Toxicity tests, antioxidant activity, and antimicrobial activity of chitosan

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Abstract. Chitosan is a naturally occurring cationic biopolymer, obtained by alkaline deacetylation of chitin. This research aims to investigate the toxicity, antioxidant activity and antibacterial activity of chitosan from shrimp chitin. In this study, chitin extracted from shrimp waste material. Chitin is then deacetylation with 60% NaOH so that chitosan produced. Degrees of deacetylation, molecular weight, toxicity test, antioxidant activity and antimicrobial activity of chitosan then evaluated. Toxicity test using Brine Shrimp Lethality Test. The antioxidant analysis was performed using DPPH method (2,2-diphenyl-1-picrylhydrazyl) and FTC method (ferric thiocyanate) in which the radical formed will reduce Ferro to Ferri resulting in a complex with thiocyanate. To determine the antibacterial activity of Staphylococcus aureus, antifungal in Candida albicans and Aspergillus niger by measuring antimicrobial effects and minimum inhibitory concentrations (MIC). Based on the result of research, the value of degrees of deacetylation, molecular weight, and LC50 values of chitosan synthesis was 94.32, 1052.93 g/mol and 1364.41 ppm, respectively. In general, the antioxidative activities increased as the concentration of chitosan increased. MIC value of chitosan against S. aureus, C. albicans, and A. niger was 10 ppm, 15.6 ppm, and 5 ppm, respectively.

1. Introduction
Nowadays people use only a small portion of the waste of shrimp shells as flavorings in the process of making chips and shrimp paste [1]. Therefore, the waste needs a treatment, mainly because the waste contains a substance, chitin, which further can have potential use. One of the derivatives of chitin is chitosan. The main commercial sources of chitin are the shell wastes of shrimp, lobster, krill, and crab [2].

Chitosan is attained through the process of chitin deacetylation, in which the acetyl groups in chitin changed into amino groups by adding high concentration alkali [3]. Chitosan is bio-active, biodegradation and non-toxic [4] polymer from nature. Chitin and chitosan are often applied in industry as well as health. Chitosan consists of three reactive and functional groups, namely amino group, a primary hydroxyl group and secondary hydroxyl group [5]. Chitosan has a free positively-charged amino group so that it can bind with negatively-charged one. Chitosan and its derivatives had some biological activities, like the anti-microbial feature [6] because chitosan has an active group inside it that reacts with microbes so that it can inhibit their growth.
Free radicals are relatively unstable species, have unpaired electron on their outside orbit, so they are reactive in finding another electron. Free radicals found in environments, several metals (iron, copper), smoke from cigarettes, air pollution, medicines, toxic substances, packaged food, additives, and UV rays from both the sun and radiation [7]. Antioxidants can prevent the presumed deleterious effects of free radicals in the human body, and prevent the deterioration of fats and other constituents of foodstuffs [8]. According to a study conducted by [9], chitosan can inhibit DPPH radical (1,1-diphenyl-2-picrylhydrazyl) in the concentration ranging 0.125 – 1.0 mg/mL with the inhibition of 28.37 – 38.03%. According to the study by [10], hexanoyl chitin could trap peroxide radicals in the organic solvent when the chain reaction of radicals is initiated 2,20-azobis (2,4-dimethylvaleronitrile). Based on the above background, a study was conducted to investigate the toxicity, antioxidant activity and antibacterial activity of chitosan from shrimp chitin.

2. Experimental

2.1. A Chitin Isolation [11]
Shrimp shells are cleaned, dried, and mashed until smooth. The powder, which is 100 mesh, is used for demineralization process using HCl 1.5 M solvent with the ratio of 1:15 (b/v) at 60-70°C for 4 hours while stirred once in a while. The result, which is in the form of solids, is then cleaned using distilled water for several times until neutral. The solids dried at 80°C. The obtained powder cooled then it weighed. The protein from the demineralized solids is then removed using NaOH 3.5% (1:10) (b/v) at 60-70°C for 4 hours while stirred once in a while. The resulted solids are cleansed using distilled water for several times until neutral. The resulted solids dried in an oven at 80°C then they are cooled inside a desiccator and are weighed. The resulted solids are then conducted depigmentation using NaOCl 4% (1:10) while stirred at room temperature for 1 hour. The result is then cleaned using distilled water and then dried at 60°C, so obtained chitin.

2.2. Chitosan Synthesis [3]
Chitin is dissolved using NaOH 60% with the ratio of 1:10 (b/v). The mixture is stirred and heated at 120°C for 3 hours. The resulted solids are cleansed using distilled water for several times until neutral. The solids dried at 80°C then they are cooled inside a desiccator and weighed until they reach the constant weight.

2.3. Chitosan Characterizing
Chitosan is characterized using FITR to count the degree of deacetylation (DD). DD is calculated using baseline method [12]. Measurement of water content and ash content chitosan refers to [13].

2.4. Molecule weight determined
The molecule weight of chitosan is measured based on the intrinsic viscosity (ƞ). Chitosan solution is made in a concentration ranging 0.02-0.1% in acetic acid solution 0.1 M and sodium chloride 0.2 M (1:2) and put into an Ostwald viscometer. The data mapped in a graph ƞsp/C toward C. Intrinsic viscosity is a point in the graph that shows C=0. The molecular weight is determined based on Mark-Houwink equation:

\[ [\eta] = K_m M_w^\alpha \]

[ƞ] is intrinsic viscosity, Km is solvent constant (1.8 X 10^-3), α is constant (0.93), and Mw is the molecule weight [2].

2.5. Antioxidant activity test

2.5.1 DPPH Method (1,1-diphenyl-2-picyrylhydrazyl radical). Four mL of 0.05mM DPPH solution added to one mL methanol. After that, it left for 30 minutes in a dark place. The uptake measured at 400-600 nm of wavelength using spectrophotometer UV-Vis. Four mL of 0.05mM DPPH solution is added with one mL chitosan 100 ppm. The solution is measured for its absorbance with a 5-minute
interval to determine the operating time of chitosan solution test. Four mL of 0.05mM DPPH solution is added to 1 mL chitosan solution with different concentration 500, 1000, 2000, 3000 and 4000 ppm. Then incubated for 300 minutes at room temperature; then it is measured for its absorbance at the maximum wavelength. The percentage of inhibition calculated as follows:

\[
\text{Inhibiting percentage (\%) = 1 - \frac{\text{sample absorbance}}{\text{control absorbance}} \times 100}
\]

2.5.2 FTC (ferric thiocyanate) method. The sample about to test is made by adding 8 mL buffer phosphate 0.1 M pH 7 and 10 mL linoleic acid 0.05% (b/v) into 2 mL chitosan solution 500, 1000, 2000, 3000 and 4000 ppm. Linoleic acid with the same treatment without adding chitosan solution used as a controller. As the comparison to the sample used antioxidant BHT 500 ppm and α-tocopherol 500 ppm. This solution is then kept in an oven at 60°C for seven days [14].

Two mL of BHT solution which has been kept in an oven for 24 hours added with 4.7 mL of 75% ethanol, 0.1 mL of 30% NH₂CNS and 0.02 M FeCl₃H₂O to 0.1 mL of 3.5% HCl. The solution measured for its absorbance at 470 – 570 nm of wavelength. The highest absorbance tells the maximum wavelength of the solution. The solution is measured for its absorbance with a 5-minute interval to determine the balance time of chitosan solution test.

The sample is taken out after being kept in an oven, let it reach the room temperature. As many as 0.1 mL of 30% NH₂CNS and 0.02 M FeCl₃H₂O in 0.1 mL of 3.5% HCl is allowed to reach the balance time after the mixing; it then measured for its absorbance using a spectrophotometer UV-Vis at the maximum wavelength. The measurement is conducted every 24 hours for seven days [14]. Antioxidant activity is based on the oxidation inhibition of linoleic acid toward a sample that has antioxidant trait based on the calculation,

\[
\text{% of inhibition} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{sample absorbance}} \times 100\%
\]

2.6. Toxicity test using Brine Shrimp Lethality Test (BSLT) method
The first step is breeding A. salina Leach larvae. Seawater put into a small container that has been divided into two chambers using a net partition. Some shrimp eggs of A. salina Leach put into one of the chambers; then it is closed. The other chamber is kept open and given light to attract hatched shrimps through the partition so that they can separate from the eggs and the unhealthy shrimps. The eggs will hatch into little shrimps after two days and are ready for testing.

As many as 0.1 grams of chitosan is taken and added with two drops of tween 80, then dissolved in 5 mL of seawater. The adding of tween 80 is to help dissolve chitosan in the seawater to obtained 10000 ppm of chitosan solution. Some main solution of chitosan is taken using micropipette and diluted into several test solutions with 1400, 1200, 1000, and 800 ppm at the final concentration.

Each tube is filled with ten shrimp larvae and seawater, is given two drops of yeast (3 mg/mL seawater) as their nutrition than the tube is given test sample in a certain amount and diluted with seawater until five mL. Control tube is the same content as the test tube but without a sample. The test tube and control tube is incubated openly and given light for 24 hours. The number of living larvae for each concentration calculated and recorded to determine the percentage of shrimp mortality as well as LC₅₀. If there is a dead larva in the control solution, the number of larvae killed in control solution subtracted by the number of larvae killed in the test solution. Base on [15], the percentage of the mortality calculated as follows:

\[
\text{% of mortality} = \frac{\Sigma \text{controlling living larvae} - \Sigma \text{treated living larvae}}{\Sigma \text{controlling living larvae}} \times 100\%
\]

2.7. Antimicrobial activity test
S. aureus (ATCC 25923), Candida albicans and Aspergillus niger used for the measurement of antimicrobial activity. C. albicans is a clinical blood isolate of the Candida species collected in our laboratory were used at an inoculum size of 1-5 x 10⁵ CFU/ml. Aspergillus niger strain used in the
present study was previously isolated and identified [16]. Bacteria cultures incubated in Luria Broth and agar Medium at room temperature. The concentration of the bacteria controlled from 10^7 to 10^8 CFU/mL. Yeast and the filamentous fungi maintained on potato dextrose agar (PDA).

2.7.1. Measurement of antibacterial activity and Antifungal susceptibility testing. Antibacterial activity measurement was performed by the agar dilution method using 90mm × 15mm Petri dishes. Luria agar medium prepared with the bacteria cultures which have a hole in the center (10 mm diameter). Ten microliters (10 ppm) of chitosan added in the hole of Luria agar medium. The LB medium incubated for 24 h for S. aureus, PDB medium during 24 h for C. albicans, and 48 h for A. niger at room temperature. The bacterial and fungal growth checked for a visible growth, control plates prepared without any chitosan. The antibacterial effects of chitosan measured by growth suppression exchange = total growth suppression exchange−diameter of the hole (8 mm). Inoculums without treatment as negative control also included.

2.7.2. Measurement of minimum inhibitory concentration (MIC). Different concentration of chitosan (0, 5, 10, 50, 100 ppm) and added in Luria Broth medium. The bacterium culture (S. aureus) controlled at 10^3–10^6 CFU/mL. The minimum inhibitory concentration of chitosan for S. aureus, C. albicans, and A. niger determined by the optical density of the bacterial culture solution containing different concentration after 24 h.

2.7.3. Measurement of the colony-forming unit (CFU). A bacterial culture medium containing chitosan solution spared on LB medium. The bacterium (S. aureus) was incubated its cultivation temperature for 48 h after that colony-forming unit (CFU) was measured. The LB medium incubated the same method of above measurement of the MIC. The decrease of bacterial at each LB medium was measured. MIC defined as the lowest concentration of extract that inhibited visible growth on agar.

3. Results and discussion

3.1 Chitosan

Chitosan is deacetylation product of chitin which is a polymer long chain of glucosamine (2-amino-2-deoksi-β-(1→4)-D-glucose) [17]. Chitin, however, is a linear polymer most of which comprises of β-(1→2)-acetamide-2-deoksi-β-D-glucopyranose units [18] and can be isolated from shrimp shells. Chitin isolation usually conducted in three stages, namely demineralization, deproteinization, and depigmentation. Demineralization is to eliminate minerals or inorganic compounds in the shells. Calcium will react with hydrochloric acid and result in calcium chloride and phosphoric acid dissolved in water [3]. CO2 bubbles from the demineralization process show that there has been a reaction between HCl with minerals in the shells [19]. Deproteinization is to eliminate protein using alkali solution and proper heating [3]. The last stage of chitin isolation is depigmentation. This stages to eliminate color so that whiter chitosan with the higher degree of deacetylation compared to that without depigmentation obtained. Pigments in the shells are astaxanthin pigment which can be eliminated using NaOCl solution. NaOCl acts as the bleaching agent that can produce white chitin [20]. This process will produce chitin. Based on the results, % of the yield of chitin is 31.92 %.

Chitosan is obtained by chitin deacetylation using high concentrate alkali solution [3]. Deacetylation process is the eliminating process of acetyl groups (–COCH3) of chitin using alkali solution to change it into amino groups (–NH2). Chitin has a long crystalline structure with a strong bond between the nitrogen atom and acetyl groups. Therefore, in the deacetylation process, it takes a long time, a high concentrate of NaOH solution and high temperature to break the bond between acetyl groups and the nitrogen atom to produce amino groups [21]. The final product from chitin deacetylation is chitosan and sodium acetate as the byproducts. Based on the results, % of the yield of chitosan is 16.63 %.
3.2. Chitosan characteristics

The chitosan from the synthesis process is characterized using FTIR instruments. The result of chitosan IR spectrum shown in Figure 1. There is an expanding uptake in the wave area of 3452 cm\(^{-1}\) where there is overlapping on vibration uptake of \(-\text{OH}\) groups. The moderate uptake in 2877 cm\(^{-1}\) shows that there is vibration range C-H on \(-\text{CH}_2\)- aliphatic. The uptake in the area of 1419 cm\(^{-1}\) is the bending vibration of \(-\text{CH}_2\)-. The range of C=O (\(-\text{NHCOCH}_3\)) occurs in 1651 cm\(^{-1}\). The uptake in 1597 cm\(^{-1}\) shows that there is a bending vibration N-H from \(\text{NH}_2\). The wavelength of 1381 cm\(^{-1}\) indicates that there is bending uptake in \(-\text{CH}_3\) with the weaker intensity; this happens because of the deacetylation process causing the loss of most of the methyl groups, \(-\text{CH}_3\). The range of C-O of the C-O-C bond detected in 1033 and 1087 cm\(^{-1}\). The 894 cm\(^{-1}\) shows that there is moderate uptake as a result of the vibration change of \(\text{NH}_2\) from primary amide.

![Figure 1. IR spectrum of chitosan](image)

The obtained IR spectrum can be used to calculate the degree of deacetylation of chitosan. The level of deacetylation shows the diminishing of acetyl groups in chitin to become amine groups in chitosan [12]. The value is affected by several factors, like the alkali concentration, temperature, the ratio of chitin and alkali solution, and the particle size [21] and the reaction time [22]. The use of high concentration of alkali solution as well as high temperature during the deacetylation process can affect the results. The more NaOH to use, the more substances that will react and there is a high possibility that impact will occur. However, if the NaOH concentration is more than 60%, the solution will become thick. Consequently, a part of chitin that imperfectly reacts with NaOH solution so the resulted amino groups are little or the deacetylation level decreases. The longer deacetylation time, resulting in more reduction of acetyl groups in chitin. This results in the less output of chitosan but better quality of chitosan. The calculation degree of deacetylation chitosan using baseline b [12] is 94.32%.

Water content shows how much water in the chitosan in percent. It obtained by heating several grams of samples into the oven at 100-105°C until constant weight reached. The result of the study is 0.33%. The obtained chitosan has a proper water content according to the quality standard of chitosan water content, which is ≤10%. Water content also depends on the humidity level around the storage area of chitosan [23]. The ash content shows that there is an inorganic compound in chitosan. How high and low the level indicates how pure the chitosan is. If the content is high, then chitosan is less pure, and
vice versa. The ash content determines how successful the demineralization process in chitin isolation. The study results show 1.53%.

The use of a very high temperature in the chitosan synthesis causes the breaking in polymer bond (depolymerization) of chitosan molecule chains so that their mass loses. The mass can be determined using Ostwald viscosity method. The measurement conducted by determining the time needed by a certain amount of solution to flow between two calibrating signs. The flow time then compared to the flow time of the pure solvent, so specific viscosity values obtained. The mass of chitosan molecule is determined using 0.1 M acetic acid and 0.2 M sodium chloride solvent. The varied chitosan samples used are 0.02; 0.04; 0.06; 0.08; and 0.1%. From the measurement, the relative viscosity and specific viscosity can calculate (Table 1). From the chart of the correlation between reduced viscosity (ηsp/C) and various concentration (C) of chitosan, there is a regression equation y = 8.7412x + 1.1646. The molecule mass is calculated using Km value (solvent constant) and α (constant) as much as 1.81 x 10⁻³ and 0.9 [2]. Based on the calculation, the molecule mass (Mw) of chitosan is 1052.93 g/mol.

Table 1. Molecule mass determination of chitosan

| Concentration | Flow time (seconds) | ηintrinsic | ηspecific (ηsp) | ηreduction (ηsp/C) |
|---------------|---------------------|------------|----------------|-------------------|
| Solvent       | 25.51               | -          | -              | -                 |
| 0.02%         | 26.12               | 1.0239     | 0.0239         | 1.195             |
| 0.04%         | 27.21               | 1.0666     | 0.1043         | 1.665             |
| 0.06%         | 28.17               | 1.1043     | 0.1043         | 1.738             |
| 0.08%         | 29.37               | 1.1513     | 0.1513         | 1.891             |
| 0.1%          | 30.50               | 1.1956     | 0.1956         | 1.956             |

3.3 Antioxidant activity using DPPH method

The maximum wavelength (λmax) measurement shows the substance absorption or maximum absorbance at a specific wavelength. The analysis uses DPPH 0.05 mM; then the solution is measured for its absorbance at 400-600 nm wavelength using spectrophotometer UV-Vis. The result shows that 515.6 nm wavelength with 0.560 absorbances is λmax. Determining the operating time is conducted by reacting sample solution with 0.05 mM DPPH solution, and then it is observed for its absorbance at λmax 515.6 nm from 5 until 60 minutes with a 5-minute interval. Operating time is chosen when the decreasing absorbance is relatively stable [8]. The result obtained at minute 30 – 40.

Antioxidant activity of chitosan solution is conducted using DPPH method. The measurement of antioxidant activity from chitosan conducted by reacting four mL of 0.05 mM DPPH solution with one mL chitosan solution with varying concentrations: 500, 1000, 2000, 3000, and 4000 ppm. It is then incubated for 30 minutes and measured for its absorbance at λmax. The absorbance is used to know the antioxidant activity by calculating the damping percentage. The free radical inhibition percentage by chitosan solution can see in Figure 2. According to Figure 2, the solution inhibition percentage chitosan increases from 27% to 36.9%. The bigger the concentration, the bigger the percentage.

The higher the concentration of chitosan solution is, the lower the uptake is. The principle of DPPH method that antioxidant compound will release its hydrogen atom in DPPH radical so that DPPH becomes a non-radical reduced form. A non-radical DPPH will lose its purple intensity proportionally to the number of electrons it captures. The fading color marked with the decreasing DPPH absorbance at the maximum wavelength [8].
Figure 2. Free radical inhibition percentage by chitosan solution

3.4 Antioxidant activity using Thiocyanate method

Determination of maximum wavelength (\(\lambda_{\text{max}}\)) aims to obtain maximum absorption. Measurement of sample uptake at wavelength 470 nm to 570 nm due to the color of the solution orange. The results show the \(\lambda_{\text{max}}\) is 481 nm. The measurement of complex stability time uses 500 ppm BHT solution, and it conducted during the minute 5 until 60 with 5-minute interval using 481 nm wavelength. The time showing the stable absorbance in the minute 35 – 40.

Thiocyanate method is a method that measures the number of free radicals based on lipid peroxidation, which is the forming of the alkoxy radical [24]. This method uses linoleic acid, which is unsaturated fatty acids acting as free radicals [25]. The linoleic acid used as the substrate, and oxygen will oxidize it. The linoleic acid oxidation accelerated by the existence of light, temperature, pH, oxygen, metal ions and lipid radicals. Therefore, linoleic acid incubation is set at 60°C so that the high temperature can catalyze linoleic acid oxidation. After being incubated for 24 hours, a measurement of peroxide number conducted by reacting sample with ammonium thiocyanate and Ferro (II) chloride to produce reddish orange Ferri thiocyanate using spectrophotometer UV-Vis at \(\lambda_{\text{max}}\) 481 nm.

The resulted of absorbance 500, 1000, 2000, 3000, 4000 ppm chitosan; 500 ppm BHT; 500 ppm \(\alpha\)-tocopherol shows that the uptake for control (linoleic acid) with the incubation time is quite big. For chitosan samples has a less uptake than the control. \(\alpha\)-tocopherol and BHT as the comparates have a little uptake compared to the control or the sample. According to [26], the absorbance value is directly proportional to the amount of peroxide in samples because of the oxidation. The bigger the peroxide values, the bigger the absorbance values and the more reddish orange intensity. The ability of thiocyanate method can see from the absorbance values compared to the values of control absorbance. The obtained result of absorbance is used to calculate the inhibition percentage of linoleic acid oxidation so that the activity of the antioxidant sample is known. The percentage shown in Table 2.
Table 2. The inhibition percentage of linoleic acid oxidation

| Days to | Cs 500 ppm | Cs 1000 ppm | Cs 2000 ppm | Cs 3000 ppm | Cs 4000 ppm | a-tokoferol BHT |
|---------|------------|-------------|-------------|-------------|-------------|-----------------|
| 1       | 2.02       | 27.77       | 30.95       | 35.08       | 35.45       | 55.25           | 63.80           |
| 2       | 13.33      | 45.43       | 53.95       | 51.00       | 47.77       | 65.42           | 62.63           |
| 3       | 26.48      | 46.31       | 46.45       | 55.57       | 48.48       | 76.63           | 70.69           |
| 4       | 39.46      | 60.83       | 57.26       | 57.69       | 58.55       | 75.06           | 74.81           |
| 5       | 57.67      | 77.41       | 77.07       | 75.95       | 75.26       | 70.54           | 79.97           |
| 6       | 55.50      | 75.70       | 74.60       | 76.90       | 74.00       | 55.11           | 77.22           |
| 7       | 32.28      | 50.72       | 54.99       | 64.82       | 57.84       | 73.95           | 80.37           |

Table 2 shows that chitosan (Cs) has inhibition percentage ranging from 2.02% to 77.41%, while BHT and α-tocopherol range from 55.11% to 80.37%. On day one there is a correlation between the increasing concentration. This is that the inhibitory power is getting bigger by the concentration adding. On day two until day 7, the inhibitory power fluctuates when there is concentration adding. This is because of the uncontrollable oxidation process. The correlation of adding the days is seen in 3000 ppm concentration which reaches the 6th day and shows that the longer the storage time, the bigger the inhibitory power and it reaches its peak then goes down on day 7. This means that on day seven peroxide begins to experience the decomposition to become another secondary compound. Peroxide is an unstable product of primary oxidation and will be decomposed gradually to become a secondary product (e.g., alkane, alcohol, and aldehyde) [26]. The inhibitory power of BHT and α-tocopherol fluctuates from day 1 to day seven caused by uncontrollable oxidation reaction that influences ferric thiocyanate complex [27].

BHT and α-tocopherol have the antioxidant ability to inhibit linoleic acid oxidation process. BHT is phenol compounds that are substituted in the ortho position (tertiary butyl group) and can expand the ability as an antioxidant. On the other hand, α-tocopherol has methyl group substituents bound to aromatic rings in the meta position. This causes the reactivity as the antioxidant to weaken. Chitosan can inhibit linoleic acid oxidation process although its inhibitory power is not as strong as BHT or α-tocopherol. The inhibition process varies depending on the chemical structures and mechanisms. In this mechanism, the most important thing is the reaction with free-lipid radicals which form non-active products.

3.5. Chitosan toxicity

Toxicity test is a test conducted to know the toxicity level of a compound. The study uses Brine Shrimp Lethality Test (BSLT) method [15] because this method is one method that mostly used, cheap, fast, easy (does not require aseptic conditions) and reliable. The result can be known from the mortality Artemia salina Leach larvae because of the effect of certain compounds with a certain dosage that has been specified previously. This method conducted by determining the value of LC50 for 24 hours. The data are analyzed using Probit Analysis on a computer to determine the value of LC50. The result from the 24-hour observation of Artemia salina Leach larvae toward chitosan is shown in Table 3.
Table 3. The 24-hour observation of *Artemia salina* Leach larvae toward chitosan

| Concentration (ppm) | Living larvae | The average of living larvae | Mortality percentage | Concentration Log |
|---------------------|---------------|-------------------------------|----------------------|-------------------|
| K                   | 10            | 10.00                         | 0.00                 | -                 |
| 800                 | 9             | 8.67                          | 13.33                | 2.90              |
| 1000                | 7             | 7.33                          | 26.67                | 3.00              |
| 1200                | 7             | 6.33                          | 36.67                | 3.08              |
| 1400                | 4             | 4.67                          | 53.33                | 3.15              |

Based on Table 3, a curve is then made to show the correlation between the mortality percentage of larvae and chitosan concentration log (Figure 3). The linear regression equation from the above chart is then used to determine LC$_{50}$. The obtained line equation is $y = 4.8323x - 10.149$, $R^2 = 0.992$. Based on the result, the value of LC$_{50}$ at probit (Y) = 5, the concentration (X) 3.1349 is $10^{3.1349} = 1364.41$ ppm.

![Figure 3. Curve of the correlation between the mortality percentage of larvae and chitosan concentration log](image)

As it can see in Figure 3, the value of chitosan concentration is directly proportional to the mortality percentage of *Artemia Salina* Leach larvae. The existence of larvae test in control is to find out the mortality percentage of *Artemia Salina* Leach larvae caused by natural death. According to [28], a compound shows its toxicity activity in BSLT if the compound can cause 50% mortality of the animal tests on the concentration less than 1000 ppm. Based on the statement, it can conclude that chitosan is not toxic because the LC$_{50}$ value is more than 1000 ppm. Chitosan reaches LC$_{50}$ at the 1364.41 ppm concentration. The result proves that chitosan is not toxic.

3.6. Antimicrobial activity
The bacteria test used in the antimicrobial activity test is *S. aureus* ATCC 25923 (for bacteria) and *Candida albicans* and *Aspergillus niger* (for fungi). Antibacterial and antifungal tests are conducted...
aseptically to avoid contaminants. The antibacterial activities of chitosan show for S. aureus. Figure 4, figure 5 and figure 6 showed the antibacterial activities of chitosan for S. aureus.

![Figure 4](image)

**Figure 4.** Antibacterial activity of (a) control and (b) chitosan

![Figure 5](image)

**Figure 5.** curves of *S. Aureus* Growth inhibition of chitosan with different concentrations in LB medium

Table 4 showed *antibacterial activity and antifungal susceptibility testing* of chitosan. According to [29], acetic acid has antibacterial activity. Therefore, when it uses as chitosan solvent, the acetic acid cannot be ignored. Based on the previous studies, the result should use chitosan. In the bacteria test acetic acid solvent with a concentration less than 100 ppm is used [30]. In this study, used acetic acid with 50 ppm concentration.

| Sample  | Antifungal activity MIC (ppm) |
|---------|-------------------------------|
|         | *S. aureus* | *C. albicans* | *A. niger* |
| Chitosan| 10          | 15.6          | 5          |
4. Conclusion
The synthesized chitosan from *vanamei* shrimp shells has the output as many as 17.83% and the deacetylation 94.32%. The chitosan from synthesis has weak antioxidant activity. The chitosan gives antimicrobial activity toward *S. aureus* bacterium as well as *C. albicans* and *A. niger* fungi.

Acknowledgments
This research supported by *Penelitian Produk Terapan* Research Program funded by the Ministry of Research, Technology and Higher Education of the Republic of Indonesia in 2017.

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