Synthesis, Characterization and Antibacterial Activity Assay of Carboxymethyl Chitosan

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Abstract. Chitosan is a natural biopolymer with the second largest abundance after cellulose. This compound was obtained from the deacetylation of chitin from crab shell waste obtained in province of Lampung. It is known that chitosan has antibacterial properties because it has an amino group. However, a modification process needs to be carried out to produce compounds with more specific properties and benefits. The purpose of this study was to synthesize and to determine the characterization and antibacterial activity of carboxymethyl modified chitosan. Based on the research results obtained as much as 10.24% chitosan (per weight of crab shell powder) and carboxymethyl chitosan as much as 72.9% (per weight of chitosan). FTIR analysis of the derivative confirmed the incorporation of the carboxymethyl groups. Chitosan has a water content of 0.49% while carboxymethyl modified chitosan has a water content of 0.67%. The ash content of chitosan was 0.33% and the ash content of carboxymethyl modified chitosan was 0.69%. The synthesized chitosan has antibacterial activity against E.coli bacteria with the inhibition zone of 10 mm while against S. Aureus it had an inhibition zone of 11.89 mm. Meanwhile the inhibition zone of carboxymethyl chitosan against E.coli bacteria was 11.50 while that for S. Aureus was 12 mm.

1. Introduction

Chitosan is a natural product which is a derivative of the polysaccharide chitin. Chitosan is also an abundant cationic polymer after cellulose and is antibacterial, nontoxic, biodegradable and compatible. Chitin which is a source of chitosan can be obtained from marine biota, especially from crustaceans and arthropods such as shrimp and crabs. The process of isolation of chitin and chitosan from natural sources and the utilization of the antibacterial properties of chitosan have been carried out in previous studies.

[1] isolated chitosan from crab shell waste and has potential antimicrobial properties on various bacteria such as *Escherichia Coli, Straphylococccuc aureus etc*. [2] also stated that chitosan derived from shrimp has antibacterial activity against both gram-positive and gram-negative bacteria. Chitosan as an edible film has also been studied by [3], where chitosan effectively inhibits bacterial growth and effectively reduces water evaporation and product oxidation.

In this study, chitosan derivative compounds will be used, namely carboxymethyl chitosan from crab shell waste and tested for its antibacterial activity against gram-positive and gram-negative bacteria. Later this compound can be used as an antibacterial agent that is easily soluble in water media and can be applied in various fields such as; food, cosmetics, agriculture and pharmaceutical industries.

2. Methods

2.1. Material

The materials to be used are crab shell waste, NaOH, HCl, Acetic Acid, Methanol, Monochloroacetic Acid, Aquades, Filter Paper, gram positive bacteria (*Staphylococcus aureus*) and gram negative bacteria (*Escherichia coli*), aquades, Whatman 42 ashless circles filter paper 110 mm, universal indicator.

2.2. Chitosan synthesis

Crab shell powder was mixed with 4% NaOH solution and stirred for 60 minutes for the deproteination step. The precipitate obtained was washed and dried. The deproteinized precipitate was reacted with 1M HCl solution for demineralization. The chitin obtained was then converted into chitosan through a deacetylation process with 60% NaOH for 3 hours. The chitosan obtained was then washed with distilled water and dried at 60°C.

2.3. Synthesis of carboxymethyl chitosan

The synthesized chitosan was alkaline esterified using 20% NaOH solution for 15 minutes. Monochloroacetic acid was then added to the chitosan solution and stirred for 2 hours at 40°C. The solution mixture was then neutralized with acetic acid solution, then poured into an excess of 70% methanol solution. The solution mixture was then filtered and washed with methanol. The carboxymethyl chitosan product obtained was then dried at 55°C.

2.4. FTIR characterization of chitosan and carboximethyl chitosan

Chitosan and carboxymethyl chitosan were analyzed for water content and ash content using the AOAC method (1970) and characterized using an FTIR spectrophotometer to determine the functional groups characteristic of chitosan.

2.5. Antibacterial test of chitosan and carboximethyl chitosan using diffusion assay

S. aureus and *E. coli* were used as targeted Gram-positive and Gram-negative bacteria, respectively. Antimicrobial characteristics of the chitosan and carboxymethyl chitosan were determined using an agar diffusion assay (paper disk). In brief, 7 ml of soft-layer nutrient agar containing approximately 106 CFU/ml of the tested bacterial strains were poured over the plate count agar layer. The 1 cm in diameter shaped paper disk coated chitosan and carboxymethyl chitosan were placed on the plate and all plates were incubated at 37°C, and the zone of inhibition in contact and around the test samples were recorded after 24 h of incubation.

3. Results and Discussion

3.1. Chitosan synthesis

Chitosan preparation from crab shell waste is generally carried out through 4 stages of treatment, namely preparation, deproteination, demineralization, and deacetylation. The preparation stage includes washing, drying, and grinding the crab shells to obtain a powder that passes a 100 mesh sieve.

The deproteination stage aims to remove the protein contained in the crab shell. In the deproteination stage, the yield was 79.21%. This shows that the protein that is lost in the deproteination process is 20.79%.

The next stage is the demineralization stage, namely the removal of minerals in the crab shell. At this stage, the yield of 21.32% was obtained. This shows that most of the components that make up crab shells are metal minerals, especially calcium carbonate ($CaCO_3$) and calcium phosphate [$Ca_3(PO_4)_2$] [4]. The mineral removal process is estimated according to the following reaction:

$$2HCl(aq) + CaCO_3(s) \longrightarrow CaCl_2(aq) + H_2O(l) + CO_2(g)$$

$$Ca_3(PO_4)_2\ (s) + 6HCl(aq) \longrightarrow 3CaCl_2\ (aq) + 2H_3PO_4\ (aq)$$

The last step is deacetylation, which is the removal of the acetyl group. At this deacetylation stage, the yield of 60.67% was obtained. In the deacetylation process, the bond between the carbon in the acyl group and the nitrogen in the chitin is broken into an amino group. When the deacetylation process uses a high concentration of NaOH solution, in the solution NaOH will decompose into Na^+ and OH^- ions. The hydroxyl ion then attacks the electropositive carbonyl carbon. The final product of this reaction is chitosan and sodium acetate salt as a by-product.

Based on the yield data from the stages of deproteination, demineralization, and deacetylation, it can be concluded that in the preparation process of chitosan from crab shell powder, the total yield is 10.24%. So from the initial weight of 100 grams of crab powder, 10.24 grams of chitosan will be obtained.

Synthesis of carboxymethyl chitosan. Carboxymethyl chitosan, which is a chitosan derivative compound, was synthesized from chitosan raw material obtained through the preparation process from crab shell powder. Chitosan modified carboxymethyl was synthesized by reacting chitosan with monochloroacetic acid. The reaction for the conversion of chitosan to chitosan modified carboxymethyl is estimated as follows:

$$R\text{-}O\mathbf{H} + Cl\text{-}\mathbf{CH_2\text{-}COOH} \rightarrow R\text{-}O\text{-}\mathbf{CH_2\text{-}COOH}$$

Chitosan carboxymethyl chitosan

In this study, the yield of carboxymethyl chitosan was 72.9% by weight of chitosan powder. This shows that chitosan which is the raw material for the synthesis of carboxymethyl chitosan has decreased in mass because according to the theory, the mass of carboxymethyl chitosan should be greater than the mass of chitosan due to the substitution of hydroxyl groups with carboxyl groups. The phenomenon of decreasing the mass of chitosan may occur when mixing chitosan with 20% NaOH solution which causes the dissolution of protein residues and the possibility of breaking the acetyl group into an amine group [5] Thanakkasaranee, et al (2021).

Characterization of carboxymethyl chitosan. The formation of carboxymethyl chitosan is indicated by changes and shifts in the IR spectra of chitosan and carboxymethyl chitosan, as shown in Figure 1.

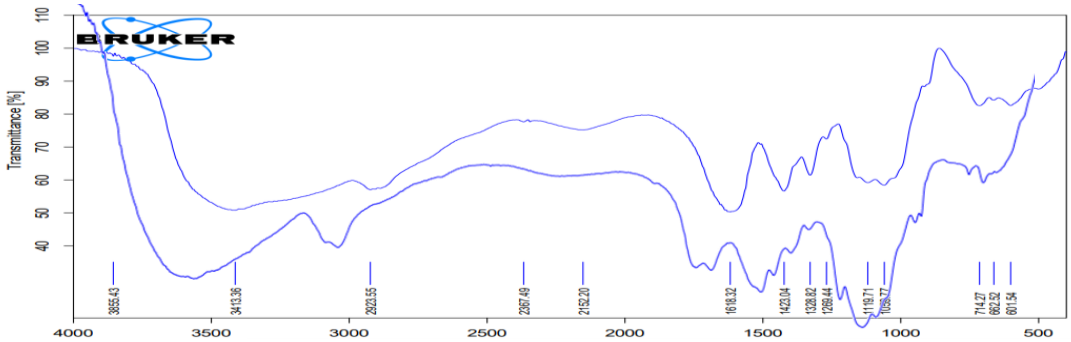

Figure 1. IR spectra of (A) Chitosan and (B) carboxymethyl chitosan

Based on Figure 1, it can be seen that in the IR spectra of carboxymethyl chitosan, an absorption peak appears at a wave number of 3448.72 cm-1 which is the absorption of the stretching vibration of the -OH group which overlaps the absorption of the stretching vibration of -NH. The absorption peak of the C-H stretching vibration shifted from 2854.65 cm-1 to 2885.51 cm-1 and the intensity decreased, possibly indicating the re-disconnection of the acetyl group from the chitosan polymer chain due to treatment with 20% NaOH solution. The increase in the absorption peak of the amide I band (C=O stretching) that appears at wave numbers 1658.78 cm-1 and 1627.92 cm-1 indicates an increase in the

C=O group due to the addition of a carboxylate group (-COOH) which means that CS-O has been formed carboxymethyl chitosan[6] Bukzem, et al (2021). In addition to the evidence of the appearance of two C=O absorption peaks, the formation of Carboxymethyl chitosan from chitosan can be strengthened by the widening of the CO stretching vibration absorption peak that appears at wave numbers 1072.42 cm-1 and 1026.13 cm-1, which indicates that there has been addition of a carboxylate group.

Based on the comparison analysis of functional groups between chitosan and carboxymethyl chitosan above, it is concluded that carboxymethyl chitosan has been formed due to the appearance of characteristic peaks of carboxymethyl chitosan, while characteristic peaks of chitosan are reduced.

Water content. The synthesized carboxymethyl chitosan in this study had a water content of 0.67%, which was higher than the initial water content of 0.49%. This shows that the carboxymethyl chitosan compound has the ability to bind water molecules stronger than the ability to bind water molecules by chitosan.

The greater ability to bind water molecules in carboxymethyl chitosan than chitosan is probably due to the presence of carboxymethyl groups in the structure of carboxymethyl chitosan. The presence of a carboxymethyl group that replaces the H atom in the hydroxyl group on the C-5 atom of the chitosan structure causes more hydrogen bonds to occur with water molecules, causing more hydrated water molecules surrounding the carboxymethyl chitosan chain than those surrounding the chitosan chain.

Ash content. The carboxymethyl chitosan synthesized in this study had an ash content of 0.69%. The ash content of carboxymethyl chitosan was higher than that of chitosan, which was 0.33%. These data indicate that chitosan derivative compounds have a higher carbon content than chitosan. The increase in ash content of carboxymethyl chitosan compounds was most likely caused by the substitution of H atoms in the hydroxyl group on the C-5 atom of the 23 chitosan structure with a carboxymethyl group so that the number of carbon atoms in the carboxymethyl chitosan chain was more than in the chitosan chain.

Antibacterial activity of chitosan and carboxymethyl chitosan. The inhibition zone of chitosan alone against E.coli and S.aureus is 10 mm and 11.89 mm respectively. While carboxymethyl chitosan with concentration of 1% has inhibition zone of 11.50 mm againts E.coli and has inhibition zone of 12 mm againts S. aureus. As is known, chitosan has an amino group (NH_2) which will become ammonium (NH^{3+}) in an acidic medium [7](Hoseinnejad, et al. 2018). The positive charge of this ion will interact with the negatively charged bacterial cell wall, so that it can inhibit the growth of bacteria, both gram-positive and gram-negative[8](Tanpichai, et al 2020). When viewed from the antibacterial activity of chitosan and carboxymethyl chitosan, there was no significant difference. However, the zone of inhibition against E. coli bacteria is smaller than that of S. aureus bacteria, this is because the cell wall structure of E. coli bacteria has a more complex cell wall structure (three layers). This inhibits the antibacterial agent from penetrating the bacterial cell wall.

4. Conclusions

Based on data analysis and discussion, it can be concluded that: (1) Carboxymethyl chitosan can be synthesized from chitosan derived from crab shell waste by reacting chitosan with monochloroacetic acid in 20% NaOH solution with 72.9% in yield from chitosan powder.; and (2) Carboxymethyl chitosan can act as antibacterial agents that have an inhibitory zone against E.Coli bacteria 11.50 mm and 12 mm againts S. Aureus.

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