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Biorecovery of chitin from shrimp shell waste *(Litopenaeus vanamme)* using fermentation and co-fermentation of *L.plantarum* and *B.thuringiensis*

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Abstract. *Litopenaeus vanamme* shrimp shell waste, which is abundantly available in Indonesia, could be optimally utilized through chitin extraction. The high-temperature chemical process is a fast and simple conventional method for extracting chitin. However, for economical and environmentally friendly process, it need a green extraction method through bacterial fermentation. Therefore in this research, biological process was carried out through *Litopenaeus vanamme* shrimp shell fermentation using various bacteria, such as *Lactobacillus plantarum* (A), *Bacillus thuringiensis* (B), and the combination of both bacteria (C) compared to chemical method (D). The yield of method A, B, C and D were: 80.67±0.43%; 71.25±0.45%; 63.91±1.20%; and 31.46±0.06% respectively. Co-fermentation method (method C) resulting in brighter color, higher deproteination and demineralization degree than single-fermentation methods (method A and B). The deproteination and demineralization degree of method A; B; C; D were: 28.51±0.69%, 17.41±0.89%; 47.71±1.01%, 15.08±0.36%; 50.29±1.14%, 34.33±1.04%; and 85.17±0.10%, 50.13 ± 0.72% respectively. Chitin produced from biological and chemical method had total nitrogen less than 7%. Based on the calculations through FTIR data, chitin from biological method resulted in higher degree of acetylation than chemical method. The higher the degree of acetylation obtained, the less the polymer is degraded which can preserve chitin structure.

1. Introduction

Indonesia is a maritime country with fishery products as the main commodities. Shrimp dominate the fishery product exports (1.423.844.000 US$ in 2017) [1]. The abundance amount of shrimp in Indonesia has an impact on the increasing amount of shrimp shell waste. That waste has negative impact on environment. However, we can explore the benefit of this waste because one of the main constituent of shrimp shell waste is chitin. Chitin is a superior substance for industry since it can be applied in various fields, especially for medical and environmental purposes. For environmental purposes, it can be used as an adsorbent for heavy metal removal. So far, the most widely used method of isolating chitin is through chemical process that involves the use of strong acids for mineral removal or demineralization, strong bases for protein removal or deproteination, and other concentrated chemicals (such as NaOCl and H_2O_2) for decolorization at high temperatures [2]. By using hazardous chemicals, the chemical extraction process has several drawbacks, such as environmental problems, increasing the cost of production, equipment maintenance, and liquid waste's handling. Chemical extraction process also cause hydrolysis in chitin polymers, which will affect the physiological properties of chitin, resulting an easily

degraded chitin [3]. Biological methods using protease and lactic acid enzymes can produce better quality chitin with environmentally friendly process. However, it increases production cost and has a low success rate of extraction. Therefore, the biological method through fermentation is cost saving and environmentally friendly method. Proteolytic bacteria such as *Bacillus thuringiensis* produces protease enzymes, which degrade protein in shrimp shell waste [4]. As for mineral removal process, the use of lactic acid bacteria such as *Lactobacillus* species was applied [5].

In this research, three methods of bacteria adding for bio-chitin extraction were carried out, i.e: lactic acid fermentation using *L.plantarum*, non-lactic acid fermentation using *B.thuringiensis*, and co-fermentation using *L.plantarum* and *B.thuringiensis*. Those two bacteria are easily isolated from natural materials, and have the ability to produce bacteriocins, which inhibit the growth of pathogenic microorganisms [6]. The results of chitin that obtained from three biological methods were compared with chitin obtained from chemical method.

2. Materials

All chemical reagents were analytical grade (Sigma-Aldrich Co.Ltd.) and used with no further purification. The main material used in this research was shrimp shell waste that obtained from PT. Sekar Katokichi, Sidoarjo which was stored in the ice box during the trip, then washed and dried at 102^0C for 12 hours, then grinded with a blender and sifted to 80 mesh. The microorganisms used was *Lactobacillus plantarum* bacteria that was obtained from the Biology Department, Brawijaya University, isolated from intestinal of razor clam (*Solen spp*) and *Bacillus thuringiensis* which isolated from silkworm cocoons, obtained from the Soil Department, Faculty of Agriculture, Brawijaya University. The growth media used were MRSB (de Man, Rogosa and Sharpe Broth) for *L.plantarum* and NB (Nutrient Broth) for *B.thuringiensis*. Each growth medium was added with shrimp head extract shells (SHES) (10% v/v). SHES was obtained by boiling shrimp head (50% w / v) in distilled water for 30 minutes, then filtered to obtain a yellowish SHES solution.

3. Experimental Methods

The extraction of chitin from *L.vanamme* shrimp shell was conducted on four different methods: method A (3.2.1) : single fermentation with L.plantarum, method B (3.2.2): single fermentation with *B.thuringiensis*, method C (3.2.3) : co-fermentation with *L.plantarum*, continue with *B.thuringiensis*, and method D (3.1): chemical method.

3.1. Chemically chitin extraction

Eighty mesh of shrimp shell powder was heated in 4% NaOH (1:10 w / v) for 1 hour, then washed with distilled water and weak acid to gain neutral pH. The solution was filtered and the precipitate was dried in an oven at 60 ^{0}C. Dried precipitate was heated in 1 M HCl solution (1:10 w / v), then washed with distilled water and weak base to gain neutral pH. After filtering with fine filter paper, the precipitate was added with 4% NaOCl (1:10 w / v) for 1 hour at 80 ^{0}C and then filtered. The precipitate then heated in oven at 60 ^{0}C for 24 hours.

3.2. Biological chitin extraction:

3.2.1. Method A: Fermentation with L.plantarum. Eighty mesh of shrimp shell powder was fermented using 15 hours *L. plantarum* inoculum co-culture (according to the bacterial growth curve), with ratio of inoculum to the substrate was 1:1 (w/v). In each inoculum 10% glucose 1 M and 1% NaCl 1 M were added. Fermentation was held for 30 hours in the shaker incubator at 37 ^{0}C and 80 rpm. The fermentation results washed by hidrobat then filtered and the precipitate was dried in an oven at 80 ^{0}C for 24 hours

3.2.2. Method B: Fermentation with B.thuringiensis. Eighty mesh of shrimp shell powder was fermented for 72 hours at 37 ^{0}C using 30-hours *B.thuringiensis* co-culture (according to the bacterial growth curve). Inoculum was added with 10% glucose 1 M and 1% NaCl 1 M. Fermentation was held

for 72 hours in the shaker incubator at 37 ^{0}C and 200 rpm. The fermentation results washed by hidrobat then filtered and the precipitate was dried in an oven at 80 ^{0}C for 24 hours.

3.2.3. Method C: Co-Fermentation with L.plantarum and B.Thuringiensis. Eighty mesh of shrimp shell powder was fermented using 15 hours *L. plantarum* inoculum co-culture, with ratio of inoculum to the substrate was 1:1 (w/v). Inoculum was added with 10% glucose 1 M and 1% NaCl 1 M. Fermentation was held for 30 hours in the shaker incubator at 37 ^{0}C and 80 rpm. The fermentation results washed by hidrobat, then filtered and the precipitate was dried in an oven at 80 ^{0}C for 24 hours. The dried precipitate then mashed and sifted until 80 mesh and continued fermentation using 30-hours *B.thuringiensis* co-culture which added with 10% glucose 1 M and 1% NaCl 1M. The fermentation results washed by hidrobat then filtered and the precipitate was dried in an oven at 80 ^{0}C for 24 hours.

3.3. Characterization of Biochitin and Chemically Extracted Chitin

3.3.1. Water Content Analysis. Chitin samples were weighed as much as 0.5 grams and put in a porcelain dish (whose empty weight was known), then weighed again. After that, it was heated at 105 ^{0}C for 2 hours, then cooled in a desiccator for 30 minutes and weighed again. This treatment was carried out until the weight was constant. Water content can be calculated by the following formula [7]:

$$\%Water\ content = \frac{(B1-B2)}{B1}\ x\ 100\% \quad (1)$$

notes: B1 = initial mass of chitin (g), B2 = final mass after dried (g)

3.3.2. % Total Nitrogen Content Analysis. Analysis of total nitrogen content in shrimp shell powder and chitin was carried out through the Kjeldahl method [8]. The calculations for % nitrogen take into account which type of receiving solution was used and any dilution factors used during the distillation process. In the equations below, "N" represents normality. "mL blank" refers to the millilitres of base needed to back titrate a reagent blank with standard acid as the receiving solution [9].

$$\%\ Nitrogen = \frac{(ml\ standard\ acid - mL\ blank)\ x\ N\ of\ acid\ x\ 1.4007}{weight\ of\ sample\ in\ grams} \quad (2)$$

3.3.3. Deproteination. Analysis of protein content in shrimp shell powder and chitin was carried out through the Kjeldahl method [8], with the same calculation as % total nitrogen content multiplied by 6.25 for the protein-nitrogen conversion factor for chitin. The degree of deproteination was determined by calculating the initial nitrogen content of shrimp waste and the nitrogen content of shrimp waste after fermentation process. The efficiency of deproteination (% DP) was determined by the following equation [10]:

$$\%\ DP = \frac{[(PO\ x\ O) - (PR\ x\ R)]}{(PO\ x\ O)}\ x\ 100\% \quad (3)$$

PO and PR are protein concentrations before and after biorecovery, O and R are the initial shrimp shell mass and chitin mass (g) respectively.

3.3.4. Demineralization. Analysis of mineral content on shrimp shell powder and chitin were carried out through the method of ignition [7]. Chitin samples obtained from the isolation were weighed 0.5 grams and put in a porcelain dish (which had known the empty weight) then weighed. After that, the sample was released in furnace up to 500 ^{0}C for 45 minutes. From 500 ^{0}C it was raised to 900 ^{0}C for 60 minutes. Then, it was inserted in the desiccator to be cooled to room temperature and weighed. The equation (4) can be used to calculate the ash content.

$$\%Ash\ content = \frac{(m2-m1)}{m}\ x\ 100\% \quad (4)$$

notes : m is sample weight (g), m1 is porcelain dish weight, m2 = m+m1 after furnace.
Demineralization efficiency (% DM) was determined through the equation [10]:

$$\%\ DM = \frac{[(AO\ x\ O) - (AR\ x\ R)]}{(AO\ x\ O)}\ x\ 100\% \quad (5)$$

where AO and AR are concentrations of ash from the sample before and after biorecovery process respectively, O and R are the initial shrimp shell mass and chitin mass (g) respectively.

3.3.5. Degree of Acetylation. Analysis of functional groups contained in chitin compounds was determined through FT-IR analysis at 4000 to 400 cm^{-1} using KBr pellets. The degree of acetylation (DA) was calculated from the ratio of absorbance according to the equation [11]:

$$\%\ DA = \frac{A1650}{A3450}\ x\ 100\ /\ 1.33 \quad (6)$$

whereas A1650 is the absorbance at 1655 cm^{-1} of the amide band as a measure of the N-acetyl group content, A3450 is the absorbance at 3450 cm^{-1} of the hydroxyl band as an internal standard, and 1.33 is a factor that represents the ratio of A1650/A3450 for fully N-acetylated chitin.

4. Result and Discussion

Figure 1. Chitin powder color comparison: A. Chitin from method A (single fermentation with *L.plantarum*); B. Chitin from method B (single fermentation with *B.thuringiensis*); C. Chitin from method C (co-fermentation with *L.plantarum*, continue with *B.thuringiensis*); D. Chitin from method D (chemically extracted chitin).

Based on the research, several chitin (Figure 1) were obtained from biological and chemical method. Chitin that was obtained from co-fermentation and chemical method have brighter color than single fermentation method. Bright color indicates more mineral removal in shrimp shell waste which increases the demineralization percentage, resulted in table 1. Demineralization efficiency was obtained through equation (6) with ash content calculation from ignition method as in equation (5). The demineralization and deproteination efficiency in the co-fermentation method was more optimal than single fermentation method because there were two step of gram-positive bacillus bacteria fermentation which can optimize the enzyme production. Lactic acid enzyme which resulted from *L. plantarum* fermentation, which can react with Ca mineral contained in shrimp shell to form calcium lactate according to figure 2. Calcium lactate deposit can dissolved in water so it can be removed from shrimp shell through washing using hidrobat. According to the table 1, single fermentation using *B. thuringiensis* (method B) also involved in demineralization process. It is because *Bacillus* belongs to the group of *Microbially induced calcium carbonate precipitation* (MICCP) bacteria which has the ability to induce the deposition of calcium carbonate contained in shrimp shell.

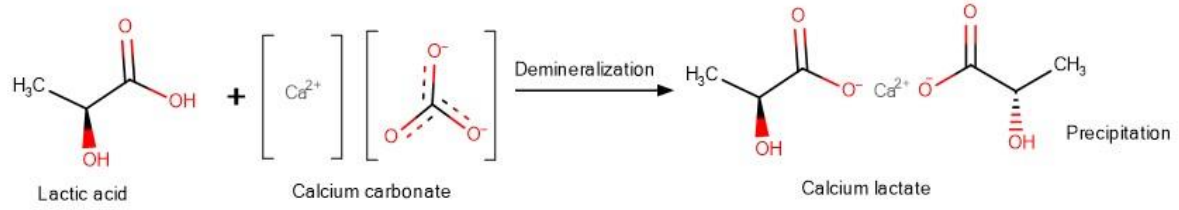

Figure 2. Demineralization in shrimp shell by fermentation with *L.plantarum* [12].

Table 1. Efficiency of deproteination and demineralization of biological and chemical method.

Sample	%N Total	%Deproteination	%Demineralization
Method A	6.53 ± 0.06%	28.51 ± 0.69%	17.41 ± 0.89%
Method B	5.41 ± 0.10 %	47.71 ± 1.01 %	15.08 ± 0.36%
Method C	5.74 ± 0.19 %	50.29 ± 1.14%	34.33 ± 1.04%
Method D	3.45 ± 0.02%	85.17 ± 0.10%	50.13 ± 0.72%

Table 2. % yield and water content of chitin from biological and chemical method.

Sample	% Yield	%Water Content
Method A	80.67 ± 0.43%	6.39 ± 0.95%
Method B	71.25 ± 0.45%	5.07 ± 0.43%
Method C	63.91 ± 1.20%	4.26 ± 0.64%
Method D	31.46 ± 0.06%	6.75 ± 0.32%

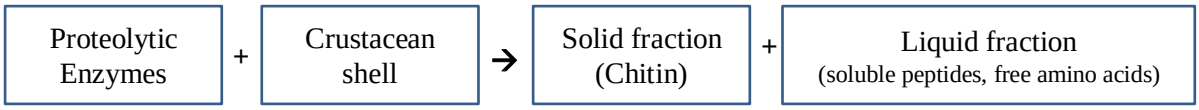

Figure 3. Deproteination in crustacean shell by fermentation [12].

Deproteination efficiency can be obtained through equation (3). As shown in table 1, deproteination efficiency in method B was higher than method A because *Bacillus thuringiensis* as a proteolytic bacteria produce a protease enzyme, which help protein catabolism by hydrolysis of peptide bond for deproteination process (according to Figure 3). Single fermentation with *Lactobacillus plantarum* also involved in deproteination process, because *Lactobacillus* is a probiotic bacteria that also has proteolytic activity, but not as much as *Bacillus* species. On fermentation, *Lactobacillus* has main role to turn sugars into lactic acid, so it could not optimally hydrolysis the peptide bond from protein media. Both bacteria can produce bacteriosin which can kill pathogenic microorganisms that contained in shrimp shell waste.

According to table 1 using equation (2), total nitrogen content of chitin from all methods were less than 7%, suggesting the good quality chitin that is similar with reference [5]. This is also supported by percentage of water content as in table 2 using equation (1), chitin from all methods had water content less than 10% and insoluble in water.

The efficiency of deproteination and demineralization of biological methods were smaller than chemical methods. It can be caused by several factors, including pH conditions for *L.plantarum* growth and the additional of shrimp head extract shell (SHES) for growing media. In this study, the media that used for *L.plantarum* growth had neutral pH (pH 7), so that *L.plantarum* could not grow optimally. The optimal condition of *L.plantarum* growth is in the range of pH 5.3 - 5.6. The additional SHES on MRSB and NB media also increase the mineral (Ca) and protein levels in shrimp shell, so that the limited

number of *L.plantarum* bacteria needs extra effort to produce more lactic acid, which will react with calcium minerals for demineralization process.

Based on the data in table 2, biological methods have higher yield and degree of acetylation than chemical method. Method A (single fermentation with *L.plantarum*) shows the highest yield compared to another method since it has lowest degree of deproteination and low degree of demineralization, so it still contain higher protein and mineral in the chitin than another method. In contrast, method D (chemical method) shows the lowest yield compared to another method since it has highest degree of deproteination and demineralization.

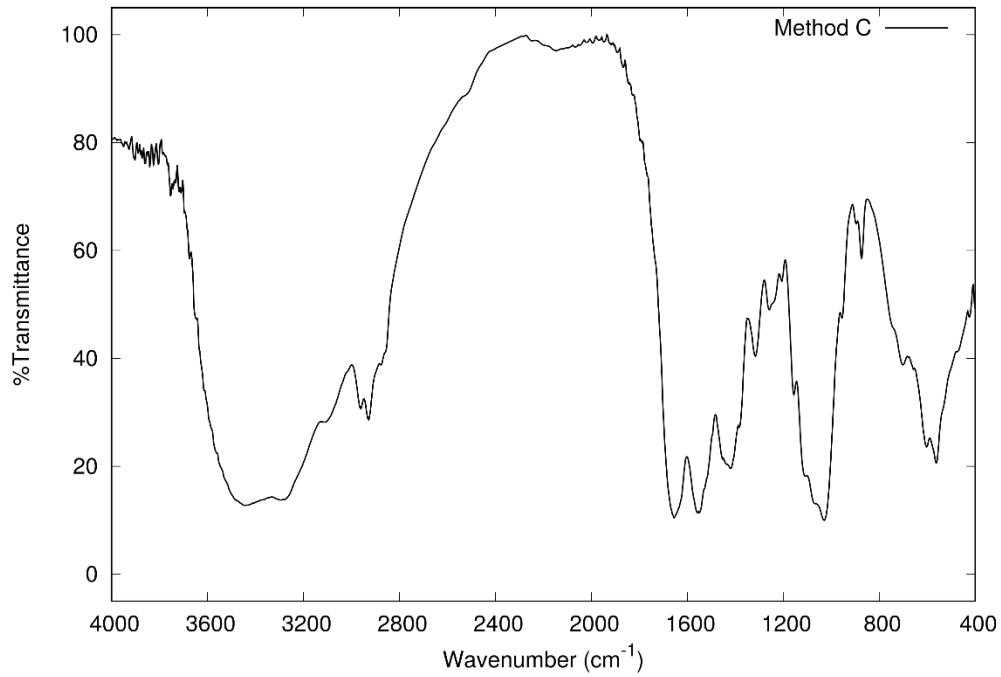

Figure 4. FT-IR Spectra from method C (co-fermentation method)

Based on the figure 4 and 5, it can be conclude that bio-chitin and chemical chitin that extracted from *L.vanamme* have similar absorption patterns with reference (Mudasir, 2008), indicating that good quality of chitin had been obtained. Strong peak at 3.389 - 3.451 cm^{-1} indicates the stretching vibration of O-H, also observed at 3.104 cm^{-1} and 3.121 cm^{-1} each belongs to asymmetric and symmetric stretching vibration of N-H group from acetamide ($-NHCOCH_3$), respectively. Absorption peak at 2.840-2.940 cm^{-1} is from −C-H stretching vibration of $–CH_3$, which is supported by the existence of the absorption at 1.316 cm^{-1} for the bending vibration of $–CH_3$. Strong peak at 1.630-1.655 $–CH_3$ represents the stretching vibration of the carbonyl group, C=O from acetamide ($-NHCOCH_3$). Absorption at 1.156-1.157 cm^{-1} indicating the –C-O vibration of polysaccharide and absorption at 1.026-1.030 cm^{-1} is the stretching vibration for –C-O-C– of the glucosamine ring. Another characteristic absorption for chitin are at 1.559 cm^{-1} and 1.311-1.381 cm^{-1}, indicating the bending vibration of –NH and stretching vibration of –CN from acetamide group, respectively.

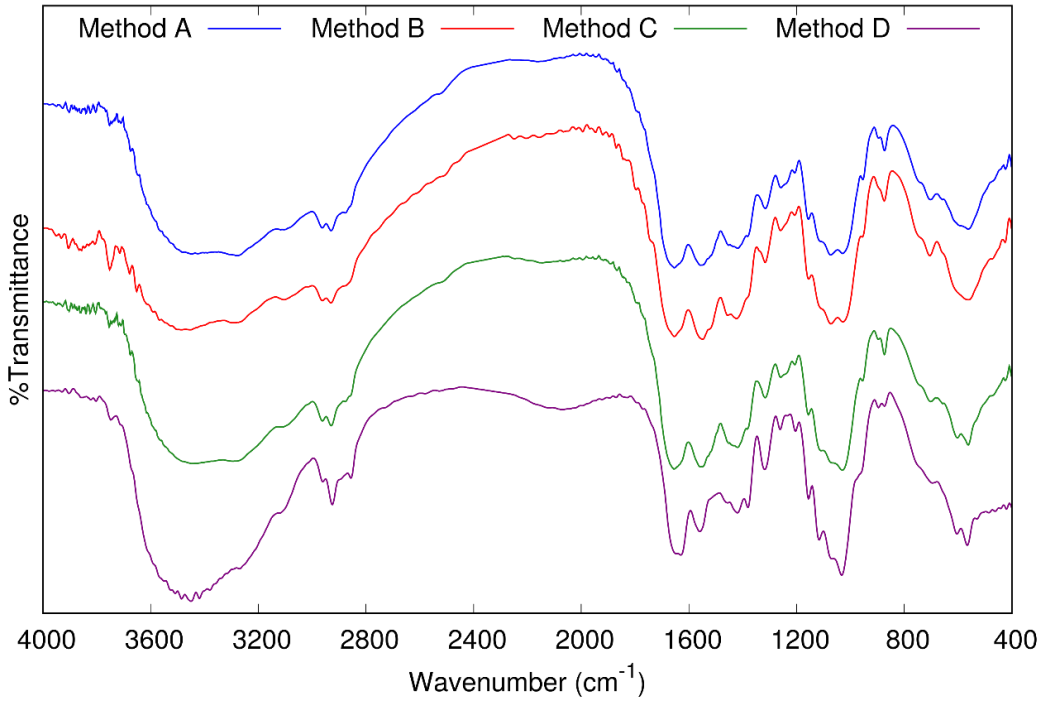

Figure 5. FT-IR Spectra from Bio-Chitin (red line: chemically extracted chitin, black line: chitin from method A, green line: chitin from method B, blue line: chitin from method C)

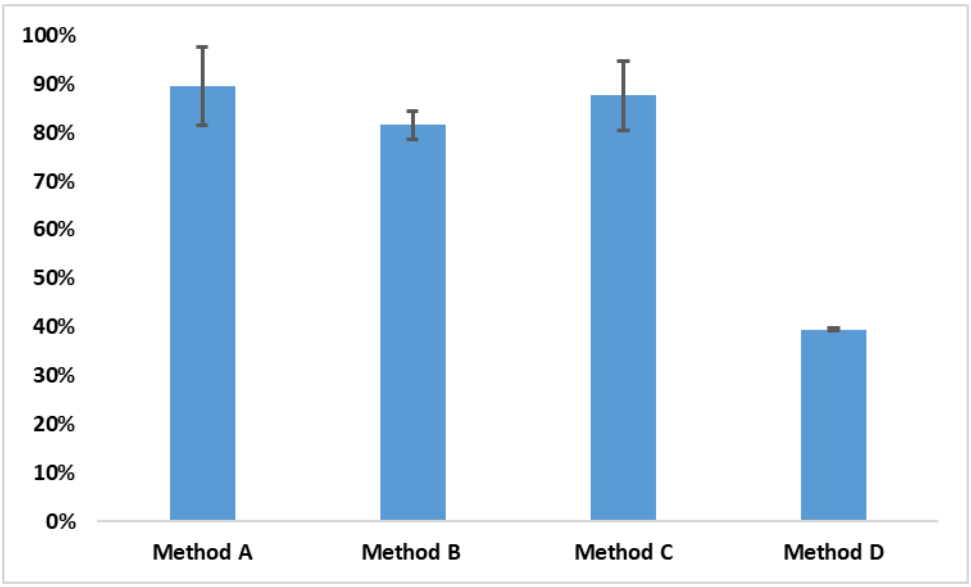

Figure 6. Degree of acetylation (%DA) from biological (method A, B, C) and chemically extracted chitin (method D).

Comparing the absorption value at wavenumber 1650 to 3450 cm^{-1} from FTIR spectra as in equation (6), the degree of acetylation was obtained (figure 6). Chemical extracted chitin has lower acetylation degree (39.47%±0.21%) than biological extracted chitin (86.22±6.1%). According to figure 6 and one way Anova test between biological methods (method A, B, and C), there is no real difference in %degree of acetylation since F value (0.834) < F critical table (9.55); otherwise there is real difference between

% degree of acetylation between chemical (method D) and biological methods (method A,B, and C) since F value (35.2) > F critical table (6.59) Degree of acetylation is determine as an indicator for chitin degradation. The higher the degree of acetylation obtained, the less the polymer is degraded. The chemical deproteination process can damages the bonds between chitin and proteins. It was causes the production of heterogeneous chitin and depolymerize that biopolymer. The use of strong acid and strong base, such as HCl and NaOH, can also cause chitin to partially deacetylate and hydrolyzed the biopolymer, which can reduce chitin molecular weight.

From the data, it can be concluded that biological process can preserves the structure of chitin. Therefore from environmentally friendly biological process, we get a better chitin structure than chemical method. The filtrate that produced from bacterial fermentation contains SHES that contains high levels of protein and minerals which is suitable for animal feed or human nutrition. In addition, fermented by-products filtrate which contain *L. plantarum* and *B. thuringiensis* bacteria can be recovered and reused in the next shrimp shell waste fermentation process, so it can decrease the production cost of chitin.

5. Conclusion

From this research, it confirmed that bio-chitin with good characteristic can be obtained from environmentally friendly bio-recovery method using fermentation method. The bio-chitin that obtained from co-fermentation process are similar with the properties of reference chitin, such as: bright color powder with total nitrogen content < 7% and water content <10%. The biological method has a higher percentage of chitin yield and degree of acetylation compared to chemical method. Co-fermentation method has a higher efficiency of deproteination and demineralization than the single-fermentation methods, but still lower than chemical methods. From this study, it can be concluded that bio-recovery method using co-fermentation can be an option for good quality, reproducible, economical, and environmentally friendly chitin production.

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