IMMOBILIZATION OF a-AMYLASE FROM BACILLUS SUBTILIS ITBCCB148 USING BENTONIT

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Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 20 (2) : 2018 : 487-492
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ISSN-0972-3005

IMMOBILIZATION OF α-AMYLASE FROM *BACILLUS SUBTILIS* ITBCCB148 USING BENTONIT

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(Received 6 Novemebr, 2017; accepted 31 December, 2017)

Key words : *α-amylase, B. subtilis ITBCCB148, Bentonite, Immobilization*

Abstract – This research aims to increase the stability of α-amylase obtained from *B. subtilis* ITBCCB148 by immobilization process using bentonite matrix. The research phases performed were: production, isolation, purification, immobilization and characterization of purified and immobilized enzymes. The results showed that the purified α-amylase had optimum temperature of 60°C, K_M = 6.18 mg mL^{-1} substrate, and $V_{max.}$ = 909.09 µmol mL^{-1} $min.^{-1}$. The immobilized α-amylase gave optimum temperature 75°C, K_M = 12.19 mg mL^{-1} substrate, and $V_{max.}$ = 88.50 µmol mL^{-1} $min.^{-1}$. The residual activities of the purified and immobilized enzymes on thermal stability test at 60°C for 100 minutes were 12 and 43%, respectively. The repetition used of the immobilized enzyme was 5 times. The thermodynamic data of the immobilized enzyme was $t_{½}$ = 88.85 min., ki = 0.0078 $min.^{-1}$, and ΔG_i 106.65 kJ mol^{-1}. Based on the decrease of k_i and the increase of ΔG_i and half-life ($t_{½}$) the immobilization process with bentonite has successfully increase the stability of α-amylase from *B. subtilis* ITBCCB148.

INTRODUCTION

α-amylase or α-1,4-glucan-4-glucanohydrolase belong to endoenzyme that catalyzes the hydrolysis reaction α-1,4 glycosidic bond on the amylose unit to produce maltose and glucose, or amilopectin to yield dextrin (Rajagopalan and Krishnan, 2008; Fogarty and Kelly, 1979). The commercial need of α-amylase annually reached 30% of the total enzyme production globally (Choubane *et al.*, 2014). This enzyme is the most required enzyme used as biocatalysator in many industrial processes such syrup, bread, candy, paper, sugar, detergent, bioethanol, drugs and waste processing (Bal *et al.*, 2016; Li *et al.*, 2012; Singh, 2014).

The weakness of this enzyme is that it is easily dissolved in water especially on batch industrial process causing the enzyme stability decreases and the enzyme is only able to be used for one cycle. This condition leads to the fact that the cost production of this enzyme is not economic. For example, the industry of amylum processing requires $622 million annually to cover the need of this enzyme (Bal *et al.*, 2016). Thus, one of the ways to reduce this cost is by immobilizing the α-amylase. The use of immobilized enzyme reduces the cost in industrial process since the enzyme can be used many times and has higher stability (Sirisha *et al.*, 2016).

The immobilization of α-amylase has been studied extensively by various methods and supporting matrices. In this work, the immobilization was performed using bentonite matrix. Bentonite is clay containing silica, aluminum oxide and hydroxide that forms two tetrahedral layers and one octahedral layer (Ohtsuka, 1997). Bentonite was chosen as supporing matrix due to some reasons that it : is insoluble in water, has big ion exchange ability, has pH range of 4-7 which is suitable to the optimum pH for α-amylase, is containing bivalent cation (Ca^{2+}) which stabilizes the enzyme, inexpensive, wide particle surface, easily activated, rigid, stable and non-toxic (Sedaghat *et al.*, 2009).

The immobilization method depends on the character of immobilizing matrix used. In this work, the enzyme immobilization using bentonite was performed with carrier binding method by physical absortion. This method was applied based on the character of bentonite matrix which is insoluble in

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water, big ion exchange power and wide interlayer surface which are able to protect the enzyme structure. In this method the enzyme will be absorbed to the matrix surface via hydrophobic bond, hydrogen bond and Van der Waals force.

MATERIALS AND METHODES

Materials

All chemicals used were of high grade (pro analysis) reagents. Local bacteria isolate *B. subtilis* ITBCCB148 was obtained from Microbiology and Bioprocess Technology Laboratory, Chemical Engineering Department, Bandung Institute of Technology, Bandung, Indonesia.

Research procedure

The following research phases were performed: production, isolation, purification, immobilization and characterization of the native and immobilized enzymes. The procedures used were based on our previous work (Yandri *et al*., 2010).

Activity test of α-amylase and determination of protein content

Activity of α-amylase was determined based on the Fuwa method using iodine reagent (Fuwa, 1954) and using dinitrosalicylic acid reagent (Mandels *et al*., 2009). The protein content was determined based on the method by Lowry *et al.* (1951).

Immobilization of purified enzyme

The immobilization of purified emzyme was performed based on the procedure used by Bollag *et al.* (1996) and Ghiaci *et al.* (2009) as follows: 0.25 g bentonite powder was stabilized with phosphate buffer 0.1 M with pH variation of 5.0; 5.5.; 6.0; 6.5; 7.0; and 7.5. The matrix was separated from the solution by centrifugation and then 0.5 mL purified enzyme and 2 mL phosphate buffer 0.1 M were added based on each pH variation. The mixture was separated by centrifugation. The enzyme was then eluted from the matrix using 2 mL mixture of phospate buffer pH 8.5 and NaCl 1 M in 1 : 1 ratio, then centriguated. The activity of supernatant obtained was then determined by Fuwa method. The enzyme bound to bentonite matrix at optimum pH binding, was added with amylum substrate and incubated at 60°C for 30 minutes. The immobilized enzyme then separated from the matrix by centrifugation for 45 minutes and the activity was tested by Mandels method.

Characterization of enzyme before and after immobilization

Determination of optimum temperature

The determination of optimum temperature of purified and immobilized enzyme was performed by varying the temperature at 55, 60, 70, 75. 80 and 85°C for 30 minutes. The residual activities (%) of the puriried and immobilized enzyme were then evaluated and compared using Mandels method.

Repetition used of Immobilized Enzymes

The immobilized enzyme which has been used washed with phospate buffer 0.1 at binding optimum pH, then centrifugated. The solid of immobilized enzyme was reacted again with new substrate. The residual activity (%) of the immobilized enzyme before and after repition used were then tested and compared using Mandels method..

Determination of K_M dan V_{max} values

The Michaelis-Menten (KM) constant and maximum reaction rate (V_{max}) values of immobilized enzyme were determined by varying the substrate concentration 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 % 60°C for 30 minutes. The enzyme activity was then measured using Mandels method. The data of relation between reaction rate of enzyme towards substrate concentration was plotted on Lineweaver-Burk curve.

The thermal stability test

The stability of enzymes was performed by measuring the residual activity of the enzyme after being incubated for 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 minutes at temperature of 60°C (Yang *et al*., 1996).

Determination of half-life ($t_{½}$), k_i and ΔG_i

Determination of ki value (thermal inactivation rate constant) of the enzyme was carried out using the first order of inactivation kinetics equation (Eq. 1) (Kazan *et al*., 1997):

$$\ln(E_i / E_0) = -k_i t \qquad (1)$$

Where E_i and E_0 are the activity of the inactivated and initial forms of the enzyme, respectively: k_i is the inactivation rate constant of the enzyme and t is the time.

The denaturation energy change (DGi) of the enzymes was performed using Eq. 2: (Kazan *et al.*

1997):

$$D G_i = -RT \ln (k_i h/k_B T) \quad .. (2)$$

Where ki is the inactivation rate constant of enzyme, kB is the Boltzmann constant, h is Planck's constant and T is the absolute temperature and R is the universal gas constant.

RESULTS AND DISCUSSION

Enzyme Immobilization

Determination of Enzyme Binding pH on bentonite matrix

The determination of binding pH enzyme-bentonite was perfored by binding the α-amylase from dialysis having the highest activity unit on bentonit matrix using phosphate buffer 0.1 at various pH. The results showed that the enzyme bound on matrix at acidic pH between 5 to 6.5. The highest activity unit of immobilized enzyme was at binding pH of 6.5.

Characterization of purified and immobilized enzymes

Determination of Optimum Temperature

The optimum temperature was determined based on the highest activity of the purified and immobiled enzymes at various incubation temperature. The optimum temperature of all enzymes is shown at Fig. 1.

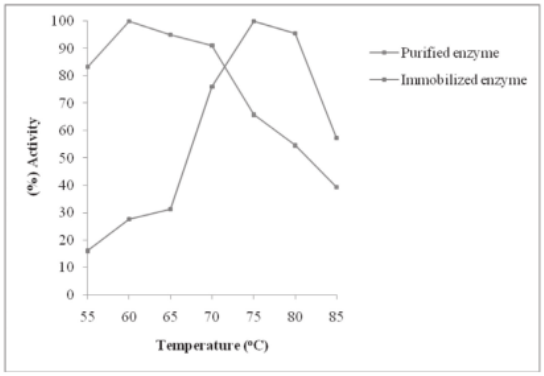

Fig. 1. Optimum temperature of purified and immobilized enzyme

It can clearly be seen that the optimum temperature of the purified enzyme was 60°C and the immobilized enzyme was 75°C (Fig. 1). These data indicate that immobilized enzyme requires higher temperature to convert substrate to the product. The shift of optimum pH is caused by the presence of steric hindrance by bentonite matrix toward enzyme molecule, thus the enzyme is protected from the denaturation effect due to heat given.

Determination of K_M and V_{max}

Michaelis-Menten (K_M) constant shows the afinity the enzyme toward substrate, while V_{max} is maximum reaction rate. When the substrate concentration is very high, so that all enzymes form complex of enzyme-substrate, thus the rate reaction is maximum. The determination of K_M and V_{max} values aiming to know the concentration of substrate to produce maximum rate reaction. These values were obtained from Lineweaver-Burk equation as seen in Fig. 2.

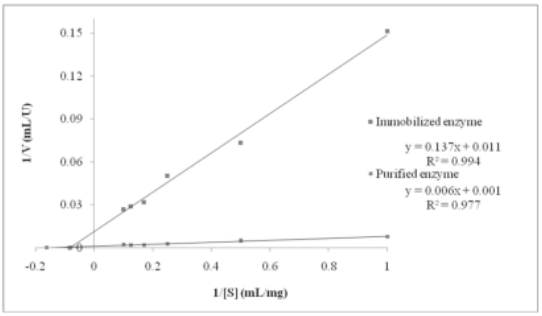

Fig. 2. The graph of Lineweaver-Burkenzim of á-amylase and immobilized enzyme

Based on Figure 2, the rate reaction of enzyme increases by the increase of substrate concentration. When the substrate concentration is increased, there will be more substrate interact with enzyme, as a result the complex of enzyme-substrate formed will also increase. Table 1 tabulates the values of K_M and V_{max} of the purified and immobilized enzymes.

Table 1. The values of K_M and V_{max} of the purified and immobilized enzymes

Enzyme	V_{max} (µmol mL^{-1} $min.^{-1}$)	K_M (mg/mL substrate)
Purified	909.09	6.18
Immobilized	88.50	12.19

The increase of K_M value on immobilized enzyme indicates that the affinity of immobilized enzyme toward substrate is lower, thus higher concentration of substrate is needed to reach the maximum rate

reaction or the same rate reaction as the purified one. The conformational change on the enzyme molecule can increase the K_M value due to the decrease of the ability to form complex between enzyme and substrate.

The conformational change of the enzyme may be caused by the chemical change on the covalent binding process of the enzyme and bentonite matrix. The big decrease of V_{max} of the immobilized enzyme relates to the hindrance of bentonite matrix on the interaction between centre site of the enzyme and substrate, a similar observation to previous result reported by Mozhaev and Martinek (1984) that enzyme immobilization has weakness since there are decrease of binding capacity and enzyme reactivity due to the mass transfer inhibition by immobile matrix.

Repeated used of immobilized enzyme

The immobilized enzyme which physically bound to a matrix is still having catalytic activity and can be used few times in continues process (Sirisha *et* 2016). The repeated used of the enzyme causing the decrease of the enzyme activity is shown in Fig. 3.

Figure 3 shows that the immobilized enzyme may be repeatedly used up to 5 times due to the decrease of enzyme activity. The decrease of enzyme activity after immobilitation process can be known from the residual activity of the enzyme after repetead used. The residual enzyme activities from the first used till the fifth used were 100; 81; 75; 49 and 40%, respectively.The decrease of activity is caused by the loss of enzyme after repeated washing.

Thermal Stability test

Thermal stability test is determined based on the residual activity of the purified and immobilized enzymes which were measured at various incubation times: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 minutes at 60°C using Mandels method. Figure 4 represents the result of thermal stability of the both enzymes.

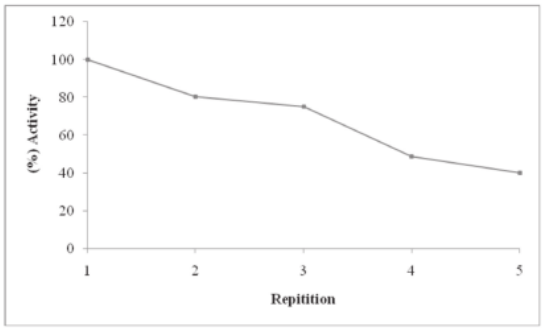

Fig. 3. The repeated used of immobilized α-amylase with bentonite matrix

Figure 4 shows that the purified enzyme has lower residual activity compared to the immobilized one i.e. 12 % compared to 43 %. This is because the immobilized enzyme was protected by immobile matrix from the extreme temperature condition, thus the immobilized enzyme is more stable. Therefore, it can be said that the immobilized á-amylase has better stability than the purified one.

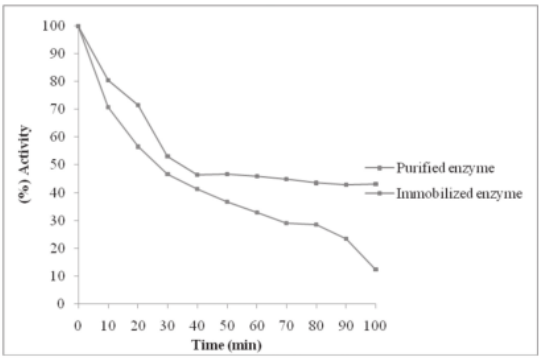

Fig. 4. Thermal stability of purified and immobilized enzyme

Determination of half-life ($t_{1/2}$), the constant of thermal inactivation (k_i), and the change of energy due to denaturation (ΔG_i) of purified and immobilized enzymes

Figure 5 illustrates the result of determination of $t_{1/2}$ value. The values of half-life ($t_{1/2}$), the constant of thermal inactivation (k_i), and the change of energy due to denaturation (ΔG_i) of purified and immobilized enzymes are shown in Table 2.

The half life of immobilized enzyme is increased 2.12 times compared to the purified one (Table 2). This means the immobilized enzyme requires longer time to decrease its activity 50%. Further more, the decrease of inactivation rate constant on the immobilized enzyme indicate the decrease of enzyme denaturation rate. The ΔG_i of the immobilized enzyme is also increased indicating that it becomes more rigid and less flexible thus the energy required to denaturate the enzyme is higher.

CONCLUSION

The immobilization with bentonite on α-amylase obtained from *B. subtilis* has successfully increased

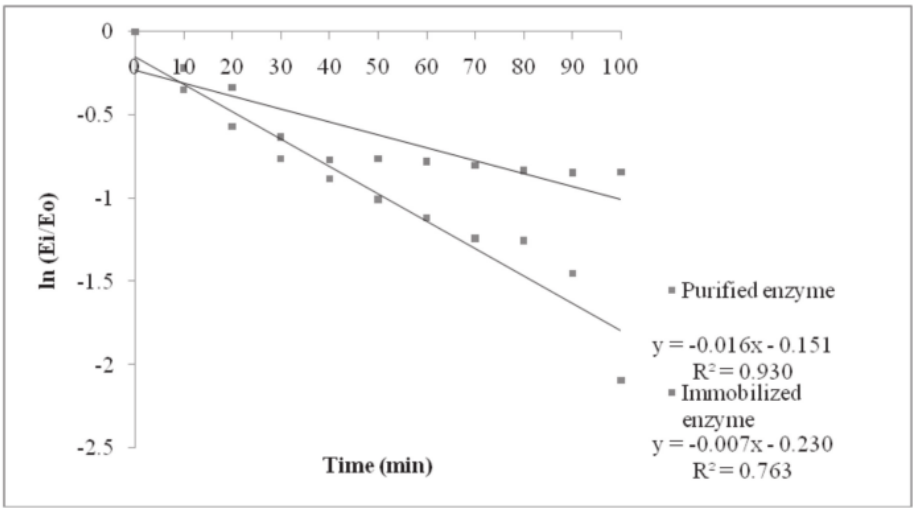

Fig. 5. The graph of $\ln(E_i/E_o)$ of purified and immobilized enzyme

Table 2. The values of k_i, ΔG_i, and $t_{½}$ of purified and immobilized enzyme

Enzyme	k_i(min.$^{-1}$)	ΔG_i (kJ/mol)	$t_{½}$ (min.)	Increase
Purified	0.0165	104.57	42.00	1
Immobilized	0.0078	106.65	88.85	2.12

the thermal stability of the native enzyme. The thermal stability of the modified enzyme was increased 2.12 times compared to the purified enzyme. The decrease of k_i value, the increase of half-life and ΔG_i values indicated that the immobilized enzyme was more stable than the purified enzyme.

ACKNOWLEDGMENTS

The authors would like to thank The Rector Universitas Lampung and Head of Institute of Research and Community Services that provided funds for this project to be undertaken through the Professor Research Grant Scheme 2017 with contract number 806/UN26.21/PP/2017.

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