Antibacterial Potential of Chitosan Extracted from Rama Shrimp (Thalassina Anomala) Carapace

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Abstract. The added value of the waste of Rama shrimp (Thalassina anomala) carapace could be increased by extracting it to be a chitosan through the stages of demineralization, deproteination and deacetylation. The aim of the research was to determine the ability of chitosan extracted from the carapace to inhibit the growth of pathogenic bacteria. Chitosan extracted from the shrimp carapace was showing its chemical characteristics, namely: the content of water 6.74%, ash 4.13%, nitrogen 7.5%, and deacetylation degree 71%. Chitosan was dissolved in 1% acetic acid. Antibacterial activity testing was carried out by using the well diffusion method, conducted by addition of chitosan at varied concentration (0%, 1%, 2%, 4% and 6%). The results showed that the chitosan had an ability to be antibacterial against Salmonella thypi, Escherichia coli, Staphylococcus aureus and Bacillus cereus. It was indicated by the clear zones formed around the well. The clear zone formed was indicating that the chitosan extracted from the carapace could inhibit the growth of pathogenic bacteria at a concentration of 1%. The average of inhibition zone diameter for each bacterium was 11.54 mm, 11.46 mm, 7.78 mm, and 9.84 mm, respectively.

1. Introduction
Rama shrimp (Thalassina anomala) called as “mud lobster” is a semi-aquatic animal belonging to the Crustacean class commonly found in coastal environments that have mangrove vegetation. The shrimp are very abundant fishery commodity often found in the Meranti Islands of Riau Province along the coast of the mangrove coastal area in the District of Selat Panjang. However, up to date the shrimp has not been used optimally because it has a low economic value around IDR 4,000-7,000 / Kg [1].

Most of the body parts of the shrimp is carapace which reaches 50-70%. In general, carapace is not utilized by the community because it has no sale value and will become the waste. This waste has not been used properly and merely became to pollute the environment. Increasing the value added of carapace waste can be done by utilizing it by extracting into chitin and chitosan substances. The chitin substance can be deacetylation which will produce chitosan. Recently, there have been many studies on the using of chitosan in various fields, because chitosan has some advantages. Chitosan is a natural substance, used in small amounts in a concentrate form, has a strong positive charge that can bind the negative charge of other compounds, easily biodegradable, and non-toxic [2].

Chitosan is one of the natural compounds that can be used in the pharmaceutical field as an antibacterial. Chitosan compounds have properties that interfere with the activity of the outer membrane of gram-negative bacteria. However, its ability to inhibit bacterial growth is strongly influenced by the degree of deacetylation. The higher the degree of deacetylation, the greater the
number of amine groups in chitosan. Consequently, the number of protonated amine groups under acidic conditions also increases and eventually dissolves completely, thereby increasing the chance of chitosan interacting with negative charges on the cell walls of microorganisms [3]. In previous study on the use of chitosan in the pharmaceutical field [4] was stated that the chitosan has a degree of deacetylation of 70-100%.

Although the shrimp carapace has the potential to be used as chitosan, the ability of chitosan extracted from the Rama shrimp carapace is still unknown. It because every raw material used in making chitosan will produce different quality chitosan. In order to have good marketing value, this chitosan needs to be tested. One of the quality testing is its potential as an antibacterial in pathogenic bacteria purposing to determine the ability of chitosan to inhibit the growth of pathogenic several bacteria.

2. Methodology

2.1. Materials
The main raw material used in this study was the waste of Rama shrimp (Thalassina anomala) carapace collected from the District of Selat Panjang, Riau Province. The chemicals used were HCl 1.5N, NaOH 3.5N, NaOH 50%, CH₃COOH 1%, concentrated H₂SO₄, catalyst Na₂S₂O₅.5H₂O, solution of NaOH 45%, indicator PP, boric acid 2%, mixed indicator, HCl 0.01N. The materials used for the testing to bacteria were Nutrient Agar, Nutrient Broth, generic chloramphenicol, ethanol 96%, and specific bacterial media cultures for Escherichia coli, Bacillus cereus, Staphylococcus aureus and Salmonella thyphi.

2.2. Methods
The research used descriptive method that explains the process of making chitosan through the steps of demineralization, deproteination, and deacetylation, and then testing to apply it as an antibacterial to pathogenic bacteria by well diffusion method.

2.2.1. Chitosan extracting
Chitin and chitosan extraction refers to the procedure [4] (2004). Chitin preparation process includes the process of demineralization, deproteination, and drying. The chitosan is obtained from deacetylation of chitin.

Demineralization step. Shrimp carapace powder was demineralized with a 1.5N HCl solution at a ratio of 1: 7 (w/v) for 1hr at temperature 90 °C while being stirred with a magnetic stirrer. The results of the demineralization process were then precipitated to separate solids and liquids by settling. The solids obtained are washed with distilled water until the pH is nearly neutral.

Deproteination step. The solid was added with a NaOH 3.5N solution in a ratio of 1:10 (w/v). The mixture is heated at about 90 °C for 1 hr while being stirred with a magnetic stirrer. The results of the deproteination process were then remainly precipitated. The obtained solid was washed repeatedly with distilled water until it reached a neutral pH. The solid was dried in an oven at 60 °C for 6 hrs. Chitin formed was analyzed by water content, ash content, nitrogen content and the degree of deacetylation.

Deacetylation step. Chitin obtained was deacetylated by using 50% NaOH solution at a ratio of 1:10 (w/v) for 1 hr at temperature around 130 °C. The results of the deacetylation process were precipitated to separate solids and liquids by settling. The obtained solid was washed repeatedly with distilled water until it reaches a neutral pH. The solid is dried in an oven at 60 °C for 6 hours. The chitosan formed was analyzed for water content, ash content, nitrogen content and the degree of deacetylation.
2.2.2. Antimicrobial testing

Sterilization. The tools used in this testing, glassware and media, were sterilized in an autoclave at 121°C for 15 minutes. The ose and tweezers are burned by burning them upon the flame.

Media Preparation. NA media or NB was mixed with distilled water and then homogenized and boiled by using a hot plate stirrer until it boils. The medium was then sterilized by using an autoclave at a temperature of 121°C, in pressure of 2 atm for 15 minutes.

Bacterial rejuvenation. The species of testing bacteria were Escherichia coli, Bacillus cereus, Staphylococcus aureus and Salmonella thyphi. The bacterial rejuvenation aimed to increase the number of bacteria by etching one ose of test bacteria on NA media, then each bacterium was incubated at 37 °C for 24 hrs.

Preparation of bacterial suspension test. The bacteria culture was taken using an ose needle, and put into NB media, and then incubated for 24 hours at 37 °C. The bacterial suspension to be used is equivalent to the Mc Farland 1 standard solution which bacterial density was equal to 10^8 CfU/mL.

Preparation of bacteria inoculated with test bacteria. The 0.5 mL of the bacterial suspension was put into a petri dish, added with 15 ml of NA media, and then homogenized by turning the petri dish and allowed to condense.

Preparation of the concentration of chitosan solution. The concentration of chitosan solution used were 6%, 4%, 2% and 1% (w/v) dissolved in 1% acetic acid (v/v). Used as the positive control was 2% chloramphenicol solution, while as the negative control was 1% acetic acid solution.

Determination of antibacterial activity. The method used for determining the antibacterial activity was the well diffusion. The solidified culture media were made aseptically as many as 6 pieces with a diameter of 6 mm. The wells that have been made are put into 60 μL chitosan solution with a concentration of 6%, 4%, 2% and 1% (w/v). 60 μL of 1% acetic acid was added for negative control and 60 μL of chloramphenicol was added for positive control. Petri dishes are closed, then incubated for 24 hours at 37 °C. Bacterial growth was observed and their diameter inhibition growth was measured indicated by the clear zones around the well by using a calipers.

3. Results and discussion

3.1. Characteristics of the chitin and chitosan

Characteristics of the chitin and chitosan extracted from the Rama shrimp carapace was showing the color changing after processing. The chitin was pinkish and slightly brownish in a form of amorphous and biodegradable solids. Chitin is insoluble in water, dilute inorganic acids, organic acids, concentrated alkalies and organic solvents but soluble in concentrated acids such as sulfuric acid, nitric acid, phosphoric acid, and anhydrous formic acid. Chitin in concentrated acid can be degraded into its monomer and break off the acetyl group [5]. Meanwhile, chitosan was in semi-transparent cream colored, in the form of amorphous solids and odorless. The chitosan color produced was in appropriate to the National Indonesian Standard [6], which was light brown to white colored.

The physico-chemical characteristics of chitin and chitosan were indicated by the content of moisture, ash, Nitrogen, and deacetylation degree, as seen in Table 1 below.

| No. | Compound                  | Chitin (%) | Chitosan (%) |
|-----|---------------------------|------------|--------------|
| 1.  | Yield (%)                 | 17.50      | 11.50        |
| 2.  | Moisture (% wb)           | 10.26      | 6.74         |
| 3.  | Ash (% db)                | 6.21       | 4.13         |
| 4.  | Nitrogen (%)              | 6.70       | 7.50         |
| 5.  | Deacetylation degree (%)  | 43.00      | 71.00        |

The yield of chitin and chitosan are 17.5% and 11.5%, respectively. The chitosan yield was obtained from the percentage of the weight of chitosan compared to the initial weight of the carapace.
flour used. The different yield of chitin and chitosan produced was caused by the breaking process of the acetyl bonds (deacetylation) in chitosan. The breaking acetyl bonds in the process of chitosan hydrolysis causes a decrease in molecular size so that the molecular weight of chitosan is lighter than the molecular weight of chitin. The yield of 17.5% chitin was almost equal to the yield of common shrimp was containing chitin in a yield of 18.7-32.9% [7] and in previous study [8] which stated that the shrimp carapace contained 15-20% chitin.

Chitin should have a moisture content not more than 10% in order to prevent damage due to molds [9]. The moisture content in the chitosan yielded was 6.74%, lower than this in the chitin. That is because the process of transforming chitin into chitosan uses sodium hydroxide which is a hygroscopic compound, so that the water content of chitosan is decreasing [10]. However, the moisture content in chitosan is in appropriate to this standardized by National Indonesian Standard (SNI) [6], that the maximum permissible water content of chitosan is 12%. The water content can affect to the resistance of chitosan against microorganisms.

The content of ash in the chitin was 6.21% and this in the chitosan was 4.13%. That is because the deacetylation process uses high NaOH concentrations. The high concentration of NaOH solution can reduce the remnants of minerals that are bound to the polymer [11] and appropriate to the maximum standard 5% determined by SNI [6]. Nitrogen content in chitin and chitosan of rama-shrimps was 6.7% and 7.5%. Nitrogen content in commercial chitosan ranges in 7 - 8.4 %. The nitrogen element in each chitosan monomer is associated as an active group because it is associated with the high content of nitrogen in the polymer chain. Nitrogen content in chitosan produced is appropriate to the chitosan quality for commercial use. The value of chitosan deacetylation degree of the shrimp carapace was 71%. This value is appropriate to this for the pharmaceutical use around 70-100% [4]. The degree of deacetylation of chitosan of Rama shrimp is not too different from chitosan shellfish at 69.11% and crab shell chitosan at 69.67%. However, it is lower than it in a crab shell which has a deacetylation degree of 88% [12].

3.2. Antimicrobial capacity of chitosan

Antimicrobial test of chitosan showed the presence of inhibitory zones, marked as clear zone, on the bacteria of Salmonella thypi, Escherichia coli, Staphylococcus aureus, and Bacillus cereus as seen in Figure 1 below.

![Figure 1. Inhibition zones to bacteria S. thypi (a), E. coli (b), S. aureus (c), and B. cereus (d)](image)

Meanwhile, the results of the measurement of the diameter of the clear zone formed around the well filled with chitosan at various concentrations as seen in Table 2 below.
The antibacterial activity varies depending on the type of test bacteria and the concentration of chitosan. The wider the clear zone formed, the stronger the ability of chitosan to inhibit bacterial growth. Figure 1 showed that the clear zone formed is different for each chitosan concentration. In the negative control solution of 1% acetic acid was also formed a clear zone, because acetic acid solution has the capacity of antibacterial activity [13], therefore, when it was used as a solvent, the role of acetic acid cannot be ignored. Chitosan does not dissolve in water above pH 5.5, bases and most organic solvents, but can dissolve in dilute organic acids [14]. Chitosan can dissolve and active in an acidic solution but cannot react in an alkaline one.

Chitosan extracted from the shrimp carapace at a certain concentration can act as an antibacterial compound to all pathogenic bacteria: Salmonella thyphii, Escherichia coli, Staphylococcus aureus, and Bacillus cereus. Positive control using chloramphenicol showed the greatest inhibition. Chloramphenicol is a broad-spectrum antibiotic. The broad-spectrum antibiotics are antibiotics that are effective against procaryotics both killing and inhibiting gram-positive and gram-negative bacteria [15]. Clear zone area shows how much the ability of chitosan to inhibit the growth activity of bacteria tested. The wider the clear zone formed, the stronger the ability of chitosan to inhibit bacterial growth. The antibacterial activity varies depending on the type of test bacteria and the concentration of chitosan [16].

Table 2 also shows that the lowest inhibitory zone is varied for each bacterium, but for all bacteria tested, the lowest inhibitory zone was achieved at a concentration of chitosan solution 1%. The lowest inhibition zone in Salmonella thyphii was 11.54 mm, Escherichia coli was 11.46 mm, Staphylococcus aureus was 7.78, and Bacillus cereus was 9.84 mm. At a concentration of 1% the bacteria Salmonella thyphii and Escherichia coli which are gram-negative bacteria showed the formation of a larger inhibitory zone compared to Staphylococcus aureus and Bacillus cereus which are gram-positive bacteria. The presence of clear zones contained in petri dishes proved that chitosan solutions can inhibit the activity of the tested bacteria, so it acts as a bacteriostatic.

Chitosan solution at a concentration of 1% has shown potential as an antibacterial and is increasingly apparent at a concentration of 2% for all types of bacteria. The optimum inhibition zone diameters were varied for each type of bacteria tested at a concentration of chitosan solution 2%, namely 13.82 mm, 16.92 mm, 15.91 mm and 14.16 mm, respectively. The higher the concentration of an antimicrobial substance the higher the antimicrobial power, which means that more bacteria are killed faster when the concentration of the antimicrobial compound is higher [17]. The antibacterial activity of chitosan is related to the absorption ability of bacterial cell walls. Chitosan can absorb better in gram negative bacteria compared to gram positive because the negative charge on the surface of gram negative bacterial cells is higher than that in gram positive. The positive charge from chitosan can be neutralized by the negative charge on the bacteria cell wall which result in bacterial death. At a certain concentration can act as an antibiotic.
more and more chitosan is absorbed, it will produce major changes in the structure of cell walls and bacterial cell membrane permeability [19].

Chitosan includes preservatives and the mechanism of inhibition of bacterial growth by chitosan, one of which is through reactions that occur in cell walls or membranes that can change cell permeability. It can interfere or block the way of nutrients enter to the cell, and interfere with the release of substances making up cells and metabolites from the cell. Damage to cell membranes can occur due to the reaction between chitosan with the active side or dissolution of lipid compounds. Cell wall is a complex compound. Therefore, chitosan can mix with the cell wall compilers, so that it affects the cell wall simple components and will inhibit the polymerization of the cell wall constituents. If it develops further, as a result, cell needs cannot be fulfilled properly [20].

The mechanism of action of antimicrobial substances in general is to damage the main structures of microbial cells such as cell walls, cytoplasm, ribosomes and cytoplasmic membranes [21]. Damage to the cell wall results in weakening of the strength of the cell wall, the shape of the cell wall becomes abnormal, and the pores of the cell wall become enlarged. It results in the cell wall not being able to regulate the exchange of substances from and into the cell. The cell membrane becomes damaged and undergoes lysis, so that the metabolic activity will be inhibited and eventually will experience death.

4. Conclusions
Chitosan has been proven having the capacity to inhibit the growth of pathogenic bacteria Salmonella thypi, Escherichia coli, Staphylococcus aureus, and Bacillus cereus. The inhibitory activity is marked by the forming of a clear zone around the well filled by chitosan solution. The optimum inhibition zone diameters were varied for each type of bacteria tested at a concentration of chitosan solution 2%, namely 13.82 mm, 16.92 mm, 15.91 mm and 14.16 mm, respectively. The lowest inhibition zone was also varied for each type of bacteria. The lowest inhibition zone in Salmonella thypi was 11.54 mm, Escherichia coli was 11.46 mm, Staphylococcus aureus was 7.78, and Bacillus cereus was 9.84 mm, all were achieved at a concentration of chitosan solution 1 %. Generally, it can be concluded that the chitosan extracted from the carapace of Rama shrimp has a potential use as an antibacterial.

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References
[1] Ghazali T M 2019 Karakteristik dan efektivitas kitosan karapas udang rama-rama (Thalassina anomala) sebagai senyawa antibakteri [tesis]. Pekanbaru: Sekolah Pasca sarjana, Universitas Riau.
[2] Kaho A R 2006 Chitosan "magic of Nature". http://totalwellness.blogspot.com/2006/ 02/10/ formalin-chitosan-. [accessed on 10 December 2018]
[3] Helander I M, Numiaho E L, Ahvenainen R, Rohoades J and Roller S 2001 Chitosan disrupts the barrier properties of the outer membrane of gram negative bacteria. International Journal of Food Microbiologi 71:235-244.
[4] Suptijah P 2004 Tingkatan kualitas kitosan hasil modifikasi proses produksi. Jurnal Teknologi Hasil Perairan 7(1):56-67.
[5] Einbu A 2007 Characterisation of chitin and a study of its acid-catalyzed hydrolysis, thesis for the degree of philosophiae doctor. Norwegian University of Science and Technology. Dept. Of Biotechnology. Norwegian
[6] [SNI] Standar Nasional Indonesia. 7387:2009. Batas Maksimum Cemaran Logam Berat dalam Pangan. Jakarta: Badan Standardisasi Nasional.
[7] Marganov 2003 Potensi limbah udang sebagai penyerap logam berat (timbal, kadmium, dan tembaga) di perairan [disertasi]. Bogor: Sekolah Pascasarjana, Institut Pertanian Bogor.
[8] Focher B, Naggi A, Tarri G, Cosami A and Terbojevich M 1992 Structural differences between chitin polymorphs and their precipitates from solution evidence from CPMAS 13 C-NMR, FT-IR and FT-Raman Spectroscopy. *Charbohydr Polymer* **17**(2):97-102.

[9] Liu, D, Y Wei, P Yao and L Jiang 2006 Determination of the degree of acetylation of chitosan by UV spectrophotometry using dual standards. *Carbohydrate research*. **341**(6): 782-785.

[10] Purwatiningsih 1992 Isolasi Kitin dan Komposisi Senyawa Kimia Limbah Udang Windu (*Panaeus monodon*). TESIS. ITB. Bandung.

[11] Kusumaningsih T, Masykur A, Arief U 2004 Pembuatan kitosan dari kitin cangkang bekicot (*Achatina fulica*). Jurusan Kimia Fmipa, Universitas Sebelas Maret, Surakarta. Page 64-68.

[12] Pusawati N M and Simpen I N 2010 Optimasi Deasetilasi Kitin Dari Kulit Udang Menjadi Kitosan Melalui Variasi Konsentrasi NaOH. Universitas Udayana. Jurnal kimia **4**(1): 79-90.

[13] Solihah, M 2009 Identifikasi dan uji aktivitas antibakteri minyak atsiri dari daun secang (*Caesalpinia sappan* L.) [skripsi]. Surakarta: Universitas Sebelas Maret.

[14] Pelczar W J, Chan E C S 1998 *Dasar-dasar mikrobiologi*. Jakarta: UI Press.

[15] Volk and Wheeler 1990 *Mikrobiologi dasar*, jilid 2. Edisi kelima. Erlangga. Jakarta.

[16] Suprianto 2008 Potensi ekstrak sereh wangi (*Cymbopogon nardus* L.) sebagai anti *Streptococcus mutans*. IPB. Bogor.

[17] Sulistiyoningrum R S 2013 Aktivitas antibakteri kitosan dari cangkang kerang simping pada kondisi lingkungan yang berbeda: kajian pemanfaatan limbah kerang simping (*Amusium* sp). *Journal of marine research*. **2**(4): 111-117.

[18] Meidina, Sugiyono, Jenie, and Suhartono M T 2006 Aktivitas antibakteri oligomer kitosan yang diproduksi menggunakan kitonase dari isolat b. *Licheniformis MB-2* [tesis]. Bogor: Sekolah Pascasarjana. Institut Pertanian Bogor.

[19] Islam M, Masumb S, Mahbuba K R, Haque Z 2011 Antibacterial activity of crab-chitosan against *Staphylococcus aureus* and *Escherichia coli*. *Journal of Advanced Scientific Research*. **2**(4): 63-66.

[20] Butarbutar E 2018 Uji aktivitas antibakteri kitosan berbahan baku cangkang rajungan (*Portunus pelagicus*) terhadap bakteri *Staphylococcus aureus* dan *Escherichia coli* [skripsi]. Medan: Sekolah Sarjana, Universitas Sumatera Utara.

[21] Fajrina I H 2008 Potensi kitosan sebagai bahan antibakteri. Laporan akhir PKM, Institut Pertanian Bogor.