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Chitosan of Shrimp Shell as a Natural Antibiotic Candidate for Bacteria *Vibrio harveyi* and *Vibrio alginolyticus* Causes Vibriosis in Tiger Shrimp (*Penaeus monodon*)

Petrus Lapu*, I.G.N.G. Bidura, I.W. Suarna***, & I.G. Mahardika******

**Faculty of Mathematics and Natural Sciences, Pattimura University, Ambon, Indonesia*
,,&****Faculty of Animal Science, Udayana University, Denpasar, Indonesia*

ABSTRACT

This research was conducted with the aim to determine the potential of chitosan derived from shrimp shells as a candidate for natural antibiotics in Vibrio harveyi and Vibrio alginolyticus. Shrimp shells are extracted into chitin through the process of deproteination, demineralization and depigmentation, then the chitin obtained is transformed into chitosan through a deacetylation process. The chitosan produced was then measured in the form of water content, ash content, and the degree of deacetylation and then compared with the chitosan quality standard issued by the protan laboratory. The chitosan produced in this study fulfilled the chitosan quality standard that is the water content of 10.77%, ash content of 1.0839%, and the degree of deacetylation of 70.04%. Furthermore, the chitosan was applied in the inhibitory test of Vibrio harveyi and Vibrio alginolyticus using agar diffusion method with chitosan concentration of 1.00%, 0.75%, 0.50%, 0.25% and control acetat acid 2%. It was concluded that shrimp shells chitosan has a minimum inhibitory concentration on Vibrio harveyi and Vibrio alginolyticus bacteria at a concentration level of 0.25% each of 1.2000 mm and 2.8000 mm

Keywords: *Chitosan, vibriosis, antibiotics, shrimp shells*

INTRODUCTION

Various crop failures that occur in shrimp ponds in Indonesia become a phenomenon that is very detrimental to pond farmers. Failure to harvest is usually caused by *Vibrio* bacterial attack which results in the death of shrimp in a fast time and in large numbers. Shrimp that are attacked by *Vibrio* are generally characterized by clinical symptoms, where the shrimp look weak, dark red or pale, the antenna and swimming legs are red. *Vibrio* is a pathogenic bacteria that infects and causes disease when shrimp conditions are weak and extreme environmental factors. The potential for such a large spread of *Vibrio* should be addressed immediately by making various countermeasures (Felix *et al*., 2011).

Research on disease management in aquaculture is still limited to the use of chemicals such as formaldehyde, malachite green and some types of antibiotics such as chloramphenicol, oxytetracyline and prefuran (Brown, 1989). However, the use of antibiotics still leaves the problem of environmental pollution because of residues that are difficult to decompose, even antibiotic residues can contaminate exported fishery products. In addition, the use of antibiotics with the wrong dose can cause resistance to bacteria that can cause new strains. As stated by Sukenda (2008) efforts to control disease by using antibiotics for long-term

uncontrolled and improper doses can have a negative impact. This impact can not only cause bacterial strains that are resistant to antibiotics that can harm humans (zoonotics), but can also pollute the aquatic environment, and even have an impact on health by the presence of chemical residues from antibiotics in consumed fishery products.

The use of antibiotics in the cultivation of tiger shrimp (*Penaeus monodon*) has been banned by the government due to leaving residues. An alternative replacement is a natural antibiotic. Until now, alternatives are still being sought to treat fireflies caused by the bacterium *Vibrio harveyi*. Cultivators still depend on the use of chemicals to improve the health status of tiger prawns, an antibiotic that has been banned by the government, resulting in Indonesian products finding it difficult to compete in the global market due to the issue of antibiotic use that leaves residues (Trismawanti *et al*., 2014; Muliani *et al*., 2014). Therefore it is necessary to try to replace chemical antibiotics with natural antibiotics.

Hargono *et al*. (2008) in Agustina *et al*. (2015) said that one alternative to overcome this environmental disturbance phenomenon is to utilize shrimp shells containing chitin and subsequently transformed into chitosan that can be applied in various fields. Rochima (2014) reported that natural ingredients containing antimicrobial compounds, one of which is chitosan. Chitosan is a polysaccharide obtained from chitin deacetylation. Chitin is found in the head, skin, tail and carapace of *Crustaceae*. Chitosan application development is very potential because the amount of shrimp, crab and crab production as chitin raw material continues to increase.

The large number of shrimp production will produce waste which is a byproduct of production in the form of head, skin, tail and feet at 35-50% of the initial weight. Waste generated from shrimp freezing, shrimp canning, and shrimp cracker processing ranges from 30-75% of the weight of shrimp (Susilowati *et al*., 2015). In Indonesia, shrimps undergo a process of "cold storage" in which the head, tail and skin are removed as waste. This shrimp waste can pollute the environment around the factory so it needs to be utilized. So far, shrimp shells have only been used as ingredients for making crackers, shrimp paste, and animal feed ingredients, even though 20-30% of the waste contains chitin compounds that can be converted into chitosan (Isnawati *et al*., 2015).

Chitosan is the result of fishery waste originating from crustacean skin after demineralization, deproteination, depigmentation and deacetylation. The basic ingredients of chitosan are easily obtained, available in large quantities, and have not been utilized optimally. Approximately 80-90% of shrimp exports are carried out in the form of frozen shrimp without head and skin, estimated to produce waste weighing up to 50-60% of the weight of whole shrimp (*Hernawati et al*., 2013). Chitosan as a natural polymer that has a high molecular weight, and non-toxic can stimulate the immune system, accelerate wound healing, and is antibacterial (Suptijah, 2006).

Chitosan has antimicrobial properties because its activity can inhibit pathogenic bacteria and spoilage microorganisms, including fungi, gram-positive bacteria, and gram-negative bacteria (Hafdani, 2011; Nurainy *et al*., 2008). Chitosan is bacteriostatic which inhibits bacterial growth, antimicrobial compounds in chitosan are able to prolong the adaptation phase and slow down the logarithmic growth phase of microbes (Damayanti *et al*., 2016). Chitosan is widely used in various purposes, chitosan can be used as a food preservative, antimicrobial, metal absorber and water purifier (Sarwono, 2010).

Harvest failures that harm pond farmers due to *vibriosis* attacks on shrimp culture both at the level of seed homes (hatcheries) and rearing ponds, the use of chemical antibiotics that are very environmentally unfriendly and leave residues on shrimp products can even cause resistance to bacterial *Vibrio* and trigger the emergence of strains new, the amount of shrimp shell waste that pollutes the environment even though the shrimp shell waste contains a lot of chitin which can be transformed into chitosan that has antibacterial activity that can be used as a natural antibiotic candidate in suppressing populations of *Vibrio harveyi* and *Vibrio alginolyticus* that cause *Vibriosis* in black tiger shrimp (*Penaeus monodon*) is an important reason for this research to be carried out immediately.

MATERIAL AND METHODS

Material and Equipment

The materials used in this study were shrimp shells taken from the head, skin and tail, hydrochloric acid (HCl), acetic acid (CH_3COOH), sodium hydroxide (NaOH), sodium hypochloride (NaOCl), aquades, pure culture bacteria *Vibrio harveyi* and *Vibrio alginolyticus*, tripthone soya broth (TSB), tripthone soya agar (TSA). The tools used in this research are mill, analytical balance, hot plate magnetic stirrer, oven, furnace, decoator, pH indicator, thermometer, autoclave, UV-Vis spectrophotometer, shaking incubator, incubator, and supporting equipment such as erlenmeyer, glass chemicals, measuring cups, test tubes, pipettes, pipettes, and others.

Chitin extraction from shrimp shells: Chitin extraction from shrimp shells was carried out used the Hong method (Kusumaningsih *et al*., 2004) in the following manner: (i) Preparation: shrimp shells were washed with water until clean, then dried in the sun. The clean shell were smoothed to get a size of 50 mesh; (ii) Deproteination: to the 250 ml round bottom flask containing shrimp shell powder 3.5% NaOH solution was added in a ratio of 10:1 (v/w), then heated while stirring with a magnetic stirrer for 2 hours at 65 ^{0}C. After chilling, filtered and neutralized with distilled water. The solids obtained were dried in an oven of 60 ^{0}C until dry; (iii) Demineralization: Shrimp shell powder from deproteination plus 1N HCl solution in a ratio of 15:1 (v/w) in 500 ml round bottom flask and refluxed at 40 ^{0}C for 30 minutes, then cooled. After chilling, filtered and solids neutralized with distilled water, then dried in an oven 60 ^{0}C; and (iv) Depigmentation: NaOCl 0.315% solution was added to the demineralized powder at a ratio of 10:1 (v/w) in a 250 mL round bottom flask. Reflux is carried out for 1 hour at 40 ^{0}C, then the solid is filtered and neutralized with distilled water. The neutralized solids are dried in an oven at 80 ^{0}C until the weight remains.

Transforming chitin into chitosan from shrimp shells: chitosan were made from shrimp shell chitin were done through the process of deacetylation of chitin by using the Knorr method (Kusumaningsih *et al*., 2004) by adding 60% NaOH with a ratio of 20:1 (v/w) and refluxing it at 100-140 ^{0}C for 1 hour. After chilling was filtered and the solids obtained are neutralized with distilled water. The solid was then dried in an oven at 80 ^{0}C for 24 hours and chitosan was ready to be analyzed. Chitosan was identified using a UV-visible spectrophotometer instrument at a wavelength of 201 nm.

Characterization of chitosan: Chitosan characterization includes determining the degree of deacetylation, moisture content, and mineral content, with the following description the degree of deacetylation (Liu *et al*., (2006). Chitosan has two distant chromophore groups

namely N-acetyl glucosamine and glucosamine so that it is possible to analyze its absorbance using a UV-Vis spectrophotometer at a wavelength of 201 nm which can then determine the degree of deacetylation using equations (1) and (2).

$$DA = \frac{161.1AV - 0.0128M}{3.3615M - 42.1AV} \quad \text{..... (1)}$$

$$DD = (1 - DA)100\% \quad \text{..... (2)}$$

Note: A): absorbance; V): the volume of the solution and M): the weight of chitosan.

Moisture content is calculated by measuring the reduction in sample weight before and after heating. A number of samples were dried in an oven at 105 ^{0}C for 3 hours, then cooled in a dexicator for 30 minutes to room temperature, then removed to be weighed and calculated using equation (3).

$$\text{Moisture content} = \frac{B1 - B2}{Bs} x100\% \quad \text{.....(3)}$$

Note: B1 = initial weight (g)

B2 = final weight after drying (g)

Bs = sample weight (g)

Ash content was calculated by inserting chitosan samples into a porcelain cup which has weighed before, then ash at 900 ^{0}C in the furnace for 3 hours. After it was cooled in a dexicator for 30 minutes to room temperature, then removed to be weighed and calculated using equation (AOAC, 1995).

Chitosan Inhibitory Test on *Vibrio harveyi* and *Vibrio alginolyticus* Bacteria: Based on the standard curves were made for each of the *V. harveyi* and *V. alginolyticus* test bacteria, 110µL of pure liquid culture inoculum was determined *V. harveyi* (equivalent to 10^6 cells/ml) and 60 µL of pure liquid culture inoculum *V. alginolyticus* (equivalent to 10^6 cells/ml). A total of 10 petri dishes (5 petri for *V. harveyi* and 5 more petri for *V. alginolyticus*) containing 10 mL of frozen TSA media each as a bottom layer were prepared. Then for *V. harveyi* as much as 110 uL from liquid culture was put into a tube containing 10 ml of TSA which is still runny then shaken and poured into a petridish containing lower layer TSA, let stand until frozen. After freezing, each petridish was made into a well with a diameter of 6 mm (agar diffusion method), then put in 40 uL chitosan with a concentration according to the treatment ie 1.00%; 0.75%; 0.50%; 0.25%, and control acetat acid 2%. Then grown in an incubator at a temperature of 37^{0}C for *V. harveyi* and a temperature of 30^{0}C for *V. alginolyticus* for 24 hours to then observe the area of inhibition zone formed.

After incubating at 37^{0}C for *V. harveyi* and 30^{0}C for *V. alginolyticus* for 24 hours, the clear zone formed around the well was measured. The area of inhibition formed is the area of the clear zone minus the area of the well (ø 6 mm). Clear zone areas that form around the wells are areas that were not overgrown with bacteria which was called an area of growth retardation.

Statistical analysis: Data analysis for the inhibitory test was performed using one-way analysis of variance at a 95% confidence level using the SPSS 16.0 program.

RESULTS AND DISCUSSION

The content of protein, mineral content ($CaCO_3$ and $Ca_3(PO_4)_2$), pigment (red orange-castaxanthin), and chitin yield from shrimp shells can be seen in Table 1. After going through the process of deproteination, demineralization, and depigmentation of shrimp shell waste of 400 g can be extracted into chitin at 93.84 g with chitin yield value of 23.46%. This is consistent with the results of research by Haryani and Budiyati, 2007 in Isnawati *et al*., 2015 that 20-30% of shrimp shell containing chitin can be converted into chitosan, as well as Agustina *et al*. (2015) the results of his research prove that shrimp shells containing chitin by 15-30%.

Table 1. Protein, mineral, pigment, and chitin content in shrimp shells

Variables	Weight (g)	Percentage (%)
Shrimp shell (powder)	400	100
Protein levels (Na-protein)	130.40	32.60
Mineral content ($CaCO_3$ and $Ca_3(PO_4)_2$)	144.50	36.12
Pigment levels (red orange-astaxanthin)	31.26	7.81
Chitin yield	93.84	23.46
Decomposed shrimp shell	306.16	76.54

High chitin content in shrimp shell shows that shrimp shell is very high economic value because it has the potential to be transformed into chitosan which is very useful in various fields, especially those related to aquaculture because it has antibacterial activity that can be used as a natural antibiotic in overcoming problems *vibriosis*.

In addition to chitin, shrimp shells also contain 32.60% protein (Na-protein), $CaCo_3$ and $Ca_3(PO_4)_2$ mineral content 36.12%, and pigment (red orange-astaxanthin) content of 7.81 % (Table 1.). The results of this study when compared with the results of research Marganov (2003) in Agustina *et al*. (2015) have relatively similar results, where Marganov (2003) in Agustina *et al*. (2015) states that shrimp shells contain protein of 25-40%, minerals at 45-50%, and chitin at 15-30%, but the amount of the content depends on the type of shrimp. Comparison of the two results of this study can be seen in table 2.

Table 2. Comparison of protein, mineral and chitin content the results of this study with previous research

Parameter	Research results	Marganov (2003) in Agustina *et al*. (2015)
Protein (%)	32.60	25 – 40
Minerals (%)	36.125	45 – 50
Chitin (%)	23.46	15 – 30

In Table 2, it was appears that the mineral content in this study is slightly lower than the results of research Marganov (2003) in Agustina *et al*. (2015), this is thought to be likely due to the different types of shrimp used, the agitation process (stirring) which imperfect so that the heat generated is uneven as stated by Hartati *et al*. (2002) in Zahiruddin *et al*. (2008) that the removal of minerals in the demineralization process is influenced by the agitation process

(stirring) during the process, so that the heat generated becomes homogeneous. A constant stirring process will cause heat to be evenly distributed so that the solvent (HCl) can bind minerals perfectly. If the stirring is not constant, the heat produced is not evenly distributed so that the reaction of binding of minerals by the solvent will also be imperfect.

Table 3, shows the results of the research and the calculation of chitin yield, chitosan yield from shrimp shells, and the total number of decomposed shrimp shells, while Figure 1., is a chitosan product isolated from shrimp shells in this study.

Table 3. Chitin yield, chitosan yield from shrimp shells and the total number of decomposed shrimp shells

Variables	Weight (g)	Percentage (%)
Shrimp shell (powder)	400	100
Chitin yield (% of raw material)	93.84	23.46
Chitosan yield (% chitin)	27.10	28.87
Decomposed shrimp shell	372.90	93.22

In the stages of transformation of chitin into chitosan which only involves one stage, deacetylation is able to transform chitin by 93.84 g into chitosan by 27.107 g or about 28.8% (chitosan yield). The mechanism that works in transforming chitin into chitosan is through the process of deacetylation in strong bases and heat causing loss of acetyl groups in chitin resulting in positively charged chitosan so that it can dissolve in organic acids such as acetic acid or formic acid. The reaction of chitosan formation from chitin is the hydrolysis reaction of an amide by a base. Chitin acts as an amide and NaOH as its base. First the addition reaction occurs, in this process the OH group enters the $NHCOCH_3$ group, then the CH_3COO group eliminates - resulting in an amine called chitosan (Mahatmanti, 2001 in Agustina *et al*., 2015).

Table 4. shows that in addition to the water content, chitosan produced from shrimp shells in this study has the quality of ash content and the degree of deacetylation meet the chitosan quality standards set by the protan laboratory.

Table 4. Comparison of Chitosan Characteristics of Shrimp Shells Results of this Research with the Chitosan Protan Laboratory Quality Standards

Variables	Results of research	Protan laboratory standards*)
Water content	10.77 %	≤ 10 %
Ash content	1.08 %	≤ 2 %
Degree of deacetylation	70.04 %	≥ 70 %

*) Dompeipen *et al*. (2016)

The water content of chitosan results of this study is slightly higher at 10.77% of the chitosan quality standard set by the protan laboratory that was ≤ 10%, this is due to the less optimal drying process in this case the drying time is too fast and the amount of chitosan which is drained too much while the container used is too small. This is consistent with what was stated by Saleh *et al*. (1994) in Zahiruddin *et al*. (2008) that the water content contained in chitosan is affected by the drying process, drying time carried out, the amount of chitosan that is dried and the surface area of the chitosan site dried up. Likewise Agustina *et al.* (2015) stated that the water content in chitosan is influenced by the process during drying, drying

time, the amount of chitosan that is dried, and the surface area where the chitosan is dried. In addition, the high water content of the results of this study is due to the absorption of water vapor when chitosan is open because chitosan contains amine groups that have the ability to bind water molecules, this is in accordance with the opinion of Walke *et al*. (2014) in Cahyono (2018) which states that chitosan is a compound that is hydroscopic in nature therefore chitosan samples have the ability to absorb water during storage. High water content is also influenced by the uneven laying of chitosan in the drying area, so that there is chitosan clumping together and will complicate the drying process.

The ash content in the shrimp shell chitosan in this study amounted to 1.0839%, this shows that the chitosan produced meets the chitosan ash quality standard set by the Protan Laboratory that was ≤ 2%. Low ash content indicates low mineral content. The lower the ash content produced, the quality and purity level of chitosan will be higher because the ash content is a parameter to determine the minerals contained in a material that characterizes the success of the demineralization process carried out. This is in accordance with the opinion of Nugroho *et al*. (2011) that ash content indicates the presence of inorganic compound components contained in the raw material of shrimp shells, chitin and chitosan. According to Nugroho *et al*., (2011) ash content is a measure of the success of the demineralization process in the process of chitin isolation from its raw material. In addition, a good washing process to obtain a neutral pH also affects the ash content. Minerals that have been released from the material and bound to the solvent can be wasted and dissolved with water. Imperfect leaching will result in released minerals which can be reattached to the surface of the chitin molecule (Angka and Suhartono, 2000 in Zahiruddin *et al*., 2008).

The results of the measurement of the degree of deacetylation (DD) chitosan of shrimp shells in this study amounted to 70.04%, fulfilling the chitosan quality standard set by Protan Laboratory that was ≥ 70%. Thus chitosan produced in this study is suitable for various needs, one of which is feasible to be used in bacterial inhibition testing. According to Knoor (1982) in Rochima (2007) the degree of deacetylation is a chitosan parameter that shows the percentage of acetyl groups that can be removed from the chitin yield. The higher the degree of deacetylation of chitosan, the lower the acetyl chitosan group so that the interaction between ions and hydrogen bonds will be stronger. Chitosan is said to be completely de-methylated if the DD value> 90% (Srijanto, 2003 in Agustina *et al*., 2015).

The average of inhibition zones formed on *Vibrio harveyi* and *Vibrio alginolyticus* and the results of analysis of variance and different tests can be seen in Table 5.

Table 5. The results of the analysis of variance and test differences between treatments to the average inhibition zone formed on *Vibrio harveyi* and *Vibrio alginolyticus*

Chitosan concentration (%)	Average zone inhibitor (mm) at *Vibrio harveyi* ($\bar{x}$ ± SD)	Average zone inhibitor (mm) at *Vibrio alginolyticus* ($\bar{x}$ ± SD)
1.00	0.8000 ± 0.44721a	1.6000 ± 0.54772a
0.75	2.2000 ± 0.44721b	2.2000 ± 0.44721ab
0.50	1.2000 ± 0.44721a	2.4000 ± 0.54772ab
0.25	1.2000 ± 0.44721a	2.8000 ± 0.4472b

Note: Means with different superscripts within column values are significantly different (P<0.05)

Table 5, shows that on *V. harveyi* the concentration of chitosan 1.00%, 0.50%, and 0.25% are not significantly different from each other on the average inhibition zone formed but significantly different from chitosan concentration 0.75% at 95% confidence level. Different things were shown in *V. alginolyticus* where the concentration of chitosan 1.00%, 0.75%, and 0.50% were not significantly different from each other on the average inhibition zone formed but significantly different from the concentration of 0.25% in 95% confidence level. Meanwhile, the concentration of 0.25% was not significantly different from the concentration of 0.50%, and 0.75% but significantly different from the concentration of 1.00%.

Chitosan concentration of 0.25% was the minimum concentration that can inhibit bacterial growth or commonly known as MIC (Minimum Inhibitory Concentration) against *V. harveyi* and *V. alginolyticus* bacteria by forming inhibition zones of 1.2000 mm and 2.8000 respectively mm. In general, the results of the inhibitory test showed that the higher the concentration of chitosan, the smaller the inhibitory zone formed in each *Vibrio harveyi* and *Vibrio alginolyticus* bacteria. This is because the higher the concentration of chitosan, the thicker the solution so that it is difficult to diffuse into TSA media so that the effect of chitosan is reduced marked by the smaller inhibitory zone formed. The inhibition zone formed indicates that chitosan has the ability to inhibit the growth of *Vibrio harveyi* and *Vibrio alginolyticus* bacteria.

Chitosan can inhibit the growth of *V. harveyi* and *V. alginolyticus* bacteria in this research because chitosan has a positively charged functional amine (-NH2) group that is very strong which can attract negatively charged amino acid molecules forming protein in *V. harveyi* and *V. alginolyticus*. The amine functional group also has free electron pairs so that it can attract Mg^{2+} minerals found in ribosomes and Ca^{2+} minerals found in the cell walls of *V. harveyi* and *V. alginolyticus* form coordinating covalent bonds. This makes chitosan can cause leakage of intracellular constituents so that the microbes will die. Chitosan breaks down cell walls from microbes so that they cannot develop and eventually die (Sarwono, 2010).

Still according to Sarwono (2010) the antibacterial from chitosan is a functional group of amines and the ability to absorb from chitosan which has a positive charge, while microbial cell membrane is negatively charged. Positive and negative charges interact electrostatically which causes the membrane to undergo permiable pressure which causes osmotic pressure in the unbalanced cell which inhibits the growth of microbes. Inside the cell there is also a hydrolysis process in the cell wall which causes the release of cell electrolytes which causes the death of a cell. Hui (2004) in Killay (2014) states that chitosan compounds can kill bacteria by damaging the bacterial cell membrane. Likewise Dewi *et al*. (2006) in Damayanti *et al*. (2016) stated that the bactericidal mechanism of various cationic antibacterials in general is through interaction and destruction of the membrane/cell wall structure.

The small inhibition zone formed in both Vibrio harveyi and Vibrio alginolyticus were caused by the result of chitosan which has a small degree of deacetylation of 70.04% although it meets the minimum standard quality of chitosan released by protan laboratory, were ≥70%. This is consistent with the statement of Cureo (1999) and Rabeo (2003) in Sarwono (2010) that the higher the level of acetylation of chitosan the more active the antibacterial. The level of antibacterial activity of chitosan can be increased by increasing the

degree of deacetylation, this is very influential on the antibacterial because more and more functional groups (Sarwono, 2010).

CONCLUSION

We conclude that shrimp shells chitosan has a minimum inhibitory concentration on *Vibrio harveyi* and *Vibrio alginolyticus* bacteria at a concentration level of 0.25% each of 1.2000 mm and 2.8000 mm

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