



Isolation and Activity Testing of Proteolytic Bacteria Associated with The Sponge *Stylotella sp.* and Its Potential as an Antibacterial

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Abstract

Background: Background: The province of East Nusa Tenggara (NTT), Indonesia, boasts a significant expanse of marine territory, making its waters a potential treasure trove of aquatic resources. One such marine resource is the *Stylotella sp.* sponge, which is found in this region. Sponges like *Stylotella sp.* often have intricate relationships with microorganisms, including bacteria. **Method:** This research was to characterize and identify proteolytic bacteria and test bioactive compounds from isolates of proteolytic bacteria from the sponge *Stylotella sp.* Proteolytic bacterial strains were rejuvenated first in 2% TSA + NaCl medium and then characterized microscopically and biochemically using catalase and oxidase tests. The diffusion method used the antibacterial activity test against *Escherichia coli* and *Staphylococcus aureus*. **Result:** The results showed that proteolytic bacterial isolates had irregular and rounded colony shapes, wavy and intact colony edges, hilly and convex colony elevation, and white color, while biochemically, it was declared positive for the catalase and oxidase tests. Antibacterial activity tests on proteolytic bacterial strains showed the ability to inhibit the growth of *S. aureus* with an inhibitory capacity of 9.5 mm and *E.coli* with an inhibitory capacity of 7 mm. **Conclusion:** Hasil isolasi bakteri proteolitik menunjukkan bahwa keenam isolat bakteri yaitu SP0, SP1, SP3, SP4, dan SP5 umumnya mempunyai bentuk koloni bulat dan tidak beraturan, berwarna putih, tepinya berwarna putih.

Keywords: Antimicrobial; Proteolytic Bacteria Isolate; *Stylotella sp.* Sponge

Introduction

Indonesia is the largest archipelagic country in the world, with an area of around 3.1 million square kilometers. This means that Indonesia also has a vast sea. The sea contains various kinds of natural resources in it. Natural resources in the sea are natural resources that have great potential and need to be developed and managed optimally (Jo et al., 2016). Marine resources are biological resources with great potential that must be developed and managed optimally. Indonesia's vast sea areas contain a wealth of marine diversity that can be explored, including mangroves, coral reefs, and seagrass, along with other types of marine biota such as fish, mollusks, crustaceans, sponges, algae, and turtles (Suparno, 2005). One of Indonesia's provinces with a giant sea area is the province of East Nusa Tenggara (NTT). This makes NTT waters rich in potential marine resources such as fisheries, seaweed, coral reefs, and sponges (Pardosi et al., 2022). Sponges are a type of porous invertebrate and belong to the genus Porifera. Sponges act as filter feeders, looking for food, among other things, by filtering or absorbing sea water containing food through pores (ostia). Sponges or porifera are animals from the phylum Porifera, one of the components in coastal and marine ecosystems, especially on coral reefs. Sponges have bioactive potential, including



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antibacterial, anticancer, and antifungal properties (Rumampuk, 2017). This is by (Utami et al., 2016), which states that bioactive compounds from sponges can be used as medicine, for example, antibacterial, anticancer, antifungal, anti-inflammatory, cytotoxic, antibacterial, antiviral, antimalarial, anti-inflammatory, cancer and immunosuppressive.

Exploration of microbes in symbiosis with sponges from various regions in Indonesia is one thing that needs to be done. Isolation of bacteria in symbiosis with sponges, molecular characterization, and characterization of bioactive compounds produced by bacteria from sponges are strategies that can be used to explore the role of sponges. According to Mahdiyah (2021), Sponges produce compounds that have the potential to act as antibacterials on a large scale. Sponges harbor various types of bacteria, up to 40% of their biomass (Sable et al., 2017). Bacteria in symbiosis with sponges serve as a source of extracellular enzymes (Maharsiwi et al., 2020). Extracellular enzymes are active outside bacterial cells and have various functions, such as breaking down large molecules into smaller ones, an essential process in degrading nutrients sponges need (Wicaksana & Rachman, 2018). This shows that there is a complex symbiotic relationship between sponges and bacteria that can provide benefits to sponge organisms. With the presence of bacteria, sponges become more resistant to bacterial infections than other land and marine organisms (Mahdiyah, 2021).

In addition, the extracellular enzymes these bacteria produce are used to break down nutrients and act as chemical defenses against predators (Wicaksana & Rachman, 2018). Proteolytic bacteria are a type of bacteria that can produce extracellular protease enzymes (Agrawal et al., 2016). Protease enzymes are a group of enzymes that have an essential role in the breakdown or digestion of proteins, but they also have wide applications in the industrial field (Rizaldi, 2016). Regarding this, it is necessary to identify and characterize proteolytic bacteria and test bioactive compounds from isolates of proteolytic bacteria from the sponge *Stylotella sp.*

Methods

Tools and materials

The tools used in this research are scissors, incubator, laminar airflow, micropipette, knife, dropper pipette, test tube, beaker, petri dish, autoclave, microscope, object glass, oven, analytical balance, Erlenmeyer, Bunsen, tube needle, horn spoon, mortar, match, mask, tweezers, tube rack. The materials used in this research were *Stylotella sp.* sponge samples and distilled sodium chloride (NaCl) salt solution with a concentration of 0.9%, cotton, label paper, iodine solution, tissue, 96% alcohol, disc paper, crystal violet dye, solution dye Lugol, Methylene Blue (MB) dye, and safranin dye.

Sponge Sampling

Sponge samples were taken from Oenggae Waters, Pantai Baru District, Rote Ndao Regency. They were taken randomly by diving into the seabed. The samples were then put into sterile plastic. Next, the sponge was taken to the laboratory to be characterized and isolated (Rizaldi, 2016).

Media Creation

The media used is Tryptic soy agar media, made by dissolving 6 grams of TSA media in 150 ml of distilled water and 3 ml of NaCl and heating it on a hot plate. Then, the press is sterilized in an autoclave for 15 minutes at a temperature of 121°C (Retnowati et al., 2011).

Bacterial Isolation

Isolation of bacteria associated with the sponge was carried out by washing it with running water and rinsing it with sterile distilled water. Then, the sample was cut into small pieces, weighed 10 grams, and crushed using a mortar. Next, a 10⁻⁰ to 10⁻⁵ dilution is made. The results of the dilutions 10⁻⁰, 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵ were then

taken 0.1 ml and inoculated in TSA media + 3 ml NaCl, incubated for 24 hours at 30°C (Pastra et al., 2012; Marzuki et al., 2014). The growing bacterial isolates were observed for colony morphology, including shape, edges, elevation, and color.

Purification of Bacterial Isolates

Take one cycle of bacterial culture that grows on TSA media in a petri dish, streak it on Tryptic soy agar medium + 3ml NaCl, and incubate at 30°C for 24 hours (Massinai, 2013).

Microscopic Identification of Potential Isolates

Microscopic observations include gram-staining biochemical tests, catalase tests, and oxidase tests.

Gram stain

Identification of proteolytic bacterial isolates includes gram staining using the smear method. Bacterial cultures are taken in one dose and spotted on a glass object to be made into smears and added with dye. The smear that has been made is then observed under a microscope to be observed under a microscope at a magnification of 10 x 0.25.

Catalase Test

Take a bacterial sample from the culture medium and place it on an object glass cleaned with alcohol. Drip 1-3 drops of 3% H₂O₂ solution onto the bacterial sample in the object glass. When H₂O₂ is added, oxygen gas bubbles will form. These gas bubbles will appear as foam or air bubbles rising upward from the bacterial sample. If gas bubbles form, this indicates that the bacteria have the catalase enzyme and the test result is positive (catalase positive). If no gas bubbles form, then the test is negative.

Oxidase Test

The bacterial culture to be tested is planted on Trypticase Soy Agar and then incubated at 37°C for 1x24 hours. After 24 hours, bacterial colonies growing on the TSA medium were flooded with oxidase reagent. If these bacteria produce the oxidase enzyme, the colony will experience a color change after the application of the oxidation reagent, which will become light red, dark red, and black.

Proteolytic bacterial activity assay

The proteolytic bacterial activity test was carried out using the disk diffusion method. The bacterial isolate to be screened is first cultured for 1x24 hours in TSA+ 2% NaCl media. Next, isolate cultures were made as dots on the surface of Skim Milk Agar media and incubated for 1x24 hours. The clear zone around the bacterial isolate is then observed and measured (Hudzicki, 2013; Rizaldi, 2016).

Antibacterial activity test

Antibacterial testing of proteolytic bacterial isolates was done by culturing proteolytic bacterial isolates in TSA+2% NaCl media, testing bacteria in NA median, and incubating for 1x24 hours. Proteolytic bacterial isolate cultures were then prepared in suspension form following McFarland standards. Then, 10 µl of the suspension was pipetted and dropped onto a paper disc with a diameter of 0.6 cm, which was placed on Mueller Hinton Agar, which had been etched with *S. aureus* and *E. coli*. The diameter of the inhibition zone is measured using the formula:

$$\frac{(DV - DC) + (DH - DC)}{2}$$

Information:

DV: Vertical diameter

DH: Horizontal diameter

DC: Disc diameter

Data Analysis

Data from research on proteolytic bacterial activity tests and antibacterial activity tests against *E. coli* and *S. aureus* was obtained from the sponge *Stylotella sp.* The data obtained and analyzed is descriptive. The analysis results are presented in various formats, explained narratively in text form, and presented in tables and figures.

Result and Discussion

Isolation and Identification

The isolation of bacteria associated with the *Stylotella sp.* sponge resulted in 6 bacterial isolates. The results of observations of proteolytic bacterial colony isolates from the sponge *Stylotella sp.* have morphological characteristics, including shape, edges, elevation, and color. SP0 bacterial isolates have colony characteristics that include irregular shapes, wavy edges, and hilly elevations and have a white color, whereas SP1, SP2, SP3, SP4, and SP5 bacterial isolates have round-shaped colonies, flat edges, and convex elevations and colors. White. This agrees with [Pahriyani et al. \(2020\)](#), who stated that bacterial isolates associated with sponges, which include colony color and colon shape, orange, yellow, and white colony colors, and circular colony shapes with a nucleus in the middle, the edges of the isolate are smooth. , irregular, has curved elevations and convex elevations, and elevations like drops, raised, and hilly ([Pardosi et al., 2022](#)). The results of colony morphology identification of 6 bacterial isolates associated with the *Stylotella* sponge can be seen in (Table 1).

Table 1. Bacterial isolates associated with the sponge *Stylotella sp.* macroscopically.

Bacterial isolate	Colony morphology			
	Shape	Margin	Elevation	Pigmentation
SP ₀	Irregular	Undulate	Hilly	White
SP ₁	Circular	Entire	Convex	White
SP ₂	Circular	Entire	Convex	White
SP ₃	Circular	Entire	Convex	White
SP ₄	Circular	Entire	Convex	White
SP ₅	Circular	Entire	Convex	White

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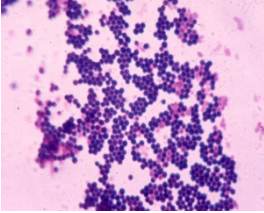
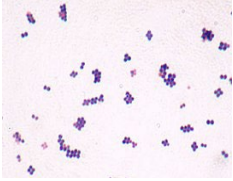
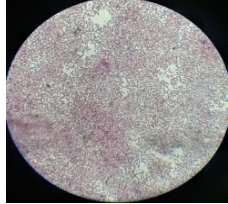
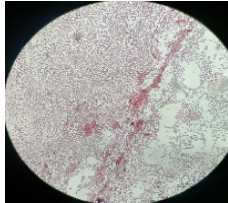
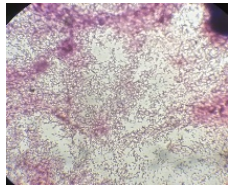
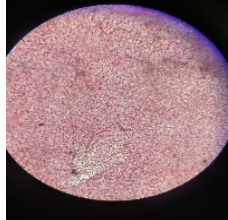
Identification of proteolytic bacteria microscopically

The results of the gram test on bacterial isolates SP0 and SP1 were found to have a cocci form and were gram-positive, while isolates SP2, SP3, SP4, and SP5 were in the form of bacilli with gram-negative characteristics (Table 2). [Pelczar and Chan \(1986\)](#) stated that 95% of marine bacteria are gram-negative, some are motile, motile nature refers to the ability of bacteria to move actively, which can be used to find sources of nutrition or respond to environmental changes, 70% contain pigments. The pigment content can give bacteria a specific color and role in bacterial photosynthesis or adaptation to the marine environment ([Kuddus, 2019](#)).

The results of observations showed that bacillus-shaped bacteria were the dominant ones found in this study. This is probably because these bacteria have flagella, so they can stick to sponges, whereas bacteria not equipped with flagella, non-flagellum, can move by gliding. [Aryulina \(2005\)](#) suggested that bacteria that have flagella can help these bacteria find environmental conditions that are more suitable for their growth. According to [Fardiaz \(1989\)](#), marine bacteria associate with various solid surfaces, including algae, sponges, seagrass, coral, and mangrove plants, to obtain the necessary attachment places. Bacteria in cocci form do not have locomotion, such as flagella. Therefore, bacteria tend to stick to objects in the water as a survival strategy ([Rizaldi, 2016](#)). Bacteria in the form of coccus, which do not have locomotion, tend to live attached to specific substrates, including sponges. The coccus shape of this bacteria is caused by the presence of a slimy

material, which causes the bacterial cells to bond or combine to form a firm or solid surface (Megawati et al., 2019).

Table 2. Results of Gram Staining and Biochemical Tests

D e s c r i p t i o n : (+) P o s i t i v e ; (-) N e g a t i v e	Isolate Code	Gram staining		Biochemical Test		
		Picture	Form	Gram	Catalase Test	Oxidase Test
	SP ₀		Cocci	+	+	+
	SP ₁		Cocci	+	+	-
	SP ₂		Basil	-	+	-
	SP ₃		Basil	-	+	-
	SP ₄		Basil	-	+	-
	Sp ₅		Basil	-	+	-

Biochemical testing is carried out using 2 test methods: the catalase and oxidase tests. In the catalase test results, six isolates of proteolytic bacteria were found, producing air bubbles on the colony's surface. Air bubbles indicate positive bacteria produce the catalase enzyme (Figure 2. a). Proteolytic bacterial isolates that do not produce air bubbles on the surface of their colonies can be declared catalase-negative, meaning that the bacteria do not degrade the H₂O₂ provided and, therefore, does not produce oxygen. According to Cappuccino & Sherman (2017), positive catalase test results for all isolates tested indicated the presence of air bubbles on the colony's surface when dipped with 3% H₂O₂ solution. This shows that these bacteria live in aerobic conditions, such as *S. aureus*, *Bacillus sp.*, and *Pseudomonas sp.* (Kuddus, 2019).

The results of the oxidase test on proteolytic bacterial isolates carried out on six bacterial isolates showed that the isolate that produced the cytochrome oxidase enzyme was isolate code SP0, which was marked by colonies that changed color to red. Meanwhile, isolates SP1, SP2, SP3 and SP4, SP5 showed no color change. According to Lay (1994), positive bacteria in the oxidase test contain the enzyme (Kuddus, 2019).

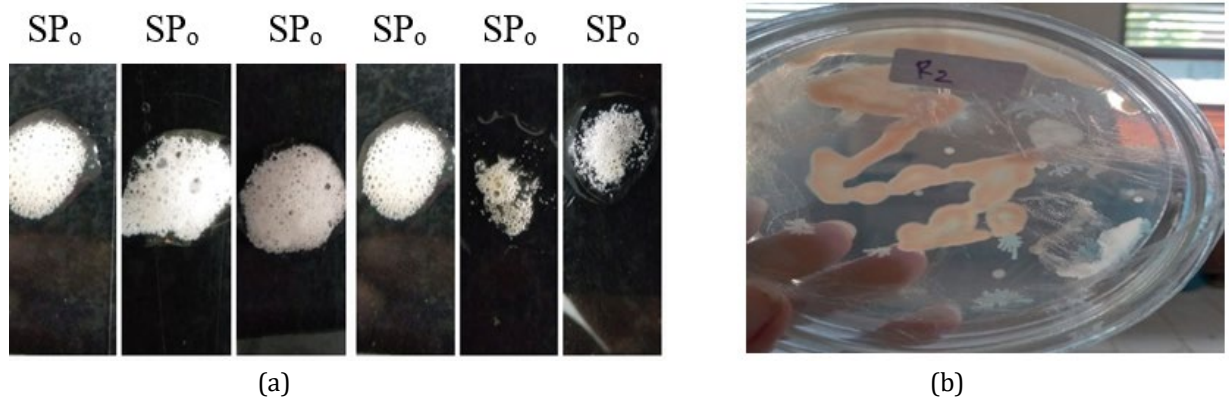


Figure 2. (a)Catalase test results; (b) Oxidase results.

Proteolytic Bacterial Activity Test

Table 3. Proteolytic activity test results of associated bacterial isolates with the sponge *Stylotella sp.*

Isolate code	Proteolytic activity test	Proteolytic index
SP0	+	37,5 mm
SP1	+	40 mm
SP2	-	0
SP3	-	0
SP4	-	0
SP5	-	0

Description: (+) exists, (-) does not exist

Based on the results of the proteolytic activity test, it showed that the isolates producing the protease enzyme were isolates SP0 and SP1, which were characterized by explicit zone activity around the paper disc with a proteolytic index for isolate SP0 of 37.5 mm and bacterial isolate SP1 with a proteolytic index of 40 mm (Figure 3). This can be calculated using the following formula:

$$IP = \frac{\text{Clear Zone Diameter}}{\text{Bacterial Colony Diameter}}$$

The results of precise zone measurements on proteolytic bacterial isolates showed significant variations between these isolates. The high proteolytic index of the SP0 isolate may be due to the isolate's rapid ability to synthesize and degrade amino acids. According to (Rizaldi, 2016), bacteria from the genus *Bacillus*, such as *Bacillus sp.*, produce protease enzymes. The formation of clear zones in media containing proteins, such as skim milk, is a typical sign of this proteolytic activity. Therefore, clear zones in proteolytic bacterial isolates associated with the sponge *Stylotella sp.* indicate these bacteria' ability to produce protease enzymes.

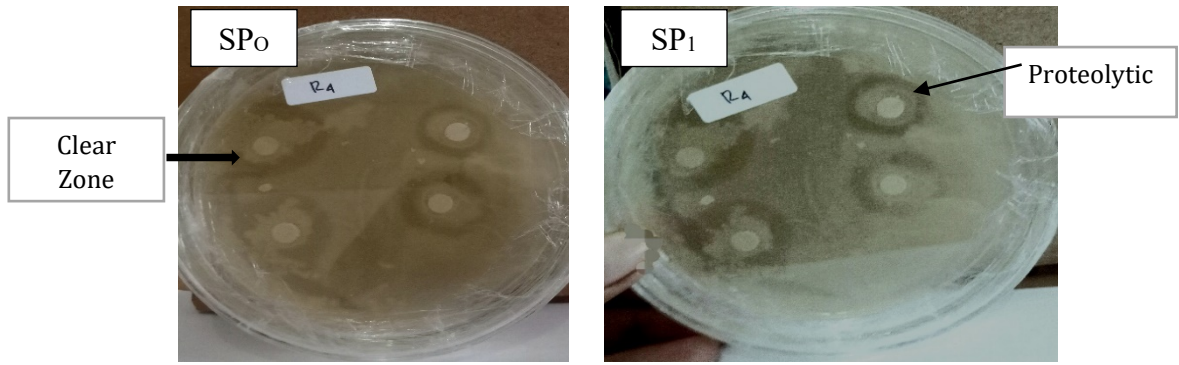


Figure 3. Proteolytic activity assay

Antibacterial Activity Test

Testing the antibacterial activity of proteolytic bacterial isolates is an essential step in evaluating the potential of these isolates to inhibit the growth of pathogenic bacteria (Nioede et al., 2021). The results of this test show that proteolytic bacterial isolates can produce effective antibacterial active compounds. This clear zone indicates that the antibacterial compounds produced by these isolates can inhibit the growth of the pathogenic bacteria tested (Figure 4).

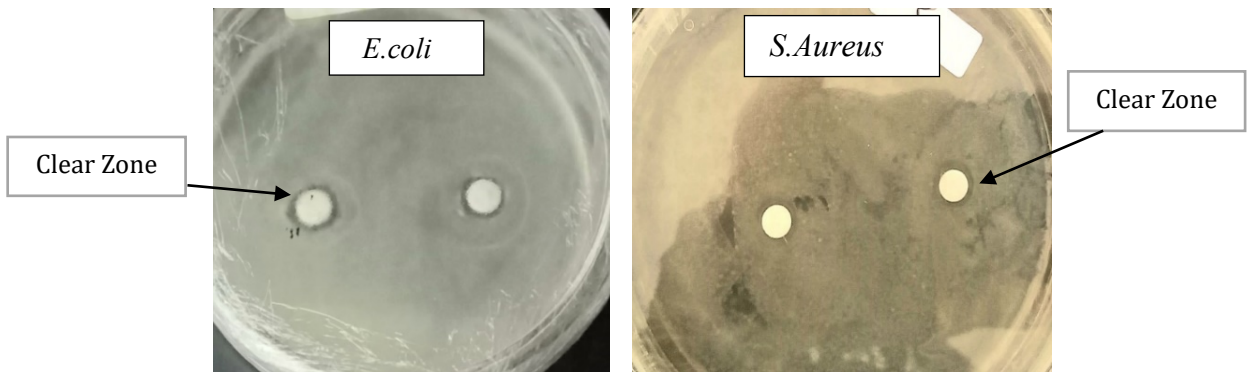


Figure 4. Antibacterial Activity Test Results of Proteolytic Bacterial Isolates

Proteolytic bacterial isolates tested against pathogenic bacteria had antibacterial activity, with a barrier diameter of 7 mm against *E. coli* and 9.5 mm against *S. aureus* (Table 4).

Table 4. Antibacterial activity test results of proteolytic bacterial isolates

Isolate Code	Tested bacteria	Repetition	Inhibition Zone Diameter (mm)
Bacteria Proteolytic	<i>E. coli</i>	1	8
		2	6
		Average	7
	<i>S. aureus</i>	1	10
		2	9
		Average	9.5

According to Abubakar et al. (2011), isolated from the mesohyl and superficial parts of *Jaspis* sp. show antibacterial properties because it can inhibit the growth of *S. aureus*, *Vibrio harveyi*, *E. coli*, and *P. aeruginosa* (Pardosi et al., 2022). Gratia et al. (2019) stated that bacterial isolates associated with sponges had antibacterial activity against the test bacteria *E. coli* at 13.5 mm and *S. aureus* at 14.25 mm.

Conclusions

Based on the research results, it was concluded that the results of proteolytic bacterial isolation showed that the six bacterial isolates, namely SP0, SP1, SP3, SP4, and SP5, generally had round and irregular colony shapes, were white, the edges of the isolates were wavy and flat, the colony elevations were hilly and convex and were colored. White. The proteolytic bacterial activity test results showed two bacterial isolates, SP0 and SP1. The SP0 bacterial isolate had a proteolytic index of 37.5 mm, while the SP1 bacterial isolate had a proteolytic index of 40 mm. The results of testing the antibacterial activity of bacterial isolates SP0 and SP1 showed an average resistance of 9.5 mm against *S. aureus* and 7 mm against *E. coli*.

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Declaration statement

The authors reported no potential conflict of interest.

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