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# Screening of Bioactive Metabolites *Actinomycetes* to Evaluate Potential Sources of Sustainable Marine Natural Products

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# Screening of Bioactive Metabolites *Actinomycetes* to Evaluate Potential Sources of Sustainable Marine Natural Products

**T G P Utami[1], A Setiawan[1] and N L G R Juliasih[1]***

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**Abstract.** This study aimed to screen the activity of methanol extract of sponge-derived actinomycetes as an anti-biofilm and antibacterial agent to *Staphylococcus aureus*. Nine actinomycetes isolates were selected from the UPT LTSIT deposit. *S. aureus* was obtained from the skin of patients at Abdul Moeloek General Hospital. An antibiotic susceptibility test was performed by the disk diffusion method. Biofilm formation of *S. aureus* was tested using the crystal violet method. The viability of pathogenic bacteria was measured using the indicator resazurin. The results of the biofilm formation test *in vitro* revealed that the organic extracts 33A1T2, 33A2T3, 21A1T11, and 38A1T12 inhibited bacterial growth at 0.5 mg/mL. Meanwhile, 50A2T9, 21A1T11, and 38A1T12 significantly inhibited the formation of staphylococcal biofilm on polystyrene at a concentration of 0.25 mg/mL. This information is very important as a basis for further understanding of the mechanism of action of antibiofilm agents.

## 1. Introduction

The biofilm protection system in the microorganism community is closely related to the symptoms of resistance of pathogenic bacteria to antibiotics [1]. Bacterial communities protected by biofilms can be several times more resistant to various types of antibiotics than planktonic bacteria. This is certainly a serious problem in the field of medicine related to the persistence of bacterial infections. But so far, studies that specialize on anti-biofilm compounds are still few. Therefore, there is an urgent need to obtain bioactive compounds that can prevent the formation of biofilms. Based on studies related to the mechanism of biofilm formation, there are four main categories that can be developed in the study of anti-biofilm compounds including prevention of biofilm formation, biofilm attenuation, disruption or spread of biofilms, and killing of bacteria, especially sub-pollution that persists [2]. This study aims to obtain anti-biofilm compounds from crude extracts from sponge-derive actinomycetes.

Until now, natural materials are still relied on as potential sources of new bioactive compounds. Sponges are one of the potential sources to obtain new bioactive compounds. The results of this final project have reported new bioactive compounds that have antibacterial and antibiofilm properties from sponges, *Psamosnia* sp. and *Hyattella* sp. [3]. However, the relatively small content of bioactive compounds and the slow growth of sponges make intensive exploitation difficult. Another alternative to find new sources of bioactive compounds is focused on marine microorganism metabolites. The extreme and dynamic conditions as well as the biological diversity of waters such as Indonesian waters

are the strong reasons often associated with the potential for secondary metabolites of marine microorganisms. Actinomycetes are examples of microorganisms that have diverse and unique types with the ability to produce different secondary metabolites such as alkaloids, amino phenols and peptides. Several researchers have also succeeded in isolating bioactive compounds from *Streptomyces* sp. capable of inhibiting the formation of *Staphylococcal* biofilms [4].

In an effort to search for anti-biofilm agents, we used clinical isolates of *S. aureus* to identify the antibiofilm activity of crude extracts from actinomycetes. The use of biofilms from clinical isolates of S. aureus as a model for screening crude extracts of actinomycetes isolates from Bali and Gorontalo sponges has not been reported. Here we report the inhibitory effect of methanol extracts from marine sponge-associated actinomycetes on *S. aureus* biofilm formation on polystyrene surfaces. The results obtained highlight that the extract exhibits a strong anti-biofilm effect on *S. aureus*.

## 2. Method

### *2.1. Biomaterial*

*2.1.1. Isolate actinomycetes*. In this study, nine strains of actinomycetes were selected from a deposit of the Technical Service Unit of the Integrated Laboratory for Innovation and Technology Center (UPT-LTSIT) Lampung University. Originally, actinomycetes were isolated from marine organisms taken randomly by the SCUBA diving technique at a depth of 5-30 meters in the waters of Buleleng, Bali in 2018 and off the coast of Oluhuta, Gorontalo in 2019. Isolation was carried out by the dilution method and maintained on 1% chitin media and artificial seawater (ASW) [5]. Microscopic analysis was carried out with a light microscope using the cover slip [6].

*2.1.2. Clinical pathogenic bacteria*. The clinical pathogenic *S. aureus* used in this study was collected from Abdul Moeloek Hospital, Bandar Lampung, Indonesia. The isolate was maintained in 2% (w/v) NA [9]. Disk diffusion [10] method was used to determine antibiotic resistance patterns of *S. aureus* with some modifications, according to the CLSI guidelines [7].

### *2.2. Preparation extract of isolate actinomycetes*

*2.2.1. Preparation Colloid Chitin:* Shrimp shell waste is obtained from the free market in the Teluk Betung, Bandar Lampung. Preparation of chitin colloid refers to the method of Hsu S and Lockwood J [8] with several modifications.

*2.2.2. Cultivation and Extraction:* Isolate actinomycetes were cultured in liquid media. The inoculum was prepared using 1% (w/v) chitin colloid medium in artificial sea water (ASW). Isolates were inoculated for seven days, then the inoculum was added (1:10) to the new media. The cultures were allowed to grow for up to 14 days at room temperature under static conditions in 20 mL of liquid chitin colloid medium at 100 mL Erlenmeyer. The cultivation results were extracted using methanol, then centrifuged at 6000 rpm. The filtrate obtained was concentrated using a vacuum rotary evaporator at a temperature of 40°C and a pressure of 122 mbar. For each extract obtained, 0.5 mg/mL stock solution was made for anti-bacterial test and 0.25 mg/mL for anti-biofilm test.

### *2.3. Antibiofilm Assay*

*2.3.1. Biofilm formation assay*. The biofilm formation test method refers to Kwasny and Opperman [9] using microplate 96-well. Briefly, the inoculum was prepared by diluting the overnight culture(s) 1:100 in the amount of an appropriate assay media required for the experiment. A total of 200 µl of inoculum was added to each of the 96 test plate sterile test wells, which corresponded to an inoculum of approximately $5 \times 10^6$ cells, as well as a control for biofilm growth. The inoculum on the test plate was incubated for ± 16-18 hours at 37°C. After incubation, the media was removed, and removed non-adherent bacteria by washing each well 3-4 times. Staining was done by adding 200 µl of 0.1% (w/v) crystal violet solution. After the colouring reaction is complete, remove any excess stain with repeated

washing (3-4 times of washing) with distilled water. Then add 200 µl of ethanol, let the staining be done for at least 60 minutes. OD values were measured using a plate reader at 630 nm. The values of OD were measured at 0, 4, 8, 1 2, 16, 20, and 24 hours.

*2.3.2. Anti-biofilm Screening*. *S. aureus* was cultured in 2% (w/v) NA dissolved in distilled water. Inoculum was prepared by diluting the culture (1:100) as required for the experiment. Transfer 200 µl of inoculum to the assay well of a sterile 96-well test plate. Similarly, for the control of biofilm growth, the inoculum was set at about 5 x $10^6$ cells/well. To the test compound, added 100 µl of extract 0.5 mg/mL and 100 µl of inoculum, incubation of the inoculated test plate for ± 16-18 hours at 37°C. After incubation, the media and planktonic bacteria were removed by washing each well 3 times with distilled water. Staining was done by adding 200 µl of 0.1% (w/v) crystal violet solution and allowed to stand 10 minutes. After the staining reaction is complete, remove the remaining stain by washing with distilled water 3 times. Then add 200 µl of ethanol, let the staining is done for at least 60 minutes. The OD values were measured using a plate reader at 630 nm.

*2.3.3. Antibacterial Screening.* The assay was performed using a sterile 96-well microplate. *S. aureus* was cultured on 2% (w/v) NA media, one colony of pure *S. aureus* was taken, put into 3-5 mL TSB, and incubated for 2-4 hours until the bacterial density matched the McFarland 0, 5 (OD between 0.08-0.1). The inoculum was prepared by diluting it at 1:100 in the amount of test medium required for the experiment. The test was carried out in 3 replications, 2 mg/mL of chloramphenicol solution was used as a positive control and 2 mg/mL of sample extract was used as the test compound. Incubate the test plate for 18 hours at 37°C. The OD value was measured using a microplate reader. Staining was done with resazurin then incubated for 2-8 hours, and every 2 hours the OD value was measured at 630 nm.

## 3. Results and discussion

### *3.1. Isolate Actinomycetes*

In this study, nine strains of actinomycetes were selected from the UPT-LTSIT deposit. The actinomycetes isolates were originally isolated from seven sponges. Three of the seven types of sponges taken are shown in Figure 1.

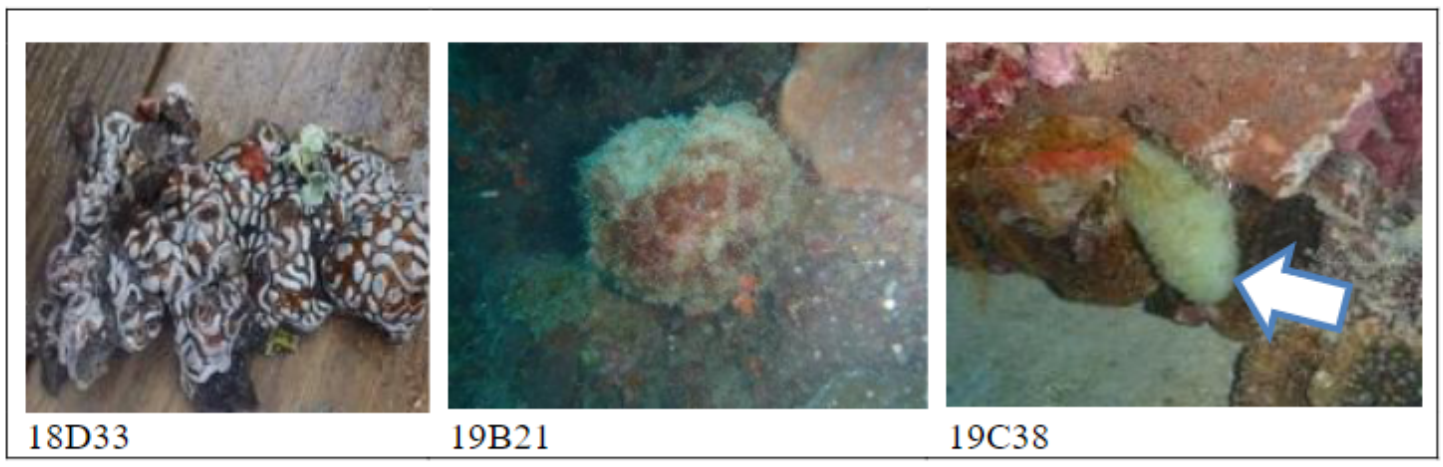


**Figure 1.** Collection of sponges from Buleleng, Bali (18D33) and off the coast of Oluhuta, Gorontalo (19B21 and 19C38).

Five types of sponges were obtained from the waters of Buleleng, Bali in 2018, 18D32, 18D33, 18D35, 18E45, 18F50 and successfully isolated 7 types of actinomycetes in sequence 18D32A2, 18D33A1, 18D33A2, 18D35A2, 18D35A3, 18E45A2, 18F50A2. Meanwhile, two other actinomycetes strains 19B21A1 and 19C38A1 were isolated from sponge off the coast of Oluhuta, Gorontalo namely 19B21 and 19C38 as showed in Table 1.

**Table 1**. Isolate Actinomycetes.

| No. | Sponge Code | Phylum | Isolate actinomycetes | Color |
|---|---|---|---|---|
| 1. | 18D32 | Porifera | 18D32A2 | White |
| 2. | 18D33 | Porifera | 18D33A1 | White |
| | | | 18D33A2 | White |
| 3. | 18D35 | Porifera | 18D35A2 | Greyish |
| | | | 18D35A3 | White |
| 4. | 18E45 | Porifera | 18E45A2 | White |
| 5. | 18F50 | Porifera | 18F50A2 | Greyish |
| 6. | 19B21 | Porifera | 19B21A1 | Peach |
| 7. | 19C38 | Porifera | 19C38A1 | Greyish |

Sponges are marine invertebrates and filter feeders [10]. In general, most sponges can be characterized by the presence of spicules composed of silica or calcium carbonate. Actinomycetes can be found in sponges, some of which have been isolated. Chitin media can be used to isolate actinomycetes in sponges. Actinomycetes can grow on chitin selective media because actinomycetes are able to produce chitinase enzymes, so actinomycetes can obtain carbon sources from chitin [11].

In general, isolates appear white and gray in color, but often their activities are different. Microscopic analysis of the seven isolates using light microscopy observed the presence of 5 types of actinomycetes including *Streptosporangium* sp. (18D32A2), *Actinoplanes* sp. (18D33A1, 18D33A2, 18D35A3), *Streptomyces* sp. (18D35A2, 18E45A2, 18F50A2, 19B21A1), and *Nocardiopsis* sp. (19C38A1) as shown in Figure 2.

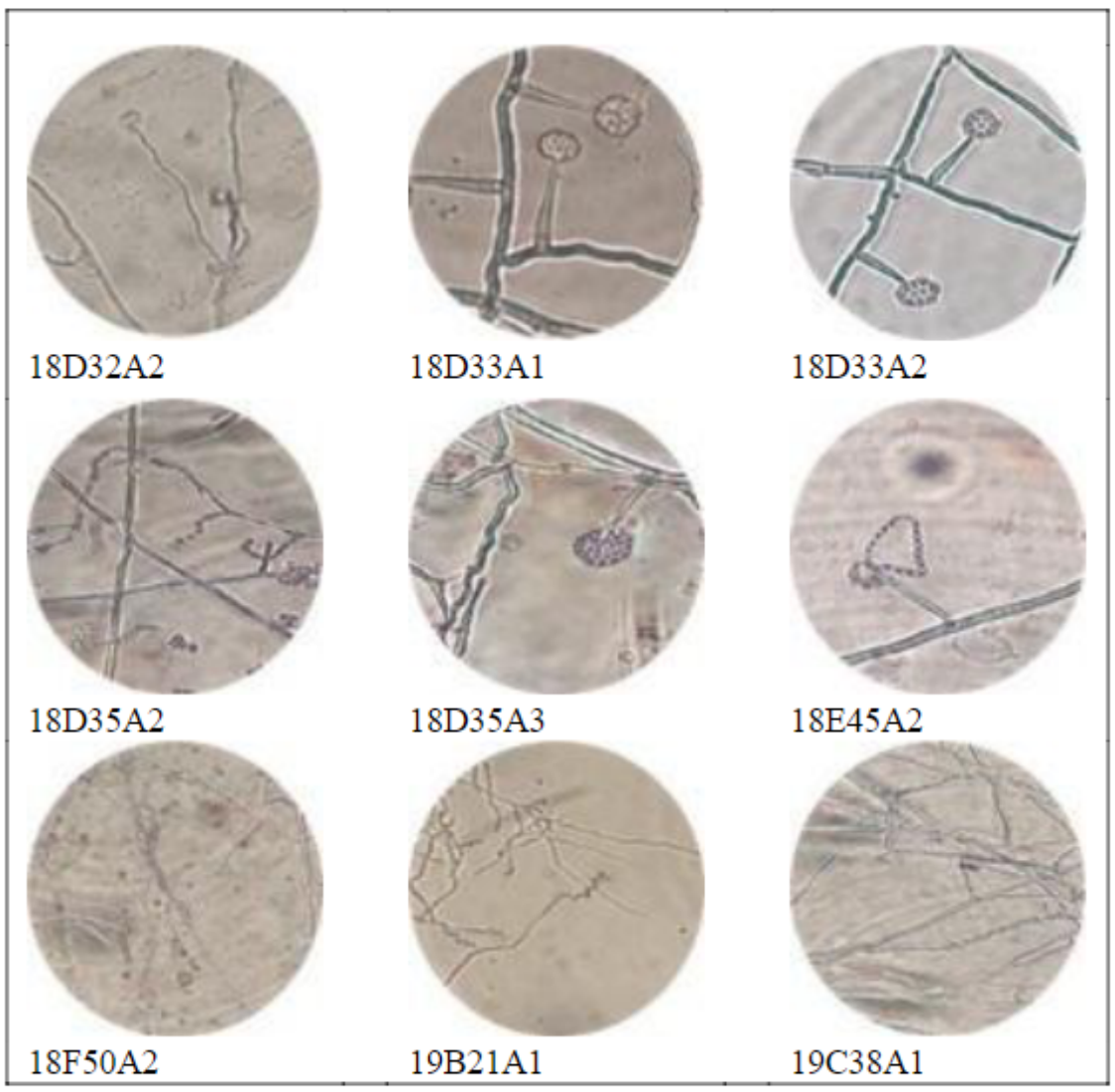


**Figure 2**. Isolate actinomycetes from marine sponge.

*Nocardiopsis* is an actinomycetes species that has a nocardioform mycelium substrate (mycelium that breaks down into bacilli and cocci) and aerial hyphae that are able to develop straight-curved or zigzag to form long spore chains [12]. The genus *Streptomyces* is a polysporous actinomycetes that forms long chains with more than 50 spores. aerial hyphae *Streptomyces* sp. Varieties are Rectiflexibiles type (straight or flexible spore chain), Retinaculiaperti type (spore chains with hooks, open loops or short, irregular), Spira type (spiral spore chain with two types, namely closed and open), Verticillati type (circular spore chain and open-loop). branching) [13].

*3.2. Susceptibility Test*

In this study, the pathogenic bacteria, *S. aureus*, obtained from the skin of patients were tested against several types of antibiotics. Based on the results of inhibition zone measurements on agar media, our finding showed that clinical isolate *S. aureus* are susceptible to doxycycline hyclate (DCH), and cefadroxil (CFDX). *S. aureus* is resistant to amoxicillin-clavulanate (AMX-CL) in the intermediate category. *S. aureus* is resistant to clindamycin (CDM), ciprofloxacin (CFX), erythromycin (ERH), lincomycin (LC), and amoxicillin (AMX).

**Table 2.** Standard of susceptibility test of *S. aureus* to antibiotics.

| No. | Antibiotic | Potency (µg) | Resistant (mm) | Intermediate (mm) | Susceptible (mm) | Inhibition Zone *S. aureus* (mm) |
|---|---|---|---|---|---|---|
| 1. | Clindamycin (CDM) | 2 | ≤ 14 | 15 – 20 | ≥ 21 | 6,3 ± 0,6 |
| 2. | Doxycycline hyclate (DCH) | 30 | ≤ 12 | 13 – 15 | ≥ 16 | 16,7 ± 1,2 |
| 3. | Ciprofloxacin (CFX) | 5 | ≤ 15 | 16 – 20 | ≥ 21 | 7,0 ± 1,0 |
| 4. | Erythromycin (ERH) | 15 | ≤ 13 | 14 – 22 | ≥ 23 | 6,7 ± 0,6 |
| 5. | Cefadroxil (CFDX) | 30 | ≤ 14 | 15 – 17 | ≥ 18 | 25,3 ± 0,6 |
| 6. | Lincomycin (LC) | 2 | ≤ 14 | 15 – 20 | ≥ 21 | 7,0 ± 1,0 |
| 7. | Amoxicillin (AMX) | 25 | ≤ 14 | 15 – 20 | ≥ 21 | 10,3 ± 0,6 |
| 8. | Amoxicillin-clavulanate (AMX-CL) | 30 | ≤ 13 | 14 – 17 | ≥ 18 | 13,3 ± 0,6 |

The resistance test that has been carried out shows that the S. aureus isolates used to have a category of resistance to CDM 14 mm; CFX 15 mm; ERH 13 mm; LC 14 mm; and AMX 14 mm. Antibiotics such as clindamycin, erythromycin, and lincomycin are able to inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit which can inhibit the formation of peptide bonds in bacterial cells, causing bacteria to die [14]. Other antibiotics such as ciprofloxacin have the ability to inhibit the common mechanism of action of the DNA gyrase enzyme that plays a role in bacterial cell division [15]. Penicillin antibiotics (amoxicillin) are able to inhibit bacterial growth by interfering with cell wall synthesis, especially during the transpeptidation process in cell wall peptidoglycan synthesis [16]. The resistance of an isolate to 5 antibiotics is classified as multidrug resistant, namely a bacterium that is resistant to three or more types of antibiotics [17].

*3.3. Biofilm formation*

The biofilm formation test method refers to Kwasny and Opperman using microplate 96-well. Based on the *S. aureus* biofilm growth curve analysis (Figure 3), it can be seen that the optimal biofilm formation after 16-24 hours of observation.

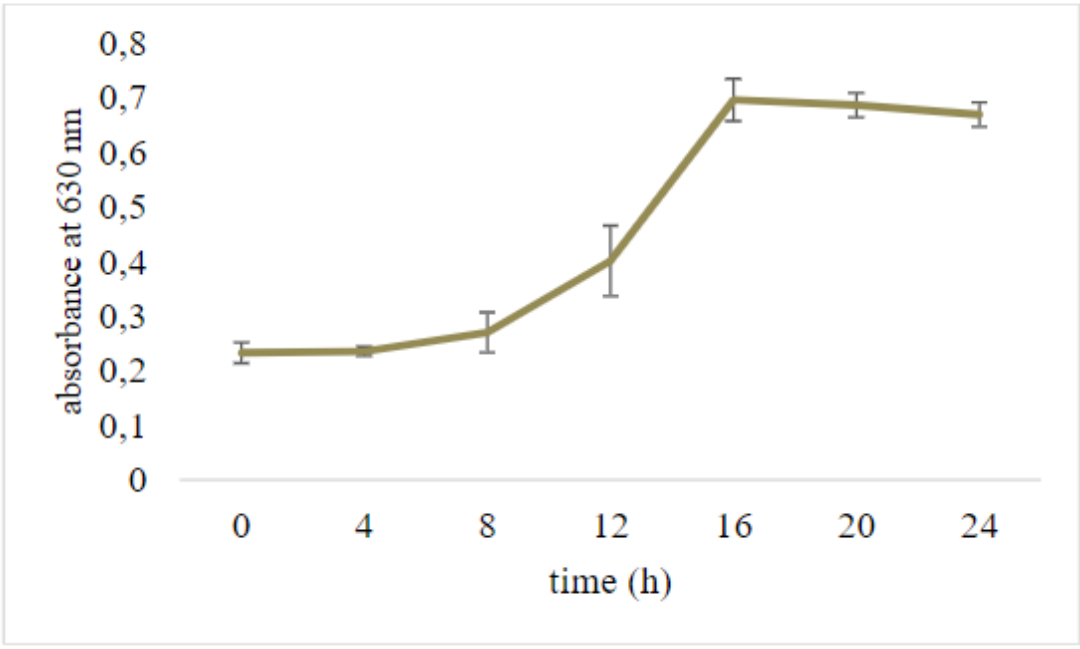


**Figure 3.** Growth curve biofilm formation *S. aureus*.

Based on the analysis of *S. aureus* biofilm growth curve (Figure 3), the optimal biofilm formation after 16-24 hours of observation can be seen. This confirms that *S. aureus* is feasible to be used as an antibiofilm test model which refers to the literature curve of the biofilm formation method.

*3.4. Antibiofilm assay*

The tests were carried out based on the results of the biofilm formation test and were carried out using a 96-well microplate. The results of the antibiofilm test showed that the 50A2T9, 21A1T11, and 38A1T12 strains had the potential to inhibit *S. aureus* biofilm formation at a concentration of 0.25 mg/mL (Figure 4).

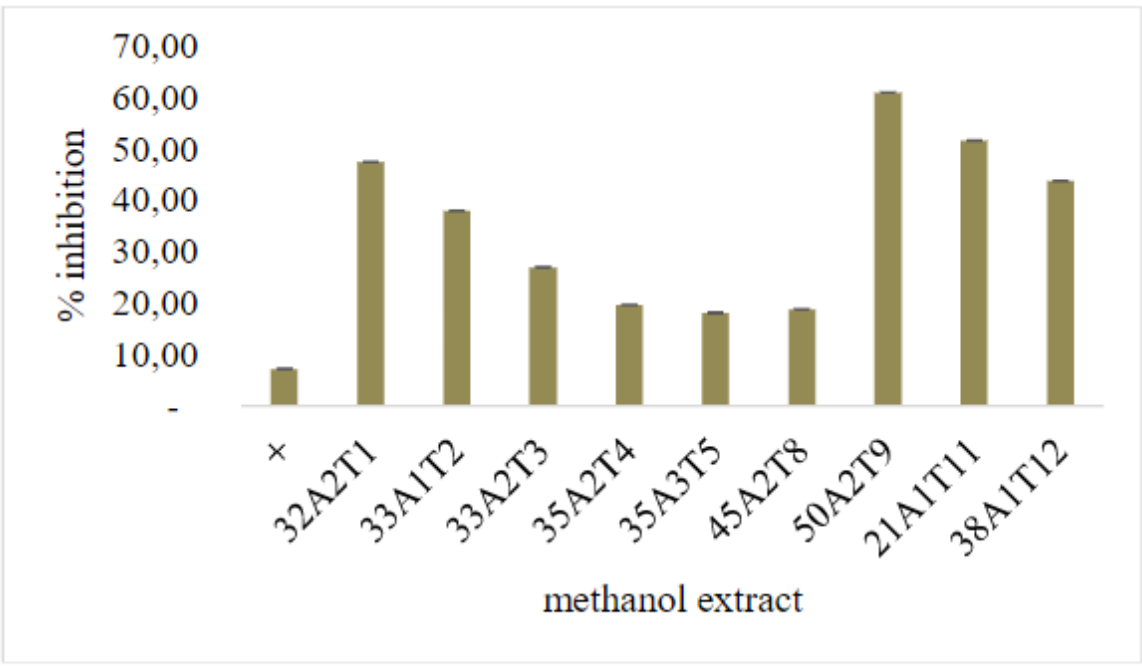


**Figure 4.** Anti-biofilm assay of MeOH extract actinomycetes.

Another study also reported that *Streptomyces* sp. isolated from sponges were able to produce bioactive compounds that have potential as antibiofilm compounds against *Staphylococcus epidermis* biofilms. *Nocardiopsis* sp. from sponges that can produce bioactive compounds as antibiofilm agents that can inhibit the growth of the biofilm, *K. pneumoniae* [18].

*3.5. Antibacterial assay*

To determine the activity of the methanol extract of actinomycetes cultured using 2% chitin liquid media, *S. aureus* bacteria were screened using the well plate reader method 96 method. The results of the antibacterial test showed that the methanol extract 33A1T2; 33A2T3; 38A1T12; and 21A1T11 had the activity of inhibiting the growth of *S aureus* bacteria at a concentration of 0.5 mg/mL (Figure 5). Meanwhile, the isolate strains were 32A2T1; 35A2T4; 35A3T5; 45A2T8; and 50A2T9 at a concentration of 0.5 mg/mL did not show the ability to inhibit bacterial growth. Potency shown in methanol extract 33A1T2; 33A2T3; 38A1T12; and 21A1T11 proved that Actinomycetes isolates isolated from sponges were able to produce bioactive compounds as antibacterial agents. *Streptomyces tirandamycinicus* from a sponge capable of producing bioactive compounds that have the potential as antibacterial agent against *Streptococcus agalactiae* [19].

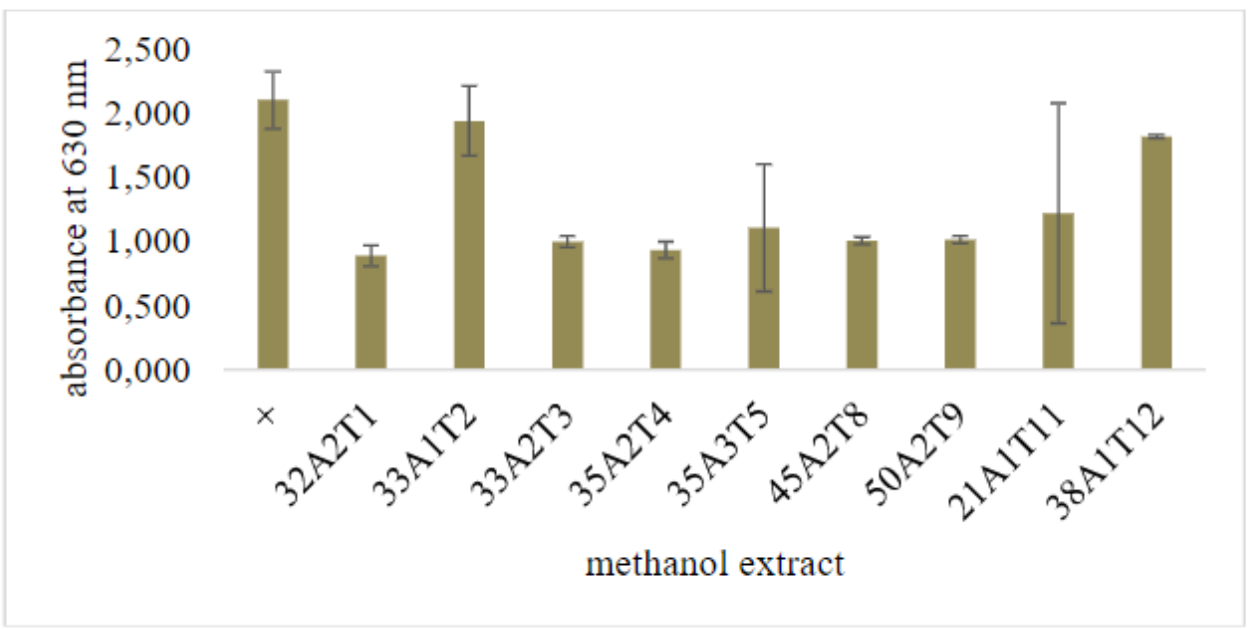


**Figure 5**. Antibacterial assay of actinomycetes extracts.

## 4. Conclusion

Based on the data above, it can be concluded that actinomycetes from sponges are a potential source of bioactive compounds with varying levels of activity. Furthermore, the results of the screening of methanol isolate extracts showed that 21A1T11, and 38A1T12 extracts had potential as anti-bacterial and also as anti-biofilms. This information is very important for further studies related to the molecular structure of bioactive compounds and the study of the relationship between structure and activity.

## Acknowledgements

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