ISOLATION OF BIOACTIVE SECONDARY METABOLITES FROM MARINE *STREPTOMYCES* SPECIES

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Abstract: *Marine Microorganisms and animals are gaining attention biotechnologically for the isolation of bioactive metabolites. An antagonistic marine microorganism was collected from Sundarban, mangrove forest of Bangladesh. The microorganism was identified on the basis of its morphological characteristics as Streptomyces species. It was grown in the yeast-extract glucose broth media at 37.5°C for 7 days for production bioactive metabolites.Antibacterial activity of ethyl acetate and chloroform extracts of the culture filtrate was performed against Bacillus subtilis and Escherichia coli. Cytotoxic activity of the extracts wes also determined by brine shrimp lethality bioassay. The LC_{50} values were 2.2, 0.9, and 1.6 µg/ml for chloroform and ethyl acetate extract and cephalosporin, respectively. Further analysis is going on to isolation of the active constituents from the extracts.*

Keywords: *Streptomyces spp, antimicrobial compound, marine microorganism, susceptibility testing &brine shrimp lethality bioassay*

Introduction

Microbial natural products are an important source of both existing and new drugs. Among the producers of commercially important metabolites, bacteria have proven to be a prolific source with a surprisingly small group of taxa accounting for the vast majority of compounds discovered till date[1]. Among these, Actinomycetes are the most economically and biotechnologically priceless prokaryotes. However, *Streptomyces* is the major producers of commercially important biomolecules.

Secondary metabolites produced by actinomycetes possess a wide range of biological activities[2-4]. The genus *Streptomyces* alone produces a large number of bioactive molecules[5-7]. It has an enormous biosynthetic potential that remains unchallenged without a potential competitor among other microbial groups. A large number of *Streptomyces* spp. have been isolated from soil and screened in the past several decades[8-9] for biologically important molecules. Above 500 species of *Streptomyces* account for 70–80% of relevant secondary metabolutes. An important reason for discovering novel secondary metabolites is to circumvent the problem of resistant pathogens, which are no longer susceptible to the currently used drugs[10-11].

The number of deaths due to these pathogenic organisms is on the rise. Secondary metabolites from marine actinomycetes may form the basis for the synthesis of novel therapeutic drugs, which may be efficient to combat a range of resistant microbes. Existence of cousins of terrestrial actinomycetes has been reported in the relatively

untapped marine ecosystem.The immense diversity of this habitat along with it'sunderexploitation is the fundamental reason for attracting researchers towards it for discovering novel metabolite producers. Researchers are finding new genera from marine environments on a regular basis and discovering new metabolite producers. Marine actinomycetes have proven to be efficient producers of new secondary metabolites[12-15],which show a never reported earlier range of biological activities such as antifungal, antitumor, antibacterial, immunosuppressive, insecticidal and enzyme inhibition, to name a few.

With the aim of finding new bioactive compounds from marine microorganisms and to investigate the bioactive metabolites, we started a program to identify *Streptomyces* species with antimicrobial activity and further to isolate their active metabolites

Materials and Methods

Collection of Soil Sample: Soil is the major source of microorganisms. It has the appropriate conditions for microbial growth. However, condition of soil as well as its microbial content and type varies from place to place and with its depth. Thus, for screening purposes soil samples were collected from different location of Sundorbon, Khulna, Bangladesh depth ranging from 0.25 to 1.5. The soil samples were stored in small clean polyethene bags and were tested for antagonistic species by "Crowded plate technique".

Isolation of the *Streptomycetes* organism: The developed colonies on the suitable plates of actinomycetes enumeration were picked up and transferred to Yeast Extract Glucose agar slants.

Morphological properties of *Streptomycetes*isolated: All strepyomycetes cultures isolated from different localities were examined to determine the color of (substrate mycelium) conidiospore and diffusible soluble pigments. These isolates were divided into different series according to Bergey's Manual of Determinative Bacteriology.

Production of antibiotic in liquid media: The isolated organism was grown in liquid Yeast Extract Glucose medium and the broth medium was separated using a cotton filler. To get the secreted bioactive principles in the liquid medium half of the filtrate was extracted with ethylacetate while the rest half was extracted with chloroform. After evaporation of the solvent, the extract was tested for their biological activity.

Sensitivity tests: To select the most promising antibiotic producing strepyomycetes, diffusion plate method was used.

Disk diffusion assay method: The nutrient agar medium for each test organism was poured in test tube and inoculated with one ml of bacterial, spore suspension (test organisms). These standard inoculums contained 10^4 -10^5 cells or spores/ml. Then, the inuculum was distributed to the petridishes in laminar flow cabinet to dry the surface of inoculated medium. The crude extracts were dissolved in DMSO (as it is nontoxic) in such a concentration that 25μl of it contained 200μg. Then 25μl of extract was placed on sterile paper discs. Thus, each disc contained 200μg. The discs were dried at low

temperature. The plates were refrigerated at 5°C for 30 minutes for complete diffusion of antimicrobial principles and thereafter they were incubated for 20 hours at 37 °C. The size of inhibition zone was used as an indicator of effectiveness. A comparison of inhibitory zone measurement showed the relative effectiveness on various test organisms[16].

Determination of cytotoxicity: The cytotoxic potentiality of the extracts were performed on brine shrimp nauplii using Mayer's method[17-18]. The eggs of brine shrimp (*Artemiasalina* Leach) were collected and hatched in a tank containing 1L of simulated seawater at a temperature around 37°C and pH 8.4 with constant oxygen supply. Two days were allowed to hatch and mature the nauplii. Pure DMSO solutions of the extracts were applied to *Artemiasalina* in a one day *in vivo* assay. 2 mg of each of the extract and antibiotic were accurately weighed and dissolved in 200 μl of DMSO. From stock solution 10, 20, 30, 40 and 50μl were taken and diluted each up to 5ml with sea water to give concentration of 20, 40, 60, 80 and 100μg/ml, respectively. The prepared test solutions were addad to the pre-marked vials containing 20 live brine shrimp nauplii in 5 ml simulated seawater and incubated for 24 h. After incubation period, the vials were examined using a magnifying glass in order to the number of survived nauplii in each vial. From this data, the lethality percent of the brine shrimp nauplii was calculated for each concentration. The median lethal concentration, LC_{50} of each tested sample was calculated from the plotted graph of percentage of the shrimp mortality *vs.* logarithm of the sample concentration, which was defined as the amount of extract required to kill 50% of brine shrimps within 24of exposure. From this data, percentage of mortality of nauplii was calculated at each concentration for each sample.

Results

Isolation of antagonistic species: The organism under investigation was obtained from the marine soil of Sundarbon, is the largest single block of tidal halophytic mangrove forest in the worldat a depth of 0.5 meter. Among a number of culture medium, yeast-extract glucose agar media was found to be most suitable for the isolation purpose.

Identification of the organism: Visual observations of the organism: The visual observation of the organism on 3 and 7 day are shown in the Figure 1 and 2, respectively. The following characteristics were observed: Upper surface of the organism white (upto 3 days) then turn to brown, the surface was velvety, the background was reddish and the colony of the organism was almost round.

Microscopic observations of the organism: The characteristics under microscope in yeast-extract glucose agar media are shown in figure 3 and 4 on spore stain after 3 and 15 days, respectively and were recorded at various time intervals as follows:

After 24 hours: Vegetative mycelia, no aerial mycelium, no sporulation were observed

After 48 hours: Branched vegetative mycelia, insufficient aerial mycelium and no spiral in aerial mycelia were observed.

After **72** hours: Round spores, branched vegetative mycelia, coiled or spiral aerial mycelia, abundant sporulation and reddish diffusible pigment was observed.

The Taxonomic Position of the Organism:

Order-Actinomycetales

Family- Strptomycetaceae

Genus- *Streptomyces*

Figure 1: Visual observation of colony of the isolated organism after 3 days of incubation

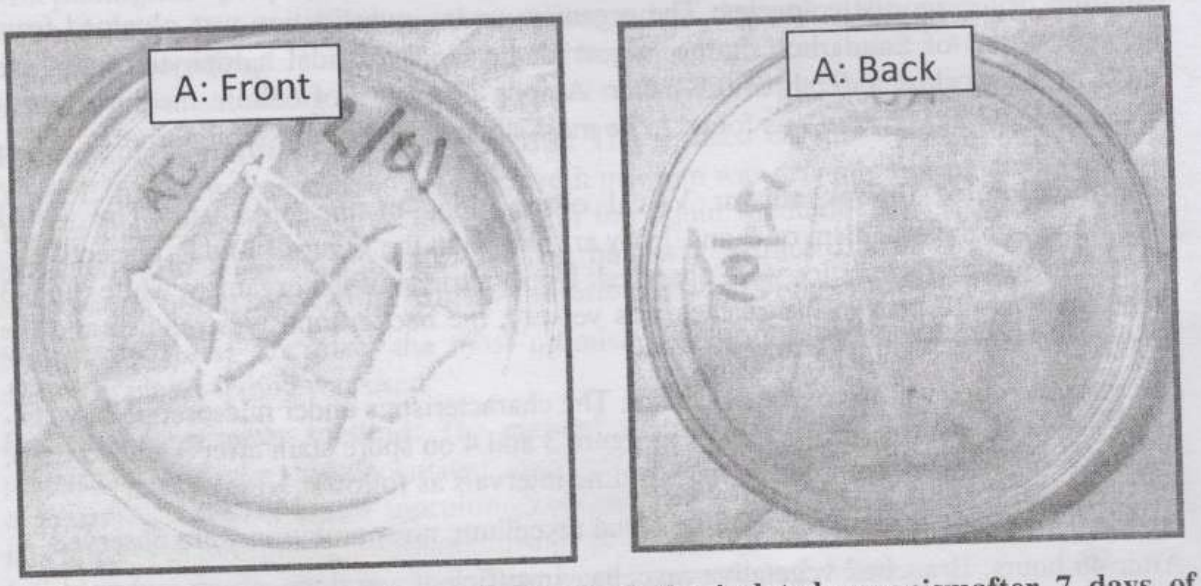

Figure 2: Visual observation of colony of the isolated organismafter 7 days of incubation.

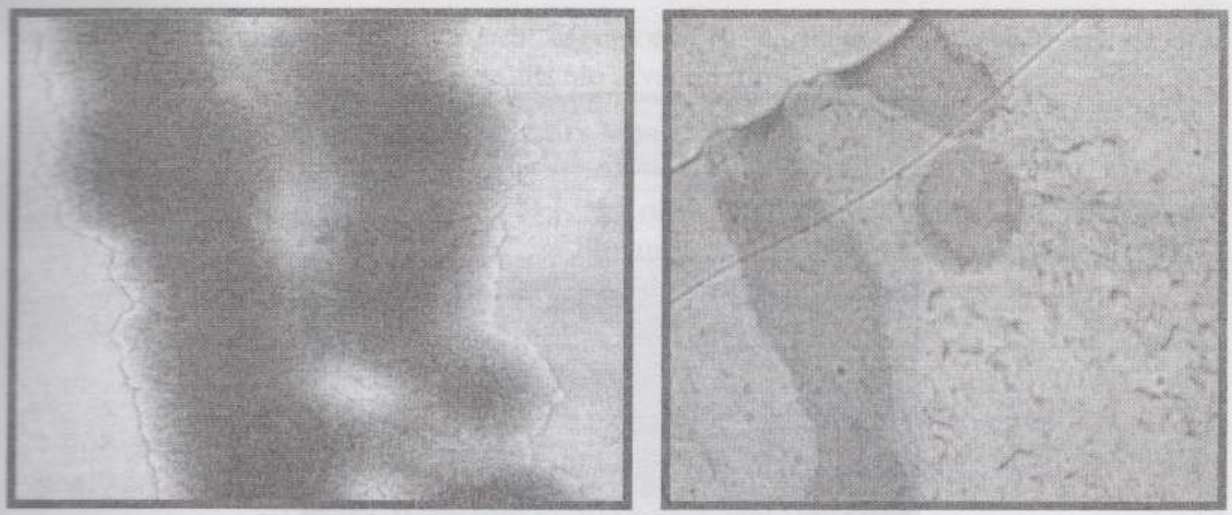

Figure 3: Microscopic view of the isolated organism after 3 days of incubation (staining spores , ×1000)

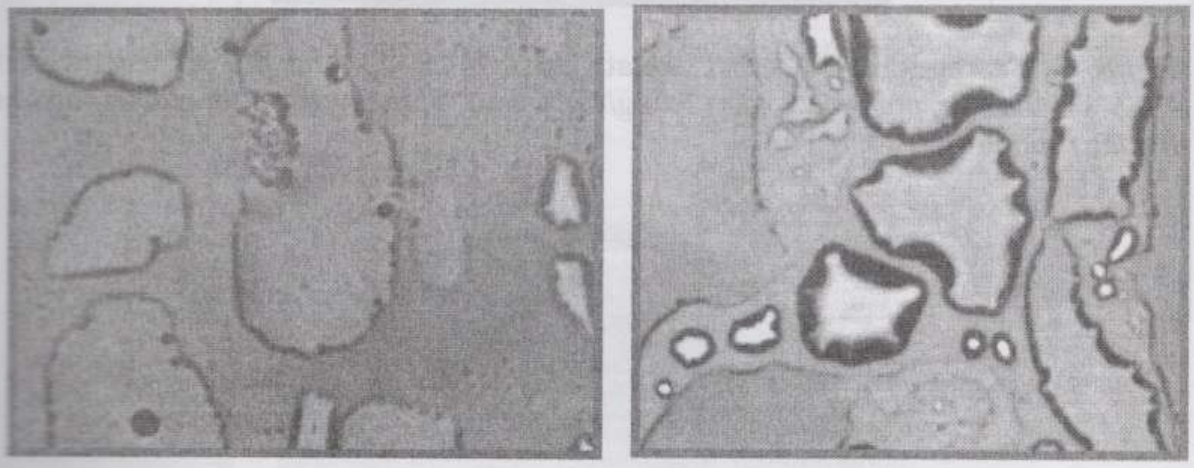

Figure 4: Microscopic view of the isolated organism after 15 days incubation of (staining spores, ×1000)

The results of extracts obtained from the isolated organism by disc diffusion method are shown in Table 1. The extracts showed almost similar inhibitory activity against the tested stains of gram positive and gram negative bacteria in the study. The antibacterial activity of the extracts was promising as compared to standard.

Table 1: Antibacterial screening of chloroform extracts of the isolated microorganism.

Test Bacteria	Diameter of zone of inhibition (mm)		
	Chloroform extract 200µg/disc	Kanamycin 30µg/disc	Ethyl acetate extract 200µg/disc
Bacillus subtilis	25	30	28
Escherichia coli	27	33	27

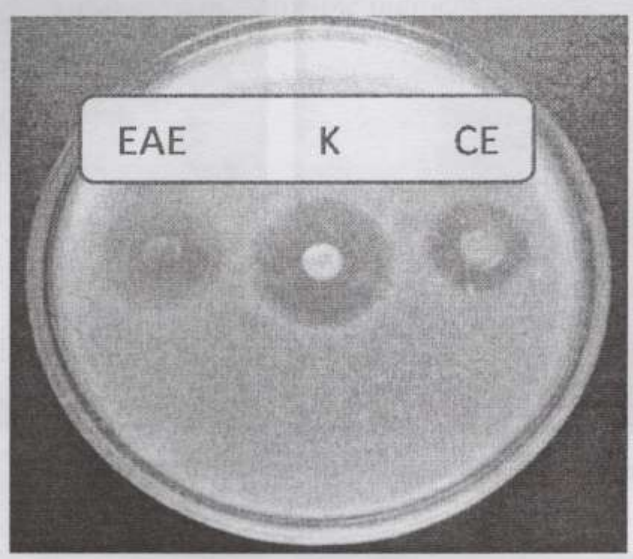

Figure 5: Susceptibility of ethyl acetate extracts (EAE) and chloroform extracts (CE) of the *Streptomyces* sp. (200µg/disc) and Kanamycin (30µg/disc) against *Bacillus subtilis*

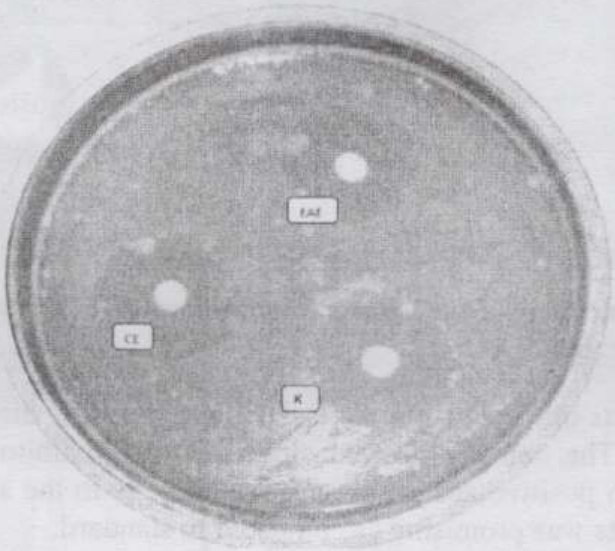

Figure 6: Susceptibility of ethyl acetate extracts (EAE) and chloroform extracts (CE) of the *Streptomyces* sp (200µg/disc) and Kanamycin (30µg/disc) against *E coli.*

Brine shrimp lethality bioassay: The mortality rate of brine shrimp nauplii was found to be increased with the increase of concentration of the samples and a plot of log of concentration vs. percent mortality gave an almost linear correlation. The chloroform extract and ethyl acetate extract positive results indicating that the extracts were cytotoxic. From the graph, the LC_{50} was determined by extrapolation (Figure 6) and

found to be 2.2, 0.9, and 1.6 μg/ml of chloroform extract, ethyl acetate extract and Cephalosporin, respectively. The results are given in the Table 2.

Table 2: Result of brine shrimp lethality bioassay

Test sample	Cone. of sample (C) (μg/ml)	Log C	No. of Shrimp added	% mortality after 24 hour	LC50 from the graph (μg/ml)
Control	0	0	20	0	
Chloroform extract	5.0	0.69	20	20	2.2
	10	1.00	20	25	
	20	1.30	20	35	
	40	1.60	20	40	
	80	2.00	20	45	
Ethyl acetate extract	5.0	0.69	20	45	0.9
	10	1.00	20	50	
	20	1.30	20	60	
	40	1.60	20	75	
	80	2.00	20	80	
Cephalosporin	5.0	0.69	20	35	1.6
	10	1.00	20	30	
	20	1.30	20	45	
	40	1.60	20	55	
	80	2.00	20	60	

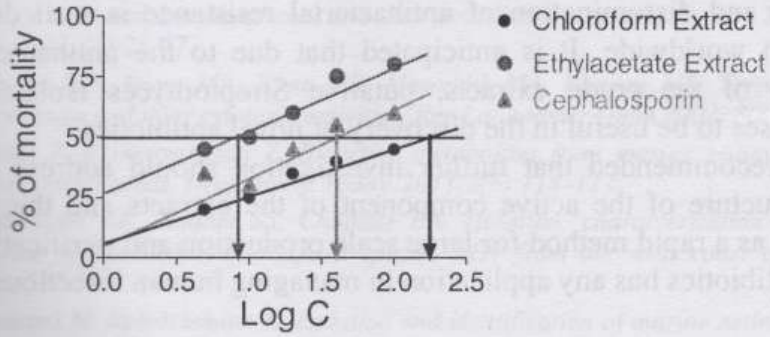

Figure 7: Determination of LC_{50} of CE, EAE and cephalosporin

Discussion

Streptomyces comprise 10% of the total bacteria colonizing marine aggregates. Marine habitat has been proven as an outstanding and fascinating resource for innovating new and potent bioactives producing microorganisms. Marine microbes are particularly attractive because they have the high potency required for bioactive compounds to be effective in the marine environment, due to the diluting effect of sea water. Members of the *Streptomyces*, which live in marine environment, are poorly understood and only few

reports are available. *Streptomyces* account 70% of the earth's surface and represent attractive source for isolation of novel microorganisms and production of potent bioactive secondary metabolites[19].

According to Kokare[20] during the screening of the novel secondary metabolites, *Actinomycetes* isolates are often encountered which showed more active antimicrobial activity against gram positive bacteria than gram negative bacteria. In the current study, also the *Streptomyces* species showed a good antimicrobial activity against *Bacillus subtilis*, than gram negative *E. coli*. The present study agreed with the earlier findings of Devi[21]in which it has been reported that *Streptomyces* species showed significant antimicrobial activity. The promising antibiotic producing isolates were identified as *Streptomyces* species[22]The present study also showed similar findings. In the present investigation, it has been observed that compared to other *Actinomycetes*, *Streptomyces* species showed efficient antagonistic activity. Only very few reports are available on the occurrence and distribution of antagonistic *Streptomyces* in the marine environment. The marine *Streptomyces* have not received much attention. Recent investigations indicate that the tremendous potential of marine *Actinomycetes*, particularly *Streptomyces* species as a useful and sustainable source of new bioactive natural products. Thus, the results of the present investigation reveal that the marine *Streptomyces* species from coastal environment are a potent source of novel antibiotics. It is anticipated that isolation, characterization and study of *Streptomyces* species can be useful in the discovery of novel species of *Streptomyces* species. *Streptomyces* species are the most important resources of these secondary metabolites.

Conclusion

The emergence and dissemination of antibacterial resistance is well documented as a serious problem worldwide. It is anticipated that due to the antibacterial profile and characterization of the crude extracts, putative Streptomyces isolated from marine organism promises to be useful in the discovery of novel antibiotics.

It is therefore recommended that further investigation should address the relationship between the structure of the active component of the extracts and the broad spectrum activity, as well as a rapid method for large scale production and purification and whether this group of antibiotics has any application in managing human infectious disease.

Acknowledgement

The authors wish to thanks Dr. Zakir Sultan, Research fellow, Centre of Excellence, University of Dhaka for helping to take microscope view.

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