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Original Article

Stability enhancement of *Bacillus subtilis* ITBCCB148 originating α-amylase by immobilization using chitin

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ABSTRACT

This study aimed to analyze the influence of chitin as immobilization agent on the stability of purified α-amylase obtained from *Bacillus subtilis* ITBCCB148, which was immobilized the adsorption method. A series of characterization of immobilized and purified enzymes was conducted to determine optimum temperature, pH, K_M, V_{max}, thermal stability, and reusability of immobilized enzyme. The enzymatic activity of free and immobilized α-amylase was determined on the basis of glucose formed using Mandels method, and protein content using the Lowry method. The experimental data demonstrated that the immobilized α-amylase can be used up to five times and has an optimum temperature of 75°C, K_M=6.04 mg.mL^{-1} substrate, and V_{max}=1277.14 µmol.mL^{-1} min^{-1}. Stability test conducted at 65°C for 80min indicated that the immobilized enzyme has 76.26% residual activity, which is equivalent to the unit activity of 582.61 U/mL. Purified enzyme had an optimum temperature of 65°C, K_M of 1.9 mg/mL substrate, and V_{max} of 3,508.77 µmol/mL.min^{-1}. Stability test performed at 65°C for 80min indicated that the residual activity of the native enzyme results in a stability test at 65°C for 80 min was 15.50% with a unit activity of 42.34 U/mL. The immobilized enzyme had $t_{1/2}$-value=346.5 min, k_i=0.002 min^{-1}, and ΔG_i was 115.51 kJ.mol^{-1}, while for the purified enzyme, tha data obtained were t½=28.88 min, ki=0.024 min^{-1}, and ΔG_i=105.14 kJ.mol^{-1}. Increased stability of the enzyme as a result of immobilization was also indicated by decreased value of ki and increased values of ΔGi and t½.

Keywords: α-amylase, *B. subtilis* ITBCCB148, Chitin, Immobilization

Introduction

Enzymes in the free state (solution) can react with the substrate to produce a product and then cannot be reused. This problem can be overcome by enzyme immobilization methods, because immobilization has been acknowledged to offer several improvements such as higher economic beneficial [1]; increased biomolecular stability under various reaction conditions [2]; increased endurance of the enzyme against various application variables, such as temperature, solvents, pH of the reaction system, and the presence of unwanted components (contaminants and impurities) [3-5]; easier separation of the enzyme from the product with minimized or free from protein contamination from the product; and reduced enzyme and enzymatic product costs [6]. Moreover, immobilized enzymes allow easy recovery of the enzymes from the products, repeated use of enzymes, and continuous enzymatic processes [7]. In this study, α-amylase (EC.3.2.1.1) was used is as an enzyme that is able to break down starch and glycogen molecules by cutting the glycosidic α-1,4 bonds in starch molecules (carbohydrates) to form shorter carbohydrate molecules [8]. Immobilized enzymes are stronger and more resistant to changing circumstances.

Previous studies have reported that immobilized β-amylase on Chitopearl BCW 3505 produces maltose with the activity of 142 U/g matrix [9], with the determination of optimum conditions

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and characterization of immobilized α-amylase from several types of immobilizer matrices being carried out. Immobilized amylase has an optimum activity of 179.8 U/mL using a substrate with a concentration in the range of 1.5–2.5% (w/v), at pH= 6, incubating for 20 min. It was also found that the enzyme retained its residual activity of 52.7% after six times application [8]. Glucose syrup was successfully obtained from wheat hydrolysis using α-amylase enzyme from *Bacillus cereus*, immobilized with calcium alginate, with an activity of 421.73 U/mL [10]. It was also observed that the immobilized enzyme displayed thermal stability as high as 2–3.8 times more than that of the enzyme without immobilization. To improve the stability of purified α-amylase obtained from *B. subtilis* ITBCCB148, researches have been carried out by Yandri *et al.* [11, 12], by immobilizing the enzyme on chitosan and bentonite matrices, and chemically modifying using dimethyladipimidate [13].

Another potential enzyme immobilizing material is chitin. This natural biopolymer is an attractive material for its availability in large quantity, high stability, biodegradability, non-toxicity, and low price [14]. The enzyme that binds to chitin has been reported to exhibit 335 U/g matrix activity. This is due to the stable attachment of the enzyme onto the matrix, increasing the local surface of matrix and, consequently, directly reducing steric resistance (hindrance) around the enzyme molecule and contribute to a better understanding of enzymes in regards to the structural transformation after being hosted in a confinement environment, particularly to the orientation and conformation change [15]. Systems that use chitin and its derivatives-based supports offer stability and cost-effective bioprocessing [16].

Based on the previous reports that show successful utilization of chitin as immobilizing material for enzymes, in this study, this material was used for purified α-amylase isolated from a local isolate of *Bacillus subtilis* ITBCCB148, with the main purpose to investigate the performance of the immobilized enzyme.

Materials and Methods

Materials and Microorganism

The isolate of *B. subtilis* ITBCCB148 was obtained from the Laboratory of Microbiology and Fermentation Technology ITB. Reagent grade chemicals were used in the study. The equipment used in this study were: Beckman Centrifuge, J2-21, New Brunswick Scientific Co. Gyrotory shaker Inc. Edison N. J. USA 610; Shimadzu UV-Visible Spectrophotometer; Socorex micropipette; Oven Memmert-Germany; Sartorius-Germany analytical balance; pH meter Fisher-Canada; NUOVA II-USA magnetic stirrer; Autoclave model N25X; Eppendorf pipette; and glasswares as required.

Research Procedures

Production of α-amylase

Amylase was produced in fermentation media containing starch (0.5%), yeast extract (0.5%), KH_2PO_4 (0.05%), $MgSO_4.7H_2O$ (0.02%), and $CaCl_2.2H_2O$ (0.01%) with a pH of 6.5. Fermentation temperature was 32°C with 72 h fermentation time [17, 18].

Isolation of α-amylase

α-amylase produced was isolated from the fermentation medium using a cold centrifuge at 5000 rpm for 20 min to obtain a crude extract of the enzyme [17].

Purification of α-amylase

Enzyme purification was carried out in stages, namely: fractionation of the crude extract of the enzyme with ammonium sulfate salts of various saturation levels and dialysis so that the enzyme purification was obtained [17].

α-Amylase Activity Test and Protein Content Determination

Activity test of the α-amylase was performed according to the methods reported by Fuwa [19] and Eveleigh *et al.* [20]. The Lowry method [21] was applied to determine protein content.

Immobilization of the Purified A-Amylase by Chitin

The immobilization of the α-amylase was carried out with the following procedure [22]. Chitin powder with the mass of 0.25 g was stabilized with phosphate buffer (0.1 M) with pH varied from 4.5 to 8.0, with 0.5 scale step. Using centrifugation, the matrix was recovered from the solution, followed by addition of 0.5 mL of purified enzyme and 2 mL of the buffer of specified pH. The mixture was stirred and then separated by centrifugation. The supernatant obtained was used as a control. The precipitate (immobilized α-amylase by chitin) acts as a sample to be tested for its enzyme activity using the Mandels method.

Characterization of Native and Immobilized Enzymes

Optimum pH determination

The optimum pH for the enzyme was determined by measuring the concentration of glucose produced at different pH using the Mandels method.

Determination of the optimum temperature of the native and immobilized enzymes

The optimum temperature for the enzyme was determined by measuring the concentration of glucose resulted from the experiments carried out at different temperatures of 55, 60, 65, 70, 75, and 80 °C.

Thermal stability test

A general method to determine the thermal stability of enzymes is by experimenting at different incubation times [23]. In this study, the experiments were conducted with incubation time from 0 to 80 min with a 10-minute increase. The experiments were conducted at the optimum temperature, and the glucose formed from each of the experiments was measured.

Determination of the kinetics data of the native and immobilized enzymes

To determine the Michaelis-Menten constant (K_M) and maximum reaction rate (V_{max}) of the α-amylase investigated, a series of experiments were undertaken using substrate (starch solution) with different concentrations of 0.1, 0.2, 0.4, 0.6, and 0.8% at optimum temperature for 30 min. The concentration of glucose from each experiment was measured using the Mandels method to determine the activity of the enzyme. Activity data were then used to draw the Lineweaver–Burk curve, from which K_M and V_{max} were obtained.

Reusability of immobilized enzymes

Used immobilized enzymes (reacted with 0.1% substrate) were washed using buffer solution (0.1 M phosphate) with optimum pH and then centrifuged. Immobilized enzyme precipitates were reacted with the new substrate to determine the residual activity (%) of the enzymes using the Mandels method after repeated uses.

Determination of half-life ($t1_{/2}$), inactivation rate constant (k_i), and free energy change (ΔG_i) of denatured enzyme

Calculation of k_i of α-amylase as a result of purification and immobilization was done using the kinetic equation of the first order inactivation [24].

Results and Discussion

Production and Isolation of α-Amylase

Crude extract of α-amylase obtained from fermentation at 32°C, pH 6.5, for 72 h had unit activity and specific activity of 138.65 U/mL and 1,765.25 U/mg, respectively. The native α-amylase has unit and specific activities of 1,096.53 U/mL and 28,834.13 U/mg, respectively.

Characterization of Native and Immobilized Enzymes

Determination of the optimum pH of the native enzyme

The activities of the enzyme purified using 0.1 M phosphate buffer measured at various pH are presented in **Figure 1**. The experiments were conducted at an optimum temperature of 65°C for 30 min.

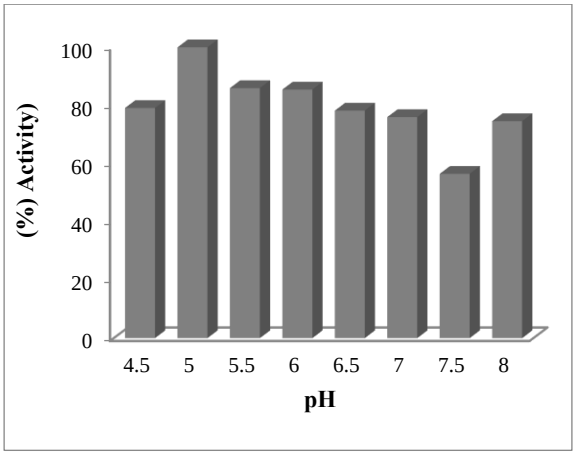

Figure 1. Residual activity (%) of native α-amylase at various buffer pH

As can be seen in **Figure 1**, the native enzyme exhibited 100% activity, which is equivalent to the unit activity of 804.00 U/mL at buffer pH of 5.0, implying that this is the optimum pH for the enzyme.

Determination of binding pH of native enzyme onto chitin matrix

The relationship between pH and the activity of the remaining immobilized α-amylase chitin matrix at various binding pH is presented in **Figure 2**, showing that the highest unit activity was displayed by the α-amylase enzyme immobilized on the chitin matrix is at pH 5.5. With this condition, 100% activity and unit activity of 563.21 U/mL were achieved, and therefore for the immobilization process, the binding pH of 5.5 was used.

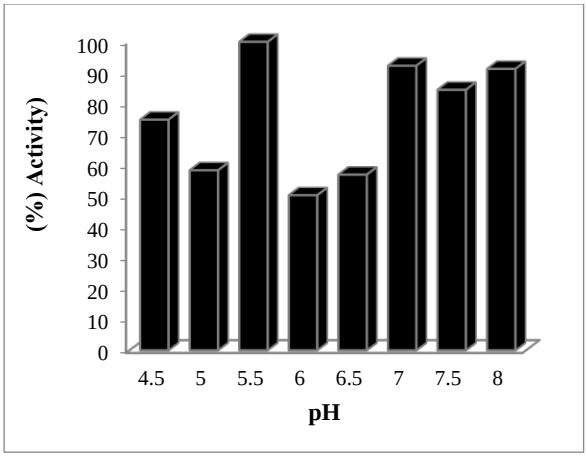

Figure 2. Residual activity (%) of α-amylase immobilized onto chitin matrix at various binding pH

Determination of the optimum temperature

Temperature is closely related to the activation energy and enzyme stability, and increasing the temperature can cause an increase in reaction speed and simultaneously increase enzyme activity. Enzymatic reactions are affected by temperature. Below the optimum temperature, the activity of the enzyme increases with increasing temperature, whereas above the optimum temperature, the opposite is true.

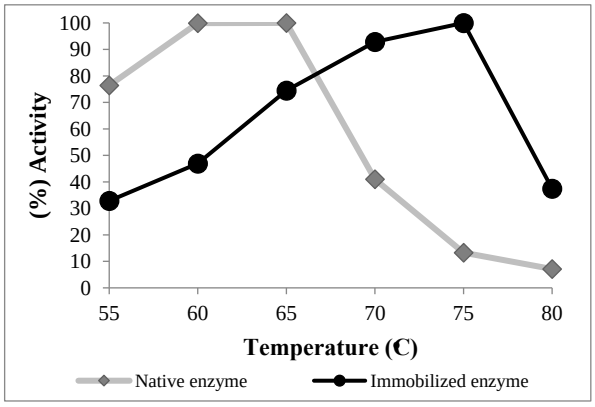

Figure 3. The activity of native and immobilized enzymes at different temperatures

To find out at what temperature the enzyme has the highest activity, the activity of native and immobilized enzymes at various incubation temperatures were determined, and the results are presented in **Figure 3**. In **Figure 3**, it can be seen that for the native enzyme, the optimum temperature of 65°C is observed, while for the immobilized enzyme higher optimum temperature (75°C) is observed. This shifting of the optimum temperature is most likely because the three-dimensional structure of the immobilized enzyme, especially its active center, is protected by chitin matrix.

The chemical bonds in the enzyme molecule play a significant role to enhance the stability of the tertiary structure of the enzyme molecule since the enzyme molecule is composed of amino acids with weak hydrogen bonds in the polypeptide structure. These hydrogen bonds are vulnerable to the increased temperature of the environment around the enzyme, causing the bonds to stretch and eventually broken. The determining factor for the ability of the enzyme to resist heat is the non-covalent forces that exist on the protein molecule, which maintains the secondary and tertiary structure of the enzyme. This force is contributed by three main factors, i.e. hydrogen bonds, electrostatic forces, and hydrophobic interactions. Other factors that also contribute to the thermostable properties of the enzyme are the interaction of non-polar groups of the amino acids and the existence of a disulfide bridge, capable of sutain the active conformation of the enzyme.

Compared to the free enzyme, the immobilized enzyme was found to have better catalytic activity. As can be seen, at 75°C, the immobilized α-amylase enzyme in chitin can still maintain its catalytic activity well. At 80°C the immobilized enzyme showed good activity with 37.47% residual activity. Free enzymes have optimum temperatures at 65°C, and at 80°C, the enzyme activity decreased dramatically with residual activity of 7.11%. Above the optimum temperature, destruction of the conformation of the enzyme might lead to a decreased activity of the enzyme, resulting in a less amount of glucose produced. The enzyme will experience denaturation at this point due to the conformation damage at high temperatures. The α-amylase enzyme immobilized in chitin has better catalytic activity at high temperatures compared to the free α-amylase enzyme. The presence of chitin as a supporting solid causes the enzyme to be protected from heat and not easily denatured so that its thermal stability increases [25].

Determination of thermal stability

The ability of the enzyme to overcome the influence of temperature can be determined by determining its thermal stability. Thermal stability is determined based on the residual activity of both enzymes investigated (native and immobilized) subjected to inactivation at optimum temperature for various incubation times, then the starch substrate was added and incubated at optimal temperature (at 65°C for the native sample and 75°C for immobilized sample) for 30 min and analyzed using the Mandels method and the remaining activity (%) was calculated. The results obtained are presented in **Figure 4**.

In **Figure 4**, it is shown that residual activity (%) of the immobilized enzyme is higher than that of a native enzyme. As can be seen for the immobilized enzyme, no significant decrease was observed in the activity of the immobilized enzymes at the incubation time 0-80 min, implying that the immobilized enzymes were protected from the influence of extreme temperature conditions by the chitin matrix, making the enzyme more stable and minimizing denaturation of the enzyme proteins. As a comparison, after 80-min incubation, the immobilized enzyme retained the residual activity of 76.26% with a unit activity of 582.61 U/mL, while for the native enzyme, the residual activity was only 15.50% with a unit activity of 42.34 U/mL. These significantly different results are in agreement with the general findings showing the advantage of the immobilized enzyme [26].

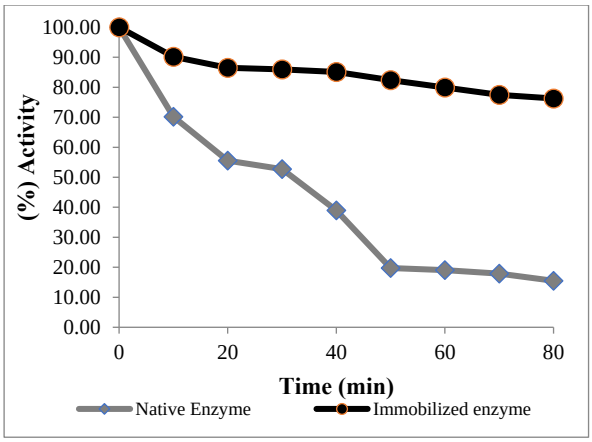

Figure 4. The activity of the native and immobilized enzyme at different incubation times

Determination of the kinetics data of the native and immobilized enzymes

The affinity of the enzyme toward the substrate is shown by the Michaelis-Menten constant K_M, and the maximum reaction rate by the V_{max}. The reaction rate reached if the concentration of the substrate was sufficient for all enzymes to form a complex with the substrate (the enzyme-substrate complex).

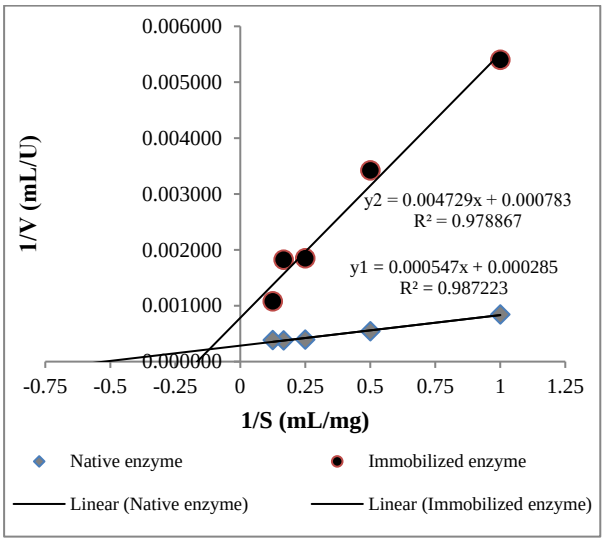

Figure 5. Lineweaver-Burk graph for the enzymes studied

Determination of K_M and V_{max} aimed to determine the substrate concentration in order to produce the maximum reaction rate. These two constants were determined based on the enzyme activity measured using the Mandels method at the optimum temperature of the native and immobilized enzymes for 30 min with various substrate concentrations. The substrate concentration used were 0.1, 0.2, 0.4, 0.6, and 0.8%. The equations derived from the Lineweaver-Burk graph presented in **Figure 5** were used to calculate K_M and V_{max}.

As displayed in **Figure 5**, the native enzyme has V_{max} and K_M values of 3,508.77 μmol mL^{-1} min^{-1} and 1.90 mg mL^{-1} substrate, while the immobilized enzyme has values of 1,277.14 μmol mL^{-1} min^{-1} and 6.40 mg mL^{-1} substrate, respectively. As can be seen in **Figure 5**, the immobilized enzyme has higher K_M, which implies that a substrate with a higher concentration is required to achieve the maximum reaction rate or the same reaction rate for pure enzymes. This higher K_M also means that the affinity of the immobilized enzyme toward the substrate was lower, most likely due to a change in the shape of the immobilized enzyme as a result of immobilization treatment, which reduced the accessibility of the active center of the enzyme [27].

V_{max} is the maximum rate of reaction of the enzyme, in which the enzyme has become saturated and therefore cannot function at a faster rate. The decrease in the value of V_{max} on the results of immobilization shows that the maximum rate of the enzyme decreases due to immobilization, which can result in changes in the shape of the enzyme.

The K_M of α-amylase immobilized on chitin is higher than that found for native α-amylase but for V_{max}, the opposite is true. This situation is caused by the changes in the conformation of the molecule of the enzyme attached to the chitin matrix, thereby reducing the combining power of the enzyme and the substrate. The immobilized enzyme reaction speed is also very low due to the effect of substrate diffusion, which is long enough to interact with the enzyme because it must pass through the chitin matrix before reacting with the active site of the enzyme.

Reusability of Immobilized Enzymes

Defined as enzymes that are physically attached to a matrix, immobilized enzymes still have catalytic activity and can be used repeatedly for continuous processing. Enzymatic conversion of potato starch using immobilized enzymes was carried out with a sample solution concentration of 0.1% and an incubation time of 30 min. The advantages of using immobilized enzymes are that it is easier to separate the resulting product, a more stable system, and reuse of biocatalysts.

After the first incubation, the immobilized enzyme was recovered by filtration followed by washing with 2 mL of 0.1 M phosphate buffer (pH 5.5) to remove any substrate and product that may have been carried away. Furthermore, the chitin immobilized amylase enzyme was inserted into a test tube filled with 0.5 mL substrate (0.1% potato starch) and subjected to 30 min incubation at 75°C. The experimental results are shown in **Figure 6**.

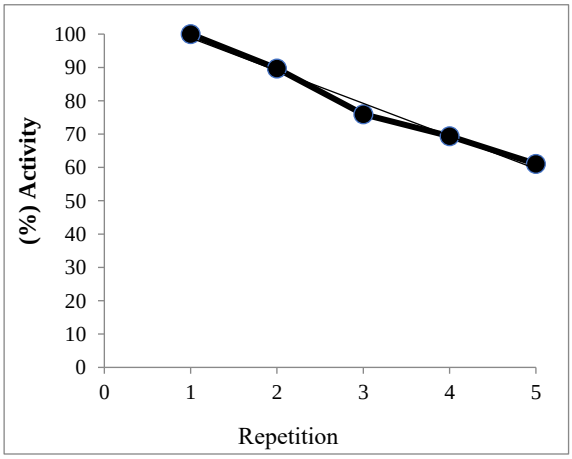

Figure 6. Reusability of α-amylase immobilized on chitin matrix

As shown in **Figure 6**, the activity of the enzyme decreases with repeated uses, from 100% to 90.26%, 77.32%, 71.09%, and 63.26% after 5 times reuse. The activity of residual enzymes in the fifth use looks quite large at 63.26%, so there is a possibility that the enzyme can still be used more than five times.

The decrease in enzyme activity resulting from immobilization on repeated use is probably due to the washing of the chitin matrix that binds to the enzyme by phosphate buffer so high molecular weight chitin is needed so that it is easier to separate during washing [16]. In addition, because there is no chemical binding between the enzyme and the chitin used, this decrease in activity occurs due to the release of enzymes from the polymer (immobilizer) that is used, so the enzyme is easily released and comes out with the products formed.

Constant Inactivation Rate (k_i), Half Time ($t_{½}$), and Free Energy Change Due to Denaturation (ΔG_i)

Figure 7 represents the determination of the k_i values of the native and immobilized enzymes, i.e. 0.024 and 0.002 min^{-1}, respectively. Based on the values of these ki, the values of half-life ($t_{½}$), and free energy changes due to denaturation (ΔG_i) of the native and immobilized enzymes can be calculated as shown in the next discussion.

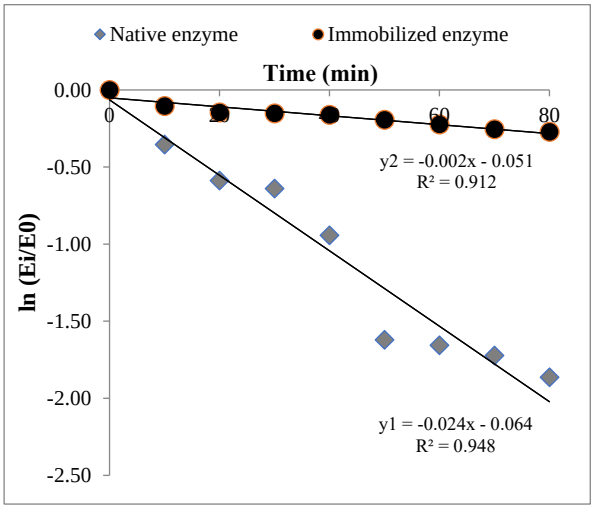

Figure 7. Graph of $\ln(E_i/E_o)$ of native and immobilized enzymes

Half-life ($t_{½}$) and thermal inactivation rate constant (k_i)

Based on **Figure 7**, it can be calculated that the value of half-life ($t_{½}$) of the immobilized enzyme is 346.50 min, while the $t_{½}$ of the native enzyme is 28.88 min, thus $t_{½}$ of immobilized enxyme has increased 12 times. The longer the half-life of the enzyme, the better its stability [28, 29], while, in addition, a decrease in the inactivation rate constant (k_i) in immobilized enzymes indicates that there is a decrease in the denaturation rate of proteins (enzymes). Half-life indicates the time at which the enzyme activity is reduced to 50% of its original activity. Thereby, the half-life is shown to be inversely related to the denaturation rate. A decrease in the value of k_i is estimated due to the condition of enzymes that is less compatible with water, leading to reduced protein unfolding and increased stability of the enzyme [28, 29]. In addition, a decrease in the inactivation rate constant (ki) in immobilized enzymes indicates that there is a decrease in the denaturation rate of proteins (enzymes) [23].

Free Energy change due to denaturation (ΔG_i)

The free energy change due to denaturation (ΔG_i) was higher for the immobilized enzyme, in which for the native enzyme the ΔG_i is 105.14 kJ mol^{-1} while for the immobilized enzyme ΔGi is 115.51 kJ mol^{-1}. The increase in the value of ΔG_i indicates that in the immobilized enzyme, the conformation of the protein structure is more folding than that for the native enzyme, which has a more rigid structure and stronger bond so that the conformation of the enzyme is not easy to open, thus maintains the tertiary structure of the enzyme, resulted in more rigid structure and less flexibility in water and therefore more energy is needed for denaturation of the enzyme [23]. Higher values of the ΔG_i and $t_{½}$, but smaller k_i, justify increased stability of the enzyme as a result of immobilization using chitin.

Conclusion

The experimental of this study demonstrated a significant improvement of the stability and reusability of the enzyme investigated by immobilization using chitin. The immobilized enzyme was found to have 10°C higher optimum temperature, and 12 times increased in thermal stability, compared to those found for native enzyme. The results also indicate that the immobilized enzyme can be reused up to 5 times with reasonable activities.

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Conflict of interest: None

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Ethics statement: We declare that this submission follows the policies of the Journal of Advanced Pharmacy Education and Research as outlined in the Guide for Authors and in the Ethical Statement. This article does not contain any studies with human participants or animals performed by any of the authors.

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