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Identification of Bacteria Isolated from Sea Urchin (*Diadema setosum*) Gonads Inhibiting *Pseudomonas fluorescens* and *Staphylococcus aureus* Growth

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Abstract. Sea urchins are organisms that have antibacterial, antitumor, and anticancer abilities. The gonads can be used as a food source because they store 28 kinds of amino acids, vitamin B complex. This research aims to determine the type of bacteria from the gonads of sea urchins (*Diadema setosum*) which have inhibitory power against the growth of *Pseudomonas fluorescens* and *Staphylococcus aureus* bacteria. This research used an exploratory method which was analyzed descriptively, using stages in which sea urchin samples were taken and antibacterial tests against *P. fluorescens* and *S. aureus* from sea urchin gonads using the spread method. The media used for the process of isolating bacteria from the gonads of the sea urchin *D. setosum* is Nutrient Agar (NA) media. Isolate colonies that grew after incubation for 24 hours were separated based on differences in colony morphology. Morphological characterization of bacterial cells begins with gram staining. The diameter of the inhibition zone in the test bacteria shows different shapes. The research obtained 8 bacterial isolates, namely isolates B1-B8 which were gram-negative. The eight bacterial isolates have the potential as antibacterials against the test bacteria *P. fluorescens* and *S. aureus*. Isolates B1, B2, B3, B7 are similar to the genus Proteus, while isolates B4, B5, B6, and B8 are similar to the genus Citrobacter. The diameter of the largest inhibition zone in the *P. fluorescens* test bacteria, namely isolate B1, was 9.03 mm. Meanwhile, the smallest diameter for isolate B7 was 6.97 mm. In the *S. aureus* test bacteria, the largest diameter in isolate B1 was 11.09 mm. Meanwhile, the smallest diameter for isolate B4 was 8.57 mm.

Keywords: antibacterial; *Diadema setosum; Pseudomonas fluorescens; Staphylococcus aureus*

I. INTRODUCTION

Sea urchins are organisms that have antibacterial, antitumor, and anticancer abilities. The gonads can be used as a food source because they store 28 kinds of amino acids, and vitamin B complex. Processing sea urchin gonads as a food source in Indonesia has been carried out for a long time. Generally eaten fresh or fried, baked, and boiled [1]. *Diadema setosum* is one of the sea urchin species that is distributed in all parts of coral reefs, including in the sand zone, seagrass zone and the tip zone [2]. *Diadema setosum* contains unsaturated fatty acids, along with omega-3 and omega-6 and is classified as an amino acid. Omega-3 fatty acids are effective in reducing cholesterol levels in the blood. A research stated that sea urchin gonad extract (*D. setosum*) has a potency as an antibacterial agent and as a regulator of the body's immune response [3]. Another research [4] stated that sea urchin gonad extract has the highest antibacterial activity compared to extracts of sea urchin spines and bodies, namely it has an inhibition zone of 8.5 mm against *Escherichia coli* bacteria and 14 mm against *Staphylococcus aureus* bacteria. Thus, sea urchins, especially sea urchin gonads, are safe for consumption.

Pseudomonas fluorescens is a gram-negative bacterium with non-pathogenic saprophytic properties and lives in soil and water, along with plant roots or the root zone (Rhizosphere). Pseudomonas is the trigger for all kinds of damaging effects on food parts related to the potential to produce enzymes that can break down both fats and proteins in food.

Some Pseudomonas bacteria can live at refrigerator temperatures and can cause the formation of mucus and pigment on the contaminated part of the meat. *Staphylococcus aureus* is a gram-positive bacterium that has a round shape. This bacterium is one of several important pathogenic bacteria associated with toxin virulence, invasiveness, and resistance to antibiotics [5]. *Staphylococcus aureus* bacteria can cause all kinds of infections. The effects of poisoning on what we eat or drink due to Staphylococcus are stomach cramps and vomiting which is often also followed by diarrhea [6].

II. METHODS

Location, Tools, and Materials

This research was conducted at the Microbiology Laboratory, Faculty of Mathematics, Natural, and Earth Sciences, Manado State University, North Sulawesi from 11 November to 17 December 2022.

The tools used in this research include an orbital shaker, laminar air flow, analytical balance, micropipette, tip, autoclave, petri dish, test tube, spectrophotometer, Bunsen, incubator, vernier caliper, laminar, sterilization oven, 6 mm corkborer, drying oven, and bowl.

The material used in this research was *Diadema setosum* sea urchins taken from Lembeh Beach at Bitung City, North Sulawesi. *Pseudomonas fluorescens* and *Staphylococcus aureus* bacteria, Nutrient Agar (NA), Nutrient Broth (NB), Mueller Hinton Agar (MHA), distilled water, 70% alcohol and 96% ethanol.

Research Methods

The research method used to obtain sea urchin gonads used an exploratory method which analyzed descriptively, using the stages of sampling sea urchin gonads, and antibacterial tests against *P. fluorescens* and *S. aureus* from sea urchin gonads. The clear zone of inhibition that forms around the well media is then measured.

Research Procedure

Sample Preparation

The samples were sea urchins (*D. setosum*) taken from Lembeh Beach, Bitung City, Lembeh Island, North Sulawesi Province. Sea urchins are placed in jars that have been previously sterilized. All tools and materials used in this research were sterilized in an autoclave for 15 minutes at a temperature of 121^0C at a pressure of 1 atm.

Bacteria Purification

The test bacteria for gram-positive bacteria are *S. aureus* and gram-negative *P. fluorescens* are purified from the preparation by taking one loop and then streaking it on the slanted NA medium that has been made in a test tube, after which it is incubated for one day.

Medium Preparation

The bacterial suspension was put into three sterile Petri dishes of 1 ml each using a micropipette, then filled with 20 ml of sterile NA medium at a temperature of around 40ºC. Then it was homogenized and allowed to stand until the medium froze.

Analysis of Inhibitory Power Activity

Testing of inhibitory activity against test bacteria used the well diffusion method. Wells with a diameter of 6 mm were made in MHA which had been mixed with 50 µL of test bacterial inoculum using a micropipette. 50 µL of bacterial isolate was dropped into the well. The media was incubated at 37ºC for 24 hours, and observations of antibacterial activity were carried out every two hours to determine the properties of the extract against the test bacteria. Inhibitory activity is characterized by the formation of a clear zone around the well and is measured using a Vernier Caliper [7].

Bacterial Purification

Purification aims to obtain the desired pure culture without any contaminants from other microbes. The selection of purified microbial colonies is based on differences in the morphological appearance of the colonies, both in terms of color, elevation, surface texture, radial lines, concentric circles and exudate drops to obtain pure isolates. Purification of bacterial isolates is carried out by transferring the bacteria using the line method which is then grown on NA media [8].

Bacterial Identification

Identification of colony forms of bacterial isolates

Characterization of lactic acid bacteria includes colony morphology (shape, elevation, edge shape, size, surface, and color) and cell morphology (cell shape and Gram stain). Macroscopic characteristics include colony diameter, colony color, colony elevation, colony edges, and the presence or absence of exudate drops and concentric circles. Microscopy includes hyphae shape, hyphae pigmentation, and spores.

Morphological characterization of bacterial isolate cells

All pure isolates that have been obtained will then be gram-stained. The first stage of gram staining is to sterilize the test preparation using alcohol. Bacterial glass preparations are made by aseptically taking 1 dose of potential bacterial culture suspension and then spreading it over the surface of the glass preparation, then passing it over a Bunsen flame for a moment.

When it is cold, sprinkle it with crystal violet solution evenly, 2-3 drops, and let it sit for 1 minute. Washed with running water for 5 seconds then dripped with Lugol's solution on a glass object and dried for 1 minute. After that, wash the slide again with running water, then wash with 96% alcohol for 30 seconds until there is no more Lugol dye, then wash again with flowing distilled water. Then dripped with safranin solution for 10-30 seconds, then washed again using running water, then filtered by placing the slide on top of filter paper, then observed the preparation under a microscope.

Biochemical characterization of bacterial isolates

pH

The pH test is carried out using the first step of the procedure, namely placing one piece of litmus paper by placing it on the NB media in the tube and then waiting for 30 seconds. The result of a pH number that is good for bacteria will show a pH number of 5 to 7.

Catalase Test

Take one bacterium from the pure culture then scratch the bacterial isolate on a glass plate on which the name of the bacterium has been written, then drop a 3% H2O2 solution on the surface of the colony. If there are air bubbles above the surface of the colony, it produces a positive test.

Carbohydrate Fermentation Test

The carbohydrate fermentation test was carried out by aseptically inserting one tube of bacteria into each glass preparation and then leaving it for 1 day for the bacteria to grow in a petri dish containing nutrient agar media and then dripping it with Lugol. Positive results will show a red color in the media.

Sulfate Indole Motility (SIM) Test

Motility indole sulfide is weighed first to get a weight of 4.5 grams, then poured into an Erlenmeyer flask containing distilled water with a measurement of 100 milliliters, and then heated on a hotplate while stirring until it boils. After that, pour the sim agar into a test tube to be sterilized at a temperature of 121ºC using an autoclave which takes 15 minutes, then cool the media by leaving it in the room until it becomes solid.

After that, transfer the bacterial isolate into a sterile test tube filled with sim agar and then give the test tube a name so it can be identified. Then let it sit at room temperature 37ºC which takes 1-2 days.

Data Analysis

Data obtained from the identification results are presented in a qualitative descriptive manner including the morphological and biochemical characteristics of each bacterial isolate isolated from sea urchins (*D. setosum*) and the bacteria that have the potential to act as antibacterials produced.

III. RESULTS AND DISCUSSION

Results

Morphological Characterization of Bacteria

In the results of bacterial isolation, there were different morphologies of bacterial colonies and further observations were made including the shape of the colony, the surface of the colony, the color of the colony and the edges of the colony after incubation for 24 hours in a petri dish. The morphology results of bacterial isolates can be seen in Table 1.

TABLE 1.
COLONY MORPHOLOGY RESULTS OF BACTERIAL ISOLATES IN THE GONAD SECTION OF SEA URCHINS (*D. setosum*)

Isolate	Morphology					
	Color	Shape	Edge	Elevation	Appearance	Colony Formation of Endophytic Bacteria
B1	Dull	Circular	Raised	*Entire*	Dull	*Punctiform* (dot)
B2	Dull	Circular	Umbonate	*Entire*	Dull	*Circular*(round)
B3	Dull	Circular	Raised	Arise	Dull	*Undulate* (wavy)
B4	Dull	Circular	Raised	*Lobate*	Dull	*Glossy* (shiny)
B5	Dull	Circular	Convex	*Lobate*	Dull	*Dull*
B6	Dull	Punctiform	Convex	*Undulate*	Dull	*Lobate* (notched)
B7	Dull	Punctiform	Raised	Arise	Dull	*Undulate* (wavy)
B8	Milky White	Circular	Umbonate	Arise	Dull	

Morphological Characterization of Bacterial Isolate Cells

Bacterial isolates characterized by cell morphology can be seen in Table 1. The gram staining results of 8 isolates of gonad bacteria from sea urchins (*D. setosum*) were that isolates B1-B8 were gram-negative.

Table 3 shows the morphological characterization of all bacterial isolate cells from the gonad of the sea urchin *D. setosum*, which is in the form of bacilli. The gram-staining results of all endophytic bacterial isolates were gram-negative. Biochemical characterization through the catalase test showed that isolates B1, B3, B4, and B7 were negative, while B2, B5, B6, and B8 showed positive results.

The starch hydrolysis test on all isolates showed positive results. The SIM (Sulfate Indole Motility) test on isolates B1, B2, and B4 showed negative results, while isolates B3, B5, B6, B7, and B8 showed positive results. The citrate test for isolates B1, B3, and B7 showed negative results, while isolates B2, B4, B5, B6, and B8 showed positive results. The gelatinase hydrolysis test on all isolates showed negative results.

Table 4 shows that isolates B1, B2, B3, and B7 are suspected to be bacteria of the genus Proteus. Isolates B4, B5, B6, and B8 are thought to be bacteria from the genus Citrobacter.

Investigating Bacteria in Sea Urchin Gonads for Potential Antibacterial Properties

Tests of bacteria that have antibacterial activity showed that seven bacterial isolates had antibacterial properties against *P. fluorescens* and *S. aureus*, namely isolates B1, B2, B3, B4, B5, B6, B7, and B8. From the results of measuring the inhibition zone, complete data was obtained. can be seen in Table 5.

TABLE 2.
BACTERIAL ISOLATES ISOLATED FROM SEA URCHIN GONADS

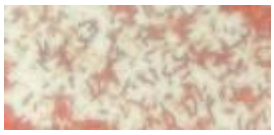
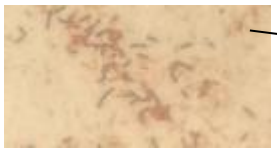
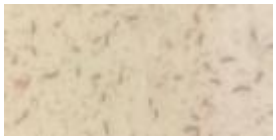
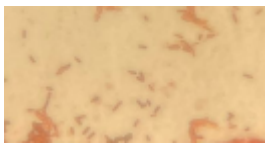
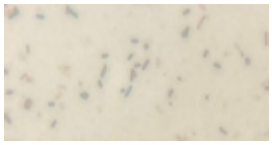
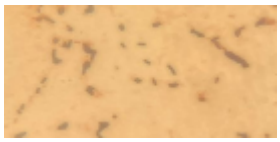
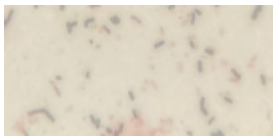
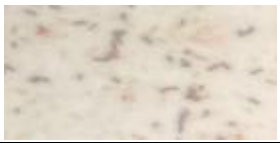
Isolate	Figure	Gram Stain
B1		gram-negative
B2		gram-negative
B3		gram-negative
B4		gram-negative
B5		gram-negative
B6		gram-negative
B7		gram-negative
B8		gram-negative

TABLE 3.
RESULTS OF BIOCHEMICAL CHARACTERIZATION AND MORPHOLOGY OF BACTERIAL CELLS IN THE GONADS OF THE SEA URCHIN *D. setosum*

Parameters	Isolate							
	B1	B2	B3	B4	B5	B6	B7	B8
Cell Shape	Basil	Basil	Basil	Basil	Basil	Basil	Basil	Basil
Gram Stain	-	-	-	-	-	-	-	-
Catalase	-	+	-	-	+	+	-	+
Starch Hydrolysis	+	+	+	+	+	+	+	+
Sulfate Indole Motility (SIM) Test	-	-	+	-	+	+	+	+
Citrate Test	-	+	-	+	+	+	-	+
Gelatinase Hydrolysis	-	-	-	-	-	-	-	-

TABLE 4.
GENUS CONJECTURE OF BACTERIA

GenusConjecture	Positive Results in Biochemical Tests	Isolate	Genus Conjecture Show	References
Proteus	Sulfate Indole Motility (SIM) Test	B1, B2, B3, B7	There is motile movement and can produce the enzyme amylase.	[9]
Citrobacter	Catalase, Starch Hydrolysis, Sulfate Indole Motility (SIM), and Citrate Test.	B4, B5, B6, B8	Hydrogen peroxide is toxic to bacterial cells because this material can deactivate enzymes in cells and is very dangerous for the bacterial cells themselves.	[10]

TABLE 5.
DIAMETER OF THE INHIBITORY ZONE OF BACTERIAL ISOLATES AGAINST *P. fluorescens* AND *S. aureus* (mm)

Isolate	*Pseudomonas fluorescens*	*Staphylococcus aureus*
B1	9.03	11.09
B2	8.04	9.74
B3	7.43	9.00
B4	7.77	8.57
B5	8.45	9.63
B6	7.11	10.05
B7	6.97	10.98
B8	8.08	9.77

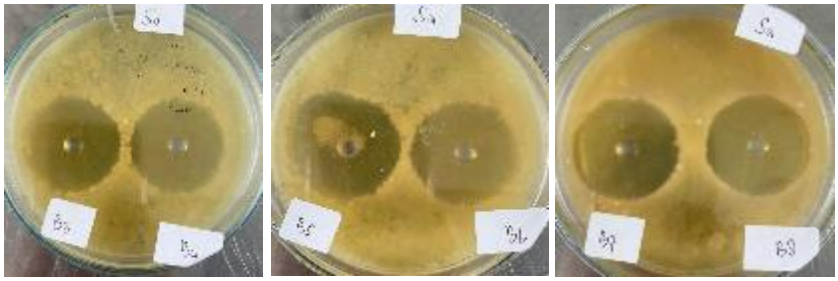


Fig. 1. Diameter of the Bacterial Inhibition Zone against Staphylococcus aureus

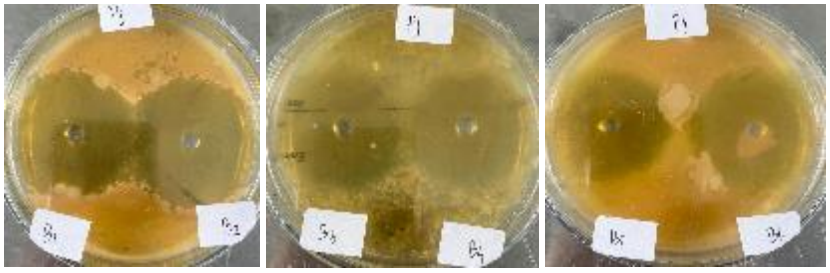


Fig. 2. Diameter of the Bacterial Inhibition Zone against *Pseudomonas fluorescens*

Discussion

The first stage in this research was the isolation of bacteria from the gonads of sea urchins using the spread method. The media used in the process of isolating bacteria from the gonads of the sea urchin *Diadema setosum* is Nutrient Agar (NA) media. NA media is a universal media that is often used to grow and reproduce bacteria. NA media is composed of peptone, meat extract, and agar. Peptone functions as the main organic nitrogen source and contains vitamins and carbohydrates. Meat extract is a source of nutrition to grow bacteria, a source of organic vitamins, organic nitrogen, organic carbon, and inorganic salts. Agar is used as a media compactor but does not contain the nutrients needed by bacteria. Isolate colonies that grew after being incubated for 24 hours were separated based on differences in colony morphology and 8 bacterial colonies were obtained. Purification is carried out to obtain a pure culture using the quadrant method.

Characterization of bacterial isolates begins with characterization of cell morphology, namely cell staining (gram staining) to determine cell shape, and cell arrangement and to determine whether the isolate is gram-positive or gram-negative bacteria [11]. Before staining, fixation is carried out in order to stick the bacterial cells to

the glass object, kill microorganisms quickly without causing changes in their shape and structure, change the binding capacity of the dye and prevent cell rupture caused by their own enzymes.

Based on the gram staining carried out, all bacterial isolates were gram-negative (B1-B8). Gram-negative bacteria have a cell wall component, most of the cells of which consist of a lipid layer, as a result, when staining, they are unable to retain the main dye, especially when washing with alcohol (lipids are damaged by alcohol), so that the bacterial colonies appear red in the gram staining results.

The inhibition zone is the activity of bacterial secondary metabolites in inhibiting the development of test bacteria by disrupting the metabolism of the test bacteria, inhibiting bacterial cell wall synthesis, disrupting the permeability of bacterial cell membranes, inhibiting bacterial protein synthesis, and damaging the nucleic acid synthesis of test bacteria.

Observation of colony morphology and cell morphology was not sufficient to identify the eight bacterial isolates. Therefore, further identification is needed to determine the metabolic activity caused by the workings of the enzymes of the seven bacterial isolates. Bacterial biochemical testing is a method or treatment carried out to identify and determine a pure isolated culture of bacteria through its physiological properties. A bacterium cannot be determined just based on its morphological characteristics, so it is necessary to look at its biochemical characteristics and factors that influence its growth. The biochemical tests carried out were catalase test, starch hydrolysis test, citrate test, Sulphate Indole Motility test and gelatinase hydrolysis test.

After the identification process is prepared according to the results of each biochemical test, it is then matched with the suspected genus in Bergey's Manual of Systematic Bacteriology [12]. From the results of the biochemical tests that were carried out, four isolates were obtained that were similar to the Proteus genus, namely isolates B1, B2, B3 and B7. Proteus has the characteristics of being gram negative and bacillus shaped. Four isolates, namely B4, B5, B6 and B8, are similar to the Citrobacter genus with the character of being gram negative and using citrate as the only carbon source.

After carrying out the identification stage on indigenous bacterial isolates, the next step was to carry out antibacterial testing on the growth of the test bacteria, namely *P. fluorescens* and *S. aureus*. Both types of bacteria are suitable for use as models for testing new active compounds and also have pathogenic properties for humans. The two test bacteria came from different types of bacteria, namely gram-negative (*P. fluorescens*) and gram-positive (*S. aureus*) bacteria [13].

Based on the results of the inhibition test on the first day after incubation for 24 hours, the bacteria *S. aureus* and *P. fluorescens* showed the appearance of a clear zone in the well hole area with different inhibitory index sizes. On the second day with an incubation period of 48 hours, the diameter of the inhibition zone around the *S. aureus* and *P. fluorescens* bacteria isolates began to decrease. On the third day of the incubation period, the *S. aureus* and *P. fluorescens* bacterial isolates no longer had a clear zone around the well media.

The antibacterial compound produced from gonad bacteria isolated from the sea urchin *D. setosum* shows that the antibacterial compound produced from it is only bacteriostatic with activity that inhibits the development of bacteria (inhibits the multiplication of the bacterial population), but is not deadly. The mechanism of action is to interfere with the protein synthesis of pathogenic bacteria. This was shown in the test media on the first day to the third day, the smaller the resistance zone.

The mechanism for inhibiting bacterial growth by secondary metabolites can occur through inhibiting the formation of compounds that make up the bacterial wall, increasing the permeability of the cell membrane so that the cell loses components that make up the cell and inactivating enzymes [7].

The results of the antimicrobial activity test showed different activity for each tested-bacteria. This is indicated by the different diameters of the inhibition zones formed. The differences in the inhibition zones produced are influenced by several factors, namely the sensitivity of the organism, pH, type of microbe, antimicrobial agent used, culture medium, incubation conditions, and agar diffusion speed. Factors that influence the speed of agar diffusion are the concentration of microorganisms, media composition, incubation temperature, and incubation time [14]. These factors are likely to cause differences in the diameter of the resulting clear zone.

The results of the SIM (Sulfate Indole Motility) test showed that isolates B1, B2, B3 and B7 were suspected to be bacteria of the genus Proteus which showed motile movement and were able to produce the amylase enzyme. Isolates B4, B5, B6, and B8 are thought to be bacteria of the Citrobacter genus which show that the presence of hydrogen peroxide is toxic to bacterial cells because this material is able to deactivate enzymes in cells and is very dangerous for the bacterial cells themselves.

CONCLUSION

There were 8 bacterial isolates isolated from the gonads of *Diadema setosum* sea urchins which had the potential to act as antibacterials against the growth of *Pseudomonas fluorescens* and *Staphylococcus aureus* bacteria. The largest diameter of the inhibition zone is 9.03 mm (*P. fluorescens*) and 11.09 mm (*S. aureus*). The diameter of the smallest inhibition zone was in *P. fluorescens*, namely 6.97mm (isolate B7) and 8.57mm in *S. aureus* bacteria (isolate B4). Four isolates were suspected to be of the Proteus genus (B1, B2, B3 and B7) and four other isolates were suspected to be of the Citrobacter genus (B4, B5, B6 and B8).

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