

Antibacterial Activities of *Pseudomonas orientalis* APD 16 Isolate Sponge-Associated *Aplysina* sp. from Enggano Island Against *Escherichia coli* and *Staphylococcus aureus*

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Abstract

Sponge is one of the invertebrates from the Porifera phylum. Sponge bodies have structural complexity with different cell layers. The sponge has many pores (ostium) on the surface of their body as a filter feeder. Sponge is recognized as organisms that have the potential because they can produce metabolites. Secondary metabolites produced by sponges are the result of the association of sponges with bacteria. The sponge used in this study is the *Aplysina* sp. sponge collected from Enggano island, Bengkulu Province. *Aplysina* sp. sponge is known to contain metabolites with antibacterial, antifungal and cytotoxic activity on cancer cells. This study aims to identify of potential isolate associated with *Aplysina* sp. sponge collected from Enggano island. Isolation of bacteria from *Aplysina* sp. sponge using Sea Water Complete (SWC) media. The isolate was screened by antagonistic test, morphological characters, Gram-staining, biochemical test and molecular identification. Based on the antagonistic test, APD 16 isolate could inhibit *Escherichia coli* and *Staphylococcus aureus* in Vitro. APD 16 isolate was identified molecularly using of 16S rRNA genes analysis and it genetically close with *Pseudomonas orientalis*.

Keywords: Antibacterial activity, *Aplysina* sp., Enggano island, molecular identification

1. Introduction

One of the important problems in the world health is infectious diseases. Infectious diseases are diseases caused by pathogenic microbes such as bacteria, viruses, parasites, or fungi. Pathogenic bacteria that cause important infectious diseases include *Escherichia coli* and *Staphylococcus aureus*.

Enggano island is one of the regions in Indonesia that has a high megadiversity of plants, animals, and microbes (Sipriadi et al., 2020). The island is still well-maintained because it has not been exposed to human exploitation. One of the biodiversity objects in Enggano island is preserved (Sipriadi et al., 2021). Marine biodiversity in Enggano provides an opportunity to utilize marine life as a search for new bioactive compounds (Belarbi et al., 2003). Various studies have shown that marine life has great potential in producing bioactive compounds that can be used as medical raw materials, one of the known marine biota, namely sponges (Ismet, 2007). Sponge is one of the secondary metabolites producers that are most active in producing various chemical compounds, as well as a variety of bioactivity which is pharmaceutically and medically relevant (Abdelmohsen et al., 2013). Sponges that can produce bioactive compounds are due to the symbiotic association with microbes such as bacteria because they can produce of natural bioactive compounds, such as antibiotics, anticancer and immunosupresants (Du and Loxu, 2010). Based on the description above, it is necessary to conduct research on the identification of sponge-associated bacteria and their potency to produce of antibacterial compounds.

2. Materials and Methods

2.1. Research Materials

The tools and materials used in this study were Petri dishes, test tubes, mortar and pestle, Erlenmeyer, measuring cups, test tube racks, beakers glass, bunsen burner, loop needles, drop pipette, analytical scales, centrifuge, UV-Laminar flow-hood, incubators, refrigerators, autoclave, disc paper (Whatman 6 mm), hot plate, magnetic stirrer, spreader rod, glass objects, cover glass, micropipette, serology pipete 10 mL, digital calipers, *Polimerase Chain Reaction* (PCR), electrophoresis, *Gel Documentation*, label papper, cotton, tissue, marker, ruler, *Aplysina* sponge, *Sea Water Complete* (SWC), confectionery medium, test microb isolates consisting of *Escherichia coli* and *Staphylococcus aureus*, 63F/1387R primers of 16S rRNA, *Presto™ Mini gDNA Bacteria* (Geneaid) kit protocol, *GoTag Green Mastermix* kit PCR, *nuclease free water* (NFW), agarosa 1%, *ethidium bromide*(EtBr) dye, alcohol 70%, alcohol 96%, distilled water, spritus, crystal violet, safranin, lugol, immersion oil, wrapping paper, and aluminum foil.

2.2. *Aplysina* sp. Sponges Collection

Aplysina sp. sponge samples were collected previous research have been done from the coastal waters of Dua Island, Enggano Island located in North Bengkulu Regency (Wibowo et al., 2020). The samples obtained were immediately carried out in the preparation. Furthermore, the potential isolates were tested in the Microbiology Laboratory, Biotechnology and Genetics Laboratory, University of Bengkulu.

2.3. Isolation of Sponge-associated Bacterial

1 gr of sponge body crushed with a sterile mortar and pestle, then diluted with NaCl 0.85% as much as 9 mL and made a suspension from dilution 10^{-1} to 10^{-3} . Each dilution 0.1 mL pipette aseptically spread on to a Petri dish containing sterile *Sea Water Complete* (SWC) media, then, incubated at 30 °C for 48 hours (Newbold et al., 1999).

2.4. Purification of Sponge-associated Bacterial Isolates

Purification of isolates associated with *Aplysina* sp. sponges was carried out by streak the colonies on a Petri dish containing sterile SWC media. Isolates of bacteria were incubated in the incubator at 30 °C for 48 hours. The morphology of the growing bacterial colonies were observed based on their margin, texture, form, elevation, and color of the colony.

2.5. Antagonistic Test of Antibacterial-Producing Isolates using Culture, Pellets and Supernatant Methods

Screening of bacteria were carried out using the test of pathogenic bacteria, such as *Escherichia coli* and *Staphylococcus aureus*. Each test bacterial was cultured and homogenized on solid media. Furthermore, the pure isolates were streaked on the surface of the media containing the tested bacterial culture, then incubated at 37 °C for 24 hours. Positive result indicated by a clear zone around the colony. The area of the clear zone is measured using a digital caliper. The isolates which have the largest zone of inhibition and inhibit the test pathogens will be selected for the furture test.

The selected isolates from the previous method were cultured on liquid SWC media. Then, the bacterial culture was centrifuged at 10.000 rpm for 5 minute. The pellets obtained are dissolved in 150 µL the supernatant. The pellets were used in the antagonistic test against the test microbes by dropped it on the disc paper and placed on the SWC media containing the test microbes, then incubated at 37 °C for 24 hours.

Supernatant in the use for antagonistic test against the test microbes. The antagonist test was carried out with a sterile disc papper dropped with supernatant. The disc paper was placed on the SWC media containing the test microbial, then incubated at 37 °C for 24 hours. Positive results by used of the pellet and supernatant methods were indicated by the formation of a clear zone around the paper disc. The clear zones formed by the these methods were compared with 4 categories of inhibition of antimicrobial compounds according to Davis and Stout (Davis and Stout, 1971).

2.6. Identification of APD 16 Isolate (Morphological Characters, Gram Staining and Biochemical Test)

Gram staining was done by placing 1 loop of bacterial isolate from the previous test into the object glass aseptically. Then it was given a solution of crystal violet, lugol, alcohol 96% and safranin respectively, then rinsing under running water in each stage (Lay, 1994). Identification of bacterial colonies using biochemical tests, such as carbohydrate fermentation test, catalase test, motility test, urea test, and citrate test were done.

2.7. Genomic Extraction of APD 16 Isolate

APD 16 isolate were purified in solid SWC media and incubated at 30°C for 48 hours. 3-4 use of these isolates were put into a micro sterile tube/*Eppendorf* tube containing 0.5 ml *nuclease free water* (NFW) and centrifuged at a speed of 12000 rpm for 5 minutes and DNA genom was isolated using *Presto™ Mini gDNA Bactexria* (Geneaid) kit protocol. Procedures are carried out according to the manufacturer's instructions.

2.8. Molecular Identification of the APD 16 Isolate Based on 16S rRNA Genes

16S rRNA genes were amplified using the *forward* primer 63F (5'-CAGGCCTAACACATGCAAGTC-3') and *reverse* primer 1387R (5'-GGGCAGGTGTACAAGGC-3') with target fragment of 1300 pb (Marchesi et al., 1998). The total volume of the reaction used was 40 μ L consisting of 20 μ L *Go Taq Green Mastermix 2x*, each of 2 μ L primers, DNA template 4 μ L (~100 ng) and *nuclease free water* (NFW) 12 μ L. Amplification was performed under PCR conditions at pre-denaturation (94 °C, 5 minutes), denaturation (94 °C, 45 second), *annealing* (55 °C, 1 minutes), elongation (72 °C, 1 minutes 10 second). Denaturation, annealing and elongation were repeated for 30 cycles. At the next stage, post elongation (72 °C, 7 minutes), and *cooling* (15 °C, 15 minutes).

3. Results and Discussion

3.1. Bacterial Isolates from Sponge *Aplysina* sp.

Bacteria colonies from sponge *Aplysina* sp. obtained after grew at each dilution. The colony was screened based on morphological characters such as their margin, texture, form, elevation, and color of the colony. Total of 16 isolates were successfully purified and screened base on the colony morphological characters.

3.2. Antibacterial Activities of Bacteria Associated with the Sponge *Aplysina* sp.

There were 16 isolates with clearly showed antibacterial activity against the test bacteria, both in *Escherichia coli* and *Staphylococcus aureus*. One of the selected isolate that has antagonistic activities namely APD 16 isolate. Growth inhibiting activities against the tested pathogenic bacteria were showed by the presence of a clear zone around the colony/disc paper. The antagonistic activities of APD 16 isolate against the test pathogens bacteria by culture, supernatant and pellet can be seen in Table 1 and Figure 1.

Table 1. Antibacterial activities of APD 16 isolate.

Sampel code	Method	Microb test	Disc/culture Diameter (mm)	Total diameter (mm)	Diameter of inhibition zone (mm)	Inhibition zone activity
APD 16	Culture	<i>E. coli</i>	3.9	9	5.1	Medium
		<i>S. aureus</i>	3.9	5.3	1.3	Weak
	Extract	<i>E. coli</i>	6	8.4	2.4	Weak
		<i>S. aureus</i>	6	9	3	Weak
	Supernatant	<i>E. coli</i>	6	8.2	2.2	Weak
		<i>S. aureus</i>	6	7.3	1.3	Weak

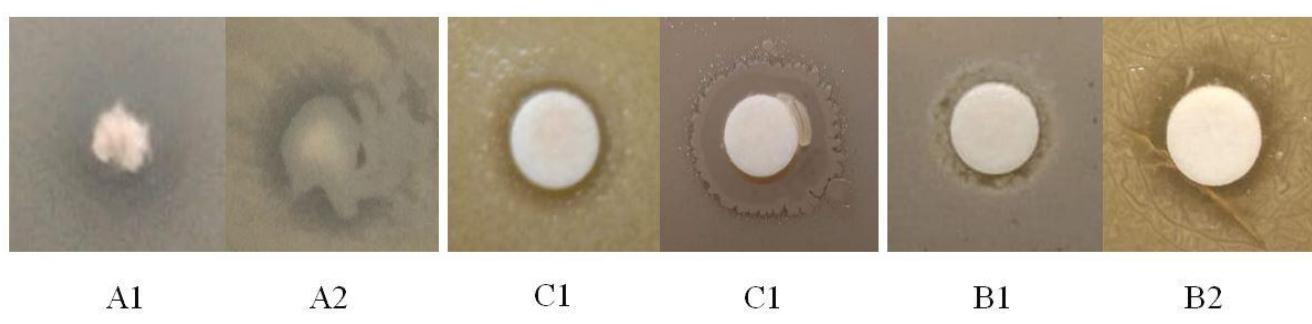


Figure 1. The clear zone produced by APD 16 isolate against *Escherichia coli* (1) and *Staphylococcus aureus* (2); A = Clear zone form by culture, B = Clear zone formed by pellets, and C = Clear zone formed by supernatant

Different clear zone area showed varies of bioactive compounds produced by sponge-associated bacteria. These different compounds are thought to have different mechanisms in inhibiting colonization of the test pathogens. Indraningrat *et al.*, (Indraningrat et al., 2016) reported that sponge-associated bacteria can produce antimicrobial compounds by 90%.

3.3. Identification of the APD 16 isolate by Gram Staining and Biochemical Test

Gram staining were conducted to distinguish two groups of bacteria, namely Gram-positive bacteria and Gram-negative bacteria. Differences in the response of bacterial staining are based on their structure and chemical components of cell wall (Harley, J. P., & Prescott, L. M., 2002). APD 16 isolate was grouped into Gram-negative bacteria and has the shape of a bacil with monobacil arrangement. Gram-negative staining is indicated by bacteria that are red, while Gram-positive bacteria are purple. The characters of Gram-staining of APD 16 isolate can be seen in Figure 2.

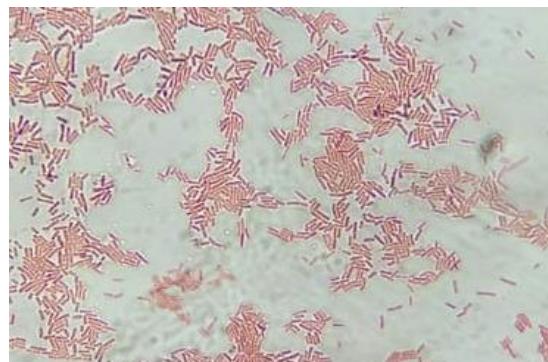


Figure 2. Gram-staining of APD 16 isolate observed under a Binocular Microscope with a magnification of 1000x.

Biochemical tests were carried out to determine the physiological characteristics of bacteria. Bacteria used the nutrients obtained from the environment to carry out biochemical activities. Morphological characterization and biochemical tests on APD 16 isolate associated with *Aplysina* sp. sponge were shown in Table 2.

Table 2. Morphological observations and biochemical tests of APD 16 isolate sponge-associated *Aplysina* sp.

Sample code	Characterization of bacterial morphology				
	Margin	Texture	Form	Elevation	Color
Entire	Smooth	Pinpoint	Flat	White	
Biochemical Test					
APD 16	Carbohydrate Fermentation Test				
C	Si	Mo	U	G	M
+	+	+	-	+	+
				L	S
				+	+

Note: C = Catalase test; Si = Citrate test; Mo = Motility test; U = Urea test; G =Glucose test, M = Maltose test, L = Lactose test, S = Sucrose test

In the catalase test, APD 16 isolate reacted positively after dropping with H_2O_2 3%, characterized by the formation of air bubbles. From the catalase test showed that APD 16 isolate had a catalase anzyme which could degrade hydrogen peroxide into water and O_2 .

In the citrate test, APD 16 isolate showed a positive reaction with a change in color from green to blue. According to Lay (Lay, 1994) of the color change in the media because in the Simon's citrate medium there was a bromine thymol blue indicator pH.

APD 16 isolate showed a positive in the motility test. Motility test is used to see the ability of bacteria using flagella or cilia. According to Harley and Prescott (Harley and Prescott, 2002), that bacteria are said to be motile if their movement is not only along the inoculation line.

APD 16 showed a positive reaction to 4 types of carbohydrates, such as glucose, sucrose, lactose and maltose. The positive test for carbohydrate fermentation is indicated by a color change in the liquid medium from red to yellow. According to Lay (Lay, 1994), in the fermentation process, bacteria carry out biochemical activities in a liquid medium containing carbohydrates. Then, the fermentation results are in the form of acids.

3.4. 16s rRNA genes Molecular Identification of APD16 Isolate

16S rRNA genes are used as the genetic marker in studying bacterial phylogeny and taxonomy. Total DNA from APD 16 isolate was used as a DNA template for amplification of the 16S rRNA gene using 63F and 1387R primers using PCR techniques. Furthermore, PCR products were analyzed using 1% agarose gel electrophoresis technique. APD 16 isolate was successfully amplified and showed DNA fragments measuring ~1300 pb (Figure 3).

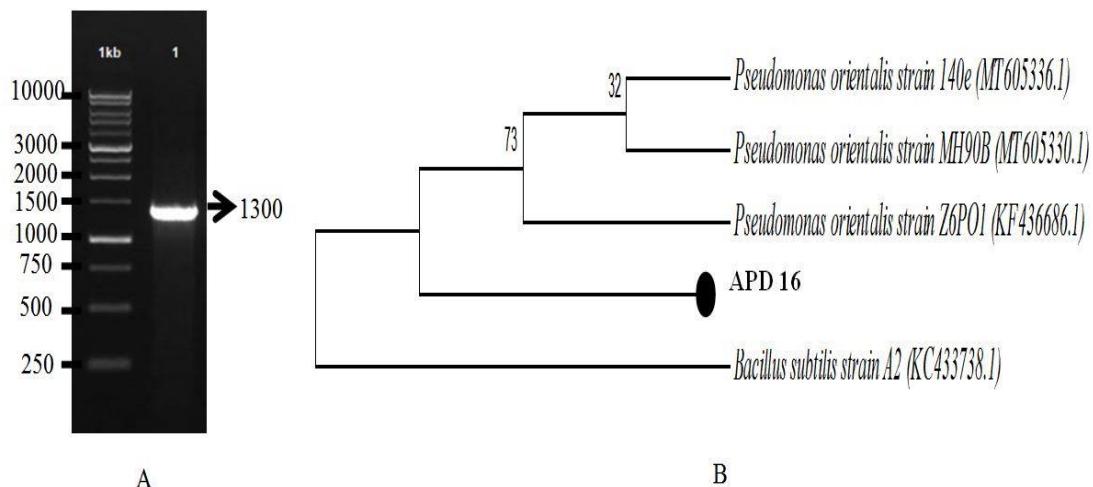


Figure 3. 16S rRNA gene electrophoresis resulted in sized DNA bands ~1300 pb. (1kb) marker 1 kb (A), and Phylogenetic tree of APD 16 isolate based on 16S rRNA genes sequences using the *Neighbor Joining Tree* method with a bootstrap value of 1000x replicates (B)

Table 3. Molecular Identity of APD 16 isolate based on 16S rRNA genes using BLASTn

Isolate	Homology	Query Cover (%)	E-value	Similarity	Access Number
	<i>Pseudomonas orientalis</i> strain Z6PO1	100	0.0	99.68%	KF436686.1
APD 16	<i>Pseudomonas orientalis</i> strain 140e	100	0.0	99.68%	MT605336.1
	<i>Pseudomonas orientalis</i> strain MH90B	100	0.0	99.68%	MT605330.1

Based on BLASTn APD16 isolate had genetically similarities with *Pseudomonas orientalis* strain Z6PO1, *P.orientalis* strain 140e and *P. orientalis* strain MH90B with a value of 99.68% (Table 3). According to Stackebrandt and Goebel (Stackebrandt and Goebel, 1994), that the similarity of the 16S rRNA genes sequences which is less than 97% can be indicated as a new species, the similarity of the sequences between 93-97% can be indicated at the same genus level but different species. And according to Hagström (Hagström et al., 2020), that the similarity of 16S rRNA gene sequences which is more than 97% can be said to be the same species.

Phylogenetic tree is constructed to determine the relationship of a bacterial isolate with other bacteria. The phylogenetic tree design (Figure 3) showed that APD 16 isolate had homology of 16S rRNA gene sequences with *Pseudomonas orientalis* which is Gram-negative in nature and is clearly far apart from *Bacillus subtilis* strain A2 (*outgroup*) which is Gram-positive.

4. Conclusion

A total of successfully 16 bacterial isolates were collected from the sponge *Aplysinasp*. Antimicrobial compounds from bacteria-associated with the sponge *Aplysinasp* against *Escherichia coli* and *Staphylococcus aureus* with the code APD 16. Based on their Gram-staining and biochemical test APD 16 isolate have close relative with *Pseudomonas*, and based on molecular identification use 16S rRNA gene, APD 16 isolate had genetically similarities with *Pseudomonas orientalis*.

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