Bacillus thuringiensis HCB6 Amylase Immobilization by Chitosan Beads

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Abstract. The purpose of this study was to optimize the amylase immobilization using a chitosan bead and to characterize immobilized amylase of *Bacillus thuringiensis* Bacteria HCB6. This study was started of amylase production, continued by immobilization optimization including ratio of chitosan:enzymes, enzyme-matrix contact time, substrate concentration, pH effect, incubation temperature effect, reaction time, and stability of immobilized enzyme. Amylase activity assay was dinitro salicylic (DNS) method. The results showed the optimum chitosan:enzyme ratio was 2.5: 1 (v/v), immobilization contact time of 18 hours and immobilization efficiency of 87.93%. Furthermore, immobilized amylase of *B. thuringiensis* HCB6 showed optimum substrate concentration of 1.5%, optimum pH of 6, optimum incubation temperature of 37 ° C, and the reaction time of 30 minutes. The Michaelis-Menten constant K_M value for free and immobilized amylase were 5.30% and 1.33% respectively. Immobilized amylase can be used up to five times with the remaining activity of 43.3%.

1. Introduction

Amylase is an enzyme catalyse the breakdown of glycoside bonds of starch into simple sugars. Amylase plays an important role in the industrial application. This enzymes can be produced from plants, animals and microorganisms [1]. Amylase was also reported can be isolated from a waste, for example the liquid waste of tapioca, which rich of complex carbohydrate. The amylase producer bacteria from tapioca liquid waste has been previously reported as *Bacillus thuringiensis* HCB6 [2].

Generally, enzyme production costs is relatively high, due to the complex procedure of their isolation from living organism which would increase the cost of enzymatic base process. In another side, the conventional enzymatic process of single-use enzymatic reaction was also contributed to increase the cost of enzymatic base application. One strategy to reduce the cost of enzymatic process is to use the enzyme in immobilized form, which allow to reuse the enzyme in several reactions. Many strategies have been reported in the enzyme immobilization, including the immobilization matrix, crosslinker and the form of immobilized enzyme-matrix. Several material for enzyme immobilization such as bentonite [3], sheparose, gelatin and chitosan were generally used [4]. Chitosan is one of the interesting material, which a natural polymer allowed high biocompatibility, low cost, renewable, and easy to obtain from

chitin by deacetylation with alkali [5]. The lower level of N-acetyl groups (<40%) provides greater solubility in pH below 6.5, nontoxic, available in different forms (powder, gel, fibers, and membranes), and easy to derivate and demonstrating high affinity protein.

Enzyme immobilization on the chitosan matrix could be performed based on the formation of chemical bonds between free amine groups of the chitosan and carboxyl groups of the enzyme, tailored with crosslinkers such as tripolyphosphate (TPP), glutaraldehyde, isocyanate derivatives, bisdiazobenzidina, N, N-ethylene bismalemida and N, N-polymethyl bisoodoaseomida. The efficiency of enzyme immobilization using a chitosan supporting matrix with TPP as a crosslinking was influenced by various factors. This report studied several factors in the amylase immobilization using chitosan matrix, such as the ratio chitosan-enzyme and contact time of enzyme-chitosan beads. Immobilized amylase was also studied including an optimal substrate concentration, pH effect, temperature effect, reaction time and stability of the immobilized amylase.

2. Materials and Methods

2.1. Materials

Chitosan from crab shell and sodium tripolyphosphate were from Sigma-Aldrich (Steinheim, Germany), acetic acid, nutrient agar, sodium chloride, starch, DNS (dinitro-salicylic acid), sodium hydroxide, potassium sodium tartrate, disodium hydrogen phosphate, hydrogen disodium phosphate, sodium acetate, citric acid and sodium citrate were Merck (Germany). Bacteria isolate of *Bacillus thuringiensis* HCB6 was collection of Biochemistry Laboratory, Department of Chemistry, Jenderal Soedirman University, Indonesia.

2.2. Apparatus and Measurements

UV-Vis spectrophotometer (Shimadzu UV-1800, Japan) was used to measure the glucose concentration using DNS method. Bacteria as amylase sources was growth in the incubator (Memmert, Schawbach, Germany). Extracellular amylase separation was perform using centrifuge (Vulcan Quantum Q100).

2.3. Amylase production

Amylase used in this study was produced from *B. thuringiensis* HCB. In brief, bacterial isolate was inoculated in a 100 ml inoculum medium, incubated on a shaker incubator for 18 hours at room temperature. This inoculum medium was then poured into 400 mL of production medium and incubated on a shaker for 48 hours at room temperature. Crude amylase was separated by centrifuging for 15 minutes, with speed of 7,000 rpm, at 4 °C. Supernatant as a crude amylase was kept at 4 °C when it is not in use.

2.4. Enzyme Assay

Determination of amylase activity was performed by using the DNS method. Solution of 400 mL crude extract enzyme was added to the sample test tube. Starch solution (1%) as a substrate of 350 mL was added in the other test tube. Both tubes were then incubated at 37 °C for 10 minutes. Both solutions were mix and incubated for next 15 min at 37 °C. DNS reagent of 750 mL was added into the solution and incubated for 5 min at 100 °C. The mixture was then cooled for 20-60 minutes. Control assay was also prepare by the absent of starch solution as substrate. The solution both samples and control was diluted with 3 mL of distilled water and mix well, continued by measuring using spectrophotometer at 575 nm. Enzyme activity was then calculated by one unit amylase activity defined as 1 µmol formation of reducing sugar per mL (0.18 mg reducing sugar) per minute

$$Amylase\ activity = \frac{reducing\ sugar\ of\ sample - reducing\ sugar\ of\ control}{0.18\ x\ incubation\ time\ (min)}$$

2.5. Chitosan bead preparation

Chitosan solution of 2% (w/v) was prepare in dilute acetic acid of 1% (v/v), dissolve with heating at 60°C and filtered. This solution was then dropped in 50 mL of 1% TPP using micropipette and allowed for 30 minutes to complete the bead formation. Beads formed was then filtered, rinse with distilled water and then stored at -20 °C overnight. The chitosan beads was then storage at 4 °C when it was not in use. Amylase immobilization was performed by activation of chitosan bead in a solution of 1% TPP for 20 minutes. The beads were separated by filtration and then rinsed with distilled water, and immerse in the amylase solution for immobilization. This amylase immobilization was performed at 4 °C for overnight.

2.6. Amylase immobilization Optimization

2.6.1. Enzyme – chitosan ratio

The amylase on the chitosan bead preparation was used the same procedure of chitosan beads preparation. The chitosan beads were then activated using TPP solution for 20 min, and soaked in the enzyme solution to allow enzyme immobilization with various volume ratio of chitosan:enzyme of 0.5: 1; 1: 1; 1.5: 1; 2: 1; 2.5: 1 and 3: 1.Enzyme-chitosan with the highest amylase activity was then selected for further study. Immobilized amylase activity was also calculated its immobilization activity by comparing to the free amylase activity.

2.6.2. Immobilization contact time

Using the optimum enzyme-chitosan ratio, the immobilization contact time was then studied. Longer enzyme-chitosan beads contact time should better enzyme-chitosan bonding. Various contact times were studied, including 6, 12, 18, 24 and 30 hours. Optimum immobilization contact time was determined by the highest activity with the shortest contact time.

2.6.3. Immobilized Amylase Activity

The immobilized amylase activity assay was performed using a simple enzyme reactor composed of substrate container, pipe flow, tube reactors, flow control valves, and products container. The tube reactor was filled with 150 units of immobilized enzyme, pre-incubation at 37 °C for 10 min. Starch solution of 1% (w/v) was filled into the substrate container which was higher position than the enzyme reactor to allow substrate flow by the gravitation. Substrate of 5 mL was allowed to flow to the reactor containing chitosan beads by opening flow controller. The process of enzymatic reactions performed at 37 ° C for 15 minutes. The flow controller was reopened after 15 minutes to remove the product which was then kept in products container. Reducing sugar release was then determined using DNS method.

2.7. Amylase characterization

Substrate concentration variations was first studied in the amylase characterization. Starch solution as substrates concentration studied were 0.5 1.0, 1.5, 2.0, and 2.5% (w/v). The substrate concentration effect was studied similar to the enzyme activity assay. Furthermore, under the optimum substrate concentration, the pH effect was also studied. Various pH 3, 4, 5, 6 and 7 were used in this study. Besides substrate concentration and pH, temperature effect on the amylase activity was also studied. The study was performed under the optimal substrate and pH with various incubation temperature of 32 °C, 37 °C, 42 °C and 47 °C. The incubation time was then also studied in the various incubation of 5, 10, 15, 20 and 25 minutes.

2.7.1. Immobilized amylase reusability
This study was similar to the immobilized amylase activity assay, under the optimum condition, the immobilized amylase was reused to catalyse of 5 mL substrate uninterrupted catalysing, which each step of 15 min incubation time. The resulted reducing sugar was tested and compared each catalysing batch relatively compared to the first enzyme reaction.

3. RESULTS AND DISCUSSION

3.1. Immobilization of Amylase
Immobilization of enzymes was performed to enhance the enzyme efficient as biocatalyst, which can be reusable. Amylase was immobilized using TPP which had multivalent anions to allow reaction between the anion groups with positive charged of amine groups of chitosan. This polycations of chitosan solution formed when it was dissolved in acetic acid [6]. The chitosan beads was prepared using an ionic gelation method which was the process of beads formation of particles by electrostatic interactions between the positive charge and negative charge polyanion polymer that act as crosslinker [6]. The chitosan beads were activated using the cross-linker to prepare the amine group needed on the enzyme immobilization [7]. The amylase immobilization was the optimized to improve the enzyme activity.

3.2. Optimization of Amylase Immobilization

Enzyme immobilization optimization studied was the enzyme-chitosan ratio. Results showed that increasing chitosan-enzyme ration would also increase the immobilization efficient from 0.5:1 to 2.5:1. However, the immobilization coefficient was decrease in the higher ratio of chitosan of 3:1 (**Fig. 1**). The addition of chitosan volume would lower the efficiency of immobilization. This decline was because the increase in support may increase of the steric hindrance and diffusion resistance of the enzymes, leading to some inhibition in catalyzing the reaction, thus, the enzymatic activity recovery gradually decreased [8].

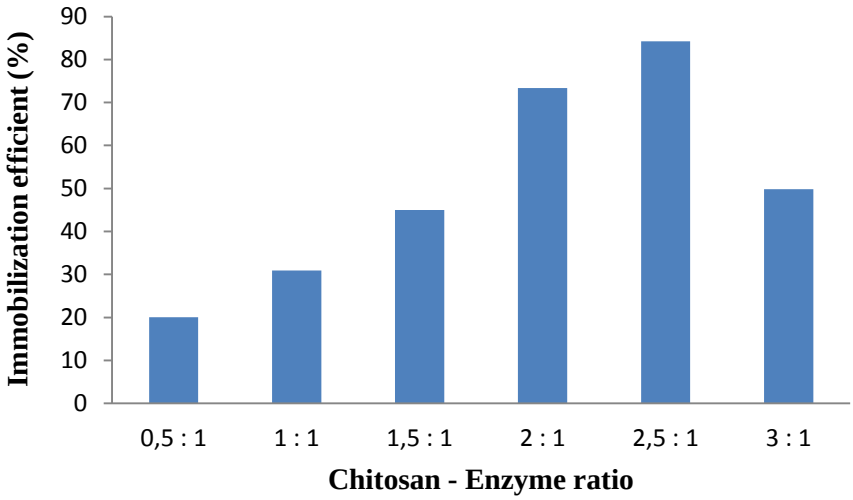

Figure 1. Enzyme-chitosan ratio effect on the immobilized *B. thuringiensis* HCB6 amylase immobilization coefficient.

3.3. Immobilization contact time optimization

Amylase immobilization efficiency increased with the increasing contact time between chitosan beads and the enzyme (**Fig. 2**). In the short time, the degree of crosslinking was not enough, the cross-linked

enzyme-chitosan was low and lead to the low immobilized enzyme activity. The higher enzyme-chitosan contact time would increase the cross-linking degree, thus, enzyme activity was also increased. Extra contact time was decreased the efficiency of immobilization, which may due to the configuration of the immobilized enzyme changed by the excessive cross-linking, promoted the denaturation of enzyme protein [9].

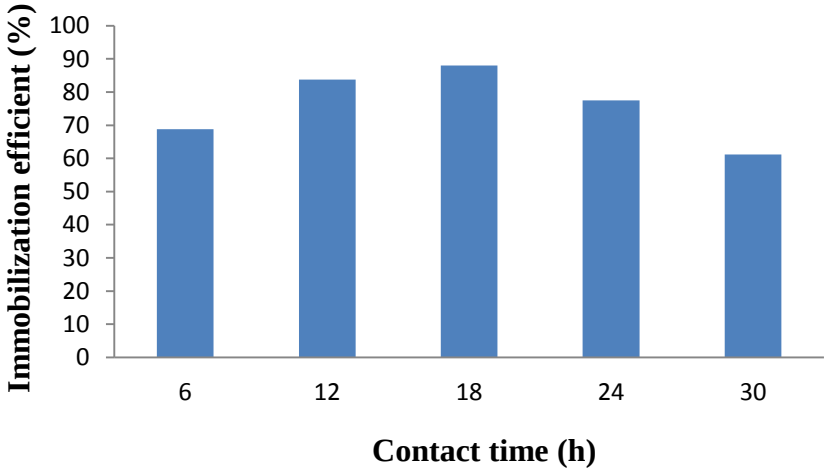

Figure 2. Immobilization contact time effect on the immobilized amylase activity.

3.4. Characterization of Immobilized Amylase

Immobilized amylase was tested in an enzyme reactor assembled using continuous flow system. This immobilized amylase was studied including substrate concentration, pH effect, temperature effect and stability.

3.4.1. The effect of substrate concentration on the immobilized amylase activity

Both free and immobilized amylase activity were increase with the substrate concentrations, up to rich their maximum enzymatic reaction rate of about 1.5% for free amylase and about 3.5% for immobilized amylase (**Fig. 3**). Furthermore, amylase kinetic constant of Michaelis-Menten constant (K_M) was calculated using Lineweaver-Burk equation, by plotting the invers of substrate concentration and inverse of enzyme activity. The result showed that K_M for free and immobilized amylase were respectively 5.30% and 1.32%. %. K_M value of the immobilized amylase was lower than that of free amylase. The lower K_M may be due to the amylase affinity change by the enzyme conformation change which was higher accessibility of the substrate to the immobilized amylase [10].

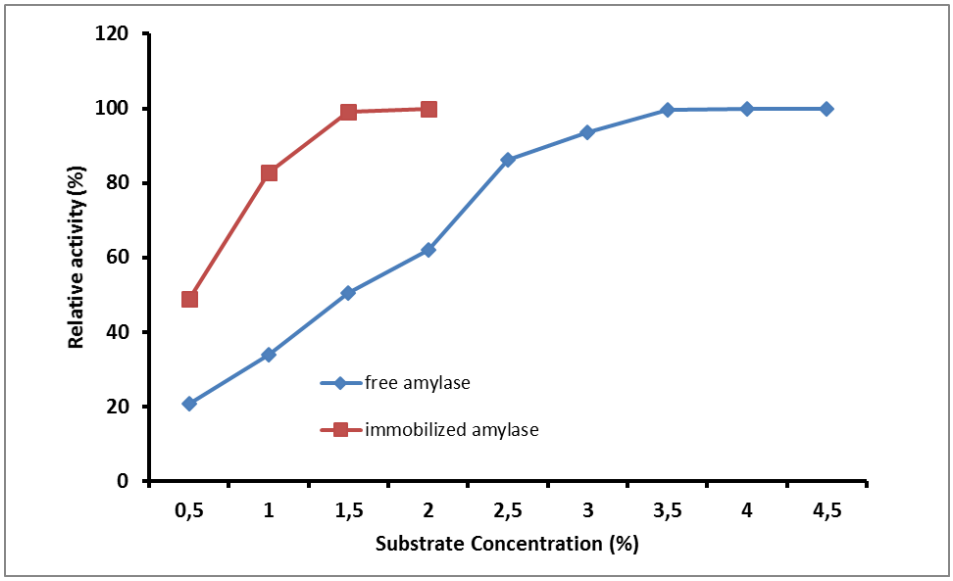

Figure 3. Substrate concentration effect on the *B. thuringiensis* HCB6 free and immobilized amylase.

3.5. *The Effect of pH on amylase activity*

The optimum pH on the amylase immobilization was shifted from 6.0 of free enzyme to 7.0 of immobilized enzyme (**Fig 4.**). This could be resulted from the change in acidic and basic amino acid side chain ionization in the microenvironment around the active site, which was the caused by the newly formed interactions between basic residues of enzyme and TPP during cross linking [11]. Shi-Lin Cao [12] suggests that the shift in the value of the optimum pH may be related to the strong interaction between the enzyme and matrices such as hydrogen bonding and electrostatic interactions. These shifts may be attributed to secondary interactions, such as ionic, polar interaction and hydrogen bonding, between the enzyme and the polymeric matrix [12].

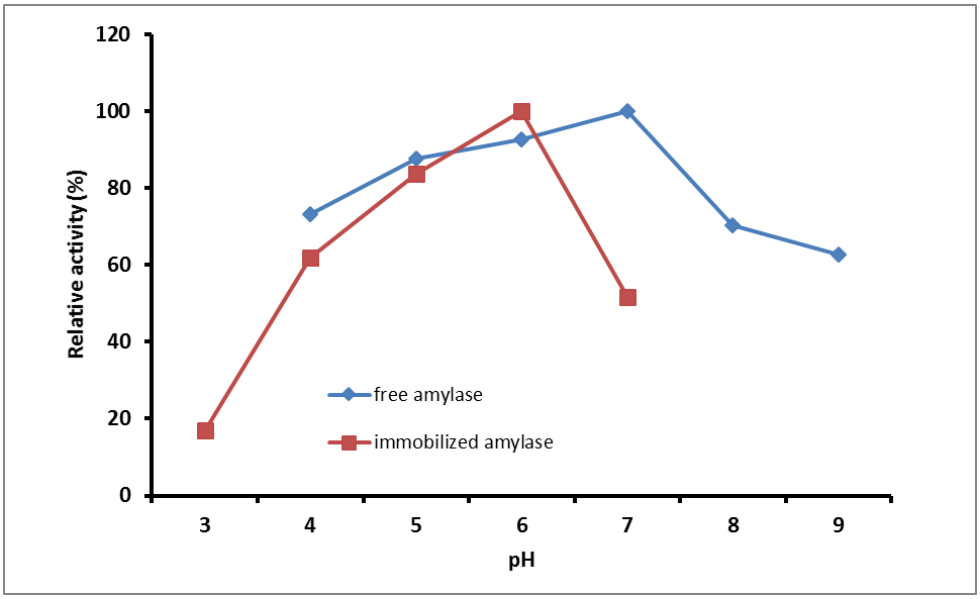

Figure 4. pH effect on the *B. thuringiensis* HCB6 amylase activity of free and immobilized enzyme.

3.6. *The effect of temperature on the amylase activity*

Both free and immobilized amylase showed optimum activity at 37 °C and subsequently enzyme activity decreased. Increasing temperature from 27 °C to 37 °C resulted increasing energy of each molecule, thus, resulted high enzymatic reaction product. However, temperature above 37 °C may started to degrade the enzyme structure, resulted lower amylase activity (**Fig. 5**). The optimum temperature of the free and immobilized enzyme was at 37 °C, which was similar to the previously reported [13-15].

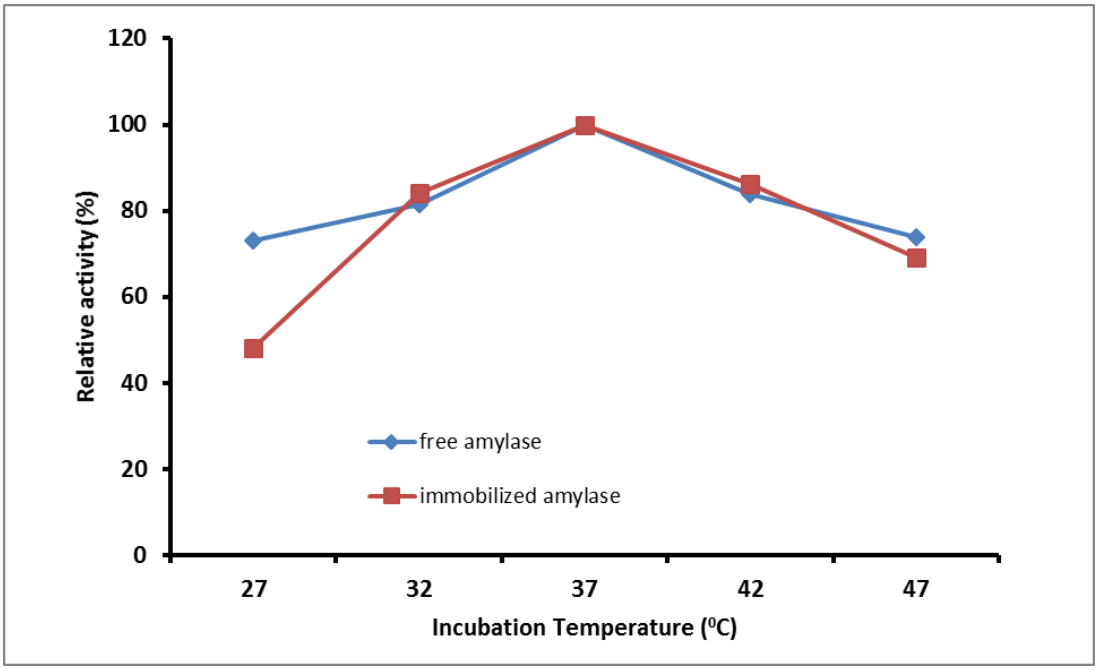

Figure 5. Temperature effect on the *B. thuringiensis* HCB6 amylase.

3.7. *The effect of reaction time on amylase activity*

The contact between the enzyme and the substrate to form a complex of enzyme substrate is affected by the reaction time, in which the short time would result in the least complex of the enzyme substrate formed and vice versa. The decreasing of amylase activity after the optimum reaction time may due to the damage of enzyme structure from environmental exposure, such temperature, light, and chemicals [16]. Immobilized amylase showed longer optimum reaction time of 30 min that that free amylase of 25 min. This may due to the diffusion effects which were caused by the supporting matrix in the form of chitosan, so the substrate-enzyme requires a longer time to contact and react [17].

3.7.1. Reuse of immobilized enzyme

The activity of immobilized amylase was then studied their stability by uninterrupted use to convert the given substrate. The immobilized activity was decrease during this reusability, with up to the fifth used of 43.3% remaining activities. This decreasing of catalyst ability in the repeatedly using may due to the release of enzymes from the matrix, which totally reduce the amount of enzyme bound on the chitosan beads.

4. CONCLUSION

Amylase from *B. thuringiensis HCB6* has been immobilized using a supporting matrix of chitosan bead, with the optimum ratio of chitosan: enzyme was 2.5: 1 and immobilization contact time of 18 hours, and resulted immobilization efficiency of 87.9%. Furthermore, immobilized amylase showed optimum substrate concentration of 1.5%, optimum pH of 6, optimum incubation temperature at 37 °C and optimum reaction time of 30 min, with amylase activity of 12.2 U / mL. Calculated K_M for free and immobilized amylase were 5.30% and 1.32% respectively. Immobilized amylase showed high stability can be used up to five times with the remaining activity of 43.3%.

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