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# *In vitro* Insecticidal and Time-Kill Profile of Ethyl Acetate Extract of Marine *Streptomyces* sp. Isolated from Sundarbans, Bangladesh

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**Abstract**

The marine soil and sediment samples were collected from different locations of mangrove forest Sundarbans, Bangladesh the largest tidal halophytic mangrove forest in the world. A total of twenty nine *Actinomycete* strains (AIAH-1 to AIAH-29) were isolated by serial dilution method using isolation media. Among twenty nine strains, AIAH-10 was selected for further study due to its potent antibacterial activity against a wide range of pathogenic bacterial strains. On the basis of morphological, cultural and biochemical studies, the strain AIAH-10 was assigned to *Streptomyces* sp. The present study was designed to investigate the *in vitro* insecticidal and time-kill profile of ethyl acetate extracts of marine *Streptomyces* sp. A dose dependent mortality was observed against the larvae of *Sitophilus oryzae.* The larval mortality was recorded as 100% in the concentration of 80 µg/ml and higher concentrations, $LC_{50}$ was found as 11.48 µg/ml. The minimum inhibitory concentration was recorded as 8 to 32 µg/ml against six different pathogenic bacterial strains. Average $Log_{10}$ reductions in viable cell counts for the extracts ranged from 1.91 $Log_{10}$ and 2.86 $Log_{10}$ cfu/mL after 3 h interaction and 2.10 $Log_{10}$ and 2.93 $Log_{10}$ after 6 h interaction at MIC, 2 × MIC, 3 × MIC and 4 × MIC concentrations**.** This investigation reveals that the *Streptomyces* species isolated from Sundarbans, Bangladesh may be interesting source for the isolation of potent bioactive compounds.

**Key words:** Marine, *Streptomyces*, Insecticidal, Time-kill profile, Antibacterial

## Introduction

As the frequency of novel bioactive compounds discovered from terrestrial microorganism decreases with time, much attention has been focused on screening of microorganism from diverse environments like marine source for their ability to produce new secondary metabolites. Studies have shown that microorganism isolated from the marine environment are metabolically active and have adapted to life in the sea (Valli *et al.,* 2012).

In the past, it was presumed that the marine environment was a "desert" with scarcity of life forms. However, it is now clear that the oceans are thriving with tremendous diversity of living microorganisms, with cell counts of $10^6$ - $10^9$ cells per milliliter (Fenical *et al.,* 2006) and levels of species diversity and richness predicted to exceed many of the Earths rainforests (Sogin, *et al.,* 2006; Stach *et al.,* 2005). This microbial diversity is presumed to translate into metabolic diversity resulting in the potential for new bioactive to be discovered.

Marine sediment is an inexhaustible resource that has not been properly exploited. It is estimated that less than 1% of potentially useful chemicals from marine environment has been screened so far, with microbial products representing approximately 1% of the total number. Exploration of microbial secondary metabolites has led to the discovery of hundreds of biologically active compounds. Members of the actinomycetes, which live in marine environment, are poorly understood and only few reports are available pertaining to microorganism from mangroves (Vikineswari 1997; Rathana and Chandrika, 1993).

Early evidence supporting the existence of marine microorganism came from the description of Rhodococcus marinonascene, the first marine microorganism species to be characterized (Helmke *et al.,* 1984). Actinomycetes account for approximately 10% of the bacteria colonizing marine organic aggregates (Grossart *et al.,* 2004; Alldrege

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*et al.,* 1988) and that their antagonistic activity might be highly significant in maintaining their presence, which affects the degradation and mineralization of organic matter.

Marine microbial biotechnology has opened up unexpected new horizons for finding novel organism for trapping their potential resources. More than 70% of our planet's surface is covered by oceans and life on Earth originated from the sea. As marine environmental conditions are extremely different from terrestrial ones, it is summarized that marine microorganism have different characteristics from those of terrestrial counterparts and therefore might produce different types of bioactive compounds (Imada *et al.,* 2007).

In addition to structural variety, bioactive compounds obtained from marine microorganisms are known for their broad range of biological effects, which include antimicrobial, antiprotozoan, antiparasitic, and antitumor activities (Matz *et al.,* 2004; Feling, 2003; Fremlin *et al.,* 2009), as well as antifouling activities that prevent the surface-settlement of various marine organisms (Xu *et al.,* 2010; Dash, 2009).

Recently, the marine derived microorganism has become recognized as a source of novel antibiotic and anticancer agent with unusual structure and properties. Moreover, marine derived antibiotics are more efficient at fighting microbial infections than the terrestrial bacteria have not developed any resistant against them (Jensen *et al.,* 2005).

The present study was performed to evaluate the insecticidal and and time-kill assay (antibacterial activity) of marine *Streptomyces* sp. (AIAH-10) isolated from mangrove forest Sundarbans, Bangladesh.

## Materials and Methods

*Collection of marine soil samples:* Marine soil samples were collected from the marine sediments and different locations of mangrove forest (Sundarbans), like Kochikhali, Jamtoplapoint, Tigerpoint, Dublarchor, Koramjol of Bangladesh, from the layers beneath the upper surface to the 1.5 cm depth. Samples were collected in plastic bag with proper labeling. Sixteen soil samples were collected within eight days (March 05 to March 12, 2011) and allowed to dry in hot air oven at 60-65°C for about 3 hours and kept in 4ºC until use.

*Isolation and characterization:* For the isolation of antimicrobial compound producing marine microorganisms from soil samples, the protocol developed by Dr. Jerry Ensign, Emeritus Professor, Bacteriology Department, University of Wisconsin, USA was followed. From seven soil samples, 29 pure isolates were separated and screened for antibacterial activity. Among them 79.31% isolates inhibited the test bacteria with variable activity-low, moderate to high. One isolate namely AIAH-10 was selected for further study for their high antibacterial activities. The organism belongs to *Streptomyces* genus on the basis of their morphological, cultural and biochemical characteristics (Shirling and Gottlieb, 1966). Pure culture of the strain was preserved in 4ºC, Yeast Extract Glucose Agar media.

*Extraction of ethyl acetate crude extracts:* The *Streptomyces* suspensions were prepared by suspending a loopful of pure *Streptomyces* colony in 2 ml sterile normal saline, vortexed to homogenize and stored at 4ºC until ready for use. This suspension was used as *Streptomyces* inoculants in all cultivations. Fermentation for production of bioactive was done as described by Ilic *et al*. (2007) with modification. Yeast extract Glucose broth (YEG) was prepared and 20 ml dispensed into 100 ml conical flask, sterilized, allowed to cool and inoculated with 0.5 ml *Streptomyces* isolate suspension and incubated at 37°C for 48 h at 250 rpm. About 500 ml of YEA was prepared in 1L conical flask and inoculated with the 48 h old pre-culture of *Streptomyces* isolate and incubated for 8 days at 37°C at 250 rpm. At the end of the incubation period, the culture was harvested by centrifugation at maximum speed for 15 min. The culture supernatant was extracted twice with equal volumes of ethyl acetate (1:1 v/v) and vaporized to dryness in a rotary evaporator at 50ºC. The extract was re-constituted in 50% filter sterilized ethyl acetate to obtain the desired concentration at every stage of screening.

*Collection of test species:* A total of six pure strains of pathogenic bacteria (three Gram-Positive bacterial strains, *Bacillus cereus*, *Streptococcus agalactiae* and *Agrobacterium* and three Gram-Negative bacterial strains, *Pseudomonas aeruginosa*, *Escherichia coli*, *Shigella dysenteriae*) were collected from the "International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B)

while the larvae of *Sitophilus oryzae* was collected from the Department of Zoology, University of Rajshahi, Bangladesh.

*Determination of minimum inhibitory concentration (MIC):* Sterile nutrient broth tubes containing different dilutions of extract (0.05 mg to 1.0 mg/ml) were specifically inoculated with 0.1 ml of standardized inoculums ($10^7$cfu/ml). The tubes were incubated aerobically at 37ºC for 18-24 h. Two control (tube containing the growth medium, saline and the inoculum) tubes for each organism were maintained. The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with the control tubes were regarded as MIC (Hassan *et al*., 2009).

*Determination of insecticidal activity:* Insecticidal activity of different concentrations of ethylacetate extracts of marine *Streptomyces* sp. *(*AIAH-10) was tested against the larvae of *Sitophilus oryzae.* Twenty larvae were placed in each of the beakers containing different concentrations of extracts (5, 10, 20, 40, 80 and 160 µg/ml). A control was kept without adding extract. The larvacidal effect of extracts was determined by counting the number of dead larvae after 24 hours. Dead larvae were identified when they failed to move after probing with a needle in siphon or cervical region. Each test was repeated thrice; the percentage of larvae mortality was determined (Khanna *et al*., 2007).

*Determination of time-kill profile:* The method of Spangler *et al*. (1997) was adopted for the time-kill assay against *Bacillus cereus* and *Escherichia coli* bacteria. Viable counts of the test bacteria were first determined. Initial inocula of $10^5$ to $10^6$ cfu/ml were prepared from 100 µl aliquots of test bacteria in normal saline and this was verified by performing colony counts. Eighty microliter (80 µl) of suspension of known cell density of selected test bacteria was added to 10 ml of Nutrient broth in McCartney bottles containing known concentration (relative to MIC) of each of the extracts. Time kill assay of each extract against *Bacillus cereus* and *Escherichia coli* bacteria were determined using the following extract concentrations: MIC, 2 × MIC, 3 × MIC and 4 × MIC and the reactants bottles were incubated in a rotary incubator at 37ºC. The time kill kinetics were determined at 0, 3 and 6 h. Exactly 0.5 ml volume of the reaction mixture was withdrawn at the appropriate time and transferred to 4.5 ml of nutrient broth recovery medium containing 3% sodiumthioglycolate or 3% "Tween-80" to neutralize the effects of the extracts carry-overs from the test suspensions and diluted serially in sterile nutrient broth. Approximately 100 µl aliquots of each dilution were plated out for viable counts by pour plate technique. Each experiment was done in duplicate, and the mean of two almost identical results was calculated. Only plates yielding 30 to 300 colonies were selected for counting. Data were analyzed by expressing the reduction of growth as the $Log_{10}$ colony forming unit per milliliter (cfu/ml). For the Time-kill end point determination; bacteriostatic activity was defined as a reduction of 0 to 3 $Log_{10}$ cfu/ml, and bactericidal activity was defined as a reduction of ≥ 3 $Log_{10}$ cfu/ml at 3 and 6 h compared to that at 0 h (Spangler *et al.,* 1997). McCartney bottles containing broth and test organism without extract was used as growth control in each experiment.

**Results**

Actinomycetes have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds. The activities of bioactive compounds from *Streptomyces* are categorized as pharmacologically, agrobiologically active agents and autoregulators. Thus, it is obvious that the activity profile of *Streptomyces* products is very broad.

The minimum inhibitory concentration of the ethyl acetate extracts of AIAH-10 was determined against three gram-positive bacteria such as *Bacillus cereus, Staphylococcus agalactiae* and *Agrobacterium* sp. and three gram-negative bacteria such as *Pseudomonas aeruginosa, Escherichia coli* and *Shigella dysenteriae* species by serial dilution method (Tyler, *et al*., 1988). The results of MIC are shown in the Table 1. The MIC value of extracts was varied between 8-32 µg/ml. The lowest MIC value 8 µg /ml was found against *Bacillus cereus* and *Staphylococcus agalactiae.* The MIC value of 16 µg/ml was found against *Agrobacterium, Pseudomonas aeruginosa* and *Escherichia coli* whereas MIC values for a *Shigella dysenteriae* were found to be 32 µg/ml.

**Table 1. MIC and minimum bactericidal concentration (MBC) of test samples.**

| Name of bacteria | MIC (µg/ml) | MBC (µg/ml) |
|---|---|---|
| 1. *Bacillus cereus* | 8 | 16 |
| 2. *Streptococcus agalactiae* | 8 | 32 |
| 3. *Agrobacterium* sp. | 16 | 64 |
| 4. *Pseudomonas aeruginosa* | 16 | 32 |
| 5. *Escherichia coli* | 16 | 128 |
| 6. *Shigella dysenteriae* | 32 | 256 |

As MIC is lower than the values of MBC, so it is evident that the extracts exhibit bacteriostatic rather than bactericidal activity.

Insecticidal activity of the ethyl acetate crude extracts against the larvae of *Sitophilus oryzae* in different concentrations was shown in the Table 2. Concentration dependent mortality was observed. The larvae mortality was recorded as 100% in case of 80 µg/ml and higher concentrations. The lethal concentration 50 ($LC_{50}$) of the extracts was found to be 11.48 µg/ml (from the Figure 1).

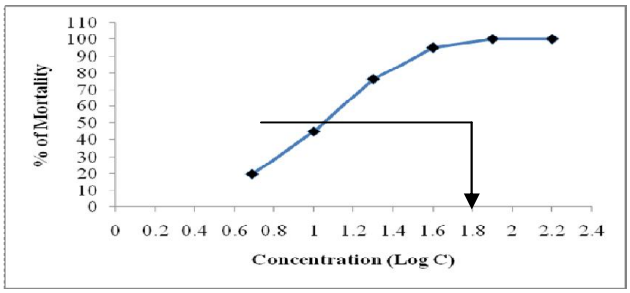


Figure 1. $LC_{50}$ of crude extracts against *Sitophilus oryzae* larva.

Time kill profile of the crude extracts obtaining from microorganisms isolated from marine sediments and soil samples of mangrove forest, sundarbans (Bangladesh) against the susceptible test bacteria are shown in Table 3. The extracts exhibited varying degrees of bacteriostatic activities depending on the test bacteria. A singnificant decrease in mean viable count of test bacteria was observed at each time interval. Results were presented as $Log_{10}$ cfu/ml.

**Table 2. Insecticidal activity of the ethyl acetate extract of test samples.**

| Group | Conc. of sample (µg/ml) | LogC | No. of larvae added | No. of death in each beaker | | | Average no. of death | Mortality (%) | $LC_{50}$ (µg/ml) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | | | |
| Control | 0 | 0 | 20 | 0 | 0 | 0 | 0 | 0 | 0 |
| Extracts | 5 | 0.69 | 20 | 3 | 7 | 2 | 4 | 20 | 11.48 |
| | 10 | 1 | 20 | 8 | 12 | 7 | 9 | 45 | |
| | 20 | 1.30 | 20 | 14 | 17 | 15 | 15.3 | 76.5 | |
| | 40 | 1.60 | 20 | 19 | 18 | 20 | 19 | 95 | |
| | 80 | 1.90 | 20 | 20 | 20 | 20 | 20 | 100 | |
| | 160 | 2.20 | 20 | 20 | 20 | 20 | 20 | 100 | |

**Table 3. Time-kill profile of ethyl acetate extract.**

| Test bacteria | MIC ($Log_{10}$) | | 2 x MIC ($Log_{10}$) | | 3 x MIC ($Log_{10}$) | | 4 x MIC ($Log_{10}$) | |
|---|---|---|---|---|---|---|---|---|
| | 3h | 6h | 3h | 6h | 3h | 6h | 3h | 6h |
| *Bacillus cereus* | 2.08 | 2.32 | 2.41 | 2.55 | 2.61 | 2.8 | 2.86 | 2.93 |
| *Escherichia coli* | 1.91 | 2.10 | 2.17 | 2.32 | 2.41 | 2.56 | 2.69 | 2.84 |

Mean Log reduction in viable cell count for the extracts ranges between 2.08 $Log_{10}$, 2.41 $Log_{10}$, 2.61 $Log_{10}$ and 2.86 $Log_{10}$ cfu/ml after 3h interaction and between 2.32 $Log_{10}$, 2.55 $Log_{10}$, 2.8 $Log_{10}$ and 2.93 $Log_{10}$ cfu/ml after 6h interaction in MIC, 2×MIC, 3×MIC and 4×MIC, respectively against *Bacillus cereus.*

Log reduction in viable cell count varied from 1.91 $Log_{10}$, 2.17 $Log_{10}$, 2.41 $Log_{10}$ and 2.69 $Log_{10}$ cfu/ml after 3h interaction and between 2.10 $Log_{10}$, 2.32 $Log_{10}$, 2.56 $Log_{10}$ and 2.84 $Log_{10}$ cfu/ml after 6h interaction in MIC, 2×MIC, 3×MIC and 4×MIC, respectively against *Escherichia coli.* For the time-kill end point determination; bacteriostatic activity was defined as a reduction of 0 to 3 $Log_{10}$ cfu/ml, and bactericidal activity was defined as a reduction of $\geq 3$ $Log_{10}$ cfu/ml at 3h and 6h compared to that at 0h (Ellof, 1998). From this

standpoint, the crude extracts exhibit bacteriostatic activity.

## Discussion

Marine ecosystems are poorly unexplored areas in terms of drug discovery. The ocean hosts the richest diversity of life forms and they may be a potential source of new molecules with unknown pharmacological and ecological functions. In this study, we explored the presence of antimicrobial compounds producing microbes in soils of Sundarbans, the largest tidal halophytic mangrove forest in the world. The soils samples were collected from the different regions of mangrove forest that is frequently inundated with moderate saline water. From seven soil samples, 29 pure isolates were separated and screened for antibacterial activity. Among them 79.31% isolates inhibited the test bacteria with variable activity-low, moderate to high. One isolate namely AIAH-10 was selected for identification for their potent antibacterial activities. The organism belongs to *Streptomyces* genus on the basis of their morphological, cultural and biochemical characteristics.

Killing of *Sitophilus oryzae* is a challenge to protect the paddy (*Oryza sativa*). It is reported that every year more than 5 to 10 % of total rice is destroyed all over the world including Bangladesh due to the attack of this insect. However now days, the killing of *Sitophilus oryzae* is largely depend on the use of synthetic chemical insecticides. But their repeated use has caused environmental problems and widespread development of resistance. Marine *Streptomyces* offers an alternative source of insect-control agents because they contain a range of bioactive chemicals, many of which are selective and have little or no harmful effect on non-target organisms and the environment.

The effectiveness of an antibacterial agent is measured by its ability to inhibit and kill bacteria (Nostro *et al*., 2001). *In vitro* time-kill assays are expressed as the rate of killing by a fixed concentration of an antimicrobial agent and are one of the most reliable methods for determining tolerance (Nostro *et al.*, 2001). Generally, the effect of the crude ethyl acetate crude extract of marine *Streptomyces sp.* on the test bacteria in this experiment is time and concentration dependent, as it is evident from the data presented. At higher concentration (4×MIC) and longer duration of interaction (6 h), more bacteria were killed. The *in vitro* data corroborates the reported efficacies of the several different crude extracts of marine *Streptomyces* sp. on a wide range of microorganisms and this will encourage the researchers to engage themselves towards the marine microorganisms to discover novel antimicrobial compounds.

## Conclusion

Natural compounds obtain from marine source plays significant input to identify various novel drug molecules. Bioactive compounds from *Steptomyces* sp. having diverse applications. Hence these compounds are serves as basic platform to find out new antibiotics and other natural products. The present evaluations emphasize the significance of *Steptomyces* sp. as potential sources of powerful insecticidal and antimicrobial agents. However further study is required to isolate the compounds and determination of their structures.

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