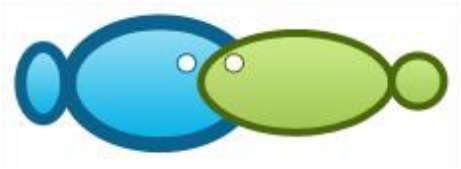

Antibacterial activity of micro-chitosan obtained from vannamei shrimp (*Litopenaeus vannamei*) in Southeast-Sulawesi, Indonesia

[1]Laode M. H. Nadia, [2]Pipih Suptijah, [1]La Ode Huli, [3]Nurmaladewi, [4]Vit N. Satrah

[1] Department of Aquatic Product Technology, Faculty of Fisheries and Marine Sciences, Halu Oleo University, Kendari, Southeast Sulawesi, Indonesia; [2] Department of Aquatic Product Technology, Faculty of Fisheries and Marine Sciences, Bogor Agriculture University, Bogor, Indonesia; [3] Department of Public Health, Public Health Faculty, Halu Oleo University, Kendari, Southeast Sulawesi, Indonesia; [4] Department of Plant Protection, Agriculture Faculty, Halu Oleo University, Kendari, Southeast Sulawesi, Indonesia. Corresponding author: L. M. H. Nadia, hazairinnadia@uho.ac.id

Abstract. Vannamei shrimp (*Litopenaeus vannamei*) presents potential as a raw material of micro-chitosan production, because its shell contains chitin and chitosan. Chitosan is a natural polysaccharide with specific properties, including non-toxicity, biodegradability, and biocompatibility. It is obtained from the process of chitin deacetylation and has a free amino group (NH_2) that makes this polycationic polymer a suitable antibacterial agent. Micro-chitosan is chitosan that has been modified into a micro-polymer with stronger ions and antibacterial properties. The aim of this study was to produce micro-chitosan and to evaluate the effectiveness of antibacterial properties of micro-chitosan obtained from vannamei shrimp. Proximate analysis, deacetylation degree (Fourier Transform Infrared Spectroscopy) vannamei shrimp shell characteristics, chitosan and micro-chitosan, particle size (Scanning Electron Microscopy), and antibacterial activity were evaluated. The results showed that the produced chitosan met the quality standards of chitosan with a moisture content of 4.61% (dw), ash content of 1.57% (dw), the nitrogen content of 3.14% (dw), and degree of deacetylation of 86.23%. Also, micro-chitosan proximate analyses, namely ash content and nitrogen levels were 1.27% (dw), and 3.82% (dw). FTIR and SEM measurement results showed that the deacetylation degree of micro-chitosan was 86.39% and its size 36.4-288 μm. The results of micro-chitosan antibacterial activity showed that a higher concentration of micro-chitosan will produce a greater growth inhibition zone for *S. aureus* and *E. coli*. The greatest inhibition zone by the micro-chitosan was found at a concentration of 0.3%, namely 20.1 mm in *E. coli*, categorized as very strong, and 16.3 mm in *S. aureus*, categorized as strong. The inhibitory spectrum of micro-chitosan was 0.3% greater than alcohol 70%.
Key Words: deacetylation degree, *Escherichia coli*, SEM, *Staphylococcus aureus*.

Introduction. Vannamei shrimp (*Litopenaeus vannamei*) is one of the important commodities of Indonesian aquaculture, with production currently increasing. Production of *L. vannamei* shrimp cultivation was 517397 tons in 2019, and 1290000 tons in 2020 (KKP 2020). Southeast-Sulawesi is one of the regions in Indonesia that has potential for vannamei shrimp cultivation. Vannamei shrimp production in Southeast-Sulawesi amounted to 1720 tons in 2019 and 4922.94 tons in 2020 (BPS 2020). It is mostly exported in a frozen form, where 60 to 70% of the shrimp weight is waste. The wasted shrimp shell is easily degradable and can cause environmental pollution (Azhar et al 2010). In this study, we elaborate a preparation of chitosan from wasted shrimp shell. Chitosan can be synthesized from shrimp shells because it contains chitin.

Chitosan is a natural polysaccharide with specific properties, including non-toxicity, biodegradability, and biocompatibility, obtained from the process of chitin deacetylation. It has a free amino group (NH_2) that makes this polycationic polymer suitable to be used widely either in the food industry or in other industries (Nadia et al 2018). Prior studies have demonstrated that chitosan has many advantages, and can be

used in various different fields, like biotechnology (Mao et al 2001), food industry (Chien et al 2007) or wastewater treatment (Ramnani & Sabharwal 2006). It has many properties, such as anti-tumor (Chaiyakosha et al 2007), antioxidant (Rajalakshmi et al 2013), anti-inflammatory (Oliveira et al 2012), pharmaceutical (Kumar et al 2004), anticancer (Kuppusamy & Karuppaiah 2012) and antibacterial activities (Mahea et al 2011; Aliasghari et al 2016). Chitosan antibacterial activity is affected by several factors including bacterial species (No et al 2002), pH, molecular weight (Liu et al 2006), concentration (Zheng & Zhu 2003), and chitosan solubility (Qin et al 2006).

Chitosan has antibacterial properties that can inhibit the growth of pathogenic bacteria including gram-positive and gram-negative bacteria (Costa et al 2013). The positively charged polycation of chitosan can prevent bacterial growth (Islam et al 2011). The mechanism of antibacterial activity of chitosan is the electrostatic communication between positively charged polycations with the negatively charged cell membranes of bacteria. This interaction affects intracellular membrane permeability. Chitosan binds DNA, and inhibits RNA and protein synthesis (Kong et al 2010).

Previous studies of chitosan had been done by modifying chitosan chemically by increasing the degree of deacetylation, as well as physically by changing the size of chitosan. One of the exceptional development methods is the modification of chitosan into micro-chitosan (micro-polymer) that has stronger ions. Ibrahim et al (2012) noted that a smaller particle and polymer size of chitosan will boost its ability as a rectifier or binder to the surrounding toxic components, including pollutants, microorganisms (including viruses), and toxic materials including exhaust gases, whether in the air, on hands or on surfaces. Micro-chitosan of vannamei shrimp has attracted great attention because of its antibacterial properties. The aim of this study was to produce micro-chitosan and to evaluate the effectiveness of antibacterial properties of micro-chitosan obtained from vannamei shrimp.

Material and Method. The study was conducted from January to March 2021. The formulation and testing of antibacterial activity of micro-chitosan were done in the Laboratory of the Department of Plant Protection, Faculty of Agriculture, Halu Oleo University. The isolates of bacteria *S. aureus* ATCC 8095 (Gram-positive) and *E. coli* ATCC 25922 (Gram-negative) were prepared by the Laboratory of the Faculty of Mathematics and Natural Sciences, Halu Oleo University. Media and chemicals were obtained from Merck.

Formulating chitosan. The manufacture of chitosan from vannamei shrimp referred to the procedure of Nadia et al (2014), which included three processes, namely: deproteination, demineralization and deacetylation. Deproteination was carried out to remove proteins from the shell of shrimp. 100 g of shell received a solution of NaOH 3N in a ratio of 1:10 and was heated at a temperature of 90°C for 1 hour while stirring. It was then filtered and washed with aqueous solution until a neutral pH was reached. Demineralization was carried out to remove minerals. The deproteination result received HCl 1 N solution with a ratio of 1:7 and it was heated at a temperature of 90°C for 1 hour while stirring, then filtered and washed until a neutral pH was reached. The deacetylation of chitin was carried out in a 50% NaOH solution with a ratio of 1:20. It was then heated at 140°C while stirring for 2 hours. Washing was carried out with aqueous solution until a neutral pH was obtained. The product was dried, obtaining chitosan soluble in acid.

Formulating micro-chitosan. This study was commenced by formulating chitosan. Micro-chitosan was obtained using the modified method of Yuliani et al (2018). The first step of the manufacture process of micro-chitosan was done by dissolving 3 g of chitosan in 1% acetic acid, then adding 1000 mL aquades and homogenizing with a magnetic stirrer at a speed of 5000 rpm for 2 hours (to form a homogeneous micro polymer sol). Furthermore, stabilization was carried out with 10 mL of citric acid 3%, which was slowly added to the chitosan solution, so that a chitosan micro-suspension was formed.

Characterization of the shell of shrimp. The characterization of the shell of vannamei shrimp included the determination of moisture content, ash content, and protein content according to the standard methods of AOAC (2005).

Characteristics of chitosan and micro-chitosan. The characteristics of chitosan, and micro-chitosan consist of moisture content, mineral content, and nitrogen content (AOAC 2005). The degree of deacetylation (DD) was calculated using Fourier Transform Infrared Spectroscopy (FTIR) (Domszy & Robert 1985). The particle size of micro-chitosan was determined by using scanning electron microscopy (SEM) (Khan et al 2002).

Preparation of bacterial growth media. Nutrient agar (NA), nutrient broth (NB), and Muller Hilton agar (MHA) media were placed into different test tubes. 5 mL of the prepared NA as bacteria growth media was placed into the test tube. 9 mL of NB, as bacteria growth liquid media, was placed into the test tube. 20 mL of the prepared MHA, as bacteria growth media, for antibacterial activity test was placed into a test tube. Then the NA, NB, and MHA test tubes were sterilized into autoclaves at 121^{o}C for 15 minutes.

Testing of antibacterial activity. The testing of micro-chitosan antibacterial activity was conducted with the agar diffusion method (Nadia 2014). 20 µL of tested bacteria was inoculated into the MHA, then slowly spun to become homogeneous. The mixture was poured into a sterile petri dish and then stored for a few minutes until the MHA solution hardened. Next, a well was made so that the MHA was 5 mm in diameter. After that, 20 µL of micro chitosan (0.1%, 0.2%, and 0.3%), 70% alcohol, and 0.1% acetic acid as solvents were introduced into the agar well. The petri dish was incubated at 37^{o}C for 24 hours. Observations were conducted by measuring the clear zone around the agar well. The diameter of the inhibition zone formed due to the antibacterial properties of micro-chitosan, alcohol 70%, and acetic acid 0.1%. The inhibition zone was measured from the left to the right side using the caliper. The results of the measurement of the inhibition zone were tabulated.

Statistical analysis. Antibacterial activity was determined by descriptive analysis aimed to obtain objective exposure about tested parameters in *S. aureus* and *E. coli* bacteria.

Results and Discussion. The raw material used in this study was the vannamei shrimp shell. Proximate analysis results of the shrimp shells can be seen in Table 1.

Table 1

Proximate analysis results of shrimp shells

Specifications	*Result*
Ash content (dw)	14.13
Nitrogen levels (dw)	17.24
Degree of deacetylation	32.78

Note: dw - dry weight.

Quality characteristics of chitosan. The moisture content, ash content, and nitrogen levels of chitosan had the values of 4.61%, 1.57%, and 3.14%, respectively. The chitosan had a degree of deacetylation of 86.23%. Proximate analysis results and the degree of deacetylation of chitosan can be seen in Table 2.

Table 2

Results of proximate analysis and degree of deacetylation of chitosan

Specifications	*Chitosan test*	*European Food Safety Authority (EFSA 2010)*
Moisture content (dw)	4.61%	≤10%
Ash content (dw)	1.57%	≤2%
Nitrogen levels (dw)	3.14%	≤5%
Degree of deacetylation	86.23%	≥70

Note: dw - dry weight.

Proximate analysis and deacetylation degree (Fourier Transform Infrared Spectroscopy) of micro-chitosan. Chitosan was modified into micro-chitosan by reducing the particle size. The quality characteristics of micro-chitosan included ash content (1.27% dw) and nitrogen levels (3.82% dw). The FTIR measurement results showed that micro chitosan had a degree of deacetylation of 86.39%. The proximate analysis and the degree of micro-deacetylation of chitosan can be seen in Table 3.

Table 3

Proximate analysis and the deacetylation degree of micro-chitosan

Specifications	*Test result*	*European Food Safety Authority (EFSA 2010)*
Ash content (dw)	1.27%	≤2%
Nitrogen levels (dw)	3.82%	≤5
Degree of deacetylation	86.39%	≥70

Note: dw - dry weight.

Analysis of Scanning Electron Microscopy of micro-chitosan. Scanning Electron Microscopy was used to observe the morphology of micro-chitosan. The formulation of micro-chitosan had a particle shape resembling a sphere and exhibited a fairly homogeneous particle size. The SEM test showed that micro-chitosan had a size of 36.4-288 μm. The SEM test can be seen in Figure 1.

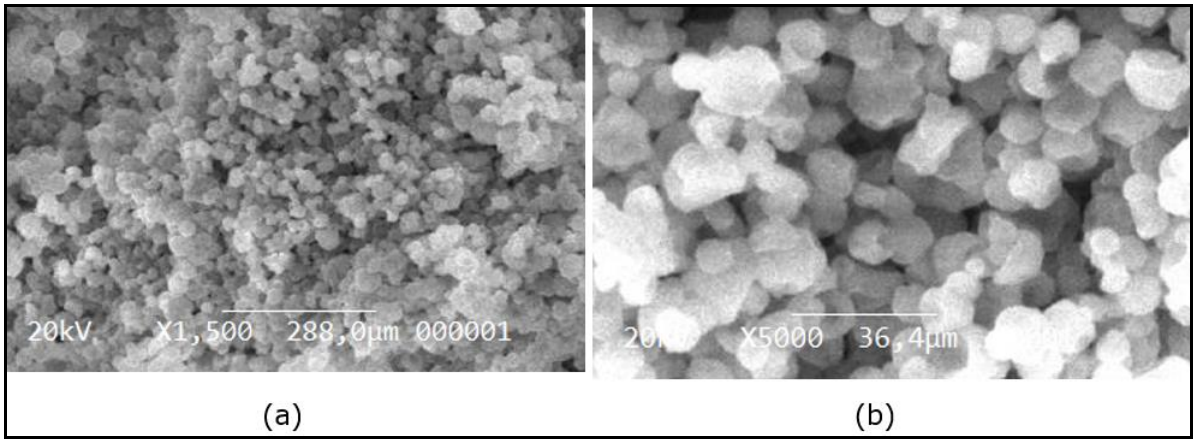

Figure 1. The morphology of micro-chitosan; a - magnification of 1500x; b - 5000x.

Antibacterial activity of micro-chitosan. The diffusion results indicated that 0.1% acetic acid solvent did not inhibit the growth of the tested bacteria. The results of the antibacterial activity test indicated that media containing *E. coli* with micro-chitosan concentration of 0.3% had the largest inhibition zone, 20.1 mm, even greater than the inhibition zone produced by alcohol 70% (positive control), which was 18.4 mm. On the other hand, distinct results were indicated in the micro-chitosan concentration of 0.1%, which had the smallest inhibition zone in media containing *S. aureus*, namely 6.1%. The

results of antibacterial activity against *S. aureus* bacteria growth can be seen in Table 4. The results of antibacterial activity against *E. coli* bacteria growth can be seen in Table 5.

Table 4

The inhibition zone of micro-chitosan, alcohol 70%, and acetic acid 0.1% against *S. aureus*

Concentrations	*The inhibition zone for Staphylococcus aureus (mm)*	*Categories of inhibition activity**
Micro-chitosan 0.1%	6.1	Medium
Micro-chitosan 0.2%	9.7	Medium
Micro-chitosan 0.3%	16.3	Strong
Alcohol 70% (+)	15	Strong
Acetic acid 0.1% (-)	0	Weak

Note: positive control (+); negative control (**-**); * - Nadia et al (2021).

Table 5

The inhibition zone of micro-chitosan, alcohol 70%, and acetic acid 0.1% against *E. coli*

Concentrations	*The rate inhibition zone of Escherichia coli (mm)*	*Categories of inhibition activity**
Micro-chitosan 0.1%	9,3	Medium
Micro-chitosan 0.2%	14,4	Strong
Micro-chitosan 0.3%	20.1	Very strong
Alcohol 70% (+)	18.4	Strong
Acetic acid 0.1% (-)	0	Weak

Note: positive control (+); negative control (**-**); * - Nadia et al (2021).

The ash content of the shrimp shell was lower than the ash content determined by Ravichandran et al (2009) of 21.5% (dw), and Suptijah et al (2011) of 18.02% (dw). According to Ravichandran et al (2009), the difference in ash content was caused by differences in habitat and the environment.

The chitosan used in this study has characteristics that meet EFSA standards (2010). Chitosan purity can be seen from the moisture, ash and low nitrogen content, but has a high degree of deacetylation. A higher degree of deacetylation means more amine groups (NH_2) in the chitosan molecular chain, so that chitosan is more reactive (Nadia et al 2014). Based on the results of this study, analysis of micro-chitosan showed smaller moisture, ash, and nitrogen levels compared to EFSA chitosan quality standards (2010). The degree of deacetylation was 86.39%. A degree of deacetylation above 70% indicates that the modification of chitosan to micro-chitosan did not change the functional properties of chitosan. According to Lewandowska et al (2014), a higher degree of chitosan deacetylation will induce more amino groups in the chitosan molecular chain. Chitosan is more reactive due to the number of amine groups replaced by acetyl groups. The degree of deacetylation is determined by the number of vanished acetyl groups during the deacetylation process. A high degree of deacetylation indicates chitosan purity. Ibrahim et al (2012) obtained a moisture content of microcrystalline chitosan of 3.92%, a nitrogen content of 4%, ash content of 1.4%, and a deacetylation degree of 88.69%. The results were similar to the results of this study.

The process of modifying chitosan into micro-chitosan with the gel sol method has produced micro and even nano sizes. Nadia et al (2014) demonstrated that nano-chitosan is chitosan that had a solid particle with a size between 10-1000 nm. Suwarda & Maarif (2012) suggest that chitosan in the form of nanoparticles has better absorption and better ability as antibacterial and antifungal agent than chitosan with regular size. This makes the nanoparticles more reactive.

The results of the antibacterial activity test showed that the higher micro-chitosan concentration will enlarge the inhibition zone in the tested bacteria. Micro-chitosan concentration of 0.3% had the largest inhibition zone compared to micro-chitosan

concentrations of 0.1%, 0.2%, and alcohol 70%. The inhibition spectrum of micro-chitosan was 0.3% greater than alcohol 70%. Nadia (2014) stated that nano-chitosan antibacterial activity test on *E. coli* with a concentration of 100 ppm had a large inhibition zone of 13 mm, greater than the nano-chitosan concentrations of 25 ppm, 50 ppm, and 75 ppm, with 8, 9 and 9.7 mm, respectively. This distinct bacterial inhibition power occurred at each level of micro-chitosan concentration in the study of Liu et al (2006), who demonstrated that antibacterial chitosan activity relies on the concentration of chitosan in liquid. Antibacterial activity of chitosan in media will increase if the concentration of chitosan increase. Micro-chitosan antibacterial activity of 0.3% against *E. coli* was categorized as very strong, and against *S. aureus* it was categorized as strong. Alcohol 70% activity against the pathogens was categorized as strong. Nadia et al (2021) stated that if the inhibition zone formed in the diffusion test is less than 5 mm, then the inhibitory activity is categorized as weak. The formed inhibition zone of 5 to 10 mm is categorized as medium, 10 to 19 mm is categorized as strong and over 20 mm is categorized as very strong. Micro-chitosan was more effective against Gram-negative bacteria, because the surface of Gram-negative bacteria has a greater negative charge than Gram-positive bacteria, which will interact with positively charged amino groups. Zheng & Zhu (2003) declared that the antibacterial mechanism of chitosan occurs through the interaction of very strong positively charged amino groups with negatively charged cell walls. Goy et al (2016) suggest that antibacterial interactivity in chitosan is related to the absorption ability of bacterial cell walls. Chitosan can be absorbed better in Gram-negative bacteria compared to Gram-positive bacteria, because the negative charge on the cell surface of Gram-negative bacteria is higher than in Gram-positive bacteria.

Conclusions. The resulting chitosan and micro-chitosan quality characteristics did not have important distinctions from the standards set by EFSA 2010. Micro-chitosan modified with the gel sol method generated particle sizes ranging from 36.4 to 288 μm. A higher concentration of micro-chitosan used will enlarge the inhibition zone against *Staphylococcus aureus* and *Escherichia coli* bacteria. Antibacterial activity at micro-chitosan concentrations of 0.3% presented greater inhibition than alcohol 70% (positive control).

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

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Authors:
Laode Muhamad Hazairin Nadia, Department of Aquatic Product Technology, Faculty of Fisheries and Marine Sciences, Halu Oleo University, 93231 Kendari, Southeast Sulawesi, Indonesia, e-mail: hazairinnadia@uho.ac.id
Pipih Suptijah, Department of Aquatic Product Technology, Faculty of Fisheries and Marine Sciences, Bogor Agriculture University, 16680 Bogor, Indonesia, e-mail: suptijah@yahoo.com
La Ode Huli, Department of Aquatic Product Technology, Faculty of Fisheries and Marine Sciences, Halu Oleo University, 93231 Kendari, Southeast Sulawesi, Indonesia, e-mail: laodehuli5@gmail.com
Nurmaladewi, Department of Public Health, Public Health Faculty, Halu Oleo University, 93231 Kendari, Southeast Sulawesi, Indonesia, e-mail: nurmaladewi@uho.ac.id
Vit Neru Satrah, Department of Plant Protection, Agriculture Faculty, Halu Oleo University, 93231 Kendari, Southeast Sulawesi, Indonesia, e-mail: vitnerusatrah@uho.ac.id

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