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Bioprospecting of marine derived actinomycetes with special reference to antimycobacterial activity

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Present study consists the bioprospecting of marine actinomycetes with special reference to antimycobacterial activity. Totally 49 actinomycetes were isolated from sediment samples collected from mangrove area of Pitchavaram coastal areas of Parangipettai and Andaman & Nicobar Islands and by using starch casein agar medium. Based on the cultural and microscopic characteristics, 30 isolates were identified as *Streptomyces* sp. and 19 as rare actinomycetes including 11 *Micromonospora* sp. Bioactive substance from all the isolates were produced by shake flask fermentation method using yeast extract malt extract broth. Culture filtrate and mycelial methanol extracts were tested against drug sensitive and drug resistant clinical isolates of *M. tuberculosis* and standard strain *Mycobacterium tuberculosis* H37Rv by luciferase reporter phage (LRP) assay. Culture filtrate and/or mycelial methanol extracts from 41 out of 49 actinomycetes inhibited at least one of the *M. tuberculosis* strains tested. Culture filtrates from actinomycetes strains viz., R6 (*Streptomyces* sp), M1A6 (*Micromonospora* sp), M1A15 (*Actinosynnemma* sp), M1A18 (*Micromonospora* sp) and M1A23 (rare actinomycete) showed more than 90% reduction in RLU by LRP assay. Isolation of active compounds from these potent strains probably would lead to the discovery of novel antiTB drugs.

[Keywords: marine actinomycetes, antimycobacterial activity, LRP assay]

Introduction

The demand for new antibiotics continues to grow due to the rapid spread of antibiotic-resistant pathogens causing life-threatening infections. Although considerable progress is being made in the filed of chemical synthesis, nature still remains the richest and the most versatile source for new antibiotics[1]. Screening of microbial products continues to represent an important route to the discovery of novel chemicals for development of novel therapeutic agents[2].

Actinomycetes are the most economically valuable prokaryotes which are well known to produce chemically diverse metabolites with wide range of biological activities. It has been estimated that about half of the microbial bioactive metabolites notably antibiotics, antitumor agents, immuno suppressives and enzyme inhibitors have been isolated from actinomycetes. Recently the rate of discovering new compounds from terrestrial actinomycetes has decreased but the rate of re-isolation of known actinomycetes and antibiotics is on the increase. This precluded the study of normal terrestrial sources particularly for actinomycetes and has led researchers to explore unique and extreme habitats such as marine environment for potentially new biosynthetic diversity. Marine actinomycetes are the promising source for secondary metabolites[3]. In the past 10 years, 659 marine bacterial compounds have been described in which 256 compounds have originated from actinomycetes[4]. Studies on compounds such as salinosporamide and abyssomycin isolated from unique marine actinomycete genera Salinispora and Verrocosispora suggest that these bacteria add an important dimension to marine natural product research[5].

Tuberculosis (TB) remains one among the leading causes of infectious disease worldwide. One third of the world population is infected with *Mycobacterium tuberculosis* and hence at a risk of developing active TB[6]. A number of efficacious antitubercular agents were discovered in the late 1940s and 1950s, with rifampicin getting introduced in 1960s[7]. Streptomycin was the first drug to be introduced in 1940s for the treatment of TB but immediately after its introduction many patients started showing resistance to this

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antibiotic[8]. The combined resistance to isoniazid and rifampicin (multi drug resistant) impacts strongly on control programmes. The additional acquisition of resistance to a fluoroquinolone and one of three injectable second line drugs (capreomycin, kanamycin, amikacin) has defined the extensively drug resistant (XDR) tuberculosis. There is an urgent need to discover novel antibiotics against drug resistant *M. tuberculosis.* With this view the present study was initiated for bioprospecting of marine actinomycetes with special reference to antimycobacterial activity.

Materials and Methods

Actinomycetes were isolated from marine sediment samples collected from mangrove area of Pitchavaram and coastal area of Parangipettai, Andaman & Nicobar Islands and Rameswaram. All the samples were dried at room temperature for 5 d and 5 gm of dried sediment samples were kept at 55°C for 6 min[9]. Actinomycetes were isolated using starch casein agar medium (SCA) supplemented with nalidixic acid (20µg/mL) and cycloheximide (100 µg/mL) and incubated at 28°C for 30 d. Colonies showing actinomycete morphology were selected and purified on yeast extract-malt extract agar (yeast extract 0.4%; malt extract 1.0%; glucose 0.4%; pH 7.0±0.2) (ISP2 medium). Morphologically different actinomycetes were preserved both as slant culture in ISP2 medium and as glycerol stock in 20% glycerol. All the media used in this study were prepared in 50% sea water.

Cultural and microscopic characteristics of actinomycetes were studied by adopting the methods described by Shirling and Gottileb[10]. All the actinomycete isolates were cultured on ISP2 agar at 28°C for 7-14 d. The recorded growth characteristics include growth level, colony consistency, aerial mass color, reverse side pigment, soluble pigment including melanoid pigment using tyrosine agar (ISP7 medium). Micromorphological characteristics were studied by slide culture method. Slides were observed under bright field microscope at 40X magnifications on 14th day of incubation. Micromorphological characteristics recorded include aerial mycelium, substrate mycelium and spore chain morphology.

Bioactive substances from all the actinomycetes were produced by adopting submerged fermentation. The well grown actinomycete culture from ISP2 agar medium was inoculated to each 25 mL of yeast extract malt extract broth and incubated in rotary shaker with 120 rpm for 5 d for streptomycetes and 10 d for rare actinomycetes. After fermentation, culture medium was separated by centrifugation at 10,000 rpm for 10 min at 4°C. The culture supernatant was filtered through 0.45 µ filter. Intracellular compounds from dried mycelium were extracted using 20 mL of methanol at 4°C for 24 h and concentrated by eppendorf evaporator. Mycelial methanol extracts were dissolved in 1 mL of 10% DMSO and filtered through 0.45 µ filter.

Actinomycete culture filtrates and mycelial methanol extracts were screened for antimycobacterial activity by Luciferase Reporter Phage (LRP) assay. A reduction by 50% in relative light units (RLU) as measured by luminometer is considered as sensitive. Standard laboratory strain *M. tuberculosis* $H_{37}Rv$, SHRE sensitive and SHRE resistant clinical strains of *M. tuberculosis* were used as test organisms[11].

Results and Discussions

Totally 49 actinomycetes were selected from starch casein agar plates based on their colony morphology. About 90% of the actinomycete isolates showed good growth on ISP2 agar medium. Majority of the isolates were white or dirty white in colour (36.73%), powdery in consistency (55.10%) and possessed both aerial and substrate mycelium (69.39%) with rectus flexibile spore chain arrangement (22.44%). Based on the cultural and microscopic characteristics, 30 isolates were identified as *Streptomyces* sp. and 19 isolates as rare actinomycetes, out of which 11 isolates were tentatively identified as *Micromonospora* sp. *Streptomyces* sp. was the most common actinomycetes genera isolated from marine sediments and mangrove ecosystems followed by genus *Micromonospora*. Antagonistic streptomycetes are well documented from Parangipettai, Pichavaram and also from Andaman and Rameswaram to some extent[12]. The present work is the first report on rare actinomycetes from Pichavaram mangrove rhizosphere sediments including 11 *Micromonospora* sp.

Culture filtrates or extracts showing more than 50% RLU reduction is considered as antimycobacterial activity. In the present study culture filtrates from 19 strains of *Streptomyces* sp. and 19 rare actinomycetes inhibited one or more of the *M. tuberculosis* strains tested. However, results of the study by Yilmaz et al.[13] reveal that majority of the aqueous extracts of the actinomycetes tested by them had no effect on bacterial pathogens, whereas ethyl acetate extracts showed remarkable activity.

Most of the secondary metabolites including antibiotics are extracellular in nature and extracellular products of actinomycetes exhibit potent antimicrobial activities[14]. In the present study, culture filtrates and mycelial extracts from 14 out of 30 *Streptomyces* sp. and 4 out of 19 rare actinomycetes showed antimycobacterial activity demonstrating the high antimycobacterial potential of *Streptomyces* sp.

Majority of *Streptomyces* isolates in the present study belonged to white and dirty white colour series followed by ash and grey colour series. However isolates representing all the colour series showed antimycobacterial activity. In an earlier study, Patil et al.[15] reported that majority of the antagonistic *Streptomyces* isolates belonged to grey series.

Overall, culture filtrates and/or mycelial extracts from 41 actinomycete strains inhibited atleast any one of the three *M. tuberculosis* strains tested. Ndonde and Semu[16] obtained similar results in which 96% of the isolates were found to inhibit anyone or more of the plant pathogens tested. Alexander[17] also reported that over 75% of all soil streptomycetes were capable of producing antibiotics.

In this study, culture filtrates from five actinomycetes showed good activity (>90% RLU reduction) against all the three *M. tuberculosis* strains tested (Table 1). Mycelial extracts of strain R6 and M1A23 also showed activity. Characteristics of five potential actinomycetes strains are given in Table 2. In Indian peninsula 41 species of marine actinomycetes were reported in which the genus *Streptomyces* was more frequently recorded[12]. Of the five potential actinomycetes reported in this study, strain M1A15 was tentatively identified as *Actinosynnema* sp. which was not previously reported from Indian marine ecosystems. There are only few reports so far on *in vitro* antituberculous activity of microorganisms of marine origin. Messetolide A and viscosin are cyclic depsipeptides from cultures of two *Pseudomonas sp* isolated from a marine alga and tube worm, respectively[18]. Marine derived antibiotics may be more efficient in fighting infections because the terrestrial bacteria have not developed resistance against them[19]. The urgent need for the development of new drugs to help reduce the global burden of TB is well documented in the current biomedical literature. Actinomycetes strains R6, M1A6, M1A15, M1A18 and M1A23 reported in this study show promise as a good source for novel antimycobacterial compounds from marine origin.

Table 1—Antimycobacterial activity of potential actinomycetes strains by LRP assay

	% RLU reduction					
	Culture filtrate			Mycelial extract		
Strain No	H37Rv	SHRE Sensitive MTB	SHRE resistant MTB	H37Rv	SHRE Sensitive MTB	SHRE resistant MTB
R6	96.0	98.9	93.4	76.3	96.4	94.0
M1A6	98.9	99.1	93.4	40.0	0.00	0.00
M1A15	98.5	99.0	94.0	28.2	31.1	46.1
M1A18	99.0	99.0	94.5	23.6	0.00	42.8
M1A23	98.3	95.7	94.0	62.7	86.5	83.4

Table 2—Characteristics of potential marine actinomycetes

Strains	Ecosystem	Growth	Consistency	Mycelial color	RSP	SP	MP	Aerial mycelium	Substrate mycelium	Spore chain morphology	Suspected genera
R6	Rameswaram, Tamilnadu (TN)	Good	Powdery	Dirty white	-	-	-	+	+	RF	*Streptomyces* sp.
M1A6	Pitchavaram, (TN)	Good	Mucoid	Brown	Brown	-	-	-	+	M	*Micromonospora* sp.
M1A15	Pitchavaram (TN)	Good	Powdery	Orange	Orange	-	-	-	+	-	*Actinosynnema* sp.
M1A18	Pitchavaram (TN)	Good	Mucoid	Brown	Brown	-	-	+	+	M	*Micrormonospora* sp.
M1A23	Pitchavaram (TN)	Good	Mucoid	Orange	Orange	-	-	+	-	-	Rare actinomycete

RSP – Reverse side pigment; SP – soluble pigment; MP – Melanoid pigment; RF – Rectus flexibile; M - Monosporic

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