



Diversity and Potential Active Compound of the Sponge-Associated Bacteria from Lemukutan Island, West Kalimantan Indonesia as New Type of Antibiotics

¹M. Fiqri Baihaqi, ²Faika Nabila, ³Adelia Triwahyuni, ⁴Annisa Nasywa Fahira, ⁵Yuyun Nisaul Khairillah

^{1,2,3,4,5}Biotechnology Study Program, Institut Teknologi dan Kesehatan Muhammadiyah Kalimantan Barat, Kubu Raya, Indonesia

*Corresponding Author e-mail: yuyunkhairillah@itekesmukalbar.ac.id

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Abstrak: This study aims to determine the diversity of sponge symbiont bacteria and the effectiveness of active compounds of bacteria that are symbiotic with marine sponges from Lemukutan Island waters. The methods in this study are isolation of sponge symbiont bacteria, characterization of sponge symbiont bacteria, antagonistic tests, activity tests of the best sponge symbiont bacterial isolate extract culture filtrate, and GCMS/MS tests. The results showed that nineteen isolates of sponge symbiont bacteria were successfully isolated from sponge types from *Theonella cylindrica* and *Hyattella intestinalis* sponges in Lemukutan waters. Seven isolates of sponge symbiont bacteria are included in the Gram-positive bacteria genre. While the other twelve are included in Gram-negative bacteria. Ten of the nineteen isolates of sponge symbiont bacteria have antibacterial activity for *S. aureus*. Isolate Sp4 10-6 B, based on the results of antagonistic tests, has an inhibition diameter of 10.28 mm, which is categorized as solid inhibition. Ethyl acetate extract of Sp4 10-6 culture filtrate has a minimum inhibitory concentration value of 0.5% against pathogenic bacteria *S.aureus*. The active compound with the potential for antibiotic development is the Tetradecane compound, which has the highest peak area of the eight other types of compounds. This shows that the concentration of the compound contained is higher than the other compounds. This compound is included in the aliphatic hydrocarbon and alkane groups.

Keywords: Antibiotics; bioactive compounds; concentration; inhibition diameter; resistance

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INTRODUCTION

Infectious diseases are one of the biggest causes of death in the world. Every year infectious diseases can cause the death of 3.5 million people. Infectious diseases can attack the host's body tissue, and the treatment of infectious diseases generally uses antibiotics. However, over time the use of inappropriate and uncontrolled antibiotics can cause resistance to antibiotics known as Multi Drug Resistance (MDR). The existence of high levels of resistance, especially to types of antibiotics, has encouraged various studies to explore new types of bioactive compounds that are used as alternatives to overcome existing levels of resistance, one of which is by utilizing potential marine resources. One of the marine biota that is a potential candidate for a new type of antibiotic is sponges (Migue et al., 2020).

Sponges are a group of animals from the phylum Porifera. It is known that sponges have various active compounds including alkaloids, terpenoids, polyketides and peptides. The sponge bioactive compounds have a role as, antiviral, anti-HIV and anti-inflammatory, antibacterial, antifungal, antimalarial, antileukemia, enzyme activity inhibitors and several other properties (Luo et al., 2020). In addition, sponges are also known to contain peptides, terpenoids, steroids, acetogenins, alkaloids, cyclic halides

and nitrogen compounds. These compounds have pharmacological activities such as antifouling, antitumor, anti-inflammatory, antiviral, antibacterial, and antimalarial (Kurimoto et al., 2020).

The ability of sponges to produce bioactive compounds has been widely reported and is the result of symbiosis with bacteria that live commensally with them. Bioactive compounds resulting from symbiosis are a contribution from bacteria as a sponge defence against predators and pathogenic bacteria. It is known that bacteria that are symbiotic with sponges play a role in helping sponges produce antibiotic compounds (Wang, 2020). This underlies the assumption that symbiotic bacteria can produce bioactive compounds like sponges and cause the existence of symbiotic bacteria to continue to be explored. The results of the exploration of symbiotic bacteria have great benefits in the search for the potential of marine sponge symbiotic bacteria (Hayes et al., 2022).

The *Vibrio* sp. which is symbiotic with the sponge *Pseudoceratina purpurea* can inhibit bacterial growth (Yu et al., 2023). Sadahiro et al. (2020) reported that marine bacteria have the potential as a source of antibiotic rifampicin. Holland et al. (2020) reported that sponge symbiont bacteria have the potential to produce bioactive materials. Li et al., (2020) reported that a bacterial isolate code 6A3 from a sponge from Panggang Island, Seribu Islands which was identified based on the 16S rRNA gene showed 96% similarity to *Chromohalobacter* sp. Maeyama et al., (2021) reported that a bacterial isolate symbiont of the sponge *Aaptos* sp. from the North Java Sea code SPA1 had a similarity of 99% to *Halomonas aquamarine*, the isolated code SPA2 had a similarity of 100% to α -proteobacterium D21, and the isolated code SPA3 had. The similarity of 100% with *Pseudoalteromonas luteoviolacea* and has antibacterial activity. Moosmann et al., (2021) reported that *Jaspis* sp. sponge symbiont bacteria from Waigeo Island, West Papua, namely the genus *Pseudomonas* and the genus *Bacillus* have antibacterial activity against test bacteria.

Research on sponge bacterial symbionts has been widely conducted, however, studies of bacterial diversity to the molecular level and exploration of the potential for active compounds of symbiont bacteria, especially from the Lemukutan Island as a source of new types of antibiotics have not been carried out, considering that Lemukutan Island is one of the islands with a high abundance of marine resources. Based on the above, the aim of the research is study diversity and exploration of the potential for active compounds of sponge symbiont bacteria from the waters of Lemukutan Island as a source of new types of antibiotics in overcoming the existing level of antibiotic resistance.

METHOD

Sponge samples in this study were taken from the waters of Lemukutan Island, West Kalimantan and the research was conducted from April to July 2024 October the Biotechnology Laboratory, Institut Teknologi dan Kesehatan Muhammadiyah Kalimantan Barat. The procedure for conducting this study, namely:

1. Isolation and Purification of Sponge Symbiont Bacteria

Sponge samples were processed under aseptic conditions. Sponge samples were cut into small pieces measuring 1 cm³ using a sterile Scapel. The sterilized sponge samples were then placed in a petri dish containing NA seawater medium and incubated at room temperature for 24-48 hours. Furthermore, the bacterial colonies that grew were purified (Mohan et al., 2016). Pure cultures were then grown on slant agar and stored at 4 °C until ready to use (Tarazona et al., 2022).

2. Morphological Identification of Sponge Symbiont Bacteria

Pure bacterial isolate colonies were then morphologically identified based on the size, shape, colour, edge (margin) and slope (elevation) of the colony. Morphological observations were carried out until pure isolates were obtained. Furthermore, after pure isolates were obtained, each pure isolate was then transferred to a marine agar slant medium (Guzii et al., 2022).

3. Screening of Sponge Bacteria Potential as Antibiotic Compounds

In this test, there are several processes that must be carried out to obtain antibacterial activity test results. Such as making Mec Farland 0.5 solution, making suspensions and test bacteria, preparing endophytic symbiont bacteria, testing antibacterial activity, and observing and measuring inhibition zones (Guzii et al., 2022).

4. Production of Secondary Metabolites of Sponge Bacterial Isolates

The selected potential isolates were cultivated and then extracted. The ethanol extract of the symbiont bacteria was concentrated with a vacuum rotary evaporator until a thick extract was obtained. The thick extract was then dried in an oven at a temperature of 40-50, then the dry extract was used for antibacterial potential testing

5. Identification of Sponge Bacterial Compounds by the GC-MS Method

The best bacterial extract was analyzed for its bioactive compound content using the Shimadzu Gas Chromatography-mass Spectrometry (GCMS)-QP 2010 instrument (Narayana et al. 2018).

The data obtained were analyzed using the Analysis of Variance (ANOVA) method with the SPSS 20 program. Significant differences between treatments were continued with the Duncan Multiple Range Test (DMRT) at the 5% level ($p < 0.05$). The molecular data were analyzed for homology with sequences in the Gen bank and aligned using Muscle software in MEGA version 5 (Tamura et al., 2017).

RESULTS AND DISCUSSION

Diversity of Sponge Symbiont Bacteria Based on Morphological Characteristics

The results of isolating sponge symbiont bacteria isolated from the sponge species *Theonella cylindrica* and *Hyattella intestinalis* were then identified based on their morphological characteristics through Gram staining. Twenty-nine sponge symbiont bacteria were successfully isolated from the *Cylindrica* and *Hyattella intestinalis* sponge species. The results of identifying bacteria through Gram staining obtained seven sponge symbiont bacteria included in the Gram-positive bacteria. While the other twelve are included in the Gram-negative bacteria. From the sponge species *Theonella cylindrica*, the sponge species in the *Demospongiae* class is an isolate from species one (Sp 1). From the sponge species *H. intestinalis*, the sponge species, which is also included in the *Demospongiae* class, is an isolate from Species one (Sp 4). Nine isolates were successfully obtained from the sponge species. From the sponge species *H. intestinalis*, 10 isolates were obtained.

Based on the results of Gram staining on the two sponge species, namely *T. cylindrica* and *H. intestinalis*, it shows that species 1 (Sp 1) of the *T. cylindrica* sponge type consists of six isolates categorized as Gram-positive bacteria and four isolates categorized as Gram-negative. At the same time, species 1 (Sp 1) of the *H. intestinalis* sponge type consists of one isolate categorized as Gram-positive bacteria; the other eight isolates are categorized as Gram-negative bacteria. In identifying Gram-positive and Gram-negative bacteria, the two sponge species, namely *T. cylindrica* and *H. intestinalis*, also show the form of bacteria present. In Sp 1 of the sponge species *T. cylindrica*, there were six isolates of *Coccus*-shaped bacteria, including Sp 4 10-6 c1,

Sp 4 10-6 D, Sp 4 10-6 A3, Sp 4 10-6 D, and Sp 4 10-5 A. Meanwhile, there were four isolates of *Bacillus*-shaped bacteria, including Sp4 10-6 A2, Sp 410-6 C2, Sp 4 10-6 B, and Sp 4 10-6 B. Meanwhile, in sp 4 *H. intestinalis*, there were five isolates of *Coccus*-shaped bacteria, including Sp 1 10-5, Sp1 10-6 B, Sp 1 10-6 A, Sp1 10-5 B, Sp1 10-5 A, and Sp1 10-6 E. Meanwhile, there were four isolates of *Bacillus*-shaped bacteria, including Sp1 10-6 A1, Sp1 10-5 Aq, Sp1 10-5A, and Sp1 10-6 Aq.

Antagonistic activity of sponge symbiont bacteria against pathogenic bacteria 19 isolates of sponge symbiont bacteria were tested against the pathogenic bacteria, namely *Staphylococcus aureus*. There were 10 isolates of bacteria that had antibacterial activity against *S. aureus*. In this study, the isolates of sponge symbiont bacteria that were successfully isolated had an inhibition range of 10.28 -1.08 mm (Table 1). In this study, two isolates were classified as potent inhibitors, namely isolate Sp4 10-6 B with an apparent zone diameter of 10.28 mm, which was categorized as potent inhibition and Sp4 10-6 Bq with an apparent zone diameter of 10 mm, which was categorized as potent inhibition. Both isolates were isolated from the marine sponge *H. Intestinalis* (Figure 1).



Figure 1. Antagonistic activity of sponge symbiont bacterial isolates against the pathogenic bacteria *Staphylococcus aureus*

Table 1. Inhibitory activity of ten isolates of sponge symbiotic bacteria against pathogenic bacteria *Staphylococcus aureus*

Species name	Isolate code	Inhibition Zone \pm SE	Inhibition category
<i>H. intestinalis</i>	Sp 4 10 ⁻⁶ (B)	10.28 \pm 0.37 cd	Strong
<i>H. intestinalis</i>	Sp 4 10 ⁻⁶ (Bq)	10.00 \pm 0.20 cd	Strong
<i>H. intestinalis</i>	Sp 4 10 ⁻⁵ (A)	6.84 \pm 0.68 bc	Medium
<i>H. intestinalis</i>	Sp 4 10 ⁻⁶ (A1)	4.84 \pm 0.58 bc	Weak
<i>H. intestinalis</i>	Sp 4 10 ⁻⁶ (C1)	3.28 \pm 0.50 bc	Weak
<i>H. intestinalis</i>	Sp 4 10 ⁻⁶ (D)	2.38 \pm 0.48 ab	Weak
<i>H. intestinalis</i>	Sp 4 10 ⁻⁶ (C)	2.22 \pm 0.24 ab	Weak
<i>T. cylindrical</i>	Sp 1 10 ⁻⁶ (E)	2.11 \pm 0.76 ab	Weak
<i>H. intestinalis</i>	Sp 4 10 ⁻⁶ (A3)	2.01 \pm 0.44 ab	Weak
<i>T. cylindrical</i>	Sp 1 10 ⁻⁶ (A2)	1.08 \pm 0.44 ab	Weak

The Best Activity of Sponge Symbiont Bacterial Isolate Extract Culture Filtrate

The results of the sponge symbiont bacterial culture filtrate Sp4 10-6 B using n-hexane solvent resulted in a yield of 0.89%, while the extraction using ethyl acetate

solvent produced a smaller yield of 0.70%. The presence of culture filtrate from sponge symbiont bacteria Sp4 10-6 B inhibited the growth of *S. aureus* bacteria with an apparent diameter zone of 8.90 mm (Table 2). The ethyl acetate extract Sp4 10-6 has a minimum inhibitory concentration value of 0.5%, which is more active than the n-hexane extract with a minimum inhibitory concentration of 15% (Table 2).

Table 2. Antibacterial activity of *Staphylococcus aureus* filtrate and crude extract of isolate Sp4 10-6 B

Sample	Mean (mm) ± SE	Minimum inhibitory concentration (MIC) %
Culture filtrate	8.90 ± 1.20c	-
n-hexane extract	6.40 ± 0.33ab	16.00
Ethyl acetate extract	7.60 ± 0.88b	0.50
Positive control	7.40 ± 0.44 bc	1.00
Negative control	0.00	-

There are differences in the results of staining of various types of sponge bacteria that have been successfully isolated, which can be caused by differences in response to the gram staining mechanism in bacteria, which is based on the structure and composition of the bacterial cell wall. Gram-positive bacteria contain protein, and gram-negative bacteria contain fat in a higher percentage. The structure of the cell wall also affects the colour of Gram-negative bacteria. The walls of Gram-negative bacteria have a high lipid content compared to Gram-positive bacteria. Gram-negative bacteria have three layers of cell walls. The outermost layer, namely lipopolysaccharide (lipid), is likely to be washed away by alcohol so that when stained with safranin, it will be red. Lugol iodine causes a bond between crystal violet and iodine, which increases the affinity of the dye by bacteria. Absolute ethanol person causes the formation of pores in Gram-negative which have many layers of fat (lipid is soluble in ethanol) so that the crystal violet iodine complex remains attached to the cell wall, and Gram-negative cells become clear (Fitriani & Herdiansyah, 2016).

The mechanism of Gram staining using crystal violet dye cannot attach to gram-negative bacteria. This also causes Gram-negative bacteria to be able to bind the red colour of safranin. The function of safranin here is only as a differentiator (contrast) to crystal violet dye. In addition, Gram staining requires four reagents: the primary dye (crystal violet), Lugol, alcohol, and counter dye (fuh sin). Lugol's solution functions to intensify the primary colour. Alcohol functions to remove the leading dye. In addition, the function of alcohol can also cause lipid extraction, which can increase the permeability of the cell wall. This increase in permeability can cause fuchsin to enter the cell, which causes the cell to turn red in Gram-negative bacteria. In contrast, the Gram-positive cell wall is hydrated by alcohol so that the pores shrink and reduce the cell wall's and membrane's permeability so that safranin cannot enter, which makes the cell purple (Sari et al., 2019).

The presence of a clear zone indicates positive antibacterial activity against test bacteria. Clear zones of ten, called inhibition zones, are formed due to several factors, such as the production of antibiotics, hydrogen peroxide, lysosomes, siderophores, proteases and other enzymes. Bacteriocins can be affected by the pH of the media due to the formation of specific organic acids. Several factors, including the composition of the culture medium, the incubation process, the rate of agar diffusion and the organism's sensitivity, cause the low inhibition power. The difference in the diameter of the inhibition zone produced by each isolate against the test fungus

indicates differences in the secondary metabolite compounds produced by each isolate (Sari et al., 2019). The antagonistic activity of sponge symbiont bacteria in producing clear zones indicates that the sponge symbiont bacteria contain metabolite compounds that can inhibit the growth of pathogenic bacteria. In this antagonistic test, an inhibition zone or clear zone is produced that inhibits the growth of pathogenic bacteria.

Ethyl acetate extract is even better than the positive control using chloramphenicol with a greater minimum inhibitory concentration of 1.0%. This shows that the active ingredient Sp4 10-6 is semipolar. Then, the bacterial isolate culture Sp4 10-6 extract will be used for further GCMS testing. The culture filtrate of the sponge symbiont bacterial isolate Sp4 10-6 B inhibited the pathogenic bacteria *S.aureus* with a diameter of 8.90 mm. The inhibition value of this culture filtrate was higher than the positive control value of chloramphenicol, which was 7.40 mm. Chloramphenicol is the most widely used broad-spectrum antibiotic drug and has properties that can inhibit the growth of pathogenic bacteria by inhibiting the formation of peptide bonds. While the negative control used was 10% DMSO, this negative control did not produce inhibition (Miso et al., 2017).

All sponge symbiont bacterial isolates Sp4 10-6 B extracts can inhibit the growth of pathogenic bacteria *S.aureu* with different inhibition values. Ethyl acetate solvent produces excellent inhibition values for pathogenic bacteria *S.aureu*. Ethyl acetate in this study is a solvent that can extract the most active metabolite compounds from the Sp4 10-6 culture filtrate. Ethyl acetate is the most widely used solvent for extracting secondary metabolites because its semipolar nature allows it to bind more diverse chemical compounds. Ethyl acetate is the most efficient solvent for secondary metabolites (Mohan et al., 2016). This study also showed that ethyl acetate in the extract of the sponge symbiont bacterial isolate Sp4 10-6 B was able to produce a higher extract than its n-hexane extract.

The results of the GC-MS analysis showed ten compounds reported to have antibacterial and antifungal activity. These compounds come from the saturated hydrocarbon, fatty acid, and aliphatic aldehyde groups. Eight of the compounds were reported as antibacterial compounds, namely Tetradecane, 3-tetradecane, Pentadecane, Nanodecane, n-Hexadecanoic acid, Bis (2-ethylhexyl) phthalate, Hexacosane, 2-Methyltetracosane, 1-Hexacosene (Francois et al., 2020; Kamiyama et al., 2013; Silva & Wansapala, 2016). These compounds are known to have antibacterial activity. GC-MS analysis of the isolate of Sponge Symbiont Bacteria Sp4 10-6 B showed the presence of various compounds with different concentrations. The higher the peak, the higher the concentration and there are eight compounds among them reported as antibacterial compounds: Tetradecane, 3-tetradecane, Pentadecane, Nanodecane, n-hexadecanoic acid, Bis (2-ethylhexyl) phthalate, Hexacosane, 2-Methyltetracosane, 1-Hexacosene (Li et al., 2020).

The presence of compounds identified at the peak also shows a difference in retention time. This retention time shows how long a compound needs to be identified. The Tetradecane compound identified from the Sp4 10-6 B ethyl acetate extract has a retention time of 10,502 minutes, with the highest peak area of the eight other compounds with antibacterial properties of 1.38%. This shows that the compound concentration is higher than the other compounds. This compound is included in the aliphatic hydrocarbon compound group and is included in the alkane group. The mechanism of this compound in killing bacteria is by entering aliphatic and aromatic hydrocarbon compounds into bacterial cells. This can also be achieved by increasing the compound's bioavailability through the solubilization and emulsification process. So that, it will damage the bacterial cell wall. This working mechanism will damage the

structure and cell membrane so that it can act against the pathogenic bacteria *S. aureus* (Tarazona et al., 2022).

CONCLUSION

Based on the research result, it can be concluded that (1) Nineteen isolates of sponge symbiont bacteria were successfully isolated from sponge species *Theonella Cylindrica* and *Hyattella intestinalis* in lemukutan waters. Seven isolates of sponge symbiont bacteria are included in the Gram-positive bacteria. While the other twelve are included in the Gram-negative bacteria. (2) Ten of the nineteen isolates of sponge symbiont bacteria have antibacterial activity against *S. aureus*. Isolate Sp4 10-6 B based on the results of the antagonistic test, which has an inhibition diameter of 10.28 mm and is categorized as potent inhibition. Ethyl acetate extract of Sp4 10-6 culture filtrate has a minimum inhibitory concentration value of 0.5% against pathogenic bacteria *S. aureus*. (3) The active compound that has the potential to develop antibiotics is the Tetradecane compound, which has the highest peak area of the eight other types of compounds. This shows that the concentration of the compound contained is higher than the other compounds. This compound belongs to the aliphatic hydrocarbon compound group and the alkane group.

RECOMMENDATION

Based on the results of the research that has been conducted, the research team recommends that further research be conducted on molecular testing, related to potential bacteria as antibiotics to determine the type of bacteria down to the genus level specifically.

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