

Squid (*Loligo edulis*) ink raw extract as an anti-vibriosis substance in grouper (*Epinephelus fuscoguttatus*) juvenile culture infected by *Vibrio alginolyticus*

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Abstract. In mariculture *Vibrio alginolyticus* is frequently isolated from vibriosis sick grouper, *Epinephelus fuscoguttatus*. The high mortality of grouper larvae in grouper hatchery as vibriosis cases need a way to overcome with other antibacterial products. Squid ink, as a squid waste product, which contains a variety of bioactive components which are environmentally friendly was used as an anti vibriosis substance. The methods used in this research were: Phase 1: extraction of squid ink; Phase 2: Paper Disk Test, Scanning Electronic Microscope (SEM) observation, and GC-MS chromatography test for the active ingredient; Phase 3: in-vivo trials on squid ink extract against *V. alginolyticus* in tiger grouper juvenile. An experimental method with completely randomized design using three treatment doses of squid ink extract (365.5; 312.5; 265.5 mg L⁻¹), after preliminary test using Minimum Inhibitory Concentration (MIC), to treat the infected juvenile with *V. alginolyticus* each with three replications was used. Blood samples were investigated for hematological profile i.e.: erythrocyte, leukocyte, monocyte, lymphocyte, and neutrophile. The conclusion of the research results were squid ink extract can be used as bactericidal for *V. alginolyticus* at a dose of 265.5 mg L⁻¹. Squid ink contains 9-octadecenoic acids/oleic acids as an antibacterial agent. The tiger grouper juvenile which was infected with *V. alginolyticus* reached 100% survival rate after squid ink extraction treatment and gives a highly significant effect to hematological profile.

Key Words: squid ink, *V. alginolyticus*, *E. fuscoguttatus*, survival rate, hematological profile.

Introduction. Grouper (*Epinephelus fuscoguttatus*) is one fish species that has a very high economic value in Indonesia. Therefore, it is widely cultivated and especially important for export to the country of Singapore, Taiwan, and Hong Kong. Therefore, this species has great potential and is promising as an aquaculture product. The limited seed supply, both in terms of quantity and quality is still a major problem (Sugama et al 2012).

One of the obstacles encountered in grouper aquaculture is primarily pathogenic bacteria attack on the larval stadia and seeds. The occurrence of these pathogens omitted causes loss of quality and production efforts on grouper hatchery, harvest failure and even death (Hatmanti et al 2009).

Vibrio illness is called vibriosis, penaeid bacterial septicemia, penaeid vibriosis, luminescent vibriosis or red leg disease (Aguirre-Guzman et al 2004). This disease is a major bacterial disease, especially at the seed can lead to mortality up to 100% within 2 weeks. Several species of *Vibrio* bacteria are frequently isolated from diseased groupers (i.e. *E. coioides* (Huang 2005), Polkadot grouper, *Cromileptes altivelis* (Nitimulyo et al 2005) and tiger grouper, *E. fuscoguttatus* (Desrina et al 2006)): *Vibrio alginolyticus*, *V. anguillarum*, *V. vulnificus* (Nagasawa & Cruz-Lacierda 2004; Nitimulyo et al 2005). Signs of the disease include fatigue, tissue death, slow growth and metamorphosis, disability, bolitas negricans, bioluminescence, muscle opacity and melanization. In many cases, opportunistic *Vibrio*, which only cause disease when the host organism immunity

declining or experiencing physical stress, the frequency of infection is common in intensive cultivation and poor environmental conditions (Alderman & Hastings 1998).

Vibriosis often leads to a critical problem for the life of shrimp and fish. In *Vibrio anguillarum* (Norqvist et al 1990), *V. vulnificus* (Kothary & Kreger 1987), *V. proteolyticus* (David et al 1992), a zinc metalloprotease is an important virulence factor, which causes tissue damage in fish and shrimp. *V. alginolyticus* virulence factors that play a role are the phospholipase, hemolysin, protease (cysteine proteases, metalloproteases, serine proteases), lipopolysaccharide, a bacteriophage, bacteriocin-like substance (BLIS) and chitinase (Arachchige 2010).

Several types of antibiotics, such as streptomycin, chloramphenicol, and cotrimazole have been used to control the disease, particularly the bacterial disease (Jayasree et al 2006). But prolonged use of antibiotics caused the pathogenic bacteria to be resistant and reduced the effectiveness of antibiotics (Yulvizar et al 2015). Therefore, it is crucial to explore the alternative techniques to control bacterial diseases, and one of the promising techniques is by using the squid ink, which is considered as fishery waste product. Various amine bioactive components simple and paralyzing protein are found in chepalopods. These bioactive components can be used for the prevention of pathogenic bacteria (Cariello & Zanetti 1977; Walker & Masuda 1990). Hence, the aim of this study was to examine the effect of squid ink as an anti vibriosis substance to improve the tiger grouper aquaculture production.

Material and Method

Squid ink extract. The squid, *Loligo edulis*, was obtained from the fish landing place in Lamongan, East Java. Squid ink was taken from squid ink sac and centrifuged on 15.000 g for 15 min, the supernatant was collected and stored at -20°C for further utilization.

V. alginolyticus. *V. alginolyticus* was obtained from a pure culture of Fish Quarantine Office, in Surabaya, Indonesia; cultured in Nutrient Agar before used.

Paper disk test. Mc Farland standard test was used to get the exact dose of squid ink liquid extract to inhibit the Vibriosis infection. Paper Disk Test was done using a circular paper (6 cm in diameter) which was depth in different doses of squid ink liquid extract after preliminary test using Minimum Inhibitory Concentration (MIC), 365.5; 312.5; 265.5 mg L⁻¹. The thin paper then laid on the plates that the *V. alginolyticus* grown up and scratched on solid medium of TCBSA with density of 10⁸ cfu mL⁻¹.

Scanning electronic microscope (SEM). To know the effect of squid ink extract to *V. alginolyticus*, the reaction was observed using SEM-EDS merchandise FEI type Inspect S50, EDAX AMETEK.

Gass Cromatography-Mass Spectrometry (GC-MS). Squid ink raw extract for the best dose of test results was sent to the laboratory for chromatography analysis using GC-MS merchandise, Aligen type 7890 A.

Infecting the tiger grouper with *V. alginolyticus*. The tiger grouper juvenile was put into sea water media which has *V. alginolyticus* in density of 10⁷ cfu mL⁻¹ for 24 hours. The next day the tiger grouper juvenile were put into aquarium with density of 5 fish L⁻¹ which has given squid ink raw extract, 365.5; 312.5; 265.5 mg L⁻¹, for treatment dose and negative control without squid ink raw extract. The tiger grouper were reared until 7 days. Mortality data was written every day.

Hematological profile. Blood sample was taken at the first (D+1) and seventh (D+7) day since the fish were infected with *V. alginolyticus* and put into aquarium with squid ink raw extract treatments. Examination of hematological profile covered leukocyte, erythrocyte, monocyte, lymphocyte and neutrophile based on Bastiawan et al (2001).

Survival rate of grouper juvenile. Survival rate of juvenile was measured at the end of research using formula:

$$SR = \frac{N_t}{N_0} \times 100\%$$

Where: R = survival rate (%), N_t = amount of fish at the end of research (fish), N_0 = amount of fish at the beginning of research (fish).

Time and place. This research was done in Fish Disease Laboratory, Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, Indonesia; Biology Laboratory of State University of Malang, Malang, Indonesia for SEM; and Forensic Laboratory of East Java Police Region, Surabaya, Indonesia for GC-MS. The hole research was conducted from January 2013 to October 2015.

Statistical analysis. Completely randomized design was used for paper disk test and analyzed using SPSS 16 series for ANOVA.

Results and Discussion. Squid ink liquid extract can inhibit the activity of *V. alginolyticus* bacteria In paper disc test, inhibition effect was characterized by the formation of clear zone around the hole sinks bacterial culture media, and it appeared outside the paper disc diameter. The diameter of inhibition zone describes the ability of antibacterial substance in different concentrations (Syakti et al 2008).

The average value of the inhibition zone located on 265.5 mg L⁻¹ treatment squid ink liquid extract was 10.85 mm, for 312.5 mg L⁻¹ squid liquid extract was 11 mm, while for 362.5 mg L⁻¹ squid liquid extract was 11.27 mm. These inhibition zone discribed the ability of inhibitory activity, as mentioned by Suriawiria (2005) i.e.:

- a. less then 5 mm diameter categorized as weak inhibitory activity;
- b. 5-10 mm diameter categorized as intermediate inhibitory;
- c. diameter of 10-19 mm categorized strong;
- d. 20 mm or more was considered very strong.

Statistical result of the challenging test showed that the squid ink liquid extract at different doses provides greater inhibitory power when compared to controls (0%), but no significant differences among the different doses of treatment were observed. Even all of the concentration can be used as inhibition concentration but for efficiency the concentration of 265.5 mg L⁻¹ can be used as anti-vibriosis against *V. alginolyticus*. The higher the concentration of squid ink extract give the more broad inhibitory activity. This was due to the higher concentration of treatment, and the higher number of the antibacterial compounds. Jawetz et al (1982), explained that the higher concentration will make faster ability to kill bacteria.

The images of Scanning Electronic Microscopy (SEM) showed that after *V. alginolyticus* was given exposure of squid ink extract, the bacterial cell was broken, which resulted in the death of the bacteria (Figure 1).

Abou-Elela et al (2009), show the results of GC-MS of marine natural products *Cytosoria* brown algae and sponge *Spongia compressa officinalis*, which showed antimicrobial effect derived from amino acids and esters (acids hexadecanoic and octadecanoic acids). Some of molusks ink secretion are protein and the protein mass range is between 62 and 249 kDa which have specific role in chemical defens mechanism (Vennila et al 2011). One day after infected with *V. alginolyticus*, the fish got vibriosis symptoms such as anorexia, darkening of the skin (Hendrickson & Zenoble 1983), ulcerated skin lesion, and all of the control fish (without squid ink raw extract treatments) died in the second day. But the symptoms didn't appear in the fish with different treatments of squid ink raw extract. At the seventh day, all the treated fish reached 100% of survival rate.

V. alginolyticus produces anhydrotetrodotoxin which in low pH condition will be changed into tetrodotoxin (Noguchi et al 1987; Bordas et al 1996). Tetrodotoxin is a neurotoxin which blocks the sodium channel. It binds to the sodium channels of the excitable tissues of the fish (muscle and nerves), and the inhibition of sodium ions through the channels effectively immobilies these tissues (Denac et al 2000 in Bane et al

2014). The GC-MS results showed that squid ink extracts has the highest abundance of 9-octadecenoic acids/oleic acids (Figure 2) in 18.242 retention time.

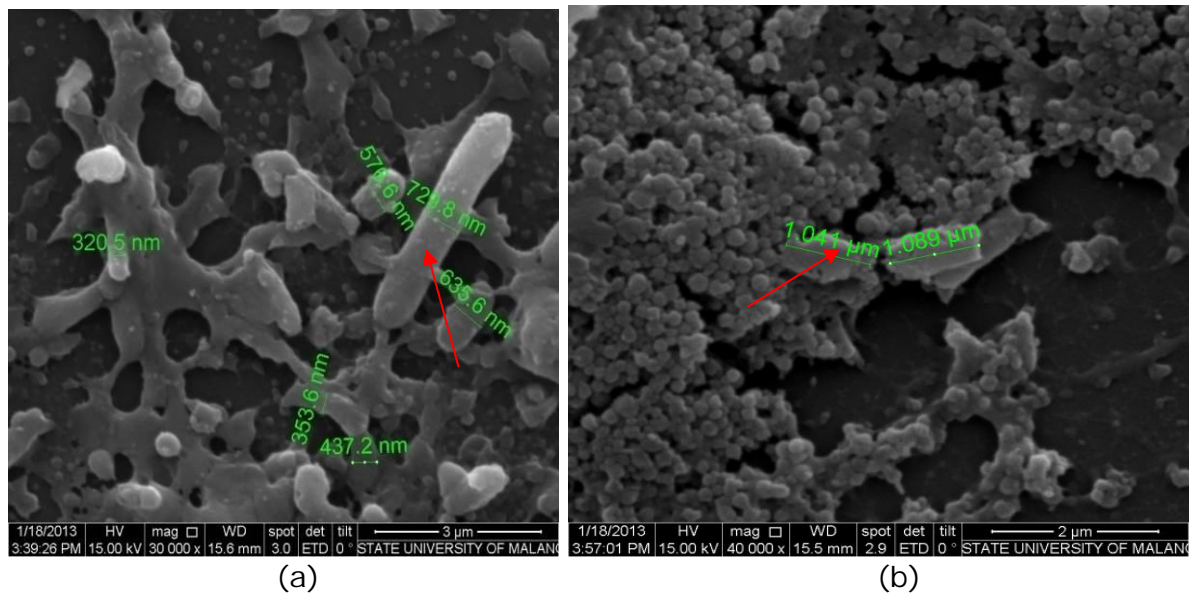


Figure 1. *V. alginolyticus* before and after treatment in SEM. Description: (a) *V. alginolyticus* before treatment, (b) *V. alginolyticus* after treatment with squid ink extract.

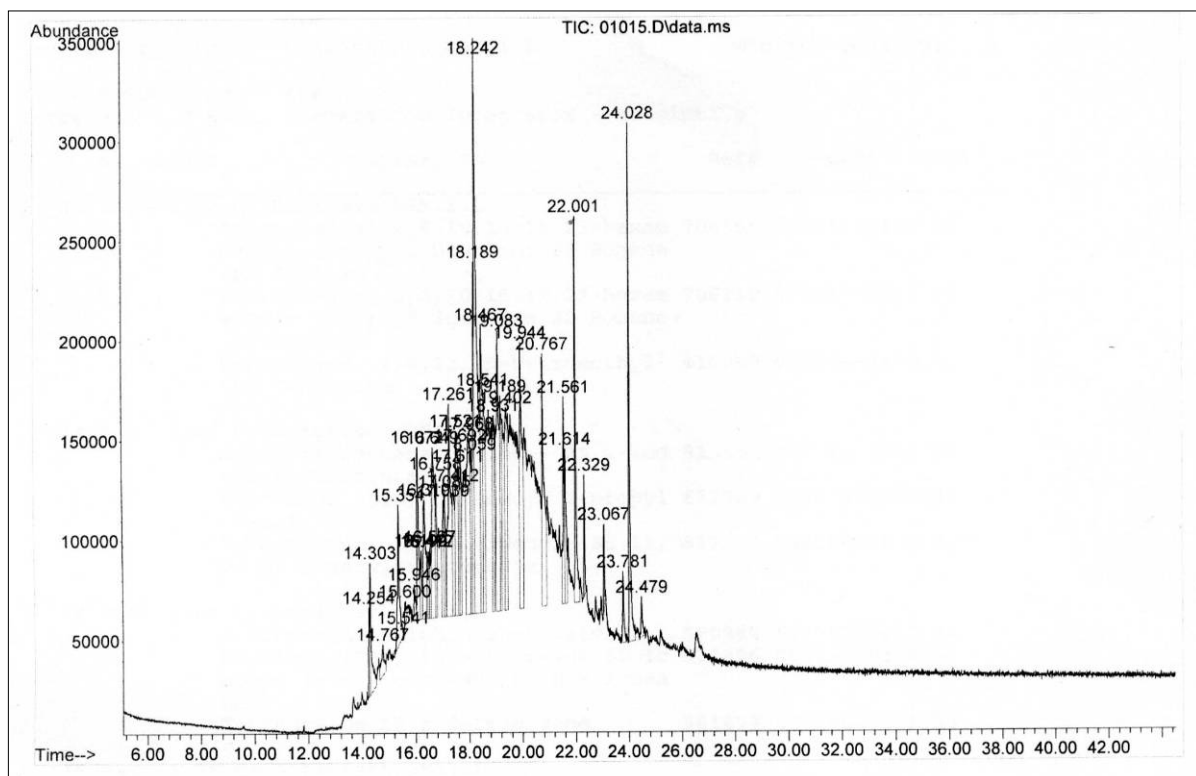


Figure 2. GC-MS result.

Erythrocyte and leukocyte were investigated after vibriosis infection and treated with squid ink raw extract. Leukocytes consist of monocytes, lymphocytes, and neutrophils. According to Bastiawan et al (2001) monocyte was functioned as phagocytes against foreign objects that act as agent of disease. Lymphocyte was functioned as antibody-producing immunity from the disease. And neutrophils play a role in the immune response to attack pathogenic organisms and possessed in phagocytic properties.

Neutrophils of the blood will increase in the event of infection and act as the body's first defense (Dellman and Brown 1989 in Bastiawan et al 2001).

Data in Table 1 show the amount of erythrocyte, leukocyte, monocyte, lymphocyte and neutrophile at the first (D+1) and seventh (D+7) days. Amount of erythrocyte, monocyte, lymphocyte and neutrophile were increased in the seventh day of treatment while leukocyte was decreased. These signs proved that using squid ink raw extract for vibriosis treatment showed response of the fish against Vibriosis infection which gave an increasing of immune response to the tiger grouper juvenile.

Table 1
Average amount of erithrocyte, leukocyte, monocyte, limphocyte and neutrophile at the first (D+1) and seventh (D+7) days

Treatment dose (mg L ⁻¹)	Erythrocyte (10 ⁶ cells mL ⁻¹)		Leukocyte (10 ³ cells mL ⁻¹)		Monocyte (10 ³ cells mL ⁻¹)		Lymphocyte (10 ³ cells mL ⁻¹)		Neutrophile (10 ³ cells mL ⁻¹)	
	D+1	D+7	D+1	D+7	D+1	D+7	D+1	D+7	D+1	D+7
Control	8.27	0	29.85	0	14.33	0	55.00	0	7.67	0
265.5	7.77	8.90	31.78	28.92	10.33	14.67	64.00	73.33	9.33	10.67
312.5	7.50	9.27	31.95	28.83	15.67	21.00	65.00	74.00	9.67	11.67
365.5	8.07	10.17	31.50	27.52	18.33	27.00	66.67	79.00	10.67	12.67

Increasing of squid ink raw extract dose gave a higher impact than the lowest dose (265.5 mg L⁻¹), but the lowest dose (265.5 mg L⁻¹) can be recommended for efficiency because statistically this dose was not significantly different with the highest dose (365.5 mg L⁻¹) based on the average amount of erithrocyte, leukocyte, monocyte, limphocyte and neutrophile at the first (D+1) and seventh (D+7) days.

The result between control and treated fish was significantly different. Two days after *V. alginolyticus* incubation, all the control fish were died but all the treatment fish with raw ink extract were 100% survive.

9-octadecenoic acids/oleic acids content in squid ink raw extract could kill the bacteria directly and maintaining an acidic pH condition for the bacteria (Fluhr et al 2001; Takigawa et al 2005).

Oleic acids in squid ink can stick in the bacterial membranes (e.g., ceragenins and lipopeptides) and proteic (lipoglycopeptides) or lipidic (glycodepsipeptides) cell wall precursors, damaging the cell wall structure (Kenny et al 2009). This activity will break the cell wall and kill the bacteria, *V. alginolyticus*. The death of bacteria will reduce mortality risk of tiger grouper.

Statistical analysis showed highly significant effect of using squid ink raw extract to the hematological profile and survival rate of tiger grouper juvenile against *V. alginolyticus*.

Conclusions. Squid ink raw extract can be used as bactericidal against *V. alginolyticus* at a dose of 265.5 mg L⁻¹, giving a highly significant effect to hematological profile and survival rate of tiger grouper juvenile. Squid ink raw extract contains 9-octadecenoic acids/oleic acids as antibacterial agent.

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