TIK-217 Synthesis, Characterization, and Insilico Nanochitosan of Pupa Black Soldier Fly (Hermetia Illucens) As Bone Graft Material for Bone Remodeling Post Tooth Extraction

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Synthesis, Characterization, and Insilico Nanochitosan of Pupa Black Soldier Fly (Hermetia Illucens) As Bone Graft Material for Bone Remodeling Post Tooth Extraction

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KEYWORDS
Black soldier fly (*Hermetia illucens*), Chitosan, Insilico, Ionic gelation methods, Nanochitosan.

ABSTRACT

Objectives: This research aims to make nanochitosan BSF pupae through ionic gelation and size reduction with a magnetic stirrer and to determine the bonding of chitosan compounds and their derivatives through the insilico method for forming alveolar bone.

Methods: BSF chitosan is made through demineralization, deproteination, depigmentation, and deacetylation, then formulated into nanochitosan using the ionic gelation method. The reduction in particle size uses a magnetic stirrer by adding Na-TPP 0.1%. The bonding of chitosan compounds and their derivatives uses the insilico method through the Autodock Vina program, which is integrated into the PyRx application version 0.8. Docking results of chitosan compounds using Biovia Discovery Studio V21.1.0.20298 software.

Results: The Deacetylation of chitosan reached 80%, indicating the purity of chitosan and according to SNI No.7949-2013. The nanochitosan formed has an average size of 204.9 nm, is reasonably uniform, relatively stable, and has a spherical shape resembling a ball. The Glycol Chitosan and Aminoethyl-Chitosan compounds have many hydrogen bonds. The lowest binding affinity value is -9.8 kcal/mol in Glycol Chitosan and has identical amino acid residues as the comparison ligand in Aminoethyl-Chitosan.

Conclusions: Chitosan extracted from pupa BSF (Hermetia illucens) has fulfilled SNI No.7949-2013 including water content test, ash content and degrees of deacetylation with a yield of 13.2%. The degree value of the deacetylation of chitosan used to make nanocitosan is 80%, showing that the resulting chitosan is pure chitosan. The characterization of nanoparticles is formed an average size of 204.9 ± 48.7 nm, quite uniform and relatively stable.

INTRODUCTION

The Black Soldier Fly (Hermetia illucens) is a type of insect distributed almost all over the earth's surface, including Indonesia. It has a black body physiology in the transparent basal segment of the abdomen (wasp waist), which resembles the shape of a bee's abdomen. BSF fly length ranges from 15-20 mm and has a lifetime of five to eight days [1,3]. Black Soldier Fly (BSF) larvae

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are included in the decomposer fly larvae, which are not disease vectors. BSF flies are widely cultivated because of their high nutritional value, with a protein content of 44.26% and a fat content of 29.65%. The life stages of BSF flies are egg-larvae-pupae-adult flies. The pupa shell contains chitin, which can be converted into chitosan. Chitosan has biocompatible, biodegradable, and non-toxic properties, can be the second most abundant cationic polyelectrolyte available in nature, and has high binding capacity so that it can be applied as an absorbent and drug delivery medium composed of amino groups and hydroxyl groups –OH [2,4].

According to several studies, BSF pupae contain chitin, which can be converted into chitosan. Chitosan is a natural polysaccharide synthesized from chitin extracted from the shells of crustaceans and insects. The BSF life phase starts from larva, prepupa, and pupa to adult flies. In the pre-pupae to pupal stage, BSF flies can be used as a potential chitin source because the BSF flies' exoskeleton contains 35% chitin. Chitosan is multipurpose and widely used in industry and health because of its excellent biological and chemical properties. In dentistry, it is used as an ingredient in root canal dressings, antibacterial, wound healing or bone regeneration, and is used to improve the properties of other dental materials [28].

According to a 2014 Bosch study, the protein content in BSF is 40-50% high. Wardhana's research in 2016 stated that the high protein content contained in BSF is believed to be able to help the wound healing process. [9] Marsela Mangunsong's research in 2021 revealed that the effect of methanol extract of BSF maggot given to open wounds in male white guinea pigs was able to influence wound healing.[10] According to Wang and Shelomi in 2017, BSF pupae contain chitin which can be converted into chitosan. Chitosan is a natural polysaccharide that is synthesized from chitin as a result of the deacetylation process. [11] BSF is a type of insect that can be commercialized until now because it contains chitin and chitosan which can be used as added value in the manufacture of an ingredient. The 2022 Kautsar research concluded that the chitin content (Black soldier fly larvae) had been shown in larvae (3.6%), prepupae (3.1%), adults (2.9%), and pupal shells (14.1%) [13].

The most important factors influencing the successful extraction of chitin and chitosan are sample type and extraction method. Simple chemical processes can extract chitin and chitosan. [11] Chitosan is a polysaccharide consisting of N-glucosamine and N-acetylglucosamine units. [12] Chitosan has advantages such as high osteoconductivity, easy application, and biodegradability, which makes it a good candidate as a graft material bone in bone regeneration. The bone graft material is good, one of which is if it can stimulate the production of osteoprotegerin (OPG) to stimulate the differentiation and proliferation of osteoblasts, which can support the process of bone formation. OPG was found to bind to RANKL with approximately 500-fold higher affinity than RANK. Therefore, OPG prevents RANKL from binding to its RANK receptor, inhibits osteoclastogenesis, and protects bone from excessive osteoclast-mediated resorption, making it very suitable as an alternative to bone grafts if there is damage to the alveolar bone after tooth extraction.

The ability of chitosan to be applied in various modern industrial fields encourages the continued development of multiple studies using chitosan, including modifying chitosan chemically or physically by changing the size of the chitosan particles or granules to become smaller in the form of nanoparticles. Nanoparticles have a greater ratio between surface area and volume than similar materials in large sizes, making nanoparticles more reactive. Nanoparticles are particles measuring 1-100 nanometers. Nanoparticles aim to overcome the solubility of difficult substances to dissolve, improve poor bioavailability, modify drug delivery systems, increase the stability of active substances, and improve absorption. One of the advantages of nanoparticles is the ability to penetrate spaces between cells that colloidal particles can penetrate. Nanoparticles are flexible for combination with other technologies. This capability opens up broad potential to be developed for various purposes and targets.

The ionic gelation method is used to manufacture inorganic materials through a chemical reaction in a solution at a relatively low temperature. The advantages of making nanochitosan using this method are the simple process, the relatively low temperature, the high purity level, and the absence of organic solvents. In this method, interactions between the positively charged amino groups in chitosan and polyanions will trigger the formation of inter- and intra-network structures in three-dimensional polymer chains. Magnetic stirrer and determine the characteristics of nanochitosan based on the morphology and size of the nanoparticles.

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MATERIAL AND METHODS

Tools and materials

The main material used in this study was 140gram BSF Pupa obtained from CV Maggo Banua Prima Banjarmasin, South Kalimantan. The materials used for the manufacture of chitosan and nanochitosan consist of 2% NaOH, HCl, KMnO4, Calcium carbonate (CaCO3), Calcium Phosphate (Ca3(Po4)2), acetic acid (CH3 COOH), H2SO4, distilled water, Tripolyphosphate (TPP). The tools used in this research were Retsch brand 40 mesh sieve, oven, magnetic stirrer (Gibernity Europe), spray drying (Labconco), Particle Size Analyzer (PSA) (DelsaTM Nano, Cordoun), Viscometer (Brookfield LV), Fourier Transform Infrared Spectrophotometer (FTIR) (Bruker Tensor Tipe MBQ00).

Research methods

Preparation of BSF pupae

BSF pupae were cleaned of feed impurities, weighed with a digital scale to obtain 102.8 grams of BSF pupae, and then dried at 110-120°C in an oven for 1 hour to reduce the water content.

Chitin Isolation

Demineralization

Dried and mashed BSF pupae samples were processed through several stages. The first step is demineralization by immersing the sample in 3 M HCl 1:10 (w/v) for 36 hours at room temperature (25-30°C), then rinsing with distilled water on filter paper until the pH is neutral and drying in an oven at 60 °C for 1 hour.

Deproteination

The residue was soaked in 2 M NaOH 1:10 (w/v) for 36 hours at room temperature (25-30°C). This resulted in a change in the color of the BSF pupa samples from dark brown to yellowish brown.

Depigmentation

The residue was soaked in 2% 1:10 (w/v) KMnO4 solution for 2 hours, then in 2% 1:10 (w/v) oxalic acid for 2 hours. Then, it was filtered, washed until it reached a neutral pH, and dried at 105°C for 48 hours. The resulting particle is called chitin.

Chitin Deacetylation

Using a magnetic stirrer, chitin samples were immersed in 50% NaOH solution (1:10) at 80°C for 12 hours. Then, the model was filtered and rinsed with distilled water until it reached a neutral pH and then dried for 48 hours at 60°C in an oven.

Chitosan Characterization

Test Water Level

As much as 1-2 g of chitosan was spread in a pan tested for water content at 105°C; the water content was obtained when it reached a constant weight. The test was carried out in 3 repetitions.

Ash Rate Test

Heat a porcelain crucible in the oven at 105°C for 30 minutes. Weigh the porcelain crucible and chitosan, heat the sample in a furnace at 800 for 3 hours, cool it in a desiccator, and weigh it.

Determination of Degree of Deacetylation (DD)

2.5 g of chitosan was dissolved in 30 mL of 0.1 M HCl at 20°C. While stirring in a 250 mL beaker, two drops of methyl orange indicator are added and titrated with 0.1 M NaOH. The endpoint of the titration is indicated by a color change from pink to yellow-orange.

The degree of chitosan deacetylation was measured using the Fourier Transform InfraRed (FTIR). The spectrum was taken by scanning in the wave number region 4000-500 cm-1. Measure the degree of deacetylation using the baseline method on the FTIR results. This method's calculation method is to measure the highest peak and record it from the line obtained, and the absorbance is calculated using the formula. Determination and analysis of the degree of deacetylation were carried out at absorbance values of 1655 cm-1 and 3450 cm-1 using the formula:

$$A = \text{Log}\left[\frac{Po}{\ }\right]$$

Information:

Po = Distance between baseline and tangent line

P = Distance between the baseline and the lowest trough

$$\%\text{N-deasetilasi} = \left[100 - \left(\frac{A_{1655}}{A_{3450}} x \frac{100}{1{,}33}\right)\right]$$

Information:

A1655: Absorbance value at 1655 cm-1

A3450: Absorbance value at 3450 cm-1

1.33: A1655/A3450 ratio at N - 100% deacetylation.[15]

Based on the results of DD, chitosan produced meets commercial quality standards from Protan Laboratories

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Inc. (1987), namely ≥70%. The higher the DD value, the more amino groups (NH2) in the chitosan molecule, so the chitosan is more reactive.

Functional Group Analysis

Chitosan samples in powder form were prepared and tested using the KBr-disk method, analyzed by FTIR at a wavelength of 4000-500 cm-1.[15]

Yield

The determination of chitosan yield is based on comparing the weight of the chitosan produced and the weight of the BSF pupa. The formula for determining the result is as follows: [17]

$$\% \text{ Yield} = \frac{\text{Weight of chitosan produced}}{\text{BSF pupal weight}} \times 100\%$$

Studies in Silicon

The interaction between chitosan and its derivatives and Osteoprotegerin (OPG), a bone-forming protein, was analyzed using the Autodock Vina program integrated in the PyRx version 0.8 application. The docking results of chitosan compounds and their derivatives with the targeted Osteoprotegerin will be visualized using the BIOVIA Discovery Studio V21.1.0.20298 software.

Manufacturing of BSF Nanochitosan Pupae

Dissolve Pupa BSF 100% chitosan in 1% acetic acid as much as 50 mL poured into a beaker, then stirred using a magnetic stirrer. Add 0.1% Na TPP (Sodium Tripolyphosphate) at a ratio of 5:1 (chitosan:TPP). TPP was added drop by drop into the chitosan solution while stirring using a 1200 rpm magnetic stirrer for 2 hours at room temperature to form a suspension of nanoparticles.

Characterization of Nanochitosan

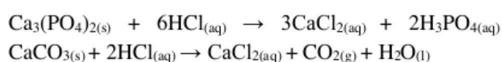
Particle Size Examination

5 mL of nanochitosan suspension was dropped on the identification lens of the PSA instrument until there was data on size information and size distribution on the monitor screen of the PSA instrument.

RESULTS AND DISCUSSION

The basic material for BSF pupae was obtained from 102.8gram BSF Pupa obtained from CV Maggo Banua Prima Banjarmasin, South Kalimantan. The BSF fly (Hermetia illucens) was determined at the Laboratory of the Faculty of Agriculture, University of Lambung Mangkurat. The initial process of chitosan synthesis is demineralization, and this process aims to remove the mineral content contained in the BSF pupa samples. The indicator of the mineral release process in the BSF pupa is characterized by the formation of CO2 gas bubbles when the sample is mixed with HCl solution.

Mineral salts in CaCl2 can dissolve in solvents, so they are easily removed during washing. The results obtained in this treatment were BSF pupa extract, which had lost minerals such as calcium phosphate [Ca3(PO4)2] and calcium carbonate (CaCO3). The demineralization reaction is estimated as follows:



$$Ca_3(PO_4)_{2(s)} + 6HCl_{(aq)} \rightarrow 3CaCl_{2(aq)} + 2H_3PO_{4(aq)}$$
$$CaCO_{3(s)} + 2HCl_{(aq)} \rightarrow CaCl_{2(aq)} + CO_{2(g)} + H_2O_{(l)}$$

The residue from chitin demineralization was obtained as a dark brown powder with a coarse texture.

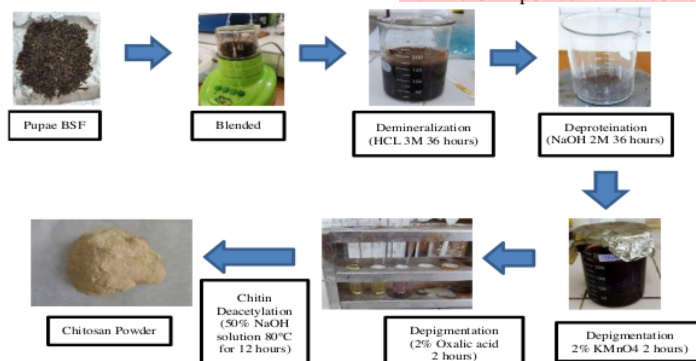


Figure 1. Chitosan synthesis process from BSF Pupae

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The next stage is deproteinization, which aims to terminate the protein attached to chitin. The result of heating in the NaOH solution produces a solution that is slightly thickened and reddish, indicating a breakdown of the protein in the sample. This follows the research of Pratiwi, 2022 and Mirwandono, 2019 who reported that in the process of deproteinization, the solution thickens and becomes reddish as evidence that the protein in chitin is released and binds to Na+ ions in solution to form sodium proteinate [2,17].

The chitin depigmentation stage is carried out by reducing the chitosan pigment by breaking the conjugate bonds of the dye molecule, causing the color change to light brown. [2] Each isolation stage is carried out by washing the residue on filter paper until the pH is neutral, this aims to remove residual solvents, preventing damage to chitin due to extreme pH differences. The most important step in the synthesis of chitosan is deacetylation. This process aims to break the acetyl bond (-COCH3) on the amine group attached to chitin. In general, the reaction for the formation of chitosan from chitin is a hydrolysis reaction of an amide by a base. Chitin acts as an amide, and NaOH as a base. At first, the double bond between C and O will be released so that C is positively charged and O is negatively charged. OH- from NaOH, which is more electronegative, will attack C, which is more electropositive, while Na+ will bind to O from NHCOCH3. Furthermore, the lone pair of electrons from -NH will bond with H from OH. Next, an electron delocalization will occur -NH2, which lacks electrons, gets a donor from C. This causes C to lack electrons, so one electron from O is stable. It is used to bond with C. The acetyl bond with this amide will be broken to form the -NH2 group. The determination results show that the sample is a BSF fly (Black Soldier Fly) with the Latin name Hermetia illucens. The results of the chitin and chitosan phases of the BSF pupa resulted in a blackish-brown characterization because there were still pigments attached. Chitosan extraction produces a yellowish-brown color [9,17].

The chitosan produced has characteristics that meet the standards through the purity of the chitosan, which can be seen from the low moisture and ash content but has a high degree of deacetylation. The higher the degree of deacetylation, the more amine groups (NH2) in the chitosan molecular chain, so the chitosan is more reactive. The particle size of the tested chitosan was in the form of flakes to powder, according to the SNI chitosan chitosan quality standard No. 7949-2013. The mechanism of the chitosan deacetylation reaction is presented in Figure 2.

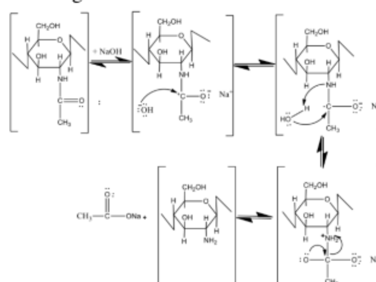

Figure 2. Chitosan synthesis reaction mechanism.[9]

Chitosan Characterization

Chitosan characterization includes yield of chitin transformation into chitosan, moisture content, ash content, and degree of deacetylation.

Analysis of Moisture Content and Chitosan Ash Content

Table 1. Results of Chitosan Characterization According to SNI No.7949-2013

Parameter	Test results	SNI No.7949-2013
Water content	6,24%	< 12%
Ash Content	1,74%	≤ 15%

Table 1 shows the results of the characterization of the BSF chitosan pupa from all parameters meeting the quality standards of chitosan according to SNI No.7949-2013, namely having a moisture content of 6.24% <12%, ash content of 1.74% ≤ 15%.

Water Level and Ash Level

The determination of the water content and ash content of chitosan is based on the gravimetric principle. The principle of determining the water content is determining the percentage of water content in chitosan by drying it in an oven at 105°C for not less than 1 hour and then weighing it until a constant weight is obtained. Table 1 shows a water content of 6.24%. The water content meets

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the SNI chitosan quality standard No.7949-2013, which is <12%. The water content of chitosan is one of the most important parameters because it indicates the quality of chitosan; the lower the water content, the better the chitosan. The water content of chitosan is generally influenced by the length of the drying process in making chitosan, the surface area used in drying chitosan, and the amount of chitosan that is dried in one drying. According to Nadia (2014), the drying process, drying time, amount of chitosan, area of drying area, and drying facilities affect the water content and the success of drying chitosan [21,2].

Determination of the ash content of chitosan is intended to determine the minerals contained in chitosan, which indicates the success of the demineralization process. Table 1 shows an ash content of 1.74%. The amount of ash content meets the SNI No.7949-2013 chitosan quality standard, namely ≤ 15%. The ash content can affect the solubility and final product of chitosan. The lower the ash content of the chitosan produced, the higher the quality and purity of the chitosan. The minerals in chitosan are affected by stirring in the demineralization process. Constant stirring will cause the heat to be evenly distributed so that the HCl can bind perfectly [28,29].

Chitosan Yield Analysis

The yield calculation results can be seen in the table:

Table 2. Chitosan yield

BSF Pupae Initial Weight	Chitosan weight	Yield Percent
102,8gram	**13,6gram**	**13,2%**

Table 2 shows that 102.8 grams of BSF pupae that went through the process of demineralization, deproteination, depigmentation, and deacetylation produced 13.6 grams of chitosan so that the yield of isolated BSF chitosan pupae was 13.2%.

The higher the NaOH used, the lower the yield of chitosan. The depolymerization of the chitosan molecular chain can be caused by the use of high concentrations of NaOH, which can cause a decrease in the molecular weight of chitosan.

Chitosan Analysis with FT-IR Method

The FTIR spectrum can be used to characterize compounds by analyzing the peaks in the spectrum corresponding to the typical functional groups in chitosan. The characteristic features of chitosan are the amide groups and hydroxyl groups. The location of the absorption of the amide group is at wave number 1620-1309 cm-1, while the hydroxyl group is located at wave number 3401-3100 cm-1. The following is the FTIR spectrum of the synthesized BSF chitosan pupa obtained.

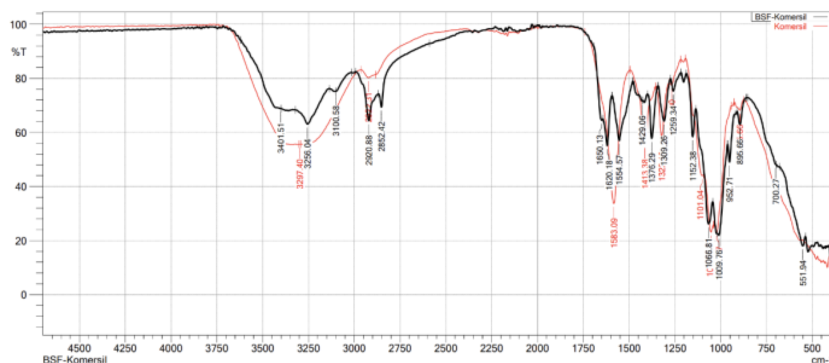

Figure 3. FTIR spectrum of BSF pupa chitosan (black color) and commercial chitosan (orange color).

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FTIR spectral analysis of BSF pupa shell chitosan at wavenumber 3256.04 and commercial chitosan at 3297.40 overlaps O-H and N-H vibrations at wave 3000. The OH group comes from the C3 and C5 positions, while the N-H group comes from the C2.

Analysis of the Degree of Deacetylation (DD) of Chitosan

The degree of deacetylation (DD) is a parameter that measures the number of acetyl groups in the loose chitin. The standard degree of deacetylation of chitosan is >70%. The greater the DD value, the better the quality of the chitosan produced. The resulting chitosan will be more reactive because more amine groups replace the acetyl groups (amine groups are more reactive than acetyl groups due to the presence of PEB on the nitrogen atom in the chitosan structure).

Determination of the degree of deacetylation is carried out by FTIR analysis. In the analysis, FTIR will detect the functional groups contained in chitosan, namely the NH, OH, and C-C functional groups—CH and C=O for chitin. The degree of deacetylation describes/indicates the removal of the acetyl group (COCH3) present in chitin. Chitin that undergoes the deacetylation process is called chitosan. The results of the FTIR detection are reflected in the form of the functional group peaks at each wave number. A1655 shows absorption in the amide band, A3450 establishes absorption in the hydroxyl band, and factor 1.33 offers the ratio A1655/A3450 value for the full degree of chitosan deacetylation.

Formula:

$$\%\text{N-deasetilasi} = \left[100 - \left(\frac{A_{1655}}{A_{3450}} x \frac{100}{1,33}\right)\right]$$

A_{1655}= Log $\frac{98}{88}$ = 0.04

A_{3450}= Log $\frac{99}{73}$ = 0.15

So the degree of deacetylation (DD):

$$\%DD = 100 - \left[\left(\frac{0.04}{0.15}\right) x \frac{100}{1.33}\right]$$

%DD = 100 – 20.0

%DD = 80

Based on the calculation results of the baseline method, the degree of deacetylation of chitosan produced in this study was 80%. These results indicate that the DD results of BSF chitosan pupae meet SNI No.7949-2013 quality standards, namely ≥ 75%. According to Natalia (2021), a 40% -100% DD value can be said to be chitosan. The higher the DD, the better the quality of chitosan. The number of acetyl groups can reduce the rate of chitosan. According to Kanto (2019), the magnitude of DD is affected by the duration and temperature of deacetylation. The higher the temperature increases the motion between molecules, so the reaction rate of breaking the acetyl group reaction runs faster.[2]

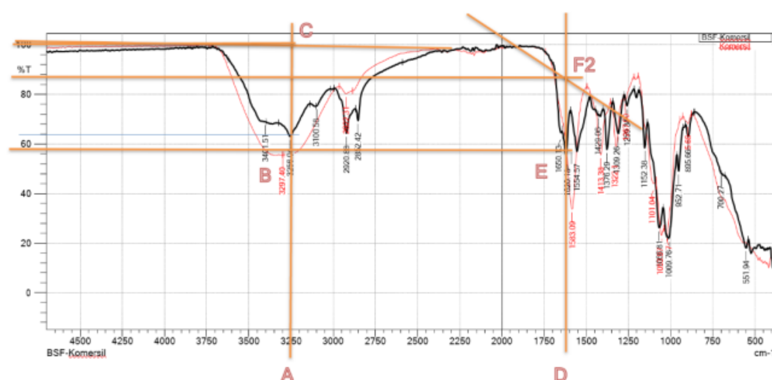

Figure 4. Calculation of the degree of deacetylation from the spectrum FT-IR for pupae BSF chitosan

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Nanochitosan Synthesis

The production of nanochitosan is carried out based on the method that has been carried out by Pratiwi (2022), namely, using the ionic gelation method using a magnetic stirrer. The advantage of the magnetic stirrer is that the homogenization process between chitosan solution and ionic gelation material can be controlled evenly at high speed to produce homogeneous, stable particles, and no accumulation occurs in the drying process. Only nanoparticles are formed, which are stable and not agglomerated. Acetic acid is used to dissolve chitosan because chitosan can dissolve in acidic conditions to form polycationic chitosan. The 1% acetic acid concentration is the right concentration so that chitosan can dissolve completely.[2]

In this study, a NaTPP solution was used with a concentration of 0.1%. Adding TPP reagent with a concentration of 0.1% is the optimum concentration to form nano-sized particles. The NaTPP solution is a crosslinking agent that will strengthen the chitosan nanoparticle matrix. [21] The reaction mechanism of chitosan and NaTPP is shown in Figure 7.

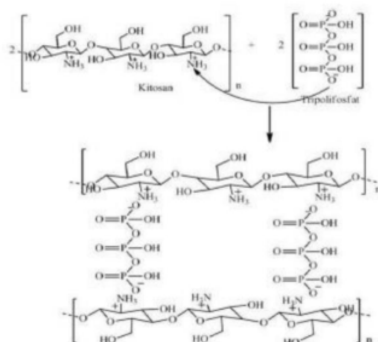

Figure 7. Chitosan Reaction Mechanism with NaTPP.15

Particle Size Analysis Nanochitosan

The preparation of chitosan to become nanochitosan in this study used the ionic gelation method, and the sizing treatment was carried out using a 1200 rpm magnetic stirrer method. The faster the rotation, the greater the intensity of the solvent molecules to come into contact with chitosan, so the greater the power of the rotational speed of the magnetic stirrer, the smaller the resulting particles. The ionic gelation method is a polyelectrolyte complexation between positively charged chitosan and negatively charged tripolyphosphate (TPP). Giving TPP, which can make chitosan homogeneous so that a nano-coating suspension is formed

In this study, the use of TPP and chitosan with a ratio of 1:5 (TPP: Chitosan), nanoparticles prepared at a volume ratio of 5 1 showed a good polydispersity index value of around 0.3, which means that the formed nanoparticles have a short size distribution range or in other words, the level of freshness is quite good. Particle size is strongly influenced by the concentration and volume ratio of chitosan and TPP used, where the particle size increases as the concentration and volume ratio of chitosan and TPP increases.

Table 4. Results of Particle Size Analysis

Peak	Diameter (nm)	Std. Dev.
1	204.9	48.7
2	0	0
3	0	0
4	0	0
5	0	0

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In this study, the particle size obtained using a magnetic stirrer was 204.9 nm with a spherical particle shape like a ball. The results of the morphology of nanochitosan are by the standards, namely in the form of spherical particles. [14] According to Tiyaboonchai (2003), nanoparticles are solid colloidal particles with 1–1000 nm diameters. Nanoparticles as particulate matter with at least one dimension smaller than 100 nm have a large surface area to volume ratio.

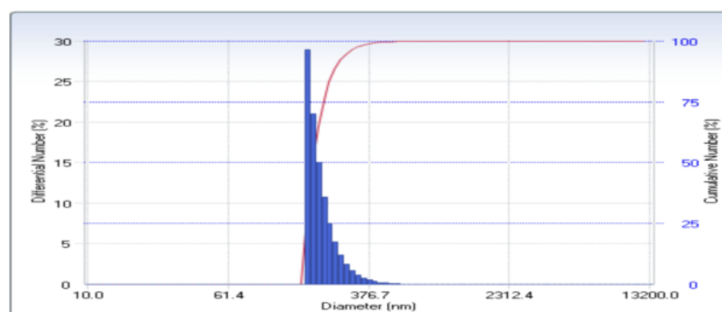

Figure 9. Graph of Number Distribution of Particle Size Analysis of Nanochitosan

CONCLUSION

Chitosan extracted from pupa BSF (Hermetia illucens) has fulfilled SNI No.7949-2013, namely the water content test, ash content and degrees of deacetylation with a yield of 13.2%. The degree value of the deacetylation of chitosan used to make nanocitosan is 80%, showing that the resulting chitosan is pure chitosan. The characterization of nanoparticles is formed an average size of 204.9 ± 48.7 nm, quite uniform and relatively stable.

CONFLICT OF INTEREST STATEMENT

The authors declare that we have no competing interests.

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