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Full Length Research Paper

Isolation and screening of actinomycetes from Sundarbans soil for antibacterial activity

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A total of 55 actinomycetes isolates from soil sample of Karanjal region in Sundarbans were characterized for morphological identification and antimicrobial activity. Four general such as *Actinomyces*, *Nocardia*, *Streptomyces* and *Micromonospora* with total numbers of isolates were 27, 14, 11 and 3, respectively, were identified from the sample. Twenty actinomycetes isolates produced antibiotic against one or more gram-negative pathogenic bacteria such as *Shigella boydii*, *Shigella flexneri*-AN-31153, *Shigella sonnei*, *Pseudomonas*, *Shigella dysenterriae* type-1, *Vibrio cholerae*-0139, *Salmonella typhi*-Ao-12014, *Plesiomonas*, *Hafnia* spp., *Vibrio cholerae*-OGET, and *Escherichia coli*-186LT. The study indicated that Sundarbans' soil had diverse group of actinomycetes and three of the tested isolates had a broader spectrum antibacterial activity which showed potential as a source of antibiotics for pharmaceutical interest.

Key words: Sundarbans, actinomycetes, isolate, antibacterial activity, identification.

INTRODUCTION

Actinomycetes are aerobic, gram-positive bacteria. They are one of the major groups of soil population and are very widely distributed (Kuster, 1968). The number and types of actinomycetes present in a particular soil would be greatly influenced by geographical location such as soil temperature, soil type, soil pH, organic matter content, cultivation, aeration and moisture content. Actinomycetes populations are relatively lower than other soil microbes and contain a predominance of *Streptomyces* that are tolerant to acid conditions (Davies and Williams, 1970). Arid soils of alkaline pH tend to contain fewer *Streptomyces* and more of the rare genera such as *Actinoplanes* and *Streptosporangium*. However, alkaliphilic actionmycetes will provide a valuable resource for novel products of industrial interest, including enzymes and antimicrobial agents (Mitsuiki et al., 2002; Tsujibo et al., 2003).

Based on several studies among bacteria, the actionmycetes are noteworthy as antibiotic producers, making three quarters of all known products; the *Streptomyces* are especially prolific (Lacey, 1973; Lechevalier, 1989; Locci, 1989; Saadoun and Gharaibeh, 2003; Waksman, 1961). A search of the recent literature revealed that at least 4,607 patents have been issued on actinomycete related product and processes (Williams and Vickers, 1988). *Streptomyces* covers around 80% of total antibiotic product, with other genera trailing numerically; *Micromonospora* is the runner up with less than one-tenth as many as *Streptomyces*. If we include secondary metabolites with biological activities other than antimicrobial, actinomycetes are still be out in front (Hopwood et al., 2000). Microbes are gaining resistance to existing antibiotic. Still, there is a desperate need of screening actionmycetes for antimicrobial compound.

The Sundarbans mangrove forest, one of the largest of such forests in the world (140,000ha), lies on the delta of the Ganges, Brahmaputra and Meghna rivers on the Bay of Bengal. It is adjacent to the border of India's Sundarbans world heritage site inscribed in 1997. The forest is unique for its agroecological condition and soil of this mangrove forest is routinely or occasionally inundated with low, moderate or high saline water. This ecosystem is ideally situated at the interphase between the terrestrial and marine environment and supports a rich and diverse group of microorganisms. The mangrove environment is

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a potent source for the isolation of antibiotic-producing actinomycetes (Rathna and Chandrika, 1993). Among thirty-two genera of actinomycetes Sundarbans' soil was rich of *Actinomyces*, *Micromonospora*, *Nocardia* and *Streptomyces*. Members of the order Actinomycetales have been the most widely exploited group of microorganisms in terms of biotechnological application and as such been the subject of prior reviews (Cross, 1982). Our investigation was aimed to screen actionmycetes from Sundarbans soil for antibacterial compounds against some gram-negative pathogenic bacteria.

MATERIALS AND METHODS

Collection and preparation of soil sample

In a systematic screening program for isolation of atino-mycetes, a total of 10 soil samples at different locations (0.5 km interval) in Karanjal region were collected from top 4 cm soil profile where most of the microbial activity take place, and thus where most of the bacterial population is concentrated. Soil sample (approx. 500 g) were collected using some clean, dry and sterile polythene bags along with sterile spatula, marking pen rubber band and other accessories. Care was taken to see that the points of collection had as widely varying characteristics as possible with regard to the organic matter, moisture content, particle size and colour of soil and to avoid contamination as far as possible. Samples were stored at -40°C (Upright Freezer, Climas) until pretreatment. Soil pretreatment was required for inhibiting or eliminating unwanted microorganisms. Moist heat treatment (Water Bath, Bibby Sterllin) had been employed to select various actinomycetes groups. One gm soil sample was serially diluted at 1:10 and 1:100 in sterile distilled water or normal saline. Soil suspensions were heated at 50°C for 10 min in a water bath.

Isolation of actinomycetes

Numerous media have been used for the isolation of actinomycetes from soil and other natural materials. Glycerolarginine medium (Porter et al., 1960), starch casein agar medium (Kuster and Williams, 1964) and colloidal chitin agar medium (Linagarppa and Luckwood, 1962) have been widely used for isolating soil actionmycetes. In this investigation, modified starch-casein agar (Mark) was used for isolating actinomycetes and pH of media used was set to 7.2. Cyclohexamide and nystatin (0.050 mg/ml) were added into the medium as antifungal agent (Porter et al., 1960; Phillips and Hanel, 1950). Dehydrated tryptone soy agar media (Oxiod) was used for routine culture of the bacteria under test. The isolation plates of actinomycetes contaminated with bacteria and fungi were purified by streak-plate technique. A small portion of typical isolated colonies were streaked on tryptone-soy agar media or starch casein agar media and incubated at 25°C for 2-7 days (Shellab). Plates were checked for the growth of typical actionmycetes colonies up to 10 days.

Morphological characterization

The Gram stain is a differential stain, which allows most bacteria to be divided into two groups, grampositive bacteria and gram-negative bacteria. Morphological characters of isolates were observed by smears from colonies up to 10 days, stained by Gram's method as described by Hucker and Conn (1923).

Microscopic characterization was done by cover slip culture method (Kawato and Sinobu, 1959). The mycelium structure, color and arrangement of conidiospore and arthrospore on the mycelium were observed through the oil immersion (1000×, Olympus) microscope. The observed structure was compared with Bergey's manual of Determinative Bacteriology, ninth edition (2000) and the organism was identified. Colonies were identified on the basis of their colony morphology and color (Shirling and Gottlieb, 1966). Color of aerial mycelium was determined from mature, sporulating aerial mycelia of the actinomycetes colonies on starch-casein agar media (Pridham, 1964).

Preservation

Nutrient agar slants were used for short time preservation of purified actinomycetes. The actinomycetes were inoculated in nutrient agar slant using a sterile loop and incubated at 25°C for 2 or 3 days. The one drum vial containing actinomycetes were kept in refrigerator at 4°C for short time storage. For longterm preservation, the expected bacterial growth were collected from subculture or purification plate by sterile inoculating loop and adequately mixed by vortex mixer. The isolates were kept at -40ºC in 10% glycerol broth.

Study of antibacterial activity

Antimicrobial activity of the selected isolates was tested against eleven gram-negative bacteria. They were *Shigella boydii*, *Shigella flexneri*-AN-31153, *Shigella sonnei*, *Pseudomonas*, *Shigella dysenterriae* type-1, *Vibrio cholerae*-0139, *Salmonella typhi*-Ao-12014, *Plesio-monas*, *Shigelloides*-Ao-12640, *Hafnia spp.*, *Vibrio cholerae*-OGET and *Escherichia coli*-186LT. *Shigell-oides*-Ao-12640 (serial number nine) was lost and did not streak as test pathogen. Some selected isolates were inoculated on trytone-soy agar along the middle line of the plate by streaking. After 5 days of incubation the cultures were broken by sterile needle, and 24 h fresh culture of the test organisms were inoculated by streaking at 90°C angle as close as possible to the streak line of actinomycetes on both sides at the 6th day of the incubation (Prescott and Dunn, 1959; Alexander, 1977; Brock et al., 1994). Five replicates were made for each of the actinomycetes isolates.

RESULTS AND DISCUSSION

Morphological and cultural characteristics of selected isolates

Isolation plates developed various types of bacterial actinomycete and fungal colonies. Fifty to sixty colonies were found per plate. Colonies selected from each plate were 5 to 20 based on colony appearance. Colonies having characteristic features such as powdery appearance with convex, concave or flat surface and color ranging from white, gray to pinkish and yellowish were selected. Colonies observed at 1st and 2nd day were eliminated because actinomycetes are considered as slow grower (Currie, 2006). Furthermore, bacterial configuration same as actionmycetes were accepted from gram staining. Fifty-five selected isolates were examined microscopically and identified by their morphological and culture characteristics. The isolates could be described under four

Table 1. Identification of actinomycetal isolates based on morphological and cultural characteristics.

Colony characteristics on starch-casein agar (after 7 days)	Microscopic characteristics (on 5th day)	Actinomycetal isolate	Total number of Isolates
Branched filamentous or 'spider-like' micro-colonies; sometime rough, heaped 'bread crumb' type colonies were also found.	Gram positive, non-acid fast; pleomorphic cells range from rods, Y and V shaped to occasional filament, which fragments readily.	*Actinomyces* (Casida, 1965)	27
Light yellow-orange to orange-red colonies, occasionally brown maroon or blue green. The dark brown to black spore colonies surface darken with spores.	Fine substrate mycelium with spores as cluster of grape, no aerial mycelium.	*Micromonospora* (Suarez, 1985)	3
Colony appears waxy, shiny; several millimeters in diameter; aerial filaments are formed, the colony surface become dull and fuzzy.	Gram positive, non-acid fast, show pleomorphic cells ranging from bacillary to coccoid structure; occasionally limited mycelium found which fragments readily to produce rod shape or coccoid cell.	*Nocardia* (Goodfellow, 1989; Gordon, 1962)	14
Powdery colony appears convex, concave or flat surface; white, gray to pinkish color colony.	Filaments long highly branched and nonfragmenting; arial filament with spirali, coils, or multiple branching & long chains spores.	*Streptomyces* (Anderson and Wellington, 2001; Locci, 1989; Wendisch and Kutzner, 1991; Williams et al., 1989).	11

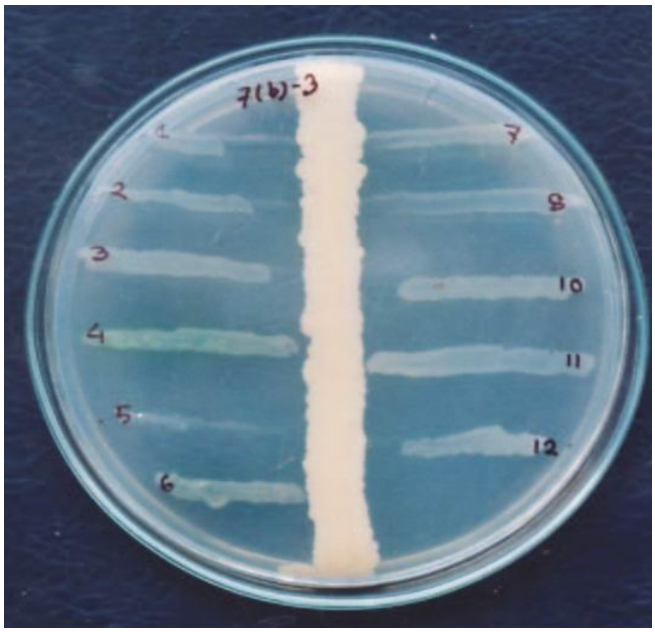

Figure 1. Positive antibacterial activity to test pathogen of isolate 7(b)-3.

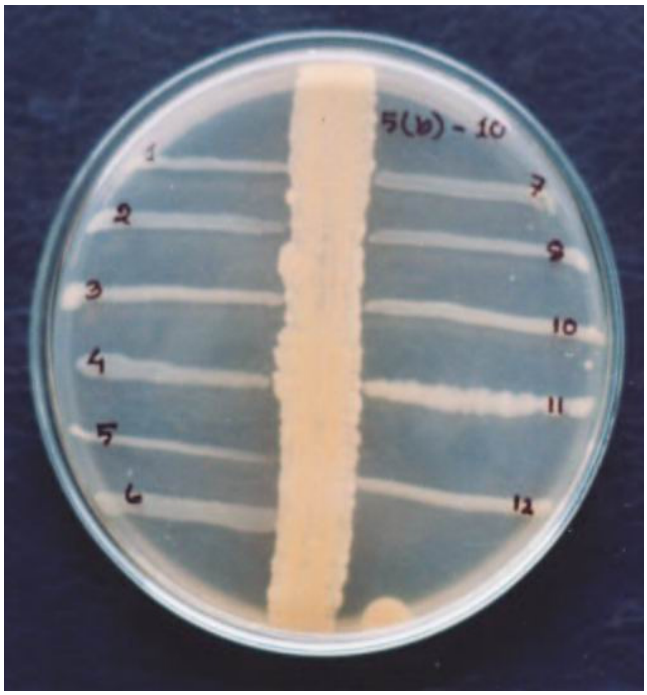

Figure 2. Negative antibacterial activity of isolate 5(b)-10 against test pathogen.

genera such as *Actinomyces*, *Nocardia*, *Micromonospora* and *Streptomyces* (Table 1).

A total of fifty-five actinomycetal isolates were screened for their antibacterial activity against eleven species of seven gram-negative pathogenic genera. No growth of the test organisms after 24 h adjacent to the streaking of Actinomycetes indicates positive antimicrobial activity of the isolates (Figure 1). If growth of the test organisms occurred in the entire streak line, then antimicrobial activity of the isolate was recorded as negative (Figure 2). The antibacterial activity of the test isolates was varied. Among the isolates tested, thirteen showed antimicrobial

Table 2. Antibacterial activity of actinomycetes isolates to tested pathogenic bacteria.

Isolates	Antibacterial activity										
	1. sSb	2. sf	3. Ss	4. Sd-1	5. Ps	6. Vc-0139	7. St-Ao-12014	8. Ple	10. Hf	11. Vc-OGET	12. *E. coli*
1(a)-6	-	-	-	-	-	+	-	-	-	-	-
3(a)-2	+	+	-	++	-	+	+	-	-	-	+
5(a)-1	-	-	-	++	-	-	-	-	-	-	-
5(a)-2	++	+	-	+++	-	+	+	+	+	+	+
1(b)-1	-	-	-	-	-	-	-	+	-	+	-
1(b)-2	-	+	-	+	-	-	+	+	-	-	+
2(b)-3	++	++	+	+	-	+	-	-	-	-	-
2(b)-7	-	-	-	-	+	-	-	-	-	-	-
3(b)-1	+	-	-	-	-	+	+	-	-	+	-
3(b)-2	-	-	-	+	-	-	-	-	-	-	-
3(b)-8	-	+	-	++	-	-	-	-	-	-	-
4(b)-9	-	-	-	-	+	+	-	-	-	-	-
4(b)-14	+	+	-	++	-	-	-	-	-	-	-
4(b)-18	-	+	-	+	-	-	-	+	-	-	-
5(b)-1	+++	++	-	++	-	-	-	+	+	-	+
5(b)-12	+	+	-	+	+	++	-	+	-	-	-
5(b)-15	+	+	-	-	-	-	-	-	+	-	-
5(b)-21	++	+	-	++	-	+	-	-	-	-	-
5(b)-24	++	+	-	++	-	-	-	-	-	-	-
7(b)-3	++	+	-	-	+++	-	-	-	+	-	+

+ = Fair, ++ = potent, +++ = highly potent, Sb = *S. boyddi*, Ss = *S. sonnei*, Sf = *S. Flexner*, Sd = *S. dysentery* type, Ps = *Pseudomonas* sp, Vc = *V. cholera,* St = *S. typhi*, Pl = *Plesiomonas* sp, Hf = *Hafania* sp, Es = *E. coli.*

activities against more than one genus of test pathogen. Isolate no. 2(b)-3 showed antibacterial activities against all four species of *Shigella* tested while some other exhibited sensitivity to all four. Moreover, seven isolates 3(a)-2, 5(a)-2, 4(b)-14, 5(b)-12, 5(b)-21 and 5(b)-24 were found similar activity against three species of *Shigella* such as *S. dysenterice*, *S. boyddi* and *S. flexneri* tested for sensitivity but ineffective against *S. sonnei*. Isolate 7(b)-3 showed antibacterial activity to four genera of the test pathogen (Figure 1). A highly broadspectrum antimicrobial activity inhibits growth of all the genera tested except *Pseudomonas* (isolate 5(a)-2). Beside isolates-1(b)-1, 1(b)-2, 3(b)-1, 5(b)-12, and 5(b)-21 were found to be effective against *Salmonella* and/or *Vibrio*. Total number of isolate which showed positive result in antibacterial activity (at least against one clinical isolate) was twenty (Table 2).

About 100 strains were isolated from a mangrove stand of Morib, Selangor and Malayasia (Vikineswary et al., 1997). In a previous study (Denizci, 1996), 356 *Streptomyces* isolates were obtained from soils in the Aegean and East Black Sea regions of Turkey and 36% of the isolates were found to be active against tested microorganisms; they were active against *Staphylococcus aureus* (20.78%), *E. coli* (2.52%), *Micrococcus luteus* (18.25%), *Mycobacterium smegmatis* (22.47%) and *Bacillus subtilis* (12.07%). Antibacterial activity of actino-mycetes isolated from Lobuche area of Nepal showed that two were active against only gram-negative organism, 8 against gram-positive organisms and 26 against both gram positive and gram-negative organisms. Among them, 31 of the isolates were active against *B. subtilis*, 27 against *S. aureus*, 17 against *E. coli*, 15 against *S. typhi* and 14 against proteus species (Bhagabati et al., 2004).

Considering the above mentioned results, it was concluded that Karanjal region of Sundarbans is rich with *Actinomyces.* From the result of the antibiogram, it could also be seen that seven from the investigated isolates exhibited higher activity against the tested pathogenic bacteria (more than four species). The widest activity spectrum and the largest inhibition zones were shown by strains 5(a)-2, 5(b)-1, and 7(b)-3 while the first exhibited the best performance.

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