IMMOBILIZATION AND CHARACTERIZATION OF *Bacillus thuringiensis* HCB6 AMYLASE IN CALCIUM ALGINATE MATRIX

AMOBILISASI DAN KARAKTERISASI AMILASE DARI BAKTERI *Bacillus thuringiensis* HCB6 DALAM MATRIKS KALSIUM ALGINAT

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ABSTRACT

Free enzyme in solution react with substrates to result in products which cannot be recovered for reuse. These problems can be overcome to a certain extent by the use of enzyme immobilization method. Immobilized enzymes are more robust and more resistant to condition changes. More importantly, the heterogeneous immobilized enzyme systems allow an easy recovery of both enzymes and products, multiple re-uses of enzymes, and continuous operation of enzymatic processes. Entrapment of enzymes in Ca-alginate is one of the simplest methods of immobilization. The aim of this research was to obtain the optimum condition of the making of immobilized amylase beads using a Ca-alginate bead and to determine its characteristics. The optimization of immobilized amylase beads includes variation of sodium alginates and variations of enzyme contact time with $CaCl_2$. The characterization of immobilized amylase includes determination of optimum substrate concentration, optimum pH, and optimum incubation time as well as amylase stability test. Amylase activity was determined by using dinitro salicylic (DNS) method. The results showed that the optimum immobilized amylase obtained at alginate concentrations of 5% (w/v), contact time of 60 minutes and immobilization efficiency of 67.5%. Furthermore, immobilized amylase showed optimum substrate concentration of 1.5-2.5% (w/v), optimum pH of 6, an optimum incubation time of 20 minutes with the activity of 179.8 U/mL. The K_M value for free amylase and immobilized amylases were 0.3 mM and 0.12 mM respectively. Vmax value for free amylase and immobilized amylases were 105.3 U/mL and 10.1 U/mL respectively. Immobilized Amylase can be used up to six times with the residual activity of 52.7%.

Keywords: Amylase, Ca-alginate, enzyme immobilization

ABSTRAK

Enzim dalam keadaan bebas (larutan) dapat bereaksi dengan substrat menghasilkan produk kemudian tidak dapat digunakan kembali. Permasalahan ini dapat diatasi dengan metode amobilisasi enzim. Enzim amobil lebih kuat dan lebih tahan terhadap perubahan keadaan. Sistem heterogen enzim amobil memungkinkan kemudahan *recovery* antara enzim dan produk, pemakaian enzim secara berulang, dan proses enzimatik secara kontinu. Penjebakan enzim dalam kalsium alginat (Ca-alginat) adalah salah satu metode amobilisasi paling sederhana. Penelitian ini bertujuan untuk memperoleh kondisi optimum pembuatan *beads* amilase amobil menggunakan matrik Ca-alginat, dan mengetahui karakteristiknya. Optimasi pembuatan *beads* amilase amobil meliputi variasi konsentrasi natrium alginat dan variasi waktu kontak enzim dengan $CaCl_2$. Karakterisasi amilase amobil meliputi penentuan konsentrasi substrat optimum, pH optimum, waktu inkubasi optimum serta uji kestabilan amilase. Aktivitas amilase ditentukan dengan menggunakan metode asam dinitrosalisilat (DNS). Hasil penelitian menunjukkan kondisi optimum pembuatan *beads* amilase amobil diperoleh pada konsentrasi alginat 5 % (b/v) dan waktu kontak 60 menit dengan nilai efisiensi amobilisasi sebesar 67,5 %. Amilase amobil mempunyai aktivitas optimum pada konsentrasi substrat 1,5-2,5 % (b/v), pH 6, waktu inkubasi 20 menit dengan nilai aktivitas sebesar 179,8 U/mL. Nilai K_M amilase bebas dan amilase amobil berturut-turut adalah 0,3 mM dan 0,12 mM. Nilai Vmax amilase bebas dan amilase amobil berturut-turut adalah 105,3 U/mL dan 10,1 U/mL. Amilase amobil dapat digunakan hingga enam kali pemakaian dengan aktivitas bersisa sebesar 52,7 %.

Kata kunci : amilase, amobilisasi enzim, kalsium alginat

INTRODUCTION

Amylase is an enzyme that catalyzes the hydrolysis of starch into simple sugars. Amylase can come from several sources, such as plants, animals and bacteria, but the enzyme from the bacteria source is preferred to use in the industrial sector (Kathiresan &

Manivannan, 2006). Amylase is widely used in industries such as food, fermentation, textiles, paper, detergents, and pharmaceutical industries (Sajedi et al., 2004). The bacteria used for the production of amylase enzyme in this study were isolated from tapioca liquid waste of *Bacillus thuringiensis* HCB6 (Zusfahair, Ningsih, Kartika, & Fatoni, 2016). Other bacteria reported their ability to produce the enzyme amylase including *B. subtilis, B. amyloliquuefacien, B. licheniformis, B. stearothermophilus, B. gavealeus, B. masentericus, B. myocodes, B. polymyxa, B. vulagates, B. aterrimus, B. stearothermophilus, B. coagulance* (Singh, Sharma, Soni, & DAS, 2011).

The use of the enzyme in the free state can only be used for one reaction. This is because of the enzyme soluble in water so difficult to separate back with the substrate to be repeatedly used. One innovation that can be done to overcome these weaknesses, namely the enzyme immobilization method, a method for placing the enzyme in a particular space and can withstand the catalytic activity so that it can be used continuously.

There are four methods of immobilization of enzymes that can be applied: adsorption, covalent binding, entrapment (entrapment) and microencapsulation. Each method has advantages and disadvantages of each. The immobilization method selection is usually based on considerations of economy and the stability of the enzyme activity. Immobilization method used in this study was entrapment. Enzyme entrapment method is more widely used than other methods because the enzyme exists in a free state and is not bound to the supporting matrix, so that the relative catalytic function and the natural structure of the enzyme molecule is not impaired. Entrapment method of enzymes immobilization require a short time preparation and also low cost. Immobilization using solid support matrix can increase resistance to various environmental changes such as pH or temperature (Homaei & Etemadipour, 2015).

One of the supporting matrices that can be used is alginate. The advantage of alginate as a supporting matrix in the immobilization of enzymes which are non-toxic, high stability mechanism, high porosity, simple procedure and relatively cheap (Anwar, Qader, Raiz, Iqbal, & Azhar, 2009). Alginate can be used as a supporting matrix for enzyme immobilization because of the alginate is an anionic polysaccharide that can form a gel when reacted with divalent cations. Ca^{2+} ion is one of the most commonly used for immobilization because of low toxicity (S. Kumar, Dwevedi, & Kayastha, 2009). Alginate has been widely used in research as a matrix supporting immobilization of enzymes including immobilization of the enzyme amylase (Riaz, Qader, Anwar, & Iqbal, 2009), protease (Anwar et al., 2009), lipase (Bhushan, Parshad, Qazi, & Gupta, 2008), urease (Maharani, Prasetyawan, & Mahdi, 2013) and glucose oxidase (Ahmad, Syaiful, & Patong, 2010)

Research on the immobilization of enzymes or cells to enhance the ability of repeated use has been widely applied. (Anwar et al., 2009) immobilized protease with Ca-alginate support matrix and protease activity obtained residual 35% in the third repetition. (Dey, Maiti, & Roy, 2015) conducted a lipase immobilization by trapping method using alginate matrix and the residual lipase activity of 72% after four times of use. (Baskar, Banu, Leuca, Gayathri, & Jeyashree, 2015) studied amylase immobilization on magnetic nanoparticles and residual activity of 75% after five times of use. Another study reported that alpha-amylase immobilized using Ca-alginate could be used six times of use and loss of activity of about 30% (Kumar, Vishwanath, Singh, & Rao, 2006). (Ahmad et al., 2010) did immobilization of glucose oxidase method using Ca-alginate entrapment and residual activity amounted to 47.06% after the fourth reaction cycle.

Various factors influence the efficiency of enzyme immobilization process using a supporting matrix of alginate. These factors include the concentration of the sodium alginate and the contact time between the beads immobilized amylase with a solution of $CaCl_2$. Optimization of these factors could improve the efficiency of immobilization process.

MATERIALS AND METHODS

Materials and Instruments

Materials used in this research were isolates of *B. thuringiensis* HCB6 Biochemistry Laboratory UNSOED collection, nutrient broth, nutrient agar, sodium chloride, starch, calcium chloride,

DNS (dinitro-salicylic acid), sodium hydroxide, potassium sodium tartrate, acetic acid, disodium hydrogen phosphate, disodium hydrogen phosphate, sodium acetate, citric acid and sodium citrate from Merck (Germany), sodium alginate, alcohol, distilled water. Intruments used in this study were a series of simple reactor system of continuous flow, autoclave (SMIC), Shaker (Kotterman 4010), Incubator (Memert), oven (Memmert), UV-Vis spectrophotometer (Shimadzu UV-1800), hot plate (Rommetsbascher), centrifuged (Quantum), analytical balance (Ohause), pH meter "Hanna Instruments", SEM (JEOL JSM type 6510-LA).

Procedures

***Production of crude extract enzyme amylase* (Zusfahair, 2015)**

Amylase used in this study resulted from *B. thuringiensis* HCB6. Bacterial isolates were inoculated in 100 ml inoculum medium (composition: Yeast extract 0.3 g, peptone 0.3 g, 1 g starch and distilled water to 100 mL), incubated in the incubator shaker for 18 hours at room temperature. Then media inoculum is poured into 400 ml production medium and incubated on a shaker for 48 hours at room temperature. Amylase crude extract was separated by centrifuging for 15 minutes, at a speed of 7,000 rpm, at 4 °C. The supernatant as a crude extract of amylase stored at 4 °C for further use.

Free state amylase activity test (Modified DNS method)

Determination of free amylase activity was performed using the DNS method. A total of 400 mL of solvent-free crude extract of amylase enzyme inserted into the sample tube. A total of 350 mL of 1% starch substrates incorporated into the control tube. Both tubes were incubated at 37 °C for 10 minutes. The solution samples were incubated for 10 minutes then add 350 mL of 1% starch substrate and then incubation was continued for 15 minutes at 37 °C. The solution in the sample and control tube was added 750 mL DNS reagent. Control tube and then added 400 mL solution of enzyme-free crude extract and mixed well. The solution in the sample and control tube is then heated in boiling water for 5 minutes, then cooled in the water for 20-60 minutes. The solution was diluted by adding 3 mL of distilled water and homogenized. Repetition done 3 times. Absorption was measured at a wavelength of 575 nm. The enzyme activity is then calculated by a unit of amylase activity is defined as the amount of enzyme that produces reducing sugar umol per minute on the experimental conditions.

Optimization of immobilization amylase

Variations in the concentration of sodium alginate

Making the beads amylase enzymes immobilized on a comparison done: sodium alginate in the buffer (1: 3) with a fixed volume of 10 mL, the amount of enzyme added about 2.5 mL and 7.5 mL of phosphate buffer. Sodium alginate is added 3% (w / v), which means that 3% of the volume of the buffer so that the powder of sodium alginate was added 0.225 grams. Making the amylase immobilized beads is done by taking the solution (a mixture of sodium alginate enzim-) using a micropipette 1000 mL then dripped slowly into a solution of 30 mL of 0.2 M $CaCl_2$, then allowed to stand with the contact time of 60 minutes at a temperature of 4 °C. Amylase immobilized beads formed and then filtered with filter paper and stored in a solution of 0.03 M $CaCl_2$ and then refrigerated and ready to be used for the hydrolysis reaction. The same steps performed to varying concentrations of sodium alginate 4, 5, 6 and 7% (w / v), then the specified value and efficiency amobilisasinya activity.

Variation of immobilization contact time

The optimum concentrations of sodium alginate beads used for the preparing of immobilized amylase with a variation of contact time between the beads immobilized amylase with a solution of $CaCl_2$. Variation of contact times used were 30; 60; 90; 120 and 150 minutes.

Immobilized amylase activity test

Amylase activity assay was performed using DNS method. Immobilized amylase of 0.5 grams (± 20 grains) in test tube was added 350 mL of 1% starch as the substrates. The tubes were then incubated at 37 °C for 10 minutes. The solution samples were incubated for 10 minutes then add 350 mL of 1% starch substrates (w / v) and incubation continued for 15 minutes at 37 °C.

Beads amylase immobilized on the sample tube were separated from the product by using a micropipette pipette products. And

control the product solution was added 750 mL DNS reagent. And control the product solution is then heated in boiling water for 5 minutes, then cooled in the water for 20-60 minutes. The solution was diluted by adding three mL of distilled water and homogenized. Absorption was measured at 575 nm and enzyme activity calculated.

Characterization of amylase immobilized

First amylase characterization was a variation of the substrate concentration. The studied was similar to the enzyme activity assay, with starch solutions as substrate concentration studied were 0.5; 1.0; 1.5; 2.0; and 2.5% (w / v). Furthermore, investigated the effects of pH and performed at optimum substrate concentration, inclunding pH of 3, 4, 5, 6 and 7. The effect of incubation time on amylase activity is also studied. Research was conducted on the optimum concentrations of substrate and pH with incubations time of 5, 10, 15, 20 and 25 minutes.

Re-usability study

The immobilized amylase activity measurement was repeated at the optimum condition. Beads amylase immobilized reused study by passing the substrate into a column reactor containing immobilized amylase and incubated for 15 minutes. The resulting product collected every 5 mL in a test tube and then tested the activity.

RESULTS AND DISCUSSION

Amylase immobilization with various concentrations of sodium alginate

Variations in the concentration of sodium alginate were used at 3, 4, 5, 6 and 7% (w/v). The results of the effect of the concentration of sodium alginate to the immobilization process can be seen in **Figure 1.** The immobilization efficiencies were low in the sodium alginate concentration of 3% and 4%, which was increased at a concentration of 5%. Furthermore, immobilization efficiency decreased with increasing concentrations of sodium alginate. A low concentration of alginate obtained pore size larger beads and less rigid, which later the entrapped enzyme amylase in the Ca-alginate gel will be easier to removed out from the matrix, finally lower the amylase activity (Anwar et al., 2009). Amylase activity immobilized on the concentration of sodium alginate 6 and 7% found to be very low, presumably due to the solution of amylase immobilized has a high viscosity, causing the pore size of beads decreases and then inhibit the transfer of the substrate into the beads amylase immobilized (Dey et al., 2015).

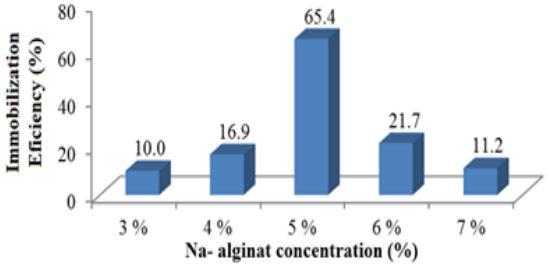


Figure 1. Amylase activity with various concentrations of sodium alginate as immobilization matrices.

Amylase immobilization with a variation of contact time

Amylase immobilization with a contact time variation performed at a concentration of 5% sodium alginate. Variation of contact time with $CaCl_2$ beads made include 30, 60, 90, 120 and 150 minutes. The effect of contact time of alginat beads with $CaCl_2$ in the immobilization process described in **Figure 2**.

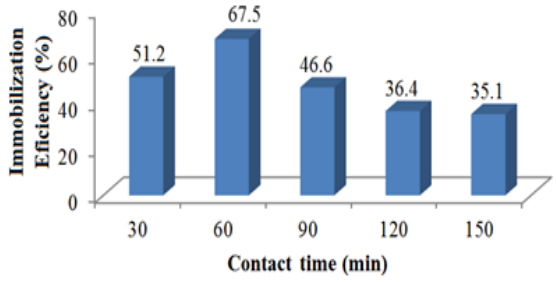


Figure 2. The relation between amylase activity immobilized with a contact time variations beads with $CaCl_2$

The results based on the physical form of beads amylase immobilized on a contact time of 30 minutes produces non-homogeneous beads which on the surface are stronger and weak bead core. This was because of the calcium can not diffuse completely into the alginate gel which finally resulted in a low enzyme activity. Extra contact time cause gel formation process will be continued towards the inside of the matrix by diffusion of calcium ions to obtain a homogenous gel at an optimum contact time of 60 minutes. The efficiency of immobilization fell back when the contact time of 60 minutes. This is due to the increased mechanical strength of the beads

immobilized amylase, causing damage to the conformation of the enzyme (Taqieddin & Amiji, 2004).

Characterization of enzyme

Amylase enzyme characterization includes determining the optimum substrate concentration, Vmax value, KM, optimum pH and optimum incubation time. Characterization of amylase immobilized done using a simple reactor in a continuous flow system.

The determination of the optimum substrate concentration

The determination of the optimum substrate concentration of amylase immobilized done on the variation of the substrate concentration of 0.5; 1; 1.5; 2 and 2.5% at 37 ° C, pH 7, and incubation period of 15 minutes. Effect of substrate concentration on amylase activity can be seen in **Figure 3.**

The optimum substrate concentration of free amylase enzyme was at concentrations of 3.5-4.0%, while the immobilized amylase was at concentrations of 1.5-2.5%. The optimum substrate concentration value used for further research. Furthermore, the value Vmax and KM were determined by plotting 1/V *versus* 1/[S] (**Figure 4)**.

The result showed the relation between 1/V with 1/[S] Vmax values obtained amounted to 105.3 U/mL on free amylase enzyme and 10.1 U/mL in the immobilized enzyme amylase. KM value obtained in free state amylase enzyme is 0.3 mM while the amylase enzyme immobilized by 0.12 mM. Generally, the value of KM enzyme showed increasements in immobilization. However, in this research is interesting that the values of amylase immobilized KM decline. Similar results were found by (Kara, Demirel, & Tümtürk, 2006) which this may be due to the electrostatic interactions between the substrate and the polymer matrix.

Determining the optimum pH

Determination of optimum substrate pH was carried on a substrate concentration of 1.5% with a variation of pH 3-7. Effect of pH to amylase activity was describe in **Figure 5**. **Figure 5** showed the optimum pH of substrate amylase immobilized and free state obtained at pH 6 and 7.

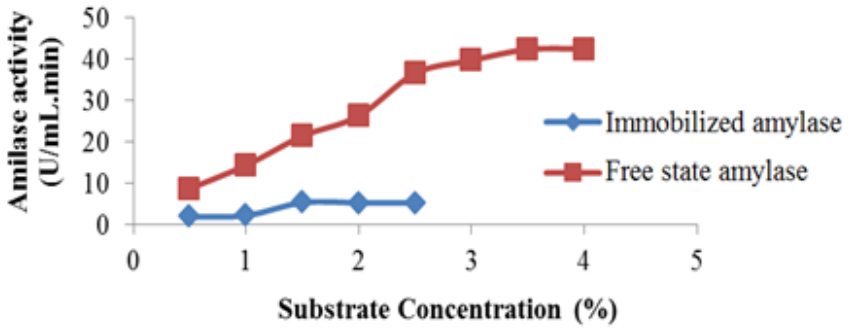


Figure 3. Effect of substrate concentration on the activity of amylase from *B. thuringiensis* HCB6

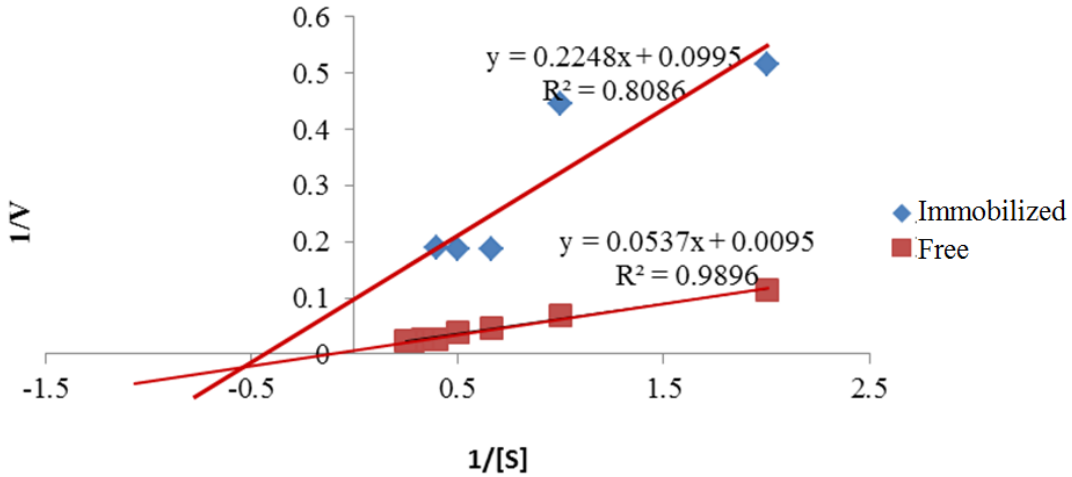


Figure 4. The relation between 1/v with 1/[S]

These results indicate that the optimum pH for amylase immobilized shifted slightly to the acid side. It has been observed in soybean (Glycine max) after immobilized urease calcium alginate (S. Kumar et al., 2009). In general, the immobilization of certain enzymes in the polycationic support matrix will result in a shift in the acid pH optimum. This shift can be explained by the theory of electrostatic potential, a matrix of calcium alginate is carrying a positive charge, so that the electrostatic potential in the immobilized enzyme was higher than in solution, the concentration of $[H^+]$ in the immobilized enzyme will be lower in a solution that causes the value of the optimum pH shifts to acid (Li & Li, 2010). The optimum pH value shifts also caused by secondary interactions between enzymes with a polymer matrix such as ionic interactions, polar interactions and hydrogen bonding (Kara et al., 2006).

In general, the nature of the enzyme molecule can be modified by the environment. The immobilized enzyme in solution can have different optimum pH of the enzyme free state as it moves on a solid matrix. Depending on the surface, and residual charge on the solid matrix and the properties of the immobilized enzyme, pH around the enzyme molecule can change, causing a shift in the optimum pH of the enzyme (S. Kumar et al., 2009).

Determining the optimum incubation time

Determining the optimum incubation time amylase immobilized performed on a substrate concentration of 1.5%, a pH of substrate 6, and the variation of the incubation time include 5, 10, 15, 20 and 25 minutes. Effect of incubation time on the amylase activity can be seen in **Figure 6**. The activity of the enzyme amylase both in a free state and immobilized hydrolyze the starch substrate at an optimum incubation time of 20 minutes. Short of incubation time results only slight enzyme binds to the substrate. Long of incubation time causes the enzyme active site has been saturated by the substrate so that no additional products and allows the backlash unraveling the complex enzyme substrates into the free enzyme and the substrate, which finally resulting a less product and enzyme activity.

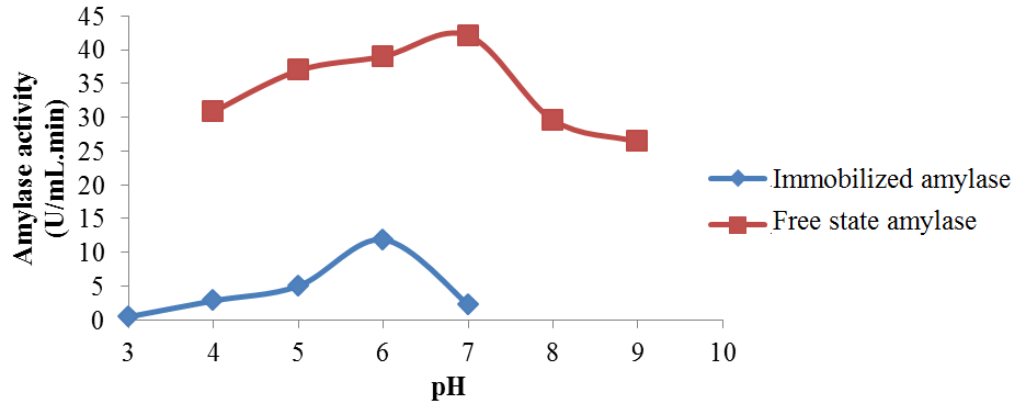


Figure 5. Effect of pH on the activity of amylase from *B. thuringiensis* HCB6

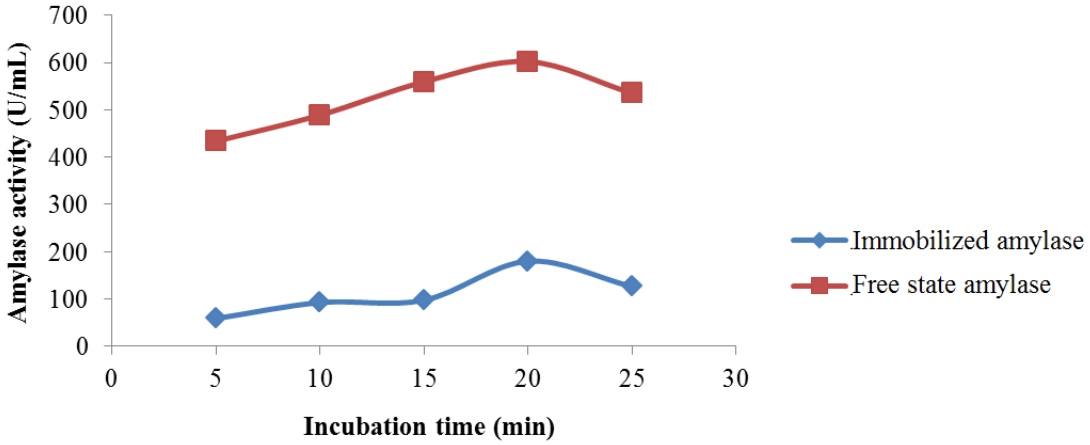


Figure 6. Effect of incubation time on the activity of amylase from *B. thuringiensis* HCB6.

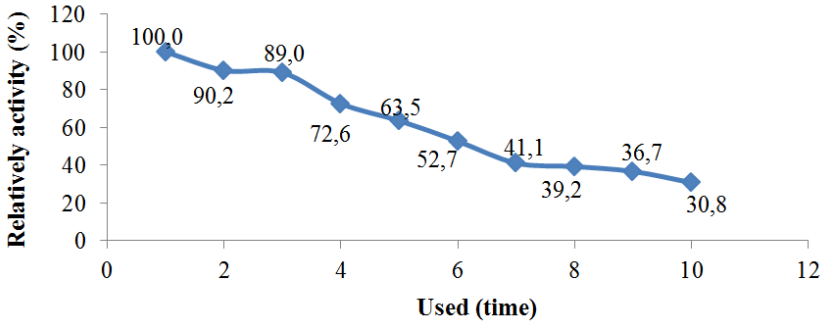
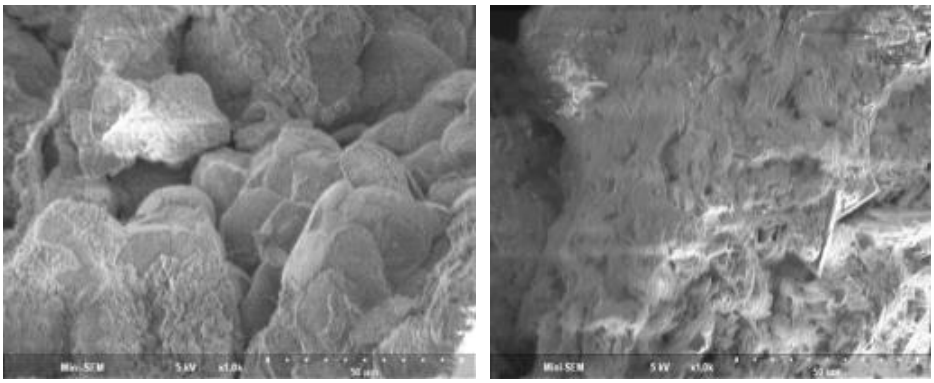


Figure 7. Repeatedly use of amylase from *B. thuringiensis* HCB6 immobilized using Ca-alginate as a support matrix.



a) b)

Figure 8. SEM morphology immobilized amylase beads of *B. thuringiensis* HCB6 using Ca-alginate matrix before (a) and after (b) repeadly use.

The use of Re-Test

Reuse of amylase immobilized beads can be seen in **Figure 7**. The immobilized amylase beads can be reused up to six times with a remain relative amylase activity of 52.7%. The decline in activity after repeated use is because of a change in the surface of the beads immobilized amylase as shown in the results of analyses using the SEM (**Figure 8**). Amylase immobilized beads surface before use corrugated causes higher surface area which can make the enzyme contact more with the substrate. Amylase immobilized beads surface more flat after repeatedly using, due to losing the enzyme on the surface resulted in the decrease of the enzyme activity. The reusability of immobilization of amylase from *B. thuringiensis* HCB6 using Ca-alginate matrix trapping method showed a potential to be applied in the industry.

CONCLUSION

The optimum conditions of amylase immobilization were the concentration of sodium alginate 5% and contact time at 60 minutes with immobilization efficiency value of 67.5%. The immobilized amylase of *B. thuriengiensis* HCB6 showed an optimum substrate concentrations of 1.5-2.5% (w / v), pH 6, incubation time of 20 minutes with the activity at the optimum conditions of 179.8 U / mL. Michaelis Menten constant (K_M) of free amylase and immobilized amylase were 0.3 mM and 0.12 mM respectively. Vmax value of free amylase and immobilized amylase were 105.3 U / mL and 10.1 U / mL respectively. Immobilized amylase of *B. thuriengiensis* HCB6 can be used up to six times with a residual activity of 52.7%.

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