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The antioxidant and antibacterial activities of chitosan extract from white shrimp shell (*Penaeus indicus*) in the waters north of Brebes, Indonesia

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Abstract. *Kusnadi, Purgiyanti, Kumoro AK, Legowo AM. 2022. The antioxidant and antibacterial activities of chitosan extract from white shrimp shell (*Penaeus indicus*) in the waters north of Brebes, Indonesia. Biodiversitas 23: 1267-1272.* Chitosan is an oligosaccharide compound produced by deacetylation of basic chitin at high temperatures. This research aimed to extract chitosan from white shrimp (*Penaeus indicus*) in the waters north of Brebes, Indonesia and determine its antioxidant and antibacterial activity compared to commercial chitosan. The functional structure of chitosan was carried out through Fourier Transformation-Infrared Spectroscopy. Determination of antioxidant activity using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method and its antibacterial activity was tested on *Staphylococcus aureus* and *Escherichia coli* bacterial strains. The chitosan extract was also tested for its average molecular weight. The degree of deacetylation of chitosan extract was higher 78.60% than that of commercial chitosan 73.46%. The IC50 of chitosan extract and commercial chitosan on DPPH radicals was 4.25 mg/mL and 5.2 mg/mL, respectively. The chitosan extract produced largest inhibition zone found at 1000 g/mL on *S. aureus* (14.40 ± 0.03 mm) and *E. coli* (13.78 ± 0.01 mm). The result of DPPH radical scavenging activity was directly proportional to the value of the degree of deacetylation produced by the chitosan extract from white shrimp (*P. indicus*), the greater the degree of deacetylation, the greater the antioxidant and antibacterial activity.

Keywords: Antibacterial, antioxidant, chitosan, DPPH, *Penaeus indicus*

INTRODUCTION

Chitosan is a form of chitin deacetylation reported by researchers to have great benefits for the industry and researchers as a natural biopolymer with various uses and functional properties (Alishahi and Aïder 2012). Chitosan is a type of oligosaccharide compound produced by deacetylation of chitin base at high temperatures. In the chitin molecule, the -NH_2 amino group binds to acetyl bonds so that the more acetyl groups in chitin are released (deacetylated), the more chitosan molecules are obtained (Daraghmeh et al. 2011; El Knidri et al. 2018). The degree of deacetylation (DD) is the process of integrating acetyl in chitin. The deacetylation process is the most essential factor in defining the chitosan's quality. Chitosan has biocompatibility, biodegradability, and non-toxic properties (Sarode et al. 2019) and can be applied as a preservative supplement, edible formation, antioxidant, antimicrobial, and antitumor (Vimaladevi et al. 2015; Sabu et al. 2020). Chitosan is also utilized in food processing, pharmaceutical medicines, and wastewater remediation in the environment (Kumar et al. 2018; Chattopadhyay et al. 2019).

Indonesia is one of the exporters of processed fishery products. The Java Sea is one of the fishing ground for shrimps in Indonesia (Tirtadanu and Suprapto 2017). In North Waters of Brebes, Central Java is one of the areas that produce *Penaeid* shrimp of *P. indicus* species. In fact, Shrimp Auction Houses and processing units in Brebes leave shell waste around 40-50%, even higher than the total shrimp weight itself. Waste disposal can be caused environmental pollution. Chitosan is an economically useful by-product of shell waste. The products can be used to help solve environmental issues and waste management (El Knidri et al. 2018). The quality and characteristics of chitosan from shrimp waste *Litopenaeus vannamei* have been studied by Mohammed et al. (2013). *Penaeus monodon* (Srinivasan et al. 2018), *Metapenaeus stebbingi* (Kucukgulmez et al. 2011) have been previously reported. One of the features of chitosan that was greatly influenced by the deacetylation and chemical modification processes was performed molecular weight (de Alvarenga et al. 2010).

Many studies have linked chitosan extract to antioxidant activity, including chitosan from shrimp shell *L. vannamei* (Arancibia et al. 2014), *Parapenaeus longirostris* (Hafsa et al. 2016), and crab shell (Fernando et al. 2016). Hafsa et al. (2016) also reported a relationship between the degree of deacetylation and antioxidant activity. The results of the degree of deacetylation (DD) of chitosan from *P. longirostris* shrimp shell were 73.68% and 83.55% with antioxidant activity of 11.45% to 21.25% and 18.27% to 44.17%. Likewise, research on chitosan on antibacterial activity (Benhabiles et al. 2012; Chang et al.

2019). Antibacterial test of chitosan extract from *Auricularia* sp. by Chang et al. (2019) showed that the extract produced a higher zone of inhibition against *E. coli* bacteria of 40.83 mm and 88 mm at DD 86.81% and 83.66%. As the very little number of scientific research has been published concerning chitosan extract from white shrimp (*P. indicus*) shells. Therefore, this study aimed to investigate the antioxidant and antibacterial activity of white shrimp shells and compare it with commercial chitosan and its molecular weight characteristics.

MATERIALS AND METHODS

Raw material

Samples of white shrimp (*P. indicus*) were obtained from Fish Auction Houses Brebes, Central Java, Indonesia. The reagents and chemicals used in this study were supplied by Brataco Chemika, Indonesia. In addition, the study was equipped with a spectrophotometer such as magnetic stirrer, 50 mesh sieve, oven, desiccator, and balance (analytic Ohaus NV2201) which were provided by the Laboratory of Microbiology and Pharmacy, at the Department of Pharmacy Pharmacy, Polytechnic Harapan Bersama.

Preparation of chitin

The dried shells were then mashed with a mortar and sieved to obtain a size of 50 mesh. These shell samples were then treated using the procedure of (Younes et al. 2014a) with slight modification. Deproteination was accomplished by placing shrimp shell waste in an extractor and M NaOH solution with a solid: solvent ratio of 1:3 (w/v), at 80ºC for one hour long with uniform stirring to make it homogeneous. After cooling, it was filtered and washed with distilled water to result a neutral pH. Demineralization was carried out by mixing the resulting solid fraction of chitin and then adding 1 N HCl solution with a liquid to solid ratio of 4:1 (v/w) to remove $CaCO_3$ and other mineral contents. The sample was then filtered and rinsed with distilled water until the pH was neutral before being dried at 55ºC for 24 hours.

Deacetylation of chitin

The acetyl group from the chitin group was removed using a concentrated alkali application at a high temperature. The resulting chitin isolate was deacetylated using a 50% (w/w) NaOH solution (the ratio of solid and base was determined 1:10 w/v) then put into the extractor at a 100±3°C of 6 hours. To achieve a neutral pH (pH: 7.0), the reaction product was filtered and rinsed with distilled water. The solid was dried for 24 hours at 100°C in an oven, chilled in a desiccator, and weighed until it reached a constant weight. A quantitative method using infrared spectrophotometry was performed based on the transmittance value (%) or absorbance. The calculation of the degree of deacetylation (%DD) from the infrared spectra of chitosan can be done by calculating the ratio of the absorbance wave number of the amide-NH group (A_{1655}). While the absorbance wave number of the primary amine group (A_{3450}), with an absorbance value of 1.33 in the complete deacetylation process (El Knidri et al. 2018). The following formula was used to compute the DD%.

$$DD\%=100-\left[\left(\frac{A_{1655}}{A_{3450}}\right)X\frac{100}{1.33}\right]$$

Where, A_{1655}: the average % absorbance after and before wavenumber 1655; A_{3450}: the average % after and before wavenumber 3450.

Average molecular weight (Mw)

One of the assessment parameters used for chitosan quality standards was molecular weight. Determination of intrinsic viscosity was carried out by dissolving 0.5 M CH_3COOH and 0.5 M CH_3COONa buffer using a viscometer at 25°C. The Mark-Houwink equation can be used to calculate the average molecular weight of all chitosan samples (Trache et al. 2020). The average molecular weight was calculated using the formula below.

[μ] = [Mw]α K, with the values of α and K were constants, their values were DD x -1.02 x 10-2 + 1.82 and DD14 x 1.64 x 10-30 (mL/g), respectively. Mw = the average molecular weight.

Antioxidant activity of chitosan

Measurement of antioxidant activity of chitosan by the DPPH radical scavenging method (Wei et al. 2019). Prepare 1 mL chitosan sample solution in 0.2 percent acetic acid at various doses (2, 4, 6, 8, and 10 mg/mL), then mix with 3 mL of 95 percent ethanol solution containing DPPH radical. The mixture was continuously shaken until homogeneous, then left in the dark for 30 minutes. Spectrophotometry was used to calculate the solution's light absorption at a wavelength of 517 nm. As a control, a DPPH solution with no sample and no standard mark was used. Ascorbic acid was employed as a positive control. The percentage of free DPPH radical scavenging activity that was inhibited was determined. The antioxidant activity of chitosan was calculated using the formula below.

$$\%\ \text{Inhibition} = \frac{\text{Acontrol-Asampel}}{\text{Acontrol}} \times 100$$

Where, A control: (ethanol and DPPH) were used as absorbance control; A sample: the sample's absorbance.

The concentration of chitosan can provide an inhibition of 50% (IC_{50}) and can be calculated from the graph by the value of the chitosan concentration and the percentage of inhibition. The IC_{50} value was calculated graphically by linear regression using a line plotted on a curve. The following formula was used to compute the IC_{50} of chitosan.

$$Y = a * X + b$$
$$IC_{50} = \frac{(0.5 - b)}{a}$$

Where, Y: % inhibition; X: Concentration; a: Slope; b: Intercept (the intersection of the lines on the Y axis).

Antimicrobial activity of chitosan

A sample solution of 1000 ppm chitosan was prepared by dissolving 1 g of chitosan in 1000 mL of 1 percent (w/v)

glacial acetic acid solution to determine antibacterial activity. In the same way, a chitosan solution was prepared at a concentration of 250 ppm and 500 ppm. The disc diffusion method was used to test the antibacterial activity of chitosan samples (Vaz et al. 2018). The two bacterial strains used were *S. aureus* as gram-positive and *E. coli* as gram-negative. Nutrient agar (NA) solid medium was prepared in distilled water with a pH of 7. The media were autoclave sterilized for 15 minutes at 120°C before being allowed to cool to room temperature. chitosan sample A total of 1 mL with a concentration of 0.1% and 0.2% was mixed with 1 mL of nutrient broth (NB) liquid medium containing the test bacteria. Each was put into a test tube, then incubated at 37°C for 24 hours. 20 mL of NA medium was inoculated with 30 µL of fresh culture aged 24 hours in NB media, then shaken evenly and then poured into a petri dish and allowed to freeze. A total of 20 µL of chitosan samples were deposited into 6 mm paper discs, which were then placed in a petri dish with solid agar medium and cultured for 24 hours at 37°C. A capillary device was used to measure the clear zone surrounding the paper disc, which indicated the quantity of antibacterial activity (Octarya et al. 2021). For antibacterial activity, the test was carried out in three replications.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 22. In addition, analysis of variance (ANOVA) was employed in statistical data analysis to evaluate the level of significance. The least significant difference (LSD) test was applied to find the smallest significant difference between treatments.

RESULTS AND DISCUSSION

Degree of deacetylation (DD) of chitosan

The results of DD chitosan, in general, can be seen following treatment with concentrated NaOH followed by 30 minutes of heating at 100°C to remove all acetyl groups from macromolecules (Kumari et al. 2017). The DD value of chitosan extract in the FT-IR spectrum was 78.60% at 6 hours of deacetylation, while the DD value of commercial chitosan was 73.46%. Rasweefali et al. (2021) also carried out the deacetylation process of Shrimp *Solenocera hextii* for 6 hours with a chitosan deacetylation degree of 87%. According to (Hossain and Iqbal 2014) the degree of deacetylation is determined by the amount of NaOH present. The presence of acetyl groups in chitin is difficult to remove, so the addition of large amounts of NaOH is needed and the temperature is quite high as well (Srinivasan et al. 2018). The FT-IR spectra and the DD of chitosan extract from white shrimp (*P. indicus*) and commercial chitosan can be seen in Figure 1.

The spectrum of the chitosan sample shows the presence of intermolecular hydrogen -OH bonds stretching in the band around 3440 cm^{-1}, and the band about 2921 cm^{-1} representing the CH_2OH groups CH_2 stretch. The presence of C-O bond stretch vibrations containing a carbonyl group can be confirmed in the band range of 1059 cm^{-1} (Shavandi et al. 2015). Figure 1 displays the FTIR spectra of chitosan, which shows a distinct absorption at wavenumbers 1615 (chitosan extract) and 1614 (chitosan commercial), which indicates a deformation of NH_2 primary amines (NH from R-NH_2) and this proves that there may be a chitin deacetylation reaction (Abdel-Rahman et al. 2015). The absorption wavenumbers of 1419 cm^{-1} (chitosan extract) and 1422 cm^{-1} (chitosan commercial) were caused by the bending and distortion of CH_2. The bands of chitosan extract (1059 cm^{-1}) and chitosan commercial (1059 cm^{-1}), chitosan was characterized by C-O bond stretch vibrations. In all samples of chitosan in chitosan extract and commercial chitosan, there was a peak of about 1325 and 1326 cm^{-1}, which indicates the presence of clarifying C-H group in pyranose ring in the chitosan structure (Khan et al. 2013; Islam et al. 2014).

Average molecular weight (Mw)

Results showed that the molecular weight (Mw) of chitosan was 116.85 with a DD of 78.60%, while commercial chitosan was 214 with a DD of 73.46%. Rasweefali et al. (2021) observed that the value of molecular weight (Mw) of chitosan from deep-sea mud shrimp (*S. hextii*) was 263.95 with a DD of 75% and 52.61 with a DD of 83%. This shows that the amount of DD produced and the average molecular weight of the chitosan produced were proportionate. The average molecular weight is influenced by the extraction process, alkali concentration, DD, raw materials, chitin experiments, functional and physicochemical characteristics of chitosan. Lower molecular weight has the potential to be selected for antioxidant and antibacterial activity, while medium weight has anticancer activity (Younes et al. 2014b).

Antioxidant activity of chitosan

Figures 2 and 3 show the antioxidant activity of chitosan extract from white shrimp (*P. indicus*). DPPH radical scavenging activity was shown by the inhibitory activity value expressed as % inhibition. The DPPH method is an in vitro and in vivo method used to determine free radical scavenging activity (Kusnadi and Purgiyanti 2021). The antioxidant activity of certain compounds can be evaluated using the DPPH method and can evaluate and estimate in vivo the dose used (Wei et al. 2019). Based on the experiment, ascorbic acid as a control showed the highest antioxidant activity compared to commercial chitosan and extract. The results reported that the antioxidant activity of chitosan extract and commercial chitosan to scavenge DPPH radicals ranged from 18.80% to 42.27% with DD (78.60%) and 13.45% to 33.86% with DD (73.46%), respectively. Due to the presence of more amino compounds in its structure, chitosan that produces a higher amount of DD has the potential to have higher antioxidant activity. The DD value in this study showed a positive correlation with the results obtained by Hafsa et al. (2016) reported DD of 73.68% and 83.55% also produced antioxidant values of 21.25% and 44.17%, therefore, the higher the DD chitosan produced, the higher the antioxidant value.

Table 1. Antimicrobial activity of chitosan extract and commercial chitosan

Samples	DD (%)	Concentrations (ppm)	Inhibition Zone (mm)	
			Staphylococcus aureus	*Escherichia coli*
Chitosan extract	78.60	250	10.65 ± 1.10[d]	9.82 ± 3.13[d]
		500	12.83 ± 1.05[b]	11.46 ± 0.04[c]
		1000	14.40 ± 0.03[a]	13.78 ± 0.01[a]
Commercial chitosan	73.46	250	9.44 ± 1,23[e]	8.80 ± 1.15[e]
		500	11.28 ± 0.02[c]	10.94 ± 1.20[d]
		1000	13.62 ± 0.05[b]	12.85 ± 0.03[b]

Description: letters from the same alphabet indicate non-significant at $p < 0.05$. The results data was expressed as mean ± standard deviation of three replications

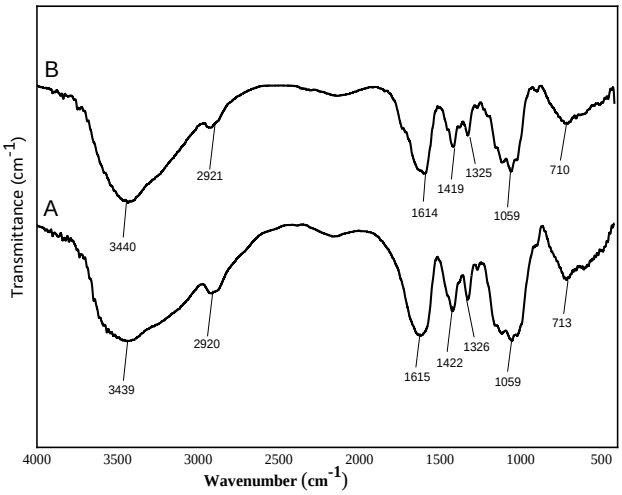


Figure 1. FT-IR spectrum of chitosan, (A) commercial chitosan with DD (73.46%); (B) chitosan extract with DD (78.60%)

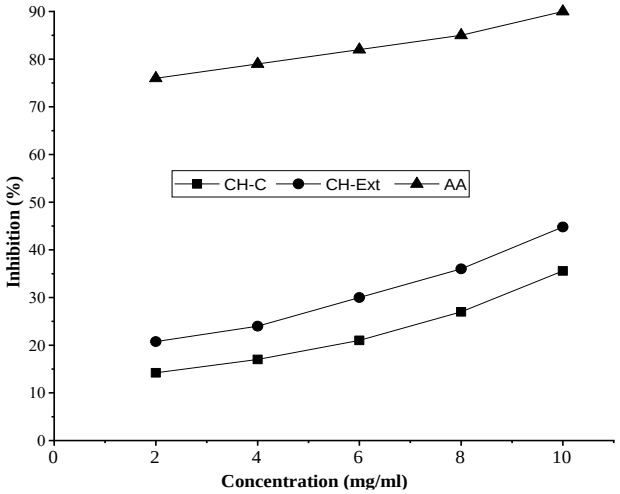


Figure 2. Antioxidant activity of CH-C: commercial chitosan; CH-Ext: chitosan extract; AA: ascorbic acid

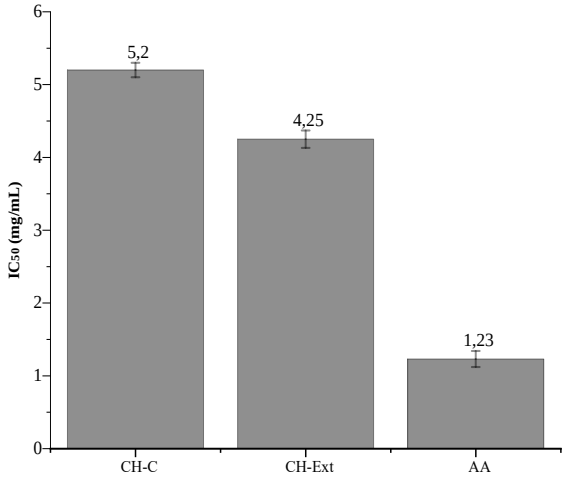
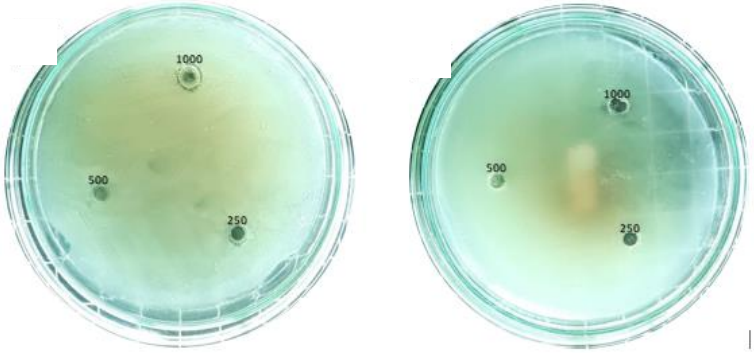


Figure 3. IC_{50} of CH-C: commercial chitosan; CH-Ext: chitosan extract; AA: ascorbic acid

Chitosan scavenging procedure can be described by the reaction of the activity of hydrogen atoms which react with superoxide and hydroxyl anion radicals which form more stable radical macromolecules. The maximum inhibitory concentration value was half (IC_{50}). Antioxidant activity can be correlated with IC_{50}. The smaller the IC_{50} value, the higher the antioxidant activity of the sample (Chlif et al. 2021). Based on the results of analysis, the scavenging ability of chitosan extract, commercial chitosan, and ascorbic acid on DPPH radicals had IC_{50} of 4.25 mg/mL, 5.2 mg/mL, and 1.45 mg/mL, respectively. Chitosan extract with lower molecular weight was predicted to be the most active radical scavenger with an IC_{50} of 4.25 mg/mL than commercial chitosan with a higher molecular weight with IC_{50} of 5.2 mg/mL.

Antibacterial activity of chitosan

Chitosan extract has been recognized for its usefulness for antibacterial activity on several types of microbes. One and is one of the most important is directly related to its application and biological mechanism. In this report, chitosan extract and commercial chitosan was investigated against two gram-positive bacteria (*S. aureus*) and one gram-negative bacterium (*E. coli*). The present study found that as the concentration of chitosan extract increased, the inhibitory activity of bacterial growth also increased. The results of antibacterial activity revealed that 1000 ppm/disc of chitosan extract showed largest inhibition zone of 14.40 mm in *S. aureus*, followed by 13.78 mm in *E. coli* (Table 1 and Figure 4).



A **B**

Figure 4. The inhibitory zone of chitosan extract against; A. *Staphylococcus aureus*; B. *Escherichia coli*

A **B**

Figure 5. The inhibitory zone of commercial chitosan against; A. *Staphylococcus aureus*; B. *Escherichia coli*

The inhibition zone of commercial chitosan at 1000 ppm/disk is shown in Table 1 and Figure 5 with the greatest inhibition zone of 13.62 mm in *S. aureus*, followed by 12.85 mm in *E. coli*. The results of the analysis showed that there were differences between the two concentrations of chitosan extract and commercial chitosan against three bacterial strains. Chitosan extract had higher antibacterial action and was able to produce greater antibacterial activity than commercial chitosan against *S. aureus*, and *E. coli*. This reveals that chitosan extract was more effective at suppressing both gram-positive and gram-negative bacteria than commercial chitosan. This was also because the DD of the chitosan extract (78.60%) was higher than DD yield of commercial chitosan (73.46%).

The level of deacetylation of chitosan is one of the factors that influence the antibacterial properties that lead to positive and negative membrane interactions (Chang et al. 2019). Chitosan with a higher DD value had a higher positive charge and could be suppressed by both gram-positive and gram-negative bacteria effectively. Regarding the antibacterial activity of chitosan, the results were from previous research reports presented by Chang et al. (2019) and Younes et al. (2014b), that the higher the DD, the higher the antibacterial power. Antibacterial activity studies against all gram-positive and gram-negative bacteria showed that lower molecular weight chitosan extract was much more effective (Benhabiles et al. 2012). Table 1 demonstrates that *S. aureus* bacteria was more susceptible to all chitosan extract than *E. coli* bacteria in terms of antibacterial activity. The effect of chitosan on the level of susceptibility of all types of bacteria was related to electrostatic interactions and cell membrane composition (Younes et al. 2014b). The natural cationic polymer content in chitosan could be bound with teichoic acid non-covalently in the peptidoglycan layer, which causes different functional disorders, resulting in cell death (Benhabiles et al. 2012). Chitosan, which contains polycationic, was regarded as the key factor in antibacterial activity. *S. aureus* had a cell wall that was not negatively charged so that it was not undergoing lysis (Arancibia et al. 2014). Gram-negative bacteria have a more complex cell wall than gram-positive bacteria. Chitosan's polycationic component could interact with gram-negative bacteria via electrostatic interactions with negative charges, causing permeability alterations (Chang et al. 2019).

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