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Research Article

Performance Comparison of α- and β-Amylases on Chitosan Hydrolysis

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The low solubility in common solvent and high viscosity resulting from its high molecular weight (MW) with fiber-like structure prevents a more widespread use of chitosan. This paper presents a performance comparison of nonspecific, commercially available enzymes, α- and β-amylases, for the hydrolysis of chitosan to lower its MW. The results showed that both enzymes demonstrate the ability to be used as catalysts in chitosan hydrolysis with β-amylase having better performance than α-amylase. The chitosan hydrolysis was influenced by not only the enzyme and the chitosan characteristics but also the hydrolysis condition. The optimum pH solution was 4 for α-amylase and 5 for β-amylase. The hydrolysis temperature was found to be optimal at 90 and 50°C for α- and β-amylases, respectively.

1. Introduction

Chitosan is a natural polysaccharide composed of β-(1-4) linked 2-amino-2-deoxy-D-glucopyranose and 2-acetamido-2-deoxy-D-glucopyranose, which is nontoxic, biodegradable, and biocompatible. Chitosan is commercially produced by deacetylation of chitin, which can be extracted from the exoskeleton of crustaceans (such as shrimp, lobster, crabs, and fish) and cell walls of fungi [1, 2]. Chitosan has been considered as a functional biopolymer, which is widely used in various industrial applications such as food and nutrition, medical and pharmaceutical, and cosmetic industries as well as environmental and agricultural industries [1–5]. Nevertheless, its low solubility in common solvent and high viscosity caused by its high molecular weight with fiber-like structure prevents a more widespread use of chitosan especially in food and medical applications. It was reported that low molecular weight chitosan (LMWC) that has an average molecular weight within the range 5000–10000 Da exhibits better biological activities than high molecular weight chitosan (HMWC) [4, 6]. Further, this LMWC had potential as a DNA delivery system [7]. Kondo et al. reported that LMWC with 20 kDa restricts progression of diabetes mellitus and displays higher affinity for lipopolysaccharides than 140 kDa chitosan [8]. Other studies showed that LMWC exhibits special biological and chemical activities such as antimicrobial activity [3, 9, 10], antifungal [11, 12], and antitumor activity [13, 14]. Therefore, lowering chitosan molecular weight to produce more water soluble chitosan (chitosan oligosaccharides) is gaining increased importance for broadening chitosan applications especially in food and biomedical industries.

Several methods have been proposed to lower chitosan molecular weight. In general, the methods include (i) chemical depolymerization via acid hydrolysis or redox reaction using O_3, $NaNO_2$, or H_2O_2, (ii) physical depolymerization using sonic radiation or hydrodynamic shearing, and (iii) enzymatic depolymerization [15, 16]. Chemical depolymerization has several drawbacks including difficult to obtain high degree of depolymerization, harsh condition of hydrolysis (e.g., high concentration and temperature), low yield of product, and side reaction can occur especially via glucose ring modification, while physical depolymerization requires special equipment, and the resulting molecular weight cannot be well-controlled [3, 4, 15–18]. Enzymatic hydrolysis offers some advantages including mild and specific reaction condition, high yield of product, no glucose ring modification, and more facile to be controlled [3, 17, 19, 20]. Furthermore,

enzymatic hydrolysis can retain original biological properties of chitosan. As a consequence of those conditions, enzymatic hydrolysis is of more relevance from the practical point of view.

Most enzymatic hydrolysis reactions for preparing LMWC used chitinases or chitosanases. Even though these enzymes are considered to be the most specific enzymes for chitosan hydrolysis, their used in practical application is limited by their high cost and limited availability. Several commercial nonspecific enzymes were then proposed, which are cellulase [17, 20], pectinase [21, 22], pepsin [17], papain [23], and protease [4, 24]. Very recently, Wu used α-amylase enzyme and showed that this enzyme can in principle be used for chitosan hydrolysis [19]. In sum, previous reported literature has used various nonspecific commercial enzymes for hydrolyzing chitosan including a wide variety in reaction condition and chitosan characteristics. Nevertheless, it is hard to determine the best nonspecific enzyme that should be used for chitosan hydrolysis. This is due to different characteristic chitosan used (e.g., molecular weight, degree of deacetylation, and crystallinity) and different hydrolysis reaction conditions. This paper uses α- and β-amylase for chitosan hydrolysis. To date, less attention has been paid for chitosan hydrolysis using α- and β-amylase. To the best of our knowledge, no study on chitosan hydrolysis using β-amylase has been found in previous reported literature. The use of both enzymes was based on the possibility of both enzymes to hydrolyze glucosidic bonds in chitosan. The performance of both enzymes was systematically compared, and the effects of process parameters on the occurring enzymatic reaction behavior were investigated.

2. Material and Methods

2.1. Material. Raw chitosan was purchased from PT. Biotech Surindo, Cirebon, Indonesia. The characterization of this raw chitosan showed that the molecular weight was ~1725 kDa, while degree of deacetylation (DD) was ~80.4%. The protein, water, and ash contents were 1%, 11%, and 1.7%, respectively. The α-amylase and β-amylase were supplied by Novozymes A/S, Denmark. Our enzyme activity measurements showed that α-amylase and β-amylase exhibited an average activity of 104,800 and 99,800 U/mg, respectively. Acetic acid glacial (CH_3COOH), NaOH (99%), HCl (37%), $C_6H_{12}O_6$, $C_4H_4KNaO_6{\cdot}4H_2O$ (99%), $CuSO_4{\cdot}5H_2O$ (99%), and Methylene Blue (MB) were purchased from Merck. Pure water was used in all experiments.

2.2. Hydrolysis of Chitosan. One percent (1%) of chitosan solution was made by dissolving 1 g of chitosan in 100 mL of 1% (v/v) aqueous acetic acid with stirring until homogeneous solution was achieved. Either NaOH (1 M) or HCl (1 N) solution was added to adjust the pH. Then, the solution was transferred into a glass hydrolysis reactor equipped with stirring temperature control system. The crude enzyme, either α- or β-amylase (0.1 mL), was added into a reactor vessel containing 250 mL of chitosan solution at a certain temperature. A reasonable volume of reaction mixture was withdrawn from the reactor at certain reaction times for the viscosity and reducing sugar analyses. The effects of reaction time, temperature, and pH on hydrolysis reaction behavior were investigated. Hydrolysis reaction was terminated by boiling the mixture for at least 15 minutes.

2.3. Analysis. The pH solution was measured using a digital multiparameter meter (Model HI 9228, Hanna, USA), while the viscosity solution was measured using viscometer DV-II+Pro Brookfield, USA. The chitosan/hydrolyzed chitosan chemistry was analyzed using the instrument IR Prestige-21 Shimadzu, Japan. Thirty-two scans were performed at a resolution of 4 cm^{-1} and a temperature of 21 + 1°C over the wavelength range of 500–4000 cm^{-1}. The IR solution 1.5 was used to record the sample spectra versus the corresponding background spectra.

Reducing sugar expressed in terms of dextrose equivalent (DE) value was used as a performance indicator of hydrolysis reaction. DE value was estimated using the method proposed by Delheye and Moreels [25].

3. Results and Discussion

3.1. Hydrolysis Reaction Behavior as a Function of Reaction Time. The behavior of hydrolysis reaction as a function of reaction time was investigated by conducting the hydrolysis reaction up to 5 hours. The results are presented in terms of dextrose equivalent (Figure 1) and relative viscosity (Figure 2). It is seen that both enzymes displayed similar hydrolysis reaction profile with β-amylase having greater dextrose equivalent (DE) than α-amylase for the same hydrolysis reaction time. This higher DE for β-amylase is probably due to similar beta bond between β-amylase and chitosan. The increase in reaction time increases the possibility of chitosan to be degraded as indicated by increasing DE. This result is supported by viscosity measurement, which is expressed in terms of viscosity ratio (measured viscosity to initial viscosity). The viscosity of the chitosan solution decreased significantly in the beginning of hydrolysis reaction followed by relative constant value after 2 hours reaction. A control experiment that showed no significant changes in both DE and viscosity proved that both enzymes could really work for the hydrolysis of chitosan.

Polysaccharides hydrolysis by enzyme can take place via attacking an interior glycosidic bond (endoaction) or a susceptible glycosidic bond at terminal residue in a chain and successively release monomer units from the chain end (exoaction) [17, 24]. The molecular weight of polysaccharides will drop rapidly in endoaction hydrolysis and will decrease slightly in exoaction hydrolysis. Since DE and viscosity are related to the molecular weight (MW) of chitosan, Figures 1 and 2 suggest that chitosan hydrolysis using α- and β-amylase follows endoaction hydrolysis mechanism as indicated by rapid increase in DE or decrease in viscosity at the early stage of hydrolysis (<1.5 h). The dextrose equivalent (DE) increased linearly with increasing hydrolysis time at the beginning of reaction. The linear trend indicates that the degradation of chitosan bond was regularly distributed along the chitosan

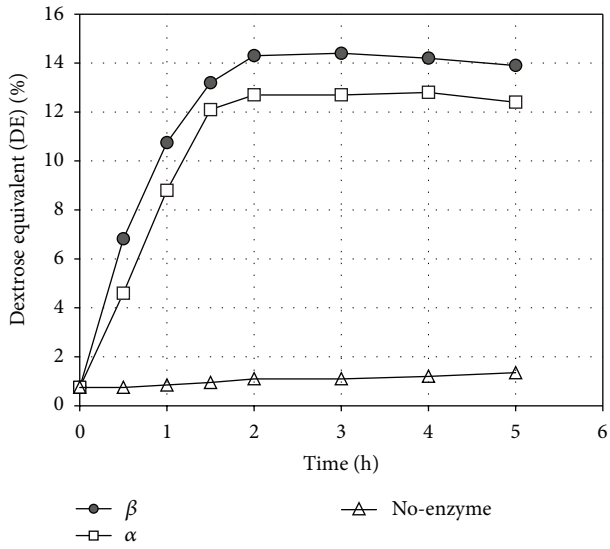

Figure 1: Hydrolysis reaction profile as a function of hydrolysis reaction time. Chitosan concentration 1%, temperature 60°C, and pH 5.

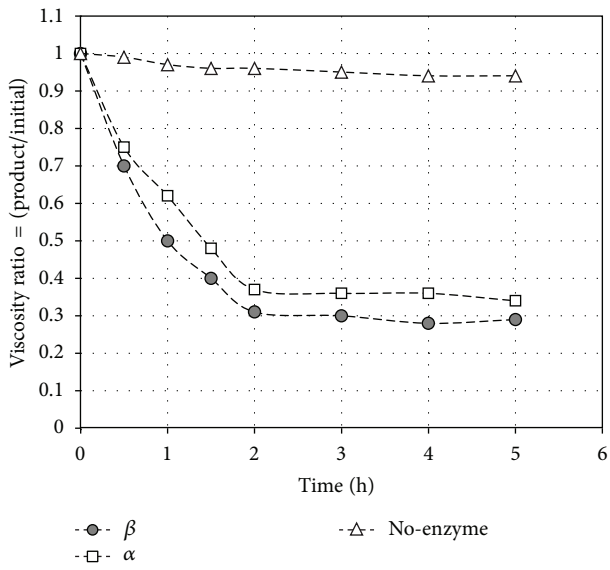

Figure 2: Relative viscosity profile as a function of hydrolysis reaction time. Chitosan concentration 1%, temperature 60°C, and pH 5.

chain (endoaction). Thereafter, slight increase in DE was observed followed by a plateau condition within the time range of 2 to 4 h. This phenomenon could be caused by decreasing enzyme activity by the end product and enzyme saturation [24]. Similar results were found by Roncal et al. [17], who investigated high DD chitosan (93%) hydrolysis using several nonamylase commercial enzymes. In addition, recombination of the two or more degraded chitosan chains is also a possible reason. This explanation is supported by decreasing DE at 5 h. In sum, the optimum hydrolysis of chitosan using α- and β-amylases was 2 h.

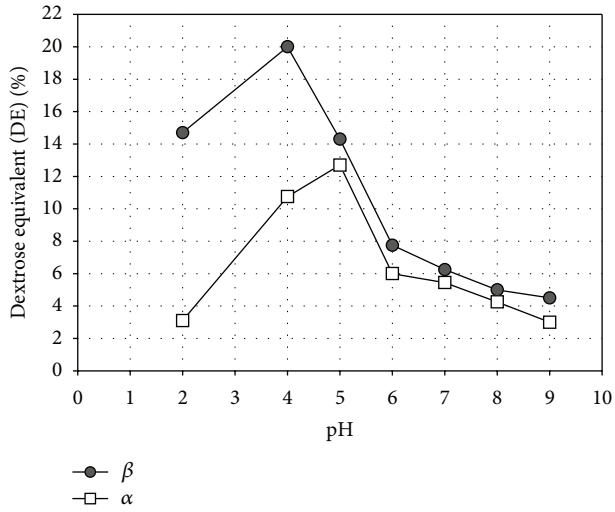

Figure 3: Influence of pH on chitosan hydrolysis using α- and β-amylase. Chitosan concentration 1%, hydrolysis reaction time = 2 h, and temperature = 60°C.

3.2. Effect of pH on Hydrolysis Reaction. It is generally known that both chitosan characteristic and enzyme activity are influenced by pH environment; therefore, the chitosan hydrolysis will also be influenced by pH solution. In this work, the effect of pH was studied within the pH range of 2–9. The results are presented in Figure 3.

It is seen from Figure 3 that the increase in pH increased DE until a maximal value at pH 5 and pH 4 for α-amylase and β-amylase, respectively. Above that pH value, the DE decreased with further increase in pH. The effect of pH on chitosan hydrolysis is mainly embodied in two respects, namely, its effect on chitosan solubility and enzyme activity. With respect to the chitosan solubility, a decrease in pH will increase chitosan solubility leading to a higher mobility and consequently higher hydrolysis reaction rate. Nevertheless, decreasing pH can also decrease an enzyme activity leading to a decrease in reaction rate. Thus, an optimum pH for hydrolysis should be different for the chitosan hydrolysis using different chitosan and enzyme characteristics. For example, Roncal et al. [17] found an optimum pH at 4.5 for chitosan hydrolysis using pepsin, whereas Wu reported that the optimum pH using α-amylase was 4 [19]. The possible reason for these different results could be difference in hydrolysis condition, enzyme, and/or chitosan used. Figure 3 also shows that at a temperature of 60°C, β-amylase could degrade better than α-amylase for all pH.

3.3. Effect of Temperature on Chitosan Hydrolysis. Beside pH, an enzyme activity is influenced by temperature. In this work, the hydrolysis reaction temperature was varied from 40 to 100°C. The results are presented in Figure 4. It is seen that the effect of temperature on chitosan hydrolysis using α- and β-amylase showed different behavior. This phenomenon indicates that both enzymes have different activity as a function of temperature. Hydrolysis using β-amylase showed that the DE increased as the temperature was increased from 40 to 50°C.

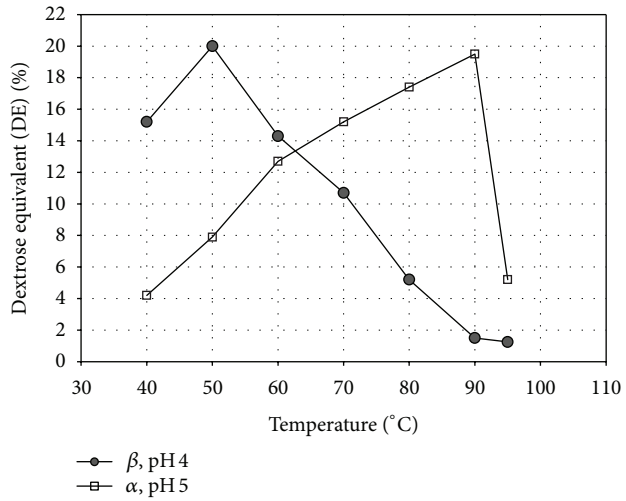

Figure 4: Influence of temperature on chitosan hydrolysis using α- and β-amylase. Chitosan concentration 1%, hydrolysis reaction time = 2 h, and pH = 4 for α-amylase and pH = 5 for β-amylase.

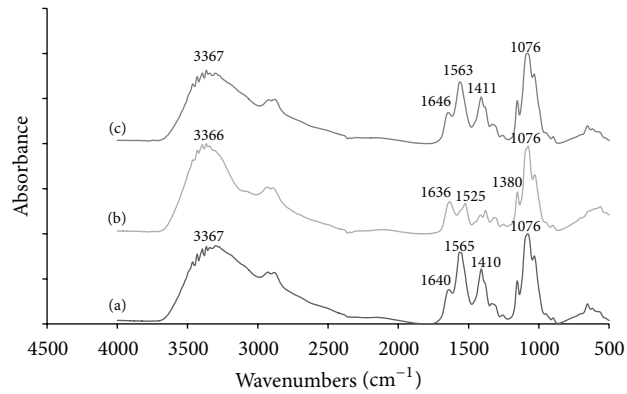

Figure 5: Chemical composition characterization of the raw chitosan (a), hydrolyzed chitosan using α-amylase (b), and hydrolyzed chitosan using β-amylase (c).

However, further increase in temperature would decrease DE. An optimum temperature for chitosan hydrolysis using β-amylase was thus considered to be 50°C. Different behavior was shown by α-amylase, where the increase in DE was observed as the temperature was increased from 40 to 90°C. The increase in temperature beyond 90°C would decrease the DE. The optimum temperature for chitosan hydrolysis using α-amylase was then considered to be 90°C. By contrast, Wu reported that the optimum temperature for chitosan hydrolysis using α-amylase was 50°C [19]. The reason for this different result is the different characteristics of the enzyme caused by different sources. This result suggests that the α-amylase used in this work should be thermophilic. It was reported in previous literature that different types of α-amylases exhibited different optimum temperatures [26].

In general, the effect of temperature on chitosan hydrolysis can be explained via competition between coalition and enzyme activity. On the one hand, the increase in temperature will accelerate the coalition between the enzyme and chitosan leading to an increase in hydrolysis reaction rate. On the other hand, the increase in temperature will first increase the enzyme activity leading to an increase in reaction rate. Further increase will provoke an enzyme inactivation and denaturation leading to a decrease in reaction rate. Thus, an optimum condition should be found from those effects.

3.4. Characterization of Product Hydrolysis. The chemistry of raw chitosan and hydrolyzed chitosan was analysed using FTIR. The results are presented in Figure 5. The FTIR spectra show that the absorption peak at ~3366 cm^{-1} indicates N–H stretching. The absorption peaks of amide (I and II) are shown at wave number of (1640 and 1565), (1635 and 1525), and (1646 and 1563) cm^{-1} for raw chitosan, hydrolyzed chitosan using α-amylase, and hydrolyzed chitosan using β-amylase, respectively. These slight differences in absorption peaks for the same functional group are probably due to the change in degree of deacetylation (DD) during hydrolysis reaction. The characteristics of absorption peak at 1380–1410 cm^{-1} are attributed to symmetrical deformation of $-CH_3$ and $-CH_2$, whereas absorption peak at ~1075 cm^{-1} indicates a stretching involving C–O functional group (typical carbohydrate). In general, FTIR spectra showed that raw chitosan and hydrolyzed chitosan demonstrated similar chemical structure. Further, no significant difference in chemical structure of the hydrolysis product resulted from enzymatic hydrolysis using α-amylase and β-amylase.

4. Conclusions

Commercial nonspecific enzymes, α- and β-amylases, have demonstrated as effective enzymes for the hydrolysis of chitosan, where β-amylase had a better performance than α-amylase. A two-hour period was considered to be an optimum hydrolysis reaction time for the hydrolysis using both α- and β-amylases. The hydrolysis reaction was influenced by pH and temperature. The optimum pH solution was 4 for α-amylase and 5 for β-amylase. The hydrolysis temperature was found to be optimal at 90°C and 50°C for α- and β-amylases, respectively. The chitosan hydrolysis performance was influenced by not only enzyme and chitosan characteristics but also the hydrolysis condition. All parameters should be considered on their impact on both enzyme and substrate characteristics.

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