Antimicrobial Activity of Endosymbiont Bacteria Isolated from Marine Sponges Collected from Kotok Kecil Island, Jakarta

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Abstract

Marine sponges-associated bacteria are among the wealthiest sources of pharmacologically active chemicals from marine organisms. Many species of endosymbiont bacteria isolated from marine sponges had been studied and showed significant bioactivity. Indonesia is very rich in marine sponges. However, only a limited number of studies have been conducted to reveal endosymbiont bacteria for their medicinal potential. This study aims to determine the antimicrobial potential of endosymbiont bacteria isolated from sponges collected from Kotok Kecil Island, Jakarta. The antimicrobial was carried out by qualitative test using the modified GIBEX method and the Kirby-Bauer disc diffusion method. The results showed that from 15 isolates of endosymbiont bacteria were obtained, and four isolates showed strong antimicrobial activity, each isolated from *Petrosia* sp., *Petrosia nigricans*, *Dasychalina* sp., and *Cinachyrella australiensis*. The fermented filtrate of isolates in Nutrient Broth media showed more potent antimicrobial activity than ethyl acetate extract. The largest average inhibition zone against *Candida albicans*, *Escherichia coli*, and *Streptococcus mutans* were 19.2 mm, 11.8 mm, and 9.1 mm, respectively. Based on the results, it can be concluded that endosymbiont bacteria isolated from marine sponges collected from Kotok Kecil Island has great potency to be further developed as a source of new antimicrobial compounds.

Keywords
Antimicrobial Activity, Bacterial Endosymbiont, Bioactivity, GIBEX, Method.

1. Introduction

Infectious diseases are one of the biggest causes of death in the world. Three million deaths were caused by respiratory infections, including 1.4 million deaths from diarrhea, 1.3 million deaths from tuberculosis, and deaths from HIV/AIDS reached 1 million people (WHO, 2016). The use of antibiotics is a powerful way to treat these infectious diseases. However, the irrational use of antibiotics has led to the emergence of antibiotic resistance, which is currently one of the most serious global public health threats (WHO, 2014).

WHO surveyed 52 countries through the Global Antimicrobial Surveillance System (GLASS) and revealed an increase in resistance to antibiotics. Resistance to penicillin to treat pneumonia ranges from 0%-51%, while resistance to *ciprofloxacin* from *Escherichia coli* bacteria associated with urinary tract infections ranges from 8%-65% (WHO, 2018). This fact explains the need to discover new antimicrobial compounds, including those derived from natural ingredients.

Biodiversity that is commonly explored is from land areas, while marine areas have not been explored optimally. Indonesia has 75% of the territorial waters located at the intersection of the Indian Ocean and the Pacific Ocean (Hanim dan Noorman, 2017). This shows the rich potential of Indonesia's marine resources that can be managed and utilized for the nation's future, one of which is sponges. Sponges are multicellular animals of the phylum Porifera. They do not have organs and tissues but have pores in their bodies (ostia) and water circulation channels in the central cavity (osculum) (Thomas *et al.*, 2016). Sponges live attached to the substrate of rocks, corals, and sand

sediments that are on the seabed (FAO, 2017). Sponges and marine microbes have a symbiotic relationship of mutualism; it is cooperation to fulfill nutrients. For example, in cyanobacteria, in addition to helping sponges get energy through photosynthesis, cyanobacteria have a function to assist the nitrogen fixation process. In contrast, cyanobacteria use sponge bodies as a place to live (Pita *et al.*, 2016).

Sponges have great potential in producing active compounds as medicinal raw materials, but their utilization is not optimal. One example of an active compound from Ircinia sp. is used as an antibiotic, analgesic, and anti-inflammatory (Dhinakaran dan Lipton, 2012). *Corticium* sp. contains a steroidal alkaloid compound in the form of *plakinamine M*, which is able to inhibit the growth of *Mycobacterium tuberculosis* (Lu *et al.*, 2013). *Xestospongia* sp. produces two active compounds*: amphimedine,* an antitumor compound and *neoamphimedine,* which inhibits the topoisomerase II enzyme in DNA replication. (Ponder *et al.*, 2011) and has potential as anticancer compounds (Li *et al.*, 2014).

Excessive use of sponges to look for new bioactive compounds can result in "overfishing", which is detrimental to the ecological system of marine biota because of their slow growth. Sponge type *Amphimedon* sp. only experienced growth (3.01 ± 1.60) – (3.43 ± 1.08) cm^3/day, while *Liosina paradoxa* only grew 3.40 cm^3/day (Sankar *et al.*, 2016; Trianto *et al.*, 2013). Therefore, the use of symbiotic microorganisms with sponges will be better because sponge endosymbiont microbes are producers of bioactive compounds, can be cultured on a laboratory and industrial scale, can be propagated in a relatively short time, and reduce the use of sponges directly.

Research related to the antimicrobial power of sponge endosymbionts in Indonesia is still very limited. Therefore, this research was conducted to explore the antimicrobial potential of endosymbiont bacteria isolated from sponges collected from Kotok Kecil Island, Seribu Islands, DKI Jakarta. Antimicrobial potency testing was carried out by the modified GIBEX (The Global Institute for BioExploration) qualitative test method and the Kirby-Bauer disc diffusion method. From the results of this study, it is hoped that new information and data can be obtained about the antimicrobial potential of sponge endosymbiont bacteria originating from Indonesia.

2. Methods

2.1 Collecting Sponge

The collecting sponge was carried out by tracing the seabed at a depth of ± 5-20 m with the SCUBA tool. The sponge was then put into a sterile bag and given sterile seawater to avoid contamination. Then, those sealed sponges were photographed for documentation.

2.2 Sponge Identification

Sponge identification is carried out based on the location of the discovery, external shape, surface texture (jagged, hairy, coarse/fine porous, etc.), and color. Identification was carried out at the Oceanographic Research Center – LIPI, Jakarta.

2.3 Isolation of sponge endosymbiont bacteria

Isolation of bacterial endosymbionts from sponges was carried out as other researchers with minor modifications (Mohan *et al.*, 2016; Santos *et al.*, 2010; Tedford, 2016). Sponge samples were cut into small pieces with 1x1 cm size using a sterile scalpel, then immersed in sterile seawater for 2 minutes. After that, the object was immersed in 70% alcohol for 3 minutes and then immersed again in sterile seawater for 2 minutes. The sample was then placed in a Petri dish containing natrium agar (NA) seawater and incubated at room temperature for 24-48 hours.

Bacterial colonies growing around the sponge samples were purified by inoculating endosymbiont bacterial isolates in seawater NA medium in Petri dishes using the Koch depletion method and incubated for 24 hours at 30°C (Cita *et al.*, 2016). The isolation process was carried out under aseptic conditions in Laminar Air Flow (LAF). Pure cultures were then divided into working cultures (on inclined NA medium) and stock cultures (on paraffin-treated upright NA medium), both of which were stored at -4°C in a refrigerator and will be used for further research (Figure 1).

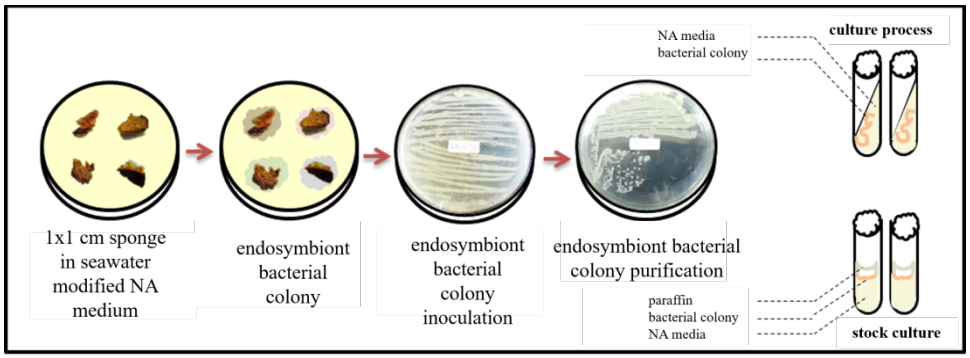

Figure 1. Stages of isolation and purification of sponge endosymbiont bacteria

2.4 Sponge Endosymbiont Bacterial Colony and Cell Morphology Characterization

The purified endosymbiont bacterial isolate colonies were characterized by size, color, shape, margin, and colony elevation. Gram staining was performed to distinguish Gram-positive or Gram-negative groups in endosymbiont bacteria, as was conducted by Asagabaldan *et al.* (2017).

Gram staining is done by making the preparation dry first. Glass slides that have been soaked in 70% alcohol are passed over a spirit fire to remove fat. Then, a sample of the endosymbiont bacteria of the sponge was taken and placed on a slide as thin as possible. This process is carried out aseptically in the LAF. After that, fixation was carried out, bypassing the preparation over the fire three times. This aims to kill, glue the endosymbiont bacteria, and facilitate staining. The preparations were then dripped with gentian violet dye to cover the entire surface and left for 5-7 minutes, after which they were washed with running water. Then, it was dripped with Lugol's solution and left for 45-60 seconds. After that, it was washed with running water. The preparations were then dripped with fuchsin water dye for 1-2 minutes, washed with running water, then dried. To observe the shape and color of the bacteria, the preparations were dripped with immersion oil and observed using a light microscope with a magnification of 1000x (Asagabaldan *et al.*, 2017; Ayitso dan Onyango, 2016).

2.5 Preparation of Test Microbial Suspension

The test microbes *Escherichia coli, Streptoccocus mutans*, and *Candida albicans* ATCC were rejuvenated on sterile NA and PDA (Potato Dextrose Agar) media. The test microbes were inoculated as much as one ose into the inclined NA medium and incubated at 37°C for 18-24 hours for bacteria and 48 hours for fungi, and the work was carried out under aseptic conditions in the LAF. The tested microbial suspension was then inoculated using an ose needle into the NB (bacteria) or PDB (*Potato Dextrose Broth*) (mushroom) media, then homogenized using a vortex. The microbial turbidity of this test was homogenized using a standard Mc Farland 0.5 (microbial density 1.5 x 10^8 CFU/mL) on a black background and bright light. Mc Farland standard was prepared by mixing 0.5 mL 0.048M $BaCl_2$ and 0.18 M H_2SO_4 (Eduardo *et al.*, 2018).

The microbial inoculation technique was carried out using a sterile swab. A sterile swab is dipped into the suspension of the test microbe, drained by gently pressing the tip of the swab, and rotated against the inner wall of the tube to remove excess fluid. The swab is then scratched on the surface of the agar in a Petri dish.

2.6 Endosymbiont Bacterial Fermentation and Extraction

Bacterial endosymbiont fermentation was carried out as conducted by Murniasih *et al.* (2018). Pure bacterial endosymbiont cultures were cultured as much as 10 mL in seawater NB media and incubated at 30°C for 72 hours. After that, the cultures were harvested. Some of the fermented products were centrifuged at 3000 rpm for 20 minutes. Meanwhile, others were extracted with ethyl acetate at a ratio of 1: 1 as done by Putra *et al.* (2017).

2.7 Modified GIBEX qualitative test

According to Raskin dan Skubel (2019), The GIBEX qualitative test is a fast and safe procedure for antimicrobial substances. This qualitative test was carried out using the chemical compound MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) as an indicator of antimicrobial activity by producing a yellow to purple color.

Each extract (NB filtrate and ethyl acetate extract) was taken as much as 50 µL using a micropipette and dripped onto discs measuring 9.6 mm and with two replications. The discs that had been dripped with extract were dried for 15 minutes aseptically with a blower in LAF. After drying, the extracted disc was put in a zip lock plastic and stored at 1-4°C (Raskin dan Skubel, 2019).

The disc containing the extract was inserted into a 6x4 well-plate using sterile tweezers, then 600 µL of LB (Lactose Broth) solution (for an antibacterial test)/600 µL of PD+LB solution (for an antifungal test) and 50 µL of test microbes were added, then homogenized. If saliva is used, then the saliva used is mixed first into the LB medium in a ratio of 1:1 and homogenized. After that, the well-plate was glued using a plastic seal to avoid contamination and incubated at 37°C for 24 hours. After 24 hours, the turbidity and color changes were observed after MTT was added. A positive result (antimicrobial activity) is evidenced by the clearness of the solution and a yellow-colored solution after adding 50 µL of MTT per well-plate and incubating at 37°C. The yellow color indicates the presence of strong antimicrobial compounds from endosymbiont bacteria so that there is no live bacterial activity in the well. In contrast, the purple color indicates the presence of dehydrogenase enzymes from the mitochondria of the test microbes, which change the yellow color of MTT to purple (Raskin dan Skubel, 2019).

2.8 Kirby-Bauer Disc Diffusion Test

The antimicrobial activity test using the Kirby-Bauer disc diffusion method was carried out as conducted by Murniasih *et al.* (2018). The test microbes were streaked evenly into a Petri dish containing MHA (Mueller Hinton Agar) media. Sterile disc paper that had been dripped with 25 µL of fermented endosymbiont bacterial extract was placed on the surface of the MHA media with two replications. Incubation was carried out at 37°C for 24 hours. As a negative control, ethyl acetate and NB solvent were used, and as a positive control, streptomycin (bacteria) and econazole nitrate (fungus) were used. The inhibition zone formed was measured using a caliper in mm.

2.9 Biochemical Characterization of Potential Endosymbiont Bacteria

Potential endosymbiont bacteria were identified based on their biochemical activity using indole, methyl red, vogues proskeur, citrate, and sugar fermentation tests (glucose, galactose, fructose, lactose, maltose, mannitol, and sucrose) as carried out by Mohan *et al.* (2016).

3. Result

3.1 Type of Sponge Used

The sponges obtained from Kotok Kecil Island, Seribu Islands, were used in this study as many as five types of sponges. The five types of sponges are *Petrosia* sp., *Dasychalina* sp., *Cinachyrella australiensis*, *Stylissa massa*, and *Petrosia nigricans* (Figure 2).

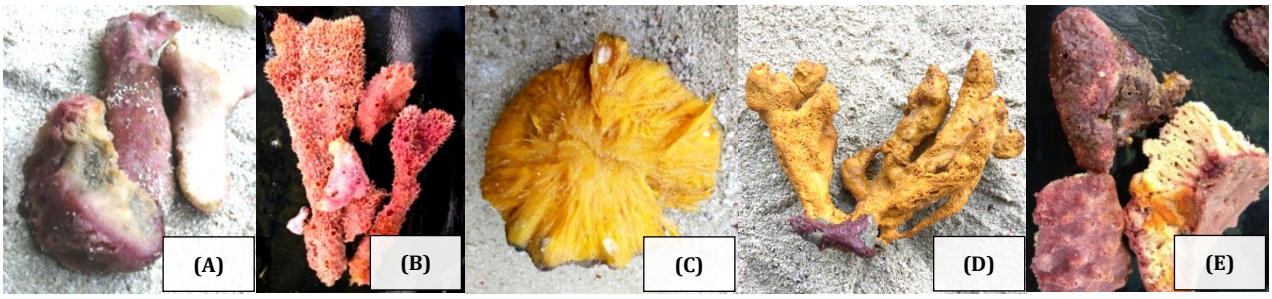

Figure 2. Sponges collected from Kotok Kecil Island: (a) *Petrosia* sp.; (b) *Dasychalina* sp.; (c) *Cinachyrella australiensis*; (d) *Stylissa massa*; (e) *Petrosia nigricans*

3.2 Endosymbiont Bacteria Found

From 5 samples of sponges collected from the waters around Kotok Kecil Island, Seribu Islands, DKI Jakarta, 15 isolates of endosymbiont bacteria were obtained. The number of each endosymbiont bacterial isolate can be seen in Table 1.

Table 1. The number of bacterial endosymbionts obtained from the sponge

No	Sponge type	The number of endosymbiont bacteria isolates	Endosymbiont bacteria isolate code
1	*Petrosia* sp.	2	1K1 and 1K2
2	*Dasychalina* sp.	3	2K1, 2K2, and 2K3
3	*Cinachyrella australiensis*	4	3K1, 3K2, 3K3, and 3K4
4	*Stylissa massa*	3	4K1, 4K2, and 4K3
5	*Petrosia nigricans*	3	5K1, 5K2, and 5K3

The purified endosymbiont bacterial isolates were then characterized by colony morphology based on size, color, shape, margins, and colony elevation. Gram staining was also performed to determine the group of bacteria (Table 2). The results of Gram staining were carried out. From 15 isolates, there were 8 isolates of Gram-positive bacteria and 7 isolates of Gram-negative bacteria with the predominance of cocobasil cell forms without spores. Some macroscopic examples of bacterial endosymbiont colonies and Gram stain are shown in Figure 3.

Table 2. Morphological characteristics of endosymbiont bacterial isolates

No	Endosymbiont Bacteria Isolation Code	Macroscopic				Microscopic		
		Form	*Elevation*	*Margin*	Color	Gram	Shape	Spore
1	1K1	*Irregular*	*Flat*	*Undulate*	Cream	+	Bacillus	+
2	1K2	*Irregular*	*Flat*	*Undulate*	White	+	Coccobacillus	-
3	2K1	*Rhizoid*	*Flat*	*Filamentous*	White	-	Coccobacillus	-
4	2K2	*Irregular*	*Flat*	*Undulate*	White	-	Coccobacillus	-
5	2K3	*Circular*	*Flat*	*Entire*	White	-	Coccobacillus	-
6	3K1	*Rhizoid*	*Flat*	*Filamentous*	White	+	Coccobacillus	-
7	3K2	*Irregular*	*Flat*	*Undulate*	White	-	Coccobacillus	-
8	3K3	*Irregular*	*Flat*	*Undulate*	White	+	Coccobacillus	-
9	4K1	*Rhizoid*	*Flat*	*Filamentous*	White	-	Coccobacillus	-
10	4K2	*Circular*	*Flat*	*Entire*	White	+	Coccobacillus	-
11	4K3	*Circular*	*Flat*	*Entire*	White	+	Coccobacillus	-
12	4K4	*Irregular*	*Flat*	*Filamentous*	White	+	Coccobacillus	-
13	5K1	*Circular*	*Flat*	*Entire*	White	-	Coccobacillus	-
14	5K2	*Filamentous*	*Flat*	*Erose*	White	+	Coccobacillus	-
15	5K3	*Filamentous*	*Flat*	*Filamentous*	White	+	Bacillus	+

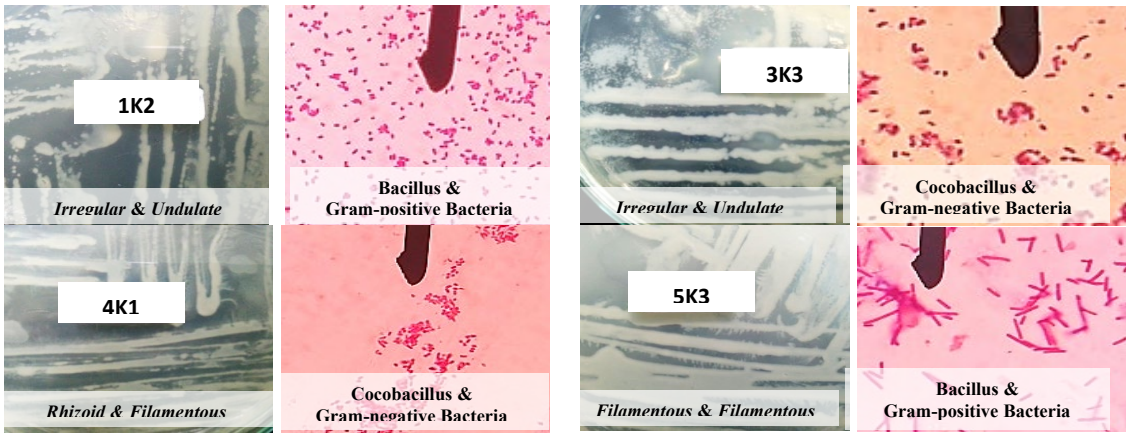

Figure 3. The results of the morphological characterization of endosymbiont bacterial isolates

3.3 Results ofAntimicrobial Activity using The Modified GIBEX Method

The modified GIBEX qualitative was performed on the NB filtrate and ethyl acetate extract. The results of the GIBEX method showed that bacterial isolates had different antimicrobial activities against each of the tested microbes used. The reaction was indicated by a change in the medium from clear to cloudy and remained clear, and

a change in color after MTT was added from yellow to purple, bright purple, and some isolates retained a yellow color. The results are presented in Table 3.

Table 3. The results of 15 endosymbiont bacterial isolates using the modified GIBEX method

Endosymbiont Bacteria Isolation Code	Saliva		*Escherichia coli*		*Streptococcus mutant*		*Candida albicans*	
	NB filtrate	extract ethyl acetate	NB filtrate	extract ethyl acetate	NB filtrate	extract ethyl acetate	NB filtrate	extract ethyl acetate
1K1	1 (+)	1 (+)	2 (+)	0 (+)	1 (++)	0 (-)	1 (++)	2 (++)
1K2	1 (+)	1 (+)	2 (+)	1 (+)	1 (++)	0 (-)	2 (++)	2 (++)
2K1	1 (-)	1 (+)	2 (+)	1 (+)	1 (-)	0 (-)	**3 (++)**	1 (+)
2K2	1 (-)	0 (-)	1 (-)	1 (+)	1 (-)	0 (-)	**3 (+++)**	1 (+)
2K3	1 (-)	1 (+)	1 (+)	1 (+)	2 (+)	0 (-)	**3 (++)**	0 (+)
3K1	1 (+)	1 (+)	1 (++)	2 (++)	2 (++)	0 (-)	2 (++)	**2 (+++)**
3K2	2 (+)	1 (+)	1 (-)	2 (++)	2 (+)	1 (+)	**3 (++)**	**2 (+++)**
3K3	1 (-)	1 (+)	1 (+)	1 (++)	2 (+)	0 (-)	1 (+)	**2 (+++)**
4K1	2 (+)	1 (+)	2 (+)	1 (+)	2 (+)	1 (++)	**2 (+++)**	**2 (+++)**
4K2	1 (+)	0 (-)	2 (+)	2 (++)	2 (+)	0 (-)	2 (++)	**2 (+++)**
4K3	1 (-)	0 (-)	0 (-)	2 (++)	2 (+)	0 (-)	**2 (+++)**	**2 (+++)**
4K4	2 (-)	1 (+)	1 (-)	1 (+)	2 (+)	0 (-)	**2 (+++)**	1 (++)
5K1	1 (+)	1 (+)	1 (+)	1 (+)	2 (++)	0 (-)	0 (-)	0 (-)
5K2	1 (+)	**3 (+++)**	1 (+)	2 (++)	2 (+)	**2 (+++)**	0 (+)	**3 (+++)**
5K3	2 (+)	2 (++)	1 (-)	1 (+)	2 (+)	0 (-)	0 (-)	0 (-)
-	1 (+)	1 (+)	1 (-)	1 (+)	2 (-)	0 (+)	0 (-)	0 (-)
+	3 (+++)	3 (+++)	3 (+++)	3 (+++)	3 (+++)	3 (+++)	3 (+++)	3 (+++)

Turbidity

-	Turbidity	=	No antimicrobial activity
+	A bit cloudy	=	Small antimicrobial activity
++	A bit clear	=	Medium antimicrobial activity
+++	Clear	=	High antimicrobial activity

MTT Color

0	Dark purple	=	No antimicrobial activity
1	Purple	=	Small antimicrobial activity
2	Light purple	=	Medium antimicrobial activity
3	Yellow	=	High antimicrobial activity

3.4 Results of Antimicrobial Activity using The Kirby-Bauer Disc Diffusion Method

All NB filtrate and ethyl acetate extract were screened using the Kirby-Bauer disc diffusion method to ensure quantitative results. The Kirby-Bauer disc diffusion method was tested on three test microbes: *E. coli, S. mutans*, and *C. albicans*. The average antimicrobial activity results are presented in Table 4 and the picture in Figure 4.

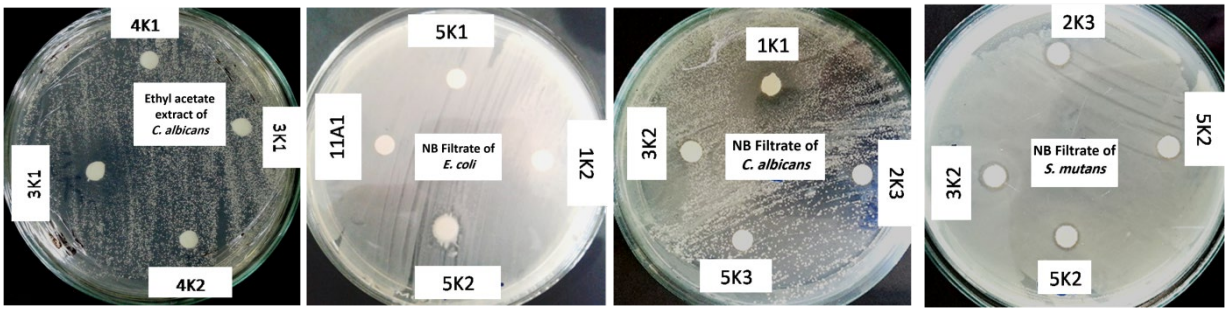

Figure 4. The results of the inhibition zone of several bacterial isolates of endosymbiont NB filtrate and ethyl acetate extract

Table 4. The average diameter of inhibition zone of 15 endosymbiont bacterial isolates against test microbes using the Kirby-Bauer disc diffusion method

Endosymbiont Bacteria Isolation Code	*E. coli* (mm)		*S. mutant* (mm)		*C. albicans* (mm)	
	NB filtrate	ethyl acetate extract	NB filtrate	ethyl acetate extract	NB filtrate	ethyl acetate extract
1K1	6,0	6,0	6,5	6,0	**19,2**	**8,3**
1K2	6,0	6,0	6,2	6,0	6,0	6,2
2K1	6,0	6,0	6,3	6,0	6,0	7,0
2K2	6,5	6,0	**8,1**	6,4	6,0	**8,1**
2K3	6,2	6,3	6,5	6,0	6,0	6,9
3K1	7,5	7.2	**9,1**	6,0	6,0	**9,2**
3K2	7,2	6,0	6,0	7,1	6,0	6,0
3K3	6,7	6,0	6,4	6,0	6,0	7,1
4K1	6,0	6,0	6,9	7,0	6,0	6,0
4K2	6,2	6,7	6,0	6,0	6,0	6,0
4K3	6,0	6,0	6,6	6,0	6,0	7,0
4K4	6,2	6,2	6,7	6,0	6,0	7,3
5K1	6,4	6,9	7,0	6,3	6,0	7,6
5K2	**11,8**	6,0	6,5	6,2	**14,2**	7,2
5K3	6,0	6,0	7,0	6,0	6,0	7,0
-	6,0	6,0	6,0	6,0	6,0	6,0
+	12,4	13,3	11,5	11,5	17	12,5

Note: The above average diameter includes a disc diameter of 6.0 mm

3.5 Biochemical Characterization of Potential Endosymbiont Bacteria

The types of endosymbiont bacterial isolates obtained can be identified by characterization based on biochemical activity, such as fermentation of various sugars (glucose, lactose, maltose, mannitol, and saccharose) methyl red reaction, Voges-Proskauer, indole reaction, citrate reaction, and TSIA reaction. The results of the biochemical characterization of endosymbiont bacterial isolates can be seen in Table 5.

Table 5. The results of the characterization of the biochemical activity of endosymbiont bacterial isolates

No	Biochemical Test	Endosymbiont Bacteria Isolation Code			
		1K1	2K2	3K1	5K2
1	Glucose	-	+	+	-
2	Lactose	-	-	-	-
3	Maltose	+	+	+	-
4	Mannitol	+	+	+	-
5	Saccharose	+	+	+	+
6	Arabinose	-	-	-	-
7	Methyl red	-	+	+	-
8	Voges-Proskauer	-	+	+	-
9	Indol	-	-	-	-
10	Citric	-	+	+	-
11	TSIA	B/B	B/A	B/A	B/B

Description: + (positive result); - (negative result); A/A (ferment all sugar); B/A (only ferment glucose); and B/B cannot ferment sugar.

4. Discussion of Results

4.1 Type of Sponge Used

Five types of sponges were obtained from Kotok Kecil Island, Seribu Islands. The five types of sponges are *Petrosia* sp.*, Dasychalina* sp.*, Cinachyrella australiensis, Stylissa massa,* and *Petrosia nigricans* (Figure 2). Sponge *Petrosia*

sp. and *Petrosia nigricans* are sponges that live at depths of 5-45 m on hard substrates. *Petrosia* sp. has a harder texture, while *Petrosia nigricans* has a softer texture. The color of these two sponges depends on the symbiotic cyanobacteria and the state of the surrounding environment, from purple to brown (Chelossi *et al.*, 2004).
Sponge *Dasychalina* sp. which is found at a depth of 5-25 m has a purple outer color and a grayish to brownish yellow inner color. This sponge has a hard and brittle texture and is prickly and slimy on the surface. This was also reported by Hooper dan Soest (2002). which states that this type of sponge has branches with a diameter of about 18 mm covered with spines. Sponge *Cinachyrella australiensis* has a gray exterior color covered with sand and yellow on the inside. This type of sponge generally has a relatively hard surface and thorns on the inside. According to the Reef Guide (2016), this sponge can measure up to 7.5 cm in depth from 0-5 m. The *Stylissa massa* sponge is a yellow sponge with a size of 5-10 cm and a diameter of 5-8 cm, this also agrees with Hooper dan Soest (2002). According to him, *S. massa* has a light yellow to orange color, measuring 7-20 cm long and 5-11 cm in diameter.

4.2 Endosymbiont Bacteria Found

Based on the results of the purification of endosymbiont bacterial isolates that had been carried out, 15 isolates of endosymbiont bacteria were obtained, which had different colony morphology from each sponge sample. The sponge with the highest number of isolates was *Cinachyrella australiensis*, with 4 isolates (3K1, 3K2, 3K3, and 3K4). Sponge *Petrosia* sp. had the least number of endosymbiont bacterial isolates, with 2 isolates (1K1 and 1K2). While the sponges *Dasychalina* sp*., Stylissa massa,* and *Petrosia nigricans* each had 3 isolates of endosymbiont bacteria.

The existence of this difference is influenced by the species of sponge and environmental conditions around the sponge. It is also supported by Webster and Thomas (2016), the diversity of bacteria associated with sponges depends on the species of the sponge. Next, according to Mehbub *et al.,* (2014), The presence of endosymbiont microbes in the sponge's body plays a role in the fulfillment of nutrients and the formation of sponge spicules. Based on the results of observations, the morphological characterization of endosymbiont bacterial colonies generally showed that the color of the colonies was generally white and cream with an elevation of "flat", meaning that the endosymbiont bacterial colonies had a thin side appearance. Colony shape varied (irregular, circular, rhizoid, and filamentous), with varying margins (undulate, entire, filamentous, and erose).

Sponge *Petrosia* sp. has endosymbiont bacterial isolates with irregular colony shape, undulating edges, and cream and white colors. Sponge *Dasychalina* sp. have endosymbiont bacterial isolates with different shapes and edges. Sponge *Cinachyrella australiensis* has endosymbiont bacterial isolates with rhizoid and irregular shape with filamentous and undulate edges. *Stylissa massa* sponge had endosymbiont bacterial isolates with rhizoid, circular, and irregular shapes with filamentous and entire edges. *Petrosia nigricans* sponge has endosymbiont bacterial isolates with circular and filamentous colonies with entire, erose, and filamentous edges.

4.3 Results of The Modified GIBEX Method of Antimicrobial Activity Screening

Based on the results of antimicrobial activity screening with the modified GIBEX method (Table 3), it can be seen that in general, NB filtrate and ethyl acetate extract have better activity against *C. albicans*, which is characterized by the media remaining clear after being incubated for one day and the color remaining yellow after MTT was added. The ability of bacterial isolates to inhibit the growth of *C. albicans* was due to the ability of endosymbiont bacteria isolates to break down *C. albicans* cells. According to Alioes *et al*. (2018), endosymbiont bacteria have antifungal potential that can denature protein bonds in the cell membrane of *C. albicans* so that the cell membrane becomes lysed and bioactive compounds of endosymbiont bacteria can penetrate the cell nucleus.

The results of the GIBEX test on microbes from saliva showed that only the ethyl acetate extract of endosymbiont bacterial isolate 5K2 had strong antibacterial activity compared to bacterial isolates endosymbiont NB filtrate and other ethyl acetate extracts. This was indicated by the media remaining clear (turbidity +++) and the media color remaining yellow after MTT was added (value 3).

The results of the GIBEX test on *E. coli* showed that there was no strong enough antibacterial activity. This was caused by the bioactive compounds of endosymbiont bacterial isolates that were not strong enough to inhibit or kill the growth of E. coli. This statement is also reinforced by Suryati *et al*. (2017), who stated that these Gram-negative bacteria have cell wall layers that are more complex than Gram-positive bacteria. These layers include lipopolysaccharides, proteins, and phospholipids.

The results of the GIBEX test on *S. mutans* showed that only the ethyl acetate extract of the 5K2 endosymbiont bacterial isolate had strong antibacterial activity compared to the endosymbiont bacterial isolate from the NB filtrate and other ethyl acetate extracts. This is indicated by the media remaining clear (turbidity +++) and the color of the media becoming light purple after MTT was added (value 2).

The results of the GIBEX test on *C. albicans* showed that the NB filtrate extract of endosymbiont bacterial isolates 2K1, 2K2, 2K3, 3K2, 4K1, 4K3, and 4K4 had quite strong antifungal activity than other bacterial isolates of endosymbiotic NB filtrate. This is indicated by the media remaining clear after two days of incubation and/or the color remaining yellow after MTT was added (score 3). Ethyl acetate extract of endosymbiont bacterial isolates 3K1, 3K2, 3K3, 4K1, 4K2, 4K3, and 5K2 also had strong antifungal activity against *C. albicans*, which was indicated by the media remaining clear after one day of incubation and/or the color remaining yellow after being dropped using MTT (score 3).

Based on the results of the GIBEX screening method, it can be concluded that from the 15 isolates of endosymbiont bacteria, there were 11 isolates with sufficient potential; they are isolates 2K1, 2K2, 2K3, 3K1, 3K2, 3K3, 4K1, 4K2, 4K3, 4K4, and 5K2. This was chosen based on the media that remained clear after being incubated for one day (turbidity +++) and or the color of the media that remained yellow after being dripped with MTT (score 3). In addition, the NB filtrate extract had a higher number of endosymbiont bacterial isolates with moderate to strong antimicrobial activity than the ethyl acetate extract isolate. This difference in results is due to the solvent used. The semi-polar nature of ethyl acetate was unable to attract the bioactive compounds of the sponge endosymbiont bacteria isolate, while the centrifuged NB filtrate was able to filter the bioactive compounds of endosymbiont bacteria more strongly than the ethyl acetate extract. This agrees with Ode *et al*.'s (2019) statement that the level of the polarity of a solvent affects the percentage of extract obtained. A compound is only soluble in solvents that have the same degree of polarity. Hutagalung *et al*.'s research (2014) showed that *Stylotella aurantium* and *Haliclona molitba* sponges in distilled water had better activity than methanol solvents.

4.4 Kirby-Bauer Disc Diffusion Method of Antimicrobial Activity Results

Based on the results of the Kirby-Bauer disc diffusion method of endosymbiont bacterial isolates on the test microbes in the table, it can be seen that, in general, NB filtrate has better activity against *E. coli* and *S. mutans*. While some isolates of ethyl acetate extract only had the best activity against *C. albicans*. This is indicated by the average diameter of the inhibition zone produced by the bioactive compounds of endosymbiont bacteria. These compounds diffuse into the growth medium to inhibit the growth of the test microbes. Based on the inhibition zone of the tested microbes' growth obtained in this study, the antimicrobial strength was classified into three groups. The mean inhibition zone diameter >11 mm was categorized as strong, 8-11 mm was categorized as moderate, and the mean inhibition zone diameter <8 mm was categorized as weak. An example of an inhibition zone image is shown in Figure 4.

In general, bacterial endosymbiont NB filtrate has diverse antimicrobial activity. This diversity of inhibition zones was also proven by the results of the SPSS analysis of variance, which showed the interaction of each bacterial endosymbiont isolate. Strong antimicrobial activity is detected on *C. albicans* and *E. coli* because it has an average inhibition zone of >11 mm. This was found in endosymbiont bacterial isolates 1K1 with an average inhibition zone of 19.2 mm and isolates 5K2 with an average inhibition zone of 14.2 mm on *C. albicans*. Meanwhile, *E. coli* was found in 5K2 isolates with an average inhibition zone of 11.8 mm. Medium antimicrobial activity with an average inhibition zone of 8-11 mm in the NB filtrate was owned by isolates 3K1 with an average inhibition zone of 9.1 mm and isolates 2K2 with an average inhibition zone of 8.1 mm on S. mutans.

Ethyl acetate extract of endosymbiont bacteria was more active on *C. albicans* because it was able to inhibit the growth of *C. albicans* in the moderate category. These results were also strengthened by the SPSS analysis of variance, which stated that there was an interaction between endosymbiont bacterial isolates and the interaction between endosymbiont bacterial isolates and the test microbes. The isolates included 1K1 isolates with an average inhibition zone of 8.3 mm, 2K2 isolates 8.1 mm, and isolates 3K1 with an average inhibition zone of 9.2 mm. Isolate 3K1 was the best isolate that could inhibit the growth of *C. albicans* with an average inhibition zone of 9.2 mm. This was also proven by the *Tukey* mean difference test.

Based on the results of the Kirby-Bauer disc diffusion method, it was proven that the NB filtrate isolate had bioactive compounds that had the potential to inhibit the activity of bacteria and/or fungi. Meanwhile, the ethyl

acetate extract isolate had the potential to inhibit the activity of fungi. These two extracts, the NB filtrate and the ethyl acetate extract, were generally able to inhibit the growth of *C. albicans*. According to Pita *et al*. (2016), bioactive compounds, especially secondary metabolites of sponge microbes, provide sponge defense against predators.

4.5 Biochemical Characterization of Potential Endosymbiont Bacteria

Based on the table of results of the biochemical characterization of bacteria (Table 5), it is known that isolate 1K1 can ferment maltose, mannitol, and saccharose. Endosymbiont bacteria isolates 2K2 and 3K1 were able to ferment glucose, maltose, mannitol, and saccharose. Other biochemical reactions tested, such as the methyl red reaction, Voges-Proskauer, and citrate, 2K2 showed positive results. This proves that the 2K2 isolate produces an organic acid end product to form acetyl methyl carbinol and uses citric acid as a carbon source in the medium. The 5K2 endosymbiont bacterial isolate, could only ferment saccharose and the TSIA test produced a red color in the slant and butt sections, which indicated that the isolate could not ferment all sugars.

5. Conclusion

Based on the research results, the following conclusions were obtained from 5 samples of sponges collected from the waters around Kotok Kecil Island, Seribu Islands, DKI Jakarta, 15 isolates of endosymbiont bacteria were obtained. There are 4 isolates of endosymbiont bacteria that are potential sources of antimicrobial compounds: the sponge *Petrosia* sp. (1 isolate), *Petrosia nigricans* (1 isolate), *Cinachyrella australiensis* (1 isolate), and *Dasychalina* sp. (1 isolate). The largest average inhibition zone of endosymbiont bacterial NB filtrate on *C. albicans* was 19.2 mm (1K1 isolate) and 14.2 mm (5K2 isolate), on *E. coli* of 11.8 mm (5K2 isolate), on *S. mutans* by 9.1 mm (3K1 isolate), and 8.1 mm (2K2 isolate). The largest average inhibition zones of endosymbiont bacterial ethyl acetate extract on *C. albicans* were 9.2 mm (3K1 isolate), 8.3 mm (1K1 isolate), and 8.1 mm (2K2 isolate).

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