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ENZIMATIC PRODUCTION OF CHITOSAN FROM WASTE OF RAJUNGAN CRAB SHELL AND IT'S APLICATION IN CHOLESTEROL REDUCTION BY *IN VITRO* TEST

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Abstrak. Kitosan adalah produk deasetilasi kitin yang merupakan suatu senyawa polimer dari glukosamin (N-amino-2-deoksi-β-D-glukopiranosa). Polimer ini bersifat nontoksik, biodegradabel dan memiliki banyak manfaat, salah satunya adalah sebagai penurun kadar kolesterol. Penelitian ini bertujuan untuk menentukan persentase penurunan kadar kolesterol oleh kitosan dari limbah kepiting rajungan secara *in vitro.* Metode penelitian dimulai dengan proses demineralisasi sampel yang berupa serbuk cangkang kepiting rajungan, kemudian dilanjutkan tahap dekolorisasi dan deproteinasi hingga diperoleh kitin. Selanjutnya kitin yang dihasilkan dideasetilasi dengan perbandingan 1 : 100 pada suhu 50^{0}C selama 2 jam hingga diperoleh kitosan dan kitosan yang dihasilkan dikarakterisasi. Hasil karakterisasi kitosan menunjukkan nilai: kadar air 5,45%, kadar abu 1,53%, N-total 6,99% dan derajat deasetilasi 78,6% dengan warna putih kekuningan yang berupa serbuk. Hasil uji kitosan secara *in vitro* sebagai penurun kolesterol menunjukkkan penurunan sebesar 31,18% pada kitosan 2 mg.

Kata Kunci: Rajungan, Kitin deasetilase, Bacillus licheniformis, Kitosan, Kolesterol.

Abstract. This research aimed to show the percentages of the level of the cholesterol decreased by the convertion product of chitosan enzimatically through *in vitro.* The research method was started by the demineralization process of the sample in the form of powder of crab shells, and then continued with the de-colorization and de-proteination steps. The next step was de-asetilation enzymatically using de-acetylated chitin which had been isolated from *Bacillus licheniformis HSA3-1a* bacteria at 50°C for 2 hours until the chitosan was obtained. The research results indicated that the characteristics of the isolated chitosan had water content of 5.45%, ash content 1.53%, N-total of 6.99% and de-acetylation degree of 78.9%, and it showed white yellowish powder form. The results of the test of chitosan as the reducer of cholesterol level by *in vitro* process showed that 31.18% of the cholesterolwas decreased by 2 mg concentrate of chitosan. Beide, 0.5 mg of simvastatin (as positive control) had decreased by 58.29%.

Keywords: Rajungan, chitosann, Bacillus licheniformis HSA3-1a, chitin de-acetylase, cholesterol

INTRODUCTION

Lifestyle patterns that occur in major cities, especially in Indonesia, the effect on the eating patterns that are less good, i.e. foods high in calories, fat and cholesterol, it does have an impact against the increased risk of various diseases. One of the diseases caused by changes in lifestyle patterns are hyperchlosterolemia, i.e., a condition that occurs with increased levels of cholesterol in the blood (Polychronopoulos et al, 2005). High cholesterol levels in the blood is a major factor that led tothe risk of coronary heart disease. Generally, people cope with the disease hypercholesterolemia with drugs synthesis which is lowering cholesterol levels in the body.But the price of these drugs are expensive, because these drugs are still raw materials imported. In addition, countermeasures with drug synthesis has a low success rate, because of the need to discipline. Almost 70% of patients hypercholesterolemia sufferers failed to reach the target cholesterol levels in accordance with the treatment. A study in Asia with a total patient hiperkolesterolemia 7,281 respondents stated that nearly half of those who run therapy, often forgetting to consume drugs lowering cholesterol levels for a period of one week or more (Pratiwi, 2011).

Some research has been done in the search for an effective natural ingredients of lowering cholesterol levels to subtitute the chemicals. Such research conducted Dibyantini and Simorangkir (2016) about testing the effectiveness of petals extracts Rosella (Hibiscus sabdariffa) against a decrease in cholesterol levels in blood serum of chicken broiler, the results showed a decrease in cholesterol levels activities as muchas 37% of the cholesterol levels without treatment. In addition Sutioso (2012), researching on pectin from guava (Guava guajava) and tests in vitro and in vivo showed a decrease in cholesterol levels in the blood of rats for 14 days of 0.5% for a dose of 0.1 g of pectin and 1.3% for doses of 0.2 g of pectin. Therefore, researchers are trying to find other natural ingredients that can be used as material for lowering cholesterol. One of the natural ingredients that can lower cholesterol levels in the blood are Chitosan. Chitosan can bind allegedly fat and cholesterol in the body so that the cholesterol in the blood can come down. Compound Chitosan carries positive electrical charge, can be fused with substances bile acids which are negatively charged so as inhibit the absorption of cholesterol, a fatty substance because that goes along with the food has to be digested and absorbed with the help ofsubstances bile acids secreted by the liver (Hargono, 2008).

Typical properties of Chitosan is having the ability of lowering levels of LDL cholesterol (Low Density Lipoprotein) while simultaneously pushing the increase of HDL cholesterol (high Density Lipoprotein) in blood serum. Chitosan is a hipokolesterolmik substance that can lower cholesterol level in serum effectively and without causing the side effects (Rismana,2001). Chitosan can be made from chitin which many compounds contained in the shell of the animal (Natsir et al, 2004), including onthe shell of the crab, shrimp or other sea animals (Watson et al., 2009). Chitosan is a type of polysaccharide that is easily degraded naturally or biologically.

Chitosan is not toxic to humans and Chitosan can be useful as drugs lowering cholesterol and weight loss as (Hargono, 2008). According to the potential of chitin and chitosan as lowering cholesterol levels as well as the availability of raw materials abundant crab waste in Indonesia, which is the crab's largest market for international value was pretty. By 2015 worth $266 million U.S. Figure pierced or Rp 2.8 trillion (Badan Pusat Statistik, 2017), need to have for the manufacture of compound-lowering cholesterol levels as an alternative medicine to lower cholesterol levels. The selection process of converting chitin becomes enzymatic chitosan in this research was conducted because the conversion process are relatively enzymatic chitosan better because its deasetilasi more evenly, easily controlled and decompose biologically (biodegradable) (Artiningsih, 2003 and Rukayadi, 2003). So the result of a decrease in cholesterol levels to be generated is also increasingly effective and very eco-friendly.

MATERIALS AND METHODS

Materials and Tools

The materials used in this study are bacteria b. licheniformis HSA3-1a, crab shell powder, chitin sigma (EC 215-744.3), NaOH, HCl, NaOCl, CH3COOH, NaCl, H2SO4, I2-KI, (NH4)2SO4.7H2O, K2HPO4, MgSO4.7H2O, NaNO2, C2H5OH, NaH2PO4, Na2HPO4, glikol kitin, Na2CO3, CuSO4.5H2O, FeCl3.6H2O, ethanol, yeast extract, bacto agar, bacto pepton, glucosamine, ammonium sulfamat, Bovine Serum Albumin indol (BSA), cholesterol powder, sodium-potassium tartrate,-foolin, akuades and white rat (Rattus norvegicus).

The tools used are the analytical balance, incubator, autoclave, ovens, water bath, sentrifuge, hotplates stirer, freeze dryer, filter, Büchner, Enkas Spektronik 20 d+, petri dish, needle ose, glasswoll, lipid ® analyzer, Cardiochek and tools commonly used glasses.

The step in this study consists of the isolation of chitin from crab shells, small crab attaching waste production of chitin enzymes deasetilase (KD), bio-convert chitin becomes chitosan, and enzimatic chitosan aplication as lowering cholesterol with in vitro test.

The Isolation of Chitin

Sample Preparation

Waste of crab shells are cleaned then dried in the Sun for 2 x 24 hours. Once dry,then digrinder until it becomes a powder and then sifted with size 80 mesh and results floured crab shells are used as raw materials in the study.

Isolation of Chitin

The process of isolation of chitin beginning with stage demineralisasi with crab shell powder dissolves into HCL 1.0 M with 1:10 ratio (b/v) for 1 hour at a temperature of 75oC, then stage dekolorisasi with 0.5% NaOCl (ratio 1:10 b/v) at a temperature of 75OC for 1 hour, and stage deproteinasi with 5% NaOH (ratio 1:10 b/v) for 1 hour at a temperature of 75OC. Chitin obtained are characterized with the parameters water content, ash content, N-total levels and FTIR analysis.

Production of Enzyme Chitin Deasetilase

Rejuvenation is performed by growing bacterial stock of b. licheniformis HSA3-1a into the medium of LA (Luria Agar) modification + 0.5% colloidal chitin (Natsir, et al., 2002; Natsir, et al., 2013). Production of de-acetylated chitin begins with the creation of inokulum was then followed by the fermentation process (enzyme production). Inokulum that have been incubated during 18-24 hours at a temperature of 50OC and then inoculated into the medium of production. Every 12 hours, conducted sampling for measuring OD at Maximum wavelength, and then centrifuged. The filtrate is obtained aubsequently performed measurements of the activity of the KD and the analysis of enzyme protein. Measurements made during 6 days to determine attainment of the optimum conditions (Natsir dkk, 2013).

The Activity Of Chitin Deasetilase Test.

Enzyme activity is determined based on the amount of KD 1 μmole Glucosamine residues released per minute during incubation time. 600 μL reaction mixture (glycol chitin 100 μl 1% + 300 μl buffer, pH 7.0 fospat + 200 μl of enzyme solution), shaken and incubated at temperature of 50o C, for 30 minutes. Then added 500 μl acetic acid 33%, added 500 μl sodium nitrite is 5%, and left on for 10 minutes at room temperature. Next 500 μL of added ammonium sulfamat 12.5% and incubated at room temperature for 30 minutes. Then added 2000 μL 0.5% HCl and 200 μl indol 1%, and dididihkan for 5 minutes in a waterbath, then cooled. Then added 96% ethanol, then measured its absorbance at wavelength spectrophotometer with a maximum of 492 nm. The amount of residu that Glucosamine is liberated is determined based on the standard curve of pure Glucosamine (Tokuyasu, et al., 1996).

The Determination of Protein Levels

Protein levels are determined by the method of Lowry, aqueous UV Vis spectrometer measured by spetronik 20D+ at a wavelength of maximum levels of proteindetermined by curve calibration relationship between protein levels and absorbance (Natsir, et al., 2010).

Chitin Deasetilation Becomes Chitosan In Enzimatic Process.

Chitin powder obtained from the results of isolation of chitin from crab shell on the working procedures (2) will be converted into Chitosan by enzymatic deasetilaseof isolates of B. licheniformis HSA3-1a (Natsir, et al., 2013). The de-asetilation process is done by mixing an enzyme with a substrate by comparison [E] : [S] = 1 : 100 (mL/mgr) then incubated at a temperature of 50OC for 2 hours. Next the Chitosan is obtained, stored in a dry container for further analysis on infrared spectroscopy (IR), analysis of water content and levels of ash (Arif, et al., 2014).

Stage of Decreased Cholesterol Levels with in vitro Test (Rudel and Morris, 1973)

Test of the Chitosan in vitro using simvastatin in 0.5 mg (positive control) that the comparison function to see a decrease in cholesterol levels with Chitosan samples. Chitosan sample weighed each 0.5 mg; 1 mg; and 2 mg dissolved in acetic acid 1%, 5 mL of the solution is then added cholestrol

with concentration of 300 ppm. Cholesterol left in supernatan taken (5 mL) and transferred into test tubes bertutup. Mix a solution using vortice and incubated at 37OC for 60 minutes, then centrifuged at 4000 rpm for 5 minutes. Each of these added supernatan 2.0 mL reagent FeCl3 then vorticed and silenced for 10 minutes, and the outer layer of blown closed with aluminum voil in order to protect from light. Then the solution is added to 1.0 mL of H2SO4 and mix a solution using the vortices, then silenced for 30 minutes and measured its maximum absorbance at a wavelength corresponding scanning results before. The standard curve is used to determine the concentration of cholesterol.

RESULTS AND DISCUSSION

Isolation Of Chitin

On the process of isolation of chitin from crab shell waste decline weights for each stage of the isolation. For the insulation. For demineralitation phase retrieved 27.31 grams of weight beginning 100 grams with percentage 27.31%, decoloriatation phase retrieved 19.23 grams with percentage 19.23%, and stage deproteination obtained at weights of 15.78 grams with rendamen 15.78%. Characteristics of chitin obtained is 5.96% moisture content, ash levels 1.39%, N-Total 7.47%. To the degree of deasetilasi chitin analyzed with spectrophotometer FTIR and retrieved DD of 65.71%. According to protan laboratories, chitin that has a good quality standard is expected to have the degree of deasetilation in the range of 15-70%.

Chitin obtained from deproteination results analyzed with FTIR spectrophotometer to know the mainfunctional grups contained on chitin, besides further FTIR measurement results are used to find out the degree of chitin de-asetylation. By using FTIR spectrum (Figure 1) can be seen the presence of regional Summit on 3000-3500 cm-1 indicating the presence of hydroxyl OH and NH2. Other summits are aliphatic C-H at 2929 cm-1, C = O amide in 1658 cm-1 (Amide I), the vibration of the C-N-H (Amide II) in 1554 cm-1, C-N strech on 1317 cm-1, the vibration of the C-O-C in cyclic 1203 cm-1, the vibration of the C-O-C stretch on dialkil at 1157 cm-1,deformation CH3 symmetric in 1377 cm-1, the vibration of the C-OH at 1072 cm-1. Based on the results of the analysis of some parameters can be deduced that the chitin obtained from the results of isolation have met the standards that have been set.

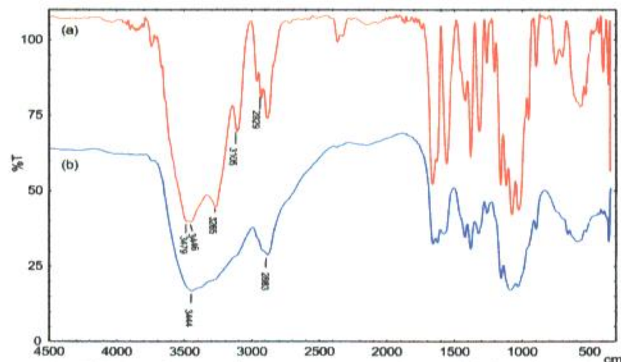

Figure 1. FTIR absorption spectrum (a) Chitin (b) Chitosan

Production Of Chitin Deasetialtion Enzymes

Bacillus licheniformis HSA3-1a grown further in the fermentation medium to know the optimal production conditions for fermentation of microbial growth in the medium and the resulting product can be monitored properly fit optimal conditions. The growth of B. licheniformis fermentation medium on the rise in the value of time intervals. Results from the withdrawal of sample done every 12 hour intervals for 1 to 6 days to define optimum enzyme production time by measuring the optical density (OD) at a wavelength of 660 nm. The increase continues until the incubation time 60 hours and began to decline thereafter. Bacterial growth shows an improvement from the 12 hours up to 48 hours, because the bacteria have been adapting to its environment and nutrients that there has been sufficient to breed, this is an exponential phase (Figure 2).

Optimal bacteria growth occurs at the time of incubation 60 hours with the value OD 0.710 which is stationary phase and begins to decrease at 72 hours incubation time value OD 0.590 which is the phase of death. In this phase of the living cells can only survive for a while, the longest generation time or even none at all. In addition, the cells will be destroyed by the influence of the enzyme itself (otolisis), the next microbial cells die (Arif, et al.,2014).

After that, the enzymes measured value of enzyme activity, it aims to find out the optimum time of chitin deasetilation enzyme production. The increase in enzyme activity occurred in a span of 36-48 hour, where the optimum time on 60 minutes incubation time with a value of 0.3471 x 10-3 U/mL, this is due to the increasing growth of bacteria so that the production of enzymes used to hydrolyze the substrate is also increasing.

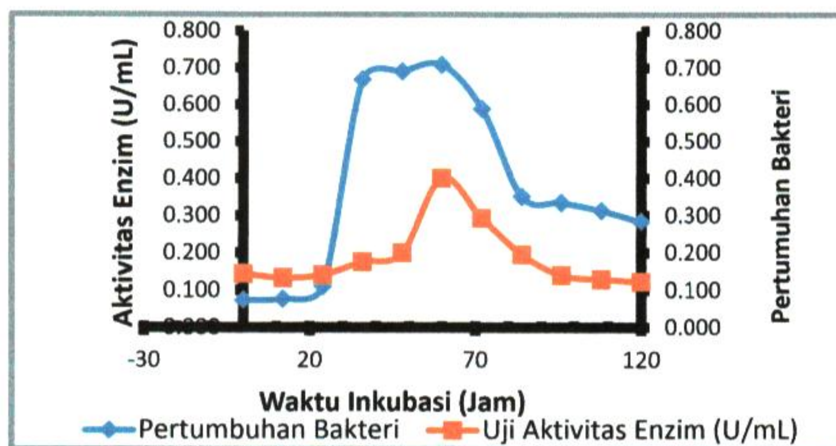

Figure 2. The growth of the bacteria b. licheniformis HSA3-1a and the activity of chitin deasetilase against time incubation

Bioconversion Chitin Becomes Chitosan In Enzimatic Process

The crude extract of chitin deasetilation obtained from the process of production of the enzyme by B. licheniformis used as converter agent chitin that obtained from crab sheel waste into chitosan. In the process of chitin deasetilation instrumental in catalyze acetyl Group on N-aseti-D-glucosamine into poly (2-amide-2-Deoxy-β-(1,4)-D-glucopyranose).

In this study, the process of deasetilation is done with optimal incubation time of 2 hours, the optimal substrate concentration by comparison of the enzyme (E): substrate (S) IE 1:100 (mL: mg) at a temperature of 50 °C is the optimum temperature of the enzyme from the isolates of b. Licheniformis HSA3-1a (Arif, et al., 2014).

On the incubation process, the optimum enzyme acetyl group can more be eliminated through termination of the bond between carbon in the acetyl group with nitrogen at the cluster of amin. This proves that the temperature is one of the factors that affect enzyme activity. Temperature changes can cause the onset of protein folding or enzymes so that the sides of active enzymes are in the right position to catalyze the substrate. The temperature was very influential in the aerodynamic motion of the molecules, similarly a molecule of protein or enzyme. Low tempertures causing lack of collisions between the enzyme's molecular with the substrate, while at higher tempertures the enzyme's molecular thermodynamic motion large enough that collisions between molecules of enzymes and substrates would occur quickly. At extreme temperatures high protein undergoes denaturation led to changes in the protein structure of the enzyme so that the side of the active enzyme changes. Thus, the enzyme becomes inactive due to changes to the active side (Natsir, et al., 2010).

Characteristics of Chitosan enzimatis obtained i.e. 5.45% moisture content, ash levels 1.53%, N-Total 6.99% and 78.6% deasetilation degrees while the standard according to protan laboratories namely water Levels < 10%, ash levels □ 1%, N-total 7-8% and deasetilation degrees > 70. Refer to the terms enzimatic Chitosan has met predetermined standards. The high degree of deasetilasi obtained indicates that the method of de-acetylatedwith enzymatically has a pretty good effectiveness in degrades the acetyl grup

attached to chitin. The results of many functional Chitosan by FTIR (Figure 1) shows that in the area of 3000-3500 cm-1 there is a peak which indicates the existence of a cluster of NH2. Other summits are aliphaticc C-H on 2883 cm-1, C = O in 1658 cm-1 (Amide I), the vibration of the C-N-H (Amide II) in 1579 cm-1, C-N strech on 1421 cm-1, C-H sym on 1379 cm-1, the vibration of the C-O-C in cyclic 1257 cm-1, the vibration of the C-O-C stretch on dialkil at 1155 cm-1.

Application Of Enzimatic Chitosan in Cholesterol Reduction Levels with in vitro Process

The binding reaction of cholesterol by Chitosan is a mechanism of in vitro method to find out the decrease in cholesterol levels (Hawab, 2002). The ability of binding of cholesterol based on measurements of cholesterol in aqueous of cholesterol-ethanol after the addition of the test sample with the incubation period of 60 minutes at 37 °C using one of the colorimetric methods of the Rudel-Morris and Zak method, namely the addition of coloring reaction between FeCl3 in acetic acid glacial and H_2SO_4 as a catalyst, so that the colored compound formed then the amount of cholesterol in the sample is determined by measuring the absorption. Uptake was measured using sample is determined by spectrophotometerr UV-Visibel certain wavelengths between 400-700 nm.

Chitosan 0.5; 1; and 2 mg reconstituted with 1% acetic acid as much as then added 300 ppm cholesterol. Furthermore each sample level using vortices and incubated for 60 minutes at 37 °C, and then centrifuged at 4000 rpm for 5 minutes. The working of chitosan mechanism when reacts with cholesterol is chitosan binds cholesterol contained in aqueous ethanol-holesterol will be bound together with Chitosan after being centrifuged. Then the supernatan resulting separated and added $FeCl_3$ reagent in glacial acetic acid and then added H_2SO_4 as a catalyst for the formation of the colors and the measured upatake λ 550 nm. The colors are sorrel. After the absorption of the solution test is read and then calculated percent of cholesterol levels reduction.

Table 1. Calculation Result Table Of Cholesterol Levels Reduction Against Samples Of Chitosan

Sampel	Early Cholester ol (ppm)	Final Cholester ol (ppm)	% Cholesterol Levels Reduction	% Cholesterol Levels Reduction with Control (+)
Control (+)	300	125,14	58,29%	100%
0,5 mg	300	288,06	4,55%	6,8%
1 mg	300	252,77	15,75%	27%
2 mg	300	206,46	31,18%	53,5%

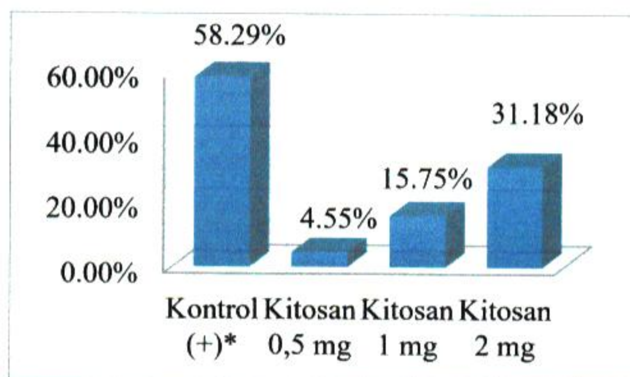

Figure 3. Graph percentage of cholesterol levels Reduction with *in vitro* Process

Upon calculation of the results assay activity decrease cholesterol levels by using the colorimetric method (Rudel-Morris, Zak) shows the concentration of Chitosan with a dose of 2 mg has the ability of cholesterol reduction as the highest percentage with decreasing levels of cholesterol i.e. 31.18% and compared to control positif i.e. of 53.5% (Figure 3). Based on data of table 1 can be seen the more doses of chitosan will will produce more cholesterol reduction activity.

Chitosan can work as lowering cholesterol through the binding mechanism. Based on the research of Xu et al (2008), which examines the effectiveness of a decrease in cholesterol levels based on the difference in molecular weight Chitosan compound, in vitro research showed that when the chitosan mixed with cholesterol binding reaction will occur (electrostatic interactions), so the cholesterol is no longer free. This is due to the amino group owned by Chitosan can bind with molecules of the cholesterol that has a negative charge is the hydroxyl (OH). Judging from the molecular weight, then the low molecular weight of chitosan has the free amino group of a more reactive compared to the high molecular weight of chitosan. So the free amino group, owned by low-molecular weight chitosan can easily react with cholesterol so binding of cholesterol by chitosan which resulted in cholesterol would no longer be free. Mechanism of decrease of cholesterol in the body is described the first catch and dissolve fat chitosan in the stomach. Chitosan fiber which has been binding the fat into a large mass in which the body cannot absorb and enhance the ekskretion into the stool (Xu et al, 2007).

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