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ORIGINAL ARTICLE

Immobilization of Candida Rugosa Lipase on the Glutaraldehyde-Activated Chitosan Beads

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ABSTRACT – An immobilized enzyme is a biocatalyst that speeds up the conversion of a chemical reaction. The application of enzymes for chemical synthesis is an effort toward a responsible production initiative to ensure the sustainability of chemical synthesis. Therefore, in the present work, Candida rugosa lipase was immobilized onto glutaraldehyde-activated chitosan beads through covalent bond linkages. Chitosan is biodegradable and contains amine groups, which serve as bases for lipase binding via cross-linking with bifunctional cross-linkers like glutaraldehyde. The immobilization of lipase on the chitosan beads was confirmed by determining lipase activity through the hydrolysis of a standard substrate. The effect of lipase and glutaraldehyde concentrations on the immobilization and activity yield was investigated. In general, lipase and glutaraldehyde concentrations have a significant effect on immobilization and activity yield. The interaction between the investigated parameters is significant toward the activity yield rather than the immobilization yield. The optimized immobilization procedures give lipase activity up to 46 IU by using 0.013 g/mL lipase and 2 %/v/v glutaraldehyde. It was found that the immobilized enzyme was rather stable and could be recycled 7 times. Therefore, immobilization of lipase onto glutaraldehyde-activated chitosan support is feasible.

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INTRODUCTION

The enzyme is a protein that acts as a catalyst to increase the rate of reaction, and it is commonly found in plants, animals, humans, and microbes. The use of enzymes gives an advantage to chemical reactions, with a reduction in energy consumption. The application of enzymes as a catalyst for an industrial process will improve productivity. Among all the enzymes, lipase has caught manufacturers' interest as it is useful for producing various types of chemicals [1]. Lipase is commonly used in the production of biofuel, lubricants, waxes, flavours, and fragrances [2]. This is due to the versatility of lipase, which is capable of catalyzing esterification, interesterification, and transesterification reactions in nonaqueous media [3]. As a result, lipase is widely used in a variety of industrial sectors, including energy, food, detergent, and pharmaceutical [4].

Nowadays, awareness has improved on the application of enzymes for fine chemical synthesis, which offers lower energy consumption, less chemical waste generated, as well as better conversion, selectivity, and yield [2, 5]. Immobilized enzymes are preferable compared to free enzymes because the handling of the immobilized enzyme is more practical for batch and continuous processes. In addition, the immobilized enzyme is reusable, which is an added advantage for increasing productivity and reducing costs. There are several methods to immobilize the enzyme. These methods include adsorption, covalent bonding, cell-to-cell cross-linking, encapsulation, and entrapment [6].

Commonly, a covalent bond method is preferable due to the strong attachment of enzymes on the surface of supportive materials. The immobilization of enzymes by covalent bonding on polymeric support is more durable compared to the other methods since the polymeric support is mechanically stable and the enzyme is irreversibly bonded [6]. The polymeric support of natural origin is more desirable for enzyme immobilization due to its biodegradable properties and provides low environmental impact. For example, polymeric support derived from chitosan, such as chitosan beads, is highly porous and suitable for enzyme immobilization through amide bond linkages [7, 8]. It provides a high surface area for the enzyme to bind and thus could yield high activity over the mass of immobilized enzyme. In a separate investigation that was conducted by Chiou and Wu using chitosan beads, they found that the wet chitosan beads have the highest enzyme activity compared to the dry chitosan [9]. Therefore, the utilization of chitosan as a polymeric support material for enzyme immobilization appears promising.

In the present work, the chitosan was prepared by the coagulation method, which resulted in the formation of microbeads. The beads were reinforced with cross-linking by the glutaraldehyde and simultaneously provided an aldehyde group terminal for enzyme attachment. The main objective of this research is to investigate the immobilization of *Candida rugosa* lipase by using chitosan beads as support. The effect of immobilization process parameters such as lipase and

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glutaraldehyde concentrations on the performance of immobilized enzymes has been investigated. The optimum conditions for immobilization have been suggested.

MATERIALS AND METHODS

Materials and chemicals

Candida rugosa lipase (unit activity of ≤700 IU/mg), glutaraldehyde solution (25 %v/v in water), ρ-nitrophenyl butyrate (PNPB), ethylenediaminetetraacetic acid (EDTA), and chitosan were purchased from Sigma-Aldrich. The Bicinchoninic acid (BCA), cupric sulfate (4%) solutions, Triton X-114, acetonitrile, and sodium triphosphate were obtained from Merck, whereas disodium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Fisher Scientific.

Preparation of chitosan beads

The chitosan beads were prepared via the coagulation method as described in the literature with a slight modification [9]. Firstly, 1.5 g of chitosan powder was dissolved in 100 ml of 1.25%(v/v) acetic acid solution. Chitosan beads were formed by dropping chitosan solution into 100 mL of 1.5%(v/v) sodium triphosphate solution by using a hypodermic needle with a tip diameter of 2.2 mm under stirring conditions. Then, the resultant mixture was allowed to stand for 3 hrs. The spherical gels formed are known as wet chitosan beads. The mixture was left to rest overnight and then the beads were removed by filtration with filter paper. Afterwards, the beads were washed with a buffer solution at pH 6.86 until the residual solution reached neutrality. Finally, the beads were restored in a buffer solution at pH 6.86 until further use.

Immobilization of lipase

In this experiment, lipase was immobilized on the chitosan beads by covalent bonding by using glutaraldehyde as a bifunctional cross-linker as described in the literature [10, 11]. The chitosan was soaked in a glutaraldehyde solution for 2 h under a static condition at room temperature to activate its surface with the aldehyde group. Then, the chitosan beads were removed and washed with a buffer solution at pH 6.86 until they reached neutrality. To immobilize the enzyme, 0.20 g of beads were added to the enzyme solution at various concentrations. Then, the resultant mixture was left for 40 h at room temperature. Finally, the immobilized enzyme was recovered and washed with a buffer solution at pH 6.86.

The immobilization yield (IY) was determined by the following Equation (1). Where Ei is the initial concentration of enzyme protein (g/mL) and Ef is the final concentration of the enzyme protein (g/mL) in the free enzyme solution during the immobilization

$$IY(\%) = \frac{E_i - E_f}{E_i} \times 100 \tag{1}$$

Protein assay

A protein assay was determined by using BSA and 4% cupric sulphate solution at a ratio of 20:1. The solution was prepared using a 20:1 volume ratio of BSA and 4% cupric sulphate. A 0.05 ml of immobilized/free enzyme solution was added to the solution. The mixture was incubated for 30 mins at 37°C. After incubation, the resultant colour change in the mixture was measured at 562 nm by a Cary 60 UV–Vis spectrophotometer (Agilent Technologies). A calibration curve was plotted for references.

Lipase activity assay

Activity assays were carried out according to a standard method [12]. Firstly, 100 mM of phosphate buffer with 150 mM sodium chloride and 0.5% Triton X-114 was prepared in a 2 mL tube, pH 7.2 at 37°C. Then, 0.1 mL of 50 mM PNPB solution prepared in acetonitrile was added to the tube. Subsequently, 0.1 mL of the free/ immobilized enzyme solution was added to the resultant mixture. The solution was incubated in a water bath for 5 min at 37°C. Then, the reaction was stopped by adding 1 mL of 0.5 M EDTA. The unit activity (IU) was calculated by using Equation (2) below.

$$Unit\ Activity\ (IU) = \frac{(A_{400}Sample - A_{400}Blank) \times V_T \times DF}{\varepsilon_{ext} \times t \times V_E} \tag{2}$$

Where A_{400}Sample and A_{400}Blank were the absorbances of the sample and blank, determined at 400 nm. Meanwhile, VT is the total volume of the assay, VE is the volume of the enzyme, DF is the dilution factor, t is the time of the assay, and ε_{ext} is the micromolar extension coefficient of p-nitrophenol at 400 nm, which is 0.0148 cm^2/μmol.

Data analysis and optimization

The response surface method (RSM) was used to investigate the effect of parameters during the immobilization of Candida rugosa lipase on the chitosan support. The parameters are enzyme (0.005 – 0.02 g/mL) and glutaraldehyde (2 – 10% v/v) concentrations, whereas the responses are immobilization and lipase activity yield. Each response was fitted to an empirical model which correlated the response using a quadratic equation as given by Equation (3),

$$Y = b_o + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j>i}^{n} b_{ij} x_i x_j \quad (3)$$

where Y is the predicted response, b_o is the constant coefficient, b_i is the linear coefficient, b_{ij} is the interaction coefficient, b_{ii} is the quadratic coefficients, x_i and x_j are the coded values of the parameters.

Recyclability

The immobilized lipase was repeatedly used in a hydrolysis reaction of a standard substrate, and its activity was measured after each cycle of the reaction. The reaction cycle was stopped after the initial activity decreased by up to 50%.

UV-Visible spectroscopy analysis

A spectrophotometer was used to determine the activity of the lipase and the immobilization yield, respectively. The analysis was carried out at a wavelength of 400 nm and 562 nm, respectively. One millilitre of each sample from the assay solution was transferred into a 2 mL cuvette. Then, the cuvette was placed into the spectrophotometer. The absorbance was measured with a light path length of 1 cm. Each sample was analyzed with three replicates.

RESULTS AND DISCUSSION

Statistical analysis of the data

The data collected was analyzed using the Design Expert-7 software as tabulated in Table 1. The experiment was designed with the response surface method and a 2-level factorial design with α=2. The resulting data were fitted to a quadratic model using Equation 3 to describe the effect of parameters on responses and any possible parameter interaction as shown in Table 1. The model only includes the most significant parameters, which are represented by linear (A and B) and quadratic (A2 and B2) terms, which represent the effect of single parameters as well as the interaction term (AB), if such interaction exists between the parameters.

Table 1. Data analysis of the effect of parameters on immobilization and activity yield

Source	Sum of squares	*df*	Mean square	F value	*p*-Value Prob>F	Assessment
Immobilization yield						
Model	1481.83	5	296.37	559.63	< 0.0001	significant
A-Lipase Concentration	1300.00	1	1300.00	2454.81	< 0.0001	significant
B-Glutaraldehyde Concentration	3.97	1	3.97	7.49	0.029	significant
AB	0.20	1	0.20	0.38	0.5559	not significant
A^2	160.49	1	160.49	303.06	< 0.0001	
B^2	0.14	1	0.14	0.27	0.6217	not significant
Residual	3.71	7	0.53			
Lack of Fit	2.28	3	0.76	2.12	0.2406	not significant
Activity yield						
Model	535.11	5	107.02	131.2	< 0.0001	significant
A-Lipase Concentration	19.51	1	19.51	23.91	0.0018	significant
B-Glutaraldehyde Concentration	366.31	1	366.31	449.07	< 0.0001	significant
AB	7.56	1	7.56	9.27	0.0187	significant
A^2	85.08	1	85.08	104.3	< 0.0001	significant
B^2	21.14	1	21.14	25.92	0.0014	significant
Residual	5.71	7	0.82			
Lack of Fit	3.4	3	1.13	1.97	0.2613	not significant

Generally, the effect of lipase and glutaraldehyde concentration is very significant to the immobilization yield with a p-value < 0.05. It also apparent that the effect of enzyme concentration is more significant (p-value < 0.01) compared to glutaraldehyde concentration (p-value = 0.03). Therefore, the quadratic term for glutaraldehyde concentration was insignificant and excluded from the model. Additionally, there is no indication of interactions between the parameters with a p-value = 0.56. Thus, the interaction term between the parameters was also excluded from the final model.
Meanwhile, lipase and glutaraldehyde concentrations are significantly affecting the activity yield, where the scale of the impact for both parameters is equally comparable with a p-value <0.01. Therefore, its quadratic terms are significant for the proposed model. It was found that there are interactions between lipase and glutaraldehyde concentrations on the activity yield with a p-value < 0.02. Thus, the final model for activity yield incorporates the quadratic and interaction terms.

The proposed models with coded factors are given by Equations (4) and (5) for the effect of parameters on immobilization and activity yield, respectively. Meanwhile, Figure 1 shows the contour plot of the results obtained where the effect of lipase and glutaraldehyde concentrations are significant.

$$IM\ Yield = 37.65 - 10.41A + 0.58B - 2.67A^2 \tag{4}$$

$$Activity\ Yield = 30.87 + 1.28A - 5.53B - 1.38AB - 1.93A^2 + 0.96B^2 \tag{5}$$

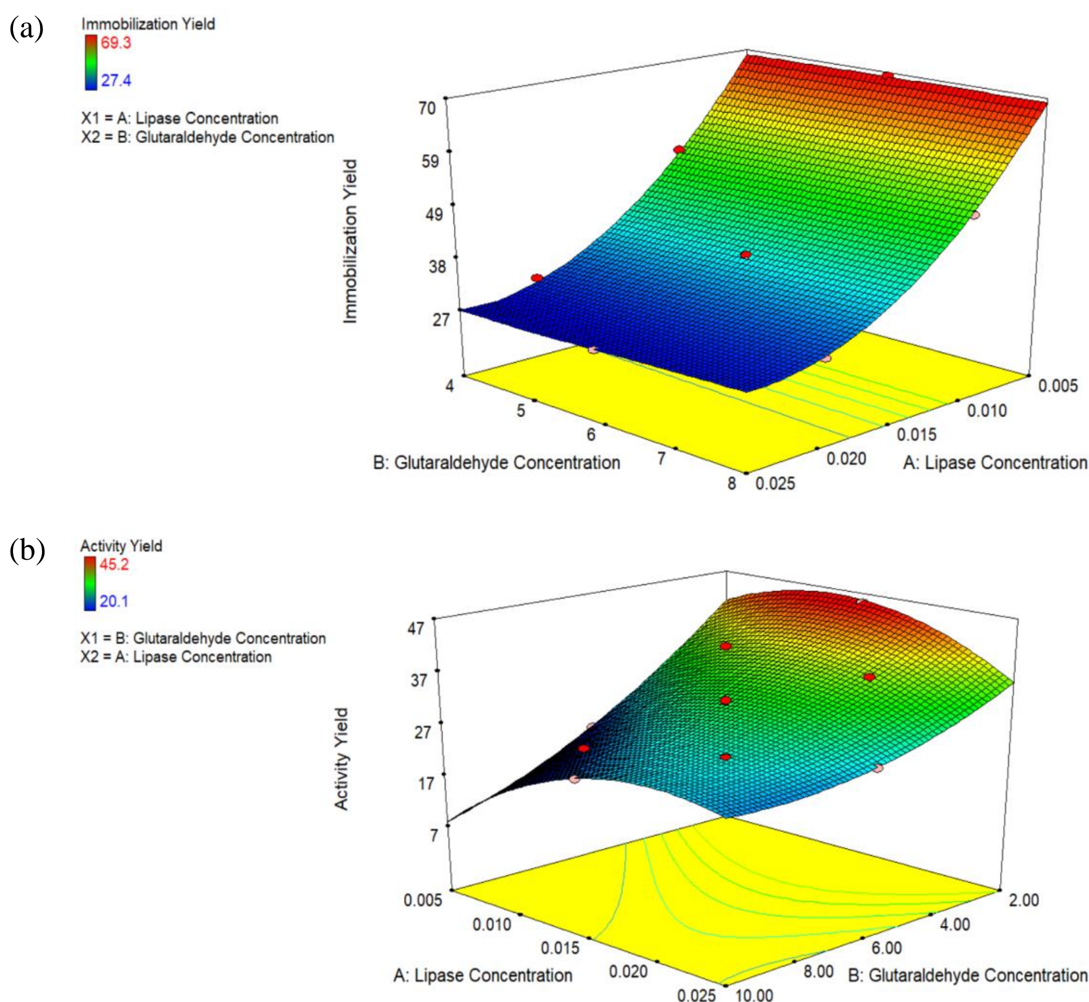

Figure 1. Contour plots of the effect of lipase and glutaraldehyde concentrations on (a) immobilization yield (%) and (b) activity yield (IU).

Effect of glutaraldehyde concentration

The glutaraldehyde consists of dual aldehyde groups which readily react with the primary amine group belonging to chitosan for surface activation. The treatment of chitosan beads with glutaraldehyde will form a free aldehyde group on their surface, which can be used to attach the lipase [13]. Figure 2 shows the effect of glutaraldehyde treatment on the

chitosan beads on the immobilization and activity yield. The immobilization yields are only slightly improved when a high concentration of glutaraldehyde is used for surface activation, whereas it significantly affects the activity yield of the lipase immobilized on the chitosan beads.

This result implies that the surface activation of the chitosan beads could be done at a lower concentration. Further increase in glutaraldehyde concentration has no effect on the activation of the chitosan beads for lipase binding. However, it reduces the activity yield. Figure 2(b) shows that treatment of chitosan beads with glutaraldehyde at 10% v/v reduces the activity yield by approximately 55%. This was probably because of the residual glutaraldehyde compounds that weakly bonded to the chitosan surfaces during the immobilization of lipase [14]. Thus, it promotes crosslinking between the enzymes through aldehyde-primary amine linkages (amide bond). Subsequently, it reduces the activity yield.

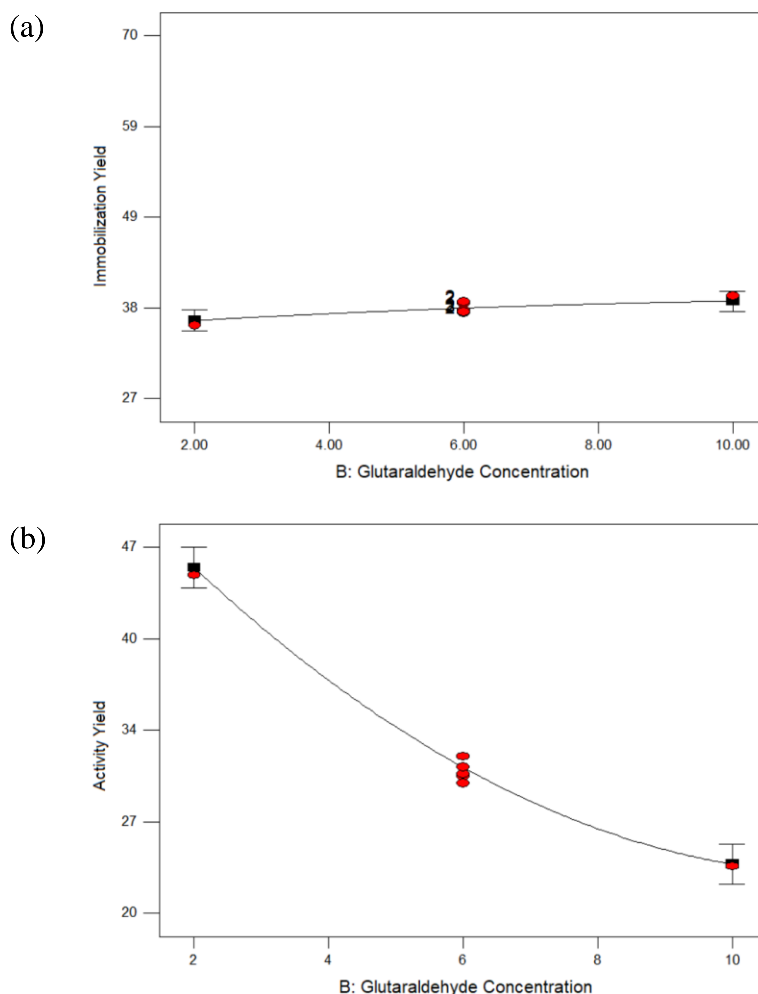

Figure 2. The effect of glutaraldehyde concentration on (a) immobilization yield (%) and (b) activity yield (IU).

Effect of lipase concentration

The immobilization of lipase was carried out after the chitosan beads were activated with the aldehyde group via glutaraldehyde treatment. The activated chitosan beads were incubated in the free lipase solution at concentrations ranging from 0.005 to 0.025 g/mL. Figure 3(a) shows that the immobilization yield was decreased by approximately 62% with added lipase concentrations. This result was probably due to surface saturation of the chitosan beads with immobilized lipase, thus further increases in lipase concentrations did not give a significant improvement. Therefore, increasing the ratio of activated chitosan beads over the concentration of lipase should improve the immobilization yield.

Figure 3(b) depicts a mixed response to lipase concentration on the activity yield. Initially, increasing the lipase concentration did increase the activity yield. However, it was slightly decreased when a lipase concentration of over 0.02 g/mL was used. Even though the immobilization yield was decreased with added lipase concentrations, the activity yield increased, especially at a lipase concentration of 0.015 g/mL. There is no conclusive reason for this behaviour. However, it could be due to the interaction between lipase and glutaraldehyde concentration on the activity yield ($p\text{-value} < 0.02$) [15].

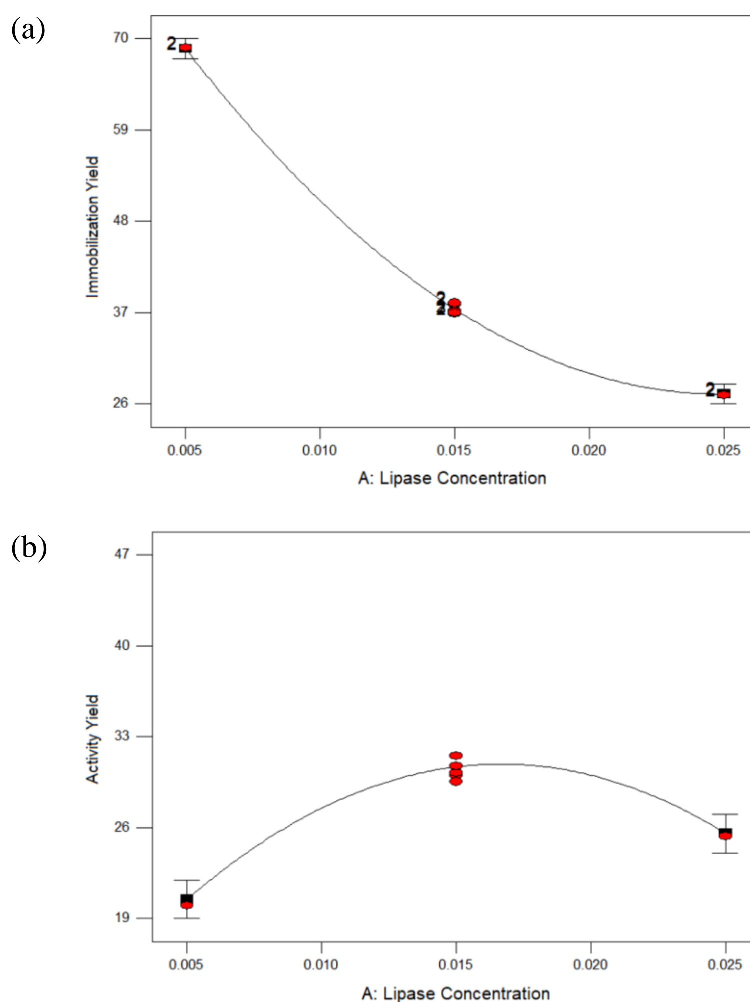

Figure 3. Effects of lipase concentration on (a) immobilization yield (%) and (b) activity yield (IU).

Parameter interaction

The interaction between the glutaraldehyde and lipase concentrations used during the immobilization of lipase on the chitosan bead is significant in the activity yield compared to the immobilization yield. Figure 4(a) shows that a change in glutaraldehyde concentration (4 to 8% v/v) did not change the immobilization yield, whereas the activity yield reduces at a higher glutaraldehyde concentration regardless of the concentrations of lipase used. The saturation of the activated chitosan bead surfaces with lipase could explain the phenomenon. It is also supported by the data in figure 3(b), which shows that the activity yield is highest at lipase concentrations ranging from 0.015 to 0.02 g/mL.

At saturation conditions, it can be assumed that the amount of lipase immobilized on the activated chitosan lipase will remain constant regardless of the initial amount of lipase used and the concentration of glutaraldehyde applied during the activation [16]. A reduction in activity remains possible, especially due to inhibition or due to the intra-crosslinking between the lipases attributed by the residual aldehyde group [17]. Any excess amount of glutaraldehyde leached out from the surface could potentially inhibit the enzyme and affect the activity determined during the activity assay. Thus, a surface deactivation procedure should be carried out to terminate the active aldehyde moieties.

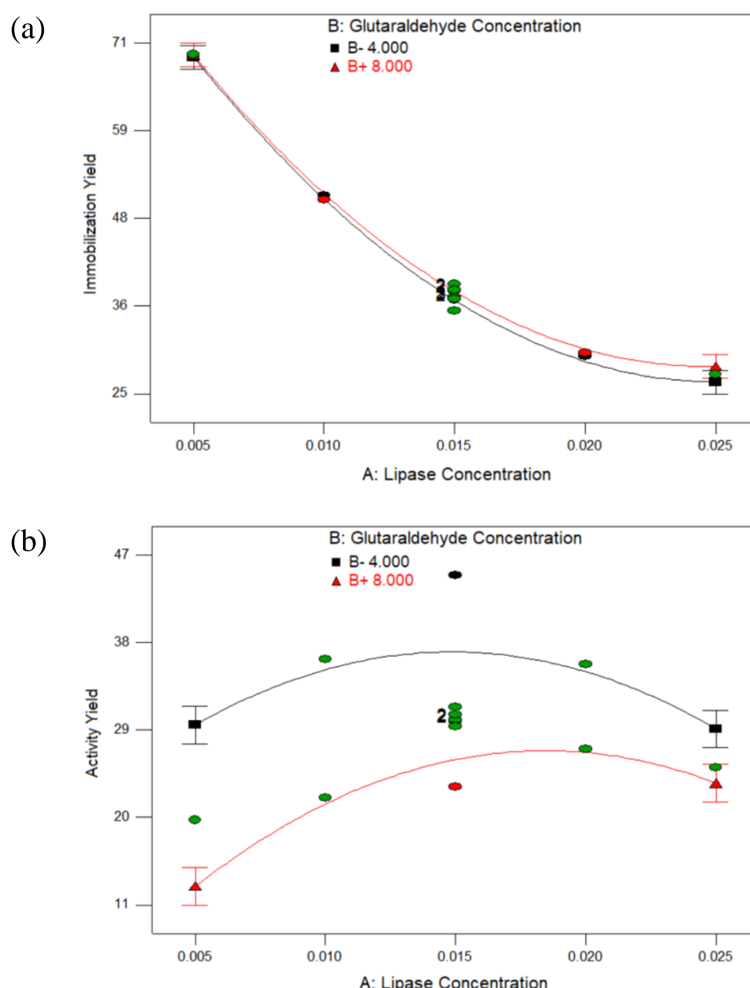

Figure 4. Interaction between glutaraldehyde and lipase concentrations on (a) immobilization yield and (b) activity yield.

Optimization

The optimization of the immobilization of lipase on the glutaraldehyde-activated chitosan beads was carried out to achieve the minimum immobilization yield with maximum activity yield. Thus, the specific activity of immobilized lipase (IU/mg lipase) is maximized, whereas the concentrations of glutaraldehyde and lipase were set at the minimal values possible. Thus, immobilization costs could be reduced. The model predicted that the optimum condition for the immobilization is a lipase concentration of 0.013 g/mL and a glutaraldehyde concentration of 2%v/v to result in a 41% of immobilization yield with immobilized lipase activity of 46 IU.

Reusability of the immobilized lipase

The immobilized lipase on chitosan beads was used for the hydrolysis reaction of the standard substrate, ρ-nitrophenyl butyrate. The recyclability of the immobilized lipase is shown in Figure 5. The recyclability of the lipase is acceptable and comparable with that reported in the literature [18]. After 7 uses, the activity of the immobilized lipase dropped by about 50% from 35 to 18 IU. The reduction of activity is rather gradual, which indicates the incomplete binding of the enzyme to the chitosan beads. In the present work, the lipase was attached to the chitosan support by glutaraldehyde intermediatory, where aldehyde groups belonging to glutaraldehyde bind to amine groups on chitosan and enzyme surfaces. Therefore, the attachment of the immobilized lipase depends on the strength of the two amides bond, which anchors the lipase to the chitosan surfaces.

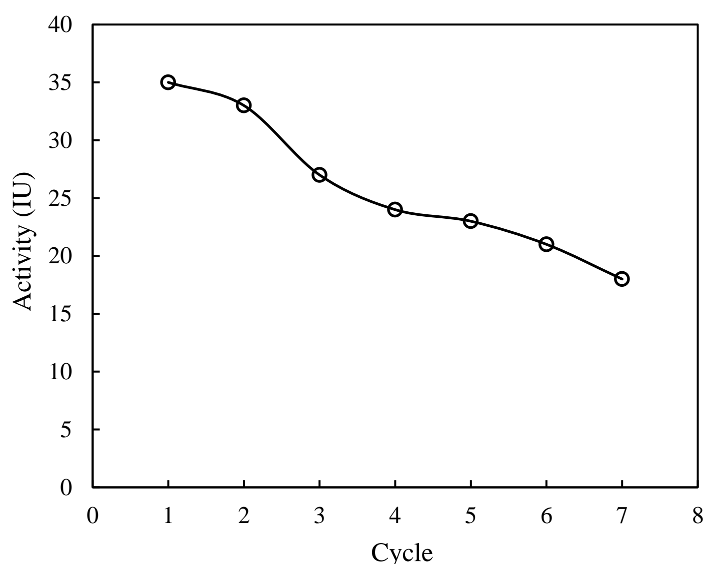

Figure 5. The recyclability of lipase immobilized on chitosan beads.

CONCLUSION

The chitosan beads for lipase immobilization were prepared by the coagulation method in a sodium triphosphate solution and then their surface was activated by glutaraldehyde. The activation process provides an active functional group on the chitosan beads for lipase attachment by covalent bonding. It was found that the glutaraldehyde and lipase concentration greatly affect the immobilization and activity yields. A higher concentration of glutaraldehyde reduces the activity yield but slightly improves the immobilization yield, whereas, a higher lipase concentration reduces the immobilization yield. The parameters did not show any interaction on the immobilization yield but did show significant interaction on the activity yield. The immobilized enzyme is recyclable, however, it loses almost 50% of its initial activity after 7 cycles. Thus, it is suggested that the surface deactivation procedure could improve the recyclability of the immobilized lipase.

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