Screening of antibiotic-producing *Streptomyces* from marine sediments of Bangladesh

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Article Info

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Abstract

Antimicrobial resistance is a rising concern in the treatment of infectious diseases and the discovery of potential antimicrobial compounds is needed to combat against it. The focus of this study was the *in vitro* antimicrobial activities of *Streptomyces* obtained from the soil samples collected from different places of Cox's Bazar, Bangladesh. A total of 156 isolates was obtained from thirty soil samples using two selective media namely yeast malt agar and starch casein agar. The isolates were morphologically distinct on the basis of spore mass color, reverse slide color, aerial and substrate mycelia formation and production of diffusible pigment. Among the isolates, 12 exhibited good antimicrobial activity against the tested micro-organisms. Isolates were subjected to biochemical characterization and identified as *Streptomyces spp*. The results suggest that the *Streptomyces* species could be a promising source for potential antibacterial agents.

Introduction

The discovery and application of antibiotics in the treatment of bacterial diseases had been a noteworthy medical success of the 20th century.[1] The demand for new antibiotics continues to grow due to the rapidly emerging of multiple antibiotic resistant pathogens causing life-threatening infection and nature still remains the richest and the most versatile source of new antibiotics.[2-4] Natural products having novel structures have been observed to perform inherent biological activities. The soil is a natural reservoir for microorganisms and their antimicrobial products. Soil *Actinomycetes* are of special interest because of their known property to produce chemically diverse compounds with a wide range of biological activities.[5] A huge number of currently used antibiotics including erythromycin, streptomycin, rifamycin, and gentamycin, are all products isolated from soil *Actinomycetes*.[6] The two major groups of soil *Actinomycetes* that serve as important sources of antibiotics are *Streptomyces* and *Micromonospora*. It has been stated that *Streptomyces* account for about 80% of the total antibiotic products; while *Micromonospora* closely follows with less than one-tenth as much as *Streptomyces*.[7]

The *Actinomycetes* are classified as a group of gram-positive bacteria with high G+C content (>55%) that are unique for their spore-forming abilities and formation of mycelia structures.[7, 8] The most important characteristic of the genus *Streptomyces* that they are potential sources for secondary metabolites possessing a variety of biological activities, including antibacterial, antifungals, antivirals, antitumoral, anti-hypertensives, and mainly antibiotics and immunosuppressives, which is used for human and animal treatment.[7]

Marine environments are a largely untapped source for the isolation of new micro-organisms with the potentiality to produce active secondary metabolites.[9] Researchers have already isolated more than 11,000 marine-derived natural products and several compounds have been shown to possess significant bactericidal activity. The extreme environment of salinity and pressure exist in the marine environment cause these *Streptomyces* to adapt and produce natural compounds.[10]

Bangladesh is a tropical country and contains great ecological diversity. Relatively few scientific studies have been carried out for new antibiotics from micro-organisms from the marine soil, where *Actinomycetes*, in particular, *Streptomyces* spp. are found abundantly. *Streptomyces bangladeshensis*, is a new species of *Streptomyces*, from the soil samples of Bangladesh producing bis-(2-ethylhexyl)-phthalate.[11] Actinomycin D was isolated from a new type strain of *Streptomyces parvulus* strain MARS-17, from the soils collected from Rajshahi, Bangladesh.[12]

Looking for the new and safe antibiotics to tackle the antibiotic resistance problem, the aim of this study was to explore few new regions of soil samples in Cox's Bazar marine ecosystems

Table I

List of sample sources and isolated colonies

Sample sources	Growth pattern		Number of isolates
	Yeast malt agar	Starch casein agar	
Moheshkhali island	Abundant	Abundant	30
Moheshkhali bridge area	Abundant	Abundant	25
Moheshkhali shooting area	Abundant	Abundant	37
Moheshkhali ghat	Abundant	Good	18
Sugondha beach	Abundant	Abundant	17
Laboni beach	Abundant	Abundant	20
Joint of beach	Good	Good	9

of the Bay of Bengal which contains a vast diversity of microbial community and also to isolate *Streptomyces* which may produce exceptional bioactive compounds as antibiotics against the selective human pathogenic micro-organisms.

Materials and Methods

Sample collection and processing

Thirty soil samples were collected from the different places of Cox's Bazar including Moheshkhali Island, Moheshkhali bridge area, Moheshkhali Shooting area, Moheshkhali ghat, Sugondha beach and Laboni beach of the Bay of Bengal (Table I).

The samples were collected from both the surface and 1.5 inch depth of the soil. The sample collection was done using grab sampler, placed in sterile polyethylene bag, closed tightly with a rubber band and then brought to the laboratory for analysis. All the samples were labeled separately using a marker. 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 color of the soil and to avoid contamination as far as possible. The samples were stored at -4°C (Upright Freezer, Walton) until pretreatment.

All samples were air-dried for 1 week, crushed and sieved. The sieved soils were then pretreated which were required for inhibiting or eliminating unwanted micro-organism. For physical treatment, dry heat treatment of soil (60-65°C for 3 hours) was used to screen out only heat-resistant microbes. This treatment inactivates the common microbes by denaturing their protein.[13] The pretreated soil was stored at -4°C until next step.

For each collected sample, 1 g of soil sample was suspended in 9 mL of 0.9% sterile saline water and agitated with the vortex at maximum speed for the detachment of spore chains. Then successive dilution were made up to 10^{-4} (1:10, 1:100, 1:1000, 1:10000).

Plating and isolation

Yeast malt agar (ISP medium 2) and starch casein agar were used. Distilled water was used for the media preparation. Nystatin (10,000 units/mL) and cyclohexamide (5% w/v) were added to both media to inhibit the fungal contamination.

A loop-full of inoculum from each of the dilution was streaked on yeast malt agar and starch casein agar separately. Plates were incubated at 30°C for 14 days and monitored after 48, 72, and 96 hours. Repeated streaking on the same media led to purify bacterial colonies that showed *Actinomycetes* like appearance. The isolated single discrete colonies were preserved at 4°C during two months and maintained for a longer period by serial subculture.

Morphological and cultural characterizations

The morphology of aerial hyphae, substrate mycelium and spore chains of a 14-day culture sample were examined by light microscopy. The cultural characteristics of the strain grown at 28°C on different media for 14 days were examined. The presence of soluble pigments and the melanoid pigment was investigated on yeast malt agar. The pure isolated colonies were studied regarding their intensity of growth, growth pattern, colony color along with the color of aerial and substrate mycelia, and the formation of soluble pigments, on starch casein agar.[14] Isolates were subjected to gram staining and examined under oil immersion (100 x).

Screening of soil isolates for antibiotic activity

Antimicrobial activities of the isolated cultures were determined by one-way streak method.[15] Antibiogram was performed against four pathogenic organisms namely *Staphylococcus aureus* (ATCC 25923), *Salmonella typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853).

Results

Isolation of Streptomyces

A total of 156 isolates were recovered from the marine soil samples collected from the different location of the Cox's Bazar in Bangladesh.

The colors of the aerial and substrate mycelium are described in Table II. Aerial hyphae arrangements, spore chain ornamentation and spore surface of the isolates were subsequently observed by light microscopy, indicating variation among the sporophore sizes and ornamentation and even in the spore surface. The morphological examination of these

Table II

Morphological and cultural characteristics of the *Streptomyces* isolates

Isolate code	Aerial mycellium	Reverse side color	Soluble pigment color	Melanin pigment	Spore chain morphology
MS6	White	Brown-White	White	-	Rectus-Flexibilis
MS27	Yellow	Egg Yellow	Brown-Yellow	-	Rectus-Flexibilis
LB63	Brown	Brown-Yellow	Brown-Yellow	+	Rectus-Flexibilis
MB92	Orange	Orange-Yellow	Orange-Yellow	-	Rectus-Flexibilis
MI104	Orange	Orange-Yellow	Orange-Yellow	-	Rectus-Flexibilis
LB105	White	Brown-Yellow	Brown-Yellow	-	Rectus-Flexibilis
LB106	Grey	Brown-Yellow	Brown-Yellow	-	Flexuous chains
SB121	Brown	Brown-Yellow	Brown-Yellow	+	Spirales
SB126	Brown	Brown-Yellow	Yellow	-	Flexuous chains
MB131	Orange	Brown-Yellow	Brown-Yellow	-	Spirales
MS136	White	Brown-Yellow	Brown-Yellow	-	Flexuous chains
MB141	Orange	Orange-Yellow	Brown-Yellow	-	Spirales

Table III

Antimicrobial activity profile of *Streptomyces* to four tested pathogenic organisms

Isolate codes	Sources	Antimicrobial activity of *Streptomycetes* isolates against selected pathogens			
		S. aureus	*E. coli*	*P. aeruginosa*	*S.typhi*
MS6	Moheshkhali shooting (surface)	++	++	+	++
MS27	Moheshkhali shooting (1.5 inch depth)	+	+	++	++
LB63	Laboni beach (surface)	++	++	+++	+
MB92	Moheshkhali bridge surface	++	++	+	++
MI104	Moheshkhali island (1.5 inch depth)	+++	++	++	+++
LB105	Laboni beach (1.5 inch depth)	+++	++	++	+++
LB106	Laboni beach (surface)	++	++	++	+
SB121	Sugondha beach (1.5 inch depth)	+	++	+	++
SB126	Mid Sugondha beach (1.5 inch depth)	+	++	+	+++
MB131	Moheshkhali bridge (1.5 inch depth)	-	+	+	+++
MS136	Moheshkhali Shooting (1.5 inch depth)	-	++	++	++
MB141	Moheshkhali bridge (surface)	-	++	++	++

Here, + = fair antimicrobial activity, ++ = potent antimicrobial activity, +++ = highly potent antimicrobial activity against tested organisms; - = no antimicrobial activity

isolates indicates that these belong to the *Streptomyces* genus.[14, 16-18] Classical biochemical test evidenced that all the isolates were positive in starch hydrolysis, casein hydrolysis, and catalase and oxidase test.

Antimicrobial activity of Streptomyces isolates

Results indicated that out of 156 isolates, only 12 were shown to have potent antimicrobial activity against the test pathogens and designated as MS6, MS27, LB63, MB92, MI104, LS105, LS106, SB121, SB126, MB131, MS136 and MB141 (Table III). No growth of the tested pathogenic organisms after 24 hours adjacent to the streaking of *Actinomycetes* was detected indicating good antimicrobial activity of the isolates. The antibacterial activity of the test isolates was varied among the 12 isolates, only 3 isolates showed no zone of inhibition against *S. aureus,* but showed good inhibition zone against *E. coli*, *S. typhi* and *P. aeruginosa.*

Discussion

Streptomyces are widely represented in nature by the largest number of species and varieties. They differ greatly in their morphology, physiology and biochemical activities in producing the majority of

known antibiotics.[19] This study was aimed to isolate antibiotic producing *Streptomyces* from the soil collected from different places of Cox's Bazar. The soil contains a diversity community of organisms differentiated by morphology, biochemical and antibacterial activity.

The collected samples were varied in their physico-chemical, biochemical and biological nature, which consisted of sediment, seawater and intestinal tract of shrimp. For that reason, pretreatment methods such as heat treatment, air drying and serial dilution were done which was an important step for reducing contamination and enhancing the isolation of *Streptomyces spp*. The present results revealed that simply air drying and heat treatment coupled with shaking successfully enhanced the isolation of *Streptomyces*. In this study, we used two selective media namely yeast malt agar and starch casein agar supplemented with antifungal and antibacterial agents (nystatin and cyclohexamide) which allowed preferential growth of *Streptomyces spp.* over other species. Commonly, media with minimal nutrients containing high molecular weight compounds are used for the isolation of actinomycetes. In this respect, the obtained results confirmed the effectiveness of yeast malt agar and starch casein agar as previously demonstrated by Okazaki and Okami.[20] A total of 156 isolates were obtained with abundant colonies with white, yellow, grey and orange/brown color. Results indicated that most of the isolates were slow-growing, aerobic, chalky, and contain both aerial and substrate mycelia with a variety of colors relevant to that of *Streptomyces*. In particular, *Streptomyces* genus can be easily distinguished from all other bacterial groups based on their distinctive phenotypic features that derived from chemotaxonomic markers and a wide range of other stable expressed features such as micro- and macro- morphology, physiology and biochemical properties.[14,21] The gross of the obtained morphological, chemotaxonomical and physiological as well as biochemical properties clearly confirmed that the marine isolates under study are belonging to the genus *Streptomyces*.

Most of the isolates were obtained from Moheshkhali shooting area (37 isolates) and Moheshkhali Island (30 isolates) and, others from Moheshkhali bridge area, Laboni beach and Sugondha beach. Since the *Streptomyces* are highly tolerant to salinity, in this study they were found abundantly in the salty soil of Cox's Bazar marine ecosystem having salinity of 32% to 34.5%. The single streak method or perpendicular streak method was used to screen the antibiotic producing *Streptomyces* using Muller Hinton agar medium. Out of 156 isolates, the antibacterial activity was exhibited by 12 of the isolates belonging to the sample source of Moheshkhali shooting area (MS6 -surface; MS27 & MS136-1.5 inch depth), Moheshkhali bridge area (MB92 & MB141 -surface; MB131 -1.5 inch depth), Moheshkhali island (MI104 -1.5 inch depth), Laboni beach (LB63 & LB106 -surface; LB105 -1.5 inch depth) and Sugongha beach (SB121 & SB126 -1.5 inch depth).

In this study, out of 12 isolates 9 were found to have potent inhibition zone against all the four tested pathogenic bacteria, although 3 isolates (MB131, MS136 and MB141) were not able to inhibit gram positive *S. aureus*, but showed good inhibition zone against three remaining gram negative pathogenic bacteria. *E. coli, S. typhi* and *P. aeruginosa* were inhibited by all the 12 *Streptomyces* isolates (100%) and *S. aureus* were inhibited by 9 isolates (75%). In this study among 12 isolates with antimicrobial activity, some exhibited highly potential results against tested organisms. For example, five soil isolates MS6, LB63, MI104, LB105, and LB106 were found to have combined potent antimicrobial activity against *S. aureus*, *E. coli* and *S. typhi*; LB63, LB106, SB121, MS136, and MB141 showed good inhibition zone against *P. aeruginosa*. Most of the highly potent isolates belong to the source of Laboni beach, Moheshkhali Island, Sugondha beach and Moheshkhali bridge respectively. This is may be due to the physical, biochemical and biological parameters of the sample sources.

The extent of inhibition zone was relatively larger and clearer leading to the conclusion that the tested pathogens are susceptible to the active compounds produced by these isolates. It is therefore suggested that a combination of several molecular analysis methods such as DNA re-association and PCR-based fingerprinting techniques may extremely help to provide broader information about the total genetic diversity of soil *Streptomyces* community. Perhaps, such methods may lead to the improvement in isolating antibiotic producing strains of soil *Streptomyces* obtained in this study.

Conclusion

Our results indicated the tremendous potential of marine *Streptomyces* species as a useful and sustainable source of powerful antibiotic and bioactive natural products. The present study was successful in selecting effective sampling site for discovery of novel antimicrobial agent.

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