PRODUCTION AND CHARACTERIZATION CHITOSANASE OF SPONGE SYMBIONT BACTERIA *Klebsiella sp* to hydrolyze CHITOSAN BE CHITOOLIGOSACCARIDES

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ABSTRACT

Chitosanase enzyme is an enzyme to catalyze the bonding endohidrolisis (1.4) β- glucoside on chitosan into a series chitosan oligomers or chitooligosaccarides. Chitosanase of microbes give good results in producing chitooligosaccarides, but very expensive for use in large-scale industry. This study aims to manufacture and characterization of crude chitosanase enzyme from Klebsiella sp used to hydrolyze chitosan into chitooligosaccarides. Chitosanase produced in the fermentation medium containing 0.5% colloidal chitosan for 60 hours with the activity of 0.309 U / mL (5,235 U / mg), work optimally at 40 ° C and pH 8, is stable at pH 6-7, activated by Co^{2+} ; Ca^{2+} and Ni^{2+}. Chitooligosaccarides obtained in the form of a mixture of monomer to octamer, which soluble in acetic acid 0.25% to 0.5%, having intrinsic viscosity decreases with increasing time of incubation with molecular weight range of 4103.12 g / mol (incubation 1 hour) ; 1483.48 g / mol (incubation 2 hours) and 1065.79 g / mol (incubation 3 hours).

Key word : Chitosanase, Klebsiella, Chitooligosaccarides

1. INTRODUCTION

Chitosan is a poly (2 - amino - 2 - deoxy - β- (1-4) -D - glucopyranose) with the molecular formula $(C_6H_{11}NO_4)_n$ [1] which can be obtained from complete or partial deacetylation of chitin . Chitosan is a biopolymer that is the main content of the D - glucosamine and some parts of N-acetyl-D-glucosamine that binds to the β-(1-4) glucoside [16]. Chitosan can be hydrolyzed to form oligomernya known as chitosan oligomers or *chitooligosaccarides* .

Chitosan received special attention as a functional biopolymer for applications in various fields . Research and utilization of chitosan and oligomernya experienced a significant improvement in various fields , especially in the fields of pharmaceutical, medical and food industries . Chitosan is more effectively absorbed into the human body when it gets converted in the form of chitosan oligomers [23].

Chitooligosaccarides is a mixture of oligomers of D-glucosamine which are formed through a process of depolymerization of chitosan by cutting β - glycosidic bonds. Kitooligomer is chitosan which has terdepolimerisasi so has the size and weight of smaller molecules. The reduced weight of the chitosan molecule makes its more soluble in water than before terdepolimerisasi [13, 26].

Chitosan oligomer application development at this time because it is soluble in water and environmentally friendly, so its use is relatively safe. Kitooligomer today applied widely in the field of health as it has bioactivity as anti-bacterial, anti- fungal, anti- viral, food supplement to boost the immune system against diseases, anti- coagulation of blood and hipokolesterolmik, anti-cancer, anti-diabetic and others [26], *Chitooligosaccarides* known biological activity of relying on its chemical structure [3].

Kitooligomer can be generated by sonic irradiation, chemical hydrolysis and enzymatic hydrolysis. Hydrolysis of chemical and sonic irradiation is random, uncontrolled, low efficiency and produce oligomers with degree of polymerization (DP) which is lower by more monomer D -

glucosamine. Meanwhile, hydrolysis enzymatic specific, controlled, produce oligomers chitosan with a DP higher and less glucosamine produced and environmentally friendly [5, 11, 15, 17, 28], so the way enzymatic this is better and generally used in producing *chitooligosaccarides*, One of the enzymes that can be used to hydrolyze chitosan is chitosanase enzyme [10]

Chitosanase functions as an enzyme to degrade the chitosan into chitosan oligomers or *chitooligosaccarides* with a high degree of polymerization. Enzyme Commission defines chitosanase (EC 3.2.1.123 or 3.2.1.99) as the enzyme that catalyzes the reaction endohidrolisis bond (1,4) β-glucoside between glucosamine residue in chitosan terdeasetilasi partly produced a series *chitooligosaccarides* oligomer or chitosan [10, 20, 22, 25]. Chitosanase among all organisms, bacteria have a particular concern because the bacterium is able to produce quickly so that the biomass content of bioactive compounds could be produced more easily, quickly, and many in the biotechnology scale [8]. Some research about *Chitosanase* and applications have been made include: Choi et al (2004) examined *Chitosanase* of *Bacillus sp*. strain KCTC 0377BP has an optimum pH 4-6 at a temperature of 30 ° C and used for the production of oligomers chitosan, Pagnoncelli et al (2010) that produce enzymes *Chitosanase* of *Paenibacillus ehimensis* and its application to hydrolyze chitosan on the temperature and pH optimum 55 °C and pH 6, Wangtueai et al (2007) to produce and characterize enzymes from *Bacillus cereus* chitosanase 12:24 TP which has a pH of 6.5 and the optimum temperature and 55 °C, Chasanah et al (2009), which isolate the enzyme chitosanase of *Acinobacter sp*. 218 Commission for the production of chitosan oligomers.

Although the enzyme *Chitosanase* of microbes give the best results in producing *chitooligosaccarides*, but very expensive for use in large -scale industry. Therefore hydrolysis with enzymes requires several steps, particularly the preparation and purification of the enzyme [20]. In this study aims to produce and characterization chitosanase enzyme used to hydrolyze chitosan into *chitooligosaccarides* with crude enzymes from bacteria sponge symbiont marine waters are rich in chitin namely *Klebsiella sp* .

1. MATERIAL AND METHODS

2.1 Rejuvenation Microbes

Inoculum medium was prepared by dissolving yeast extract (0.05 %), NaCl (0.1%), Bakto Peptone (0.1%), $CaCl_2$ (0.01%), K_2HPO_4 (0.01%), $MgSO4.7H_2O$ (0.01%), colloidal chitosan (0.5%) in distilled water. Furthermore, the solution is heated and sterilized for 30 minutes in an autoclave. Microbial isolates (*Klebsiella sp*) is inserted into the inoculum medium. The microbial culture dishaker with a speed of 150 rpm for 24 hours at 37 °C , then put in the medium LA (yeast extract 0.05% ; NaCl 0.1% ; 1.5% bacto order ; Bakto Peptone 0.1% ; $CaCl_2$ 0.01%, 0.01% K_2HPO_4 ; $MgSO_4$. $7H_2O$ 0.01% ; colloidal chitosan 0.5%) with the method of casting and incubated for 3 x 24 hours at a temperature of 37 ºC . Furthermore, the microbes grow and berzona nodes used for the rejuvenation of the bacteria *Klebsiella sp*, where 2-3 ose bacteria scratched on LA medium and incubated with the same time and temperature beforehand.

2.2. Chitosanase Enzyme Production

Chitosanase enzyme production begins with the manufacture of starter inoculum medium containing 0.5% colloidal chitosan, then bacteria *Klebsiella sp* rejuvenation results were inoculated in media starter and incubated in a shaker incubator at 37 °C, 180-200 rpm for 24 hours . Medium starter as much as 10% (v/v) was inoculated into the production medium then incubated for 6 days at the same temperature and speed to medium starter and every 12 hours of sampling for measuring OD. Enzymes produced cold centrifuged at 3500 rpm 4 °C for 30 minutes followed by measuring the activity of *Chitosanase* and protein content determined by the method of Lowry, where BSA (Bovine Serum Albumin) as a standard solution.

2.3. Chitosanase Activity Assay

The reaction mixture sample of 100 mL of 1% soluble chitosan, 100 mL of an enzyme (crude extract) and 100 mL of 50 mM phosphate buffer pH 6 were incubated for 30 min at 37 °C. Enzymatic reaction

was stopped by putting in a water bath of 100 °C for 15 minutes. Meanwhile in a separate place made mixture control consisting of 100 mL of 1% soluble chitosan and 100 mL of 50 mM phosphate buffer pH 6 was incubated as a mixture of sample and inactivated. The next stage is the coloring of the sample and control. Staining of samples was done by mixing 200 mL samples were incubated with 800 mL of distilled water and 1000 mL of dye Schales. On a separate, control solution taken as many as 133 mL then mixed with 67 mL of enzyme, 800 mL and 1000 mL of distilled water Schales dye. Control samples and then heated in a water bath temperature of 100 °C for 15 minutes. After heating, the samples and controls centrifuged at 8000 rpm at 4 °C for 10 minutes. The results of centrifugation supernatant was measured absorbance at 420 nm wavelength. To use standard glucosamine solution with a concentration of 0-100 mg / mL, and performed by the same procedure as in the measurement sample. Enzyme activity is determined by the amount of glucosamine that were released during the hydrolysis of chitosan substrate. One unit of enzyme activity is defined as the amount of enzyme required to produce glucosamine residue mol per minute after incubated with a solution of chitosan [4, 10, 12, 28].

2.4. Effect of pH

Chitosanase activity was tested on some variation of pH. The determination of the optimum pH is done by treating the enzyme with buffer at a pH ranging from 4 - 10. Buffers are used in the determination of the optimum pH is 0.2 M citrate phosphate buffer (pH 4, 5 and 6), phosphate buffer (pH 6, 7, and 8), borate buffer (pH 9 and 10) .

2.5. Effect of Temperature

Chitosanase measure activity at an incubation temperature were varied. The optimum temperature is determined by treating the crude extract of enzyme with substrate at a temperature of 30 ^{o}C , 40 ^{o}C , 50 ^{o}C and 60 ^{o}C, which is carried out at optimum pH [27]

2.6 . Effect of Substrate Concentration

Analyzing the activity of the enzyme with substrate concentration (soluble chitosan) were varied, is from 0.5% to 1.5% with an interval of 0.25% . This treatment uses the optimum pH and temperature have been obtained prior to the time of incubation for 30 minutes .

2.7 . Effect of Metal Ion Addition

Tests conducted by reacting the enzyme at pH, temperature and optimum substrate concentration. At the time of the reaction, the metal ions added to the sample tube and control. Some metal ion that is used is a divalent cation at a concentration of 10 mM, among others, Co^{2+}, Zn^{2+}, Mg^{2+}, Ni^{2+}, Cu^{2+} and Ca^{2+}. At the same time , made positive control which was not added with metal ions . Reaction results determined value of its activity [4 , 6 , 20].

2.8. Effect of pH on Enzyme Stability

Determination of pH stability of the enzyme activity by performing prainkubasi in a buffer solution at pH optimum and surrounding areas. Prainkubasi was conducted for 2 hours and tested the activity of *Chitosanase*nya each interval of 30 minutes at the optimum temperature [19].

2.9 Production of Chitooligosaccarides with Chitosanase

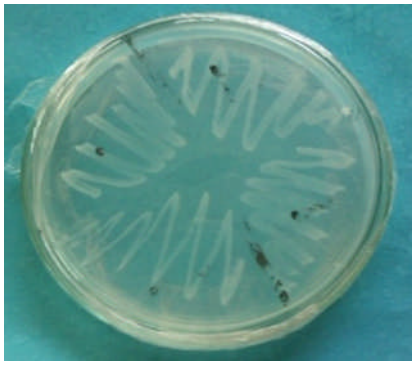
Soluble chitosan 1% prepared as a substrate. *Chitooligosaccarides* produced by reacting the enzyme and substrate at a volume ratio of 1: 1 performed at the optimum pH and temperature of the enzyme and incubated on a rocking incubator for 1, 2, and 3 hours. Enzymatic hydrolysis reaction for each incubation time was stopped by freezing the reaction mixture for 15 minutes, then heat in a water bath temperature of 100 °C for 10 minutes. *Chitooligosaccarides* solution was then dried by freeze drying [2, 6, 10] and was identified by TLC. *Chitooligosaccarides* obtained each incubation time is also determined their molecular weight by viscometry methods

3. RESULTS AND DISCUSSION

3.1 . Rejuvenation microbes

Bacteria *Klebsiella sp* is one sponge symbiont bacteria which have chitinolytic

activity . It can be seen from microbial growth on the optimal temperature conditions of 37 ºC with a pH of 7.0 in the medium LA (Luria agar) and the modification of inoculum liquid medium containing 0.5% colloidal chitosan. *Klebsiella sp* can degrade chitosan because it contains enzymes that can *Chitosanase* induced in the presence of colloidal chitosan on growth medium . *Chitosanase* activity characterized by the formation of clear areas around bacterial colonies as shown in Figure 1



Figur 1. *Klebsiella sp* colonies in solid medium LA

3.2. Enzyme Production and Activity Chitosananase Assay

Chitosanase enzyme begins to grow *Klebsiella sp* in fermentation medium containing colloidal chitosan as the substrates to determine the optimal conditions of production . The growth of the bacteria *Klebsiella sp* in the fermentation medium increased in intervals of overtime , increased growth starting from the first day of the second and began to decline in the third day .

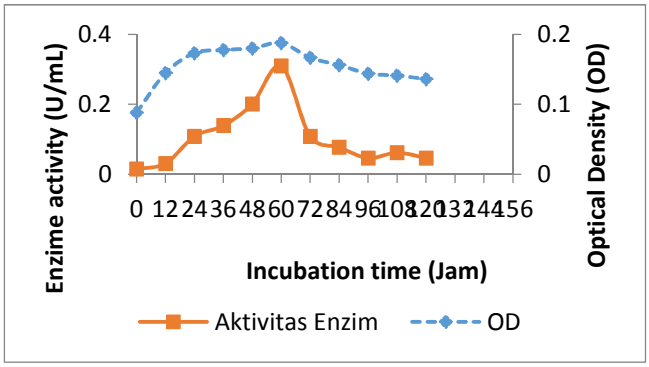


Figure 2. Chitosanase activity and optical density (OD) of the incubation time

Enzyme activity (Figure 2) is directly proportional to the rate of growth of bacteria, which with the increasing time of incubation activities and increasing the rate of bacterial growth. The increase occurred from 12 to 60 hours of incubation . Optimal growth of the bacteria with the highest activity occurred at the end of the stationary phase (60 hours) at 0.1875 OD value and the value of activity 0.309 U/mL, so the optimal production time *Chitosanase* enzyme production of *Klebsiella sp* was 60 hours of incubation . *Chitosanase* bacterial growth and activity decreased in the 72 hours up to 144 hours of incubation time. This is caused by the accumulation of toxic materials , the nutrients are very limited, so many dead cells. The number of dead cells increased exponentially or the inverse of the logarithmic growth phase. In addition, the bacterial cells will be destroyed by the effects of the enzyme itself (autolysis), then the bacteria die in total [1].

In addition to measuring the value of enzyme activity, measurement of protein content is also necessary to know the value of the specific activity of an enzyme. Based on the measurement data *Chitosanase* enzyme protein content of *Klebsiella sp* showed that the highest protein content in the first and second day of incubation, but has a low activity. This indicates that the presence of other enzymes secreted chitinolytic besides *Chitosanase*. *Chitosanase* enzyme protein content on the growth of bacteria (OD) and optimum activity in this study was 0.059 mg/mL, so that the specific activity of the enzyme obtained at 5,235 U/mg (Table 1).

Table 1. The protein content, activity and specific aktivitities of crude chitosanase of *Klebsiella sp*.

Samples	Protein content (mg/mL)	Chitosanase activity (U/mL)	Chitosanase Specific aktivitities (U/mg)
Crude Chitosanase	0,059	0,309	5,235

3.2 . Effect of pH

The increase in pH will increase the activity of *Chitosanase* to pH 8 (Figure 3), after which the increase in pH causes decreased enzyme activity significantly. *Chitosanase* of *Klebsiella sp* work optimally at pH 8 phosphate buffer (pH

optimum) with activity reaching 1.005 U/mL. The structure of the enzyme ion depends on the pH of the environment such as proteins in general. Enzymes can take the form of positive ions, negative ions or doubl -charged ions (zwitter ion). Thus the environmental pH changes will affect the effectiveness of the enzyme active site to form a complex enzyme substrate [21]. In this case the pH can affect the activity of the enzyme by altering the structure or by changing the charge of functional residues in the substrate binding or catalysis [18].

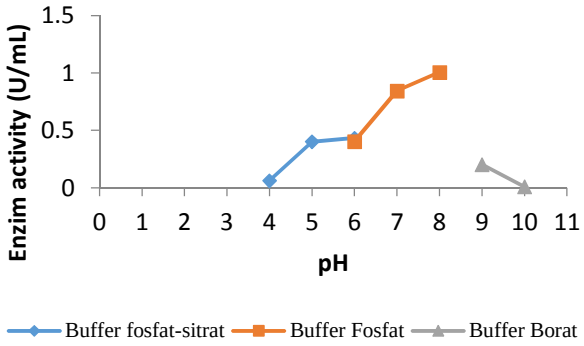


Figure 3. Graph influence of pH on the crude extract of chitosanase activity of *Klebsiella sp*

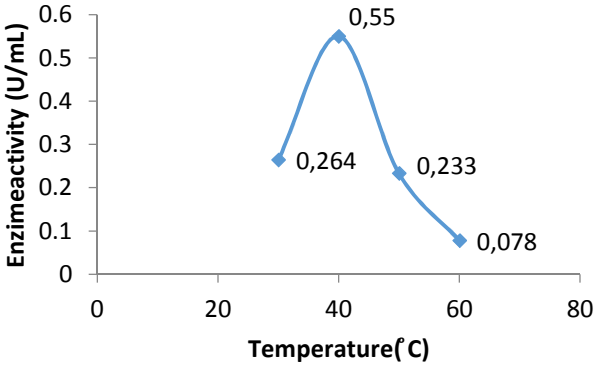


Figure 4. Graph influence of incubation temperature on the enzyme activity of crude extract *Chitosanase*

3.3 . Effect of Temperature

Crude extract of *Klebsiella sp Chitosanase* enzymes work best at a temperature of 40 ºC. At this temperature, the value of enzyme activity achieved amounted to 0.55 U/mL (Figure 4). This indicates that the enzyme works slowly at a temperature of 30 ºC, while higher temperatures the enzymes work faster in accelerating the reaction as a result of the increase in kinetic energy of the molecules that react. But with the passage of enzymatic reactions, the maximum point will be reached, in this case at the optimum temperature and after the reaction rate will decrease with an increase in temperature.

3.4 . Effect of Substrate Concentration

Based on the results obtained in this study, an increase in activity *Chitosanase* of soluble chitosan concentration of 0.5%, 0.75% and a maximum of 1% with consecutive activity was 0.17 U/mL, 0.325 U/mL and 0.401 U/mL. Activity began to decline, but not significant at concentrations of 1.25% and 1.5% as shown in Figure 5.

This shows that the concentration of the substrate also affects the speed of the reaction catalyzed by the enzyme . When the concentration of the substrate is increased at a fixed enzyme concentration, reaction speed will increase until it reaches a certain point (optimum concentration). In this study, the maximum velocity occurs at the substrate concentration of 1% with the activity of 0.401 U/mL but at a greater concentration is relatively constant. This is due to the substrate concentration limits specified, all parts have been met by the active enzyme substrates or have been saturated with the substrate. Therefore, even if the substrate concentration does not lead to the more magnified the resulting increased amount of activity [24].

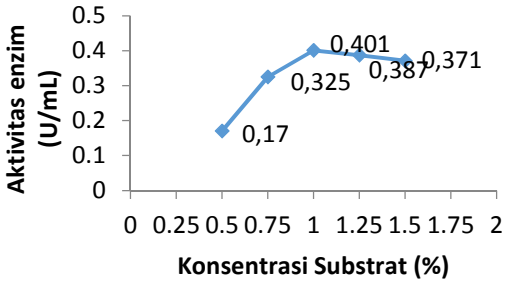


Figure 5. Effect of substrate concentration on the activity of crude chitosanase of bacteria *Klebsiella sp*

3.5. Effect of Metal Ion Addition

One substance that can function as activators or inhibitors in the process of enzyme catalysis is a metal ion. According to Palmer (1991) referred to under Natsir (2010) at certain concentrations of metal

ions can serve to activate the enzyme (as activator) and at certain concentrations also can inhibit the action of the enzyme (as inhibitors). In this study, the addition of metal ions or divalent cation at a concentration of 10 mM may increase the activity of crude enzymes *Chitosanase* of *Klebsiella sp* and also there is a slow down. In the graph (Figure 6) looks *Chitosanase* crude extract without the addition of metal ions which are control has a relative activity of 100%. Of the six metal ions are added, each of the three ions that enhance and inhibit the enzyme activity *Chitosanase* that Co2+, Ca2+ and Ni2+ with consecutive relative activity 116%, 136%, 212% (the highest relative activity). While the metal ions that give effect to the activity penghabatan *Chitosanase* is Zn2+, Mg2+, and Cu2+ with relative activity respectively 24%, 16% and 40%. Metal ion can function as an enzyme cofactor because it can play a role in the binding of the enzyme to the substrate to stabilize the active conformation of the enzyme [7].

3.6. Enzyme Stability Effect of pH Against Chitosanase

Chitosanase enzyme stability in this study, known by preinkubasi for 120 minutes at a temperature of 40 ºC with a pH of 6.0; 7.0 and 8.0. Based on test results (Figure 7) shows that crude extract chitosanase of *Klebsiella sp* at pH 6.0 is stable until the 90th minute with activity of 0.132 U/mL (relative activity 122%) and decreased to 86% at 120 minutes.

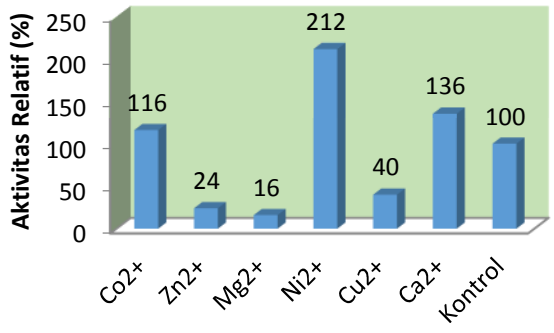


Figure 6. Influence of some metal ions (divalent cation) to extract the visible activity *Chitosanase* of *Klebsiella sp*

At pH 7.0 crude extract chitosanase can also be stable until the 90th minute with activity of 0.371 U / mL (95.9% relative activity) and minute-120 decreased by 59.9%. Whereas at pH 8.0 activity of the crude extract chitosanase can only survive until the 60th minute with the activity of 0,495 U / mL (relative activity 106, 7%) and declined to 40% in the 90th minute and then to 26.7% in minutes 120th.

This shows that the crude extract chitosanase of *Klebsiella sp* stable at pH 6.0 and 7.0, while at pH 8.0 can not maintain its conformation that causes the enzyme active site can not work optimally.

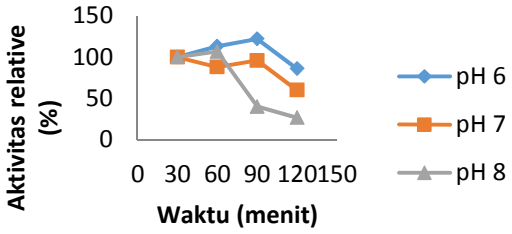


Figure 7. Effect of pH 6.0 ; 7.0 and 8.0 on the stability *Chitosanase* crude) of bacteria *Klebsiella sp* conditions : substrate concentration of 1% with a temperature of 40 ºC

3.7. Production of Enzymes Chitosanase Chitooligosaccarides

In this study the production chitooligosaccarides done enzymatically by using Chitosanase of *Klebsiella sp* isolates that have been produced. Chitosanase enzyme can hydrolyze chitosan and chitin, but the best is in the form of soluble chitosan [9]. Chitosan is used for the production chitooligosaccarides in this study had a DD of 65.6%

Hydrolysis of chitosan becomes chitooligosaccarides enzymatically affected by the chitosan concentration, temperature and pH, and agitation during the reaction. Chitooligosaccarides produced using 1% soluble chitosan reacted with Chitosanase enzyme at a temperature of 40 °C and pH 8 for 1, 2 and 3 hours in a shaking incubator. This is done so that each variation of time has the same initial conditions . Which is expected to produce oligomers with degree of polymerization different.

Dry Kitooligomer obtained after drying with freezedryer had higher levels of 57% and dissolved in acetic acid 0.25% to 0.5%. Chitooligosaccarides thus formed is then identified using thin layer chromatography (TLC).

Based on the results of the TLC observations, a product of chitosan hydrolysis with enzymes Chitosanase of

bacterial isolates of Klebsiella sp consisting of monomer to oktamer especially chitooligosaccarides at 2 and 3 hours of incubation. While the 1 hour incubation are not separate properly because the monomer chain is still longe . Oligomer cutting results are less specific and diverse, influenced by the enzyme solution used in the form of crude extract and its substrate is chitosan with a low degree of deacetylation.

Table 2. Intrinsic viscosity value and molecular weigh of Chitosan and *chitooligo saccarides* each incubation time

Samples	Intrinsic Viscosity [η]	Molecular Weigh (g/mol)
Kitosan	0,78	25.409,72
Chitooligosaccarides 1 jam	0,195	4.103,12
Chitooligosaccarides 2 jam	0,09	1.483,48
Chitooligosaccarides 3 jam	0,07	1.065,79

Chitooligosaccarides have been obtained for each incubation time is measured viscosity so that molecular weight range chitooligomer obtained can be determined by using the equation Mark-Kun-Houwik. Measurement of viscosity in Table 2, was obtained intrinsic viscosity chitooligosaccarides incubation 1 hour, 2 hours and 3 hours, respectively, namely 0.195; 0.09 and 0.07. In this case there has been a decline in the value of the intrinsic viscosity of chitosan after incubation for 1 hour (75%), 2 hours (88.5%) and 3 hours (95%). Therefore, the intrinsic viscosity is proportional to molecular weight, so as to decrease the intrinsic viscosity of chitooligosaccarides then there is also a decrease in molecular weight, wherein the percentage decrease in molecular weight from the weight of chitosan early after incubation of 1 to 3 hours in a row of about 84%; 94.2%, 95.8%. This indicates that there has been a termination of long-chain bond on chitosan, precisely on the bond β (1-4) glucoside form chitosan chains are shorter.

4. CONCLUSION

Chitosanase enzyme production of bacteria *Klebsiella sp* using a fermentation medium containing 0.5% colloidal chitosan for 60 hours with the activity of 0.309 U/mL (5,235 U/mg). Crude extract chitosanase enzyme optimum insulation results in a temperature of 40 °C and pH 8, is stable at pH 6-7, activated by Co^{2+}; Ca^{2+} and Ni^{2+} and inhibited by Zn^{2+}; Mg^{2+} and Cu^{2+}.

Chitooligosaccarides obtained in the form of a mixture of monomer to oktamer soluble in acetic acid 0.25% to 0.5%, having intrinsic viscosity decreases with increasing incubation time with a molecular weight range of 4103.12 g/mol (incubation 1 hour); 1483.48 g/mol (incubation 2 hours) and 1065.79 g/mol (incubation 3 hours).

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