



## Enzymatic Production of Chitosan from the Waste White Shrimp (*Penaeus merguensis*) and Antimicrobial Effect for Durability Against Fishballs

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### Abstract

Chitosan is a compound of poly(N-amino-2-deoxy-β-D-glucopyranose) that is widely available in nature and has an antimicrobial agent. This study aims to apply enzymatic chitosan is isolated from white shrimp waste (*Penaeus merguensis*) as antimicrobial in fish balls. Enzymatic chitosan is obtained from gradually isolation process that is demineralization, decolorization, deproteination and deacetylation. Chitin characteristics obtained by 2.14% water content, 1.05 % ash content, 6.24% N-Total content with 55.96 % deacetylated degree. Deacetylation process of chitin into chitosan by chitin deacetylase carried out with [E] : [S] 1 : 100 (mL/mg) ratio at 50° C for 2 h incubation time. Characteristics of chitosan obtained by 5.38% water content, 0.83 % ash content, 7.51% N-Total content and 82.46 % deacetylated degree. The results of chitosan as antimicrobial agent by total plate count (TPC) method of the fish balls obtained optimum concentration at 2,5% to inhibit the growth of bacteria with  $3.4 \times 10^4$  cfu/g total microbial contamination during 15 hours shelf life of fish ball.

Key words: *Penaeus merguensis*, Chitin, Chitosan, Chitin deacetylase, Total Plate Count

### INTRODUCTION

Natural polymers are currently of concern to researchers to be used because it has a wide range of benefits in the development. Chitosan is a polysaccharide that is widely available in nature after cellulose. Chitosan is a compound of poly (N-amino-2 deoxy-β-D-glucopyranose) or glucosamine resulting deacetylation of chitin / poly(N-acetyl-2-amino 2-deoxy-β-D-glucopyranose) produced in large quantities in nature, that is contained in the waste shrimp and crab which are widely available in Indonesia. Utilization of shrimp shell waste as chitosan in addition to addressing environmental problems can also increase the value added to the shrimp farmers (Ramadan et al, 2010)

Isolation results of shrimp shell chitin will produce a compound which is a polysaccharide, a polymer of glucosamine containing acetamide group, while chitosan is the result of chitin hydrolysis process by the deacetylation of acetamide groups into amine groups. In principle, the transformation process of chitin into chitosan can be done enzymatically and chemically. However, the method of enzymatically more likely to secure, easy to control and does not leave residue compared with the chemical methods. Utilization as an agent degrading enzyme acetyl groups on chitin ranging widely developed, one of which is a chitin deacetylase.

Chitin deacetylase (EC 3.5.1.41) including chitinolytic enzyme group can be isolated either from fungi or bacteria. One type of bacteria which can excrete enzymes is *Bacillus licheniformis* HSA3-1a

bacteria isolated from hot springs of Sulili Pinrang, South Sulawesi (Natsir et al, 2010)

Chitosan has a specific nature that is bioactive, biocompatible, chelating, antibacterial and can be biodegraded. The potential of chitosan as an antimicrobial refers to the content of amino-polysaccharide group that can inhibit the growth of microbes. One mechanism that may occur in food preservation that chitosan molecules have the ability to interact with compounds on the surface of bacterial cells then form a kind of absorbed layer (layer) that inhibits bacterial transport channel that substance deficient bacteria to flourish and lead to bacterial death (Tan et al, 2013).

Antibacterial properties of chitosan is a very important thing to be developed especially for the preservation of food products. One food product that is the subject of this study is the fish balls because it is one of the many food products which are popular in public.

This research has been conducted on the isolation of chitin from white shrimp waste (*P. merguensis*), the conversion of chitin into chitosan using chitin deacetylase is isolated from the bacterium *B. licheniformis* HSA3-1a that were subsequently applied as an antimicrobial chitosan on fish balls (Tan et al, 2013).

### MATERIALS AND METHODS

#### 2.1 . Materials and Equipment

Materials used in this study were isolates of *B. licheniformis* HSA3 - 1a, white shrimp waste (*P.*

*merguiensis* ), bakto agar, yeast extract, peptone bakto, NaCl, K<sub>2</sub>HPO<sub>4</sub>, MgSO<sub>4</sub>·7H<sub>2</sub>O, CaCl<sub>2</sub>, colloidal chitin, glycol chitin, citrate phosphate buffer (pH 7), CH<sub>3</sub>COOH, NaNO<sub>2</sub>, sulfamic acid, HCl, Indol, ethanol, Lowry B reagent, Lowry A reagent, glucosamine (Sigma-Aldrich), glycol chitin, Plate Count Agar (PCA), Butterfield 's phosphate buffered solution, bovine serum albumin (BSA), cotton swabs, aquadest .

The instrument used is a spectrophotometer UV-Vis (spectronic 20D+), the balance of digital analytics, cold centrifuged, shaker incubator, incubator (Memmert), autoclave, Enkas, micro pipette, needle loop, tube reaction, petri dishes and tools glass commonly used. Stages in this study consisted of enzymatic isolation of chitin from white shrimp waste (*P. merguiensis*), the production of chitin deacetylase enzyme ( KD ), bioconversion of chitin into chitosan and chitosan enzymatic applications as antimicrobial agents in fishballs .

## 2.2. Isolation of Chitin

### Sample preparation

White shrimp waste in the form of skin and tail is cleaned and dried under the sun for 2 x 24 hours. Once dried, then ground to the form of powder and then sieved to 80 mesh size and the results are floured shrimp used as raw materials in this study.

### Isolation of Chitin

Isolation or chitin production process consists of three phases: demineralization, decolorization, and deproteinasi (Purwatiningsih, 2009)

#### Demineralization

100 grams shrimp shell powder was dissolved in acid solution (1.0 M HCl) with a ratio of 1:10 (sample: solvent), and then stirred with a stirrer for 1 hour at a temperature of 75°C. Then it was filtered with a büchner filter and the resulting residue was washed with distilled water using neutral pH, and then dried in an oven at a temperature of 80 ° C for 24 hours to proceed to the next step.

#### Decolorization

Results of demineralization was weighed and dissolved into NaOCl 0.5% with a ratio of 1:10 (sample: solvent), and then inserted into the erlenmeyer and stirred with a stirrer for 1 hour at a temperature of 75°C. Then filtered with a Büchner filter and the resulting residue was washed with distilled water using neutral pH, and then dried in an oven at a temperature of 80°C for 24 hours.

#### Deproteinasi

Results of decolorization was followed by dissolving them into the enzyme. However, first the optimization process is to determine the variation of the incubation time and enzyme concentration variations. Incubation time is varied for 1, 2, 3, 4 and 5 hours while the enzyme concentration is varied with the ratio of [S]: [E] (g / mL) are 1:10, 1:20, 1:30 and 1:40 (b / v). Chitin was characterized with several test parameters including moisture content and ash content

by gravimetric method, N-total with *Kjeldhal* method and deacetylated degree with FTIR.

## 2.3. Chitin deacetylase production

Rejuvenation is done by growing the *B. Licheniformis* HSA3-1a bacteria stock into the LA (Luria Agar) modification medium + 0.5% colloidal chitin to obtain fresh isolates (Yuli et al, 2004 & Natsir et al, 2010).

Chitin deacetylase production begins with the manufacture of inoculum followed by a fermentation process (enzyme production). Inoculum had been incubated for 18-24 hours at a temperature of 500°C, 180 rpm, taken as many as 10 mL and then inoculated into 90 ml of production medium. Every 24 hours of sampling for measurement of OD at the maximum wavelength, then centrifuged at 3000 rpm at a temperature of 40°C for 15 minutes. The filtrate obtained was some crude enzyme extract (crude), which became the further KD activity measurement and analysis of the enzyme protein content. Measurements were taken for 4 weeks to determine the achievement of optimum conditions (Natsir et al, 2010).

### Chitin deacetylase activity test

KD enzyme activity is determined by the amount of 1 µmol of glucosamine residues that were released during 30 min of incubation within 50°C temperature. The mixture reaction was 600 mL (100 mL glycol chitin 1% + 300 mL phosphate buffer pH 7.0 + 200 mL enzyme solution), shaken and incubated at 50 ° C, for 30 minutes. Then added 500 mL of 33% acetic acid, 5% sodium nitrite, and left for 10 minutes at room temperature. Then added 500 mL of ammonium sulfamic 12.5% and incubated at room temperature for 30 minutes. Then added in 2000 mL of 0.5% HCl and 200 mL 1% indole, then boiled for 5 minutes in a water bath, then cooled. Then add 96% ethanol, then measured the absorbance at the maximum wavelength of spectrophotometer. The number of glucosamine residues released is determined based on a standard curve of pure glucosamine (Tokoyasu et al, 1996).

### Determination of protein content

The protein content was determined by the Lowry method, the solution is measured by spektronik 20D + at maximum wavelength. The protein content was determined by a standard curve using bovine serum albumin (BSA) (Sudarmadji et al, 1984 and Natsir et al, 2010)

## 2.3 Deacetylation chitin in to chitosan by enzymatically

Bioconversion optimization process of chitin into chitosan with KD was first performed , to determine the incubation time and the optimum substrate concentration . Incubation time is varied for 2 , 3 , 4 and 5 hours while the substrate concentration is varied with [ E ] : [ S ] ( mL / mg ) is 1 : 100 , 1 : 75 , 1 : 50, 1 : 25 . The optimization process were used in the process to obtain deacetylation chitin in to

chitosan. Chitosan was characterized with several standard parameters including water content and ash content by gravimetric method, totally Nitrogen with *Kjeldhal* method (AOAC, 1995) and deacetylated degree with FTIR. Quantitatively, the results of absorption measurements will be a reference to determine the value of the degree of deacetylation of chitosan using the base line. Calculations are based on the base line of the proposed method which combines the method of determining Baxter *baseline* absorption band in the ratio  $A_{1655}/A_{2867}$  and  $A_{1655}/A_{3450}$  (Purwatiningsih, 2009)

#### 2.4. Application of Chitosan As Antimicrobial Agent In Fishballs.

The testing of antimicrobial chitosan on fishballs was done with the total plate count method (TPC). Chitosan was dissolved in 2% acetic acid with concentration variation of 0.5%, 1%, 1.5%, 2% and 2.5%. Then, fishballs sample were soaked in a solution of chitosan for 10 minutes. Used to control acetic acid 2% and fishballs without treatment. Chitosan antimicrobial activity against microbial contamination in fish balls is calculated based on the number of colonies that grow from each fishballs sampling results every 3 hours during a 24 hour shelf life. The number of colonies that can be counted is a petri dish of bacteria colonies between 30-300 colonies (Fardiaz, 1989).

## RESULTS AND DISCUSSION

### Isolation of Chitin

In the isolation process of chitin from white shrimp waste (*P. merguensis*) there was a decrease in weight for each stage of isolation. For the demineralization we obtained a weight of 47.51 grams rendamen 47.51 % of initial weight, decolorization of 39.08 grams with rendamen 39.08 %. For deproteinasi, it's enzymatically derived enzyme concentration and incubation time on the optimum concentration [S] : [E] = 1:10 (w/v) and incubated for 2 hours at a temperature 50°C. Enzymatic chitin was obtained from the deproteinasi of 19.38 % with 19.38 % of the weight rendamen early. Characteristics of chitin enzymatic were obtained water content 2.14%, ash content 1.05 %, N - Total 6.24%.

For the deacetylated degree of chitin obtained from the deproteinasi analyzed by FTIR spectrophotometer to determine the major functional groups contained in chitin and to determine the degree of deacetylated of chitin. According to Protan laboratories chitin that have good quality standards it is expected to have a deacetylated degree in the range of 15-70 %. This is consistent with the research results obtained, in which chitin isolated from white shrimp shell waste (*P. merguensis*) has a 55.96 % deacetylated degree.

In the FTIR spectrum (Figure 2) a peak can be seen in the region 3000-3500  $\text{cm}^{-1}$  indicating the presence of OH and  $\text{NH}_2$  groups. Other aliphatic C-H

peak is at 2931  $\text{cm}^{-1}$ , CO amide contained in 1654  $\text{cm}^{-1}$  (Amide I), C-N-H vibration (Amide II) at 1558  $\text{cm}^{-1}$ , C-N stretch at 1315  $\text{cm}^{-1}$ , C-O-C vibration in the cyclic at 1205  $\text{cm}^{-1}$ , the vibrations of the C-O-C stretch dialkyl at 1157  $\text{cm}^{-1}$ ,  $\text{CH}_3$  symmetric deformation at 1379  $\text{cm}^{-1}$ , C-OH vibration at 1074  $\text{cm}^{-1}$ . Based on the analysis of multiple parameters it can be concluded that chitin obtained from the insulation has met the standards that have been set.

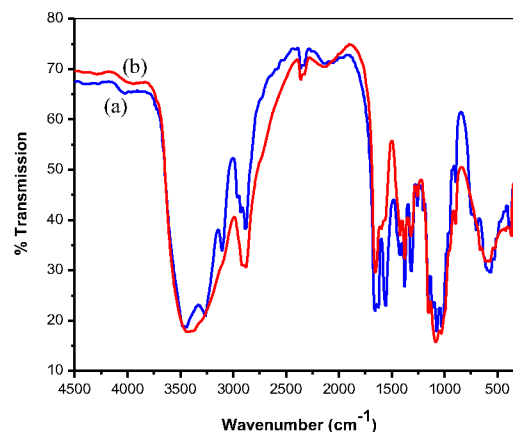


Figure 1. The FTIR spectrum of (a) Chitin, (b) Chitosan

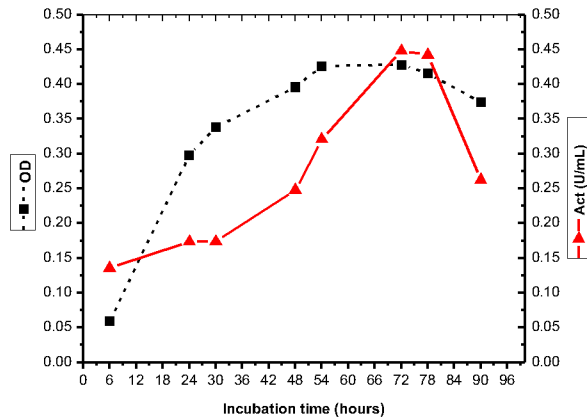
### Chitin deacetylase enzyme production

*B. licheniformis* HSA3-1a is grown in fermentation medium further to determine the optimal conditions of production, because the growth of microbial fermentation medium and the resulting product can be monitored in accordance with either optimal conditions. The growth of *B. licheniformis* HSA3-1a in the fermentation medium increased in a time interval increment. From the results of sampling performed every 6 and 18 hour intervals for 4 days, improvement continued to occur until day 3 and began to decline on day 4.

Figure 2 shows that the optimal growth of each microbial sampling interval is the incubation time of 72 hours (3 days) with value decreasing to 0.428 OD in the sampling for 78 and 80 hours (4 days). It is caused by the accumulation of toxic materials, limited nutrients and so many dead cells. The number of dead cells increases exponentially or the inverse of the logarithmic phase of growth. In this phase, the living cells can only survive for a while, a very long generation time, or even none at all. Besides, the cells will be destroyed by the effect of the enzyme itself (autolysis), here in after microbial cells are totally dead (Ali, 2005)

In line with the growth curve (OD) measurement, enzyme activity measurements were done anyway. However, the first cold centrifugation processed against bacterial suspension at 4 °C with a speed of 3600 rpm for 15 minutes. It aims to precipitate the cell so that the enzyme can obtain filtrate, besides centrifugation at cold temperatures to

avoid the heat denaturation process due to the loss of which could have an impact on the ability of the enzyme to catalyze. The measurement results showed that the optimum enzyme activity occurred on day 3 and decreased on day 4 (Figure 2)



**Figure 2. Chitin deacetylase activity, bacterial growth (OD) and KD protein content of the incubation time**

Figure 2 shows that the optimum value reached KD activity on incubation time 72 hours (3 days) and equal to  $0.0807 \times 10^{-3}$  U/mL on 4 days of incubation for 78 and 90 hours with decreasing enzyme activity, that is  $0.0796 \times 10^{-3}$  and  $0.0473 \times 10^{-3}$  U/mL. Enzymes that degrade chitin, chitin deacetylase that is produced after growth reached the stationary phase, on 3 days (72 hours) time of fermentation, in which the phase of chitin deacetylase activity was optimum. This is due to the occurrence of certain conditions in the medium, cell density conditions and availability of nutrients in the medium decrease. Reduced nutrients in the media causes chitin deacetylase enzymes to be excreted by the bacteria out in large numbers of cells to degrade chitin in the cell wall of the microbe and use it as an alternative substrate (Natsir, 2004).

#### Determination of protein content

Chitin deacetylase enzyme obtained from the cold centrifugation, is followed by protein content measurement of each sampling with incubation time variation in the production of fermentation media. The measurements results of the protein content of each coarse Extract (crude) enzyme sampling with fermentation time variations can be seen in Figure 2.

The protein content was determined by Lowry method. The protein contained in each fraction reacts with Lowry reagent molecular components to form a blue colored solution. The protein content was calculated based on absorbance values that are proportional to the protein content of maximum wavelength (640 nm) against a standard curve of BSA.

The protein content of the chitin deacetylase from *B. Licheniformis* HSA3-1a on growth (OD) was obtained optimally in 0.0616 mg/mL, the activity of

KD  $0.0807 \times 10^{-3}$  U/mL, and the specific activity of the KD is 0.0013 U/mg. Chitin deacetylase enzymes produced in this study is an crude extract (crude) enzyme which have not been fractionated with ammonium sulfate, so the chitin deacetylase activity obtained was small.

**Table 1. Protein content, activity and specific activity of KD from *B. licheniformis* HSA3-1a**

| Sample                     | Protein content (mg/mL) | Act of KD (U/mL)        | Spesific Act of KD (U/mg) |
|----------------------------|-------------------------|-------------------------|---------------------------|
| Chitin deacetylase (crude) | 0.0616                  | $0.0807 \times 10^{-3}$ | 0.0013                    |

#### Deacetylation Chitin in to Chitosan

Crude extract (crude) obtained from chitin deacetylase enzyme production by *B. licheniformis* HSA3-1a is used as an agent converting chitin obtained from white shrimp waste isolation (*P. merguensis*) into chitosan. The process of chitin deacetylase plays a role in catalyzing the acetyl groups contained in the N-asetil-D-glucosamine into poly(2-amido-2-deoxy- $\beta$ -(1,4)-D-glucopyranose). In the deacetylation process of chitin into chitosan enzymatically submarine was derived in optimum incubation time 2 h at 50 °C, while the optimum concentration of the substrate was at a concentration [E]:[S] of 1:100 (mL/mg). Chitosan is obtained by enzymatic 14.58 grams with rendamen 14.58 % of the initial weight.

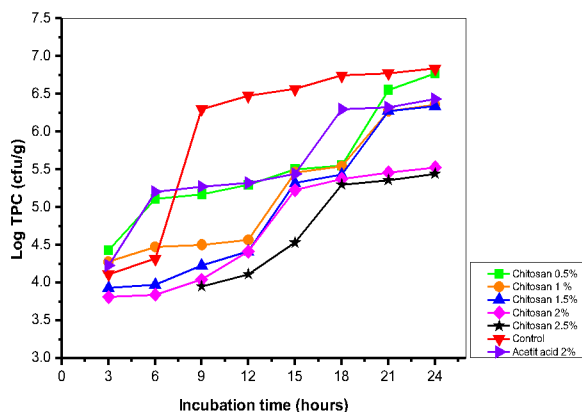
The result interpretation of functional groups of chitosan by FTIR (Figure 1) shows that in the 3000-3500  $\text{cm}^{-1}$  region there are peaks that indicate the presence of  $\text{NH}_2$  groups. Other aliphatic C-H peak at 2918  $\text{cm}^{-1}$ , CO amide contained in the 1651  $\text{cm}^{-1}$  (Amide I), C-N-H vibration (Amide II) at 1595  $\text{cm}^{-1}$ , C-N stretch at 1417  $\text{cm}^{-1}$ , C-H bend at 1377  $\text{cm}^{-1}$ , the cyclic C-O-C vibration 1251  $\text{cm}^{-1}$ , C-O-C stretch dialkyl vibration at 1153  $\text{cm}^{-1}$ .

Characteristics of chitosan were obtained by the water content 5.38%, ash content 0.83%, 7.51% N-total and 82.46% deacetylated degree while the standard used by Protan laboratories are that water content  $\leq 10\%$ , ash content  $< 2\%$ , 7-8% total-N and deacetylated degree  $> 70$ . Referring to the enzymatic chitosan that has met established standards, the high degree of deacetylation obtained indicate that the enzymatic deacetylation method has good effectiveness in degrading acetyl group attached to the chitin.

#### Application of Chitosan Enzymatic As Antimicrobial Agent In Fishballs.

Chitosan has the ability to remember some antibacterial properties, like its ability to inhibit the growth of microorganisms and their ability to provide a plating / coating of the product so as to minimize the

interaction between the products with the environment. The content of TPC in the fishball as one of the parameters to see the deterioration level of microbiological quality from the raw materials and appropriateness level for consumption. TPC analysis results (Figure 2) that an increase in the value of TPC in some samples is obtained for each time interval sampling conducted. The optimum concentrations of chitosan in inhibiting the growth of microbes on which fish balls is at a concentration of 2.5%. This is evident from the value of TPC is smaller if compared with other chitosan concentration.



**Figure 3. Chitosan concentration effect as an antimicrobial in fish balls by value Total Plate Count (TPC)**

At the 15th hour only sample with 2.5 % chitosan treatment concentration meets the standard TPC value that is equal to  $3.4 \times 10^4$  cfu/g, but after 15 hours all the samples had not met the standards for microbial contamination on the value of fish balls, where the standard contamination microbes by SNI is  $1 \times 10^5$  cfu/g. At the 18th hour the entire sample begins to show the value of which it exceeds the standard TPC. At the 24th hour TPC values to control fish balls are (without chitosan treatment), 2 % acetic acid, chitosan treatment concentration of 0.5 %, 1 %, 1.5 %, 2 % and 2.5 %, respectively, namely of  $6.9 \times 10^6$ ,  $2.7 \times 10^6$ ,  $6.0 \times 10^6$ ,  $2.3 \times 10^6$ ,  $2.2 \times 10^6$ ,  $3.4 \times 10^5$ ,  $2.8 \times 10^5$  cfu/g have passed the threshold for standard TPC predetermined value. Chitosan is a polycationic amine that will interact with the negative pole of the lining cells of bacteria. This is consistent with the statement of Chaiyakosha et al (2007) that the number reduction of bacterial cells is influenced by changes in the cell surface and loss of bacteria barrier function itself that affects inhibition of bacterial growth and death.

## CONCLUSION

Chitosan is obtained from the enzymatic isolation process gradually which are demineralization, decolorization, deproteination and deacetylation. Characteristics of chitin obtained the water content 2.14%, ash content 1.05%, N-Total 6.24% and 55.96% deacetylated degree. The process of chitin

deacetylation into chitosan by enzymes KD was carried out at a temperature of 50 °C with a ratio of [E]:[S] 1 : 100 (mL/mg) for 2 h incubation time. Characteristics of chitosan obtained the water content 5.38%, ash content 0.83%, N-Total 7.51% and 82.46% deacetylated degree. In the chitosan antimicrobial test results with the fishball TPC method optimum concentration of chitosan was obtained inhibiting the growth of bacteria at a concentration of 2.5% with a microbial contamination total of  $3.4 \times 10^4$  cfu/g during the shelf life of 15 hours.

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