Improved reuse and affinity of enzyme using immobilized amylase on alginate matrix

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Abstract. Enzyme immobilizations were widely used to increase their shelf life which is essential for the world’s industries. Therefore, amylase immobilized using Na-alginate as a matrix is necessary optimized and characterized. The parameters measured in the optimization of immobilization are the determination of the concentration of sodium alginate and contact time. Characterizations were conducted to determine the optimum concentration of substrate, the value V max, Michaelis-Menten constant (K M), pH, temperature, incubation time, and test reuse. The process of immobilized amylase activity test was performed in a continuous flow system using a reactor, and its sugar levels were determined using the Dinitro Salisilat Method (DNS). The results reveal that the immobilized amylase commercial has optimum concentration of Na-alginate of 5% (w/v) and contact time of 90 minutes with an immobilization efficiency value of 43.02%. Furthermore, the immobilized amylase has optimum activity at substrate concentrations of 3.5% (w/v), pH 4, incubation temperature of 40 °C, and a reaction time of 20 minutes with the value of the activity of 2760.4 U / mL. KM value of free amylase and immobilized amylase row are 0.18 mM and 0.15 mM, respectively. The value of KM immobilized amylase is smaller than the free enzyme. It proves that the immobilized amylase has a high affinity for the substrate. The immobilized amylase can be used up to 12 times with a value of the residual activity of 56.7%.

Keywords: enzyme amylase, immobilization, affinity, natrium alginate.

1. Introduction

Many industrial processes such as sugar, textile, paper, glucose syrup, brewing, distilling industries and pharmaceuticals have directly applied amylases as catalyst. The un-reuse able amylases are not profit for the industries. Immobilized Amylase can be used to overcome this problem. Some results have been reported on enzyme immobilization to improve the reuse of enzyme [1–5].

Enzyme entrapment within a gel matrix is one of the enzyme immobilization techniques. Enzyme a gel matrix of a specific structure permits the contact between the substrate and the biocatalyst in an appropriate way. Enzyme entrapment in calcium alginate beads has shown to be relatively safe and straightforward techniques. The enzyme immobilization using alginate beads is cheap and very easy to be applied for industrial scales such as separation between product and enzyme. The alginate matrix has been extensively used to immobilize many enzymes by entrapping [1,5–10]

Substrate concentration for enzymatic reaction is essential for the efficiency, which can be indicated by K M (Michaelis-Menten constant) value. The K M value can be used to estimate the amount of substrate. K M value is calculated by measuring the speed of the reaction catalyzed by the enzyme in different concentrations of the substrate with pH, temperature, and optimum incubation time. High K M...
value shows the lower affinity toward the substrate. Immobilized enzymes have high $K_M$ value compared with un-immobilized enzyme because the immobilized enzymes cannot freely move to reach the substrate. It is probably caused by the supporting matrix surrounded the immobilized enzyme [1,2,11–13].

In this work, characterization of immobilized amylase is conducted mainly for improving the reuse and affinity of the enzyme. The measured parameters in the optimization of immobilization are used for the determination of the concentrations of Na-alginate and the contact time between the alginate beads amylase with a solution of CaCl$_2$. Furthermore, immobilized amylase was characterized based on substrate concentration, $V_{max}$, $K_M$, pH, temperature, incubation time and the test reuse.

2. Materials and Methods

2.1 Chemicals and Materials

We used amylase from *Aspergillus oryzae* (Sigma) 36 U / mg (used 0.1 U/mL = 10 mg in 3.6 mL of phosphate buffer pH 7 0.025 M), distilled water, sodium chloride, starch, CaCl$_2$, sodium alginate, DNS (dinitro-salicylic), NaOH, Na-K tartar and buffer solution Citrate (pH 3), acetate buffer (pH 4 and 5), phosphate buffer (pH 7 and 8), Tris-HCl buffer (pH 8) as raw materials.

2.2 Instrumentation

A series of the simple reactor continuous flow system, autoclave (SMIC), Shaker (Kotterman 4010), Incubator (Memmert), oven (Memmert), hot plate (Rommetsbascher), centrifuges (Quantum), analytical balance (Ohaus) were used to synthesize the enzyme. The obtained enzyme was characterized using UV-Vis spectrophotometer (Shimadzu UV-1800), pH meter (Hanna Instruments), and scanning electron microscopy (SEM - JSM 6510-LA).

2.3 Amylase immobilization Optimization

Amylase was mixed with various percentage of sodium alginate solution (3%, 4%, 5%, 6% and 7% (w/v) of sodium alginate in 10mM of phosphate buffer) with ratio 10:1 and then extruded dropwise using a micropipette with 1000 mL sized tip into 0.2 M of CaCl$_2$ for various contact times 30, 60, 90, 120, and 150 min at 4 °C to produce beads. The beads was filtered using filter paper and stored in 0.03 M of CaCl$_2$ at 4 °C.

2.4 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 ºC for 10 min. A 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. The substrate of 5 mL was allowed to flow to the reactor containing alginate beads by opening flow controller. The process of enzymatic reactions performed at 37 ºC 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.

2.5 Amylase characterization

Substrate concentration variations were first studied in the amylase characterization. Starch solution as substrates concentration studied were 0.5 – 4.0 % (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 on the optimal substrate and pH with a various incubation temperature of 25, 30, 35, 40 and 45 ºC. The incubation time was then also studied in the various incubation of 5, 10, 15, 20 and 25 minutes.
2.6 Immobilized amylase reusability
This study was similar to the immobilized amylase activity assay. Under the optimum condition, the immobilized amylase was reused to catalyze of 5 mL substrate uninterrupted catalyzing with each step of 15 min incubation time. The resulted reducing sugar was tested and compared each catalyzing batch relatively compared to the first enzyme reaction.

2.7 Enzyme Assay
Determination of free amylase activity was performed using the DNS method. A total of 90 µL of solvent-free pure enzyme amylase (0.1 U/mL) were inserted into the sample tube. A total of 350 µL of 1% (w/v) starch substrates were incorporated into the control tube. Both tubes were incubated at 35 ºC for 10 minutes. The sample solution was incubated for 10 minutes, and then 350 µL of 1% (w/v) starch substrate was added, and then incubation was continued for 15 minutes at a temperature of 35 ºC. The solution in the sample tube and controls was added with 750 µL DNS reagent. Control tube plus 90 µL of enzyme solution and mixed well. The solution in the sample tube and the control was heated in boiling water for 5 minutes, then cooled in the water for 20-60 minutes. The solution both samples and control were diluted with three mL of distilled water and mix well, continued by measuring using spectrophotometer at 575 nm. Repetition is conducted three times. Enzyme activity was calculated by one unit amylase activity defined as one µmol formation of reducing sugar per mL (0.18 mg reducing sugar) per minute.

3. Results and Discussions

3.1. Amylase immobilization with variations of sodium alginate
Commercial amylase was successfully immobilized using various concentration of sodium alginate as a matrix. The results reveal that the best concentration of sodium alginate for the commercial immobilized amylase is 5% (w/v) as described in Figure 1. The highest immobilization efficiency, i.e., 41.3% can be achieved at this concentration. At low concentrations, the immobilized amylase beads yielded large pores led to leakage of the enzyme from the beads. The concentration of sodium alginate was increased leading to a higher viscosity of the solution for forming beads. Consequently, the pore size of the beads became smaller and thus inhibited the penetration of the substrate in beads [1].

![Figure 1: Sodium alginate concentration-dependent immobilization efficient of the immobilized amylase](image-url)
3.2 Immobilization of amylase with variations of contact time
Besides the various concentration of sodium alginate, commercial amylase was varied the contact time between CaCl₂ and beads in immobilization as shown in Figure 2. The efficiency of immobilization with the contact time of 30 and 60 minutes is still low. However, the highest immobilization efficiency value of 43.0% can be achieved at the optimum contact time of 90 minutes and afterward decreases with extra contact time. When the sodium alginate solution was dripped into a solution containing Ca²⁺, Ca²⁺ ion will expel two ions Na⁺, Ca²⁺ ions subsequently react with -COO⁻ of alginate forming immobilized amylase beads [14]. Calcium sodium ion exchange occurs only on the surface of the beads at the contact time of 30 and 60 minutes. More extended contact time, the diffusion of calcium ions into the bead can increase the strength of beads took place. Added contact time further, the activity of the enzyme decreases. It is predicted that there is damage in the confirmation of the enzyme due to the mechanical strength of the bead [15].

![Figure 2: Immobilization efficient of the immobilized amylase as a function of the contact time.](image)

3.3 Characterization of enzyme
Amylase enzyme characterization included determining the optimum substrate concentration, value \( V_{\text{max}} \) and \( K_{\text{m}} \), substrate pH optimum, optimum temperature and optimum incubation time.

3.3.1 Determining the optimum substrate concentration
Determining the optimum substrate concentration of immobilized amylase was performed under various substrate concentrations of 0.5–4% (w/v) in a continuous flow system at 37 °C, pH 7, and the incubation time of 15 minutes.
Figure 3: The effect of substrate concentrations on the amylase activity

Based on the results in Figure 3, the optimum substrate concentration of free and immobilized enzyme amylase is obtained at a concentration of 3.5% (w/v). The optimum substrate concentration value will be used for further investigation.

Figure 4: Relations between 1/v and 1/[S]

Based on the relationship between 1/V with 1/ [S], $V_{\text{max}}$ values of 357.1 U / mL.minute is obtained on free amylase enzyme and 81.3 U / mL.minute is obtained on the immobilized amylase. $K_{M}$ value obtained in free amylase enzyme is 3.4% (0.18 mM) while on the immobilized amylase the value is 2.9% (0.15 mM). A smaller value of $K_{M}$ yields a higher affinity of the enzyme to the substrate. The usage of alginate matrix as supporting the immobilization of the enzyme amylase can increase the affinity of the substrate. Generally, $K_{M}$ values of enzymes demonstrate an increase upon as shown in Table 1. However, it is interesting that the $K_{M}$ values of the immobilized amylase show a decrease upon immobilization. These results can be attributed to electrostatic interactions between the substrate and polymeric matrix [16].

Table 1. $K_{M}$ value of several enzymes

| No | Enzyme (matrix) | $K_{M}$ Free | $K_{M}$ Immobilized | Reference |
|----|----------------|--------------|---------------------|-----------|
| 1  | Poly Phenol (Bentonite) | 7.50 mM | 7.60 mM | [17] |
| 2  | Xylanase (alginate-glutaraldehyde) | 9.00 mg/mL | 14.90 mg/mL | [11] |
| 3  | Amylase (Gold nanorods) | 3.00 mg/mL | 3.40 mg/mL | [12] |
| 4  | Protease (Ca-alginate) | 130 mg/dL | 270 mg/dL | [1] |
| 5  | Urease (chitosan-alginate polyelectrolyte complexes) | 4.50 mM | 3.03 mM | [16] |

3.3.2 Determining pH of substrate and optimum incubation temperature

Determining optimum pH substrate and incubation temperature immobilized amylase was performed in a continuous flow system at the optimum condition, which was already obtained previously. Effect of substrate pH and temperature incubation on the activity can be seen in Figure 5 and 6 respectively.
Figure 5: Effect of pH substrate on the amylase activity

![Figure 5: Effect of pH substrate on the amylase activity](chart1.png)

Figure 6: Influence of incubation temperature on the amylase activity

![Figure 6: Influence of incubation temperature on the amylase activity](chart2.png)

Based on the data in Figure 5 and 6, the optimum pH of free and immobilized amylase was 4 with the optimum temperature was 40 °C. Similar findings were observed by [1] on immobilization of proteases from the newly isolated strain of *Bacillus subtilis*-HAS KIBGE where they observed no change in the pH optimum of protease before and after entrapment with calcium alginate. [6] also reported that there was no change in the optimum pH and temperature of cyclodextrin gluconotransferase (CGTase) before and after entrapment in calcium alginate beads.

Amylase from bacteria and fungi has optimum activity at acidic to neutral pH [18], [19] reported amylase from *B. cereus* MTCC 10205 has activity optimum at pH 5.5. [20] reported amylase from Bacillus sp. DR90 has optimum activity at pH 4. [4] reported the use of magnetic nanoparticles immobilization amylase has an optimum pH 4. Amylase is active at acidic pH commonly used in the industry of glucose syrup [21].

3.3.3 Determining optimum incubation time

Determining the optimum incubation time immobilized amylase was performed in a continuous flow system at an optimum substrate concentration of 3.5% (w/v), pH optimum substrate 4, and the variation of the incubation time include 5, 10, 15, 20 and 25 minutes with a free-amylase as a comparison. Figure 7 presents the effect of incubation time on the activity.
Effect of incubation time on the amylase activity was noted by varying the time course from 5 to 25 minutes. Maximum activity was found in 20 minutes of incubation, showing an increase by 5 minutes from the free enzyme. This increase in time is due to the time required by the substrate molecules to penetrate into the beads and reach the active sites of the enzyme.

### 3.4 Reuse of immobilized enzyme

Immobilized amylase in its use can be separated from the substrate and product easily because the enzyme is attached to the supporting matrix, which is not soluble in water. The nature of immobilized amylase beads is that it is easily separated from the substrate and this product makes the enzyme can be reused for further reactions. Measurement of immobilized amylase stability was performed at the optimum condition repeatedly until the decrease in amylase activity above 50%. Immobilized amylase was used again by the starch substrate tube passed into a reactor containing immobilized amylase beads, were then incubated. The products were accommodated in each period optimum contact, and then the activity test was performed. Reuse of immobilized amylase beads can be seen in Figure 8.

![Effect of incubation time on the amylase activity](image)

**Figure 7:** Effect of incubation time on the amylase activity

The immobilized amylase beads can repeatedly be used as described in Figure 8. Immobilized amylase activity remains 56.7% in the use of the twelfth. The Immobilized amylase is very potential to be used in glucose syrup industry to reduce the cost of production as it can repeatedly be used.

[4] carried out amylase immobilization on magnetic nanoparticles. Amylase activity remained over 75% after five times of use. [5] did lipase immobilization by trapping method using alginate matrix. 72% residual lipase activity after four times of usage. [1] immobilized protease enzyme with supporters of Ca-alginate matrix, and protease activity showed a trace to 35% in the third iteration. [22] conducted immobilization of glucose oxidase with entrapment method using a Ca-alginate, and...
the residual activity was 47% after application to four. The enzyme entrapped in alginate beads could be reused and retained 70% activity at the end of six cycles [23].

The immobilized amylase beads before and after usage were analyzed using SEM. The analysis shows that there are changes on the surface of immobilized amylase beads. At first, beads had rough surfaces, but after repeated usage, their surfaces become flat as shown in Figure 9. The usage causes the enzyme present on the surface of the matrix is eroded so that the amount of enzyme, which is in contact with the substrate, decreases lead to decreasing activity.

Figure 9: SEM images of the immobilized amylase bead surfaces (a) before usage (b) after usage 12 times.

4. Conclusion

Commercial amylase was successfully immobilized using various concentration of sodium alginate as a matrix. Optimum condition of the amylase with the immobilization efficiency of 43.0% can be achieved at the concentration of sodium alginate of 5% (w/v) and the contact time of 90 minutes. The amylase exhibits the optimum activity of 2760.4 U/mL at substrate concentrations of 3.5% (w/v), pH 4, incubation temperature of 40 ºC, and a reaction time of 20 minutes. It is found that the amylase can increase the enzyme affinity and repeated usage. In addition, this amylase is very promising for production process in the glucose syrup industry.

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