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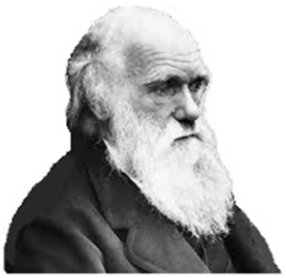

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The effectivity of metabolite secondary extract of bacteria associated with sea grass (*Cymodocea rotundata*) for Vibriosis treatment in tiger shrimp (*Penaeus monodon*)

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ABSTRACT

The natural occurrence of the shrimp (*Penaeus monodon* Fabricius, 1798) and seagrass (*Cymodocea rotundata* Asch. & Schweinf.) is found in the area of Indian Ocean, Indonesia, for central Pacific. Vibriosis can cause death in larvae, juvenile and adult shrimp almost close to 100%. Antibacterial substances from secondary metabolites are produced by plants and animals to reduce the use of synthetic antibiotics. The purpose of this research was to determine the ability of secondary metabolites contained in the bacterial extract of the association of sea grass *Cymodocea rotundata* (isolate code BA.1) to treat vibriosis in tiger shrimp (*Penaeus monodon*). This research was conducted at the Laboratory of Integrated Biotechnology and Laboratory of Aquaculture, Fisheries and Marine Sciences Faculty, Universitas Padjadajaran, and at the Brackish and Southern Ocean Aquaculture Development Center Pangandaran, from June to September 2018. The research method used was experimental, with completely randomized design (CRD) consisting of five treatments with three replications including; control treatment (concentration of 0 $mg \cdot L^{-1}$), 150 $mg.L^{-1}$, 300 $mg \cdot L^{-1}$, 450 $mg \cdot L^{-1}$ and 600 $mg \cdot L^{-1}$. Observations made include clinical symptoms (morphology, behavior) and survival rate. Clinical symptoms of infected tiger shrimp during the *in vivo* test were red spots on the abdomen and necrosis of some leg and tail segments. The behavior of tiger shrimp mostly shows less responsive movements to fish-feed on the first day, and showing responsive and active behavior on the fifth day. *In vivo* test results for 14 days showed that the highest survival rate of 51.67% was in case of the addition of antibacterial extracts of BA.1 with a concentration of 300 $mg \cdot L^{-1}$.

Keywords: bacteria association, *Cymodocea rotundata, Penaeus monodon*, secondary metabolites, survival rate, vibriosis, *Vibrio harveyi*

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1. INTRODUCTION

One of the reasons of vibriosis is that often found is *Vibrio harveyi* which can cause low survival of larvae and adult shrimp with mortality up to 100% [1]. The clinical symptoms of shrimp infected with vibriosis are red spots on the pleopod, uropod and abdominal, the gills are slightly brown red, and swim slowly [2]. The use of antibiotics has been done previously as a medicine for the treatment of infectious diseases. Provision of antibiotics continuously and use with inappropriate doses can cause pathogenic organisms to become resistant and leave residues in cultivated animals.

The use of antibacterial substances from secondary metabolites produced by plants and animals has been developed which aims to reduce the use of synthetic antibiotics. Some research has been conducted, including the sea grass. Based on the studies, a comparison of the antibacterial activity of sea grass extract and bacterial extract of sea grass *Cymodocea rotundata* species taken from Pramuka Island, Kepulauan Seribu, showed that the activity of antibacterial substances from bacterial association extract was greater than sea grass extract. Compounds contained in the secondary metabolites of bacteria associated with sea grass *Cymodocea rotundata* include steroid, alkaloid, and saponin [3].

The activity of antibacterial substances of bacteria associated with sea grass needs to be further investigated so that their use in the treatment of vibriosis disease can be well known. Therefore, it is necessary to test the effectiveness of extracts of secondary metabolites produced by bacteria associated with sea grass *C. rotundata* for the treatment of vibriosis in tiger shrimp (*Penaeus monodon*). The isolates of bacteria association from Laboratory of Integrated Biotechnology, Fisheries and Marine Sciences Faculty, Universitas Padjadjaran, were used.

2. MATERIALS AND METHODS

The research began with an *in vitro* antibacterial activity of secondary metabolites extract of bacteria associated with sea grass *Cymodocea rotundata* to analyze the inhibitory ability of the compound against bacteria of species *Vibrio harveyi*. The LC_{50}-24 hour test was used to determine the concentration of the secondary metabolites extract of bacteria associated with sea grass *Cymodocea rotundata* to treat tiger shrimp larvae infected with *Vibrio harveyi*. Tiger shrimps of age post larvae day of 12 (PL 12) infected *Vibrio harveyi* were treated with antibacterial extracts through 14 days of immersion with five different concentrations: 0 $mg \cdot L^{-1}$, 150 $mg \cdot L^{-1}$, 300 $mg \cdot L^{-1}$, 450 $mg \cdot L^{-1}$, and 600 $mg \cdot L^{-1}$.

2. 1. Extraction of Bioactive Compounds

Bacteria Associated with Seagrass *Cymodocea rotundata* (isolate code BA.1) were cultured in Nutrient Broth (NB) medium for five days. Bulk cultures that have been shredded are harvested and centrifuged (4000 rpm) for 20 minutes. The supernatant obtained was extracted in a separatory funnel with 10% mixture of methanol in ethyl acetate. Comparison of supernatants and solvents (1:1) was used. The ethyl acetate fraction is then taken and evaporated at 40 ºC [4].

2. 2. Antibacterial Activity Test (*In Vitro*)

The antibacterial activity test in this research used the Disk-Diffusion Assay method which refers to the procedure that was carried out by the Laboratory of Pests and Diseases BBPBAP Jepara, with four different concentrations extract (1 mg·L^{-1}, 10 mg·L^{-1}, 100 mg·L^{-1}, and 1000 mg·L^{-1}), positive control tests using chloramphenicol and negative controls using a 10% methanol solvent in ethyl acetate. The bacteria were incubated for 7 × 24 hours at 37 ºC. The diameter of the inhibition zone was measured using a caliper.

2. 3. LC_{50} Test for Antibacterial Extracts

The LC50 test method refers to the procedure performed by Meyer *et al.* (1982) [5] which is a test sample of acclimatized tiger shrimp larvae. Then 15 shrimp larvae were inserted in a container containing one litre of sea water and an antibacterial extract (BA. 1) with different concentrations (0 mg·L^{-1}, 50 mg·L^{-1}, 100 mg·L^{-1}, 250 mg·L^{-1}, 500 mg·L^{-1}, and 1000 mg·L^{-1}). Mortality observation was carried out for 24 hours with an observation interval of 15 minutes, 30 minutes, 1 hour, 2 hours, 6 hours, 12 hours, and 24 hours. The LC50 value was calculated using the EPA Probit program.

2. 4. Antibacterial Effectiveness Test (*In Vivo*)

The *in vivo* test was carried out with the addition of antibacterial bacteria associated with *Cymodocea rotundata* through water to tiger shrimps which were previously infected by bacteria of *Vibrio harveyi* with a concentration of 10^6 CFU· mg·L^{-1}. Tests were carried out for 14 days and observed for clinical symptoms, survival rate, and water quality.

2. 5. Data Analysis

The results of observing the survival rate of tiger shrimp larvae were analyzed using variance analysis and the real differences between treatments were followed by Duncan's multiple distance test with a level of 5% [6]. The results of observing clinical symptoms and water quality were analyzed descriptively.

3. RESULT AND DISCUSSION

3. 1. Antibacterial Activity of Secondary Metabolite of Bacteria (BA.1) Extract

Antibacterials found in the bacteria (BA.1) extract were tested for their activity against *Vibrio harveyi*. Antibacterial of bacteria (BA.1) extract showed the ability to inhibit the growth of *Vibrio harveyi* (Table 1).

Based on the results of testing the antibacterial of bacteria (BA.1) extract against *Vibrio harveyi*, it was found that each concentration has the ability to inhibit the different *V. harveyi*. The higher the concentration of the antibacterial of BA.1 extract is given, the wider the diameter of the inhibitory zone shown (Table 1). The treatment of *in-vitro* test with a concentration of 1000 mg·L^{-1}, and 100 mg·L^{-1}, had a moderate antibacterial ability, while the treatment with a concentration of 1 mg·L^{-1}, and 10 mg·L^{-1}, had a weak antibacterial ability. The criteria for the ability of antibacterial power with inhibitory zone diameters of 5-10 mm are categorized as being moderate, whereas at the diameter of the inhibition zone of 5 mm or less were categorized as weak [7].

Table 1. Antibacterial Activity of Secondary Metabolite Extract of Bacteria Associated with Seagrass *Cymodocea rotundata*

Extract	Extract Conc. ($mg \cdot L^{-1}$)	Inhibition Zone (mm) at the Incubation Day to					
		1	2	3	4	5	6
Cholramphenicol (+)	30	1.73 ± 0.230	1.82 ± 0.169	1.92 ± 0.135	1.78 ± 0.319	1.73 ± 0.436	1.71 ± 0.420
Ethyl Acetate (–)	0	1.22 ± 0.280	1.34 ± 0.147	1.55 ± 0.137	1.97 ± 0.796	1.88 ± 0.782	1.80 ± 0.717
BA.1 Extract	1	2.29 ± 0.266	2.63 ± 0.492	2.71 ± 0.508	2.85 ± 0.629	3.07 ± 0.873	3.06 ± 0.738
	10	2.36 ± 0.330	2.51 ± 0.508	2.76 ± 0.364	3.20 ± 0.567	3.36 ± 0.527	3.50 ± 0.598
	100	3.74 ± 0.606	3.95 ± 0.351	4.10 ± 0.405	5.10 ± 0.880	5.89 ± 0.852	5.92 ± 0.931
	1000	6.51 ± 0.229	6.62 ± 0.231	6.68 ± 0.232	6.82 ± 0.240	6.95 ± 0.172	7.05 ± 0.207

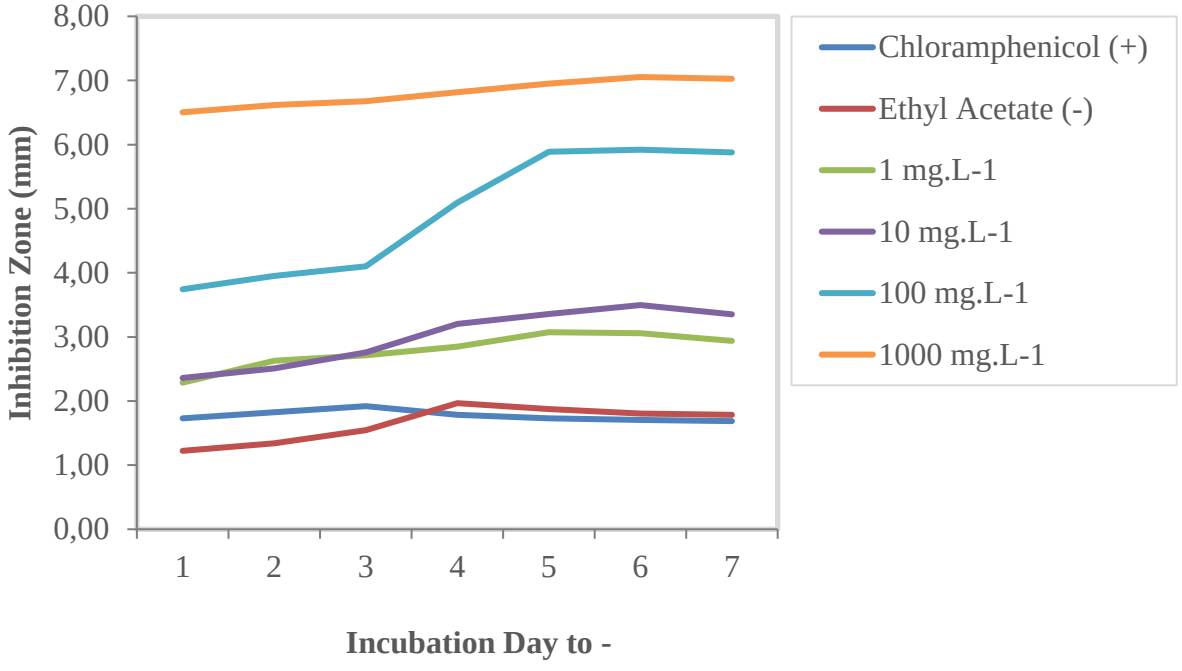


Figure 1. Effect of the extract on the inhibition zone against *Vibrio harveyi*.

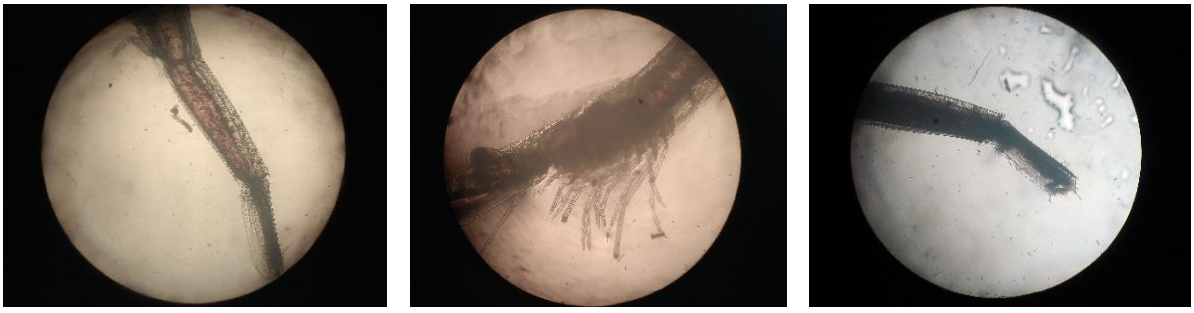
Each concentration of BA.1 extract showed the ability to inhibit *V. harveyi*, which was characterized by the gradual expansion of the inhibition zone during the incubation period (Figure 1). The antibacterial activity of BA.1 extract showed the best results on day 5 (five), while on the day 6 (six) the extract activity of BA.1 revealed the change in the area of the inhibition zone shown was very small. Based on the research, showing extracellular product activity of BA.1, the extract produced the largest inhibitory zone until day 5 (five) [3]. This shows that BA.1 extract has bacteriostatic properties. Bacteriostatic is an antibiotic that can inhibit bacterial growth, and is reversible [8]. So that BA.1 extract at concentrations up to 1000 $mg \cdot L^{-1}$ only has the ability to inhibit the growth of *V. harveyi*.

3. 2. LC_{50} test for 24 hours

Based on the results of LC_{50} monitoring for 24 hours, the antibacterial of BA.1 extract given to tiger shrimp age post larva with a concentration of 500 $mg \cdot L^{-1}$ and 1,000 $mg \cdot L^{-1}$ gave greater mortality values than a concentration of 250 $mg \cdot L^{-1}$, 100 $mg \cdot L^{-1}$ and 50 $mg \cdot L^{-1}$. The LC_{50} analysis results using the EPA Probit 1.5 program show a value of 1.743 $mg \cdot L^{-1}$.

3. 3. Clinical Symptoms

Shrimps infected with *V. harveyi* are characterized by morphological changes, including the abdomen and uropod being reddish, and the hepatopancreas in infected shrimp changing to brown. Clinical symptoms of shrimp attacked by vibriosis are changes in the color of the abdomen, pleiopod, periopod, telson and uropod to redness [9].



(a) (b) (c)

Figure 2. Morphological changes in shrimp infected with Vibrio harveyi **(a)** Red spots on the abdomen, **(b)** Necrosis on pleiopod and periopod, **(c)** Necrosis on uropod

Tiger shrimp larvae that have been infected with *V. harveyi* show morphological changes in the abdomen to redness due to red spots (Figure 2). This happens due to the entry of bacterial colonies of species *V. harveyi* into the body of tiger shrimp and attacks of the host by releasing exotoxins that trigger cell crusting in the body of the shrimp. Melanosis is a process triggered by a biochemical mechanism due to the oxidation of phenol to quinone through an enzyme complex called polyphenoloxidase [10]. The polyphenoloxidase enzyme is thought to play a role in the process of wound healing, sclerotization of the cuticle and plays a role in the process of melanisasi against foreign objects that enter the cavity between organs in the open circulatory

system (hemocoel) [11]. At the end of the treatment with BA.1 extract, the red spots on the shrimp appear slightly.

Necrocis on periopod and pleiopod tiger shrimp seen in part of the leg segment are darkened and the loss of periopodl segments caused by *V. harveyi*. Some vibrios produce a variety of destructive extracellular products, including haemolysin, protease, collagenase, phospholipase, and chitinase [12]. Tiger prawns that have undergone necrosis in parts of the body during treatment with BA.1 extract can still live but at the end of the observation the condition of shrimp body parts that have undergone necrosis has not yet recovered (Figure 2).

Changes in behavior, seen in shrimp infected with *V.harveyi***,** include responses to declining feed, with shrimp tending to be at the bottom. Changes in behavior in the treated shrimp showed slow swimming shrimp and decreased response to feed. This can be caused by infection with *V. harveyi* bacteria and the presence of antibacterial of BA.1 extracts, so the tiger shrimp need to adapt to restore health conditions.

Table 2. Observation Response to feed on Tiger Shrimp Larvae during the *In Vivo* Test.

Day to	BA.1 Extract Concentration (mg·L^{-1})														
	0			150			300			450			600		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	-	-	-	-	-	-	-	+	-	+	-	+	+	+	-
2-4	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
5-7	+	+	+	+	+	+	+	++	++	++	+	++	++	++	+
8-14	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

Note: (-) unresponsive, (+) less responsive, (++) responsive

Table 3. Observation of the Movement of Tiger Shrimp Larvae during the *In Vivo* Test.

Day to	BA.1 Extract Concentration (mg·L^{-1})														
	0			150			300			450			600		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	-	-	-	-	-	-	-	*	-	-	*	-	*	-	-
2-4	*	-	*	-	*	*	*	*	*	*	*	*	*	*	*
5-7	*	*	*	*	**	*	*	**	**	**	**	**	**	**	**
8-14	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**

Note: (-) abnormal, (*) stay on the bottom, (**): normal

Based on observations of the treatment of shrimp infected with *V. harveyi* (Table 2), the response of shrimp to feed on the first day was less responsive, which was indicated by the shrimp not eating the feed directly and the remaining feed in the maintenance container. The response of shrimp seeds after seven days of treatment has changed to be responsive, it is seen that the shrimp response directly feeds and at least the remaining feed, and tiger shrimp produces a lot of feces in the maintenance container.

Based on observations of the movement of tiger shrimp seeds after being infected with bacteria *V. harveyi* for 24 hours (Table 3), shown on the first day in a treatment container without treatment (0 $mg \cdot L^{-1}$), tiger shrimp larvae tend to be staying in the bottom, showing abnormal swimming movements, swimming sideways that was due to the effect of infection by *V. harveyi*. The movement of tiger shrimp after being infected with *V. harveyi* in each container with different concentrations showed that tiger shrimp which had originally resided at the base moved towards the water surface. This could be caused by tiger shrimp experiencing stress and requiring an adaptation because of BA.1 extract. Based on the observations, one hour after *V. harveyi* infection, tiger shrimp tend to dwell at the bottom of the aquarium, then move towards the oxygen source [13]. This shows that the shrimp is in an uncomfortable/stressful condition so it needs enough oxygen to restore its condition.

3. 4. Survival Rate of Tiger Shrimp Larvae

Observations on the survival rate of tiger shrimp larvae during the *in vivo* test until the end of the research presented different results from each treatment.

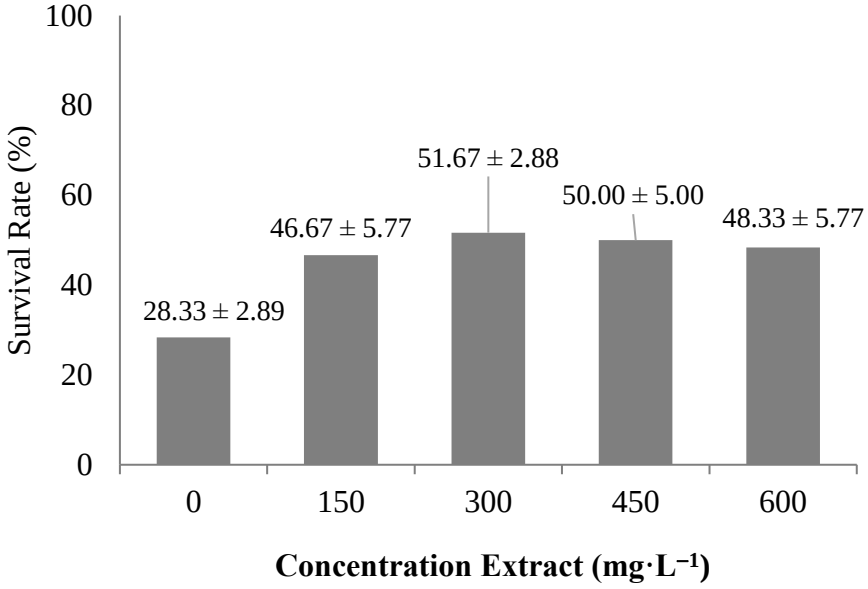


Figure 3. Histogram of survival rate of tiger shrimp larvae during the *in vivo* test

Based on the observations of survival rate results, the highest survival rate of tiger shrimp was treatment in the concentation extract 300 $mg \cdot L^{-1}$ of 51.67%, as for the high mortality caused by pathogenic bacteria activity *V. harveyi.* The survival of tiger shrimp after being infected with *V. harveyi*, which was treated with BA.1 extract, showed good results allegedly

due to the activity of secondary metabolites which could inhibit the growth of *V. harveyi*. The mechanism of antibacterial compounds is taking place generally by damaging cell walls, changing membrane permeability, disrupting protein synthesis, and inhibiting enzyme work [14]. Bioactive ingredients contained in metabolites can stimulate the formation of hemocyte cells as the body's defence for shrimp.

The metabolite compounds contained in the BA.1 extract are alkaloids, steroids and saponins [3]. The antimicrobial content of saponin compounds has the ability to cause leakage of certain proteins and enzymes from cells [15]. Saponin compounds contained in binahong leaf extract can stimulate the formation of hemocyte cells, so that the presence of a number of hemocytes affects the defense against pathogens [16]. Steroid compounds have antibacterial agents, where there is a correlation between membrane lipids and the sensitivity of steroid compounds to the mechanism of steroids causing leakage of liposomes [17]. The role of alkaloids in antibacterial activity is the presence of alkaline groups which are in contact with amino acid compounds that make up cell walls and bacterial DNA, thus causing the cell damage [18].

3. 5. Water Quality

Factors that influence the success of tiger shrimp cultivation rely on control of water quality as a medium for maintaining tiger shrimp. The condition of the aquatic environment in tiger shrimp cultivation needs to be adjusted to the tolerance range required by tiger shrimp. Water quality tested includes: Dissolve Oxygen (DO), temperature, salinity, and potential hydrogen (pH). The water in the container is not recirculated (statically treated), because there is no water change during the *in vivo* test. Water quality during research is under controlled conditions and still within the tolerance range according to the standard of tiger shrimp cultivation (Table 4).

Table 4. Water Quality During *In Vivo* Test.

Water Quality Parameters	Measurement During Research	SNI Standard
Dissolved Oxygen ($mg \cdot L^{-1}$)	6.0 – 6.4	≥ 5
Temperature (ºC)	31.1 – 31.8	29-32
Salinity (ppt)	32 – 34	15 – 34
pH	7.98 – 8.22	7 – 8.5

Note: SNI 01-6144-2006

Water quality during research is under controlled conditions and is still within tolerance range according to the standard of tiger shrimp cultivation. Water quality test results at the beginning of the research until the end of the research showed that dissolved oxygen values in maintenance media ranged from 6.0 $mg \cdot L^{-1}$ to 6.4 $mg \cdot L^{-1}$, water temperatures ranged from 31.1 ºC to 31.8 ºC, salinity ranged from 32 to 34 ppt, and pH ranged from 7.98 – 8.22 . Based

on water quality data (Table 4), water quality during research is still within the standard range for tiger shrimp cultivation, so water quality during research is not a factor that causes death of tiger shrimp larvae. The factors that cause the death of tiger shrimp are brought upon the activity of *V. harveyi.*

4. CONCLUSIONS

Based on the results of the research, the extracts of secondary metabolites from the bacteria associated with sea grass *Cymodocea rotundata* were effective for the treatment of vibriosis in tiger shrimp. The concentration of secondary metabolite extract of 300 $mg \cdot L^{-1}$ has the ability to inhibit the growth of *Vibrio harveyi* and produce the highest survival rate of 51.67%.

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