

# Antibacterial activity of chitosan of vannamei shrimp (*Litopenaeus vannamei*) shells from Molucca Sea on *Vibrio alginolyticus*

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**Abstract.** Vibriosis is one of the most prevalent bacterial diseases affecting aquatic organisms, particularly in aquaculture systems for fish and shrimp. This disease is caused by bacteria of the genus *Vibrio*, which can lead to mass mortality, reduced productivity, and significant economic losses. The control of vibriosis has traditionally relied on the use of antibiotics. However, their indiscriminate application may result in bacterial resistance, environmental contamination, and antibiotic residues in aquaculture products. Chitosan, a natural biopolymer obtained through the deacetylation of chitin abundantly found in crustacean shell waste, has gained increasing attention as an environmentally friendly alternative for disease control. Chitosan is known for its antibacterial activity, high biocompatibility, and biodegradability. The purpose of this research is to synthesize chitosan from vannamei shrimp (*Litopenaeus vannamei*) shells and to test its antibacterial activity on *Vibrio alginolyticus*. Chitin extraction goes through deproteination, demineralization and depigmentation stages, while the transformation of chitin into chitosan goes through deacetylation stages. The degree of deacetylation was determined using Fourier Transformation-Infrared Spectroscopy, whereas the molecular weight was determined using the viscometry method. The antibacterial activity test was characterized using disc paper diffusion with 5 chitosan concentration treatments, namely T1 (0.10%), T2 (0.25%), T3 (0.50%), T4 (0.75%), T5 (1.00%) and 2 controls, namely T0 (negative control, 2% acetic acid) and T6 (positive control, 30 ug tetracycline). The results of measuring the degree of deacetylation were 77.97%, and the molecular weight was 71,645.56018 g mol<sup>-1</sup>. The results of the antibacterial activity test showed that *L. vannamei* shells chitosan was able to inhibit the growth of *V. alginolyticus* at a concentration of 0.50% as the minimum inhibitory concentration, with an inhibitory zone diameter of 6.17±0.76 mm, and at a concentration of 0.75% as the optimum inhibitory concentration, with an inhibition zone diameter of 8.50±1.32 mm. This research demonstrates the possibility to inhibit the growth of *V. alginolyticus* using natural ingredients.

**Key Words:** shrimp diseases, inhibitory zone, degree of deacetylation, crustacean shells, chitin.

**Introduction.** Since 2007 until 2017 world shrimp production increased in Asia, China being the first country producing shrimp, followed by Thailand and Vietnam ranked second and third, respectively, while Indonesia was ranked fourth (Anderson et al 2019). Vannamei shrimp (*Litopenaeus vannamei*) has a high added value and is an export commodity (Adam et al 2022), being a large contributor to export revenues, besides oil and gas (Sitompul et al 2018). The high market demand for *L. vannamei* causes this species to be widely cultivated. It is very tolerant to environmental changes, has a high growth rate and a short cultivation period (Thakur 2018; Venkateswarlu et al 2019). Intensive *L. vannamei* cultivation is associated with several challenges, including disease outbreaks caused by microorganisms that can lead to mass mortality (Pangastuti et al 2010). Shrimp experiencing stress have a deficient immune system and are vulnerable to vibriosis (Aziz & Cahyadi 2020). Mass death of shrimp due to disease is the biggest problem for shrimp farmers in Indonesia (Sun & Xiang 2013). Shrimp diseases are predominantly caused by bacterial and viral pathogens (Li et al 2019; Patil et al 2021). Among these, bacteria belonging to the genus *Vibrio* are the most common causative

agents. Infections caused by these bacteria, collectively referred to as vibriosis, often result in high mortality in shrimp populations. Consequently, vibriosis leads to substantial economic losses for shrimp farmers, primarily due to significant reductions in shrimp production (Ramadhani et al 2022; De Souza Valente & Wa 2021). Zoa-2 syndrome is a major problem in rearing shrimp larvae because it can cause up to 90% mortality (Rusmana et al 2021). Loss of digestive capacity and nutrient absorption is a pathological symptom of vibriosis disease which results in shrimp molting, causing a high mortality in second stage zoea larvae (Kumar et al 2017).

The main factor causing vibriosis in shrimp is vibrio bacteria including *Vibrio alginolyticus*, *Vibrio vulnificus*, *Vibrio fluvialis*, *Vibrio anguillarum* and *Vibrio harveyi* (Rusmana et al 2021). All stages of shrimp life, from protozoa, larvae, post-larvae, even young and adult shrimp can be infected with vibrio bacteria (Zheng et al 2017). Therefore, prevention and control of vibrio populations in ponds needs to be carried out. The shortcut taken by farmers is to use synthetic antibiotics at inappropriate doses which can cause resistance to these bacteria (Chatterjee & Haldar 2012). Several previous research results showed that *V. alginolyticus* isolated from seawater sediments was 100% resistant to the antibiotics erythromycin E and penicillin P (Drais et al 2018). Meanwhile, *Vibrio parahaemolyticus* from the east coast of Saudi Arabia is 98% resistant to the antibiotic carbenicillin, 88% to ampicillin, and 76% to cephalothin (Ghenem & Elhadi 2018). Although efforts to overcome the problem of vibriosis by using natural ingredients that have the potential to act as natural antibiotics have been carried out previously, to date they have not produced optimal results to replace chemical antibiotics. Therefore, more research needs to be carried out to look for natural ingredients that have the potential to act as antibiotics in treating vibriosis. Thus, controlling vibriosis is directed at using environmentally friendly materials that are easily available (Fitri et al 2018).

One of the main fish and shrimp fishing areas in Indonesia is the Java Sea (Tirtadanu & Suprpto 2017), in particular the northern waters of Brebes. Processing shrimp leaves shell waste amounting around 40-50% of the total weight of the shrimp itself. Although shrimp shell waste can cause environmental pollution, it has economic value as it can be processed into chitosan (Kusnadi et al 2022), solving the management issues of both the waste and vibriosis (El Knidri et al 2018). Chitosan processing from *L. vannamei* shells (Mohammed et al 2013) of *Penaeus monodon* (Srinivasan et al 2018) and *Metapenaeus stebbingi* (Kucukgulmez et al 2011) have been reported previously but have not been tested on *V. alginolyticus* bacteria to overcome the problem of vibriosis in shrimp. Various shrimp wastes are produced from shrimp cultivation, including shell from molting. Chitosan and even nano chitosan are made by utilizing shrimp shell waste (Kurniawidi et al 2022). Crustacean shells are the second largest source of chitin which can be processed into chitosan, a biopolymer obtained by the chitin deacetylation process using strong alkali (Hossain & Uddin 2020; Jiménez-Gómez & Cecilia 2020). Utilization of shrimp shell waste has not been carried out optimally, since it has potential applications in various fields, especially in the field of shrimp cultivation to overcome the problem of vibriosis (Trung et al 2020). Biocompatibility, biodegradability, and non-toxicity are some of the properties of chitosan (Sarode et al 2019), which can also be used as an antimicrobial, antioxidant, antitumoral supplement (Sabu et al 2020) or in pharmaceutical drugs, as well as in wastewater remediation (Kumar et al 2018; Hardiningtyas et al 2022).

Several studies have been carried out regarding the antibacterial activity of chitosan, for example chitosan from shrimp (Benhabiles et al 2012) and chitosan from *Auricularia* sp. (Chang et al 2019) on *E. coli* bacteria, but not related to the *V. alginolyticus* bacteria, which causes vibriosis in shrimp. However, published research regarding the antibacterial activity of chitosan from *L. vannamei* shells against the bacteria *Vibrio alginolyticus* is scarce. This is the rationale of this research on determining the degree of deacetylation and the molecular weight of chitosan from *L. vannamei* shells and on the antibacterial activity of *L. vannamei* shell chitosan against *V. alginolyticus* bacteria, with minimum and optimum inhibitory concentrations as parameters.

## Material and Method

**Materials.** This study used chitosan material extracted from the shells of *L. vannamei* originating from the Maluku Sea, pure culture of *V. alginolyticus* bacteria, triphthone soya broth (TSB; CM0129, Oxoid, Basingstoke Hampshire, England), triphthone soya agar (TSA; CM0131, Oxoid, Basingstoke Hampshire, England), tetracycline (Novapharin, Gresik-Indonesia), disc paper (MN 827 ATD ø 6 mm, Macherey-Nagel, Germany). The tools used in this study were an analytical balance (Adventurer Pro AV264C, Ohaus, Pine Brook, NJ USA), hot plate magnetic stirrer (Magnetic stirrer MSH300, Boeco, Germany), oven (UFB 400, Memmert, Schwabach, Germany), autoclave (ES-315, TOMY, Tokyo, Japan), spectrophotometer (PD-303UV, Apel, Japan), shaking incubator (SI4-2, SHEL LAB, USA), and incubator (INB 500, Memmert, Schwabach, Germany).

**Extraction of chitin from vannamei shrimp shells.** The dried white shrimp shells were crushed into powder with a size of approximately 50 mesh. Deproteination was carried out by soaking the white shrimp shell powder in a 3.5% NaOH solution with a ratio of 1:10 (w/v), then heating while stirring using a hot plate stirrer at 65°C for 2 hours. After cooling, it was filtered using Whatman filter paper no. 40. Then, it was washed using aquades, until the neutral pH, and placed in the oven at 60°C for 4 hours, until the constant weight. Demineralization was carried out by soaking the deproteinated residue in 1N HCl solution with a ratio of 1:15 (w/v) for 30 minutes at room temperature to remove CaCO<sub>3</sub> and Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> levels. Depigmentation was carried out adding sufficient acetone to the demineralized residue, filtering, then in a ratio of 1:10 (w/v) adding 0.315% NaOCl and leaving for 30 minutes at room temperature, afterwards filtering and washing with aquades until the neutral pH. Subsequently, placing it in the oven at 60°C for 4 hours to dry until constant weight (No et al 2003).

**Transformation of chitin into chitosan.** Deacetylation is the process of converting chitin into chitosan. To a 50% NaOH solution, chitin was added in a ratio of 1:10 (w/v), then heated for 6 hours at 100°C, and cooled to room temperature. The cooled residue was purified using a Whatman filter paper no. 40. Also, using distilled water, the residue was washed until the neutral pH. Next, the residue was placed in an oven at 80°C for 24 hours until the weight was stabilized.

**Deacetylation degree of chitosan.** The quality of chitosan is determined by the percentage of degree of deacetylation (DD%) which indicates the level of purity of chitosan. The DD% is determined using Fourier Transform Infrared spectroscopy (FTIR), based on transmittance (%) or absorption values (Kurniawidi et al 2022). Comparison of the absorption wave number of the amino-NH group (A<sub>1655</sub>) with the primary amine absorption wave number (A<sub>3450</sub>), characteristic for an absorption value of 1.33, is used to determine the DD% value in the complete deacetylation process (Mohammed et al 2013). The following equation determines the degree of deacetylation (as percentage):

$$DD\% = 100 - \left[ \left( \frac{A_{1655}}{A_{3450}} \right) \times \frac{100}{1.33} \right]$$

Where:

A<sub>1655</sub> - the average absorption (%) after and before the wave number 1655;

A<sub>3450</sub> - the average absorption (%) after and before the wave number 3450.

**Chitosan molecular weight.** The following procedure was used to determine the molecular weight of chitosan using the viscometric method: 0.1 g of chitosan, resulting from the conversion of *L. vannamei* shell chitin, dissolved into 100 mL of 0.01M HCl, then solutions with viscosity values of 0, 1, 0.2, 0.3, 0.4 and 0.5% were prepared. The flow time was measured using an Ostwald viscometer. The viscosity of the solution was used to determine the molecular weight of the chitosan polymer, based on the specific viscosity

equation, intrinsic viscosity and the empirical Mark-Houwink equation (Sugiyanti et al 2018; Kassal et al 2020):

$$(\eta) = \lim_{c \rightarrow 0} \left( \frac{\eta_{sp}}{c} \right)$$

$$(\eta) = kM^a$$

Where:

$\eta_{sp}$  - the specific viscosity;

$c$  - the polymer concentration (in g 100 mL<sup>-1</sup>);

$a$  and  $k$  - constants for the polymer solvent,  $M$  is the molecular mass;

$\eta$  - the intrinsic viscosity.

**Antibacterial activity test.** 60  $\mu$ L of *V. alginolyticus* liquid culture inoculum were placed into a petri dish (equivalent to 10<sup>6</sup> cells mL<sup>-1</sup>) then 10 mL of TSA solid medium (temperature  $\pm$ 45°C) were poured and homogenized, then allowed to harden. Paper discs that had previously been sterilized were dipped into the chitosan of *L. vannamei* shells with concentrations of T0 (0% chitosan concentration + 2% acetic acid as negative control), T1 (0.10% chitosan concentration), T2 (0.25% chitosan concentration), T3 (0.50% chitosan concentration), T4 (0.75% chitosan concentration), T5 (1.00% chitosan concentration), and T6 (0% chitosan concentration+ 30  $\mu$ g tetracycline as positive control). After the TSA media has hardened, the paper discs were placed on the surface of the TSA medium, then incubated at 30°C for 24 hours. The paper disc diffusion method was used to determine the amount of antibacterial activity of *L. vannamei* shell chitosan (Vaz et al 2018). After incubation at 30°C for 24 hours, the clear area formed around the disc paper was measured in millimeters (mm) using a vernier caliper and then subtracted by the disc paper diameter (6 mm). The clear zone formed around the paper disc is an inhibitory zone which describes the antibacterial activity which is measured using calipers (Octarya et al 2021).

**Analysis of statistical.** The data obtained were analyzed using a one-way Analysis of Variance (ANOVA) and the Tukey's Honestly Significant Difference (HSD) test at a 95% confidence level (Musa et al 2020).

## Results

**Degree of deacetylation (DD).** Chitosan is a deacetylation product of chitin with hot concentrated NaOH. This method is used due to the thick structure of chitin cells and to the strength of the intramolecular hydrogen bonds between the amine group (source of the hydrogen atom) and the carboxyl group (source of the oxygen atom). The breaking of the bond between the carbon in the acetyl group and the nitrogen in the amine group causes the loss of the acetyl group in chitin during the deacetylation process with a heated strong base. The deacetylation stage determines the number of acetamide groups that are transformed into amines.

The chitosan resulting from the conversion was characterized using the FTIR spectrophotometric method, the spectrum results of which can be seen in Figure 1. The degree of deacetylation was calculated to determine the extent of the acetyl groups loss during the transformation of chitin into chitosan.

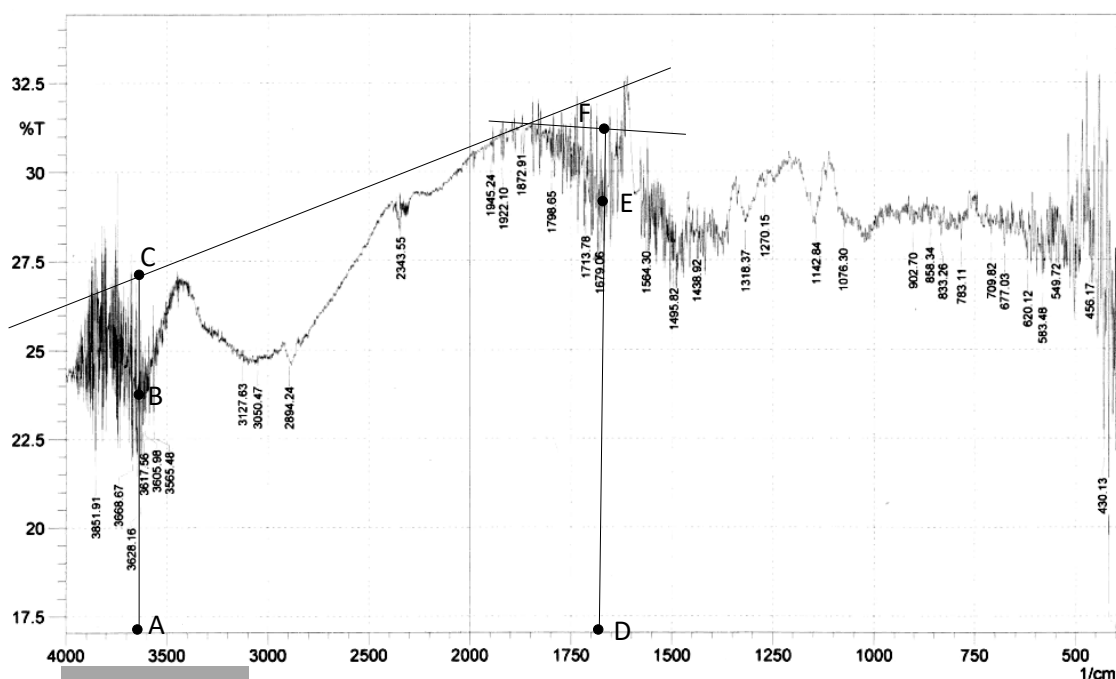


Figure 1. FTIR spectrum of *Litopenaeus vannamei* shell chitosan.

Based on the results of the FTIR spectrum of chitosan converted from *L. vannamei* shell chitin (Figure 1), the DD value can be calculated as follows  $AC=7.8$  cm,  $AB=3.7$  cm,  $DF=10.7$  cm, and  $DE=8.6$  cm.

$$A_{1655} = \log\left(\frac{DF}{DE}\right) = \log\left(\frac{10.7}{8.6}\right) = 0.094885$$

$$A_{3450} = \log\left(\frac{AC}{AB}\right) = \log\left(\frac{7.8}{3.7}\right) = 0.323893$$

$$DD\% = 100 - \left[\left(\frac{A_{1655}}{A_{3450}}\right) \times \frac{100}{1.33}\right]$$

$$DD\% = 100 - \left[\left(\frac{0.094885}{0.323893}\right) \times \frac{100}{1.33}\right] = 77.97\%$$

Based on the calculations above, the obtained DD value of *L. vannamei* was 77.97%. This DD value is not very different compared to the results of El Knidri et al (2018), who also used *L. vannamei* shells to produce chitosan with a DD value of 81.20%, or of Tirtadanu & Suprpto (2017), who used white shrimp (*Penaeus indicus*) shells and produced chitosan with a DD value of 78.60%. The chitosan resulting from this research is classified as pure, because it has a DD value above 75%. Chitin can turn into chitosan if it has a minimum deacetylation degree of 75%.

**Chitosan molecular weight (MW).** The viscosity of the polymer solution was measured using Ostwald viscosity to determine the molecular weight of chitosan. Several viscosity parameters of the polymer solution are shown in Table 1.

Table 1

Viscosity parameters of polymer solutions in determining the molecular weight of chitosan

$c$ =the polymer concentration in $g\ 100\ mL^{-1}$ (%)	$t$ =efflux time of chitosan solution in (s)	$t/t_0$ , $t_0$ =efflux time of solvent (0.01 M HCl) (s)	$\eta_{sp}$ =specific viscosity	$\eta_{sp}/c$ =reduced viscosity ( $dL\ g^{-1}$ )
0.00	97.93333	1	0	0
0.01	100.01	1.021205	0.021205	2.120490
0.02	103.83	1.060211	0.060211	3.010551
0.03	108.07	1.103506	0.103506	3.450193
0.04	113.2467	1.156365	0.156365	3.909122
0.05	119.7733	1.223009	0.223009	4.460177

The curve depicting the relationship between reduced viscosity and concentration (Figure 2) was created based on the data in Table 1. The molecular weight of chitosan was determined based on intrinsic viscosity according to the presented equations. Intrinsic viscosity shows the ability of the polymer to increase the viscosity of the solution. Intrinsic viscosity is obtained from the  $\eta_{sp}/c$  curve which is extrapolated until  $c$  approaches 0, in order to eliminate the effect of concentration.

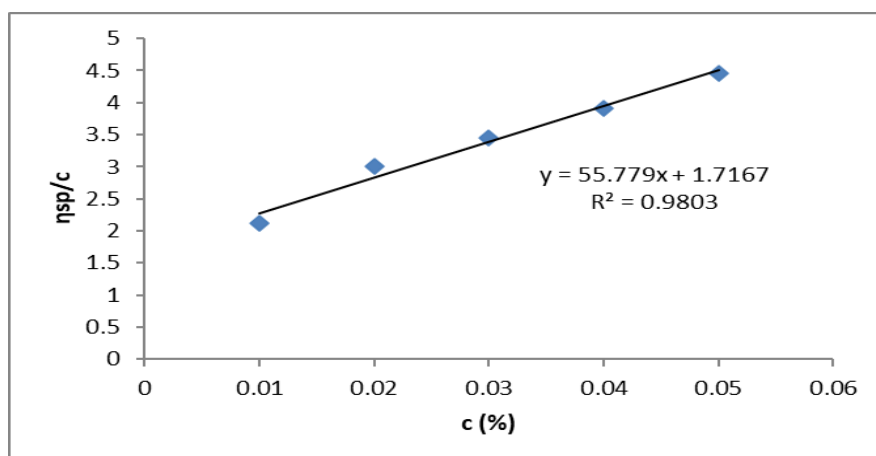


Figure 2. Curve for determining the molecular weight of chitosan.

Based on the line equation of the curve above (Figure 2), namely  $y=55.779+1.7167$ , the intercept value is 1.7167, which is the intrinsic viscosity. By using the Mark-Houwink equation, the molecular weight (MW) value can be obtained, namely  $71,645.56018\ g\ mol^{-1}$ , with a value of  $K=5.48 \times 10^{-4}$  and  $a=0.72$ . In this study, the molecular weight of *L. vannamei* shell chitosan is smaller when compared to the results of Kusnadi et al (2022), who used white shrimp shells (*Penaeus indicus*) and found a chitosan molecular weight of 116.85. This difference in molecular weight is caused by differences in the degree of DD. At larger DD, more acetamide is replaced by amine, causing the molecular weight of chitosan to decrease.

**Antibacterial activity.** The antibacterial activity test of *L. vannamei* shell chitosan on *V. alginolyticus* by the paper disc diffusion method showed that around the paper disc a clear zone was formed, indicating the presence of antibacterial activity, the magnitude of which varied in each treatment (Figure 3).

The results of statistical analysis at 95% confidence level showed that T0, T1, T2, T3 and T5 were not significantly different from each other, but they were significantly different from T4 and T6, while T4 and T6 themselves were significantly different from each other (Table 2).

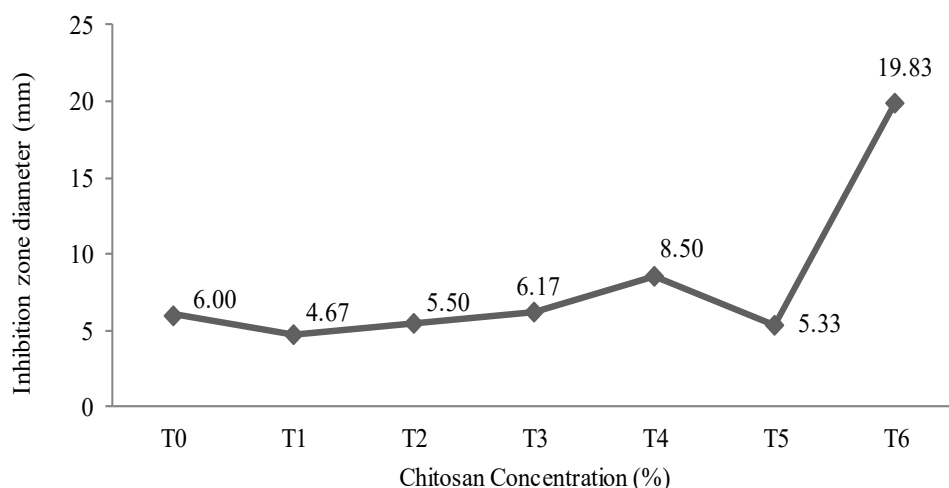


Figure 3. Graph of average inhibition zone diameter.

Table 2

The average inhibition zone diameter

Treatment	Parameter
	Average inhibition zone diameter (mm)±SD
T0	6.00±0.50 <sup>a</sup>
T1	4.67±0.29 <sup>a</sup>
T2	5.50±1.50 <sup>a</sup>
T3	6.17±0.76 <sup>a</sup>
T4	8.50±1.32 <sup>b</sup>
T5	5.33±0.29 <sup>a</sup>
T6	19.83±0.29 <sup>c</sup>

T0 (negative control), T1 (chitosan concentration 0.10%), T2 (chitosan concentration 0.25%), T3 (chitosan concentration 0.50%), T4 (chitosan concentration 0.75%), T5 (chitosan concentration 1.00%), T6 (positive control). Significant differences ( $p < 0.05$ ) are indicated in the same column with different superscripts

**Discussion.** In this study, a negative control (T0) was used in the form of 2% acetic acid (the best solvent for dissolving chitosan) and a positive control (T6) using 30 µg tetracycline. Meanwhile, tetracycline was used as a positive control (a synthetic antibiotic used to control vibriosis in shrimp). It was expected that 2% acetic acid as a negative control does not form an inhibition zone, but the results of this study indicate that 2% acetic acid also forms an inhibition zone with a diameter of 6 mm. Therefore, in determining the inhibitory concentration, a benchmark of 6 mm was used as the minimum limit for the inhibition zone diameter of the white shrimp shell chitosan.

According to Liu et al (2006), acetic acid has antibacterial activity. The higher the acetic acid concentration, the higher the antibacterial activity (Kurniasih & Kartika 2009). Therefore, when used as a solvent for chitosan, the role of acetic acid cannot be ignored. The results showed that the chitosan of *L. vannamei* shells has the potential to inhibit the growth of *V. alginolyticus* bacteria by forming an inhibition zone whose size varies depending on the concentration of the chitosan. Table 2 shows that although the T1 and T2 treatments formed an inhibition zone, the diameter was still below T0. Thus, the T3 treatment was the minimum inhibitory concentration for *V. alginolyticus* bacteria, with an inhibition zone diameter of 6.17 mm. The T4 treatment was the optimum inhibitory concentration for *V. alginolyticus* bacteria, with an inhibition zone diameter of 8.50 mm (Table 2 and Figure 4).

The results of the statistical analysis of the Tukey test showed that the T3 and T4 treatments were significantly different at the 95% confidence level ( $p < 0.05$ ). The T5 treatment actually formed a smaller inhibition zone than the T4 treatment even though

the chitosan concentration level was higher. This is because the T5 treatment has a higher solution viscosity than the T4 treatment (and the other treatments), so that the diffusion power of the T5 solution into the TSA medium is lower which causes the antibacterial effect of the chitosan to decrease. Chitosan concentration greater than 0.75% does contain higher levels of chitosan but has a small diffusion rate and cannot even diffuse into the TSA media because it is too thick, so the antibacterial effect of chitosan cannot work. Proteins in microbes can be formed as a result of negatively charged amino acid molecules being attracted by the positive charge on the amine functional group (-NH<sub>2</sub>) which is the source of the antibacterial effect of chitosan. The amine functional group also has a lone pair of electrons that attracts Mg<sup>2+</sup> to the ribosome and Ca<sup>2+</sup> to the microbial cell wall, by forming a covalent bond. These cause microbes' death due to intracellular leakage from cell walls breaking. Chitosan also prevents bacterial colonies' growth (Sarwono 2010).

Tetracycline 30 µg, as a positive control (T6 treatment), did provide the greatest inhibitory effect by forming an inhibition zone with a diameter of 19.83 mm. However, tetracycline is not recommended for use in tackling vibriosis in shrimp because tetracycline is a synthetic antibiotic which has many negative effects both on the shrimp product itself and on the environment, it is difficult to decompose, and can even cause mutations into new strains if the dosage is not correct. Chitosan can inhibit bacterial growth through the mechanism of interaction of the positive charge of chitosan with the negative charge on the bacterial cell wall, causing the permeability of the cell wall to change. This will cause the loss of several cell components such as protein, amino acids and glucose. Thus, chitosan will inhibit the metabolism of microorganisms and ultimately result in cell death. In addition, the positive charge of chitosan interacts with bacterial DNA, resulting in inhibition of RNA and protein synthesis. In this mechanism, chitosan must have a small molecular weight so that it can enter the cells of microorganisms (Kurniasih & Kartika 2009).

The antibacterial properties of chitosan lie in the amine functional group. The positive charge on the amine group will electrostatically interact with the negative charge on the microbial cell membrane. As a result of this interaction, the cell membrane experiences permeable pressure which causes an imbalance in the osmotic pressure in the cell so that microbial growth is hampered. Microbial cell death is also caused by the release of cell electrolytes in the cell wall due to hydrolysis events (Sarwono 2010). Molecular weight can affect the chitosan antibacterial activity. The lower the MW of chitosan, the better the antibacterial activity. The effect of the MW of chitosan on antibacterial activity is that if the MW increases, then penetration into the cell nucleus decreases so that the antibacterial properties will also decrease or in other words the antibacterial properties of chitosan will be better if the MW of chitosan is reduced (Liu et al 2006). The higher the DD tends to have stronger antibacterial activity.

**Conclusions.** Based on the degree of deacetylation, *L. vannamei* shells contain chitosan and have the effect of inhibiting the growth of *V. alginolyticus* bacteria. The results of this research can be used in the development of natural antibiotics to eradicate vibriosis in marine and brackish water biota, especially in the cultivation of *L. vannamei* and tiger shrimp.

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**Conflict of interest.** The authors declare that there is no conflict of interest.

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