonia* was 35.6 µg/ml.

Table 3
Antimicrobial assay of the actinomycete strain "A" extracts in different solvents by well diffusion method

Test organisms	Zone of inhibition (mm)			
	Ethanol	Acetone	Methanol	Ethyl acetate
Aspergillus flavus	4	-	-	14
Shigella sonnei	6	-	11	24
Salmonella paratyphi	19	3	10	-
Klebsiella pneumoniae	7	6	11	29
Staphylococcus aureus	8	6	12	17
Lactobacillus acidophilus	6	-	-	4
Streptococcus sp.	14	18	18	12
Streptococcus mutans	17	17	17	15
Candida albicans	8	-	9	12
Pseudomonas aeruginosa	17	8	-	-
Bacillus subtilis	22	12	12	-
Escherichia coli	-	-	9	5

Table 4
Antibiotic sensitivity test for actinomycete strain "A" extract against commercial antibiotics

Antibiotic Tested	Symbol	Concentration	Zone of inhibition (mm)
Amoxyclav	Ac	30mcg/disc	-
Amikacin	Ak	30mcg/disc	4
Erythromycin	E	15mcg/disc	-
Nalidixic acid	Na	30mcg/disc	-
Norfloxacin	Nx	10mcg/disc	19
Chloramphenicol	C	30mcg/disc	-
Nitrofurantoin	Nf	300mcg/disc	-
Tetracycline	T	30mcg/disc	-
Ciprofloxacin	Cf	5mcg/disc	-
Kanamycin	K	30mcg/disc	-
Imipenem	I	10mcg/disc	25
Penicillin G	P	10units/disc	-

The stability detection tests for the culture filtrate of strain A was performed against *Klebsiella pneumoniae* (Table 5). The actinomycete "A" strain culture filtrate responded differently when subjected to different temperatures. The culture filtrate was stable till 80^0C but the activity was good at 40^0C to 50^0C which inferred that the temperature had a significant effect on the activity of the metabolites and raising the temperature decreased the inhibitory activity [19]. The stability of the actinomycete "A" culture filtrate was tested by varying the pH levels. High pH level had greater detrimental effect on the stability of the culture filtrate than the Low pH levels. Here there was a drastic difference in the inhibitory activity of *Klebsiella pneumoniae* between pH which is in accordance with earlier report by [19,20]. The pH 6 was good for the stability of the actinomycete "A" culture filtrate. The stability of the actinomycete "A" culture filtrate was significantly different for two temperature level tested. Metabolites stored at 8^0C retained the activity for the entire testing period but its efficacy was started reducing after 30 days.

But for the culture filtrate stored in room temperature, the efficacy was started reducing from 15th day onwards and it lost activity after 45th day of storage. The stability of the culture filtrate was highest in culture filtrates stored at 8^0C and least at 30^0C.

Table 5
Effect of different temperature, pH and shelve life on the activity of antimetabolites of Actinomycetes "A" strain against Klebsiella pneumoniae

Temperature	Zone of inhibition (mm)	pH	Zone of inhibition (mm)	Shelve life	Zone of inhibition (mm)	
					8° C	30° C
40°C	20	2	11	15	24	14
50°C	19	4	15	30	24	10
60°C	18	6	24	45	21	10
70°C	10	8	10	60	20	-
80°C	10	10	6	75	19	-
90°C	-	12	6			

The actinomycete "A" strain attained their maximum growth at 25^0C and the growth was good till 40^0C but the growth of the actinomycetes was completely inhibited below 8^0C and above 40^0C so the actinomycete "A" strain was confirmed to be mesophilic in nature. The actinomycete "A" strain was growing well in alkaline pH from 6 to 8 but it could withstand upto pH 12. Since the soil was taken from coastal area, the actinomycete was growing well in alkaline condition. Actinomycete "A" strain grew well in the presence of 1% to 2% of NaCl but it was attaining maximum growth at 1% of NaCl. However the actinomycete "A" strain could withstand more than 4% of NaCl. When compared to the control (without NaCl), the growth of actinomycetes at 4% of NaCl concentration was better. So, the actinomycete "A" strain was slightly halophilic in nature. The screening of "A" strain for enzymatic activity revealed the presence of lipase, urease, protease and amylase (Table 6).

Table 6
Characteristics of the isolate

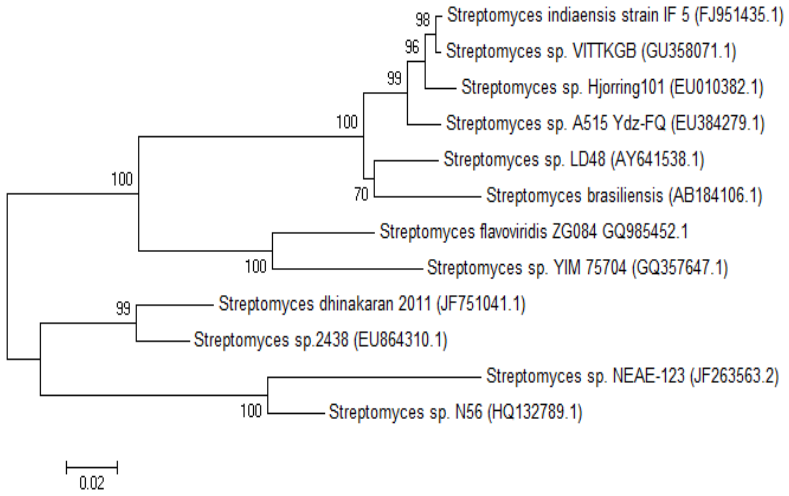
Temperature	Inference	pH	Inference	NaCl	Inference	Enzymes	Inference
4°C	-	2	-	0%	+	Urease	Present
8°C	+	4	-	1%	++++	Protease	Present
25°C	++++	6	+	2%	+++	Amylase	Present
30°C	+++	7	++++	5%	++	Lipase	Present
40°C	++	10	++++	4%	++		
50°C	-	12	+++	5%	-		

++++ Excellent +++ Good ++ Fair + Poor - No growth

The microbe was found to have 99% similarity with *Streptomyces* sp. 2438. Therefore the 16S rRNA sequence analysis and comparative analysis revealed that the selected strain was found to be *Streptomyces* sp. but does not have 100% similarity to any of the available species. Hence the new isolate actinomycete "A" strain was designated as *Streptomyces dhinakaran* 2011 and the sequence submitted in NCBI obtaining accession number JF751041 (Figure 1).

Figure 1

Neighbour joining tree based on 16S rRNA gene sequences showing relationship between the Streptomyces dhinakaran 2011 with other Streptomyces species.



The number at the nodes indicates the percent levels of boot strap support based on the analysis of 1000 replicates. Boot strap values less than 50 were not shown. The scale bar indicates number of changes per base position.

CONCLUSION

In the present study it was identified that the strain exhibited wide spectrum of antimicrobial activity against tested microbial pathogens. Specifically it could be highlighted that the *Streptomyces dhinakaran* 2011 revealed very good inhibitory activity. From the results obtained it could be inferred that specific isolation of compounds from *Streptomyces dhinakaran* 2011 could provide industrially important bioactive molecules.

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