Isolation and Characterization of *Streptomyces* sp. ANBS-15 and Antimicrobial Activities of its Secondary Metabolites

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Abstract

A total of 15 actinomycetes were isolated and purified from soil samples collected from agricultural fields of Rajshahi University, Bangladesh. 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. The strain ANBS-15 was selected for further investigation due to its strong antibacterial and antifungal activity and was identified as a member of *Streptomyces* genus based on its morphological, cultural, physiological, utilization of carbon sources and biochemical characteristics. From Czapek-dox broth fermentation medium of this strain, two active compounds, namely as MB-1a and MB-1b, were separated and purified. The compounds showed moderate to high antibacterial and antifungal activities. The antibacterial activity was in the range of 9-22 mm against 8 gram-positive and 2 gram-negative bacteria. The activity of MB-1a was higher than MB-1b against gram-positive bacteria. The antifungal activities of MB-1a and MB-1b against *Candida albicans*, *Aspergillus niger*, *Aspergillus ocherus* and *Aspergillus ustus* were in the range of 10-17 mm using 50 µg/disc. The results indicate that the soil of Bangladesh is rich source of actinomycetes having antibacterial and antifungal activities. Thus, there is potential of discovery of novel bioactive metabolites.

Keywords: *Streptomyces*, Isolation, Characterization, Secondary metabolites, Antibacterial activity, Antifungal activity.

Introduction

The use of microorganisms to produce natural products and processes that benefit and improve our socioeconomic life-styles has been a part of human history since the days of early civilization. Isolating microorganisms from the environment is the microbiologist's first step in screening for natural products such as secondary metabolites and enzymes (Demain *et al.,* 1999).

Actinomycetes are the dominant group of soil population together with bacteria and fungi and are originally considered as an intermediate group between bacteria and fungi. They are free living saprophytic bacteria and a major source for production of antibiotics. They play a major role in recycling of organic matter (Unaoguet *et al.,* 1994), production of novel pharmaceuticals, nutritional materials, enzymes, antitumor agents, enzyme inhibitors, immune-modifiers and vitamins. Around 80% of the total antibiotic production has been obtained from *Streptomuces* (Wellington, 1992).

In the present study, we emphasized on the isolation, identification and characterization of *Streptomyces* collected from soil samples of agricultural fields of Rajshahi University campus. The isolated strain, ANBS-15 was characterized based on morphological, biochemical, cultural and physiological characteristics. The antimicrobial and antifungal activities of two compounds, MB-1a and MB-1b, obtained from fermentation broth of this strain have also been discussed here.

Materials and Methods

Collection of soil samples: Rajshahi University campus was selected for the collection of soil samples at various depth of the earth surface, ranging from layers of 18 to 36 inch depth. The samples were collected in sterile small plastic tubes by using a trowel and properly labeled indicating the date of collection and the depth and transferred to the laboratory in sterile polythene bags and stored for further study.

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Isolation of Streptomyces from soil: Starch-casein-nitrate (SCN) agar medium supplemented with 100 µg/ml cycloheximide was used for isolation and enumeration of actinomycetes. In conventional dilution plate technique, 1 gm of soil sample was suspended in 9 ml of sterile water and successive dilution was made upto 10^{-4}. An aliquot (0.5 ml) of suspension from the last dilution test tube was spread over starch-agar-casein agar medium and incubated for 7-9 days at 30°C (Balagurunathan, 2001). After incubation period, the plates were examined for typical colonies of *Streptomyces*. The typical round, small, opaque, compact, frequently pigmented colonies were examined under a light microscope (100X). The colonies that bear typical *Streptomyces* morphology were purified and sub-cultured on Yeast extract-glucose agar plates and stored for further assay (Bernard, 2007).

Initial screening of the pure isolates: The pure culture of the isolates was screened against a group of test bacteria to determine their antibacterial activity. Streak-plating technique was used for initial screening.

Characterization of ANBS-15: The isolate ANBS-15 was further characterized based on morphological, biochemical, cultural and physiological features. Microscopic characterization was carried out by cover slip culture method (William, 1967). Cultural characteristics were tested in yeast extract-malt extract agar (ISP-2), oatmeal agar (ISP-3), inorganic salt-starch agar (ISP-4), glycerol-asparagine agar (ISP-5), peptone-yeast-extract-iron agar (ISP-6), tyrosine agar (ISP-7), nutrient agar and yeast extract-glucose agar. Biochemical tests including nitrate reduction, starch, cellulose and gelatin hydrolysis, peptonization and coagulation of milk were also performed as recommended by ISP. Utilization of carbon sources were also tested on starch casein agar medium.

Metabolite production in fermentation medium of ANBS-15: Czapek-dox (acidic) broth, Czapek-dox (basic) broth media were used as culture media. A loopful of the inoculums of the preserved slant culture were added to 500 ml flasks containing Czapek-dox (acidic) broth, Czapek-dox (basic) broth media for small scale liquid fermentations. The flasks were then kept at normal temperature without shaking and stirring for about 10-15 days for the growth and secretion of bioactive compounds in the liquid media.

Extraction and purification of metabolites: The metabolites were extracted from the fermentation broth using ethyl acetate. Ethyl acetate was evaporated from the crude extract using a rotary evaporator at 40ºC under reduced pressure. The crude ethyl acetate extract was separated and purified by using preparative thin layer chromatography (PTLC).

Test microorganisms: A total of 10 test bacteria viz. *Staphylococcus aureus, Stap. agalactiae. Bacillus cereus, B. megaterium, B. subtilis, Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli, Shigella flexneri* and 4 fungi- *Candida albicans, Aspergillus niger, A. ochreus* and *A. ustus* were used in this study. Test organisms were obtained from the Institute of Nutrition and Food Science (INFS), University of Dhaka and International Center for Diarrheal Disease Research, Bangladesh (ICDDR, B). All reference bacterial and fungal cultures were subcultured on nutrient agar and potato-dextrose agar media, respectively. The bacterial slants were incubated overnight at 37ºC, and the fungal slant was incubated for 48 h at 37ºC.

Antimicrobial assay: In vitro antibacterial activity of two isolated pure compounds were performed by standard disc diffusion technique (Al-Bari, 2005). The discs (50µg/disc) were prepared by dissolving the compounds in ethyl acetate and applied on the sterilized filter paper discs (5mm in diameter) with the help of a micropipette in an aseptic condition and allowed to leave these discs for few minutes in an aseptic hood for complete removal of the solvent. Kanamycin (30µg/disc) and nystatin (30µg/disc) were used as standard antibiotics for bacteria and fungi, respectively. As a negative control, a blank disc impregnated with solvent followed drying off was used. Briefly, in this study, the test discs, standard discs and blank discs were placed in a Petri dish with a particular bacteria or fungi and then left in a refrigerator at 4ºC for 12-18 hour in order to diffuse the compounds from the discs to the surrounding media in the Petri dishes. The Petri dishes were then incubated at 37ºC for overnight to allow the bacterial growth and 48-72 hour for fungal growth. The antibacterial and antifungal activities of the pure compounds were then determined by measuring the respective zones of inhibition (ZOI) in mm.

Results and Discussion

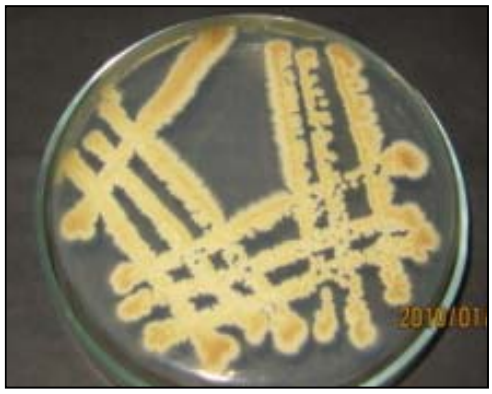
A total of 15 isolates of actinomycetes were isolated as pure culture from 5 soil samples. The colony forming units (c.f.u) were determined by counting the colonies on the dilution plates. Maximum number of colonies (0.47×10^6 c.f.u/gm of soil) were obtained in the soil collected from the cultivated land. This land is used for wheat cultivation. This cultivated land was near to a small lake and was very rich with natural composts which may be the reason for highest count. The sources of soil samples and the respective colony counts are shown in Table 1.

Table 1. Number of colonies of *Streptomyces* per gram of soil.

Collection site	Amount of soil (gm)	Number of colonies on dilution 3 plates	Number of Actinomycetes in each gram of soil (c.f.u/gm of dried soil)
Farm land, Rajshahi University (RU) Campus	1	47	0.47×10^6
Grave Yard, RU campus	1	45	0.45×10^6
Medicinal Plant Garden, RU campus	1	30	0.30×10^6
Bank of Pond, near Fine Arts, RU campus	1	0	0
Road side, near Botanical garden, RU campus	1	15	0.15×10^6

All of the isolates were screened for their ability to produce inhibitory substances against thirteen test microorganisms (data not shown). The test microorganisms included 8 Gram-positive bacteria, 2 Gram-negative bacteria and 4 fungi. Among the pure isolates, 9 isolates showed potent *in vitro* antimicrobial activity against the test organisms. The morphological examinations of these isolates indicate that these belong to the *Streptomyces* genus (Waksman, 1961; Shirling and Gottlieb, 1966; Holt, 1994). They showed good sporulation with compact, chalk-like colonies of different colors and characteristic earthy odor. The strain ANBS-15 was found to have potent antibacterial and antifungal activity against the test microorganisms and thus selected for characterization and fermentation to produce secondary metabolites.

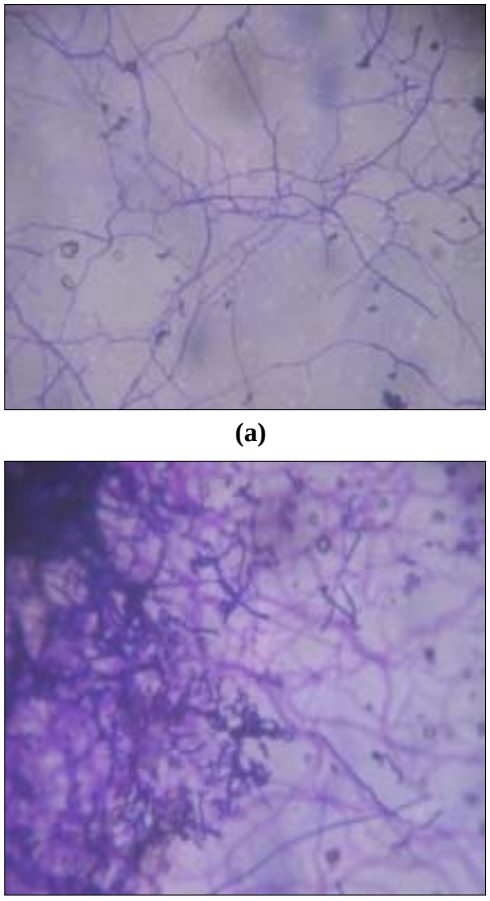
The morphological (Figure 1 and Figure 2), cultural (Table 2), and physiological characteristics (Table 3) of the strain ANBS-15 were studied. The organism formed extensively branched substrate hyphae and aerial hyphae which on maturation differentiated into spiral spore chains. The mycelia growth as well as development of spiral spore chains was studied microscopically under a light microscope at 400x magnification (Figure 2). Cultural characteristics (Table 2) were determined in yeast extract-malt extract agar (ISP-2), oatmeal agar (ISP-3), inorganic salt-starch agar (ISP-4), glycerol-asparagine agar (ISP-5), peptone-yeast-extract-iron agar (ISP-6), tyrosine agar (ISP-7), nutrient agar and yeast extract-glucose agar. Abundant



(a)

(b)

Figure 1. Aerial mycelial view (a) and reverse-side mycelial view (b) of *Streptomyces sp.* ANBS-15 grown on yeast-extract-glucose agar media after 5 days.



(a)

(b)

Figure 2. Microscopic view (400 X) of growing mycelia (a) and matured mycelia (b) of *Streptomyces sp*. ANBS-15 on yeast-extract glucose agar media (5 days old culture).

growth was observed in ISP-5 and ISP-7, moderate growths were observed in ISP-1 and ISP-2 whereas it did not grow in ISP-4 and ISP-6. Light-yellow reverse side mycelium was formed in ISP-2 and ISP-5.The physiological characteristics have also been extensively studied (Table 3). The temperature range for growth was in the range of 24-37.5˚C. Utilization of carbon sources (Table 4) were studied using nine different carbon sources. The strain ANBS-15 could utilize all carbon sources studied. Diffusible pigments were produced extensively when lactose, sucrose and L-rhamnose were used as carbon sources. These results of phenotypic, cultural, and physiological characteristics were compared with the properties of *Streptomyces* described in the literature (Waksman, 1961; Shirling and Gottlieb, 1966). The results indicated that the isolated strain ANBS-15 is a member of the genus *Streptomyces* and we named this isolated strain as *Streptomyces sp.* ANBS-15.

For small-scale liquid fermentation both Czapek-dox acidic (pH 5.3) and Czapek-dox basic (pH 8) media were used. The ethyl-acetate extract from these two fermentation media were subjected to chromatographic analysis. Two pure compounds, one from acidic medium and one form basic medium, were separated and purified by PTLC and designated as MB-1a and MB-1b, respectively. The two compounds were different in their physical characteristics. The R_f values were different under the same solvent system (data not shown).

Table 2. Cultural characteristics ANBS-15 (Incubation period: 14 days, Temp: 37.5°C.)

Medium	Growth	Aerial mycelium	Reverse side substrate mycelium	Diffusible pigment
Yeast-extract-malt extract agar (ISP 2)	Moderate	None	None	None
Oatmeal agar (ISP 2)	Moderate	Grayish brown	Light Yellow	None
Inorganic salt-starch agar (ISP 4)	None	None	None	None
Glycerol-asparagine agar (ISP 5)	Abundant	Brownish gray	Light Yellow	Moderate Yellow
Peptone-yeast-extract-iron agar (ISP 6)	None	None	None	None
Tyrosine agar (ISP 7)	Abundant	grayish brown	Brownish	Brown
Nutrient agar	Abundant	Whitish	Pale Yellow	None
Yeast-extract glucose agar	Abundant	Brownish gray	Light yellow	Moderate Yellow

Table 3. Physiological and biochemical characteristics of the strain ANBS-15. The '++' sign indicates positive, '+' sign weakly positive and '–' sign negative.

Properties	Results
Temperature range for growth	24-37.5°C
Optimum temperature for growth	29-37.5°C
Liquefaction of gelatin	-
Coagulation of milk	-
Peptonization of milk	+
Hydrolysis of starch	++
Decomposition of cellulose	+
Nitrate reduction	-
NaCl tolerance	0-2%

Table 4. Utilization of carbon sources by the strain ANBS-15. The sign '+++' indicates abundant production, '++' sign moderate, '+' poor production and '-' indicates no growth.

Carbon source	Utilization	Production of yellow pigments
D-Glucose	+	++
D-fructose	+	++
Lactose	+	+++
Mannitol	+	++
Inositol	+	+
Sucrose	+	+++
D-Xylose	+	+
L-Rhamnose	+	+++
Arabinose	+	++
No addition	-	-

Table 5. Antibacterial activity of pure compounds isolated from the organism

Test organism	Diameter of zone of inhibition (mm)		
	MB-1a (50 µg/disc)	MB-1b (50 µg/disc)	Kanamycin (30 µg/disc)
Gram positive Bacteria			
Staphylococcus aureus	16	14	22
Staph. agalactiae	15	11	23
Bacillus cereus	15	14	23
B. megaterium	22	20	23
B. subtilis	10	10	24
Pseudomonas aeruginosa	22	17	23
Proteus mirabilis	11	9	21
Gram Negative Bacteria			
Escherichia coli	10	12	22
Shigella flexneri	13	12	22

Table 6. Antifungal activity of pure compounds isolated from the organism

Test fungi	Diameter of zone of inhibition (mm)		
	MB-1a (50 µg/disc)	MB-1b (50 µg/disc)	Nystatin (30 µg/disc)
Candida albicans	10	9	22
Aspergillus niger	13	10	22
A. ochreus	11	11	23
A. ustus	17	15	22

The pure compounds were then tested for their antimicrobial activity. The results of antibacterial and antifungal activities in terms of zone of inhibition (ZOI) in mm are presented in Table 5 and Table 6. All the Gram-positive and Gram-negative bacteria showed a moderate to high sensitivity towards MB-1a and MB-1b. MB-1a (50µg/disc) was highly active against *Bacillus megaterium* and *Pseudomonas aeruginosa* producing a ZOI of 22 mm

compared to the 23 mm ZOI of standard kanamycin (30 µg/disc). Antifungal activities of both compounds (50 µg/disc) were determined in PDA media (Table 6). The compounds showed moderate to low antifungal activity with ZOI ranges from 10-17mm. *Aspergillus ustus* was most sensitive toward both MB-1a and MB-1b with a ZOI of 17mm and 15 mm, respectively, while *Candida albicans* was moderately sensitive.

Conclusion

The isolated microorganism was identified as a member of *Srteptomyces* genus but we could not identify it upto species level. 16S rDNA sequence is needed to confirm its identity. The structure of the pure compounds MB-1a and MB-1b should also be determined. As we have been successful in isolating several isolates of *Streptomyces* having antibacterial activity from soils of Rajshahi, so this indicates that the soils of this region may be an interesting source of new and potential antibiotics to tackle the emerging antibiotic resistance of the pathogenic microbes. Extensive study will be carried out in future to explore more bioactive compounds from this potential source of bioactive microbes.

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