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# Antimicrobial activity of *actinomycetes* associated with an unidentified sponge from Lemukutan island, Indonesia on various medium and incubation time

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**Abstract:** Three isolates of Actinomycetes namely MA02, MA03, and MA04 were isolated from an unidentified sponge, Lemukutan Island, Indonesia. They were identified as *Streptomyces* based on Bergey's Manual of Determinative Bacteriology. In the preliminary screening, all of the isolates showed antimicrobial activities towards 13 microorganism tests. However, the activities were not similar when their extract produced from various medium and incubation time were tested. Their cultivation was conducted on M13 agar with incubation time for 3 days, M13 broth with incubation time for 4 days, M2+ broth with incubation time for 7 days and M2+ broth with incubation time for 9 days. The result of antimicrobial assay showed difference in the ability to inhibit the microorganism tests. The best antimicrobial activity of the extracts based on cultivated condition (medium and incubation time) for *Streptomyces* MA02, *Streptomyces* MA03, and *Streptomyces* MA04 were M2+ broth for 3 days, M2+ broth for 9 days and M2+ broth for 9 days respectively.

**Key words**: Antimicrobial Activity; *Actinomycetes*, *Streptomyces*; Sponge

## Introduction

*Actinomycetes* is a potential antibiotic producer especially genus of *Streptomyces*. *Streptomyces* contributes in high production of bioactive compounds especially antimicrobial compounds which is around 80% of the total antimicrobial compounds[5]. Recently, discovery of novel antimicrobial compounds from terrestrial have been decreased so that is needed to explore another *Actinomycetes* habitat such as marine.

*Actinomycetes* isolated from marine have higher probability to get novel bioactive compounds due to characteristic difference of the structure. Generally, compound structures from marine have more halogenation with bromine and chlorine than terrestrial structures[3] which are caused by uniqueness of marine environment. Seawater contains bioactive compounds such as vitamin and inhibitor agent[4] which can probably be used to induce microorganisms to produce bioactive compounds.

Simbiosis between microorganisms and marine invertebrates is also stimulate microorganisms to produce biactive compounds. For instance, sponge associated *Actinomycetes*. Abundance of *Actinomycetes* is dominant in the sponge compared with other microorganisms which is around one-sixth from all microorganisms[7]. Micromonospora and Streptomyces isolated from the sponge-associated *Actinomycetes* from the China Seas shows antimicrobial activity[8].

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Among genus of *Actinomycetes*, marine *Streptomyces* showes higher potential antimicrobial compared with other actinomycetes (*Nocardia* sp. and *Actinolyspora* sp.) especially against *Staphylococcus aureus* and *Pseudomonas aeruginosa*[2]. Marine *Streptomyces* species from sediment Mahabalipuran sea shores and Adyar Estuary can inhibit growth more microoganism test than another *Actinomycetes*[6]. Therefore, the objective of this study is to get potential genus Actinomycetes having antimicrobial activity from an unidentified sponge Randayan Island, Indonesia. In addition, we learn effect of various medium and incubatian time towards antimicrobial substance production from Actinomycetes associated with an unidentified sponge.

## Materials and Methods

**Collection of Sponge Sample:** An unidentified sponge was collected from Lemukutan Island, Kalimantan Barat, Indonesia. The sponge was placed on polyethylen bag containing seawater then closed tightly and stored in the cool box. The depth of sponge from the surface was measured. Apart from the sponge, seawater was collected to measure salinity, pH, and temperature.

**Preparation and Isolation of Streptomyces from an unidentified sponge:** Approximately 20g sponge which was rinsed sterilized seawater was added 50mL of sterilized seawater then it was homogenized using sterilized blender to obtaine

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bacteria suspension. The suspension was inoculated into M2+ agar using pour plate method and incubated for one month. Interested colony was isolated to get a pure isolate.

**Preliminary Screening of antimicrobial activity:** Preliminary screening of antimicrobial activity was performed by inoculation of isolates in the form of circle with a diameter of 5-6mm on the appropriate medium. After 48 hours, microorganism test suspension ($OD_{600}$=0,6) was sprayed on the surface area of medium inoculated bacterial isolates then incubated 24 hours. Positive antimicrobial activity was showed by clear zones around bacterial isolates.

**Cultivation of actinomycetes on various medium and incubation time:** Actinomycetes was cultivated on various medium and incubation time namely: M13 broth for 7 days, M13 agar for 7 days, M2+ broth for 3 days, and M2+ for 9 days. M13 consists of 2.5 g/L of peptone, 2.5g/L of yeast extract, 2.5g/L of glucose, 250mL of seawater and 750mL of aquadest. $M_2^+$ consists 10 g/L of malt extract, 4g/L of yeast extract, 4g/L of glucose and aquadest until 1L.

Before cultivation of actinomycetes, the seed was prepared by inoculation of 1 colony in 10ml of liquid medium and incubated on shaker at 150RPM for 3 days. Furthermore, the seeds were incubated 1L of liquid medium and incubated on a shaker incubator at 130RPM for certain time. Finally, the culture was harvested and extracted for secondary metabolites using ethyl acetate as solvent which ratio is 1:1 between the culture and solvent. The crude extract was concentrated using rotary evaporator.

Solid culture of *Actinomycetes* was prepared by spread of seed on surface of M13 agar. After 7 days incubation at room temperature, the medium was cut to be a small piece then macerated using two kinds of solvents, methanol or ethyl acetate. The maceration was conducted for 48 hours then filtered. Furthermore, the filtrate was centrifuged at 4,000 x g for 15min. Supernatant was concentrated using a rotary evaporator and then weighed. Extracts were tested against microorganism tests.

**Antimicrobial Assay of The Extract:** Antimicrobial activity of bacteria extract was determined based on modified well-difusion agar methods. Well with 6 diameter mm was made using a punch in plate containing nutrient agar medium then its medium was spreaded with inoculum of bacterial test. Furthermore, the well was filled with 200µg/well of the bacterial extract and incubated at 37°C for 24h. The extract having antimicrobial activity was signed with formation of inhibition zone around isolate. Diameter of inhibition zone was measured from the edge of the colony to the edge of the clear zone and recorded.

## Results and Discussion

**Isolation of Actinomycetes from an unidentified sponges:** Actinomycetes was identified based on Bergey's Manual of Determinative Bacteriology. The first screening of Actinomycetes observed hyphae and spore growth. However, Actinomycetes hyphae was difficult to observed due to its high colony density and its location under the spores. Therefore, the powdery colony on the surface of agar plate is characterized as Actinomycetes. Three colonies was isolated succesfully from an unidentified sponge (Figure.1). All of the colonies exhibited powdery on colony surface which is the easiest characteristic to find out Actinomycetes. All colonies also showed positive Gram. In addition, the colony growth on slide culture exhibited that all of the colonies was Streptomyces (Table.1).

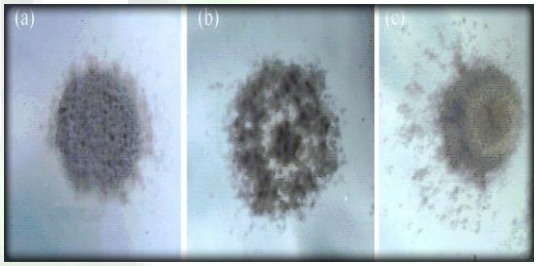


**Figure 1:** Micrograph of Actinomycetes Spore at light microscope with 10 x magnification. a: MA02; b: MA03 c; MA04

**Table 1:** Characteristic of *Actinomycetes* from an unidentified sponge

| Isolate | Gram Staining | Fenotipe Koloni | Morfology of Hyphae | Predicted Genus |
|---|---|---|---|---|
| MA02 | + | Powdery grey aerial mycelium | Chains of spores on aerial mycellium, spirals of arthrospores | *Streptomyces* |
| MA03 | + | Powdery brown aerial mycelium | Chains of spores on aerial mycellium, spirals of arthrospores | *Streptomyces* |
| MA04 | + | Powdery grey aerial mycelium | Chains of spores on aerial mycellium, spirals of arthrospores | *Streptomyces* |

**Preliminary Screening of Antimicrobial Activity:** Preliminary screening of antimicrobial activity was conducted based on cross-streak method. The bacterial colony in this method will release antimicrobial substances from the cell then diffuse it to medium. The positive assay is showed by formation of clear zone or foggy zone. Foggy zone showed lower antimicrobial activity than clear zone. The larger diameter of clear zone can also indicate higher antimicrobial activity. The

colony of Streptomyces isolated from an unidentified sponge can inhibit growth of all microorganism tests (Table 2). It was proved by formed of clear zone and foggy zone around the Streptomyces colonies (Figure 2).

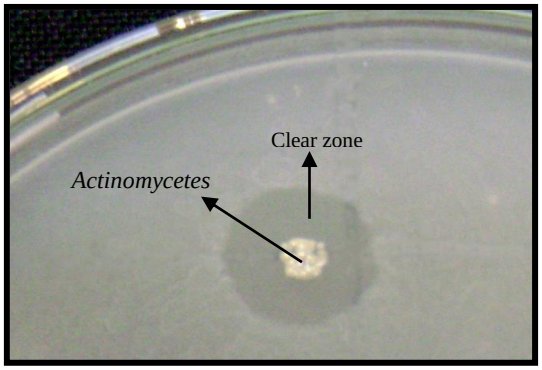


**Figure 2**: Clear Zone Formed by MA02 against *Bacillus* sp. On M13 Medium

**Table 2:** Screening of Antimicrobial Activity from Actinomycetes Associated with Sponge

| Microorganism Tests | Antimicrobial Activity of Isolate | | |
|---|---|---|---|
| | MA02 | MA03 | MA04 |
| *B. subtilis* | ++ | ++ | ++ |
| *Bacillus* sp | ++ | + | ++ |
| *Salmonella* sp | ++ | + | ++ |
| *S. aureus* | ++ | ++ | ++ |
| *P. aeruginosa*; | ± | ± | ± |
| *K. pneumoniae* | +++ | +++ | +++ |
| *A. hydrophila* | + | + | + |
| *E. coli*; | + | + | + |
| *V. harveyii* | + | ++ | + |
| *V. vara* | + | ++ | ++ |
| *V. cholerae* | ± | ± | + |

Note: (-) no clear zone, (+) clear zone with diameter 1-10 mm, (++)clear zone with diameter 11-20 mm dan (+++)clear zone with diameter 20-30 mm, (±) foggy zone.

**Antimicrobial Activity of Actinomycetes on Various Medium and Incubation Time:** In the preliminary antimicrobial activity, all of the isolates can inhibit growth of microorganism tests. Unfortunately, when they was extracted on the similar condition, the activity was absent (Table 3). It was probably antimicrobial substances contained into medium did not exist or low concentration of antimicrobial substances was very low.

All of the extracts from 3 *Actinomycetes* re-showed antimicrobial activity when they were produced by medium broths such as M13 and M2+ although the number of inhibited microorganism tests was different among the Actinomycetes extracts. However, the isolates produced by M2+ broth obtained the extracts which can inhibit more antimicrobial tests compared with the isolates produced in M13 culture. M2+ composition is richer than M13 composition especially glucose and yeast extract content. Difference of both compositions is M13 contain peptone which is as a protein source while M2+ contains malt extract which is rich in carbohydrate.

**Table 3:** Antimicrobial Activity of The Crude Extract from Actinomycetes Cultivated on Various Medium and Incubation Time

| Medium | Incubation Time | Microorganism Test | Average Diameter of Clear Zone (mm) from The Crude Extract | | |
|---|---|---|---|---|---|
| | | | MA02 | MA03 | MA04 |
| M13 Agar | 7 days | *Bacillus* sp. | - | - | - |
| | | *B. subtilis* | - | - | - |
| | | *P. aeruginosa* | - | - | - |
| | | *A. hydrophila* | - | - | - |
| | | *E. coli* | - | - | - |
| | | *C. frendii* | - | - | - |
| | | *Salmonella* sp. | - | - | - |
| | | *S. aureus* | - | - | - |
| | | *V. cholerae* | - | - | - |
| | | *V. vara* | - | - | - |
| | | *C. albicans* | - | - | - |
| | | *V. harveyii* | - | - | - |
| | | *K. pneumoniae* | - | - | - |
| M13 broth | 7 days | *Bacillus* sp. | 16,27 | 2,38 | 1,83 |
| | | *B. subtilis* | - | 3,47 | 2,17 |
| | | *P. aeruginosa* | 8,45 | - | - |
| | | *A. hydrophila* | 17,06 | 1,90 | - |
| | | *E. coli* | - | - | - |
| | | *C. frendii* | 14,35 | - | - |
| | | *Salmonella* sp. | 11,46 | ± | - |
| | | *S. aureus* | 10,90 | - | ± |
| | | *V. cholerae* | - | - | - |
| | | *V. vara* | - | ± | - |
| | | *C. albicans* | - | - | - |
| | | *V. harveyii* | 16,20 | - | - |
| | | *K. pneumoniae* | 7,08 | 2,39 | ± |
| M2+ broth | 3 days | *Bacillus* sp. | 11,48 | 13,21 | - |
| | | *B. subtilis* | 7,94 | 16,35 | - |
| | | *P. aeruginosa* | 9,36 | 14,82 | - |
| | | *A. hydrophila* | 9,07 | 9,99 | - |
| | | *E. coli* | 7,25 | 7,88 | - |
| | | *C. frendii* | - | 8,87 | - |
| | | *Salmonella* sp. | 13,54 | 10,30 | - |
| | | *S. aureus* | 13,03 | - | - |
| | | *V. cholerae* | 11,92 | 10,40 | - |
| | | *V. vara* | 6,61 | 7,11 | - |
| | | *C. albicans* | 12,59 | 10,13 | - |
| | | *V. harveyii* | 11,89 | 13,00 | - |
| | | *K. pneumoniae* | 34,11 | 30,90 | - |
| *M2+ broth* | 9 days | *Bacillus* sp. | - | 7,54 | 14,41 |
| | | *B. subtilis* | - | 6,03 | - |
| | | *P. aeruginosa* | 8,65 | 7,35 | 7,82 |
| | | *A. hydrophila* | - | 7,60 | 6,90 |
| | | *E. coli* | 10,32 | 7,79 | - |
| | | *C. frendii* | - | 6,50 | 6,32 |
| | | *Salmonella* sp. | 8,44 | 7,12 | 5,07 |
| | | *S. aureus* | 11,47 | 11,64 | 4,40 |
| | | *V. cholerae* | 9,86 | 8,77 | - |
| | | *V. vara* | 7,33 | 6,45 | - |
| | | *C. albicans* | 10,60 | 10,31 | - |
| | | *V. harveyii* | 9,33 | 9,83 | 12,86 |
| | | *K. pneumoniae* | 34,15 | 30,85 | 18,96 |

**Note:** (±) foggy zone and (-) no clear zone

On the basis of medium composition, carbohydrate content in M2+ medium was used by them to produce antimicrobial substances. Carbohydrate is a carbon source to primary secondary metabolites but it can also induce

secondary metabolite production. For instance, a marine bacterium #J292/97 shows antimicrobial activity when its growth medium is enriched by α-ketoglutarate, β-ketoglutarate, glucose, and oxaloacetate[1]. However, seawater content in M13 does not seem to affect their antimicrobial production. The best medium and incubation time of production for MA02, MA03, and MA04 were M2+ broth for 3 days, M2+ broth for 9 days,and M2+ broth for 9 days respectively.

Apart from medium composition, incubation time influences antimicrobial productions. It can be seen clearly for MA03 which was cultivated on the same medium but different incubation time. The extract MA03 can inhibit microorganism tests when is cultivated on M2+ for 9 days instead of 3 days. Generally secondary metabolites, for instance antimicobial compounds, were produced on stationary phase. Therefore, RM3 probably reached the stationary phase on day 9 in M2+ medium. From this assay, the active extracts were categorized as a broad spectrum of antimicrobial activity which can inhibit more than one microorganism test. It is caused that the extracts contain one or more active compounds. Isolation of active compounds should be carried out to characterized the active compounds

## Conclusion

*Actinomycetes* is one of pivotal antibiotic producers especially *Streptomyces*. In this study, all of the *Streptomyces* showed antimicrobial activity. However, their antimicrobial activity is influenced by medium and incubation time. Therefore, exploration of other antimicrobial substances which is not expressed by microorganisms from *Actinomycetes* was also needed to explore the medium and incubation time.

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