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Antibacterial Activity of Extracellular Compounds Produced by Bacterial Exosymbion on Sponges against *Staphylococcus aureus* ATCC 25923 Biofilm

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Abstract

Aim: The objective of this study is to evaluate the antibacterial activity of exosymbion bacteria on sponges-derived compounds against *S.aureus* biofilms.

Methods: Sponges were collected from Sancang Beach, Garut regency, West Java. Isolation and purification of exosymbion bacteria using swab and streak method. Then isolation of exosymbion bacterial extracellular product was done by centrifugation at 8000 rpm for 1 min. The Antibacterial activity of extracellular products were tested against *S.aureus* ATCC 25923 plankton using agar diffusion method, while activity test for biofilm form was conducted using turbidimetry method with violet crystal dyes for biofilm staining.

Results: The results showed that from 25 isolates of exosymbion bacteria in three sponge samples, obtained four isolates that have antibacterial activity against *S.aureus* plankton and the highest activity was come from exosymbion on *Aplysilla* sponge. From the four isolates, one isolate No. 22 with 6 days incubation had antibacterial activity against *S.aureus* ATCC 25923 biofilm. The colony morphology of isolate No. 22 was round, yellow, and has mucous structure with the coccus cell shape. The isolate No. 22 was isolated from *Aplysilla* sponge.

Conclusion: It can be concluded that the extracellular product of exosymbion bacteria on sponge could be new target for exploring natural antibiotics against *Staphylococcus aureus* infection in the future.

Keywords: *Staphylococcus aureus,* exosymbion, biofilm, sponge, extracellular

INTRODUCTION

Staphylococcus aureus is a normal flora of skin and human mucous membrane [1]. However, *S. aureus* is a significant pathogen forming biofilms among other Gram-positive bacteria [2]. Biofilm is a form of microorganism attaching to a surface in aqueous environment by forming extracellular polymeric substance (EPS) matrix [3]. Adherent bacterial populations (biofilms) present with an innate lack of antibiotic susceptibility not seen in the same bacteria grown as planktonic populations [4]. The formation of biofilms has been reported to contribute in several pathogenesis of infections, such as skin infections, damaged, chronic and recurrent airway infections, osteomyelitis, and mastitis [5-8]. *Staphylococcus aureus* can cause disease through its ability to spread widely in the tissues and its extracellular substances. Various substances that act as virulence factor can be proteins, including enzymes and toxins [9].

Biofilm resistance to antibiotics has become a global problem. The resistance is due to several factors, including the lack of antibiotic penetration into biofilms, the slow growth of organisms in biofilms, and changes in the expression of bacterial genes that probably made biofilms [10-13]. To overcome this, it is necessary to explore a natural antibacterial against *S. aureus* biofilm.

The ability of antibacterial compounds obtained from other bacteria to inhibit *S. aureus* and methicillin resistant *S. aureus* has also been tested [14-16]. This is in accordance with the definition of antibiotics as a substance produced by a microorganism to kill or inhibit the growth of other microorganisms [17]. Such potent antibacterial bacteria can be found in exosymbion bacteria. The utilization of exosymbion bacteria used in this research was derived from the sponge.

The sponge is one of the natural wealth of the sea that is quite abundant in the Indonesian sea [18]. Sponge reportedly produced bioactive compounds that have antibacterial, antifungal, antitumor and antiviral activity [19]. The ability of sponges to produce bioactive compounds is due to the symbiotic relationship with exosymbion bacteria. These relationships include the provision of nutrients by aiding the metabolic translocation and assisting chemical defenses. The exosymbion bacteria in the sponge are thought to have great potential in producing bioactive compounds that have been isolated from sponges [20]. Therefore, in this research, antibacterial screening against *S. aureus* biofilm has been done by exploring bacterial exosymbion on the sponge from Sancang beach in Indonesia.

MATERIALS AND METHODS

Materials

The sponges used in this research were obtained from Sancang beach Garut regency, Indonesia. While the chemical materials were as follows: sea water, distilled water, sodium chloride (NaCl-Merck), physiological sodium chloride 0.9% (Widatra), potassium chloride (KCl-Merck), sodium hydrogen phosphate ($NaHPO_4$-Merck), potassium dihydrogen phosphate (KH_2PO_4-Merck), crystal violet (Merck), barium chloride (BaCl-Unichem), and

sulfuric acid (H2SO4-Merck). The *Staphylococcus aureus* ATCC 25923 strain was obtained from Clinical Diagnostic and Vaccination Laboratory, PT. Biofarma, Bandung, Indonesia. marine agar (Oxoid), marine broth (Oxoid), Mueller-Hinton Agar (MHA-Pronadisa) and Mueller-Hinton Broth (MHB-Oxoid), Tryptic Soy Broth (TSB-Pronadisa) were used as a growth medium.

Sponge Collection and Determination

Sponges were collected from Sancang beach and determined at the Laboratory of Animal and Invertebrate Taxonomy, School of Biological Sciences and Technology, Bandung Institute of Technology, Indonesia.

Isolation of Exosymbion Bacteria

Before sampling, the physical parameters of water in the sampling area were measured, such as i.e. temperature, pH and salinity. Pieces of sponge were inserted in a sterile bottle containing a sterile physiological NaCl solution. Isolation of exosymbion bacteria was performed using a swab method with sterile cotton. The swab was then suspended into a dilution tube containing 10 ml of NaCl physiological sterile and diluted into a serial dilution that ranged from 10^{-1} to 10^{-6}. The last of dilution tubes were streaked on marine agar, subsequently incubated at 30ºC and observed daily for two weeks. Colonies with different shapes and colors were isolated. Each colony was isolated by using the streak plate method by scratching as a colony of zigzag direction on petri containing marine agar and followed by incubating it at 30ºC for 2 days. Each of these colonies was re-streaked on the marine agar in order to obtain a single colony.

Isolation of Extracellular Compounds-Derived Exosymbion

Isolation of extracellular compounds was done using centrifugation methods. All pure isolates were cultured in 5 ml marine broth medium and incubated at 30^0C in different time of 2, 3, 4, 5 and 6 days. Turbidity of the suspension was measured and compared to the standard level of McFarland turbidity. Then the suspension was centrifuged at 8000 rpm for 1 min. Centrifugation results are then separated between the pellets and supernatant. The extracellular product presence in the supernatant was decanted for an antibacterial activity assay against the *S. aureus* ATCC 25923 plankton and biofilm.

Antibacterial Activity Against *S. aureus* ATCC 25923 Plankton

The antibacterial activity of exosymbion's extracellular product against *S. aureus* ATCC 25923 plankton was conducted using a maceration method. The 20 µL bacterial suspensions with 0.2 McFarland in turbidity were inoculated into a sterile petri dish containing the volume of 20 ml MHA medium. The mixture of bacterial suspension and agar was homogenized until it became solid [21]. The medium was then perforated to make holes for storing a volume of 70 µl extracellular product and incubated at 37 ° C for 24 h. The diameter of inhibitory zones was observed.

Qualitative Biofilm Assay

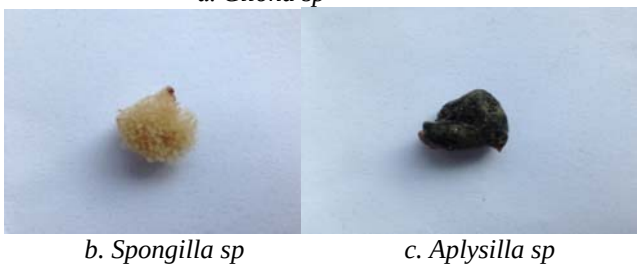
One Ose of *S.aureus* ATCC 25923 colony from slant agar were inoculated into 5 ml Tryptone Soy Broth sterile medium and incubated at 37°C for 24 h. The suspension turbidity, then compared 0.2 McFarland turbidity. The sterile TSB medium was distributed into microtiter well plates of 200 µl / well, then a volume of 0.2 µl S.aureus suspension was added into each well. The microtiter plate, then incubated at 37°C for 24 h. After incubation, the S. aureus plankton and the medium were decanted. A total of 100 µl of extracellular products of each exosymbion and 100 µl of sterile TSB media was inserted into each well, after which it was incubated at 37 ° C for 24 h. The supernatant in each well was removed and the well was rinsed with PBS 2 times. Then performed motile test was done to see the growth of bacteria by using the semisolid medium. Each pellet was taken and inserted into sterilized medium, then incubated at 37°C for 24 h to observe the viability of the *S. aureus* cells. The biofilms formed at the bottom of the well are then heated in an oven at 60^0C for 1 h to be fixed. A total of 100 µl of violet crystals was then added to each well and allowed to stand for 2 min to stain the biofilm. After which, it was rinsed with sterile distilled water until clear rinse water. After the fluids in the wells are discarded, the biofilm form at the bottom of the well was marked by the presence of a violet crystal color layer at the bottom of the well. If it has a positive antibacterial activity against the biofilm, it will not form a violet crystal color layer at the bottom of the well, because no biofilms are formed which absorb the color of violet crystals. The large number of isolated exosymbion bacteria that have antibacterial activity against the biofilm of *Staphylococcus aureus* are noted.

RESULT AND DISCUSSION

Results Of Sponge Collection and Determination

The sponge was obtained from the Sancang beach, Garut district with the physical parameters area as follows: temperature of 33^0C, pH 7.34 and salinity 35 ppm. The results of determination showed that sponges identity were Cliona sp, Spongilla sp and Aplysilla sp. The morphology of sponges could be seen in figure 1.

a. *Cliona sp*



b. Spongilla sp *c. Aplysilla sp*

Figure 1: The morphology of sponges

Bacterial isolates obtained from each sponge sample showed differences in the number of colonies. The difference in the number of colonies was thought to be caused by different types of sponges. The abundance of symbiotic bacteria is not always the same in every type of

sponge [20]. For example, certain species of sponges are known to be symbiotic with several types of bacteria simultaneously, eg Theolella swinhoei symbiotic with unicellular heterotrophic bacteria, unicellular cyanobacteria and filamentous heterotrophyl bacteria. It also found the symbiotic bacteria inhabiting the body of a particular sponge species predominantly. For example sponge Aplysina aerophoba which 40% of its body biomass is filled by bacterial symbionts [22].

Exosymbion Isolation Results

A total of 25 colonies were isolated in marine agar and purified. The type of sponge obtained and the number of isolates can be seen in Table 1.

Antibacterial Activity against *Staphylococcus aureus* ATCC 25923 Plankton Result

The purpose of the antibacterial activity test was to determine the isolates which have antibacterial activity against *S.aureus* plankton. Of the 25 isolates obtained, four isolates had antibacterial activity against *S. aureus* plankton. The results of activity screening can be seen in Table 2. Isolate no. 22 which incubated in 6 days had strong antibacterial activity. The antibacterial activity of exosymbion sponge extracellular products was shown by the clear zone around the hole in the test medium.

Antibiofilm Activity Results

The test was performed using extracellular product from exosimbion sponge bacteria which had been proven had antibacterial activity, i.e. isolates which incubated for 2 and 6 days. The results of screening of antibacterial activity against biofilm *S. aureus* ATCC 25923 can be seen in table 3.

Table 1: Bacterial Isolation Result

Isolates	Colony Morphology				Cell
	Shape	Color	Edge	Structure	Shape
Sp1	round	Light cream	circular	smooth	rod
Sp2	round	Light yellow	circular	slimy	coccus
Sp 3	round	cream	circular	smooth	rod
Sp4	round	Orange	circular	slimy	coccus
Sp5	round	Orange	irregular	slimy	rod
Sp6	round	Cream brownies	irregular	slimy	rod
Sp7	round	Cream	circular	slimy	coccus
Sp8	round	Cream brownies	irregular	slimy	rod
Sp9	round	Whitish cream	irregular	smooth	rod
Sp10	round	cream	circular	slimy	rod
Sp11	round	Orange	circular	slimy	rod
Sp12	round	cream	circular	slimy	rod
Sp13	round	Broken white	circular	slimy	rod
Sp14	round	white	circular	slimy	coccus
Sp15	round	white	circular	smooth	coccus
Sp16	round	cream	circular	smooth	rod
Sp17	round	cream	circular	smooth	rod
Sp18	round	white	irregular	slimy	coccus
Sp19	round	Whitish cream	irregular	smooth	coccus
Sp20	round	cream	irregular	smooth	rod
Sp21	round	dark yellow	circular	smooth	coccus
Sp22	round	Light yellow	circular	slimy	coccus
Sp23	round	yellow	circular	slimy	coccus
Sp24	round	white	circular	smooth	coccus
Sp25	round	Dark Orange	circular	slimy	rod

Table 2: Screening Result of antibacterial extracellular product against Plankton Form

Isolate	Inhibitory zones in periode incubation (days)				
	2	3	4	5	6
Sp1	-	-	-	-	-
Sp2	-	-	-	-	-
Sp 3	-	-	-	-	-
Sp4	-	-	-	-	-
Sp5	-	-	-	-	-
Sp6	-	-	-	-	-
Sp7	-	-	-	-	-
Sp8	-	-	-	-	-
Sp9	-	-	-	-	-
Sp10	-	-	-	-	-
Sp11	-	-	-	-	-
Sp12	-	-	-	-	-
Sp13	-	-	-	-	-
Sp14	-	-	-	-	-
Sp15	-	-	-	-	-
Sp16	+	-	-	-	-
Sp17	-	-	-	-	++
Sp18	-	-	-	-	-
Sp19	-	-	-	-	-
Sp20	-	-	-	-	
Sp21	+	-	-	-	-
Sp22	-	-	-	-	+++
Sp23	-	-	-	-	-
Sp24	-	-	-	-	-
Sp25	-	-	-	-	-

Notes: (-) = antibacterial activity absence ;
(+) = low antibacterial activity;
(++) = medium antibacterial activity;
(+++) = strong antibacterial activity

Table 3: Screening Antibiofilm Result

Isolates	Colored sediment and *S.aureus* viable	
	2 days	6 days
Sp1	+	+
Sp2	+	+
Sp 3	+	+
Sp4	+	+
Sp5	+	+
Sp6	+	+
Sp7	+	+
Sp8	+	+
Sp9	+	+
Sp10	+	+
Sp11	+	+
Sp12	+	+
Sp13	+	+
Sp14	+	+
Sp15	+	+
Sp16	+	+
Sp17	+	+
Sp18	+	+
Sp19	+	+
Sp20	+	+
Sp21	+	+
Sp22	+	-
Sp23	+	+
Sp24	+	+
Sp25	+	+

Note: (-) = antibiofilm activity presence
(+)= antibiofilm activity absence

Staphylococcus aureus infection associated with biofilm formation is of particular concern, as it can increase bacterial resistance to antibiotics compared with the plankton form requiring strong antibacterial to kill *S.aureus* biofilms [4]. Based on the data above, it showed that in the extracellular product isolates of bacteria no. 22 with 6 days incubation had antibacterial activity against *S. aureus* ATCC 25923 biofilm. Semisolid agar was used to detect the viable of *S. aureus* before crystal violet staining, indicated that *S. aureus* biofilm could be inhibited by the extracellular product of isolate no. 22. The antibiofilm and anti-plankton gave the same results that the extracellular product of isolate no. 22 showed the highest activity.

CONCLUSION

The results of this study showed the extracellular product of exosymbion on *Aplysilla* sponge gave the highest antibiofilm activity against *S. aureus* ATCC 25923.

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