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Enrichment method for the isolation of bioactive actinomycetes from mangrove sediments of Andaman Islands, India

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ABSTRACT

Various pre-treatment methods and three different media were employed for the isolation of bioactive actinomycetes from mangrove sediments of Andaman and Nicobar Islands, India. Sediments from four different sites of mangrove forest were collected and pre-treated by dry heat method, and the media were supplemented with cycloheximide 80 µg/mL and nalidixic acid 75 µg/mL. The mean actinomycetes population density in sediment samples were recorded as 22 CFU-10^{-6}/gm in KUA medium followed by 12 CFU-10^{-6}/gm in AIA medium and 8 CFU-10^{-6}/gm in SCA medium. A total of 42 actinomycetes were isolated, and all the isolates were evaluated for their antibacterial activity against pathogenic bacteria on two different media. Among 42 isolates tested, 22 species were found to be antibacterial metabolite producer against test bacteria namely, *Staphylococcus aureus, Bacillus subtilis, Salmonella typhi* and *Klebsiella pneumoniae*. Particularly, the actinomycete strains such as A101, A102, A107, A116, A121, A125, A130, F101, F102, F104, F106, De101 and De102 significantly inhibited the growth of all bacteria which were tested. Of these strains, A107 was identified as *Streptomyces* spp. This strain had the maximum activity against all used pathogens on both medium. Hence, the isolation, characterization and studies of secondary metabolites of actinomycetes from mangrove sediments in Andaman and Nicobar Island could be a pathway for discovery of antibiotics from marine actinomycetes.

Keywords: Andaman Islands, Actinomycetes, pretreatment, characterization

INTRODUCTION

Marine environments are largely untapped source for the isolation of new microorganisms with potentiality to produce active secondary metabolites. Among such microorganisms, actinomycetes are of special interest, since they are known to produce chemically diverse compounds with a wide range of biological activities (Bredholt *et al.*, 2008). The demand for new antibiotics continues to grow due to the rapid emerging of multiple antibiotic resistant pathogens causing life threatening infection. Although, considerable progress is being made within the fields of chemical synthesis and engineered biosynthesis of antibacterial compounds, nature still remains the richest and the most versatile source for new antibiotics (Kpehn and Carter, 2005; Baltz, 2006; Pelaez, 2006). Traditionally, actinomycetes have been isolated from terrestrial sources although, the first report of mycelium forming actinomycetes being recovered from marine sediments appeared several decades ago (Weyland, 1969). Recently, the marine derived actinomycetes have become recognized as a source of novel antibiotic and anticancer agent with unusual structure and properties (Jensen *et al.*, 2005). Actinomycetes represent a ubiquitous group of microbes widely distributed in natural ecosystems around the world and especially significant for their role on the recycling of organic matter (Srinivasan *et al.*, 1991).

The literatures suggested that, marine sediment sources are voluble for the isolation of novel actinomycetes with the potential to yield useful new products (Goodfellow and Haynes, 1984). However, it has been resolved whether actinomycetes form part of the autochthonous marine microbial community of sediment samples originated from terrestrial habitats and were simply carried out to sea in the form of resistant spores (Weyland, 1981; Goodfellow and Williams, 1983; Weyland and Helmke, 1988; Takizawa *et al.*, 1993; Ravel *et al.*, 1998). Microorganisms found in marine environments have attracted a great deal of attention, due to the production of various natural compounds and their specialized mechanisms for adaptation to extreme environment (Solingen *et al.*, 2001). The pre-treatment including enrichment, physical and selective media may be used to study the ecology of actinomycetes in natural habitats such as soil or water samples (Jensen *et al.*, 2005). Since marine sediments represent an environment which is markedly different from that associated with soil samples, it is not clear how effective the pre-treatment of such sediments would be for the recovery of bioactive actinomycetes. Marine sediment is an inexhaustible resource that has not been properly exploited. Few

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reports from the East Coast of India, suggests that soil is a major source of Actinomycetes (Sivakumar *et al.*, 2005; Vijayakumar *et al.*, 2007; Dhanasekaran *et al.*, 2008; Vijayakumar *et al.,* 2008). Correspondingly, the Andaman and Nicobar Island marine ecosystem is largely unexplored, and may provide a rich source of the microorganisms producing novel and efficient antimicrobial compounds. Hence, the present study was undertaken to isolate the bioactive actinomycetes from marine sediments of Andaman and Nicobar Islands by various pre-treatment methods using different media and evaluate the antibacterial potentiality of the isolates.

MATERIALS AND METHODS

Study area

Floating in splendid isolation coast of the Indian mainland is the archipelago of 572 emerald islands, islets and rocks known as Andaman and Nicobar Islands. This Union Territory is stretched over an area of more than 700 km from north to south with 36 inhabited islands. Once a hill range extending from Burma (Myanmar) to Indonesia, these undulating islands are covered with dense forests.

Collection of mangrove sediment

A total of four different mangrove sediment samples were collected from Bay of Bengal Coast (Lat. 11°38' 46 N; Long. 92°40' 71 E) and Andaman Sea Coast (Lat.11°35'47. Long. 92°45'79) of South Andaman Island, India. Samples were collected in sterile plastic containers in field and were transferred immediately to the laboratory and stored for further study.

Isolation of actinomycetes from sediments

The air dried sediment samples were ground aseptically with Pestle and Mortar, mixed thoroughly and passed through 2 mm sieve filter to remove gravel and debris. The samples were kept at 55 °C for 5 min, 55 °C for 60 min, 70 °C for 15 min and 100 °C for 1 h in separate glass container for pre-treatment (Hayakawa and Nonomura, 1987; Hayakawa *et al.*, 1991; Seong *et al.*, 2001). Then, 10 fold serial dilutions of the sediment samples were made using sterile 50% sea water (Kim *et al.*, 1994). About 0.1 mL of the serially diluted samples was spread over the Kuster's agar medium (Balagurunathan and Subramanian, 1992) g/L: glycerol 10, casein 0.3, KNO_3 3, K_2HPO_4 2, NaCl 2, $MgSO_4$ 0.05, $CaCo_3$·0.02, $FeSO_4$ 0.01, agar 16; pH 7 ± 0.1 and 50% sea water, (Difco Chemicals). Starch casein agar medium (Wellington and Cross, 1983) g/L: starch 10, casein powder 1, agar 15; sea water 50% and pH 7.2 ± 0.2 and Actinomycetes isolation agar medium g/L: sodium caseinate 2, L - asparagine 0.10, sodium propionate 4, di-potassium phosphate 0.5, magnesium sulphate 0.1, ferrous sulphate 0.001, agar 15; sea water 50% and pH 8.1 ± 0.2. All the three agar media were supplemented with 80 µg/mL of cycloheximide and 75 µg/mL of nalidixic acid (Himedia, Mumbai) to minimize the other bacterial and fungal growth. The plates were incubated at 28 ± 2 °C for 28 days. After 5 days, the actinomycetes colonies grown on Petri plates were counted at regular intervals. All the morphologically different actinomycete colonies were sub-cultured on yeast extract malt extract agar medium (ISP No. 2) g/L: yeast extract 4, malt extract 10, dextrose 4, 50% sea water, agar 20, pH 7.3 (Shirling, 1966) by streak plate technique. After growth appeared, the actinomycetes colonies were maintained in ISP No. 2 agar slants for further investigation.

Screening for antibacterial activity

All the isolated actinomycetes were tested for their antibacterial activity against several bacteria namely *B. subtilis, S. typhi*, *S. aureus* and *K. pneumoniae.* The antibacterial activity was carried by cross streak plate method (Lemos *et al.*, 1985), single streak of the actinomycetes were made on Kuster's agar and modified nutrient agar medium [g/L: glucose 5, peptone 5, beef extract 3, NaCl 5, agar 15 and pH 7 ± 0.1] and incubated at 28 ± 2 °C for 3 to 4 days (Kim *et al.*, 1994). After observing a good ribbon like growth of the actinomycetes, the bacterial pathogens were streaked at right angle to the original streak of actinomycetes and incubated at 28 ± 2 °C. The inhibition zone (mm) was measured after 24 and 48 h. Control plates were also maintained without inoculating actinomycetes/bacteria to assess the normal growth of the pathogenic bacteria and actinomycetes.

Secondary screening of A107: Preparation of antibiotic fermentation broth

The strain A107 was cultured on agar slant at 28 °C for 2 weeks, the mature spores were inoculated in fermentation broth containing 100 mL (Dextrose 2 g, soya bean meal 2 g, soluble starch 0.5 g, peptone 0.5 g, corn steep liquor 0.25 g, $(NH_4)_2$ SO_4 0.25 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, K_2HPO_4 0.002 g, NaCl 0.4 g, $CaCO_3$ 0.2 g, Sea water 50%) and incubated at 30 °C on rotary shaker at 200 rpm for 216 h. The fermented broth was centrifuged at 10,000 rpm at 4 °C for 20 min The supernatant was filtered using 0.45 µm spore size membrane filter (Millipore) the filter was collected as the antibiotic sample (Ruan, 1977). To determine the antibacterial spectrum, pathogenic bacteria were cultured on nutrient broth at 37 °C for 24 h; the cultures were swapped on Muller Hindan agar media. Three wells (6 mm in diameter) were prepared in each seeded agar plates and each well was filled with 100 µL of the antibiotic fermentation broth strain A107. The plates were incubated at 37 °C for 24 to 48 h. The diameter of the inhibition zones was measured.

Characteristics of high antibacterial active actinomycetes (A107)

The slide culture of the high active streptomycetes was prepared on Kuster's agar medium and incubated at 28 °C by using cavity slides. Periodical observation regarding

spore morphology, arrangements and mycelium structure were recorded by using Nikon Microscope in 100 x zoom. Cultural characteristics of A107 (growth, colouration of aerial and substrate mycelia, formation of soluble pigment) were tested in six different media including, yeast extract malt extract agar (ISP-2), oat meal agar (ISP-3), Inorganic salt starch agar (ISP-4), glycerol asparagine agar (ISP-5), tyrosin agar (ISP-7) and Kuster's agar medium (KU) according to the procedure of ISP. Biochemical test including IM-ViC, H_2S production, nitrate reduction, urease, catalase, starch gelatine and casein hydrolysis, haemolysis and TSI were also performed as recommended by ISP. Chemotaxanomical properties such as, analysis of cell wall sugar (Lechevalier *et al*., 1970) and cell wall amino acid analysis (Becker *et al*., 1965) were analysed. Utilization of carbon sources such as starch, dextrose, fructose, maltose, and mannitol and nitrogen sources namely D-alanine, L-arginine, and L-tyrosine were tested in Kuster's agar medium.

RESULTS AND DISCUSSION

Four different pre-heat treatments were employed for maximum isolation of actinomycetes. Of which, the soil treated at 70 °C for 15 min yielded maximum of 22 CFU of actinomycetes per gram of sediment at the dilution of 10^{-6} [Figure 2 (a)], whereas other treatments like at 100 °C for 60 min yielded only 4 CFU/10^{-6}, at 55 °C for 5 min gave 12 CFU/10^{-6} and at 55 °C for 60 min gave 15 CFU/10^{-6}. Consequently, first two treatments did not allow any bacterial and fungal colonies to grow, whereas later treatments allowed the growth of bacterial and fungal colonies as 8 and 6 CFU/10^{-6} and 6 and 7 CFU/10^{-6} were recorded in the sediments treated at 55 °C for 5 min and 55 °C for 60 min respectively (Figure 1). Hence it has been reported that, when the mangrove sediments were cultured without pre-treatment, the number of unwanted bacterial and fungal colonies were grown, [Figure 2 (b)] whereas the soil was air dried, they were decreased on culture plates. Also, the present study reported the dominance of other bacterial and fungal contamination inhibited the colonization of actinomycetes. Previously, this type of pre-treatment methods for isolation of actinomycetes has also been suggested by several researchers (Hayakawa and Nonomura, 1987; Hayakawa *et al*., 1991; Jensen *et al.*, 1991; Kim *et al.*, 1994; Seong *et al*., 2001).

Further, antimicrobial agents namely cycloheximide (80 µg/mL) and nalidixic acid (75 µg/mL) were supplemented in three different culture media namely Kuster's agar, starch casein agar and actinomycetes isolation agar. In our study, as much as 42 morphologically different actinomycetes isolates were isolated from the mangrove sediments. When compared with starch casein agar, Kuster's agar was found to be well supporting the isolation of more marine actinomycetes population. Maximum actinomycetes colonies (22 CFU/10^{-6}) were found on Kuster's agar followed by actinomycetes isolation agar (12 CFU/10^{-6}) and starch casein agar (8 CFU/10^{-6}). Thus, the present

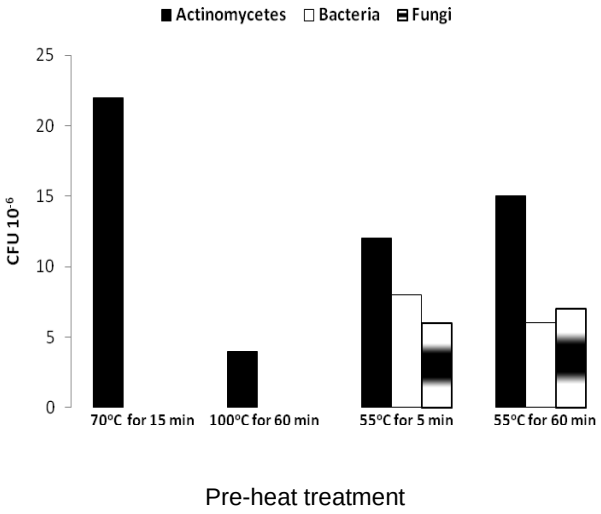

Figure 1: Various pre-heat treatments for isolation of actinomycetes from mangrove sediments

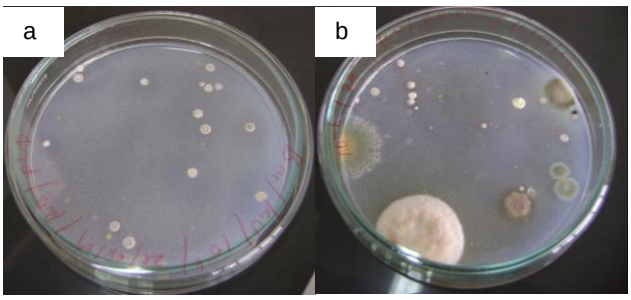

Figure 2: Isolation of actinomycetes from mangrove sediments (a) Pretreatment at 70 °C for 15 min and (b) without pretreatment

study reports that, the temperature at 70 °C for 15 min was suitable treatment method to isolate more actinomycetes colonies from the marine sediments, on Kuster's agar medium with the supplementation of cycloheximide (80 µg/mL) and nalidixic acid (75 µg/mL).

Phenotypic characteristics of selected antagonistic actinomycetes (A107)

Morphological characterization of A107, a broad spectral antagonistic isolates developed dark grey to white coloured spore mass. However, the strain A107 developed coffee brown coloured substrate mycelium. Further the strain A107 developed spiral nature spore chain in its aerial mycelium (Table 1). The details of morphological and biochemical characteristics, utilization of carbon and nitrogen sources, and chemotaxanomicals property of the test isolates are given in Table 1. Sivakumar *et al*., (2005) reported that the characters can be used as marker by which an individual strain can be

Table 1: Morphological and biochemical characteristics of high activity Actinomycetes (A 107)

Properties	*Streptomyces* spp.
Morphological characteristics	
Spore morphology	Spiral
Colour of aerial mycelium	Dark grey
Colour of substrate mycelium	Coffee brown
Spore mass	Dark grey
Biochemical Characteristics	
Indole production	-
Methyl red	-
Voges proskauer	-
Citrate utilization	+
H_2S production	-
Nitrate reduction	-
Urease	+
Catalase	+
Oxidase	-
Melanin production	-
Starch hydrolysis	+
Gelatin hydrolysis	+
Lipid hydrolysis	+
Casien hydrolysis	+
Haemolysis	+
Triple Sugar iron	alk./alk.
Chemotaxanomic characters	
Whole cell sugar analysis	-
Cell wall amino acid analysis	L-DAP
Carbon source utilization	
Starch	++++
Dextrose	++
Fructose	+
Maltose	+++
Mannitol	++++
Nitrogen utilization	
D-alanine	+++
L-arginine	++
L-phenylalanine	++++
L-tyrosine	++++

Table 2: Cultural characteristics of high active antagonistic actinomycetes on different media

Sl. No	Name of the medium	*Streptomyces* spp.
1.	Malt extract and yeast extract agar (ISP-2)	
	Aerial mycelium	Dull white
	Substrate mycelium	Dark yellow
	Pigmentation	Nil
2.	Oat meal agar (ISP-3)	
	Aerial mycelium	Grey
	Substrate mycelium	Red
	Pigmentation	Dark pink
3.	Inorganic Salt Starch agar (ISP-4)	
	Aerial mycelium	Grey
	strate mycelium	Yellowish green
	Pigmentation	Nil
4.	Glycerol asparagines agar	
	Aerial mycelium	Grey
	Substrate mycelium	Light grey
	Pigmentation	Nil
5.	Tyrosin agar (ISP-7)	
	Aerial mycelium	Grey
	Substrate mycelium	Yellow
	Pigmentation	Nil
6.	Kuster's agar	
	Aerial mycelium	Grey
	Substrate mycelium	Dark yellow
	Pigmentation	Pale pink

recognized. Particularly, chemotaxonomy plays an important role in identification of actinomycetes to generic level. In this study, the test isolates contain meso-diaminopimelic acid and there were no sugar found in their cell wall. The isolate could utilize all the carbon sources and nitrogen sources (Table 1). The present investigation concluded that the physiological characteristics of actinomycetes varied depending on the available nutrients in the medium and the physical conditions. Upon the growth of the isolates on various media, Kuster's agar was observed to be the best medium for maximal growth. Further, the colour of diffusible pigment and the aerial and substrate mycelium produced by A107 isolates varied with different media. Pridham and Tresner (1974) reported that the colour of aerial mycelium is considered to be an important character for the grouping and identification of actinomycetes. In the present study, A107, a high potential antibacterial compound producing actinomycete was cultured on six different culture media. The strain A107 produced pale pink coloured diffusible pigment on Kuster's agar medium (Table 2). Thus, the present investigation has been pre-classified as high potential producers based on the colony morphology and phenotypic characteristics of actinomycetes (Table 1). Thus, it was concluded on the basis of the present and previous studies that the nutrient compositions of the medium greatly influenced the growth and morphology of organisms.

Antibacterial activity of isolates

The actinomycetes are noteworthy antibiotic producers, making the quarters of all known pharmaceutical products; the streptomycetes are especially prolific (Waksman, 1961; Lachevalier, 1989; Locci, 1989; Saadown and Gharaibeh, 2003). In the present study, out of 42 actinomycetes from the mangrove sediments of Andaman and Nicobar Islands, 22 (58.4%) isolates had antibacterial activity against pathogenic bacteria. All the 42 isolates showed antibacterial activity with at least one test bacteria

Table 3: Antibacterial activity of actinomycetes isolates

Isolate code	Zone of inhibition (mm)							
	Modified nutrient agar				Kuster's agar			
	Staphylococcus aureus	*Bacillus subtilis*	*Klebsiella pneumoniea*	*S.* typhi	*S. aurreus*	*B. subtilis*	*K. pneumoniea*	*S. typhi*
A101	25	15	32	33	35	14	32	37
A102	12	12	12	12	30	16	30	34
A103	17	-	23	28	30	25	32	31
A104	20	-	23	27	37	19	32	35
A105	-	-	14	13	31	-	-	33
A107	30	30	30	32	33	33	35	30
A108	10	-	-	-	38	6	-	9
A110	34	19	-	-	36	16	35	35
A116	34	15	36	24	32	17	31	33
A120	9	5	5	5	47	-	-	-
A121	21	18	20	16	38	30	30	34
A125	25	12	18	19	37	18	38	37
A128	-	-	18	-	32	10	-	10
A130	24	11	30	30	32	10	35	33
F101	28	16	30	31	36	15	32	37
F102	20	20	20	20	35	16	36	36
F104	28	19	32	32	34	16	30	37
F105	-	-	15	-	44	8	12	10
F106	25	13	32	34	34	17	36	36
De101	22	18	30	32	35	15	18	37
De102	25	17	32	32	34	15	30	36
De103	15	5	13	-	36	-	-	-

- : No activity

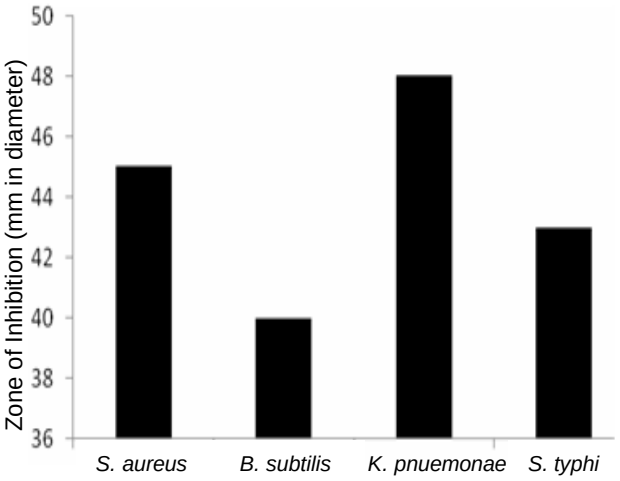

Figure 3: Secondary metabolic activity of *Streptomyces* spp. (A107)

on both modified nutrient agar and Kuster's agar media. Notably, the strains A101, A102, A107, A116, A121, A125, A130, F101, F102, F104, F106, De101 and De102 possessed supreme activity against all the bacterial pathogens tested on both modified nutrient agar and Kuster's agar. The strains A105, A108, A128 and De103 showed more activity against tested bacteria on Kuster's agar than modified nutrient agar. Actinomycete strains such as A108, A128 and F105 inhibited one bacteria each [*S. aureus* (10 mm), *K. pneumoniae* (18 mm) and *K. pneumoniae* (15 mm) respectively] on modified nutrient agar, whereas on Kuster's agar the strains A108 and A128 produced maximum to moderate inhibitory zone against all the bacteria tested except *K*. *pneumoniae*, and the strain F105 inhibited the growth of all the four bacterial pathogens in the same media. In addition, all the actinomycete strains had remarkable antibacterial activity against *S. aureus* when grown on Kuster's agar medium.

On modified nutrient agar, the strain A107 produced maximum (37 mm) zone of inhibition against *S. aureus*, whereas on Kuster's agar, both strains A121 and A125 produced maximum zone of inhibition against *S. aureus* (38 mm) and *K. pneumonia* (38 mm) respectively. Interestingly, the other isolates also showed noticeable antibacterial activity against all the tested bacteria on both media. Comparatively, all the isolates produced maximum zone of inhibition when they were cultivated on Kuster's agar medium than modified nutrient agar (Table 3). A107 was found to be highly inhibiting pathogenic bacteria on both media; hence, the culture was selected for identification. Thus, it has been reported that, there are both quantitative and qualitative variations in the antibiotics produced by different genera and species. Substrates and habitats greatly influenced the production of antibiotics by actinomycete isolates.

Secondary metabolic activity of A107 showed highest antibacterial activity against *S. aureus* (45 mm) and *K. pneumoniae* (48 mm) given in Figure 3.

Correspondingly, *Streptomyces* spp. was isolated from the marine sponges *Callyspongia diffusa, Mycale mytilorum, Tedania anhelans* and *Dysidea fragilis* (Dharmaraj and Sumantha, 2009). From the initial screening, 94 cultures of *Streptomyces* were obtained and from these 58 cultures exhibited antagonism against bacteria, 36 strains against fungi and 27 strains exhibited broad spectrum activity against both. Similar type of work has also been reported by Remya and Vijayakumar (2008), from marine soils of Kerala, West Coast of India.

Though, the results of the present study gives clear picture about the significance of pre-treatment methods for the isolation of actinomycetes and revealed the marine actinomycetes from mangrove sediments of Andaman and Nicobar Islands to be a potent source of novel antibiotics. Studies on diversity of actinomycetes desires regular visits to the sampling stations, isolation from different substrates collected from the habitat and the usage of different culture media. Such attempts need to be continued both in the same area as well as from the adjoining places during various climatic conditions as to screen more isolates for novel therapeutics.

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