Antibacterial activity of metabolites products of *Vibrio alginolyticus* isolated from sponge *Haliclona* sp. against *Staphylococcus aureus*

Happy Nursyam

Faculty of Fisheries and Marine Sciences, University of Brawijaya, Malang, Indonesia

Abstract

The objective of this study was to investigate the antibacterial activity of primary and secondary metabolites from *Vibrio alginoliticus* isolated from sponge *Haliclona* sp. against *Staphylococcus aureus*. A descriptive method was used in this research. The antibacterial activity was analysed by paper disk method. The results showed that the primary metabolites produced by *Vibrio alginoliticus* that is in symbiosis with sponge *Haliclona* sp. were able to effectively inhibit *Staphylococcus aureus* growth with an inhibition zone diameter of 12.9 mm, while the secondary metabolites of 9.9 mm. Electrophoresis analysis of the primary metabolites showed that there were 11 protein bands which were not found in secondary metabolites. Protein bands with low molecular weights presumably had an inhibiting effect on the growth of *Staphylococcus aureus*.

Introduction

Indonesia is an archipelagic country whose natural resources have not been fully explored. The Indonesia natural resources, such plant, sponge, bacteria, *etc*. have been reported (Sabdono and Radjasa, 2006; Prihanto *et al*., 2012; Nursyam *et al*., 2013). The flora and fauna found in the ocean is a huge source for producing various bioactive compunds, *i.e*.: protein, omega-3, vitamins and hormones that are beneficial to health. Sponge is one of the marine organisms that are usually associated with microorganisms. These microorganisms are potential natural resources for the pharmaceutical industry (Mehbub *et al*., 2014). The sponge itself is the biggest source of bioactive compounds (Muniasih and Rachmawati, 1998). Therefore, bacteria or microorganisms that are associated with sponge more likely have bioactive compounds that are similar to the substrate that lives and breeds.

Sponge *Haliclona* sp. has antibacterial activity, antioxidant, anti-inflammatory and anticancer properties. Secondary metabolite from *Haliclona* sp. has been characterised by gas chromatography analysis. It was found that the active fraction on the basis of spectral data with GCMS is a fatty acid mixture. With this potency, we, therefore, need to exploit bioactives compound derived from marine bioresources (Bhimba *et al*., 2013).

The emergence of bacteria resistant to antibiotics is a big concern for exploring new antimicrobial agents (Nursyam *et al*., 2016). The trend of increasing resistance to antibiotics commonly used against *S. aureus* cannot be controlled until now and unless antibiotics are used more carefully (Akindolire *et al*., 2015). Most *Staphylococcus aureus* are being resistant to antibiotics. More than 30% of *S. aureus* is methicillin resistant. This causes a major risk for infection. This phenomenon resulted in a problem on the next selection of antimicrobial agents for the treatment of *Staphylococci* infection. Therefore, prudent antibiotic use and managing antibiotic with further research worldwide is necessary (Saha and Bal, 2013). Many report on bioactive compounds isolated from sponge. The main obstacle in the final development of it is sustainable supply. The concentration of highly active compounds from marine invertebrates is very small, so it is clear that we can not exploit a sponge for this purpose. Therefore, an alternative source for the supply of marine invertebrate organisms is indispensable. Furthermore, according to Suleria *et al.* (2015), almost all marine invertebrates are hosts for marine microorganisms such as bacteria, fungi and micro-algae. The symbiosis of microorganisms and marine invertebrates plays a role in the biosynthesis of bioactive compounds. According to Majali *et al.* (2015), contained metabolites in the sponge were strongly associated with invertebrates metabolites synthesized by microorganisms' simbion. One of the symbiont sponges is *V. alginolitycus*. The aim of this study is to determine the antibacterial activity of *V. alginolitycus* associated with sponge *Haliclona* sp. against *S. aureus*.

Materials and Methods

Haliclona sp. sample was collected at depths around one meter on January, 2016 at Gili Island-Probolinggo, East Java, Indonesia. The samples were placed on sterile bag and transferred to the laboratory as soon as possible. The samples were then stored at 4ºC for the isolation of the microorganism for the next day.

Correspondence: Happy Nursyam, Faculty of Fisheries and Marine Sciences, University of Brawijaya, Jl. Veteran Malang 65145, Malang, Indonesia.
Tel: +62.341553512 - Fax: +62.341557837.
E-mail: happy_nursyam@yahoo.com

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Isolation and identification of bacteria

Isolation of *Vibrio* sp. from *Haliclona* sp. followed Gopi *et al*. (2012), with modification. The sponge was washed with tap water, 70% ethanol and sterile sea water. The sample was crushed and one gram of sample transferred to 9 mL solution of 0.9% Na-fisiologis. One mL of serially diluted samples were spreaded on selective media for *Vibrio* sp. namely, TCBS medium. After 24 hours incubation, appeared colonies were isolated.

Only the best candidate for antimicrobial compound was identified through morphological and biochemical test (Jahan *et al.,* 2015). Furthermore, additional analysis by using Microbact system was applied. Microbact 24E (Oxoid, Basingstoke, UK) was used to identify the stain.

A total of five pure colonies of *V. alginolitycus,* which have been incorporated into TSA media, were centrifuged at 3000 rpm for 15 minutes. The pellets are added with Na-fis about 5 mL and then inserted into well in microbact 24E of 0.1 mL equal to 100 mL and incubated for 18-24 hours at 30°C. The colour change in the wells of microbact 24E was analysed by comparing

the colour table and the result. Then the strain of the isolates could be determined.

Antibacterial assay

Two samples, namely metabolites primer and secunder were used. The bacteria were pre-cultured with only 5 mL on TSA medium. After 24 hour of the culture, the bacteria were transffered to 200 mL of the same media. Erlenmeyer flask was shakeed 250 rpm and incubated on 30°C.

For metabolite primer, the bacteria were harvested when the growth still in the first stage of log phase (after eight hour of main culture). The cells were separated by 10 min centrifugation for 7000 rpm with 4°C. Obtained cell were washed and sonicated for 10 min to extract the metabolite. After centrifugation with the same condition, supernatant was used for metabolite primer sample. For metabolite secunder, the bacteria was harvested when the bacteria reached nearly the end of log phase (after eighteen of main culture). The cells were separated by 10 min centrifugation for 7000 rpm with 4°C. Obtained supernatant was used for metabolite secunder sample. Density of the tested bacterium (*S. aureus*) was 10^8 CFU/mL, which was applied for antibacterial assay. Paper discs were soaked with metabolites and placed onto TSA plate. It was incubated at 37°C for 24 hours. Furthermore, diameter of formed clear zone was measured.

Electrophoresis analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) analysis followed Laemmli (1970) method. Resolved protein visualized by silver staining. To determine the molecular weight of protein samples that have been tested done by calculating the retardaction factor (Rf) value of each bands. Molecular weight was calculated based on Rf value.

Results and Discussion

Isolation and identification of bacteria

Thiosulfate Citrate Bile Salts Sucrose (TCBS) medium will select only deduced vibrio species. Hence, it is a selective medium for *V. alginolyticus*. The results of bacterial cultivation on selective media were shown in Figure 1.

It is well known that *Vibrio* sp. is fluorescent or yellow when grown on TCBS. It is because sucrose in the media is fermented. In addition, positive oxidase of *V. alginoliticus* marked by the purple colour has not changed (Mustapha *et al.,* 2012). Before characterization of the isolate, five pure colonies checked for their antibacterial activity against *S. aureus*. From this result (unpublished data), the best colony in regard of their inhibition, is further investigated.

The results of biochemical test by using manual and microbact system suggested that the isolate was *V. alginolitycus*. shown on the percentage rate of 98%. Identification of *V. alginolitycus* by manual and microbact method could be seen in Table 1. The colour change result on Microbact E-24 system was shown in Figure 2 and described in Table 2.

V. alginolitycus is a type of bacteria that live on a relatively high salinity. Most fluorescent bacteria are halophilic and optimally growth at 20-40‰ salinity of seawater. *Vibrio alginolyticus* that has been identified is yellow and has a diameter of 3-5 mm. *V. alginolitycus* is a gram-negative, fermentative, catalase, oxidase, absent from H_2S production, and positive glucose. *Vibrio* sp. is yellow/fluoresces on TCBS media for fermenting sucrose were contained in the medium (Costinar *et al*., 2010).

The observations were conducted after 12-24 hour incubation. Observation of the growth phase of *V. alginolitycus* bacterial was conducted to investigate the growth phase in order to determine harvest time of bacteria. It is important to achieve primary and secondary metabolites. The growth of bacteria was shown in Figure 3.

Primary metabolites extracted from the bacteria is on the stationary phase (Figure 2). In this phase has been known that bacte-

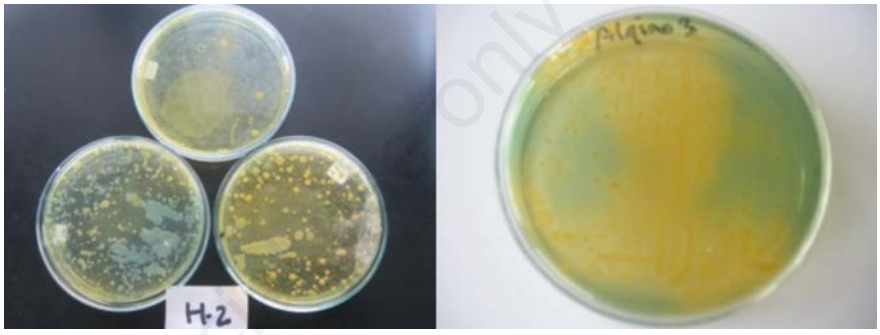

Figure 1. The growth of bacteria on Thiosulfate Citrate Bile Salts Sucrose medium. A) *Vibrio* sp. isolated from *Haliclona* sp.; B) pure isolates of *Vibrio* sp.

Table 1. Identification results of *Vibrio alginolyticus* isolated from *Haliclona* sp.

Types of tests	Methods	Results
Morphological	Naked eye investigation of colony	Circular with regular, edges, slightly convex
Microscopical	Gram staining	Gram negatif
Physiological	Catalase; oxidase; microbact system	+, +, 98% of *Vibrio algynoliticus*

Table 2. Results of Microbact E-24 analysis of *Vibrio alginolitycus.*

Biochemical test	Result	Biochemical test	Result
Lysine	+	Gelatin	+
Ornithige	+	Mallonate	-
H_2S	-	Inositol	-
Glucose	+	Rhamnose	-
Mannitol	-	Sucrose	+
Xylose	+	Lactose	-
ONPG	+	Arabinose	-
Indole	-	Adonnitol	-
Urease	-	Rafinose	-
VP	+	Salicin	-
Citrate	-	Arginin 24 h	-
TDA	-	Arginin 48 h	-

ONPG, o-nitrophenyl-β-D-galactoside; VP, Voges-Proskauer; TDA, tryptophan deaminase.

ria are in the maximum amount, which is about 28 x 10^8 CFU/mL at the 19th hours. The speed of growth and proliferation is zero, this is due to environmental conditions have changed and are not beneficial to the growth and proliferation of battery. Zhang *et al.* (2015), state the condition is caused by exhaustion of nutrients as well as the buildup of toxic metabolic outcomes. The number of newly formed cell is balanced by the number of cells that die.

Secondary metabolites are taken on a decline phase because in this phase there is an increase in bacterial cell deaths that affect the population. This is due to the reduction of nutrients and metabolic waste accumulated more bacterial cell growth is allowed to live in metabolic waste material from dead cells. Secondary metabolites released by bacteria in self-defense (Hassan *et al.,* 2015). From the observation was shown that the decline phase starts at the 20th hour with an average of 2.7 x 10^8 CFU/mL.

Antibacterial activity of *Vibrio alginolyticus*

The observation of the antibacterial activity of primary and secondary metabolites of *V. alginolitycus* symbiont *Haliclona* sp. is shown in Figure 4.

The existence of a clear zone around the paper disc indicated that the extract had an antibacterial activity. Clear zone were only found in metabolite primer extract. Zone of inhibition of primary metabolites are 12.99±0.82. This indicates that the primary metabolite *V. alginolitycus* was more useful for anti *S. aureus*.

Secondary metabolites of *V. alginolitycus* are less effective for inhibiting the growth of *S. aureus*. The diameter of inhibition zone of secondary metabolites of the bacteria *V. alginolitycus* against *S. aureus* was neglectable (Sugito *et al.,* 1997). Aydin *et al.* (2011), states that the primary metabolites of *V. alginolitycus* have a greater inhibitory zone against the target bacteria than secondary metabolites.

Electrophoresis

To compare the protein which contained in metabolite primer and secunder, the electrophoresis analysis was conducted. The results of SDS-PAGE of the primary and secondary metabolites produced by *V. alginolitycus* symbiont with the sponge *Haliclona* sp. was depicted in Figure 5, while the calculation results are shown in Table 3. Protein bands were obtained from both primary and secondary metabolite. The primary metabolite had 11 protein bands more compared with secondary metabolite (Table 3). Their molecular weight were 107.6754 kDa; 66.05299 kDa; 56.1243 kDa; 49.6701 kDa; 42.2040 kDa; 40.5199 kDa; 38.1189 kDa; 35.8607 kDa; 34 429 kDa; 23.86 kDa; 21 554 kDa, respectively. Among them are responsible for inhibiting the growth of *S. aureus*. Deduced protein which inhibited *S. aureus* is low molecular proteins. Protein with low molecular weight is usualy responsible for antibacterial activity (Sarnthima *et al.,* 2011). Furthermore, Chellaram *et al.* (2012) and Laemmli (1970), stated that there are compounds of

Figure 2. Test of Microbath 24-E system.

Table 3. Molecular weight compound (kDa) of primary and secondary metabolites.

No.	Primary metabolites	Secondary metabolites	No.	Primary metabolites	Secondary metabolites
1	107.6754	-	15	45.78512	39.70322
2	91.49033	91.49033	16	42.20401	-
3	82.63459	84.33435	17	40.5199	-
4	79.33714	80.96908	18	38.1189	-
5	76.17127	77.73809	19	35.86017	-
6	71.65775	73.13173	20	34.42921	-
7	68.79832	68.79832	21	32.38911	32.38911
8	66.05299	-	22	29.85577	30.4699
9	63.41721	63.41721	23	26.9659	26.4224
10	60.88661	59.65943	24	24.85674	22.91256
11	58.45699	54.99313	25	23.86486	-
12	56.12432	-	26	21.55488	-
13	49.6701	-	27	19.86895	19.86895
14	47.68807	47.68807			

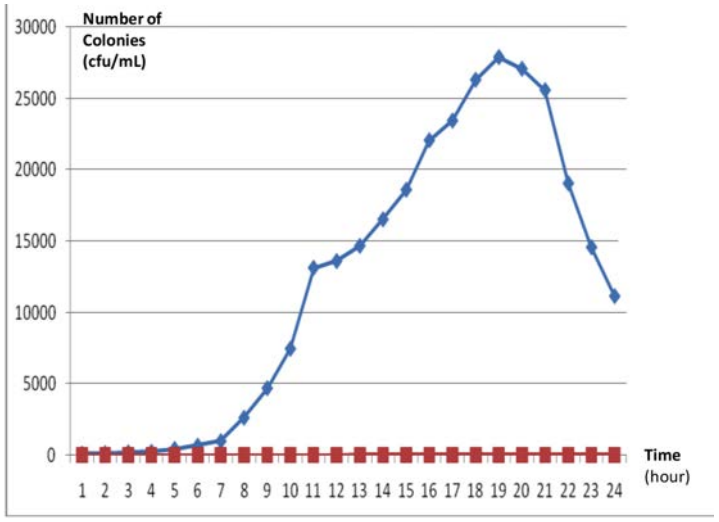

Figure 3. Bacterial growth curve of *Vibrio alginolitycus*.

Figure 4. Inhibition zone of *Vibrio alginolitycus* extract against *Staphylococcus aureus*: A) primary metabolites; and B) secondary metabolites.

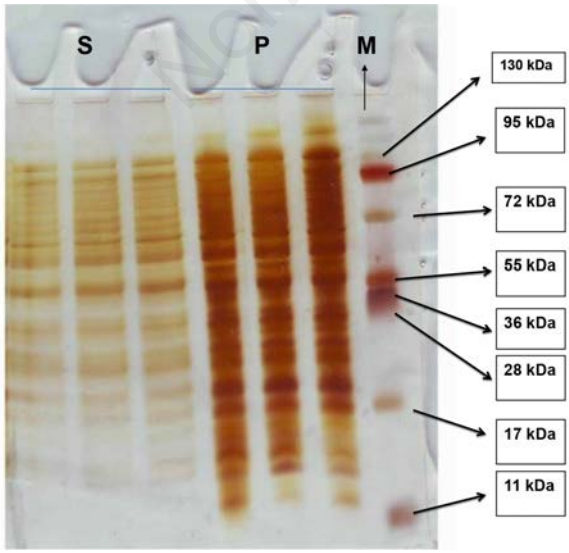

Figure 5. Results of electrophoresis analysis of the primary and secondary metabolites of *Vibrio alginolitycus*. S, secondary metabolites; P, primary metabolites; M, marker.

the class of proteins that can make-bacterial symbiosis with marine invertebrates become antagonistic towards other bacteria. Saranya and Hemashenpagam (2011), also state that microorganisms antagonistic towards other microorganisms are due to produce antibiotics, bacteriocins, siderophores, lysozyme, and protease.

Conclusions

The primary metabolite of *V. alginolitycus* that was in symbiosis with sponge *Haliclona* sp. has the ability to inhibit the growth of *S. aureus*. Low molecular weight protein is presumably able to inhibit the growth of *S. aureus* with respect to secondary metabolites. The role of the proteins regarding their inhibiting action towards *S. aureus* needs to be further clarified.

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