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Isolation of Marine *Streptomyces*, Characterization and Metabolites' Screening for Antibacterial Activity

Md. Anwarul Haque[1], Ashish kumar Sarker[1], Mohammad Sayful Islam[2], Kartik Ranjon Roy[3], Netish Kumar Kundo[3], Abu Syed Md Anisuzzaman[3,4], Md. Aktar Uzzaman Chouduri[3], Md. Anwar Ul Islam[3,*]

[1]Department of Pharmacy, Pabna University of Science and Technology, Pabna-6600, Bangladesh
[2]Department of Pharmacy, Primeasia University, Banani-1213, Dhaka, Bangladesh
[3]Department of Pharmacy, University of Rajshahi, Rajshahi-6205, Bangladesh
[4]Department of Hematology & Medical Oncology, Winship Cancer Institute, Emory University, Atlanta, GA 30322, USA
*Corresponding author: profanwarulislam@yahoo.com

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Abstract To combat drug resistant, searching of new broad-spectrum antibiotics is urgent and Marine *Streptomyces* can be potential new sources of antibacterial agents. With this aim, present study was carried out to search new, safe and effective antibacterial compounds. Twenty nine strains were isolated from marine sediments and soil samples of mangrove forest, Sundarban, Bangladesh using spread plate technique. Among them AIAH-5 and AIAH-10 were finally selected for further study owing to their promising activity against a series of pathogenic strains (done by streak plate method). The organisms were identified on the basis of cultural, morphological and biochemical properties. At least four compounds were isolated by chromatographic means, from the crude extracts of both organisms. But pure compounds, AI-5 and AI-10 were finally selected for their highest antibacterial efficacy. Antimicrobial activities of both AI-5 and AI-10 were performed against a series of pathogenic microorganisms. The minimum inhibitory concentrations (MIC) of the compound AI-5 and AI-10 varied between 8- 32 and 1- 4 µg/ml, respectively against a wide range test bacteria. The minimum bactericidal concentration (MBC) of the compound AI-5 and AI-10 were ranges from 16- 128 and 2- 16 µg/ml, respectively. The compound AI-5 and AI-10 also exhibited satisfactory activity against azithromycin and ciprofloxacin induced resistant *Escherichia coli* strain. This study demonstrates the diversity of the mangrove forest, Sundarbans, Bangladesh as a rich and interesting source of antibiotic molecules producing new and potential marine bacterial species.

Keywords*: antimicrobial activity, compound separation, time-kill, marine strepromyces*

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1. Introduction

Marine environment can offer a tremendous diversity of microorganisms which can be a rich source of potentials therapeutic chemicals [1]. The interest about marine organisms is growing in spite of being laborious culture technique. The importance of marine animals, plants and microorganisms as a source of pharmaceutically active compounds has manifested in many review articles [2,3]. A wide range of chemically unique metabolites with potentially therapeutic values were isolated from the marine sources, such as cyclomarin, an antiviral drug obtained from Streptomyces strain [4] and thiocoraline, a anticancer drug from marine Micromonospora strain [5]. That can't be easy available by the means of chemical synthesis, perhaps not reachable in sufficient amount for the therapy of mass people of third world countries, as a cheap medicine. From this viewpoint, we studied the marine actinomycetes, encompassing a large number of bacteria, exhibiting a distinct morphological, cultural and physiological properties [6], are ubiquitous in nature. The genus *Strepromyces* accounts for 50% of the total soil actinomycetes, best known for their secondary metabolites including antimicrobials, antiviral, immunomodulators, herbicides, pesticides, anti-cancer drugs production [7,8]. The worldwide bacterial resistance draws the researcher to discover new drugs to combat with. It is natural that the existing antibiotics with low spectrum of activity will be replaced by upcoming new one of wide spectrum. In this communication we describe the isolation, morphological & cultural characterization, optimization of fermentation process, purification of pure compounds and their biological assay of Streptomyces spp. AHAI-5 and AHAI-10.

2. Materials and Methods

2.1. Soil Samples Collection

Marine soil samples were collected from the marine sediments and different locations of mangroveforest

(Sundarbans), like Kochikhali, Jamtoplapoint, Tigerpoint, Dublarchor, Koramjol of Bangladesh, from the layers beneath the upper surface to the 1.5cm 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 4°C until use.

2.2. Isolation of Pure Cultures

Four sterile test tubes containing 9 ml of sterile distilled water were labeled as 1, 2, 3 and 4. One gram of dried soil sample was added into the test tube 1 and mixed well. From this solution, 1ml was transferred to test tube 2, and by successive serial dilution concentration of that soil samples were finally reached up to 10^{-3}. From each dilution, 0.1 ml amount was spread over the plate containing starch-casein- nitrate agar with cycloheximide (100µg/ml). Plates were allowed to dry before inverting and kept in incubator for a week at 30°C. Bacterial colonies were examined carefully under light microscope, and those colonies having sufficient zone of inhibition around it were scraped carefully and streaked on the transfer media by a flamed loop and re-cultivated several times for purity. Finally, 29 different actinomycetes strains were isolated and preserved in 4°C, in yeast extract-glucose agar slants.

2.3. Antimicrobial Activity Screening

Streak-plating technique [9] was used on yeast extract-glucose agar medium, for the preliminary screening of potential antimicrobial activity of those isolates. Each isolates was streaked as a single line on separate agar plate and incubated at 30°C for 5 days. Then the tested organisms were streaked vertically, going from the edge of that plate toward the fully grown *Streptomyces* isolates line. The plates were incubated for 12hrs, at 37°C and inhibited zone diameters were measured. MIC (minimum inhibitory concentration) was determined by the visual observation after incubation at lowest concentration that completely inhibited the growth of test organisms [10].

2.4. Morphological, Physiological and Color Determination Study

Morphological observations can easily be observed employing different types of standard cultivation media, namely ISP 2 (yeast extract-malt extract agar); ISP 3 (oatmeal agar); ISP 4 (inorganic salts-starch agar); and ISP 5 (glycerol-asparagine agar) medium and YEGA (Yeast extract – glucose agar). The morphological characteristics of the strains were determined in accordance with the method described by Shirling and Gottlieb [11]. Morphology was also examined under the advanced biological microscope, B1 series, Motic, China (Software: Motic image plus 1.0). Melanoid production, the characteristic has the implication in taxonomical studies were determined for different strains [12,13]. Different compounds, as carbon and nitrogen sources used to identify species level [14]. The nitrate reduction ability of these two strains were carried out in Nitrate Broth, incubating in an aerobic condition using Bacto-Nitrite Test Strips (Difco), following manufacturer's protocol. Additionally phenol (0.1% w/v) utilization test, hydrolysis of starch and milk was evaluated by the suggested media of Gordon et al. [15], optimum growth temperature, and liquefaction of gelatin [16] were carried out. After 14 days all cultural characteristics were evaluated.

2.5. 16SrRNA Amplification by PCR

A single colony was harvested from the agarplate in 500µl of saline-EDTA buffer (NaCl 150 mM; EDTA 10 mM; pH 8.2) and incubated for 1hr at 37°C. Adding, 10 µl of lysozyme solution (5mg/ml), 5 µl of proteinase K solution (15mg/ml) and 10 µl SDS solutions (25%), the mixture was again incubated for 30 minutes at 55°C. Lysate was extracted using equal volume of water saturated phenol chloroform mixture. Purified by ethanol precipitation method and dissolved in nano pure sterile water (60 µl). The 16S rRNA was amplified by the PCR in reaction mixture containing KOD FX buffer with 200 µM dNTP, 100 ng genomic DNA and 0.5 µg forward (5'-AGAGTTTGATCCTGGCTCAG -3') and 0.5 µg reverse (5'- GGTTACCTTGTTACGACTT -3') primer. Thermal cycle was performed with a model 22331 eppendrof (Germany). The samples were subjected to an initial denaturing step consisting of 2 minutes 98°C, after which 2U of Taq polymerase was added to each sample at 90°C. The thermal profile used was 30 cycle consisting of 1 min annealing at 52°C, 2 min extension at 72°C and 1 min denaturation at 94°C. The final extension step was consisted of 4 min at 65°C. PCR amplificants were detected by agarose gel electrophoresis (Figure 3) and visualized in a gel documenter (Alpha Innotech, U.S.A)

3. Results

During the screening of 29 isolates by its antibacterial activity against a wide range of Gram- positive and Gram-negative bacteria by the single line streaking technique, only strain AIAH-5 and AIAH-10 appeared to inhibit the growth of the test pathogenic organisms with a promising potency (Figure 1). The remaining isolates did not show any antibacterial activity. The aerial mycelium of AIAH-5 isolate appeared light brown on 4th day and as the time passes, it became more powdery as well as dusky reddish brown due to sporulation of the microorganism. On the other hand, thick and velvety cream colonies appeared with AIAH-10 on 4th day and turned in to the deep yellowish and powdery due to sporulation (Figure 2 and Table 1).

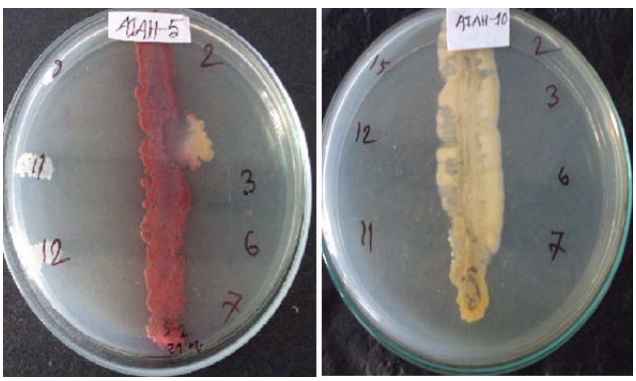

Figure 1. Antibacterial activities of the isolates (against Streptococcus agalactiae (2), Bacillus cereus (3), Pseudomonas aeruginosa(6), Escherichia coli (9), Shigella dysenteriae (11), Shigella sonnei (12) and Agrobacterium (15)) through single line streaking technique

Table 1. Cultural characteristic of AIAH-5 and AIAH-10 on 14th day

	AIAH-5			AIAH-10		
Medium	*Growth*	Aerial mycelium	*Pigmentation*	*Growth*	Aerial mycelium	Pigmentation
Trypton –yeast extract agar(ISP-1)	+	Light brown	++	+	Light brown	+
Yeast-extract-malt extract agar (ISP- 2)	++	Yellowish brown	+	++	Yellowish brown	++
Oatmeal agar (ISP -3)	++	Yellowish gray	++	++	Yellowish gray	++
Inorganic salt-starch agar (ISP- 4)	+++	Grayish yellow	++	+++	Grayish yellow	+++
Glycerol-asparagine agar (ISP-5)	++	Grayish brown	+	++	Grayish brown	++
Tyrosine agar(ISP-7)	++	Light gray	+	++	Light gray	+
Yeast-extract glucose agar (YEGA)	+++	Yellowish orange	+++	+++	Yellowish orange	+++

Note, +++, high; ++, moderate and +, Low.

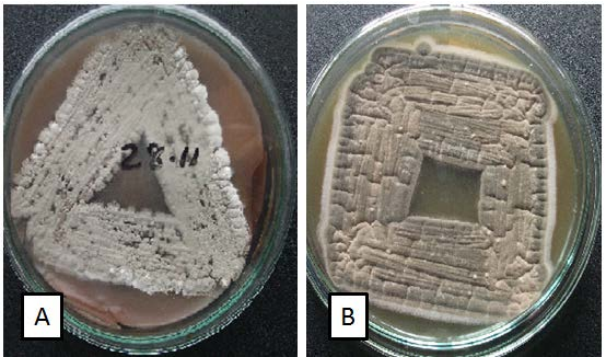

Figure 2. Aerial mycelial view on 21st day of AIAH-5 (A) and AIAH-10 (B) on yeast-extract glucose agar medium

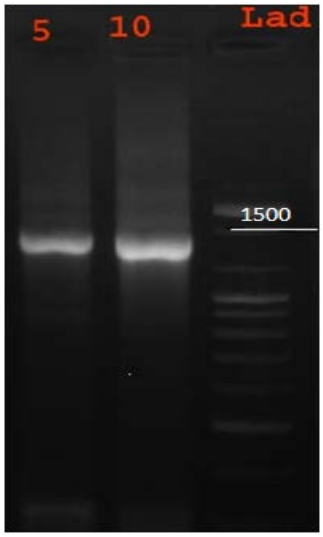

Figure 3. Agarose gel electrophoresis of 16S rRNA gene amplification, marking is for 1500 bp

16S rRNA was isolated for both of the organisms; PCR products were detected by gel electrophoresis and found in gel documenter (Figure 3). Other phylogenic data are shown in Table 2. The ethyl acetate extract obtained from the broth culture medium was subjected to chromatographic analysis to determine the number of compounds present in it. All were positive in antibacterial test against *B. cereus* (Figure 4).

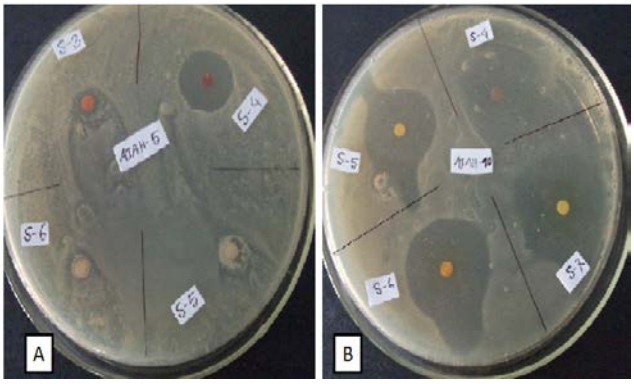

Figure 4. Antibacterial activity of the TLC fractions of metabolites from AIAH-5 and AIAH-10

The MIC value of AI-5 varies in the range between 8-32 µg/ml. The lowest MIC value of 8 µg/ml was found against *Bacillus cereus, Staphylococcus agalactiae and E. coli.* The MIC value of 16 µg/ml was found against *Pseudomonas aeruginosa,* whereas MIC values for Agrobacterium and *Shigella dysenteriae* was found to be 32 µg/ml. The MIC value against in-vitro induced ciprofloxacin resistant *E. coli* was 16 µg/ml whereas against in-vitro induced ciprofloxacin resistant *E coli* was 32 µg/ml. The minimum bactericidal concentration for AI-5 ranges in between 16-128 µg/ml (Table 3).

Table 2. Phenotypic properties of AIAH-5, AIAH-10 and a reference strain (data for reference strain was taken from Xu Ping, et al [17].

Properties	Results of AIAH-5	Results of AIAH-10	KM-4927T
Spore chain	Spiral	Flexuous	Spiral
Substrate mycelium color	Dusky reddish brown	yellowish orange	Grey
Liquefaction of gelatin	+ (weak)	+	+
Hydrolysis of starch	+ +(weak)	+	ND
Decomposition of cellulose	-	+	+
Nitrate reduction	+	+	ND
NaCl tolerance	1.5-4%	2-4%	ND
Melanoid production	+	+	±
Sucrose	+	+	+
D-Manitol	+	+	+
Inositol	+	+	+
Optimum growth temperature	33-39°C	32-41°C	ND

Note, +, positive or utilized; -, negative or not utilized; ±, ambiguous; ND, not determined

Table 3. MIC and MBC value determination of pure compound AI-5 and AI-10.

	Growth observation against							
	Bacillus cereus	*Streptococcus agalactiae*	Agrobacterium sp	*Pseudomonas aeruginosa*	*Escherichia coli*	*Shigella dysenteriae*	Azithromycin Resistant *E coli*	Ciprofloxacin Resistant *E coli*
	Gram +			Gram-			Resistant sp.	
MIC values of AI-5(in µg/ml)	8	8	32	16	8	32	32	16
MBC of AI-5 (in µg/ml)	16	32	64	64	32	128		
MIC values of AI-10(in µg/ml)	1	1	2	4	2	4	16	8
MBC of AI-10(in µg/ml)	2	4	8	8	4	16		

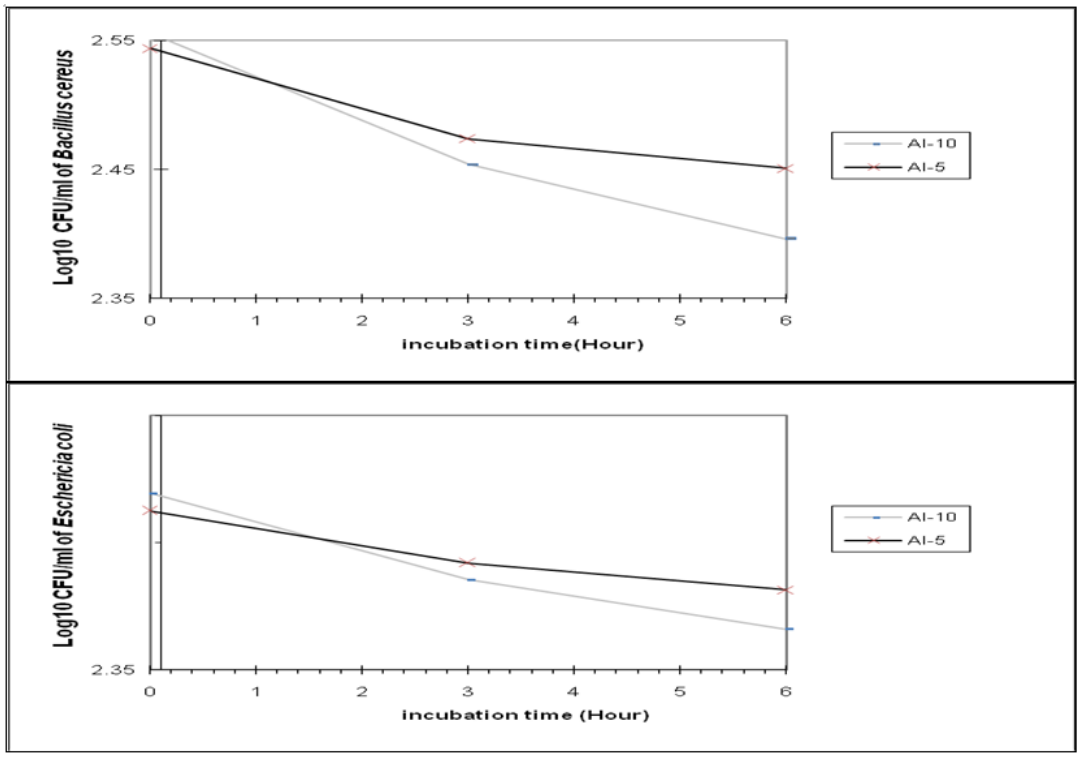

Figure 5. Log_{10} CFU of *Bacillus Cereus* and *Eschericia Coli* at various time intervals in presence of AI-10 and AI-5 at their 2MICconcentration

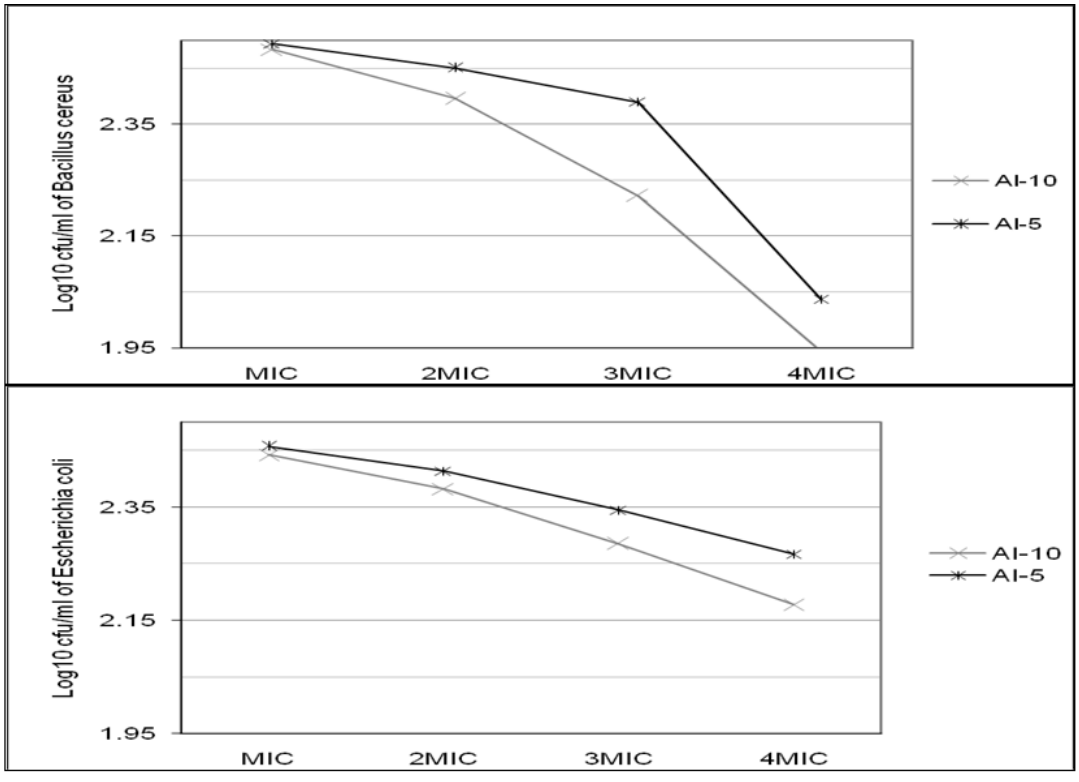

Figure 6. Log_{10} CFU of *Bacillus Cereus* and *Eschericia Coli* in presence of different concentration of AI-10 and AI-5

Time kill profile of the compounds obtained from microorganisms was shown to possess varying degrees of activity, against the susceptible bacteria. Results are presented as $\log_{10}$ cfu/ml. Similar effects were shown against both Gram+ (*B. cereus*) and Gram- (*E. coli*) bacteria in a 6hours long study and its 2MIC concentration (Figure 5 and Figure 6).

4. Discussion

Among the two potential isolates, the aerial mycelium of AIAH-5 became more powdery as well as dusky reddish brown, on the other hand AIAH-10 isolate appeared similar type powdery but deep yellowish due to sporulation of the microorganism, on their 21st day of incubation (Figure 2). Both isolates were positive in melanoid production. AIAH-5 produced brown to black diffusible pigment or a distinct brownish blank pigment modified by other color on International Streptomyces Project (ISP)-1, ISP-6 and ISP-7 medium whereas AIAH-10 produced yellowish brown diffusible pigment or a distinct brown pigment modified by other color on ISP-1, ISP-6 and ISP-7 medium(figure not shown). Many carbon sources were employed for better growth, and among them fructose, mannitol, lactose, inositol and sucrose were preferred for AIAH-5, whereas, L-rhamnose and mannitol were strongly utilized by the AIAH-10 strain. As a nitrogen source yeast extract was best utilized for AIAH-5 and yeast extract and potassium nitrate both for AIAH-10. Because of marine source, we tried to check the effect of NaCl concentration on the maximum production of antimicrobial metabolites. It was observed that the production of antimicrobial metabolites were maximum in the salt (NaCl) concentration from 3 to 4% for both of the organisms. The growth of the organisms gradually decreased either with the decreasing or increasing of NaCl concentration. And pH 7 was found to have the best condition for both isolates. Reduction ability of nitrates to nitrites was also evaluated and was found to have highly positive for AIAH-10 (Table 1). In addition to morphological, biochemical and cultural studies, 16SrRNA gene amplification by PCR and separation by agarose gel electrophoresis were also employed (Figure 3). Since fragments amplified from different species differ in their sequence, they produce different length in electrophoresis. In this study, the amplified PCR products of 16SrRNA gene of both isolates appeared similar in length, near about 1500bp, reveals that the phylogenetic relationship of the two isolates with related genera. Microbes were grown in several types of broth media to select the optimum fermentation condition and consequently the higher biomass yield. In any case, it is preferred to produce the antimicrobial metabolites in yeast extract glucose broth medium, within a temperature range 37-39°C [18]. That biomass, obtained throughout 14 day from those cultures, were investigated for the antimicrobial quality test by disc diffusion method [19] at 20µg/disc against *Bacillus cereus*, and shown to have best effect on day 8th for both isolates. The utilization of carbon sources with the antimicrobials production was also checked, that revealed that cultural broth having the glucose, as a source of carbon was most effective for both isolates. Different organic solvents were employed for the extraction of crude antibacterial metabolite, that was subjected to both air drying and rotary evaporation process and finally we choose the ethyl acetate and rotary evaporator from the highest sensitivity of that crude (20µg/disc) against Bacillus *cereus*. Analysis of the crude extract of AIAH-5 showed that it contained five compounds. All of them were antibacterial active and S-4 (Figure 4, A) was more potent than others and this was designated as AI-5. The compound AI-5 was obtained as reddish brown crystals with R_f value 0.87 in 100 % ethyl acetate and showed solubility in methanol, ethyl acetate, chloroform and DMSO, partially soluble in n-hexane. On the other hand, AIAH-10 contains four compounds. All were antibacterial active but S-5 was chosen for the best activity and (Figure 4, B) designated as AI-10. The minimum inhibitory concentrations (MIC) of compound AI-5 and AI-10, against a range of Gram + and Gram – bacteria were varied between 8- 32 and 1- 4 µg/ml (Table 3). The MIC values of AI-10 can be comparable with standard drug Cefazolin, 0.5-1.0µg/ml against *S. aureus* (#29213) and 2.0-4.0 against *E. coli* (#25922). Also with Cefoxitin, a second generation cephalosporins, 1.0-4.0 µg/ml *S. aureus* and 1.0-4.0 against *E. coli* [20].The minimum bactericidal concentration (MBC) of the compound AI-5 and AI-10 were ranges from 16- 128 and 2- 16 µg/ml, respectively. Both AI-10 and AI-5 were not shown to possess antifungal effect against three fungal species compared to nystatin (30 µg/disc) as a reference (data not shown), suggesting that the mechanism of action of those compounds are against prokaryote. According to Ell of [21], 0-3 Log_{10}cfu/ml reduction is considered to be bacteriostatic, and a reduction of $\geq$ 3 $\log_{10}$cfu/ml is bactericidal, at 3h and 6h compared to that at 0h. From this standpoint, in the time kill assay, the effect of both the AI-5 and AI-10 appears to be bacteriostatic, though there is not any superiority of bactericidal over bacteriostatic activity in clinical purpose. But AI-10 was appeared more potent than AI-5 both in time dependent killing and concentration dependent killing assay (Figure 5 and Figure 6).

5. Conclusion

Therefore it can be concluded that both of the marine *Streptomyces* spp. may be interesting sources for obtaining bioactive molecules. However further study is required to determine the structure of responsible compound and to identify the strain to its species level by sequencing of 16S rRNA.

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