Antibacterial Potention of Extract of Rotifers Fed with Different Microalgae to Control *Vibrio harveyi*

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Abstract. *Vibrio harveyi* is a pathogenic bacteria that can caused high mortality and significant loss in shrimp culture. Application of antibiotic to control vibriosis has negative impact to the environment, people and the shrimp. Therefore, it is important to explore alternative antibiotic from natural substances such as enriched rotifers. The aim of this experiment was to determine the presence of antibacterial compound in rotifer extract fed with different microalgae and to determine the ability of rotifer as an antibacterial to control *V. harveyi* in vitro. Rotifers were fed with three different types of microalgae, *Porphyridium* sp., *Tetraselmis* sp., and *Chlorella* sp.. Cultured rotifers harvested, and the crude extract then obtained with methanol extraction. The antibacterial activity was determined with Kirby-Bauer method and using tetracycline as control. The result showed that rotifer fed with microalgae have antibacterial activity against *V. harveyi* as shown by the formation of inhibition zones in the Kirby-Bauer test. However, the zone was smaller than that produce by tetracycline. It can be concluded that extract of rotifer fed with different microalgae has antibacterial compound with moderate antibiotic activity.

1. Introduction

Vibriosis, caused by bacteria in the genus Vibrio spp, is one of the most prevalent disease in brackishwater aquaculture and widely responsible for mortality in cultured fish and shrimp worldwide. Vibrios are gram-negative bacteria, ubiquitous in marine and estuarine ecosystems as well as aquaculture ponds. Vibrios also comprise as the major microbes in brackish water and marine ecosystems. *Vibrio harveyi* is one of the Vibrio that showed high virulent and frequently causing problem in the shrimp hatchery and grow out ponds. One of specific clinical signs on the infected shrimp is the glowing appearance at night because this bacterium produce bioluminescence [1]. However, non-luminous but virulent *V. harveyi* has been reported.

Controlling *V. harveyi* can be a challenge because this bacteria is naturally exist in the environment. *V. harveyi* control mostly was done through aquaculture good practices and applying biological oxidizing agents, chemicals and antibiotics. Continuous use of chemicals and antibiotics has been reported to cause resistant so that the required dose continues to increase. In addition, there will be the danger of chemical residues in the shrimp. Alternative control is using natural antibiotics produced by plants and bioactive products of microbes and lower invertebrates. One potential source of natural antibiotics is through natural-antibiotic-enriched-rotifers [1][2].

Rotifers are zooplankton which commonly used as natural feed for fish larvae. In addition, rotifers have interesting characteristics which can be used to deliver substances [2][4], because it has ability in...
absorbing various substances given to feed or from the environment that makes rotifers an effective bio-capsule. Rotifers have shown the ability to transfer nutrients, macromolecules, amino acids, unsaturated fatty acids, minerals, vitamins and antibiotics without pollutant effects (natural bio-capsules) [3][6]. However, how much of the substance incorporated as part of their cells is unknown.

The ability to produce antibiotic effect depends on the microalgae they consumed and environmental condition. Extreme environmental adaptation factors affect the production of bioactive rotifers compounds. Alleged adaptation of rotifers to extreme environmental conditions makes them produce molecules such as "temperature / salinity-shock protein", which is a class of proteins produced as defend mechanism against extreme conditions of temperature or salinity, therefore, also categorized as an anti-stress protein[4].

Several microalgae such as Chlorella sp, Porphyridium cruentum, Tetraselmis chuii, and many others have been identified to produce metabolites that potentially can be converted into bioactive antibacterial compounds in rotifers. The objective of this study is to determine the antibacterial effect of extract of rotifers –fed with different kind microalgae on V. harveyi in vitro.

2. Research Methods
Phytoplankton Chlorella sp, Tetraselmis chuii and Porphyridium cruentum were used to feed rotifers. The phytoplankton seeds were obtained from the Center of Brackish Water Aquaculture. The seeds were cultured in lab scale in the Laboratorium of Faculty of Fisheries and Marine Science, Diponegoro University, Semarang. The rotifers were cultured in sea water in 1L erlenmeyer flask with total volume 800 ml, fed with different type phytoplankton above for two weeks at which time the rotifer density was counted and subsequently the rotifers was harvested.

The extraction process of rotifers was started by filtering the rotifers using a plankton net and stored at -20 °C. The rotifers then thawed to destroy the cells. Next process is adding the 80% of methanol to the rotifers after thawing process with ratio methanol: rotifers 2:1. The process of homogenization with 80% of methanol takes 24 hours. Next step was centrifugation process at 3000 rpm for 15 minutes. The extraction stage process is done by taking the supernatant which heated for 12 hours at a temperature of 44 °C to evaporate methanol continued with freeze drying process. This process resulting in the crude extract of the rotifers.

Antibacterial activity test is done using Kirby-Bauer method. The tested bacteria used is V. harveyi (obtained from the Center of Brackish Water Aquaculture, Jepara) and cultured to Nutrient Broth (Merck) media. The antibacterial activity test was done in Mueller Hinton Agar with bacterial density 10⁸ CFU/ml. The commercial antibiotic tetracycline was used as positive control. The solvent used in the antibacterial activity test is dimethyl sulfoxide (DMSO) with the concentration of each extract is 1000 ppm. A paper disc (diameter 0,5 cm) was soaked in the extract and standard antibiotic solution, placed on the MH agar containing newly inoculated V. harveyi and incubated for 24 h at 30 °C The antibacterial activity was determined by measuring the clear zone around the disc.

3. Result
The average rotifers density obtained were 58, 86, 52 individual/ml for rotifers fed with Tetraselmis sp., Porphyridium sp., and Chlorella sp., respectively. The total amount of the extract obtained of from each treatment is presented in the table. 1.

| Sample  | Crude Extract (gr) |
|---------|--------------------|
| TreatmentA | 0,5                |
| TreatmentB | 0,6                |
| TreatmentC | 0,8                |

Notes: 
Treatment A=Rotifers fed with Tetraselmis sp.
Treatment B = Rotifers fed with *Porphyridium* sp.
Treatment C = Rotifers fed with *Chlorella* sp.

The amount of extract was varied among rotifers fed with different microalgae, with those fed with *Chlorella* sp. produce more than the rest.

The result of observation and measurement for diameter inhibition on all treatments tested towards *V. harveyi* bacteria, shown that the formation of inhibitory zones in the form of clear areas around the paper disc was observed for first time at the 18 hour incubation. The diameter of the inhibitory zone did not increase after the 24th hour. The inhibition zone diameter in treatment A was bigger than treatment B and C. (table 2).

| Repetition | Treatment A | Treatment B | Treatment C | Treatment D |
|------------|-------------|-------------|-------------|-------------|
| 1          | 8.0         | 7.0         | 7.0         | 25.0        |
| 2          | 7.0         | 8.0         | 8.0         | 26.0        |
| 3          | 8.0         | 7.0         | 7.0         | 25.0        |
| Total      | 23.0        | 22.0        | 22.0        | 76.0        |
| Average    | 7.7         | 7.3         | 7.3         | 25.3        |
| SD         | 0.5774      | 0.5773503   | 0.05774     | 0.5774      |

Notes:
Treatment A = Extract of Rotifers fed with *Tetraselmis* sp.
Treatment B = Extract of Rotifers fed with *Porphyridium* sp
Treatment C = Extract of Extract Rotifers fed with *Chlorella* sp.
Treatment D = Tetracycline (positive control)

### Table 2. Antibacterial activity of rotifer extract against *V. harveyi* based on the diameter of inhibition zone.

4. Discussion
The amount and bactericidal effect of extract obtained from each group of rotifers were different among treatments. The treatment C (rotifers fed with *Chlorella* sp.) gained more crude extract than the treatment A (rotifers fed with *Tetraselmis chuii*) and the treatment B (rotifers fed with *Porphyridium cruentum*). There are several factors influence the amount of crude extract produced according to Rumengan et al [5] that is, the amount of biomass. The type of feed of rotifers produced a different density of rotifers and the crude extract. Other factors were the amount of feed digested and used for growth by rotifers which may different according to the type of microalgae.

According to Redjeki[7], the technique in rotifers cultured either in a laboratory scale or mass scale, must consider biological and chemical aspects of the rotifers including morphology, reproduction, habitat, growth, habits and ways of eating, ways of culture and nutritional value and water quality. All of these criteria were met for growing rotifers in this study. Feed is an important factor in the pattern of growth and production of rotifers according to Hagiwara et al[8], in which feed should be able to supply protein, carbohydrates, fats, vitamins, and minerals to stimulate the rotifers growth. Under laboratory experiment, the environmental conditions are always in controlled so that it was favorable for the rotifers growth. Fluctuating environmental factors will certainly affect the quality of the culture media, that also affects the successness cultivation of rotifers[9].

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The selection of microalgae (Tetraselmis sp, Porphyridium sp and Chlorella sp) as feed for rotifers is expected to increase bioactive compounds in rotifers. It has been reported by Setyaningsih et al [11] that Porphyridium cruentum dry biomass contains the main group of chemical compounds which has antibacterial activities including alkaloids, flavonoids, and hydroquinone phenols. Tetraselmis chuii contains compounds that have the potential as antimicrobial compounds including various fatty acids, esters, aromatic hydrocarbons, alcohols, ketone, cycloalkene, alkanes, and benzenes [12]. With the capabilities possessed by rotifer as a natural bio-capsule, they were expected to absorb the content of compounds in microalgae, so that rotifer extract can be used as an antibacterial. Previous research conducted by Rimper [2] showed that rotifer extract has the potential for antibacterial compounds which were proven to inhibit V. harveyi growth in this study.

The results of the crude rotifers extract obtained then tested to the V. harveyi bacteria showed inhibitory zones formed in all crude. The zone of inhibition is smaller than that produced by tetracycline as the positive control. Tetracycline produces the inhibition zone with 25.3 mm in diameter, in contrast, the crude extract of rotifer fed with Tetraselmis sp. (treatment A), Porphyridium sp (treatment B), and Chlorella sp (treatment C) produce 7.7 mm, 7.3 mm and 7.3 mm consecutively. According to Rumengan et al [5] the inhibition zone response can be categorized into 4 stages, (<5 mm), moderate (5-10 mm), strong (11-20 mm) and very strong (21-30 mm). From the results obtained it can be conclude that crude extract of rotifers fed with different type of microalgae has moderate antibiotic activity against V. harveyi.

5. Conclusion
Findings of this study showed that extracts of rotifers fed with Tetraselmis sp, Porphyridium sp, and Chlorella sp, can inhibit the growth of V. harveyi in vitro, with moderate antibiotic activity.

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