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ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF HYDROLYSATE CHITOOLIGOSACHARIDES IN CRAB SHELL (*PORTUNUS PELAGICUS*) FROM DEGRADATION OF CHITOSANASE, A-AMYLASE, LIPASE AND CELLULASE ENZYMES

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ABSTRACT

The crab shell (*Portunus pelagicus*) has many benefits, one of the benefits is the used in the manufacture of chitooligosaccharides. The crab shell was chosen because it contains 20- 30% chitin inside. Chitin can be processed into chitosan and then hydrolyzed into chitooligosaccharides using enzymes. This study aims to determine the type of enzyme that is effective in the process of hydrolyzing chitooligosaccharides with various enzymes are the α-amylase, lipase, cellulase, chitosanase and a combination of enzymes are chitosanase α-amylase, chitosanase lipase, and cellulase chitosanase. The observation data were analyzed using ANOVA. The results showed that the best chitooligosaccharides obtained resulting from the hydrolysis of a combination of cellulase chitosanase enzymes which had the best characteristics, namely the highest degree of deacetylation of 94.95%, the lowest molecular weight of 3.54 KDa, the antioxidant activity of the DPPH method was 5.21 (µmol TE/g), the FRAP antioxidant method was 2.27 (µmol TE/g), the diameter of the inhibition area (DDH) was 7.30 mm in *Staphylococcus aureus* bacteria and 15.9 mm in *Salmonella typhosa* bacteria. From these results, it can be known that this chitooligosaccharide is more effective in inhibiting the growth. The Gram-positive bacteria was compared to gram-negative bacteria.

Keywords: chitooligosaccharide, antioxidant, antibacterial, enzyme combination

ABSTRAK

Cangkang rajungan (*Portunus pelagicus*) memiliki banyak manfaat salah satunya dalam pembuatan chitooligosakarida. Cangkang rajungan dipilih karena didalamnya mengandung sekitar 20-30% kitin. Kitin dapat diolah menjadi kitosan dan kemudian di hidrolisis menjadi chitooligosakarida. Penelitian ini bertujuan untuk mengetahui jenis enzim yang efektif dalam proses hidrolisis chitooligosakarida dengan berbagai jenis enzim yaitu enzim α-amylase, lipase, selulase, chitosanase dan kombinasi enzim diantaranya yaitu enzim chitosanase α-amylase, chitosanase lipase dan chitosanase selulase. Data hasil pengamatan dianalisis menggunakan *Analysis of Variance* (ANOVA). Hasil penelitian menunjukkan bahwa diperoleh chitooligosakarida terbaik yaitu chitooligosakarida hasil hidrolisis kombinasi enzim chitosanase selulase memiliki karakteristik yang paling baik yaitu derajat deasetilasi yang paling tinggi sebesar 94,95 %, berat molekul paling rendah 3,54 KDa, aktvitas antioksidan metode DPPH sebesar 5,21 (µmol TE/g), antioksidan metode FRAP sebesar 2,27 (µmol TE/g), nilai diameter daerah hambat (DDH) sebesar 7,30 mm pada bakteri *Staphylococcus aureus* dan 15,9 mm pada bakteri *Salmonella typhosa.* Dari hasil tersebut diketahui bahwa chitooligosakarida ini lebih efektif dalam menghambat pertumbuhan bakteri gram positif dibandingkan bakteri gram negatif.

Kata kunci: kitooligosakarida, antioksidan, antibakteri, kombinasi enzim

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INTRODUCTION

Indonesia is a maritime country that has 3.45 million km^2 ocean making the marine products very abundant, one of crustacean animal is the blue swimming crab (*Portunus pelagicus*). Blue swimming crabs have an important role in the Indonesian economy, especially in marine product export activities. In the January-February 2020 period, the export volume of Indonesian crab crabs reached 4,462 tons (BPS, 2020). However, currently, the crab is only used for meat, while the shells are simply thrown away. Even though the utilization of crab shell waste can provide added value because crab shells contain 30-40% protein, 30-50% minerals and 20-30% chitin (Amalia, 2018). One of the crab shell wastes can be made into Chitooligosaccharide (COS).

In the hydrolysis process of chitosan into chitooligosaccharides, there is a cleavage of the ß-(1,4) glycosidic chitosan bond chain, one of it using enzymatic techniques. Enzymatic hydrolysis is specific, controlled and environmentally friendly (Sarni et al, 2016). According to Abdel-Azi et al., (2014), the enzyme plays a role in specifically hydrolyze chitosan into chitooligosaccharides, the chitosanase enzyme. However, several enzymes are non-specific. Non-specific enzymes are relatively cheap and available for large-scale production, thereby reducing the cost of COS production. As for the non-specific nature of enzymatic cleavage, a series of COS products with various degrees of polymerization and high yields can be achieved by hydrolyzing chitosan through a combined enzyme system (Lin et al., 2009).

Therefore, for producing COS, this study uses treatments with different enzymes, including the enzymes chitosanase, lipase, cellulase, and α-amylase, where the combination of enzymes, namely chitosanase and lipase, chitosanase and cellulase as well as chitosanase and α-amylase to produce specific COS. This study aims to determine the type of enzyme that is effective in the process of hydrolyzing chitooligosaccharides with various enzymes and the effect of the type of enzyme and combination of enzymes (chitosanase, cellulase, lipase, and α-amylase) on the characteristics, antioxidant and antibacterial activity of chitooligosaccharide (COS) from crab shell chitosan.

MATERIALS AND METHOD

Materials

The material used in this manufacture of crab shells is crab shell obtained from PT. Kelola Mina Laut, Gresik. The enzymes are chitosanase used was imported by PT. Korean Genofocus of *Bacillus sp*. CAS no. 51570-20-8, Cellulase from novozymes viscozyme cassava CL, lipase from novozyme and α-amylase from sigma-aldrich. The chemicals used for analyzing are 2,2-diphenyl-1-picrylhydrazil (DPPH), methanol, distilled water, 96% ethanol solution, phosphate buffer, ascorbic acid, oxalic acid, TCA, K3Fe (CN)6, FeCl3, *Salmonella typhi*, *Staphylococcus aureus*, generic Amoxicillin (Kimia Farma), Nutrient Agar (Merck), Nutrient Broth (Merck), Blank paper disk (Oxoid), physiological NaCl, Aquades, Acetic Acid (Merck), pH Acetate Buffer 4.5 (Nitra Kimia).

Methods

Preparation of chitosan from crab shells (Butarbutar, 2018 Modified)

The process of isolating chitosan from crab shells was proceed chemically. First, the chitin isolation process is the demineralization process of the crab shells by adding 1.5L of 1.5M HCl into 100 g of shell powder and heating on the hotplate for 4 hours at 70^{o}C, and then the residue was filtered. Moreover, it was washed using distilled water until the pH was neutral. The residue obtained was baked in the oven for 3 hours at 70^{o}C. Furthermore, the deproteination process proceeded using 50g of the demineralized powder added with 0.5L of 3.5% NaOH, then heated for 4 hours at 70^{o}C, and then the residue was filtered and washed using distilled water until the pH was neutral. The residue obtained was baked in the oven for 3 hours at 70^{o}C to produce chitin. This process of deacetylation, namely 40 g of chitin plus 0.4L of 50% NaOH, was then heated for 4 hours. The temperature is maintained at 100^{o}C, then filtered, and the residue left behind is washed with distilled

water until the pH is neutral. The residue was baked in the oven for 3 hours at 70ºC, and dry chitosan was obtained.

Preparation of chitooligosaccharides from crab shells (Fawzya et al., 2009 Modified)

Preparation of 1% chitosan solution was carried out by dissolving chitosan into acetate buffer solution pH 4.5. Chitosan hydrolyzed with the various enzymes are chitosanase, cellulase, lipase, and α-amylase. The concentration each enzyme are α-amylase 1%, lipase 1%, cellulase 1%, chitosanase 1%, and hydrolyzed by combination enzymes are (1% chitosanase + 1% α-amylase), (1% chitosanase + 1% lipase) and (1% chitosanase + 1% cellulase) (w/w). Chitosan was incubated for 3 hours at 60ºC. The reaction was stopped by heating at 100ºC for 10 minutes. Then the sample was centrifuged at 9000 rpm to obtain a supernatant containing chitooligosaccharide. The reaction was stopped by heating at 100ºC for 10 minutes. Then the sample was centrifuged at 9000 rpm to obtain a supernatant containing chitooligosaccharide.

Physical analysis

The analysis used the Molecular Weight of the Mark-Khun Houwing method (Nasution, 2019) to determine the molecular weight of chitooligosaccharides, Degree of Deacetylation (Liu, et al., 2006) to determine the value of the number of the acetyl group or free amino groups after the deacetylation process. FTIR Functional Group (Muyonga, et al., 2004) to determine to identify functional groups in chitooligosaccharides, Antioxidant DPPH method (Munadiah, 2017) and antioxidant FRAP method (Munadiah, 2017) to determine the antioxidant activity of chitooligosaccharides, Antimicrobial disc diffusion method, Kirby-Bauer method (Nurhayati, 2020) to determine the antimicrobial activity through the zone of inhibition of bacteria.

Research design

The experimental design of crab shell chitooligosaccharide used a one-factor Completely Randomized Design (CRD), where using various enzymes with seven treatments such as 1% α-amylase, 1% lipase, 1% cellulase, 1% chitosanase, and also combination of enzymes such as (1% chitosanase + 1% α-amylase), (1% chitosanase + 1% lipase) and (1% chitosanase + 1% cellulase) (w/w) repeated three times, so there were 21 experimental units. The formulation in this design is determined based on preliminary research. The data obtained from the results of the analysis were processed using Analysis of Variance (ANOVA) and showed there were significant interactions and differences between each treatment. If there is a significant difference, a further test is carried out using the 5% DMRT (Duncan's Multiple Range Test) method. Data analysis used the help of the SPSS Statistics 17.0 for Windows program. From the data obtained, the best treatment was determined by looking at the results of each characteristic analysis performed.

RESULTS AND DISCUSSION

Molecular weight

Molecular weight is one of the essential parameters in chitooligosaccharides. The following is the table of the molecular weights of chitooligosaccharides hydrolyzed from various enzymes.

Table 1. The Molecular Weight of Chitooligosaccharides from Different Types of Enzymes

Sample	Molecular Weight (KDa)
COS α-amylase	$7.09^{a} \pm 0.09$
COS Lipase	$6.78^{b} \pm 0.06$
COS Selulase	$5.95^{c} \pm 0.11$
COS Chitosanase	$4.96^{d} \pm 0.04$
COS Chitosanase α-amylase	$4.73^{e} \pm 0.08$
COS Chitosanase lipase	$4.10^{f} \pm 0.06$
COS Chitosanase cellulase	$3.54^{g} \pm 0.07$

The data in **Table 1** shows that the chitooligosaccharides in this study have a molecular weight in the range of 7.09 KDa – 3.54 KDa. Chitooligosaccharides with the highest molecular weight were obtained from hydrolysis

with a combination of chitosanase and cellulase enzymes of 7.09 KDa. In comparison, the lowest molecular weight was obtained from hydrolysis with the α-amylase enzyme, which was 3.54 kDa. This is influenced by a specific enzyme that chitosanase. The chitosanase enzyme is only used for one reaction or the active part (the surface where the substrate is attached) is only attached to the surface of a particular substrate, which means that the specific enzyme. for chitosan hydrolysis is chitosanase. These specific enzymes can affect the hydrolysis process of breaking chitosan polymer bonds. In addition, the combination of enzymes makes the breaking of the glycosidic bond occur in two stages with a combination of specific and non-specific enzymes, it has an endo or Exo hydrolytic action so that the hydrolysis process can work optimally and affect the decrease in the resulting viscosity or molecular weight. According to the statement by Lodhi et al., (2014), chitosanase is one of the enzymes that hydrolyze chitosan to COS. According to Zhao (2019), this chitosanase has an end-type catalytic mode,endo-chitosanase often randomly breaks β-1,4-glycosidic bonds in the chitosan substrate to produce maximum chitooligosaccharides

This is supported by the statement of (Susilowati et al., 2015 in Nurhaeni et al., 2019) that the decrease in the molecular weight of chitosan after enzymatic hydrolysis occurs due to the breaking of bonds in the chitosan polymer chains to become shorter and the molecular weight of chitosan becomes lower. The combination of enzymes also influences the resulting molecular weight. According to Dong et al., (2015) that both chitosanase and cellulase enzymes are effective in hydrolyzing chitosan because their endo mode during the hydrolysis reaction process produces chitooligosaccharides. In addition, according to Je & Kim (2012), it happens because cellulase also has chitosanolitic activity in producing chitooligosaccharides. According to Poshina et al., (2020) that when compared to the amylase enzyme, the cellulase enzyme has the highest specific activity, which is shown by a rapid decrease in solution viscosity chitosan, which affects the resulting molecular weight due to the nature of this enzyme has endo and Exo capabilities that work synergistically.

Degree of deacetylation

The degree of deacetylation is also an essential parameter to determine the quality of chitooligosaccharides. According to Maidin (2017), deacetylation is the process of breaking the acetyl group from glucosamine. The degree of deacetylation indicates the number of acetyl groups that break off from the glucosamine group and the percentage number of amino groups in the polymer structure. The results of the degree of deacetylation in this study can be seen in the following table 2.

Table 2. Degree of Deacetylation of Chitooligosaccharides from Different Types of Enzymes

Sample	Degree of Deacetylation (%)
COS α-amylase	$81.78^{g} \pm 0.47$
COS Lipase	$85.01^{f} \pm 0.57$
COS Selulase	$87.90^{e} \pm 0.31$
COS Chitosanase	$90.54^{d} \pm 0.50$
COS Chitosanase α-amylase	$91.10^{cd} \pm 0.78$
COS Chitosanase lipase	$93.14^{b} \pm 0.23$
COS Chitosanase cellulase	$94.95^{a} \pm 0.77$

In **Table 2**, it can be seen that the degree of deacetylation of the chitooligosaccharides in this study ranged from 81.78% - 94.95%. Chitooligosaccharides with the highest deacetylation degree were obtained from hydrolysis with a combination of chitosanase and cellulase enzymes of 94.95%. In comparison, the lowest deacetylation degree obtained from hydrolysis with α- amylase enzyme is equal to 81.78%. The difference in the degree of deacetylation occurs due to the different types of enzymes because each enzyme has a special or specific ability to cut the existing acetyl groups. A study by Dong et al., (2015) stated that the enzymes chitosanase and cellulase were both effective in hydrolyzing chitosan because of their endo mode during the hydrolysis reaction process to produce chitooligosaccharides. The use of lipase enzymes is applied in the hydrolysis of chitooligosaccharides because the cutting mechanism can hydrolyze with endo-type and exo-

type. This is by the code (Lee, et al., 2008), namely lipase enzymes can hydrolyze chitosan into COS through exo- and endo-hydrolytic mechanisms.

Chitooligosaccharides that have a high degree of deacetylation is resulting from hydrolysis with a combination of chitosanase and cellulase enzymes. It is because the chitosanase enzyme can cut the acetyl group of glucosamine more effectively. After all, it cuts endo and Exo. The cellulase enzymes also have the characteristics of enzymes that can cut acetyl groups endo and Exo. So, they can optimally hydrolyze β-1.4 bonds and produce a large degree of deacetylation. According to Jiang et al., (2017), the degree of deacetylation (DD, %) is defined as the mole fraction of GlcN in the copolymer (chitosan), which is composed of GlcNAc and GlcN. It's also supported by Fouad's statement (2008) that the chitosanase enzyme is an enzyme that capable of endo-hydrolysis of β-1,4 bonds between GlcN (D-glucosamine) residues in the acetylation section of chitosan.

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According to Asha and Singh (2016), cellulase enzymes are enzymes glycoside hydrolase (GH) which utilize the hydrolysis acid-base catalytic mechanism. The first way, GHs with open active sites (grooves, gaps), which usually exhibit endo-cellulolysis (endo-cellulose), bind anywhere along the cellulose molecule and hydrolyze β-1,4 glycosidic bonds and other types exhibit exo-cellulolytic activity (cellobiohydrolases) which bind to the ends of the cellulose molecule and produce short chain oligosaccharides. Meanwhile, according to Liu & Xia (2006), cellulase has endo-type and exo-type activities, and the specificity of its cleavage includes GlcN-GlcNAc, GlcN-GlcN, and GlcNAc-GlcN bonds.

Antioxidant capacity DPPH method (2,2-diphenyl-1-picrylhydrazyl)

Testing the antioxidant capacity with the DPPH method is simple, fast, easy 2,2- diphenyl-1-pikrihydrazyl absorption method and uses a small amount of sample in a short time. The results of the antioxidant capacity of DPPH can be seen in the following table.

Table 3. The Antioxidant Capacity of Chitooligosaccharides from Different Types of Enzymes Using the DPPH Method.

Sample	Antioxidant Capacity (μmol TE/g)
COS α-amylase	$2.27^{g} \pm 0.05$
COS Lipase	$2.54^{f} \pm 0.06$
COS Selulase	$2.82^{e} \pm 0.04$
COS Chitosanase	$3.29^{d} \pm 0.08$
COS Chitosanase α-amylase	$3.34^{cd} \pm 0.07$
COS Chitosanase lipase	$3.72^{b} \pm 0.25$
COS Chitosanase cellulase	$5.21^{a} \pm 0.21$

Table 3 shows the average value of the antioxidant activity of DPPH resulting from the hydrolysis of chitooligosaccharides ranging from 2.27 to 5.21 μmol TE/g. In this study of antioxidant capacity using Trolox as standard, the unit of antioxidant capacity obtained was μmol TE/g. The lowest antioxidant capacity was obtained from the chitooligosaccharide treatment using the α-amylase enzyme, which was a 2.27 μmol TE/g sample. While, the highest antioxidant capacity was obtained from the chitooligosaccharide using a combination of chitosanase and cellulase enzymes, which was a 5.21 μmol TE/g sample. The greater the value of the antioxidant capacity of the sample indicates that more DPPH radicals are reduced. The

antioxidant capacity of the sample showed that the highest antioxidant capacity is chitooligosaccharide which used a combination of chitosanase and cellulase enzymes, which was a 5.21 µmol TE/g sample.

The difference in the antioxidant capacity produced is due to the different types of enzymes used. The highest antioxidant capacity of the chitooligosaccharides was produced by the combination of the enzymes chitosanase and cellulase. It's because the chitosanase enzyme can cut the acetyl group of glucosamine more effectively. After all, it cuts endo and Exo. The cellulase enzymes also have the characteristics of enzymes that can cut acetyl groups endo and Exo so that they can optimally hydrolyze β-1,4 bonds, catalyze glycosidic bonds in chitosan to produce glucosamine which has smaller monomers such as OH- (hydroxyl) groups) and amine groups (NH^+) become more numerous. The hydroxyl group and amine group play a role in the capture of free radicals. There are supported by Jung & Zhao (2012) that hydroxyl groups (OH-) and amino groups (NH^{2+}) contribute to the overall antioxidant capacity because they can react with unstable free radicals to form stable macromolecular radicals. According to the statement of Xie et al., (2001) that the mechanism from the binding of free radicals by chitosan. The OH^+ radical group from the lipid oxidation process can react with hydrogen ions from the ammonium ion group (NH^{3+}) in chitosan to produce a molecule that is more stable and produce antioxidant compounds.

Antioxidant capacity FRAP (Ferric Reducing Antioxidant Power) method

In this study, a synthetic antioxidant was used Trolox as a positive control. Trolox is similar to α-tocopherol and is used as a standard in the measurement of antioxidants. This test uses the FRAP (ferric reducing antioxidant power) method based on the ability of antioxidant compounds to reducing iron (III)-tripiridyl-triazine to iron (II)-tripiridyl triazine. This antioxidant test method used Fe $(TPTZ)_2^{3+}$ iron ligand complex 2,4,6-tripyridyl-triazine as a reagent. The blue Fe $(TPTZ)_2^{3+}$ complex will function as an oxidizing agent and will experience a reduction to yellow Fe $(TPTZ)_2^{2+}$. The results of the antioxidant capacity of FRAP are in the following table.

Table 4. The Antioxidant Capacity of Chitooligosaccharides from Different Types of Enzymes Using the FRAP Method.

Sample	Antioxidant Capacity (µmol TE/g)
COS α-amylase	$0.81^g \pm 0,03$
COS Lipase	$1.58^f \pm 0,05$
COS Selulase	$1.67^e \pm 0,01$
COS Chitosanase	$1.76^d \pm 0,03$
COS Chitosanase α-amylase	$1.89^c \pm 0,10$
COS Chitosanase lipase	$2.04^b \pm 0,03$
COS Chitosanase selulase	$2.27^a \pm 0,15$

Table 4 shows the average value of the antioxidant activity of FRAP resulting from the hydrolysis of chitooligosaccharides ranging from 0.81 to 2.27 µmol TE/g. The chitooligosaccharide treatment obtained the lowest yield using the α-amylase enzyme, which was 0.81 µmol TE/g sample. Moreover, the highest antioxidant capacity was obtained from the chitooligosaccharide treatment using a combination of chitosanase and cellulase enzymes, which is a 2.27 µmol TE/g sample. The results also show that different enzymes affect the antioxidant capacity produced. The combination of chitosanase and cellulase enzymes produced the highest antioxidant capacity of chitooligosaccharides. This is because the chitosanase enzyme itself can cut the acetyl group of glucosamine more effectively. After all, it cuts endo and Exo. Meanwhile, cellulase enzymes have the characteristics of enzymes that can cut acetyl groups endo and Exo so that they can optimally hydrolyze β-1,4 bonds, catalyze glycosidic bonds in chitosan to produce glucosamine which has smaller monomers such as OH^- (hydroxyl) groups. and amine groups (NH^+) become more numerous. The hydroxyl group and amine group play a role in capturing free radicals. This is supported by the statement of Jung & Zhao (2012) that hydroxyl groups (OH^-) and amino groups (NH^{2+}) contribute to the overall antioxidant capacity because they can

adsorb with unstable free radicals to form stable macromolecular radicals.

Antimicrobial activity with the inhibition zone diameter method

The inhibition zone test was processed using the disc-diffusion method. According to Hermawan et al., (2007), the disc-diffusion test or disc diffusion test was used to measure the diameter of the clear zone, which is an indication of a response to the inhibition of bacterial growth by an antibacterial compound. The gram-positive bacteria in this study used the *Staphylococcus aureus* bacteria, while the gram-negative bacteria used *Salmonella typhosa*. The following table presents the diameter of the inhibition zone of chitooligosaccharides hydrolyzed by various types of enzymes for two bacteria.

Table 5. The Diameter of the Chitooligosaccharide Inhibition Area of the Different Types of Enzymes for the Two Types of Bacteria

Sampel	Inhibition Zone Diameter (mm)	
	Staphylococcus aureus	***Salmonella typhosa***
COS α-amylase	$0.63^{g} \pm 0.15$	$1.03^{g} \pm 0.15$
COS Lipase	$1.17^{f} \pm 0.06$	$2.07^{f} \pm 0.15$
COS Selulase	$1.87^{e} \pm 0.06$	$3.13^{e} \pm 0.21$
COS Chitosanase	$2.47^{d} \pm 0.31$	$7.47^{d} \pm 0.51$
COS Chitosanase α-amylase	$3.00^{c} \pm 0.17$	$8.00^{cd} \pm 0.26$
COS Chitosanase lipase	$4.13^{b} \pm 0.68$	$11.47^{b} \pm 0.75$
COS Chitosanase selulase	$7.30^{a} \pm 0.79$	$15.90^{a} \pm 0.89$

In **Table 5,** it can be seen that the diameter of the inhibition zone of chitooligosaccharides against bacteria is indicated by the formation of an inhibition zone around the disc paper; for Staphylococcus aureus bacteria, the resulting inhibition zone ranges from 0.63 mm – 7.30 mm. Whereas in *Salmonella typhosa* bacteria, the range is from 1.03 to 15.90 mm. These results indicate that Chitooligosaccharide is effective in inhibiting the growth of gram-negative bacteria *Salmonella typhosa* compared to gram-positive bacteria *Staphylococcus aureus*. In this study, the effect of the type of enzyme on the hydrolysis of chitooligosaccharides on the bacterial inhibition zone was seen for two types of bacteria. Chitooligosaccharides derived from the hydrolysis of the cellulase chitosanase enzyme have the most significant inhibition zone effectiveness compared to other chitooligosaccharides. The different responses of the two groups of bacteria to chitooligosaccharides are caused by the different sensitivity of gram-negative and gram-positive bacteria. It is because gram-positive bacteria have a more straightforward cell wall structure than gram-negative bacteria, resulting in gram-positive bacterial cell walls being more easily damaged by antibacterial compounds from chitooligosaccharides than gram- negative bacteria. This follows the statement of Pilantanapak (2017), namely, chitooligosaccharides show better antibacterial activity against gram-positive bacterial species than against gram-negative bacteria. This is related to the chitooligosaccharide mechanism against gram-negative and positive bacteria, both of which are different. This is supported by Mei et al., (2015) statement that COS causes microbial cell death by changing the permeability of the cell membrane, which is a vital structure for protecting the release of cell constituents and controlling the entry of materials into cells from the environment. The positively charged COS can bind and absorb into the microbial cell wall through the negatively charged macromolecular components present in the microbial cell. ThiSampleke leads to their penetration into DNA and the blocking of RNA transcription.

Fourier-transform infrared spectroscopy (FTIR)

The change of chitosan into modified chitosan can be observed through the changes in its distinct functional groups. This FTIR test was carried out on samples of chitooligosaccharides derived from the results of hydrolysis using a combination of the enzyme chitosanase cellulase. This active group can be identified using Fourier-transform infrared spectroscopy.

Chitooligosaccharide has a distinctive FTIR spectral absorption band on the OH- and N-H

amine functional groups because the molecular formula of Chitooligosaccharide is (C6H8NO4)n. The FTIR test used the best treatment in this study, namely Chitooligosaccharide, which was the result of hydrolysis utilizing a combination of the cellulase chitosanase enzyme. The results of the OH- functional group from the FTIR absorption spectrum in this study showed a peak at a wavelength of 3417 cm^{-1}, which indicates that the wavelength of 3200-3600 cm^{-1} indicates the presence of the O-H functional group alcohol hydrogen bonding/phenol which usually appears at that wavelength. The wavelength of the O-H active group is not much different from Mourya et al., (2011), namely the O-H functional group of chitosan and its oligomers are located at a wavelength of 3450 cm^{-1}. Meanwhile, according to Singh et al., (2020), namely the FTIR chitooligosaccharide with a wavelength of 3434cm^{-1} shows the O-H functional group.

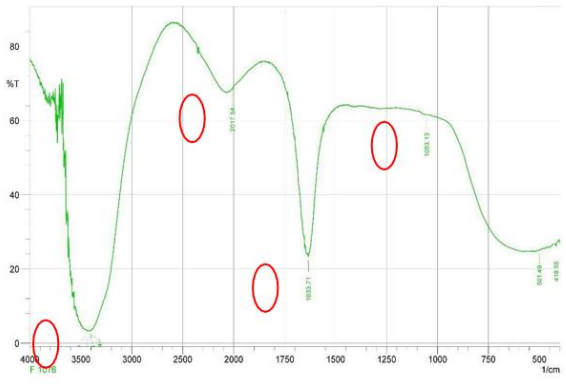

Figure 1. Chitooligosaccharide Functional Groups of Hydrolyzed Crab Shells Using a Combination of Chitosanase and Cellulase Enzymes

In this study, the peak at a wavelength of 3383 cm^{-1}, which is at a wavelength of 3300- 3500 cm-1, indicates the presence of the N-H amine/amide functional group, which usually appears at that wavelength. This amide group is also found in the statement of Mourya et al., (2011), that is, at a wavelength of 3120 cm^{-1}, it indicates the presence of a symmetrical N-H functional group. At 3320 cm^{-1}, there is an asymmetrical N-H functional group.

The peak wavelength of 1633 cm^{-1}, which is a wavelength of 1610-1680 cm^{-1}, indicates the presence of the C=O functional group, which usually appears at that wavelength. This is in accordance with the statement of Renuka et al., (2021) that Chitooligosaccharide has a C=O group at a wavelength of 1641 cm-1. In addition, there is also a functional group -C-O-C- at a wavelength of 1053.15 cm^{-1}, which indicates the presence of a 1,4-β- the glycosidic bond that has not been hydrolyzed. Thadatil (2014) explained that the enzymatic hydrolysis of chitosan into chitooligosaccharides could cut the (1,4)-β glycosidic bond from inside the chitosan structure and produce chitosan oligomers with a chain length of 2-10. This result is not much different from the results of Rokhati's research (2017), that the wavelength –C-O-C- is 1072.3 cm^{-1}.

CONCLUSION

Chitooligosaccharides from hydrolysis of the combination of cellulase and chitosanase enzymes had the best characteristics. It has the highest degree of deacetylation of 94.95% and the lowest molecular weight of 3.54 kDa. The most increased antioxidant activity of chitooligosaccharides using the DPPH method was in the chitooligosaccharides resulting from the hydrolysis of the cellulase chitosanase enzyme, which was 5.21 (µmol TE/g) and the antioxidant activity of chitooligosaccharides using the FRAP method was also found in chitooligosaccharides from the combination of the cellulase chitosanase enzyme, which was 2.27 (µmol TE/g). The best antimicrobial activity was found in chitooligosaccharides resulting from hydrolysis of the cellulase chitosanase enzyme, which had an inhibition area diameter of 7.30 mm in *Staphylococcus aureus* 15.9 mm in *Salmonella typhosa* bacteria. These results show that chitooligosaccharide is more effective in inhibiting the growth of gram-positive bacteria than gram-negative bacteria.

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