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Production and Characterization of Nano-Chitosan from Blood Clamshell (*Anadara granosa*) by Ionic Gelation

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ABSTRACT

Nano-chitosan can be produced from blood clams (*Anadara granosa*) because they contain 14-35% of chitin. The production of nano-chitosan can be conducted by a bottom-up process using sodium tripolyphosphate (Na-TPP). The aims of this study are to produce nano-chitosan from blood clamshell and to study the factor affecting the particle size of nano-chitosan such as the ratio of chitosan to Na-TPP solution (v/v) and rotation speed of the centrifuge. The research shows that The chitin content on blood clamshell is 25.42%. The yield of chitosan from chitin is about 80.92%. The degree of deacetylation of chitin from blood clamshell reaches 63.18%. The effect of the ratio of chitosan to Na-TPP solution (R) and the rotation speed of centrifuge (N) on the particlesize of nano-chitosan can be expressed by equation $dp = 0.12 (R)^{0.714} (N)^{0.99}$.

INTRODUCTION

Blood Clam (*Anadara granosa*) is one of the Indonesian traditional food obtained from rivers. The use of blood clams as food leaves waste in the form of the clamshell. Therefore, it is necessary to study the utilization of clamshell waste so that it is not polluting the environment. Several studies have been carried out on the utilization of clamshell waste in various fields.

Clamshells can be used as the raw material of amorphous calcium phosphate (CPP-ACP) paste as teeth remineralization material (Asmawati et al. 2018). The clamshells can be used as filleron polyester resin particle boards due to the high calcium carbonate content in the clamshells (Ginting et al. 2017). One of the potential uses of the clamshell is the manufacture of nano chitosan (Avadi et al. 2010, Zhao et al. 2011, Patiño-Ruiz et al. 2020).

There are two methods to produce nano-chitosan from blood clamshells. The first method is the top-down method. In this method, chitosan is milled from a micrometer particle size to obtain a nanometer particle size (Rochima et al. 2017). The second method is the bottom-up method, where nanoparticles are formed due to assembly between macromolecules from the solutions (Nugraheni et al. 2019).

This research will examine the process of production of nano-chitosan from blood clamshells using the ionic gelation method. The ionic gelation method is a bottom-up method. Furthermore, this study will examine the effect of the ratio of chitosan to Na-TPP solution (v/v), and the rotation speed of the centrifuge on the diameter of the particle size of nano-chitosan formed.

MATERIALS AND METHODS

Materials

Blood clamshell (*Anadara granosa*) was obtained from the local market at Banyumas Regency, sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium tripolyphosphate (Na-TPP), and acetic acid (CH_3COOH) were obtained from Merck.

Clamshell Preparation

Blood clamshells were washed using running water to clean the remaining dirt. The next stage was drying under the sun until they were completely dry. Then the blood clamshells were crushed until they become smaller in size. They were then milled using high-energy milling (HEM) to produce a fine powder. The blood clamshell powder was then sieved using a 100 mesh sieve.

Chitosan Preparation

There were several steps carried out for the isolation of chitosan from blood clamshells,including the deproteinization stage, demineralization stage, and deacetylation stage.

Deproteinization Stage: The blood clamshell powder was weighed to 100 g and put into a glass beaker. Then, 3.5% (w/v) NaOH solution was added in a ratio of 1:10 (w/v). Then heated at 70°C for 2 h with stirring. In the next stage, the mixture was filtered and washed using aqua dest until a neutral pH. The solid obtained was dried in an oven at 60°C to obtain a constant weight.

Demineralization Stage: The powder resulting from the deproteinization stage was put intoa glass beaker flask, and 1N HCl was added in a ratio of 1:10 (w/v). The mixture was put at room 30°C for immersion, then heated at 75°C for 1 h with stirring. In the next stage, the mixture was filtered and washed using aqua dest until a neutral pH. The solid obtained was dried in an oven at a temperature of 60°C to obtain a constant weight. Then, as a result, chitin product was obtained from blood clamshells.

Deacetylation Stage: The chitin powder obtained from the demineralization stage was weighed to 10 g, put into a third glass beaker, and then heated at a temperature of 90°C for 3 h with stirring. In the next stage, the mixture was filtered and washed using aqua dest until a neutral pH. The solid obtained was dried in an oven at a temperature of 60°C to obtain a constant weight. The next step was to weigh the chitosan from the blood clamshell powder obtained.

Preparation of Nano Chitosan Using Ionic Gelation Method

The amount of 1% chitosan solution was made in acetic acid solution. The solution was added with 1% Na-TPP solution with a variation of the ratio of chitosan to Na-TPP solution 1:1; 2:1; 3:1; 4:1; 5:1 with a rotation speed of centrifuge of 500; 600; 700; 800 and 900 rpm. The stirring was done for 1 h. The nano chitosan obtained was in the form of a dispersed solid.

Determination of Physical Characteristics of Nano-chitosan

FTIR Spectroscopy: The degree of chitosan deacetylation was determined using the Fourier Transform Infrared (FTIR) spectrophotometer analysis. In the analysis of the FTIR spectrophotometer, the chitosan sample obtained was then inserted into the sample container, and its infrared absorption spectrum was recorded at a wavenumber of 4000-400 cm^{-1}. The chitosan obtained was tested using FTIR spectroscopy, and the resulting peak was compared with the peak of commercial chitosan. Based on the peak obtained, the degree of deacetylation was calculated by comparing the absorbance of the wavelength of the amide group (1650-1500) cm^{-1} (A1655) with the absorbance of the wavelength of the amine group (3750-3000) cm^{-1}. The chitosan deacetylation degree was calculated using Equation (1) (Antonino et al. 2017).

$$DD = 100 - \left(\frac{A1655/A3450}{1.33}\right) x\ 100 \quad \ldots(1)$$

Where A1655 is an amine group and A3450 is a hydroxyl group.

Particle Size of Nano-Chitosan: The Particle size of nano-chitosan was analyzed using aparticle size analyzer (PSA).

RESULTS AND DISCUSSION

Blood Clamshell Preparation

Blood Clamshell (*Anadara granosa*) could be used as chitosan because they contain 14-35% chitin (Salsabila et al. 2022). The preparation of raw materials included washing, drying, crushing, milling, and sieving. This stage aimed to expand the surface of the blood clamshell,as shown in Fig.1. The larger the surface area of the shells produced, the easier it was for the blood clamshell powder to react during the isolation process. This was due to the increased reaction rate with increasing surface area and increased quality of the resulting product.

Chitosan Isolation

Deproteinization Stage: The deproteinization stage is the process of removing the protein contained in chitin in the shell of blood clams. Deproteinization can be done by adding chemical reactions such as sodium hydroxide (NaOH) (Antonino et al. 2017). Deproteinization using chemical reagents causes random cleavage of the acetyl group of chitin, resulting in a high degree of deacetylation. The sample used is 1:10 (w/v). After the blood clamshells were washed, dried, crushed, milled, and sieved, the blood clamshell powder was weighed 100 g and dissolved in 1000 mL NaOH with a concentration of 3.5%. Then stirred and heated for 2 h at a temperature of 70°C (kept constant). It was then cooled, and the blood clamshell powder was filtered through filter paper. The residue was filtered and neutralized using aqua dest to a neutral pH and then dried in an oven at 60°C for 1 h. The sample weight was obtained after weighing 88.68 g. Fig. 2 shows the blood clamshell after the deproteinization process. According to Percot et al. (2003), the protein content on clamshell contains amino acids, acidic amino acids corresponding to aspartic and glutamic acids, basic amino acids represented by lysine, histidine, and arginine, and nonpolar amino acids especially proline, alanine, valine, isoleucine, and leucine.

Demineralization Stage: The demineralization stage is

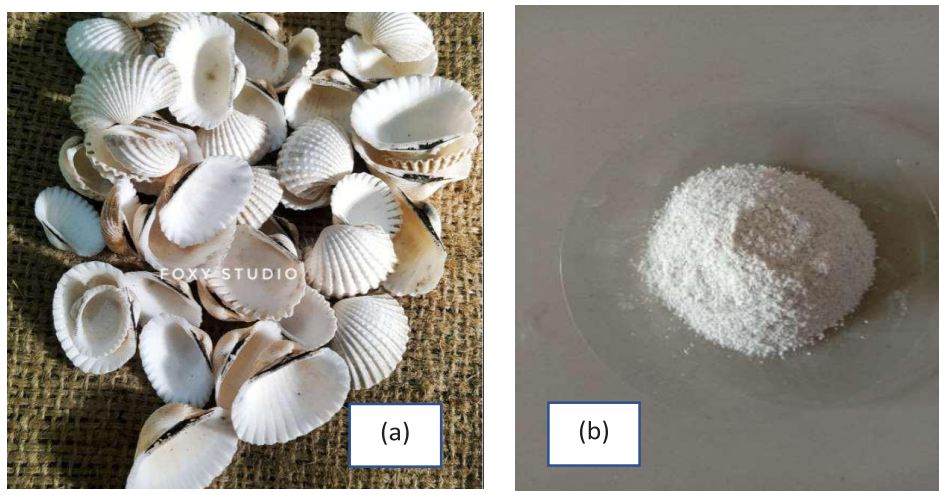

Fig.1: Blood clamshell: (a) raw material; (b) clamshell powder.

the stage of removing minerals from chitin. The minerals contained in the shells of blood clams are $Ca_3(PO_4)_2(s)$ and $CaCO_3(s)$. Demineralization can be done by adding chemical reactions such as hydrochloric acid (HCl). The use of HCl is carried out to dissolve Ca^{2+} ions in the form of $CaCO_3(s)$ to produce water-soluble $CaCl_2(aq)$ with CO_2 gas and water as byproducts. $CaCO_3(s)$ in blood clamshell powder will react with HCl to form bubbles that indicate CO_2 gas. The reaction is shown in Equation (2).

$$CaCO_{3(s)} + 2HCl_{(aq)} = CaCl_{2(aq)} + H_2O_{(l)} + CO_{2(g)} \quad \ldots(2)$$

Meanwhile, $Ca_3(PO_4)_2(s)$ will react to HCl according to the product $CaCl_2$ and H_3PO_4 which is soluble in water. The reaction is shown in Equation (3).

$$Ca_3(PO_4)_{2(s)} + 6HCl_{(aq)} = 3CaCl_{2(aq)} + 2H_3PO_{4(aq)} \quad \ldots(3)$$

Fig. 2: Blood clamshell powder after deproteinization process.

Heating in the demineralization process was carried out to accelerate the process of mineral destruction. In addition, stirring was needed to avoid the overflow of CO_2 gas during the demineralization process. This mixture was then stirred using a magnetic stirrer for 2 h at 75°C. Furthermore, the resulting residue was washed until the pH was neutral. This washing was carried out to dissolve $CaCl_2(aq)$ and $H_3PO_4(aq)$, which were soluble in water. After the blood clamshells passed through the deproteination process, the sample weight was obtained in powder, weigh and the results were 88.68 g from 100 g of the initial sample before being isolated. Next, 1 N HCL was added in a ratio of 1:10 (w/v). Then it was stirred and heatedat 75°C for 2 h (kept constant). The residue was filtered and neutralized using aqua dest toa neutral pH. Then the sample (residue) was put into an oven for 1 hour at 60°C. Then the sample was weighed, and the result was 25.42 g. It can be concluded that the chitin content on blood clamshells is 25.42%.

Deacetylation: The deacetylation process is the process of removing the acetyl group by adding an alkaline solution. The alkaline solution used was Sodium Hydroxide or (NaOH). The severance of the acetyl group in the chitin of blood clamshells was carried out through a deacetylation process using 65% NaOH at 90°C for 30 minutes. The precipitate (residue) obtained was washed and dried to a neutral pH. Blood clamshell chitosan produced was a grayish-white powder, while commercial chitosan had a yellowish flat shape, as shown in Fig. 3. The yield of chitosan from blood clamshells obtained after drying was 80.92%.

Fig. 4 shows the FTIR spectra of chitosan from blood clamshells dan commercial chitosan. Two peaks have high intensity, namely at wavelengths 1419.61 and 3356.14 cm^-

[1]. The wavelength of 1419.61 cm^{-1} corresponds to the C-H side-chain bending -CH_2OH (Varma & Vasudevan 2020). While the wavelength of 3356.14 cm^{-1} shows amino groups (Ali & Gherissi 2017). The degree of deacetylation of blood clamshell reaches 63.18%, while commercial chitosan reaches 73.35%. Varma and Vasudevan (2020) on the process of chitosan isolation from horse mussel (*M. modiolus*), the degree of deacetylation only reaches 57.43%.

Nano-Chitosan Production Using Ionic Gelation Method

The production of nano-chitosan using the ionic gelation method was conducted by making a solution of chitosan. The amount of 1% chitosan solution was made in acetic acid solution. The chitosan solution was then reacted with 1% Na-TPP solution with a variation of the ratio of chitosan to Na-TPP solution (v/v). The mixture was then stirred at various speeds for 1 hour. The nano chitosan obtained was in the form of a dispersed solid. The particle size of nano-chitosan was then measured by a particle size analyzer (PSA). Fig. 5 shows the schematic reaction of chitosan and sodium tripolyphosphate (Madni et al. 2017).

Effect of Ratio of Chitosan to Na-TPP Solution

Fig. 6 shows the particle size of nano-chitosan at the various ratio of chitosan to Na-TPP solution at the rotation speed of 700 rpm. The greater the ratio of the chitosan solution, the larger the nano size of the chitosan formed. The approxima-

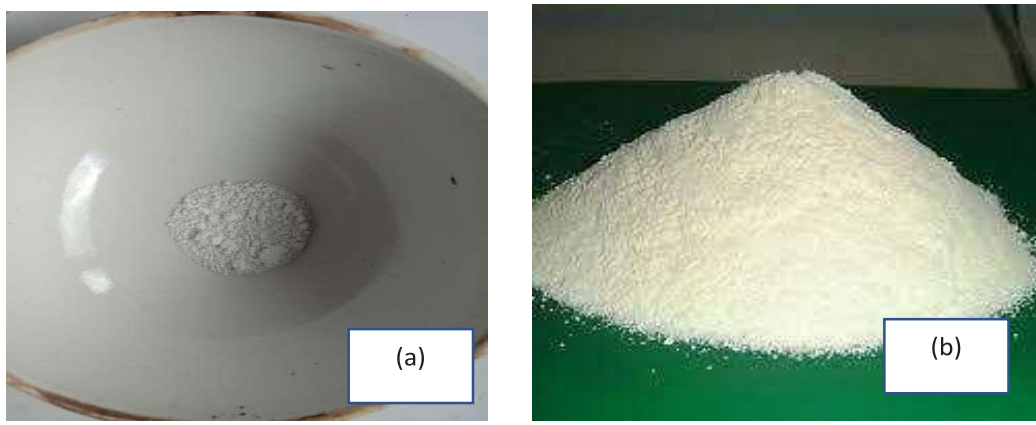

Fig. 3: Chitosan: (a) From blood clamshell; (b) commercial chitosan.

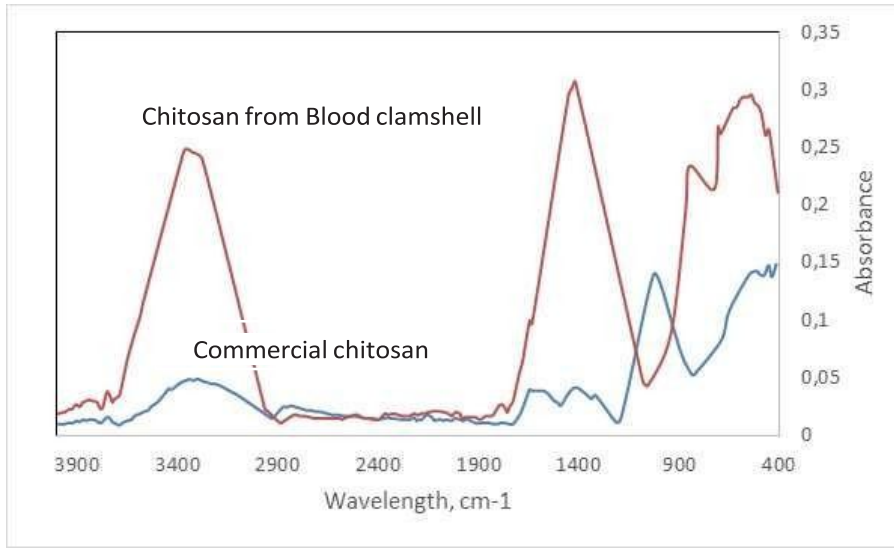

Fig. 4: FTIR spectra of chitosan from blood clamshell and commercial chitosan.

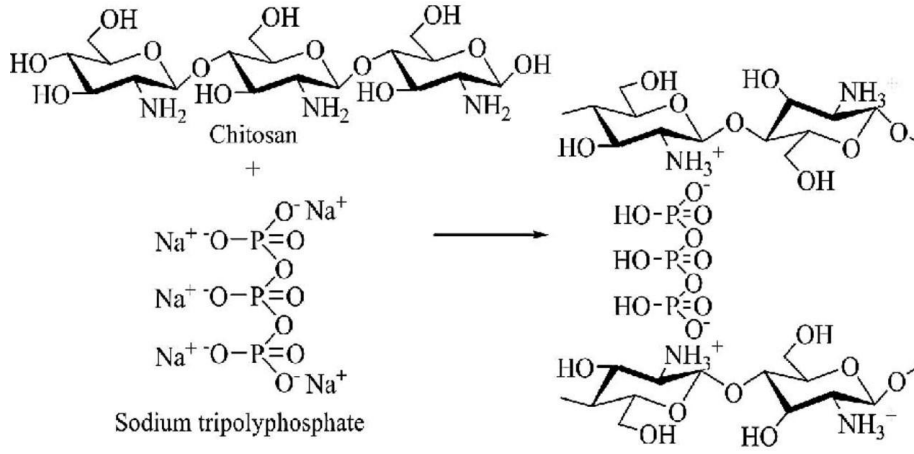

Fig. 5: schematic reaction of chitosan and sodium tripolyphosphate.

tion equation can be expressed by equation (4).

$$dp = 95.55\ (R) - 68.142 \quad \ldots(4)$$

Where dp is the particle size of nano-chitosan (nm) and R is the ratio of chitosan to Na-TPP solution (v/v).

Effect of Rotation Speed

Fig. 7 shows the particle size of nano-chitosan at the speed rotation of centrifuge at the ratio of chitosan to Na-TPP solution of 5 v/v. The greater of speed rotation, the size of the nano chitosanformed tends to be greater. The approximation equation can be expressed by equation (5).

$$dp = 61.94\ (N) - 3.6344 \quad \ldots(5)$$

Where dp is the particle size of nano-chitosan (nm) and N is the speed rotation of the centrifuge.

The effect of the ratio of chitosan to Na-TPP solution (R) and the rotation speed of centrifuge (N) on particle size of nano-chitosan can be expressed by equation (6).

$$dp = 0.12\ (R)^{0.714}\ (N)^{0.99} \quad \ldots(6)$$

CONCLUSION

Nano-chitosan can be produced from blood clamshell with the ionic gelation method by using sodium tripolyphosphate. The chitin content on blood clamshell is 25.42%. The yield of chitosan from chitin is about 80.92%. The degree of

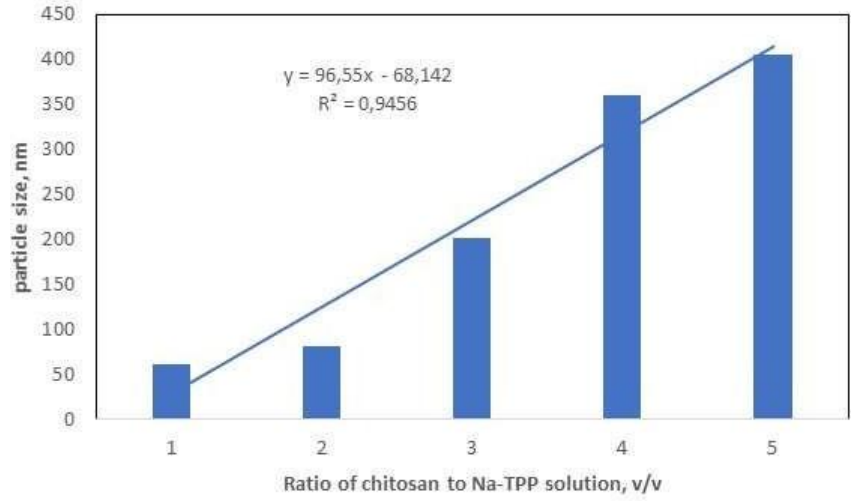

Fig. 6: Particle size of nano-chitosan at various ratio chitosan to Na-TPP solution (v/v).

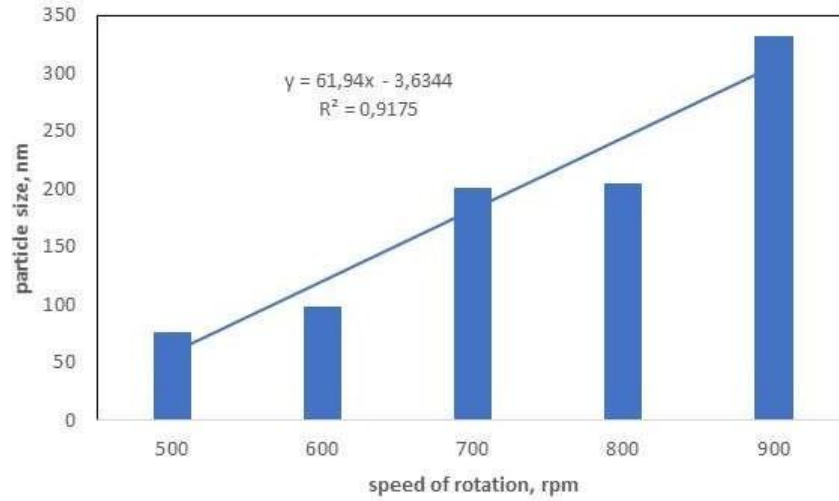

Fig. 7: Particle size of nano-chitosan at various speed rotations of the centrifuge

deacetylation of chitin from blood clamshell reaches 63.18%. The effect of the ratio of chitosan to Na-TPP solution (R) and the rotation speed of centrifuge (N) on the particle size of nano-chitosan can be expressed by equation $dp = 0.12 (R)^{0.714} (N)^{0.99}$.

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