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Antibacterial Activity of *Haslea ostrearia* Supernatant Adapted in Indonesia against Pathogenic Bacteria Relevant to Mariculture (*In-Vitro Study*)

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ABSTRACT

Haslea ostrearia has known as the only diatom which synthesizes a water-soluble blue pigment, marennine. It has some biological activities such as allelopathy, antioxidant, antibacterial, antiviral, and growth inhibitor. Marennine is available in two forms, intracellular which located in the apical of the cell and extracellular which released into the culture medium. This research aimed to test the bioactivity of *Haslea ostrearia* supernatant adapted in Indonesia as an antibacterial against pathogenic bacteria relevant to marine culture using in-vitro study. This research was using an explorative method, data analysed statistically and descriptive-comparatively observed. Observation parameters were the inhibitory zone of antibacterial activity, absorbance value of MIC test, and bacterial growth qualitatively from MBC test. Marennine concentration in the supernatant of *Haslea ostrearia* adapted in Indonesia is about 3.74 mg. L^{-1}. The highest concentration supernatant of *Haslea ostrearia* that shown antibacterial activity is 3.5 mg. L^{-1} with inhibitory zone diameter is about 6.87 mm for *Staphylococcus aureus* and 7.14 mm for *Vibrio harveyi*, correspondingly. The minimum concentration that inhibits the growth of *Staphylococcus aureus* is 0.03 mg. L^{-1}, while for *Vibrio harveyi* is 0.06 mg. L^{-1}. Antibacterial activity of supernatant *Haslea ostrearia* originally adapted in Indonesia classified into bacteriostatic.

Keywords: *Antibacterial, In vitro study, Staphylococcus aureus, Supernatant of Haslea ostrearia, Vibrio harveyi*

1. Introduction

There are two problems in the sustainability of aquaculture production, the fish mortality which due to infection of pathogenic bacteria and the degradation in environmental quality. These conditions are mutually influential along with the development of cultivation systems (Taukhid & Purwaningsih, 2011). Overall, the potential economic losses due to disease outbreaks caused by pathogenic microorganisms are significant and have an impact on sustainability, stock depletion, and income from aquaculture (Bondad-Reantaso et al., 2005).

Synthetic antibacterial agents in maricultural have been widely used and it is considered as an effective solution to maintain maricultural production. For instance,

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antibacterial which commonly used in maricultural include oxytetracycline, sulphonamide, and sulfamerazine which are used to inhibit the growth of pathogen *Aeromonas salmonicida* with significant results. However, the long-term use of these synthetic antibacterial agents can cause multidrug resistant and can cause environmental pollution. Therefore, the use of antibacterial agents originating from natural products are urgently needed due to their low side effects (Sumino, Supriyadi, & Wardiyanto, 2013).

Microalgae have been used as antibacterial agents in maricultural. Among microalgae species, diatom from the West Coast of France is the *Haslea ostrearia.* This diatom has long been known to produce marennine, a water-soluble blue generic pigment. The blue pigment shows a variety of biological activities such as antibacterial, antiviral (Bergé et al., 1999), antioxidant (J. Pouvreau, Pondaven, Morançais, Fleurence, & Guérard, 2006), allelopathy (Prasetiya et al., 2016)(J. B. Pouvreau, Housson, et al., 2007), and growth inhibitor (J. B. Pouvreau, Housson, et al., 2007). For decades, *Haslea ostrearia* has been believed to be the only diatom capable of synthesizing blue pigments at its apical end (Gastineau et al., 2014).

Naturally, marennine has been considered as the greening agent in the oyster greening phenomenon in *Crassostrea gigas* that are cultivated on the West Coast of France. There are two forms of marennine, the intracellular form and the extracellular form, where these two forms of pigment have different characteristics and biological activities (Gastineau et al., 2014). Antibacterial assay of extracellular marennine shows good activity at low concentrations (Gastineau et al., 2014) [5]. Thus, based on the results of these studies, marennine can be a solution to the problems of aquaculture activities related to disease attacks, especially by pathogenic bacteria.

Pigments are considered secondary metabolites produced by a living organism as a form of self-defence against unfavorable environmental conditions, adaptation, and self-protection from enemies and/or diseases (Sahidin, 2015). *Haslea ostrearia* which are cultivated in Indonesia certainly experiences a process of adaptation which may change its biological activities.

The purpose of this study was to test the bioactivity of the supernatant of *Haslea ostrearia* which are adapted in Indonesia as an antibacterial for pathogenic bacteria relevant to maricultural. Considering that the use of pure marennine in maricultural is not practical and cost-demanding.

2. Materials and Methods

The supernatants of *Haslea ostrearia* adapted in Indonesia was obtained from the Central Laboratory of Life Science (LSIH) Universitas Brawijaya Malang and Laboratory of Mer Molécules Santé Le Mans Université. The pathogenic bacteria isolates—*Staphyloccous aureus* and *Vibrio harveyi*—were obtained from the Laboratory of Molecular Microbiology and Biotechnology, Faculty of Fisheries and Marine Science, Universitas Padjadjaran collections.

2.1. Marennine concentration estimation

Estimation of marennine concentration from *Haslea ostrearia* supernatant was done following the method from (Prasetiya et al., 2016)(J. B. Pouvreau, Morançais, Fleurence, & Pondaven, 2007)(Robert, Morançais, Pradier, Mouget, & Tremblin, 2002). The concentration of marennine was calculated as follows:

$$[C] = \left\{ \frac{A\lambda max}{\varepsilon\lambda max.L} \right\}$$

Notes:

C = The concentration of Analyte (mg. L^{-1})
Aλmax = Absorbance value at λmax
ελmax = The absorption coefficient of marennine (12.13 l $g^{-1}cm^{-1}$)
L = Cuvette width (1 cm)

2.2. Antibacterial activity screening

Screening for the antibacterial activity was carried out on two species of bacteria, namely *Staphylococcus aureus* and *Vibrio harveyi* which represented positive- and negative-gram bacteria, respectively. Steps for antibacterial activity assay included the preparation of Nutrient Agar (NA) and Nutrient Broth (NB) medium, the preparation of bacterial suspensions, creating McFarland standard, stocking of *Haslea ostrearia* supernatant solution in various concentrations, and the antibacterial activity assay using Agar Well Diffusion method and Disc Diffusion method (Balouiri, Sadiki, & Ibnsouda, 2016). The diameter of the inhibitory zone was adjusted to the inhibitory zone classification according to David and

Stout Classification (Jannata, Gunadi, & Ermawati, 2014).

2.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum Inhibitory Concentration (MIC) is the minimum concentration as an antimicrobial which can inhibit microorganisms after 18 hours incubation period. In this study, the determination of MIC followed the method from (Soelama, Kepel, & Siagian, 2015). Briefly, 11 sterile tubes were prepared for serial dilution. The first 9 tubes were labelled with 1-9 number, the first final tube was labeled with $K^{(+)}$ which correspond to the positive control. This positive control contained bacterial suspension with turbidity equivalent to the standard solution of Mc Farland 0.5. The second final tube was labelled $K^{(-)}$ which correspond to negative control containing *H. ostrearia* supernatant solution. Furthermore, tube 1 was filled with 4 ml of *H. ostrearia* supernatant. Tubes 2-9 was filled with 2 ml NB medium. Moreover, a total of 2 ml of solution from tube 1, was placed into tube 2. A solution was then mixed and homogenized with an equal concentration of 50 % of the original concentration of supernatant (3.74 mg. L^{-1}). The similar step was done until tube 9, thus all *H. ostrearia* supernatant concentrations contain marennine with a ratio of 1:2 (v/v).

The turbidity test method was performed by adding 1 ml of the bacterial suspension to the treatment tubes (1 to 9). Then all the tubes were incubated using an incubator shaker at 30 ^{o}C for 18 hours. MIC test was carried out in duplicate for each type of bacteria.

The Minimum Bactericidal Concentration (MBC) values are the lowest antimicrobial concentration which can kill 99.9 % of the bacterial culture during a specified period. Determination of MBC concentration of an antimicrobial was carried out by placing 100 µl from each MIC solutions in the tube into a petri dish containing NA medium prior to incubation at 35 ^{o}C for ±8 hours. The MBC value was determined when bacterial growth absent in NA medium (Soleha, 2015).

2.4. Data analyses

All data concerning the diameter of the inhibitory zone were analysed descriptively and statistically using the One-Way ANOVA (Analysis of Variance) method by Statistical Product Services Solution Program (SPSS 24) with a confidence level of 95 % or α = 0.05. All statistical analyses were performed after the normality test. When significant was observed, Duncan was performed as a follow-up test. On the other hand, the absorbance value which obtained from the MIC test method and the visual data which obtained from the MBC test were analysed descriptively and comparatively to determine the antibacterial activity of supernatant of *H. ostrearia*.

3. Results and Discussions

3.1. Marennine concentration estimation

The concentration of marennine in the supernatant of *H. ostrearia* (adapted in Indonesia) was 3.74 mg. L^{-1}. This concentration was lower compared to marennine that contained in the supernatant of *H. ostrearia* which cultivated in France, which was 5 mg. L^{-1} (Gastineau et al., 2014).

The low concentration of marennine in the supernatant of *H. ostrearia* (adapted in Indonesia) can be caused by a biological response to an environmental factor, namely irradiance. *H. ostrearia* in Indonesia was cultivated on 100 µmol photons m^{-2} s^{-1} with dark/light photoperiod for 0/24 hours per day, while in France *H. ostrearia* is cultivated at 125 µmol photons m^{-2} s^{-1} with dark/light photoperiod for 10/14 hours per day. Indeed, high irradiance has several effects on the cell's ultrastructure of the *H. ostrearia*, such as shrinking the size of its chloroplast. Besides that, high irradiance also causes major changes to the composition of pigments (Mouget, Tremblin, Morant-Manceau, Morançais, & Robert, 1999).

Cells containing essential pigments for photosynthesis (chlorophyll-a and c, fucoxanthin) decrease when cells are acclimatized to high irradiance. Cells containing chlorophyll-a which are low in high irradiance is associated with a decrease in the size or number of photosynthetic units (PSU). The *H. ostrearia* seeks to minimize damage due to high radiation by closing the growth rate of the PSII, which causes a decrease in the absorption quantity of energy used for photochemistry (Mouget et al., 1999).

Based on this phenomenon, the growth H. *ostrearia* and the photosynthetic characteristics along with its pigment composition show a high degree of tolerance to light radiation. Therefore, despite the change in irradiance during the cultivation process of *H. ostrearia* in Indonesia

marennine is still produced. However, its concentration is strongly influenced by the level of irradiance and photoperiod. This results also confirmed the previous research from (Rech, Morant-Manceau, & Tremblin, 2008) (Prasetiya et al., 2016) (Mouget et al., 1999) *marennine* production can be influenced by other factors such as lack of nutrition and competition with other organisms as well as the bioactivity that has been shown in many studies (Mouget et al., 1999).

3.2. Antibacterial activity screening of H. ostrearia (Adapted in Indonesia) supernatant

a. Disc diffusion method

The antibacterial assay results using the disc diffusion method are presented in Table 1.

Table 1. Antibacterial assay of disc diffusion method

Solutions	Bacteria	Concentrations (mg. L^{-1})	Average Inhibitory Zone Diameter (mm)	
			24-h	48-h
Chloramphenicol (+ Control)	*S. aureus*	30	7.45±0.57	7.98±0.50
	V. harveyi	30	7.87±0.99	7.99±0.60
Sterile Sea Water (- Control)	*S. aureus*	0	6.58 ± 0.35	6.68 ± 0.14
	V. harveyi	0	6.49 ± 0.15	6.48 ± 0.22
H. ostrearia Supernatant	*S. aureus*	2	7.14 ± 0.25	7.17 ± 0.64
		2.5	7.75 ± 0.69	7.67 ± 0.93
		3	7.13 ± 0.18	7.20 ± 0.30
		3.5	7.27 ± 0.50	7.49 ± 0.39
	V. harveyi	2	6.60 ± 0.06	6.81 ± 0.12
		2.5	6.84 ± 0.21	7.19 ± 0.20
		3	6.79 ± 0.14	7.22 ± 0.23
		3.5	6.82 ± 0.07	6.96 ± 0.18

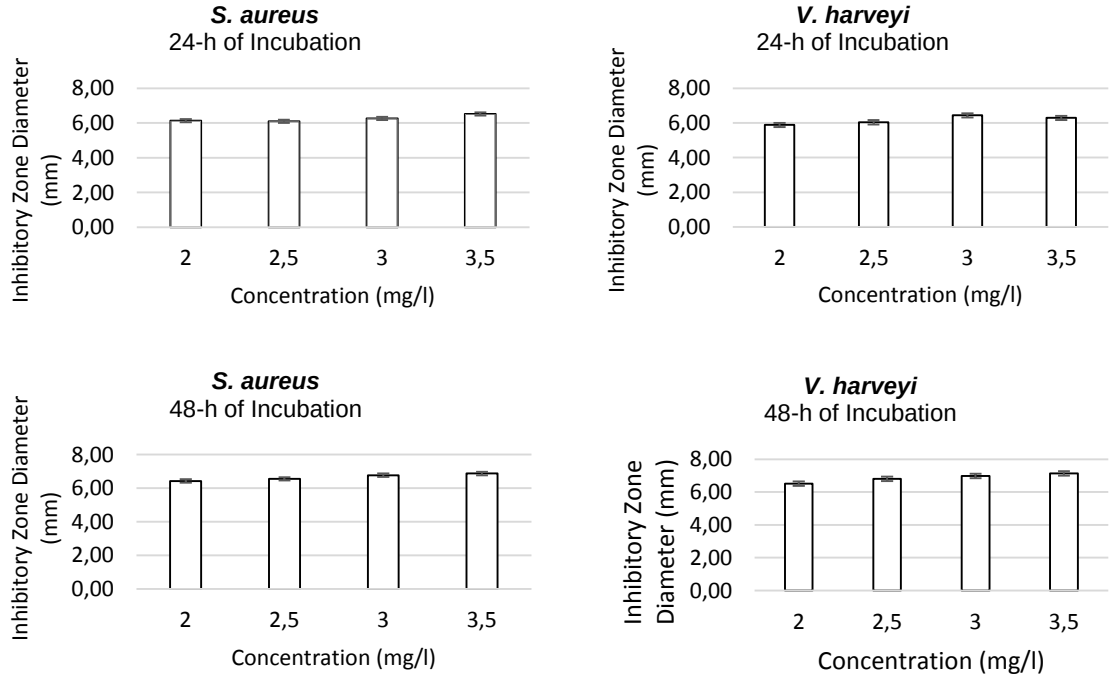

Figure 1. The diameter of the inhibitory zone using disc diffusion method

Table 1 shows that the supernatant of H. ostrearia (adapted in Indonesia) has antibacterial activity. The concentration which showed the highest antibacterial activity against S. aureus is 2 mg. L^{-1} both after 24-h incubation period and after 48-h incubation period with each diameter of inhibitory zone 7.75 mm and 7.67 mm. Whilst, the concentration which showed the highest antibacterial activity against V. harveyi is 3 mg. L^{-1} with 7.22 mm after the 48-h incubation period.

The diameter of the inhibitory zone fluctuated along with the increase of concentration (Figure 1). This showed that the variation of concentration did not significantly influence the *H. ostrearia* supernatant antibacterial ability. In addition, the high variation on these results also showed that the antibacterial activity assay of supernatant *H. ostrearia* using disc diffusion method was less effective. The difference in diameter of the inhibitory zone can be influenced by several factors such as the concentration of the solution containing antibacterial agent compounds, type and species of bacteria which used in the test, culture medium. the test method, and the speed of diffusion (Volk & Wheeler, 1989).

The diameter of the inhibitory zone created by the *H. ostrearia* supernatant using disc diffusion method ranged from 6.48 to 7.99 mm which indicates that it has a moderate category of antibacterial activity. It can be caused by the supernatant diffuses rapidly from the disc to agar medium, and also evaporation rate during the incubation process which may affect the activity of antibacterial compounds characterized by a fairly narrow diameter of the inhibitory zone (Brooks, Carroll, Butel, Morse, & Meitzner, 2013). It appeared that concentration of supernatant 2.5 mg. L^{-1} concentration affects bacterial growth on both species.

The ANOVA test on inhibitory zone diameter which formed on disc diffusion antibacterial assay did not significant ($p>0.05$). This can be caused by the narrow range of the concentrations which used in the study so that the diameter of the inhibitory zone was not significantly different.

b. *Agar well diffusion method*

The antibacterial assay using the agar well diffusion against *S. aureus* at 3.5 mg. L^{-1} showed inhibitory zone diameter as much as 6.52 and 6.87 mm after 24 and 48-h of the incubation period, respectively. Whilst, the highest concentration that showed antibacterial activity on *V. harveyi* was at 3 mg. L^{-1} with inhibitory zone diameter 6.44 mm after the 24-h incubation period and 3.5 mg. L^{-1} with inhibitory zone diameter 7.14 mm after the 48-h incubation period.

Table 2. Antibacterial activity of agar well diffusion method

Solutions	Bacteria	Concentration (mg. L^{-1})	Average Inhibitory Zone Diameter (mm)	
			24-h	48-h
Chloramphenicol (+ Control)	*S. aureus*	30	6.34 ± 0.16	4.94 ± 1.94
	V. harveyi		6.63 ± 0.68	6.99 ± 0.63
Sterile Sea Water (- Control)	*S. aureus*	0	5.28 ± 0.40	5.50 ± 0.32
	V. harveyi		5.65 ± 0.37	5.84 ± 0.96
Haslea ostrearia Supernatant	*S. aureus*	2	6.15 ± 0.17	6.43 ± 0.23
		2.5	6.10 ± 0.33	6.56 ± 0.25
		3	6.26 ± 0.22	6.77 ± 0.19
		3.5	6.52 ± 0.41	6.87 ± 0.18
	V. harveyi	2	5.89 ± 0.45	6.52 ± 0.17
		2.5	6.04 ± 0.32	6.82 ± 0.34
		3	6.44 ± 1.08	6.98 ± 0.27
		3.5	6.30 ± 0.16	7.14 ± 0.45

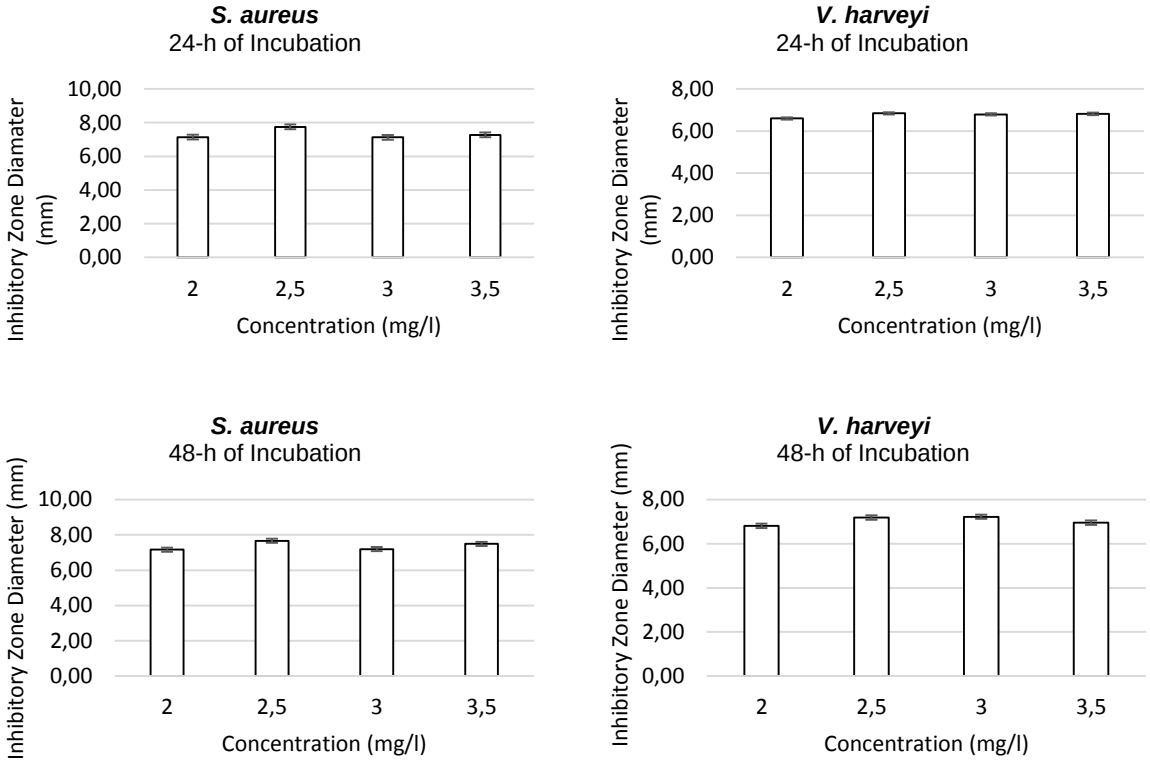

Figure 2. The diameter of the inhibitory zone using agar well diffusion method

The diameter of the inhibitory zone which formed in antibacterial activity assay using agar well diffusion method on both bacteria overall seem larger along with the increase of the concentration (Figure 2). This shows that the higher concentration of H. ostrearia supernatant, the antibacterial activity becomes stronger.

In accordance with the study from Falaise *et al.* (2016) (Falaise et al., 2016), 0.01 mg. L^{-1} of marennine slowed the growth of bacteria while the growth of bacteria was inhibited at 1 mg/ml. Additionally, the inhibition rate by marennine against *Vibrio splendidus* increased proportionally to marennine concentration. Furthermore, the supernatant of *H. ostrearia* can inhibit the growth of both positive- and negative-gram bacteria. Hence, the supernatant of *H. ostrearia* has a broad spectrum of antibacterial, but in the medium category [17].

The antibacterial activity possessed by *H. ostrearia* supernatant is thought to be caused by the marennine content consisting of polyphenols and glycosides (Gastineau et al., 2014)(Mouget et al., 1999). Polyphenols are compound which has phenol groups that have properties similar to phenol. The mechanism for inhibiting the growth of bacterial colonies caused by phenol, which is at a certain concentration of phenol can totally damage the cytoplasmic membrane and precipitate proteins. (Volk & Wheeler, 1988). Given the fact that most bacterial cell wall structures consist of proteins and fats, the reaction between phenol and bacteria's cell wall will damage the intermolecular hydrogen bonds in proteins, thus it will easily separate and binds to other compounds (Brooks et al., 2013).

Other compounds contained in marennine are glycosides (Gastineau et al., 2014). The mechanism of interactions of glycosides with microbial cells is probably due to the formation of the receptor-glycoside complex through hydrogen bonds, which will be decomposed after passing through the cell membrane. The penetration of antibacterial compounds into cell membrane will cause coagulation of proteins and cell membranes until the cells are lysis. However, along with the length of incubation time, it causes decreased activity of glycosides against microbes. This is

due to the decomposition of hydrogen bonds formed between acceptors and glycosides so that the activity decreases (Sudaryati & Naiola, 2007).

The ANOVA test on inhibitory zone diameter which formed on agar well diffusion antibacterial assay did not significant ($p>0.05$).

3.3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Haslea ostrearia Supernatant

Based on spectrophotometry results, *H. ostrearia* supernatant can inhibit the growth of bacteria. Minimum Inhibitory Concentration (MIC) of *H. ostrearia* supernatant were 0.03 against and 0.06 mg. L^{-1} for S. aureus and V. harveyi, respectively.

The maximum concentration of 3.74 mg. L^{-1} still cannot kill the bacteria. It means the highest concentration of supernatant which released to the medium by *H. ostrearia* (adapted in Indonesia) is not bactericidal but bacteriostatic.

Either from antibacterial activity screening, MIC test, or MBC test, the supernatant of *H. ostrearia* showed higher antibacterial activity on S. aureus (positive-gram bacteria). This result is supported by the fact that positive-gram bacteria are usually more sensitive than negative-gram bacteria. Moreover, the structure of a single and relatively simple cell wall of positive-gram bacteria will facilitate the entry of substances that can damage bacterial cells compared to negative gram bacteria with a three-layered cell wall structure (Nurmala, Virgiandhy, Andriani, & Liana, 2015).

Like other secondary metabolites, marennine which contained in the supernatant of *H. ostrearia* is very sensitive to the environmental changes. Marennine production strongly influenced by a large number of cells of *H. ostrearia* and irradiance, High irradiance produces high marennine but the growth of the cells is stunted (Jean Luc Mouget, Rosa, Vachoux, & Tremblin, 2005).

The supernatant production of *H. ostrearia* which adapted in Indonesia is strongly influenced by different cultivation environment. The difference of pH in the cultivation process causes changes in the chemical structure of the marennine inside. This is supported by the colorimetric study results conducted on marennine with the treatment of differences in pH and light illumination which proved that the pigments are blue-green and undergo changes in color and chemical structure. When the pH increases from 2.5 to 7, the marennine changes color from blue-green to yellow-green. These different spectral properties showed differences in the marennine chemical structure (J.-B. Pouvreau et al., 2008).

4. Conclusion

Marennine which is contained in the supernatant of *H. ostrearia* (adapted in Indonesia) is about 3.74 mg. L^{-1}. The highest antibacterial activity found at 3.5 mg. L^{-1} with inhibitory zone diameter as much as 6.87 and 7.14 mm against *Staphylococcus aureus* and *Vibrio harveyi*, respectively. The MIC of *H. ostrearia* supernatant against S. aureus and V. harveyi was at 0.03 and 0.06 mg. L^{-1}, respectively. This study showed that the supernatant of *H.ostrearia* which has adapted in Indonesia could be used as bacteriostatic for bacterial pathogens in mariculture.

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