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Investigating the Antimicrobial and Cytotoxic Properties of *Streptomyces* species Isolated from Sundarbans Mangrove Forest Soil

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Abstract

There has been a recent increase in interest in exploring marine microorganisms and fauna as sources of bioactive metabolites for biotechnological applications. This study adds to the field by exploring an antagonistic microorganism isolated from the Sundarbans mangrove forest in Bangladesh, identified as a *Streptomyces sp.* based on its morphological features. The bacterial isolates were grown in a yeast extract-glucose liquid medium for seven days at 37.5°C in order to stimulate the production of bioactive metabolites. The culture filtrate was subsequently extracted with chloroform and ethyl acetate. The ethyl acetate extract (200 µg/disc) shown antibacterial activity against gram-positive and gram-negative bacteria, including Bacillus subtilis and Escherichia coli, with inhibition zones of 28 mm and 27 mm, respectively. The chloroform extract produced smaller zones of 25 mm and 27 mm. Both extracts demonstrated promising antibacterial activity compared to Kanamycin (30 µg/disc). The brine shrimp lethality test demonstrated strong cytotoxic action, with the chloroform extract exhibiting the highest potency (LC50=2.2 µg/ml) and the ethyl acetate extract demonstrating an LC50 of 0.9 µg/ml. Cephalosporin served as a positive control with an LC_{50} of 1.6 µg/ml. These findings demonstrate the extracts potential as bioactive chemicals with cytotoxic characteristics

Key words: *Streptomyces* species, sundarbans mangrove forest; antibacterial activity; cytotoxicity.

Introduction

A vital resource for the development of new curative medications is still microbial natural products. Research has consistently shown that certain bacteria, particularly a select few species, possess significant biosynthetic potential for producing commercially valuable metabolites. (Bull, 2004). Among prokaryotes, actinomycetes stand out as a prolific source with immense biotechnological and economic significance. Notably, the genus *Streptomyces* holds the leading position within this group, serving as a primary source of biomolecules with substantial commercial value.

Actinomycetes generate secondary metabolites with a variety of biological functions (Berdy, 2005; Mann, 2001). An extensive range of bioactive compounds is produced by the species *Streptomyces* alone (Madduri *et al.*, 2001; Reeves *et al.*, 1998; Zhou *et al.*, 2006). It has a huge capacity for biosynthesis and is unopposed by any other microbial species that would pose a threat. Over the past few decades, many *Streptomyces sp.* have been isolated

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from soil and screened for biologically important molecules (Watve *et al.*, 2001; Williams *et al.*, 1988). More than 500 *Streptomyces sp.* are responsible for 70-80% of significant secondary metabolites. The problem of resistant infections, which cannot be treated with the present treatments, requires the discovery of new secondary metabolites (Ekwenye and Kazi, 2007). Antibiotic resistance has increased the global death rate, since diseases that were formerly readily curable with regular antibiotics are becoming more difficult to control. Marine actinomycetes produce secondary metabolites that serve as fundamental components for the development of novel therapeutic drugs to treat antibiotic-resistant bacterial infections. The discovery of new bioactive compounds may result from the special adaptations and biosynthetic capacities of these marine actinomycetes, suggesting the potential for new biotechnological applications in these understudied environments. The main factor drawing scientists to this ecosystem in quest of new metabolite producers is its extreme diversity combined with its underutilization. Scientists are often uncovering new metabolite producers and new taxa from marine habitats. These include enzyme inhibition, immunosuppression, anticancer properties, antifungal effects, and insecticidal actions. (Cho *et al.*, 2006; Hardt *et al.*, 2000; Macherla *et al.*, 2005; Wu *et al.*, 2007).

This study aimed to identify S*treptomyces sp.* and explore the potential bioactive properties of the culture extract. Additionally, we sought to isolate their active metabolites to uncover novel bioactive compounds from marine microorganisms and further investigate their bioactive potentiality.

Materials and Methods

Soil samples were collected from several locations in Sundarbans, Khulna, Bangladesh from a depth of 0.25 to 1.5 meters. The soil samples were examined using the crowded plate technique to check for antagonistic organisms after being kept in tiny, sterile polyethene bags.

To prepare the soil sample, 1 g of soil was poured in a 250 mL conical flask and added100 ml of distilled water. The mixture was shaken vigorously to form a homogeneous suspension. 1ml stock solution was added to 99 ml of sterile distilled water to make 1:1000 dilution. Serial dilutions were then conducted to achieve a dilution of 10^6. Following this, 50 µL of the diluted sample was inoculated into YPG agar medium by applying the spread plate technique, and the plates were kept at 37 °C for three days for incubation. During the incubation, *Streptomyces sp.* were identified and confirmed microscopically based on their colony morphology. To obtain a pure culture, a loop-full of the organism was streaked onto YPG agar plate and incubate at 37 °C for 10 days to allow for consistent growth without contamination.

The color of the conidiospores (substrate mycelium) and diffusible soluble pigments was evaluated in all *Streptomyces* cultures that were isolated from different sites. As per Bergey's Manual of Determinative Bacteriology, isolates were classified into various series and microscopic examination was carried out.

The isolated Streptomyces was cultivated in liquid yeast extract glucose (YPG) medium by inoculating one loopful into 10 mL of YPG broth and incubating it at 37°C for 10 days. After that, the culture was centrifuged at 10,000 rpm to separate the supernatant, which contains secondary metabolites. Half of the supernatant was added with ethyl acetate, and the other half with chloroform to extract the bioactive components. The biological activity of the resulting extracts was assessed after the solvents were evaporated.

*Streptomyce*s extracts were evaluated for their antibacterial activity against *B. subtilis* and *E. coli* using a disc diffusion technique. This technique aimed to identify the most promising Streptomyces strains that produce antibiotics. DMSO, which is non-toxic, was used to dissolve the crude extracts, ensuring that 25 µl of the solution contained 200 µg of extract. Subsequently, 25 µl of the extract was added to sterile paper discs, each containing 200 µg. The discs were dried using low heat and then stored

at 5°C for 30 minutes to promote diffusion. They were subsequently incubated at 37°C for 20 hours. The inhibitory zones were then measured and compared to a standard to evaluate their relative effectiveness against the various test species (Bayer *et al.*, 1966).

The extracts' cytotoxicity was assessed using brine shrimp nauplii and Mayer's technique (Hossain *et al.*, 2004; Islam *et al.*, 2002). Brine shrimp (Artemia salina Leach) eggs were collected and hatched in a 1l tank of artificial seawater, heated to 37ºC and maintained at a pH of 8.4 with a constant oxygen supply. Nauplii were allowed two days to hatch and grow. Extract solutions were produced in pure DMSO, with 2 mg of each extract and antibiotic properly weighed and dissolved in 200 µl. Dilute 10, 20, 30, 40, and 50 µl of the stock solution with 5 mL of seawater to get concentrations of 20, 40, 60, 80, and 100 µg/mL. These test solutions were mixed into pre-marked vials containing 20 live nauplii in 5 ml of simulated saltwater and incubated for 24 hours. Following incubation, each vial's surviving nauplii were counted using a magnifying lens. The lethality percentage of brine shrimp nauplii was determined at each concentration. The median lethal concentration (LC50) of each sample, defined as the quantity needed to kill 50% of the nauplii within 24 hours, was calculated using a graph that plotted percentage mortality against the logarithm of sample concentration. The mortality percentage for each sample at each concentration was computed using this information.

Results and Discussion

Streptomyces sp. was obtained from the marine soil sample. Among a number of culture mediums, yeast-extract glucose agar media was found to be most suitable for the growth of microorganisms.

The morphological observation of the growth of organisms on 3 and 7 days are shown in Figure 1 (A). Upper surface of the organism was observed to be white up to 3 days then turned to brown, the surface was velvety, the background was reddish, and the colony of the organism was almost round.

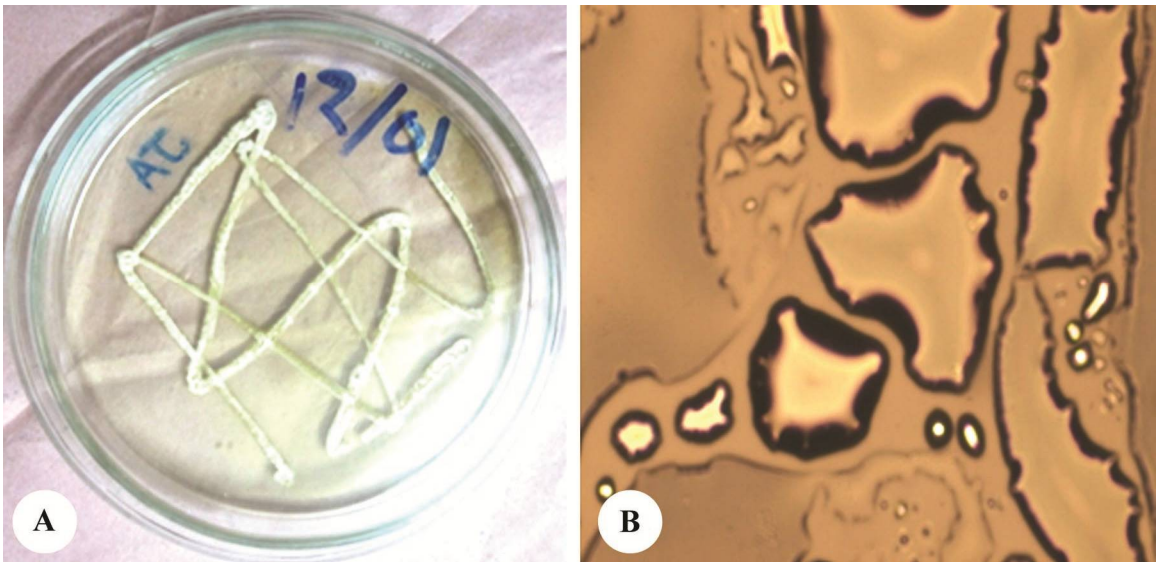

Figure 1. Morphological observation of the isolated organism. (A) Pure culture after 7 days of incubation, (B) Microscopic view of the isolated organism (stained sopres ×1000),

Under microscopic observation, we noted that this organism exhibits vegetative hyphae and branched mycelium, with spores arranged in chains. The characteristics of the *Streptomyces sp.* grown on yeast-extract glucose agar media are depicted in Figure 1 (B) following spore staining after 3 and 15

days. Observations recorded at various intervals showed that after 24 hrs, only vegetative mycelia were present, with no aerial mycelium or sporulation. By 48 hours, branched vegetative mycelia appeared, accompanied by insufficient aerial mycelium and no spirals. After 72 hrs, we observed round spores, abundant branched vegetative mycelia, coiled or spiral aerial mycelia, and significant sporulation, along with a reddish diffusible pigment (Figure 1). It was determined that the isolated microorganism was *Streptomyces sp.* based on morphological findings.

At a concentration of 200 µg/disc, the isolated Streptomyces ethyl acetate fraction inhibited gram-negative E. coli and gram-positive B. subtilis strains (28 mm and 27 mm, respectively) (Table 1). At the same concentration, the chloroform extract of the isolated Streptomyces demonstrated competitive inhibition zones of 25 mm against E. coli and 7 mm against B. subtilis. Compared to the conventional Kanamycin at 30µg/disc, the extracts demonstrated excellent antibacterial action.

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

Test Bacteria	Diameter of zone of inhibition (mm)		
	Chloroform extract (CE) 200µg/disc	Ethyl acetate extract (EAE) 200µg/disc	Kanamycin (K) 30µg/disc
B. subtilis	22.33±2.5	27.66±2.5	30.33±2.5
E. coli	29.66±3.0	28.33±4.1	35.66±3.0

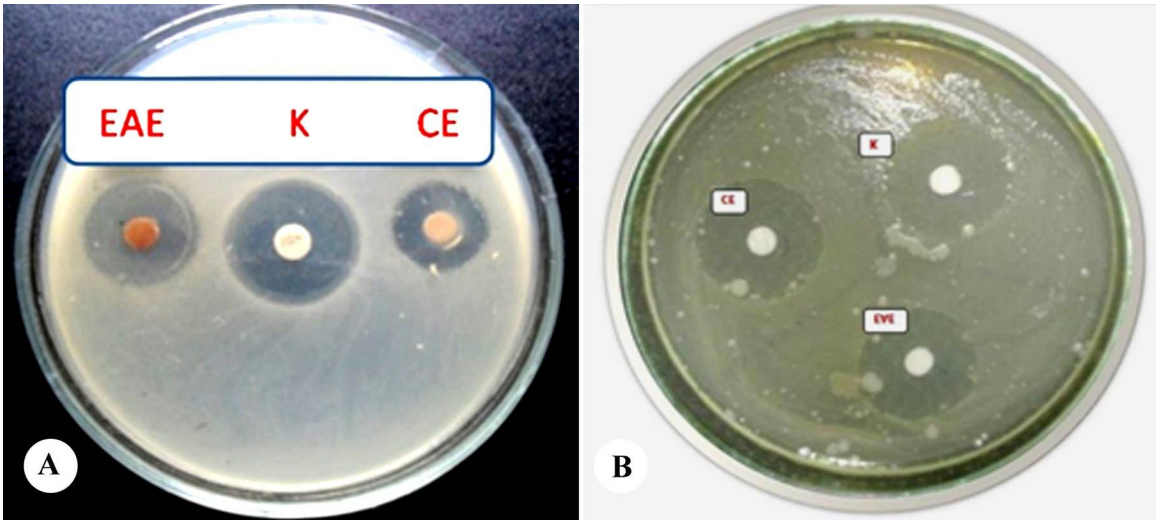

Figure 2. The susceptibility of ethyl acetate extracts (EAE) and chloroform extracts (CE) from *Streptomyces sp.* (200 µg/disc) and Kanamycin (30 µg/disc) was tested against *B. subtilis* (A) and *E. coli* (B).

In the brine shrimp lethality test, the death rate of nauplii rose with larger concentrations of the extracts, and a logarithmic plot of concentration against percent mortality revealed a roughly linear relationship. Both the chloroform and ethyl acetate extracts showed substantial cytotoxic action. The LC50 values for the chloroform extract, ethyl acetate extract, and cephalosporin were 2.2, 0.9 and 1.6 µg/ml, respectively, based on extrapolation from the curve in Figure 3. The findings are described in Table 2.

Table 2. Result of brine shrimp lethality bioassay.

Test sample	LC_{50} from the graph (µg/ml)
Control	
Chloroform extract	2.2
Ethyl acetate extract	0.9
Cephalosporin	1.6

Among the microorganisms found in marine aggregates, 10% are *Streptomyces* species. The oceanic environment is a unique and fascinating place to find microorganisms that can make potent bioactive substances. However, there are limited reports available, and our understanding of marine-dwelling *Streptomyces sp.* remains scarce (Heidarian *et al.*, 2019; Kachanuban *et al.*, 2022; Tan *et al.*, 2019). *Streptomyces* comprising 70% of the earth's surface and are an important source of powerful antibiotics (Koppula, 2011).

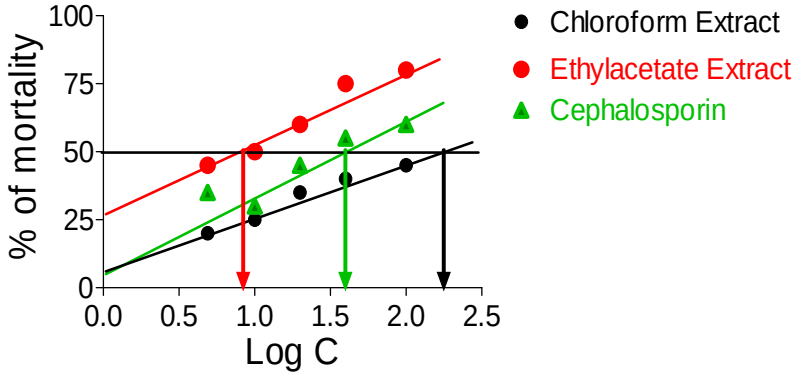

Figure 3. Determination of LC_{50} of CE, EAE and cephalosporin.

According to Kokare, when screening for novel secondary metabolites, *Streptomyces* isolates frequently exhibit stronger antimicrobial activity against gram-positive bacteria compared to gram-negative bacteria (Kokare *et al.*, 2004). The *Streptomyces sp.* examined in this study showed greater antibacterial activity against *B. subtilis* than the gram-negative *E. coli*. Current research has found that *Streptomyces* has high antibacterial activity. Some *Streptomyces sp.* were selected for possibility in generating antibiotics (Okazaki and Okami, 1972). Similar results were also obtained in the present experiment. Furthermore, it was found that *Streptomyces sp.* exhibited effective antagonistic action in comparison to other similar species. Regarding the prevalence and dispersion of antagonistic *Streptomyces* in the maritime environment, there are surprisingly few publications available.

The marine *Streptomyces* has received limited study. Recent studies show that marine Streptomyces has significant prospects. (Chakraborty *et al.*, 2022; Ibrahim *et al.*, 2023; Yang *et al.*, 2020). *Streptomyces sp.* are recognized as valuable and sustainable sources of new antibiotics. The findings of this investigation suggest that marine *Streptomyces sp.* from coastal environments could serve as a significant source of novel antibiotics. It is expected that the isolation, characterization, and study of these *Streptomyces sp.* will contribute to the discovery of new antibiotic-producing strains.

Conclusion

The development and dissemination of antibiotic resistance is now widely recognized as a significant global issue. In the quest for novel antibiotics, pure Streptomyces isolated from marine organisms is anticipated to be useful due to the antibacterial qualities and characterization of the crude extracts.

However, it is advised that more research be done to determine whether this class of antibiotics can have isolated to treat infectious diseases in human, as well as to explore the relationship between the broad-spectrum activity and the structure of the active component and a quick and efficient way to produce and purify them on a large scale.

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