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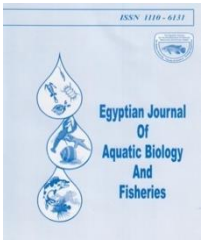

Characterization of *Bacillus altitudinis* (BSP-01) and *Bacillus thuringiensis* (BSP-06) Symbiont Associated with *Stylissa massa* Marine Sponge for Antimicrobial Agent and Multidrug Resistance Properties

Eko Efendi[1*], Maulid Wahid Yusup[2], Oktora Susanti[1], Dwi Puspita Sari[3]

[1]Department of Marine Science, Faculty of Agriculture, Universitas Lampung, Jl. Prof. Dr. Sumantri Brojonegoro No. 1 Bandar Lampung, 35145, Indonesia

[2]Department of Aquaculture, Faculty of Agriculture, Universitas Lampung. Jl. Prof. Dr. Sumantri Brojonegoro No. 1 Bandar Lampung, 35145, Indonesia

[3]Department of Marine Science, Faculty of Agriculture, Universitas Lampung, Jl. Prof. Dr. Sumantri Brojonegoro No. 1 Bandar Lampung, 35145, Indonesia

Corresponding Author: eko.efendi@fp.unila.ac.id

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ABSTRACT

The problem of antibiotic resistance has driven research to seek for new antibiotics, including those derived from the sea. Bacteria highly resistant to antibiotics include *Staphylococcus aureus* (S. *aureus*) and *Escherichia coli* (*E. coli).* Sponges have the potential to produce active compounds as raw materials for drugs. The main constraint in the final development of the potential of sponges is the issue of supply. Excessive use of sponges to search for new bioactive substances can lead to less conservative actions that may harm the ecological system of marine biota. An alternative approach is to explore the symbiotic bacteria of sponges to discover new antibiotic agents. The research aimed to screen and identify symbiotic bacteria in sponges as antibacterial agents. The study was conducted from September 2022 to September 2023 in the Oceanography Laboratory. The analyzed sponge samples belong to the *Stylissa massa* species. Based on the research, potential symbiotic bacterial isolates from *Stylissa massa* sponges were identified as BSP-01 and BSP-06. BSP-01 showed antibacterial activity in preliminary tests against *Staphylococcus aureus* strain MDR and *Escherichia coli* strain MDR with inhibition zones of 4.74 and 4.02mm against *S. aureus* strain MDR and E. *coli* strain MDR, respectively. BSP-06 exhibited inhibition zones of 5.82mm against *S. aureus* strain MDR and 4.6mm against *E. coli* strain MDR. Molecular identification revealed that the potential symbiotic bacteria from *Stylissa massa* sponges were *Bacillus altitudinis* (BSP-01) and *Bacillus thuringiensis* (BSP-06). Further research is needed to characterize the active compounds in the potential symbiotic bacteria to develop natural products that the community can utilize.

INTRODUCTION

Antibiotics are the primary treatment for bacterial infections. However, the growing resistance of pathogenic bacteria to these drugs has become a major public health concern

(Kabir *et al.*, 2024). This resistance reduces treatment effectiveness and increases the risk of severe illness and death from bacterial infection. Some bacteria commonly resistant to antibiotics include *Escherichia coli* and *Staphylococcus aureus* **(Nataraj *et al.*, 2024)**. *E. coli* is naturally found in the intestines of humans and animals **(Ortiz *et al.*, 2023)**. However, certain strains are pathogenic and can cause diarrheal and other gastrointestinal infections. These harmful strains can be transmitted through contaminated food, water, or direct contact with infected individuals or animals **(Hasani *et al.*, 2021; Zuo *et al.*, 2023)**. Studies have shown that *E. coli* exhibits resistance to several antibiotics, including ampicillin (34%), cotrimoxazole (29%), and chloramphenicol (25%) **(Jamali *et al.*, 2024)**.

In addition, *S. aureus* is known to cause nosocomial infections and food poisoning **(Aguiar *et al.*, 2024)**. Nosocomial infections occur in the hospital environment and are often difficult to treat due to antibiotic resistance. Hospitals have addressed various bacteria, such as *Staphylococcus aureus, Pseudomonas* sp., and *Klebsiella* sp. **(Devi *et al.*, 2024)**. The resistance of *S. aureus* to several antibiotics, including cephalosporins, penicillins, and carbapenems, is a serious challenge in the medical world **(Kemalaputri *et al.*, 2017)**. The irrational use of antibiotics is the main factor driving this resistance **(Camcioglu *et al.*, 2020)**. Therefore, research to find new antibiotics is needed, one of which is by looking for sources of antibiotics from the sea.

Marine sponges are a promising natural source for antibiotic development **(Beesoo *et al.*, 2023)**. They are known to produce a variety of bioactive compounds with antimicrobial, antitumor, antiviral, and antimalarial properties. Extensive research on extracting these compounds has demonstrated their potential for drug development **(Schram *et al.*, 2019; Thakur *et al.*, 2023)**. However, excessive harvesting of sponges could harm marine ecosystems since they grow slowly and have limited biomass. A more sustainable approach to exploring bioactive compounds from the sea is to utilize bacteria that live symbiotically with sponges **(Maslin *et al.*, 2021)**. These bacteria produce bioactive secondary metabolites and can be cultured in laboratories, reducing the need to harvest sponges from their natural habitats **(Fahmy *et al.*, 2021)**. Marine microorganisms, including sponge-associated bacteria, have been shown to produce a variety of antibacterial compounds **(Kiran *et al.*, 2018)**. These bacteria often form symbiotic relationships with marine organisms like sponges and corals, allowing them to thrive in nutrient-poor environments **(Kushwaha *et al.*, 2024)**.

Several previous studies have demonstrated that marine symbiotic bacteria exhibit significant antibacterial activity. **Susanti *et al.* (2021)** found that endophytic bacteria from the seagrass *Enhalus* sp. in Lampung waters also displayed antimicrobial properties. These studies suggest that the biota in Lampung waters possess the potential to produce antibacterial compounds from secondary metabolites **(Hasani *et al.*, 2022)**. Based on these findings, further research is necessary to investigate the advance of sponge-associated bacteria in producing antibacterial compounds against multi-drug resistant (MDR) bacteria. This study aimed to identify sponge symbiont bacteria to test the antibacterial

activity and molecularly identify sponge symbionts with antibacterial potential against MDR bacteria. This study is expected to contribute to advancing more effective and sustainable antibiotics.

MATERIALS AND METHODS

Time and locations

This research was conducted from September 2, 2022 to September 7, 2023 at the Oceanography Laboratory, Department of Fisheries and Marine Science and the Agricultural Biotechnology Laboratory, Faculty of Agriculture, University of Lampung. Sponge sampling was conducted in 2022 in the waters of Pahawang Island.

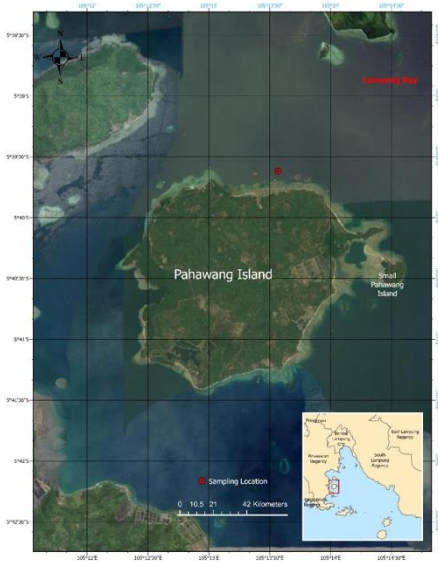

Fig. 1. Location of *S. massa* sampling

Materials

The equipments in this study included an autoclave (Hirayama), laminar airflow (Kojair), vortex (Maxi Max II), water bath shaker, measuring cup (Pyrex), Erlenmeyer (Pyrex), test tube (Pyrex), thermometer, micropipette (Thermoscientific), dropper pipette, micro tube, cool box, magnetic stirrer, hot plate, analytical balance, bunsen, tweezers, cotton, aluminum foil. The materials used in the study are *Stylissa massa* sponge, bacterial isolates of symbiont associate that have been screened and characterized morphologically, biochemically, and molecularly in studies that were isolated from *Styissa massa* sponge in South Lampung waters, NaCl, Nutrient agar (NA) (HIMEDIA), Nutrient broth (NB) (HIMEDIA), Zobell marine agar (Himedia), Zobell marine broth (Himedia), methanol, distilled water, chloramphenicol, and Nystatin 100.000 IU.

Methods

1. Sampling and isolation procedure

According to **Trianto *et al*. (2019)**, sponge samples were taken from the Pahawang Island waters, Punduh Pidada District, and Pesawaran Regency. The procedure for taking samples is as follows: sponges are taken by *skin-diving* at 2-5 meters depth into waters with coral reefs. The samples were then cleaned using sterile seawater, put into zip-lock plastic, stored in a cool box, and taken to the laboratory for isolation.

The first procedure for isolation was rinsing the sponge with sterile seawater, then chopping it into small pieces weighing 1 gram, and then crushing it with a mortar. Next, sterile seawater was added to the test tubes. The first test tube was filled with 10mL, and the 2^{nd} to 7^{th} test tubes were filled with 9mL of seawater. This isolation used dilution of 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. A sterilized marine Zobell agar medium was prepared in Petri dishes to support the growth of symbiotic bacteria. Then, the medium was put into the first test tube (100) containing 10mL of sterile seawater and dissolved. Dilutions were made from 10^{-1} to 10^{-6}. 0.1mL from dilution tubes 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} was collected and inoculated on marine Zobell agar media in ± 15 ml Petri dishes aseptically. Each dilution level was inoculated with 2x repetitions. After completion, the dishes were incubated at 37ºC in an incubator for 24-48 hours.

2. Purification and refreshment of isolates

The bacterial colonies obtained from the isolation process were purified by quadrant streaking on fresh Zobell agar medium in a Petri dish. The Petri dishes were stored in a room-temperature incubator for 24 hours for bacterial growth and colony formation. Isolates of single colonies were purified, collected in tilted Z*obell* media agar tubes and coded based on the sample. Isolates were stored in an incubator for identification and quantitative selection. Pure bacteria were stored at low temperatures below 10°C for more than 24 hours. For long-term storage of bacteria (up to 60 days), they were stored at temperatures between -20 and -70˚C with the addition of DMSO (*dimethylsulfoxide*).

3. Antibacterial assay

Antibacterial activity was screened by disc diffusion assay **(Badger *et al*., 2019)**. The first step was 100µL of *E. coli* strain MDR and *S. aureus* strain MDR bacteria in log phase (10^{9} cells/mL) were spread on NA agar media and homogenized using a cotton swab. Then, 30µL of sponge symbiont bacteria cultured on liquid marine zobell medium was taken and inoculated onto paper discs (8mm; Advantec, Toyo Roshi, Ltd, Japan). The disc paper containing the strain of symbiont bacteria was placed on the surface of the Zobell 2216E agar medium, which had been pre-inoculated with MDR strains of *E. coli* and *S. aureus*. The test was repeated three times for each bacterium. Petri dishes were then incubated at room temperature for 2x24 hours. Antibacterial activity was examined at 24 and 48 hours. The antibacterial activity was present by the inhibition zone around the disc

paper. The inhibition zone was measured using a caliper ("triple repetition"; vertical, horizontal, and diagonal), and the results were logged. The antibacterial activity of symbiont isolates can be classified based on the diameter of the inhibition zone according to the **Clinical and Laboratory Standards Institute (2018)**, as shown in Table (1).

Table 1. Category of inhibition zone

Diameter on the inhibition zone	Category
≤ 14 mm	Resistant
15 – 19 mm	Intermediate
≥ 20 mm	Susceptible

4. Morphological identification and gram test

Morphological identification and Gram staining were conducted on bacterial isolates with inhibitory activity to determine whether they are Gram-positive or Gram-negative **(Dash & Payyappilli, 2016)**. The morphological identification process began by collecting a loopful of bacterial isolates from slant media and spreading them onto a glass slide. The sample was then heat-fixed using a Bunsen burner. Finally, the bacteria were examined under a microscope at 400x magnification, and their morphological characteristics are documented and identified.

The Gram test uses both the Gram staining method and the KOH string test. In the Gram staining method, a loopful of bacterial culture was placed on a glass slide and heat-fixed using a Bunsen burner. Crystal violet solution was applied to the heat-fixed smear, left for one minute, and then rinsed with distilled water. Lugol's iodine solution was added to enhance staining, followed by decolorization with 96% alcohol for 30 seconds and another rinse with distilled water. Safranin solution was then applied as a counterstain for one minute, followed by a final rinse with distilled water. The slide was examined under a microscope to determine the Gram reaction based on color: Gram-positive bacteria appear purple, while Gram-negative bacteria appear red.

For the KOH string test, a drop of 3% KOH solution was placed onto a glass slide. A loopful of bacterial culture was mixed into the KOH drop and gently stirred. If the mixture forms a viscous, stringy consistency, the bacteria are Gram-negative. If no mucus was observed, the bacteria are Gram-positive.

These tests help differentiate bacterial cell wall characteristics, aiding in classification and further analysis.

5. Molecular identification of potential symbiont bacteria

Molecular identification was done by extracting DNA from bacterial isolates, PCR, and sequencing. The DNA extraction process started from bacterial isolates cultured in liquid marine *zobell* media in a test tube with a media volume of 10ml and shaker for 1x24 hours. Next, the bacteria from the tube were transferred to a 1.5mL microtube and centrifuged for one minute using a micro-centrifuge at 14,000 rpm. This process was

repeated until all the bacteria had been collected. After completion of centrifugation, the remaining liquid media was removed using a micropipette. Bacteria in the *microtube* were added with 567µL of TE solution and then homogenized using a *vortex*. Next, 30µL of 10% SDS solution and 3µL of proteinase K were added and mixed thoroughly. The samples were then incubated in a mini-incubator at 37^{o}C for one hour.

The incubated sample was then mixed with 100µL of 5M NaCl and homogenized. Next, 80µL of CTAB solution was added, and the mixture was incubated for 10 minutes at 65°C. After incubation, 720µL of isoamyl chloroform solution was added, the mixture was thoroughly homogenized, and centrifuged at 14,000 rpm for 5 minutes.

From the centrifuged sample, 500µL of the supernatant was collected using a micropipette in 100µL increments and transferred to a new microtube. An equal volume (1:1 ratio) of phenol:chloroform:isoamyl alcohol (PCI) solution was then added. The sample was mixed vigorously and centrifuged again at 14,000 rpm for 5 minutes.

Following centrifugation, 300µL of the supernatant was transferred (in 100µL increments) to a new microtube. Isopropanol was added at 60% of the supernatant volume (i.e., 180µL for 300µL), mixed thoroughly, and the sample was placed in a refrigerator for 10 minutes. It was then centrifuged at 14,000 rpm for 15 minutes. The supernatant was discarded, and 300µL of 70% ethanol was added. The sample was centrifuged again for 5 minutes. After discarding the ethanol, the pellet was air-dried for 24 hours. Finally, 20µL of TE buffer was added, and the DNA sample was stored in the refrigerator for subsequent analysis.

The next step was electrophoresis to verify the presence or absence of genomic DNA. For this, agarose gel was prepared and visualized using a UV transilluminator. The gel was made by dissolving 0.1g of agarose powder in 20 mL of TBE buffer and heating it in a microwave until fully dissolved. After cooling slightly, 1µL of ethidium bromide (EtBr) was added to the solution. The mixture was poured into a gel mold and allowed to solidify.

Once solidified, the agarose gel was placed into the electrophoresis chamber. For the loading process, 3µL of DNA ladder, 1µL of loading dye, and 3µL of DNA sample were mixed. A 3µL aliquot of this mixture was loaded into the wells of the gel, and electrophoresis was run for 45 minutes. The gel was then placed on a UV transilluminator to observe DNA bands. If DNA was detected, the sample proceeded to polymerase chain reaction (PCR) for amplification.

PCR was performed by adding 1µL of DNA sample to a 0.2mL microtube, followed by 12.5µL of red mix, 1µL each of forward and reverse primers, and 9.5µL of sterile water. The microtube was placed in a PCR machine and run for 90 minutes. After PCR completion, electrophoresis was repeated to confirm the presence of the amplified DNA fragment.

The amplified DNA was then sent to PT Genetika Science Indonesia for sequencing. Sequence data were analyzed using BioEdit and MEGA 11 software.

Sequencing results from forward and reverse primers were merged by generating the reverse complement of the reverse sequence. Sequence alignment was performed by comparing the obtained query sequence with existing sequences in GenBank using the BLAST (Basic Local Alignment Search Tool) search on the NCBI website (http://www.ncbi.nlm.nih.gov).

The size of the PCR-amplified fragment was determined by comparing the sample band position with that of a DNA marker. A positive result was indicated by the presence of a band at 996 base pairs (bps).

6. Extraction of potential symbiont bacteria

The extraction method used in this study is a modified version of the procedure described by **Yati (2018)**. First, symbiotic bacteria from the sponge were cultured in 500mL of nutrient broth (NB) medium and incubated in a shaker at 170 rpm for 72 hours at 37°C. The resulting culture was then centrifuged at 4,000 rpm for 20 minutes to separate the biomass **(Yati, 2018)**. The obtained biomass was extracted by maceration using 96% methanol as the solvent, in a ratio of 1:2 (biomass to solvent).

The methanol phase (upper layer) was separated by filtration using filter paper and transferred into an Erlenmeyer flask. The remaining media residue (lower layer) was subjected to further extraction. In the final step, the methanol extract was concentrated using a rotary evaporator at 35°C to obtain a thick crude extract.

This extract was then used for bioactivity testing against multidrug-resistant (MDR) pathogenic bacteria, specifically *Escherichia coli* (MDR strain) and *Staphylococcus aureus* (MDR strain), as well as for toxicity testing.

7. Toxicity test with brine shrimp lethality test (BSLT) method

The BSLT test consists of two phases: the preliminary and main tests.

7.1. Preliminary test

Preparation of containers

In the preliminary BSLT test, 18 Petri dishes were prepared for each solvent. Each dish was filled with 10mL of seawater, and the solvent and concentration were added according to the test conditions. Ten artemia were placed in each Petri dish. To ensure sterility, the Petri dishes were sanitized with ethanol to eliminate germs or bacteria.

Preparation of specimen animals

The procedure for hatching artemia eggs for BSLT testing involves an artemia hatchery containing seawater and aerators for air circulation. Eggs hatch and move actively after 24-48 hours, and the 24-hour age of the artemia is used in the BSLT test.

Preparation of stock solution

The stock solution is made using the results of the sponge symbiont bacteria extract that has been obtained. The stock solution is made by diluting the extract with its solvent to the desired concentration. The stock solution to be made is 10,000 ppm, so to make the stock solution, the amount of solvent and extract used is 1g/ 100ml.

Ten artemias were put into a Petri dish determined for each treatment. The concentration for each treatment uses the logarithmic formula: 0.1, 1, 10, 100, and 1000 ppm, with each amount of water being 10ml. The Petri dishes were then placed under lighting, and after 12 hours, the number of dead artemia larvae was counted. The standard criterion for assessing the death of artemia larvae is when the larvae show no movement during the observation time. Preliminary tests were conducted to determine the upper and lower thresholds in the BSLT test. The upper threshold concentration is the lowest concentration of test material that can cause all test animals to die at 24 hours of exposure time. The lower threshold concentration refers to the highest concentration of the test material that causes the complete mortality of all test animals after 48 hours of exposure.

7.2. BSLT main test

Preparation of containers

The main test utilized 18 Petri dishes per solvent, with concentrations set based on the threshold and lower limits identified in the preliminary test.

Preparation of specimen animals

Artemia eggs were hatched in a hatchery containing seawater and an aerator to ensure proper air circulation. The eggs hatch and become actively mobile within 24–48 hours. For the BSLT test, 24-hour-old *Artemia* was used.

Main test

The concentrations used in the main test are based on the upper and lower limits determined from the preliminary test. To ensure accuracy in determining the LC50-24 hours of the test material, a fixed logarithmic concentration range must be maintained to prevent toxic miscalculations. The LC_{50}-24-hour concentration interval was estimated using the **Hubert** formula **(1979)**, as referenced in **Yunita *et al*. (2009)**. The concentration range for the toxicity test was calculated using the following formula:

$$\text{Log}\,\frac{N}{n} = \text{k}\,\log\frac{a}{n}$$

Description:
N: Upper threshold concentration
n: Lower threshold concentration
a: 1st concentration for testing

k: Number of concentrations tested (there are 5 with values a, b, c, d, and e)

The determination of the constant values of a,b,c,d, and e was done by extracting the stock solution. The equation was used to obtain the desired concentration:

$$V_1.M_1=V_2.M_2$$

Description:
V_1: Volume of stock solution to be used
M_1: Concentration
V_2: Tested volume
M_2: Concentration desired

The experiment was conducted after 24 hours in the main BSLT test. In this main test, the data taken is the mortality of the test animals in each treatment.

Analysis of data

The isolates obtained from the isolation stage were tested for their antagonistic activity to evaluate their effectiveness against the test bacteria. Isolates exhibiting activity were assessed by measuring the clear zone using the push-pull method, and the results were recorded. The most promising sponge symbiont bacterial isolates then proceeded to the extraction stage. The condensed extracts from these potential symbiont bacteria were subsequently tested for their activity in the extract activity test. The concentration demonstrating activity was measured based on the clear zone using a vernier caliper, and the results were documented. Data analysis in this study was conducted using a descriptive approach. The collected data included results from the antagonist and the extract activity test. These data were compiled, processed, and analyzed, with the standard deviation calculated using Microsoft Excel 2019 to assess the variability of inhibition zone measurements relative to the average value.

RESULTS

1. Sample of the sponge

The sponge sample used in this study was identified as *Stylissa massa*. Sampling was conducted in Pahawang Island, Marga Punduh District, Pesawaran Regency, Lampung Province. The sponge samples collected had a length of 34 cm, found in waters with a depth of approximately 7m, with environmental conditions at a temperature of 28^{o}C, salinity 33ppt, and pH 7.7. Sponge samples can be seen in Fig. (2).

Fig. 2. Sponge samples from Pahawang Island waters

2. Inventory of *Stylisa massa* symbiont bacterial isolates

The isolation procedure was conducted to obtain pure symbiont bacterial isolates from *Stylissa massa* sponge samples from Pahawang Island waters. The data of the inventory of symbiont bacterial isolates can be seen in Table (2).

Table 2. Morphology of *Stylissa massa* sponge symbiont bacterial isolates

No.	Code	Description (shape, elevation, boundaries of colonies, color of colonies)
1.	BSP-01	Rhizoid, flat, serrated, translucent white
2,	BSP-02	Circular, flat, serrated, milky white
3.	BSP-03	Circular, convex, serrated, translucent white
4.	BSP-04	Rhizoid, flat, smooth, milky white
5.	BSP-05	Circular, flat, serrated, brownish
6.	BSP-06	Rhizoid, flat, smooth, white
7.	BSP-07	Circular, flat, serrated, white
8.	BSP-08	Circuar, flat, serrated, translucent white

After sampling, the samples were identified and isolated. The samples were isolated using a 6-level dilution method (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}). Bacterial isolation was conducted by taking 0.1mL of solution from dilution 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and inoculated on a dish containing Zobell agar media with nystatin added. The provision of nystatin aims as an antifungal to prevent fungal contamination.

3. Antibacterial assay

The collection of sponge symbiont bacterial isolates was examined against the pathogenic bacteria *Staphylococcus aureus* strain MDR and *Escherichia coli* strain MDR. The activity test of sponge symbiont bacterial isolates used the disc paper method. A positive control and a negative control were used in the test. The positive control was chloramphenicol, and the negative control was liquid marine Zobell medium diffused onto the disc paper. The diameter of the inhibition zone is shown in Table (3).

Table 3. Results of antibacterial activity test of sponge symbiont bacterial isolates against pathogenic bacteria

No.	Code of isolate	Diameter of inhibition zone against pathogenic bacteria (mm)			
		S. *aureus* strain MDR		E. *coli* strain MDR	
		Inhibition zone	K+	Inhibition zone	K+
1.	BSP-01	4.74	17.36	4.02	18.94
2.	BSP-02	0		0	
3.	BSP-03	0		0	
4.	BSP-04	0		0	
5.	BSP-05	0		0	
6.	BSP-06	5.82		4.6	
7.	BSP-07	0		0	
8.	BSP-08	0		0	

After the test, two isolates with inhibition zone activity against S. *aureus* strain MDR and *E. coli* strain MDR were successfully collected. Based on the results of the antagonist test, isolates that produce clear zones are BSP-01 and BSP-06. The activity of BSP-01 isolate has inhibition zone diameter on pathogenic bacteria *S. aureus* strain MDR 4.74mm and *E. coli* strain MDR 4.02mm. Isolate BSP-06 has an inhibition zone diameter against pathogenic bacteria *S. aureus* strain MDR 5.82mm and *E. coli* strain MDR 4.6mm.

4. Morphological identification and gram test of potential symbiont bacteria

Based on the activity test of symbiont bacterial isolates, two potential bacterial isolates, *S. aureus* strain MDR and *E. coli* strain MDR, were obtained since they inhibit pathogenic bacteria. The two potential bacterial isolates were then microscopically observed and gram-tested.

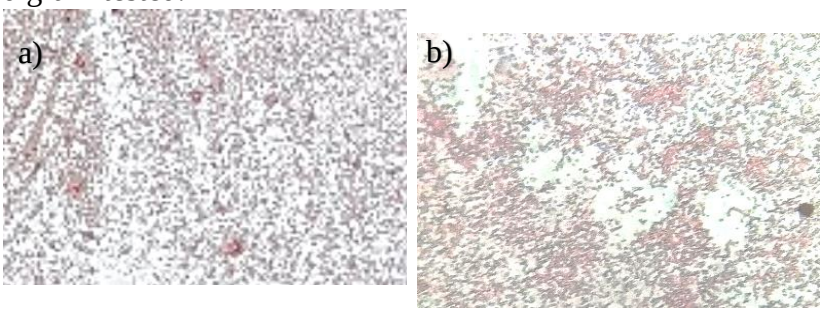

Fig. 3. Microscopic observation of bacterial isolates BSP-01 and BSP-06

Microscopic examination was done using a microscope with 10x lens magnification. The observation results can be seen in Fig. (3) for BSP-01 and BSP-06. Observations have been made to identify that the shape of the two symbionts bacterial isolates is *bacilli* with Gram-positive. Although both isolates showed crystal violet coloration, both isolates were Gram-positive.

5. Extraction and activity test of potential sponge symbiont bacteria

Potential sponge symbiont bacterial isolates were extracted using the maceration method with the methanol solvent. The results of the extract can be seen in Table (4).

Table 4. The results of extracts of potential sponge symbiont bacterial isolates

Code of isolate	Weight (gram)		Yield (%)
	Bacteria	Extract	
BSP-01	1.78	0.86	48.31
BSP-06	3.86	1.2	31.08

Bacterial isolates BSP-01 and BSP-06 were first mass-cultured using liquid marine Zobell media. The resulting cultures yielded 1.78 grams of BSP-01 and 3.86 grams of BSP-06. The harvested bacteria were then extracted through maceration using methanol as a solvent. Maceration was performed for 48 hours, with filtering conducted every 24 hours. The macerated extracts were then concentrated using a rotary vacuum evaporator. After evaporation, the final extract weights were 0.86 grams for BSP-01 and 1.2 grams for BSP-06, with extraction yields of 48.31% and 31.08%, respectively.

The extracted compounds were tested against MDR strains of *S. aureus* and *E. coli* at concentrations of 10,000, 5,000, 1,000, 100, and 10ppm. Each concentration was tested in triplicate to ensure accuracy. The results of the antibacterial activity tests are presented in Table (5).

Table 5. Antibacterial activity test of bacterial extracts

Code of isolate	Concentration (ppm)	Diameter of inhibition zone against pathogenic bacteria (mm)			
		S. *aureus* strain MDR		E.*coli* strain MDR	
		Inhibition zone	K+	Inhibition zone	K+
BSP-01	10000	9.76		7.04	
	5000	8.88		9.2	
	1000	0	18.2	0	17.38
	100	0		0	
	10	0		0	
BSP-06	10000	11.07		11.18	
	5000	9.46		8.99	
	1000	0	18.53	0	17.24
	100	0		0	
	10	0		0	

The test was performed using NA media in Petri dishes. Pathogenic bacteria were inoculated onto the medium, and extracts from potential sponge symbiont bacteria were

applied to paper disks. The disks were then positioned on NA media plates inoculated with pathogenic bacteria.

6. Brine shrimp lethality test (BSLT)

After the preliminary test, the upper threshold concentration value for the BSP-01 extract was 5000ppm, and the lower threshold concentration was 3000ppm. The results were then calculated logarithmically to get the concentration used in the main test. The concentrations of BSP-01 extract in the main test were 3319.87, 3673.84, 4065.55, 4499.02, and 4978.7ppm. The BSLT test results of the BSP-01 extract can be seen in Table (6).

Table 6. Results of brine shrimp lethality test (BSLT) of bacterial extract BSP-01

Concentration (ppm)	Log concentration	Total of test larvae	Total mortality of larvae			X ± sd	Mortality (%)	Probit Value
			1	2	3			
3319,87	3.52	10	1	1	2	1.33 ± 0.58	13.33	3.87
3673,84	3.57	10	2	3	3	2.67 ± 0.58	26.67	4.36
4065,55	3.61	10	4	4	3	3.67 ± 0.58	36.67	4.64
4499,02	3.65	10	5	7	8	6.67 ± 1.53	66.67	5.41
4978,7	3.70	10	9	9	10	9.33 ± 0.58	93.33	6.48
LC_{50} – 24h			4393,97 ppm					

A preliminary BSLT test was also conducted on the bacterial extract BSP-06. For the BSP-06 extract, the upper threshold concentration was 3000 ppm, while the lower threshold was 1000 ppm. The BSLT test results of the BSP-06 extract can be seen in Table (7).

Table 7. Results of brine shrimp lethality test (BSLT) of bacterial extract BSP-06

Concentration (ppm)	Log concentration	Total of test larvae	Total mortality of larvae			X ± sd	Mortality (%)	Probit Value
			1	2	3			
1241,65	3.09	10	2	2	1	1.67 ± 0.58	16.67	4.01
1540,88	3.19	10	3	4	3	3.33 ± 0.58	33.33	4.56
1912,22	3.28	10	4	3	5	4 ± 1	40	4.75
2372,05	3.38	10	7	6	8	7 ± 1	70	5.52
2944,93	3.47	10	10	9	10	9.67 ± 0.58	96.67	6.75
LC_{50} – 24 h			1389,5 ppm					

A logarithmic calculation of the results then yielded the concentration for the main test. The concentrations of BSP-06 extract in the main test were 1241.65, 1540.88, 1912.22, 2373.05, and 2944.93 ppm.

7. Molecular Identification of Potential Symbiont Bacteria

Molecular identification was conducted in three stages: DNA extraction, PCR, and sequencing. Electrophoresis results of the BSP-01 and BSP-06 DNA genomes after PCR are shown in Fig. (4).

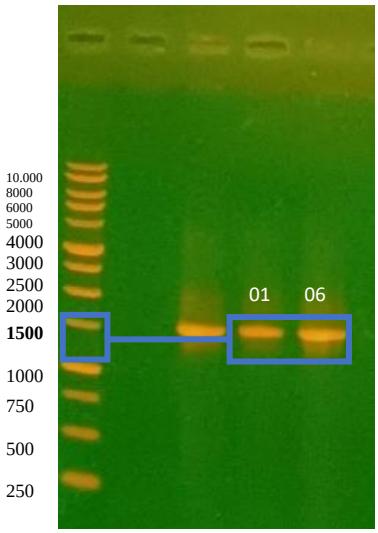

Fig 4. Results of PCR electrophoresis of BSP-01 and BSP-06 DNA

After PCR, the next step was sequencing to get DNA sequence data. DNA sequence data obtained were then processed using Bio-edit software to get DNA consensus. Next, the DNA consensus was analyzed in the gene bank with database searches on the NCBI internet site (https://www.ncbi.nlm.nih.gov/) using a *basic local alignment search tool* (BLAST). After the DNA data were analyzed, a phylogenetic tree was made using Mega 11 software, and the results are shown in Fig. (5) for BSP-01 and Fig. (6) for BSP-06.

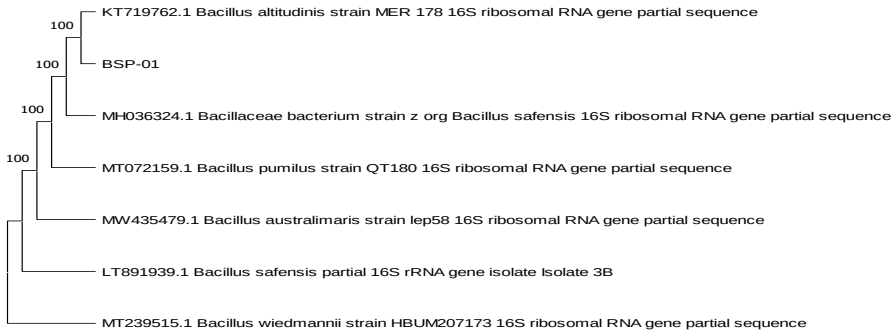

Fig. 5. Phylogenetic tree of isolate BSP-01

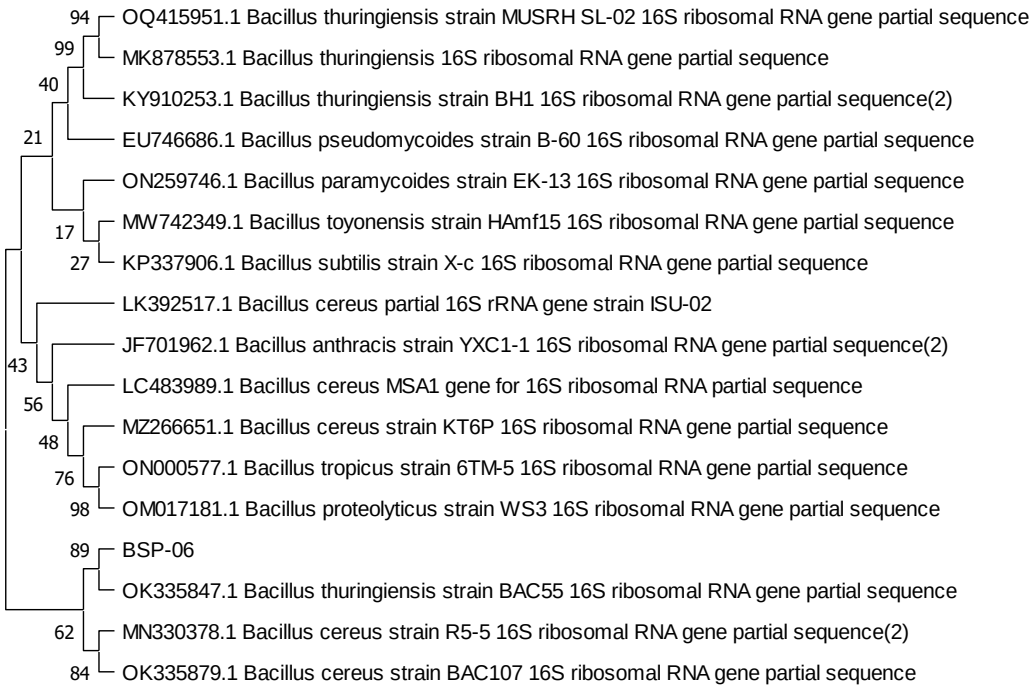

Fig. 6. Phylogenetic tree of isolate BSP-06

Phylogenetic tree analysis revealed that bacterial isolate BSP-01 is closely related to *Bacillus altitudinis* (accession number LC771232), while bacterial isolate BSP-06 is closely related to *Bacillus thuringiensis* (accession number LC79963406).

DISCUSSION

Stylissa massa **(Carter, 1889)** is a common sponge species found in various shallow marine habitats, including fringing reefs, patch reefs, lagoons, seagrass beds, and intertidal flats. This sponge is up to 75cm tall, 3-20cm wide, and 4-9cm thick. Ocula 5-10mm in diameter, prominent, membranous, but often closed and concave in appearance. The skin surface is thin with prominent spicules, a slightly fleshy texture, and soft conules. The surface appears mildly serrated or softly spiked. The spongy texture is like wet bread when pressed. When alive, it is neon orange in color, transitioning to a golden-orange shade, and releases a milky orange exudate **(Trianto *et al*., 2019)**. This species is known in regional field guides as *Stylotella aurantium* or *Stylissa aurantium*, now known as *Stylissa massa* **(Sun *et al*., 2016)**. Morphologically, these sponges vary in shape, including branching or massive forms with smooth to slightly rough surfaces. The color of these sponges is generally orange to brownish yellow. The specific size and shape may differ depending on the environmental conditions in which it grows. The sponge *S. massa* belongs to the Low Microbial Abundance (LMA) category, meaning it has a lower density of microbes than sponges with high microbial content **(Gauvin-Bialecki *et al*., 2018)**.

However, the microbial content found in *S. massa* has the potential to inhibit the growth of *S. aureus* and *E. coli* bacteria **(Trianto *et al*., 2019)**.

After obtaining the sponge, the next step is to isolate the symbiont bacteria from *S. massa*. Samples that have been isolated are then incubated for 24 hours at 37ºC. The cultivated bacteria were then transferred to a new Zobell agar media dish for purification and re-incubation. Bacteria were then selected, coded, and identified macroscopically. The code given is BSP, followed by the sequence number of the isolate. Code B means bacteria, S means sponge, and P means Pahawang. The bacteria were transferred into a tube of Zobell agar media and labeled according to the code made. The small number of isolates of sponge symbiont bacteria can be caused by the lack of salinity in the media that affects the growth of sponge symbiont bacteria, which are halophilic **(Nurama *et al*., 2023)**. Halophilic bacteria are one of the extremophile microorganisms that can grow optimally in environments with high salt content conditions. Halophilic microorganisms require these conditions to grow optimally **(Kusuma *et al*., 2024)**. Halophilic bacteria have the advantage of reducing the risk of contaminants with their ability to development in high salinity **(Wang *et al*., 2023)**. This advantage gives halophilic bacteria significant potential for further applications. Symbiont bacteria are a type of bacteria that can produce secondary metabolite compounds similar to the host. Secondary metabolites are obtained by isolating symbiont bacteria **(Leon *et al*., 2023)**. Bacterial isolates that are symbiotic with sponges have morphological characteristics by showing *rhizoid*, *circular*, and *irregular* shapes with flat or regular edges. Sponge symbiont bacteria have morphological characteristics of dominant white and brown colors **(Cita *et al*., 2017)**. This is due to the density and growth density of bacterial cells and the availability of nutrients in the media **(Subekti *et al*., 2019; Bastías *et al*., 2023)**. The results of the inventory of symbiont bacterial isolates were used for antibacterial testing against the pathogenic bacteria *Staphylococcus aureus* MDR strain and *Escherichia coli* MDR strain.

Based on the results of antibacterial tests, isolates that produce clear zones are BSP-01 and BSP-06. The activity of BSP-01 isolate has inhibition zone diameter on pathogenic bacteria S. *aureus* strain MDR 4.74mm and E. *coli* strain MDR 4.02mm. Isolate BSP-06 has a diameter of inhibition zone against pathogenic bacteria S. *aureus* strain MDR 5.82mm and E. *coli* strain MDR 4.6mm. Based on these results, the inhibition zones of BSP-01 and BSP-06 isolates against pathogenic bacteria *S. aureus* strain MDR and *E. coli* strain MDR are included in the weak category. The clear zone in the agar layer is formed because the antibacterial compound diffuses into the layer and inhibits the growth of microorganisms, namely the test bacteria. The agar layer will become overgrown with test bacteria. As a result, it will appear cloudy **(Yudha *et al*., 2024)**. The inhibitory ability of each isolate is different due to the different metabolite content of each isolate **(Agustin *et al*., 2021)**. The inhibition zone formed is also influenced by several factors such as the number of microorganisms used, the diffusion of antimicrobial ingredients into the media and their interaction with the test microbes, the growth rate of the tested microorganisms,

and their sensitivity to the tested material **(Amiin *et al*., 2023)**. After the results of the symbiont bacterial isolate activity test on two pathogenic bacteria, the next stage is to perform Gram staining on bacterial isolates exhibiting antibacterial activity. In this study, there were two isolates, BSP 01 and BSP 06, which had antibacterial properties against pathogenic bacteria.

Observations indicate that both symbiont bacterial isolates are bacillus-shaped and Gram-positive. This is because both isolates retain the crystal violet. Gram-positive bacteria are purple because they maintain the color of the crystal violet substance even though they have been given an alcohol solution. Gram-negative bacteria appear red because the crystal violet dye complex dissolves when exposed to alcohol. As a result, they absorb the red color of safranin **(Atac *et al*., 2024)**. Gram-positive bacteria can retain crystal violet dye because their cell walls have a thick peptidoglycan content **(Savadori *et al*., 2023)**. In Gram-positive bacteria, up to 90% of the cell wall is peptidoglycan, containing an acidic component known as teichoic acid **(Han *et al*., 2024)**. Wall teichoic acid is the primary of the Gram-positive cell wall and is therefore critical for cell wall integrity **(Van der Es *et al*., 2016)**. One of the well-known genera in this group is *Bacillus*. *Bacillus* bacteria are aerobic or facultative anaerobes **(Herrmann *et al*., 2023)**. These endospores allow the bacteria to survive unfavorable environmental conditions, such as nutrient deprivation or exposure to extreme temperatures. Gram-positive bacilli are essential in various ecosystems and have applications in industry, agriculture, and human health **(Liu *et al*., 2024)**.

The next step after identifying morphologically and gram staining test is to extract the two samples of symbiont bacterial isolates and then test the activity of the two isolates to two pathogenic bacteria. The yield of extracts from each isolate was 48.31% for BSP-01 and 31.08% for BSP-06. The amount of extraction yield is influenced by several factors, including the time used during the maceration process. The longer the time used in the maceration process, the more yield. Extended contact between the sample and solvent improves their interaction, allowing for the extraction of more secondary metabolite compounds **(Lotos *et al*., 2024)**. In addition to time, temperature also influences the quantity of extract yield produced. The choice of temperature must consider the condition of the sample so that there is no evaporation of volatile compounds due to high temperatures. The optimal temperature for the maceration process is room temperature **(Wanderley *et al*., 2024)**. The extracts that have been obtained are then tested against pathogenic bacteria *S. aureus* strain MDR and *E. coli* strain MDR with concentrations of 10,000, 5000, 1000, 100, and 10 ppm. The extract activity test was carried out with triplicate repetition of each concentration. The test was conducted using NA media in a petri dish. Pathogenic bacteria were inoculated into the test media. Extracts of potential sponge symbiont bacteria were inoculated onto paper disks, then the paper disks were placed on NA media cups that had been inoculated with pathogenic bacteria.

The test results on the BSP-01 extract against *S. aureus* strain MDR bacteria had an inhibition zone of 9.76mm at 10000 ppm and 8.8mm at 5000 ppm. The zone of inhibition against *E. coli* strain MDR bacteria was 7.04mm at a concentration of 10000 ppm and 9.2mm at a concentration of 5000 ppm. According to the **Clinical and Laboratory Standards Institute (CLSI) (2018)**, the diameter of the inhibition zone ≤ 14 mm has weak inhibition, 15- 19mm has moderate inhibition, and ≥ 20 has strong inhibition. Based on this, the inhibition zone successfully observed was included in the weak category. The test results of BSP-06 extract against *S. aureus* strain MDR bacteria at a concentration of 10000 ppm had an inhibition zone of 11.07mm, and at a concentration of 5000 ppm had an inhibition zone of 9.46mm. The inhibition zone against *E. coli* strain MDR bacteria at an extract concentration of 10000 ppm was 11.18mm, and at a concentration of 5000 ppm, the inhibition zone was 8.99mm. Based on the inhibition zone category according to the **CLSI (2018)**, the diameter of the inhibition zone of the BSP-06 bacterial extract at a concentration of 10000 is included in the weak category.

Several factors influence antibacterial activity, including extract concentration, antibacterial compound content, extract diffusion power, the type of bacteria being inhibited, diffusion speed, the properties of the agar medium, the number of inoculated organisms, bacterial growth rate, chemical concentration, and incubation conditions **(Mosallaie *et al.*, 2024)**. Gram-positive and Gram-negative bacteria exhibit varying sensitivity to antibacterial agents **(Fu *et al.*, 2024)**. The difference in cell wall structure between Gram-positive and Gram-negative bacteria determines the penetration, binding, and activity of antibacterial compounds **(Tansil *et al.*, 2016)**. Gram-positive bacteria have a cell wall structure with a thicker peptidoglycan arrangement, while Gram-negative bacteria have more lipid content than the peptidoglycan layer **(Savadori *et al.*, 2023)**. This study uses *S. aureus* strain MDR as a representative of Gram-positive bacteria and *E. coli* strain MDR as a representative of Gram-negative bacteria. The results showed that both the isolate antagonist and the extract test produced a larger inhibition zone against the MDR strain of *S. aureus* than to the MDR strain of *E. coli*.

The next step is the Brine Shrimp Lethality Test (BSLT). The Brine Shrimp Lethality Test begins with the hatching of *Artemia salina* larvae.. Larvae that hatch within 1x24 hours will be used for the test. The Brine Shrimp Lethality Test (BSLT) consists of two phases: an initial preliminary test followed by the main test. The preliminary test is performed to establish the upper and lower concentration thresholds. The upper threshold is the lowest concentration that causes all test animals to die at 24 hours exposure. The lower threshold is the highest concentration at which all test animals remain alive. This is observed after 48 hours of exposure. The concentrations of BSP-01 extract in the main test were 3319,87, 3673,84, 4065,55, 4499,02, and 4978,7 ppm. The test results were analyzed using the regression equation to determine the LC_{50} value at 24 hours. After calculation, the LC_{50} - 24 hours value was obtained at a concentration of 4393.97 ppm. According to **Meyer *et al.* (1982)**, the distribution of toxicity based on the LC50 value is; namely, the

concentration of <10 ppm is categorized as very strong, the concentration of 10 - 100 ppm is categorized as strong, the concentration of 100 - 500 ppm is classified as moderate, and the concentration of 500 - 1000 ppm is weak. Based on the LC_{50} value of the BSP-01 extract, it is considered non-toxic as it does not fall within the toxicity distribution.

As for the BSP-06 isolate sample, the concentrations used in the main test were 1241.65, 1540.88, 1912.22, 2373.05, and 2944.93 ppm. The test results were analyzed using the regression equation to determine the 24-hour LC_{50} value. After calculation, the LC_{50} - 24 hours value was obtained at a concentration of 1389.5 ppm. The LC50 value of BSP-01 extract is still classified as non-toxic because the LC50 price is >1000 ppm. If the LC50 value is <1000 ppm, the extract has stronger toxic activity, while extracts with an LC50 value of >1000 ppm do not have harmful activity **(Jelita *et al.*, 2020)**. The mechanism of larval death is related to the function of steroid, alkaloid, flavonoid, and terpenoid compounds that can inhibit larval feeding. Toxic compounds contained in the extract can enter the mouth of A. *salina* and are absorbed into the digestive tract so that the absorption process occurs through the cell membrane. The absorption process is followed by the distribution process of toxic compounds into the body of *A. salina,* and the process of damage to metabolic reactions occurs **(Chen *et al.*, 2024)**. The anatomical structure of the A. *salina* body at the nauplius stage is harmful, consisting of the skin layer, mouth, *anthena,* digestive tract or digestion that is still simple, and prospective *thoracopods* **(Araya *et al.*, 2024)**. Drastic concentration gradient changes between the cell's interior and exterior allow toxic compounds to spread effectively throughout the body of *A. salina*. The resulting metabolic damage is rapidly detectable within 24 hours, causing 50% mortality of *A. salina* (Athulya *et al.*, 2023).

Molecular tests followed the BSLT test to confirm the bacterial species previously identified through morphology and Gram staining. Molecular identification is conducted in three stages: DNA extraction, PCR, and sequencing **(Haryanto *et al.*, 2021)**. DNA extraction is done by harvesting bacteria cultured on liquid media. Furthermore, the processed bacteria are broken down to obtain DNA. After extraction, electrophoresis is performed to determine whether DNA was successfully collected from the cells **(Subekti *et al.*, 2021)**. If the results are attained, PCR process would be the next step to increase the amount of DNA before the sequencing process.

Furthermore, electrophoresis is again carried out to see the presence or absence of DNA after the PCR process. However, the sample DNA was sent to PT Genetika Science Indonesia for DNA sequencing **(Suryani *et al.*, 2021)**. Based on the sequencing results, bacterial isolate BSP-01 has a close kinship with *Bacillus altitudinis* with the number access LC771232. *B. altitudinis* is a rod-shaped aerobic bacterium classified in the Firmicutes phylum. It was first isolated from the air under extreme UV exposure, with samples collected from the stratosphere. Since then, *B. altitudinis* has reported diverse habitats, including the southern Indian Ocean, freshwater of Manasbal Lake, soil, and mud **(Vettath *et al.*, 2017)**. *B. altitudinis* has been used in several studies to see its benefits.

Siwach *et al*. (2024) reported that *B. altitudinis* produces xylanase enzyme and has xylanolytic activity. Xylanase is used as a biocatalyst in the food industry. For instance, xylanase is commonly used in beverages and fermented foods **(Li *et al*., 2023)**. Xylanase is also used in functional food or prebiotics with its derivative products xylose and xylooligosaccharides **(Kaur *et al*., 2024)**. Another study that examines *B. altitudinis* is the research of **Khan *et al*. (2021)**, who reported that *B. altitudinis* is an effective strain for removing Zn from the environment. **Zeng *et al*. (2021)** reported that *B. altitudinis* has potential as a biocontrol agent to control downy mildew of grapes.

Based on the analysis, bacterial isolate BSP-06 is closely related to *Bacillus thuringiensis* with access number LC79963406. *B. thuringiensis* is a Gram-positive, spore-forming bacterium that synthesizes an extraordinary diversity of insecticidal proteins and has demonstrated its potential and safety as a biocontrol agent for more than five decades **(Palma *et al*., 2014)**. Research on the potential of *B. thuringiensis* has been conducted. **Mafazah and Zulaika (2017)** reported that *B. thuringiensis* has the potential as a bioinsecticide against armyworm larvae (*Spodoptera litura*). **Pujiastuti *et al*. (2020)** reported that *B. thuringiensis* isolated in South Sumatra produced two isolates that have the potential to become active ingredients in the production of bioinsecticides that are effective in killing horned beetle larvae (*Oryctes rhinoceros*). *B. thuringiensis* is used as a potential biopesticide to control pests in agriculture due to its various insecticidal proteins **(Kumar *et al*., 2021)**. *B. thuringiensis* insecticide concentrate showed 77- 90% insecticidal efficacy against *Pieris rapae* (cabbage butterfly), *Plutella xylostella* (cabbage caterpillar), and *Heliothis virescens* (tobacco caterpillar) in field trials **(Choe *et al*., 2022)**.

CONCLUSION

Symbiont bacteria from the *Stylissa massa* sponge species were successfully isolated, yielding as many as eight isolates, two exhibited activity against the S. *aureus* strain MDR and the E. *coli* strain MDR. Isolate BSP-01 demonstrated antibacterial activity against the pathogenic S. *aureus* strain MDR and the E. *coli* strain MDR, with inhibition zones measuring 9.76 and 7.04mm, respectively, at a concentration of 10000 ppm. Isolate BSP-06 showed an inhibition zone against the S. *aureus* strain MDR of 11.07mm and against the E. *coli* strain MDR of 11.18mm, also at a concentration of 10000 ppm. The inhibition zones of these two potential bacteria were classified within the resistance categories. Through molecular identification results, it was confirmed that the two isolates share a kinship with *Bacillus altitudinis* (LC771232), presenting a similarity value of 100% for BSP-01 and *Bacillus thuringiensis* (LC79963406) with a similarity value of 89% for BSP-06. Further research is needed to characterize the active compounds in the potential symbiotic bacteria to develop natural products that the community can utilize. This research was funded by Higher Education for Technology and Innovation (HETI), Universitas Lampung, Indonesia, through a 2023 research grant under the Scheme of

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