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The Antibacterial Activity of Actinomycetes Against the Growth of *Streptococcus mutans* and *Lactobacillus acidophilus*

Deby Kania Tri Putri[1], *Fadel* Amirda[2], *Hilman* Muzadi[2], *Amy* Nindia Carabelly[2], *Isyana* Erlita[3] , *Rahmiati*[4]

1 Introduction

Indonesia is one of the countries having the most extensive peatlands compared to other tropical countries, which is around 21 million ha, mainly spread to the islands of Kalimantan, Sumatra, and Papua. Peatland is an ecosystem included as wetlands. This type of land was formed due to the accumulation of organic material over a long period, compared to the slow rate of decomposing organic matter in the wet or inundated parts of the forest. This process of organic materials, decomposition, or breakdown can occur without the role of microbes [1]. One of the most abundant soil microbes is *Actinomycetes* [2].

Actinomycetes can produce various secondary metabolites acting as an antibacterial agent [3]. Several studies on *Actinomycetes* confirmed their ability to produce antibacterial compounds. Adriani [10] isolated *Actinomycetes* from soil samples taken in Takalar. Two isolates were shown to have antibacterial activity against *E. Coli* and *Staphylococcus aureus*. Rante et al (2017) studied the *Actinomycetes* isolated from the soil rhizosphere. They examined the effect of *Actinomycetes* extract with a concentration of 5%, 10%, and 20% to *Staphylococcus aureus*. The result concluded that *Actinomycetes* extract with a concentration of 20% had the widest inhibitory zone against *Staphylococcus aureus*. *Staphylococcus aureus* is a normal flora inside the mouth cavity [4].

The oral cavity is the perfect habitat for various bacteria. The most common bacteria associated with dental caries are *Streptococcus mutans* (*S. mutans*) and *Lactobacillus acidophilus* (*L. acidophilus*). *S. mutans* is lactic acid-producing bacteria and with *L. acidophilus* plays a role in developing advanced carious lesions. These bacteria can metabolize the glucose in the food into organic acids rapidly, thus increasing the acidity inside the oral cavity and speed up the process of caries [5]. Dental caries is a disease of the hard tissue of the teeth commonly found in Indonesia. Indonesian Basic Health Research in 2013 stated that there was a 9.8% increase in the prevalence of dental caries in Indonesia, from 43.4% in 2007 to 53.2% in 2013, and the experience of caries patient increased by 5.1%, from 67.2% in 2007 to 72.3% in 2013. Based on Indonesian Basic Health Research in 2018, dental and mouth problems in Indonesia had a very significant increase from 25.9% in 2013 to 57.6% in 2018 [6,7].

The growth inhibition of *S. mutans* and *L. acidophilus* could be performed by giving antibacterial agents. Many have developed various types of antibacterial compounds that originated from natural extracts, included biochemistry. Researches in recent years have also shown the ability of bacterial cells to produce secondary metabolites as antibacterial agents that act as self-defense against unfavorable environmental conditions. Bacteria that can produce antibacterial compounds are generally isolated from food ingredients. In recent years the study of antibacterial compounds from soil bacteria has become a concern [8]. No research has been done to assess the antibacterial activity of *Actinomycetes* against oral bacteria *S. mutans* and *L. acidophilus*. Based on the description, it is necessary to

research the antibacterial activity of Actinomycetes against the growth of *S. mutans* and *L. acidophilus* bacteria.

2 Materials and Methods

This study used a true experimental post-test only with control group design method. The treatment groups in this study were *Actinomycetes* extract with various concentrations: 0.625%, 1.25%, 2.5%, 5%, 10%, 20%, 40%, positive control (chlorhexidine gluconate 0.12%) and negative controls (aquadest). Based on the results of the Federer formula, the minimum number of repetitions obtained per group is 3 times.

2.1 Sterilization

The initial procedure of the study was to sterilize the tools that had been washed clean and coated with aluminum foil using an autoclave for 15 minutes at 121°C.

2.2 Rejuvenation of Actinomycetes culture

Actinomycetes InaCC A156 culture obtained from the INACC laboratory of the Indonesian Institute of Sciences Bogor was rejuvenated by taking a colony using sterile ose and then transferred to YSA media and incubated for 14 x 24 hours at 37°C.

2.3 Extraction of Actinomycetes

After 14 days of incubation, the agar plate overgrown with microbes was mashed using a blender. The media was then extracted using a maceration method with ethyl acetate solvent in a 1: 5 ratio. Extraction was carried out for 8 hours on a maceration vessel and stirring every 2 hours. The macerate was then filtered using filter paper, obtaining the liquid extract. The extract subsequently was evaporated using a rotary evaporator to obtain the dry extract.

2.4 Preparation of Bacterial Testing

In the preparation of bacterial testing, *Streptococcus mutans* and *Lactobacillus acidophilus* colonies from pure isolates were planted on Nutrient Agar (NA) media and incubated for 1x24 hours at 37°C, then the bacteria were inoculated into 0.5 ml of liquid BHI, incubated for another 1x24 hour at 37°C. The suspension solution was then added with sterile distilled water until the turbidity was proportional to the McFarland standard of 0.5 or the bacteria equivalent to the amount of $1.5x10^8$ CFU.

2.5 Minimum Inhibitory Concentration (MIC)

The extracts from the *Actinomycetes* culture were made into 7 different concentrations: 0.625%, 1.25%, 2.5%, 5%, 10%, 20% and 40%. Hereafter, the testing bacteria standardized with McFarland 0.5 ($1,5x10^8$ CFU/ml) were diluted using a test tube filled with liquid media and several testing bacteria. The test tube was measured for its turbidity before and after incubation for 24 hours using a UV-Vis spectrophotometer. The difference in the turbidity value was measured to determine the minimum inhibitory content (MIC).

2.6 Minimum Bactericidal Concentration (MBC)

Actinomycetes extracts and test bacteria were taken from the test tubes. The samples would be inoculated and spread in agar Nutrient (NA) media and undergo incubation for 24 hours at 37°C. Finally, the number of *Streptococcus mutans* and *Lactobacillus acidophilus* was observed using the *Colony counter* to get the minimum bactericidal concentration.

3 Result and Discussion

3.1 Results

In this study, the results of antibacterial activity tests were obtained by measuring the difference between the absorbance values in anaerobic conditions. Each sample from each treatment group was calculated using UV-Vis Spectrophotometry. The wavelength value used before and after incubation, was 420 nm, are provided in Table 1.

Tabel 1. The minimum inhibitory concentration (MIC) test of *Actinomycetes* on *Streptococcus mutans* growth.

Sample	0 Hour	24 Hour	Deviation	Note
1	0.38	0.33	-0.05	Decreased
2	0.57	0.47	-0.10	Decreased
3	1.23	0.93	-0.30	Decreased
4	1.28	1.46	0.18	Increased
5	1.45	1.59	0.13	Increased
6	2.38	2.49	0.11	Increased
7	2.52	2.73	0.20	Increased
8	2.11	1.90	-0.20	Decreased
9	0.35	1.23	0.87	Increased

Note : 1-7 : Actinomycetes 0.625%; 1.25%; 2.5%; 5%; 10%; 20%; 40%; 8 = Chlorhexidine gluconate 0.2%; 9 = Aquadest.

Tabel 2. The minimum inhibitory concentration (MIC) test of *Actinomycetes* on *Lactobacillus acidophilus* growth.

Sample	0 Hour	24 Hour	Deviation	Note
1	0.52	0.47	-0.04	Decreased
2	0.75	0.64	-0.10	Decreased
3	1.87	0.85	-1.02	Decreased
4	2.02	2.25	0.23	Increased
5	2.23	2.34	0.10	Increased
6	2.44	2.55	0.10	Increased
7	2.56	2.69	0.13	Increased
8	1.87	1.12	-0.75	Decreased
9	0.31	0.87	0.56	Increased

Note : 1-7 : Actinomycetes 0.625%; 1.25%; 2.5%; 5%; 10%; 20%; 40%; 8 = Chlorhexidine gluconate 0.2%; 9 = Aquadest.

Tables 1 and 2 showed the *Actinomycetes* inhibitory properties of the testing bacteria measured using UV-Vis spectrophotometry by calculating the value difference.

Actinomycetes extract with a concentration of 0.625% was found to be ably inhibiting the growth of *S. mutans* and *L. acidophilus,* marked by the decrease in the absorbance value. In this study, it was found that *Actinomycetes* extract concentration of 2.5% had the most effective inhibition compared to other treatment groups including positive control of 0.12% by chlorhexidine gluconate. The minimum bactericidal concentration was measured next using a *colony counter*. The result was provided in Table 3.

Tabel 3. The minimum bactericidal concentration (MBC) test on *S. mutans* after 24-hour incubation.

Sample	Replication	Mean ± SD
1	3	1216 ± 94.5
2	3	861 ± 20.1
3	3	453 ± 21.0
4	3	216 ± 10.6
5	3	44 ± 4.0
6	3	14 ± 3.0
7	3	0 ± 0.0
8	3	3 ± 0.6
9	3	2112 ± 79.5

Note : 1-7 : Actinomycetes 0.625%; 1.25%; 2.5%; 5%; 10%; 20%; 40%; 8 = Chlorhexidine gluconate 0.2%; 9 = Aquadest.

Tabel 4. The minimum bactericidal concentration (MBC) test on *L. acidophilus* after 24-hour incubation.

Sample	Replication	Mean ± SD
1	3	232 ± 22.34
2	3	165 ± 12.12
3	3	48.67 ± 4.94
4	3	5 ± 1
5	3	0 ± 0
6	3	0 ± 0
7	3	0 ± 0
8	3	3 ± 1
9	3	472 ± 24

Note : 1-7 : Actinomycetes 0.625%; 1.25%; 2.5%; 5%; 10%; 20%; 40%; 8 = Chlorhexidine gluconate 0.2%; 9 = Aquadest.

Tables 3 and 4 provided a result that no *S. mutans* colony growth was found in *Actinomycetes* concentrations of 40% in the Petri dishes. Hence, *Actinomycetes* extract 40% was the concentration that acts as bactericidal for *S. mutans* bacteria. On the other hand, in *L. acidophilus* colonies, *Actinomycetes* at a concentration as small as 10% could inhibit the growth of bacterial colonies in Petri dishes completely, thus, a concentration of 10% is the minimum bactericidal concentration for *L. acidophilus* bacteria.

Based on the Shapiro Wilk normality test results, the data were normally distributed (p-value > 0.05). The homogeneity test using the Levene test showed that the data was non-homogeneous. Subsequent analysis using parametric tests One Way ANOVA results on p-value = 0,000 (p < 0.05) meaning there were significant differences. Data analysis was carried on using the Post Hoc Dunnett T3 test. The results provided significant differences (p <0.05) in several groups, while no significant difference in the remaining groups (p > 0.05).

3.2 Discussion

The result of this Antibacterial Activity of *Actinomycetes* on The Growth of *Streptococcus mutans* and *Lactobacillus acidophilus* study showed that *Actinomycetes* extracts with concentrations of 0.625%, 1.25%, 2.5%, 5%, 10%, 20%, and 40% could inhibit growth (MIC) of *Streptococcus mutans* and *Lactobacillus acidophilus*, starting from a concentration of 0.625%. The bactericidal, however, had a difference in the concentration needed between *Streptococcus mutans*, (MBC) 40% and *Lactobacillus acidophilus* (MBC) 10%. *Actinomycetes* extract with concentrations of 0.625%, 1.25%, and 2.5% measured with Spectrophotometer had decreased absorbance values, in contrast to increased absorbance values found in concentrations of 5%, 10%, 20% and 40%. This increase did not fully indicate the growth of bacterial colonies in the media and could be influenced by the color density of the extract of the test material.

In this study, an increase in pigment was found starting white the turned brownish-yellow. According to Wulandari [9], *Actinomycetes* were able to change its color based on the composition of the media used. This statement was also supported by Adriani [10] findings that the white-colored *Actinomycetes* colonies could turn into black. The reasonable explanation was that the change was due to secondary metabolites of secreted compounds containing pigments. The secreted color pigment would vary according to the type of the *Actinomycetes*. Thus the color changes could affect the absorption of light in turbidity measurement by using the UV-Vis Spectrophotometry method, leading to the smaller concentrations Actinomycetes having better values due to more optimal light absorption and lack of secreted pigment [10].

This study also observed that *Actinomycetes* extract with a concentration of 0.625% was found to be the Minimum Inhibitory Concentration (MIC) because of its ability to inhibit the growth of testing bacteria. The presence of antibacterial activity in this study was indicated by the difference in absorbance values which indicates a decrease in the growth of testing bacteria. Actinomycetes were able to produce extracellular enzymes and antibiotic compounds [11]. Extracellular enzymes could degrade complex compounds into simpler compounds to be used in *Actinomycetes* growth, whereas antibiotic compounds could inhibit the growth of other microbes by interfering with the process of cell wall synthesis and protein synthesis of other microbes [12].

The results of this study also indicated that *Actinomycetes* extract with a concentration of 2.5% was the best in terms of inhibiting the growth of *S. mutans* and *L. acidophilus* bacteria due to the decrease in absorbance value of 0,300 for *S. mutans* and the decrease in absorbance value of 1,02 for *L. acidophilus*. *Actinomycetes* extract was effective in inhibiting the growth of testing bacteria by targeting the peptidoglycan of the cell wall. Peptidoglycan is an important layer of bacteria to survive in hypotonic conditions [13].

Damage to this layer could disrupt the stiffness of cells, leading to cell death. The difference between gram-positive and gram-negative bacteria is in the location and content of the peptidoglycan layer. The gram-positive bacterial cell wall consists of 90% peptidoglycan and is located on the outside, while gram-negative bacteria only have 10% located between lipopolysaccharide and phospholipids. Therefore, common antibacterial compounds have to pass through a narrow channel before reaching peptidoglycan [13]. *Streptococcus mutans* and *Lactobacillus acidophilus* are both gram-positive bacteria, thus it is reasonable that Actinomycetes could inhibit the growth of both bacteria. Chlorhexidine gluconate 0.12% as a positive control was only able to disrupt bacterial cell transport, creating small holes in the cell membrane and leakage of *S. mutans* and *L.Acidophilus* cells, causing slow cell death [14].

The antibacterial activity of *Actinomycetes* was yielded by secondary metabolites. Secondary metabolites are molecules and metabolic products produced by microorganisms through secondary metabolic processes where the metabolic product is not a primary requirement for microorganisms to grow and develop. Although not always needed for growth, secondary metabolites could act as an emergency nutrient for survival [15]. There are several functions of secondary metabolites for secondary metabolites-producing bacteria, for example in maintaining life from other bacteria, fungi, insects, animals, and other species [14]. The secondary metabolism is only active at certain times, mainly during unfavorable periods for bacterial growth due to lack of nutrition or microbial invasion [17].

Bactericidal activity in this study was observed from the number of growing bacterial colonies in the media incubated for 24 hours using the Colony counter. The bactericidal activity could be indicated in the absence of bacterial colony growth completely or equal to 0 CFU/µl. MBC test results showed that *Actinomycetes* extract was effective for killing *S. mutans* (concentration of 40%) and Lactobacillus acidophilus (concentration of 10%). Accumulated bacterial colonies would generally form a biofilm. The biofilms are a defense mechanism of bacterial colonies against external factors. Biofilms were found to have an extracellular matrix called Extracellular Polymeric Substance (EPS) to support its function [17].

Antibacterial agents are unable to penetrate the biofilm layer due to its extracellular matrix which inhibits the antibacterial diffusion and binding [17]. Several types of secondary metabolite compounds are known to have antibiofilm properties as they can inhibit the formation of intercellular adhesion genes icaA and icaD [18]. The icaA and icaD genes will synthesize Polysaccharide Intercellular Adhesion (PIA) which plays an important role in biofilm formation, namely cell aggregation and the formation of Extracellular Polymeric Substance (EPS), which will facilitate antibacterial interference easily diffusing into the biofilms layers [19].

Actinomycetes extract had a potency to be used as a topical antibacterial agent in the oral cavity either in the form of gels, pastes or mouthwash with the other additional ingredients due to its secondary metabolite extracts particularly at a concentration of 2.5% having a high inhibitory effect/activity exceeding the positive control itself (Chlorhexidine gluconate 0.12%). It can be concluded that there was an antibacterial activity of *Actinomycetes* against the growth of *S. mutans* and *L. acidophilus*.

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