Inhibitory Rate of Chitosan Against Vibrio harveyi use in vitro method

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Abstract. The intensification of the penaeid shrimp culture industry have been accompanied over the last two decades by an increased incidence of infectious pathogens. Vibrio sp. has become a major source of concern for shrimp culture because of their close association with low survival rates in hatcheries or growout ponds. The antibioctic use to aim this disease but it harmful to use continuously. Resistance of pathogen and retention are such side effect left after antibiotic used. Chitosan is a biocompatible and biodegradable natural polymer with established antimicrobial properties against specific microorganisms. This research demonstrates its antibacterial activity to inhibit growth of Vibrio harveyi comparing with oxolonic acid. Inhibition growth of Vibrio harveyi was tested using diffusion method on TSA added 2% NaCl by additional disc paper which has been soaked in chitosan suspension concentrations 0.8; 1.0; 1.2; 1.4; 1.6% (w/v), asetic acid 1%, and oxolonic acid disc (2µg), each treatment was repeated three times. In this study chitosan showed an antibacterial activity. The lowest antibacterial activity showed by the lowest concentration 0.8% (w/v) and the higest antibacterial activity showed by the highest concentration 1.6% (w/v).

1. Introduction
Chitosan is a chemical compound derived from the biological material chitin, an organic compound that is abundant in nature after cellulose [1]. Chitosan can be produced from chitin by removing the acetyl group (CH3-CO). In the deacetylation process, the bond between the carbon in the acyl group in chitin is broken into an amino group [2]. Chitosan is a biocompatible and biodegradable natural polymer with antimicrobial properties against certain microorganisms.

Chitosan impairs the protection of the outer membrane of Gram-negative bacteria [3]. Electron microscopy showed that chitosan caused changes to the cell surface and covered the outer membrane of bacteria with a vesicular structure. Chitosan binds to the outer membrane and causes a loss of barrier function of the bacterial cell membrane. Research conducted by [4] showed a positive correlation between the concentration of chitosan and the antibacterial activity of chitosan on 48 isolates of Vibrio species from shrimp larvae rearing media.

2. Materials and Methods
2.1. Materials
The material to be used in this study is chitosan with a degree of deacetylation (DD) of 85.96% obtained from the Bogor Agricultural University (IPB) and Vibrio harveyi bacteria obtained from BBPAB Jepara. Other materials used in this study were sterile physiological NaCl solution, Triptic
Soy Agar (TSA), NaCl, 70% alcohol, spirit, disc paper, 1% acetic acid, oxolinic acid, and distilled water.

2.2. Methods
This study used an experimental method to determine the inhibitory power of chitosan on the growth of *Vibrio harveyi* bacteria, by comparing the treatment with the control. The concentration of chitosan used in this study was 0.8%, 1.0%, 1.2%, 1.4%, 1.6%. The negative control in this study was 1% acetic acid while oxolinic acid 3 g/ml was the positive control.

2.3. Experimental Research
The first stage in this research is the process of dissolving chitosan into 1% acetic acid with five different concentrations (0.8%, 1.0%, 1.2%, 1.4%, 1.6%) and sterilized at 121 °C for 15 minutes. The inhibition of chitosan on the growth of *Vibrio harveyi* was tested using the disk diffusion method. The number of bacteria used is according to the standard of 0.5 McFarland, which is equivalent to 1.5 x 10 CFU/ml [5]. *Vibrio harveyi* bacterial suspension as much as 1 ml and put in a Petri dish containing Triptic Soy Agar (TSA) media, then leveled with a spreader. Disc paper was dipped in chitosan solution with five different concentrations, 1% acetic acid, and oxolinic acid, placed on the surface of the agar medium and then incubated at 29°C. Observations were made after incubation to see the inhibition zone (no growth) around the paper disc [5].

3. Results and Discussion
The results of the five treatments showed the presence of inhibition against *Vibrio harveyi* bacteria. The smallest inhibition was shown by treatment A (0.8%) while the largest inhibitory power was shown by treatment E (1.6%). Table 1 showed that treatment E was categorized as moderately sensitive (intermediate) to inhibit the growth of *Vibrio harveyi* bacteria. The main factors that are believed to contribute to the antibacterial properties of chitosan are the concentration of chitosan in solution, molecular weight, degree of deacetylation, and level of protonation [4]. The mechanism of antibacterial activity of chitosan is to flocculate bacteria and thereby depriving them of nutrients and oxygen (i.e. restriction of mass transfer) [8]. According to [3], chitosan impairs the protection of the outer membrane of Gram-negative bacteria. Electron microscopy showed that chitosan caused changes to the cell surface and covered the outer membrane of bacteria with a vesicular structure. Chitosan binds to the outer membrane and causes a loss of barrier function of the bacterial cell membrane.

| Treatment     | Inhibition zone diameter (mm) | Criteria for Antibiotic Inhibitory Zone Diameter Oxolinic acid [5]. |
|---------------|------------------------------|------------------------------------------------------------------|
|               | Resistance (<14 mm) | Intermediate (14-18 mm) | Sensitive (>18 mm)   |
| A (0.8%)      | 6.2                        | √                                         |                      |
| B (1%)        | 9                          | √                                         |                      |
| C (1.2%)      | 12.2                       | √                                         |                      |
| D (1.4%)      | 13.5                       | √                                         |                      |
| E (1.6%)      | 15.2                       |                                           | √                     |
| Acetic Acid 1%| 7.4                        | √                                         |                      |
| Oxolinic Acid 10 µg | 21.3               | √                                         |                      |

The activity of inhibiting bacterial growth (antibacterial) of chitosan, which was not found in this chitin, came from the free amino group (NH₂) in chitosan. Free amino groups in acid solution will be
protonated to form polycationic, so that the positively charged polysaccharide chitosan is different from the polysaccharides in general which have a negative charge, including one of them is protein. This natural polycationic chitosan exhibits a high charge density which is a cationic polyelectrolyte which is very effective in interacting with negatively charged biomolecules and surface biomolecules such as the negatively charged surface of bacterial cells [8].

The most widely accepted mechanism is the interaction of the positive charge of chitosan with the negative charge on the bacterial surface [9,10,11]. This is because the cations in the chitosan react with the anionic polymer and form an electrolyte complex [12].

4. Conclusion
Based on results of research that has been carried out, it showed that chitosan has an inhibitory effect on the growth of *Vibrio harveyi* bacteria. The inhibitory power of chitosan at a concentration of 1.6% (0.016 gram / 1 ml) can be categorized as an antibacterial agent that is sensitive enough to inhibit the growth of *Vibrio harveyi* bacteria after being compared with standard oxolinic acid antibiotics.

5. References
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