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Optimization of immobilization conditions of *Thermomyces lanuginosus* lipase on styrene–divinylbenzene copolymer using response surface methodology

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ABSTRACT

Microbial lipase from *Thermomyces lanuginosus* (formerly *Humicola lanuginosa*) was immobilized by covalent binding on a novel microporous styrene–divinylbenzene polyglutaraldehyde copolymer (STY–DVB–PGA). The response surface methodology (RSM) was used to optimize the conditions for the maximum activity and to understand the significance and interaction of the factors affecting the specific activity of immobilized lipase. The central composite design was employed to evaluate the effects of enzyme concentration (4–16%, v/v), pH (6.0–8.0), buffer concentration (20–100 mM) and immobilization time (8–40 h) on the specific activity. The results indicated that enzyme concentration, pH and buffer concentration were the significant factors on the specific activity of immobilized lipase and quadratic polynomial equation was obtained for specific activity. The predicted specific activity was 8.78 μmol *p*-NP/mg enzyme min under the optimal conditions and the subsequent verification experiment with the specific activity of 8.41 μmol *p*-NP/mg enzyme min confirmed the validity of the predicted model. The lipase loading capacity was obtained as 5.71 mg/g support at the optimum conditions. Operational stability was determined with immobilized lipase and it indicated that a small enzyme deactivation (12%) occurred after being used repeatedly for 10 consecutive batches with each of 24 h. The effect of methanol and *tert*-butanol on the specific activity of immobilized lipase was investigated. The immobilized lipase was almost stable in *tert*-butanol (92%) whereas it lost most of its activity in methanol (80%) after 15 min incubation.

1. Introduction

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) catalyze the hydrolysis of triglycerides to free fatty acids, diacylglycerols, monoglycerols and glycerol [1]. The use of lipases as industrial catalysts is a very promising alternative to those used in conventional industrial chemistry. They are also efficient in various reactions such as ester synthesis, transesterification, interesterification, acidolysis and aminolysis in organic solvents or on support materials [2,3]. Therefore, microbial lipases are currently receiving much attention for their potential industrial applications in the detergent, food, flavor industries, biocatalytic resolution of pharmaceuticals, esters and amino acid derivatives, making of fine chemicals, agrochemicals, use as biosensor, bioremediation and cosmetics, perfumery and biodiesel production [4–6]. However, the drawbacks of the extensive use of lipases as biocatalyst compared to classical chemical catalysts can be found in the relatively low stability of enzyme in their native state. Consequently, there is a great interest in methods trying to develop competitive biocatalysts for industrial applications by the improvement of their catalytic properties such as activity and stability. Such an improvement can be carried out by chemical or physical modifications of the native enzyme.

Immobilized enzymes are versatile catalysts in the laboratory and on an industrial scale. Enzyme immobilization is the most commonly used strategy to impart the desirable features of conventional heterogeneous catalysts onto biological catalysts [7,8]. There are different methods for the immobilization of enzymes. Immobilization facilitates the efficient recovery and reuse of costly enzymes [9]. Besides enhanced stability, enzymes can acquire additional advantageous properties via immobilization: (1) immobilized enzymes can be used repeatedly or continuously in a variety of reactors and (2) they can be easily separated from soluble reaction products and unreacted substrate, thus simplifying work-up and preventing protein contamination of the final product [10].

Lipases have been successfully immobilized on many different types of supports, such as celite [11], Nylon-6 [12], chitosan, agarose [13], hydrotalcite, zeolites [14], and cross-linked enzyme aggregates (CLEAs) [15]. Styrene–divinylbenzene (STY–DVB) copolymer has peculiar physicochemical and hydrophobic characteristics. It has been shown as a good potential support material for lipase immobilization. Lipase from *Candida rugosa* has been successfully immobilized on the STY–DVB copolymer by adsorption and had

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high activity to perform hydrolysis and esterification reactions [16]. The *C. rugosa* has also been immobilized by activating the hydroxyl groups of chitosan using carbodiimide coupling agent. The process has the advantage of high ability to activate carboxyl groups and low toxicity to enzymes [17]. Lipases have been immobilized on different supports by covalent binding, adsorption, entrapment or cross-linking [18–21]. The scope of enzyme stabilization depends on the support used and selection of the immobilization process. On the other hand, by the selection of suitable immobilization method, support material and the operational cost of biocatalytic reaction can be significantly reduced [22]. Glutaraldehyde activation of supports is one of the most popular techniques to immobilize enzymes. The glutaraldehyde is used to activate supports by physical adsorption and covalent binding, immobilizing the enzyme on a glutaraldehyde pre-activated support [23–25]. Multipoint covalent attachment of enzymes on highly activated supports promotes a rigidification of the enzyme structure of the immobilized enzyme [26]. This rigidification reduces any conformational change involved in enzyme inactivation and increases the enzyme stability.

In the presence of any hydrophobic support, the lid moves to permit the interaction between its hydrophobic face and the hydrophobic residues that usually surround the lipase active center with this hydrophobic surface. This way, the lipase becomes adsorbed onto this hydrophobic surface, and the active center is exposed to the reaction medium. This mechanism of action is called interfacial activation of the lipases. The other strategy that has been quite successful in the immobilization of *Thermomyces lanuginosus* is the use of the peculiar catalytic mechanism of the lipases to adsorb the enzyme on different hydrophobic supports via interfacial activation. Thus the immobilized enzyme will stabilize its open conformation [5]. For interfacially active lipases, it is evident that the selectivity is often conformation-controlled due to the fact that the lid opening is often a prerequisite for higher activity and selectivity for the carrier-bound immobilized enzyme [27].

T. lanuginosus lipase has been immobilized on hydrophobic supports to produce biodiesel with canola oil and methanol. The immobilized lipase proved to be stable and lost little activity when subjected to repeated uses [6,28,29]. It has been demonstrated that enzymes show higher activity in relatively hydrophobic organic solvents such as *n*-hexane and petroleum ether [30,31]. However, short chain alcohols especially methanol have poor solubility in these hydrophobic solvents, so the negative effects on lipase activity caused by methanol cannot be eliminated and lipase still exhibits poor stability in such reaction medium. The effect of various alcohols (methanol, ethanol, *iso*-propanol, *iso*-butanol, acetone, chloroform and acetonitrile) as well as alkanes (*n*-pentane, *n*-hexane and *n*-heptane) has been studied on the activity of immobilized lipase [12,32]. Among alcohols, an exposure to *iso*-propanol increased the activity of bound enzyme while ethanol, methanol and *iso*-butanol inhibited the activity. A moderate polar solvent, *tert*-butanol was adopted as the organic solvent in which lipase expressed quite high catalytic activity and operational stability. It has been confirmed that *tert*-butanol is inert in the immobilization system. The toxicity of methanol on lipase activity can also be eliminated in the *tert*-butanol system.

Response surface methodology (RSM) has been a widely practiced approach for the production and optimization of different industrially important biotechnological and biochemical products such as enzymes and chemicals [33,34]. Optimization with RSM not only allows quick screening of wide experimental field, but also shows the role of each of the components. The graphical representation of RSM function is called response surface, which is used to describe the individual and cumulative effects of the parameters on the response. Central composite design has been the most successful factorial design for the optimization of parameters with a limited number of experiments and estimates the response surface [35–37].

In the present study, immobilization was carried out by circulating the enzyme solution by a peristaltic pump through novel STY–DVB–PGA beads in a cylindrical column made of stainless steel. The lipase enzyme is a single chain protein consisting of 269 amino acids. Its molecular weight is 31,700 g/mol and its isoelectric point is 4.4. The lid is an alpha-helical mobile surface loop consisting of amino acids 86–93 that covers the active site [5]. Although the STY–DVB has been already shown as a good potential matrix for lipase immobilization by physical adsorption, covalent attachment of lipase on the copolymer and full characterization of support before and after immobilization have not been investigated to our knowledge in literature. We provide data on the properties of lipase immobilized on the STY–DVB by covalent attachment using scanning electron microscopy (SEM) and Fourier transform infrared (FTIR) spectroscopy. Immobilization of lipase is affected by many factors such as enzyme concentration, pH, buffer concentration and immobilization time. Most of the studies on immobilization changed one separate factor at a time. However, immobilization system can be influenced by simultaneously changing more than one factor. The main objective of this work is the development and evaluation of a statistical approach to better understand the relationship between the parameters and specific activity of the lipase immobilized on a novel STY–DVB–PGA support. Furthermore, lipase stability as a function of incubation time in methanol and *tert*-butanol was investigated to understand the effect of these solvents on lipase activity.

2. Experimental

2.1. Materials

Lipase from *T. lanuginosus* (lipozyme TL 100 L) was a gift of Novozymes Enzim Dış Ticaret Ltd. Şti. (Istanbul, Turkey). Glutaraldehyde (25% aqueous solution), divinylbenzene, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, Coomassie brilliant blue G 250 were obtained from Merck. Span 80 and potassium peroxidosulfate were purchased from Fluka. Triton X-100, gum arabic, *p*-nitrophenyl palmitate (*p*-NPP), *p*-nitrophenol (*p*-NP), and bovine serum albumin (BSA) were purchased from Sigma. All other chemicals were of analytical grade.

2.2. Synthesis of polyglutaraldehyde

10 mL of glutaraldehyde (25% aqueous solution) was diluted to 50 mL with distilled water. pH value was adjusted to 10.5 with 1 M NaOH solution. The solution was then mixed at room temperature with a magnetic stirrer for 30 min [38].

2.3. Synthesis of styrene–divinylbenzene copolymer containing polyglutaraldehyde

Polymerized glutaraldehyde solution with potassium persulfate (1.4%, w/v) as starter was added to the organic mixture containing 59% (v/v) of styrene, 26% (v/v) of divinylbenzene, 15% (v/v) of span 80. The ratios of the aqueous and organic phases were 90% and 10%, respectively. Polymerization was conducted at 80 °C for 3 h and waited at 60 °C until dry. Polymerization procedure resulted in an activated polyglutaraldehyde styrene–divinylbenzene copolymer. The porosity of the resulting polymer was 90% since the aqueous phase was removed from the polymer [38].

2.4. Lipase immobilization

Lipase from *T. lanuginosus* was immobilized on the STY–DVB–PGA (0.6–1.4 mm particle size) by covalent linkage method in a column with continuous circulation by a peristaltic pump. Synthesis of STY–DVB–PGA and immobilization are illustrated in Fig. 1. Different concentrations of lipase solutions were prepared using different concentrations and pHs of phosphate buffer solutions. Before immobilization, polymeric beads were washed with 200 mL of distilled water. The enzyme solution was circulated at 25 °C throughout the reactor with 5 mL/min flow rate for up to 40 h within the experimental range (Table 2). The reactor has 2.8 cm outer diameter, 2.3 cm inner diameter and 10 cm length. The immobilized enzymes onto polymeric beads were finally washed with 200 mL buffer solution which is the same phosphate buffer used during immobilization.

2.5. Protein assay

Protein concentration was determined using the Bradford assay method [39]. The amount of immobilized protein on the support material was determined by measuring the initial and final concentrations of protein within the enzyme solutions. Coomassie brilliant blue solution was used as a dye reagent. Bovine serum albumin (BSA) was used as a standard to construct the calibration curve.

2.6. Measurement of lipase activity

Spectrophotometric measurement was based on the capability of lipases to cleave *p*-nitrophenyl palmitate (*p*-NPP), thus releasing an amount of *p*-nitrophenol (*p*-NP) which has a yellow color that can be measured by absorbance at 410 nm. The enzymatic reaction mixture contained 1.5 mL of 20 mM of *p*-nitrophenyl palmitate solution in 2-propanol and 13.5 mL of 60 mM phosphate pH 7.0 containing 0.2% (w/v) gum arabic and 0.6% (v/v) Triton X-100 as detergent. The reaction was initiated by the addition of 70 mg lipase immobilized support. The mixture was stirred with a magnetic stirrer for 5 min. The support was separated from the reaction mixture by filtering with filter paper. 0.4 mL of filtrate was diluted to 12-folds with 10% 2-propanol and 90% phosphate buffer solution (60 mM, pH 7.0). The absorbance of *p*-nitrophenol was measured at 410 nm. One lipase unit was expressed as the release of 1 μmol *p*-nitrophenol per minute under the assay conditions. Specific activity was defined as the number of enzyme units per milligram protein.

2.7. Stability of immobilized lipase in organic solvents

Immobilized lipase (150 mg) was incubated in 15 mL of methanol and *tert*-butanol each at 25 °C for 60 min. The residual activity was assayed for different times following the same procedure as described above.

2.8. Operational stability of immobilized lipase

Immobilized lipase (3.30 g) was used for transesterification of crude canola oil with methanol. 100 g of crude canola oil and three-step addition of methanol, 27 mL with 9 mL in each step (a 1:4 molar ratio of oil/methanol), were circulated into the reactor by a peristaltic pump at 5 mL/min flow rate for 3 h at each successive step, then the reaction was continued at 40 °C for 24 h. The reactions were conducted with 10 batches. One batch reaction time was 24 h. The immobilized lipases were rinsed with *tert*-butanol between each batch. The residual activity determined after 24 h was expressed as relative activity.

2.9. Characterization of the support

FTIR spectra of the STY–DVB–PGA and lipase immobilized polymeric beads were obtained using FTIR spectrophotometer (Thermo Nicolet 6700). The dry support was thoroughly mixed with KBr, and pressed into a tablet form, and the spectrum was then recorded in the 4000–400 cm^1 range with 32 scans at a resolution of 4 cm^{-1}.

The STY–DVB–PGA was imaged in dry state by SEM (Carl Zeiss EVO 40). For this purpose, the support was coated with 80% gold and 20% palladium on argon atmosphere for enhancing conductivity. The coated support was then imaged.

2.10. Experimental design

Five-level-four-factor central composite design was employed in this study, requiring 30 experiments for the optimization of immobilization parameters. The parameters and their levels are enzyme concentration (4–16%, v/v), pH (6.0–8.0), buffer concentration (20–100 mM) and immobilization time (8–40 h). The experimental and predicted data in terms of specific activity are shown in Table 1.

Second-order polynomial equation (1) which includes all interaction terms was used to calculate the predicted response:

$$y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} b_{ii} x_i^2 + \sum_{i=j}^{3} \sum_{j=i+1}^{4} b_{ij} x_{ij} \quad (1)$$

where y is the specific activity, b_0 is the offset term, b_i is the linear effect, b_{ii} is the squared effect, b_{ij} is the interaction effect and x_i is the ith independent variable.

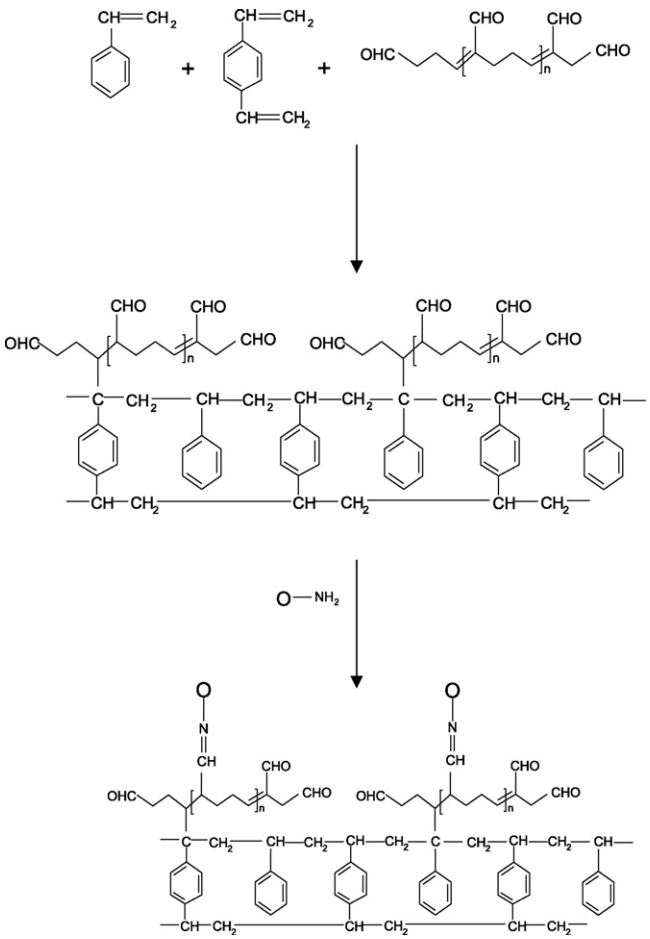

Fig. 1. Scheme of lipase immobilization on the STY–DVB–PGA beads.

Table 1
Central composite design of factors with coded values and specific activity.

Treatment	Factors				Specific activity (μmol *p*-NP/mg enzyme min)	
	Enzyme concentration (%, v/v), x_1	pH, x_2	Buffer concentration (mM), x_3	Immobilization time (h), x_4	Experimental	Predicted
1	−1	−1	−1	−1	3.45	3.73
2	1	−1	−1	−1	2.59	3.10
3	−1	1	−1	−1	4.58	4.82
4	1	1	−1	−1	4.34	4.19
5	−1	−1	1	−1	5.00	4.71
6	1	−1	1	−1	4.55	4.08
7	−1	1	1	−1	5.23	5.80
8	1	1	1	−1	5.54	5.17
9	−1	−1	−1	1	3.30	3.70
10	1	−1	−1	1	3.39	3.06
11	−1	1	−1	1	4.62	4.79
12	1	1	−1	1	4.32	4.15
13	−1	−1	1	1	4.40	4.68
14	1	−1	1	1	3.82	4.04
15	−1	1	1	1	6.14	5.77
16	1	1	1	1	4.66	5.13
17	−2	0	0	0	7.93	7.53
18	2	0	0	0	5.88	6.27
19	0	−2	0	0	3.12	3.30
20	0	2	0	0	5.20	5.48
21	0	0	−2	0	2.33	2.10
22	0	0	2	0	3.83	4.06
23	0	0	0	−2	3.29	3.38
24	0	0	0	2	3.41	3.32
25	0	0	0	0	4.59	4.39
26	0	0	0	0	4.21	4.39
27	0	0	0	0	4.66	4.39
28	0	0	0	0	4.64	4.39
29	0	0	0	0	4.18	4.39
30	0	0	0	0	4.07	4.39

2.11. Data analysis

The data were analyzed using Design Expert program (version 7.1.4) and the coefficients were interpreted using *F*-test. Three main analytical steps: analysis of variance (ANOVA), regression analysis and plotting of contour plot were performed to establish the optimum conditions for specific activity.

3. Results and discussion

3.1. Characterization of STY–DVB–PGA

The STY–DVB–PGA beads were characterized before and after immobilization using lipase from *T. lanuginosus* by FTIR spectroscopy and SEM. FTIR spectra of the polymeric support before and after immobilization of lipase are shown in Fig. 2. The STY–DVB–PGA beads show OH absorption band of water at 3436 cm^{-1} formed during polymerization step. The spectrum shows aromatic C–H stretching at 3059 and 3025 cm^{-1}, aliphatic C–H stretching at 2924 and 2853 cm^{-1}, aromatic C=C stretching at 1603, 1492 and 1451 cm^{-1}. Aromatic C–H in-plane bending at 1064, 1029 and 902 cm^{-1}, aromatic out-plane bending at 829, 758 and 699 cm^{-1} for *p*-substitute benzene. The peaks at 1740, 1725 and 1707 cm^{-1} show C=O stretching corresponding to aldehyde group that indicates the presence of polyglutaraldehyde in the copolymer (Fig. 2a). The lipase immobilized on the STY–DVB–PGA beads shows an absorption peak at 1678 cm^{-1} due to the formation of imine bond (–C=N) during covalent immobilization (Fig. 2b). The peaks at 1640 and 1630 cm^{-1} are for amide I and amide II, respectively, indicating the presence of enzyme in the polymeric beads [40]. The support was washed after immobilization process with buffer solution to remove unbound enzyme. Hence, amide I and amide II peaks originate covalent bound enzyme.

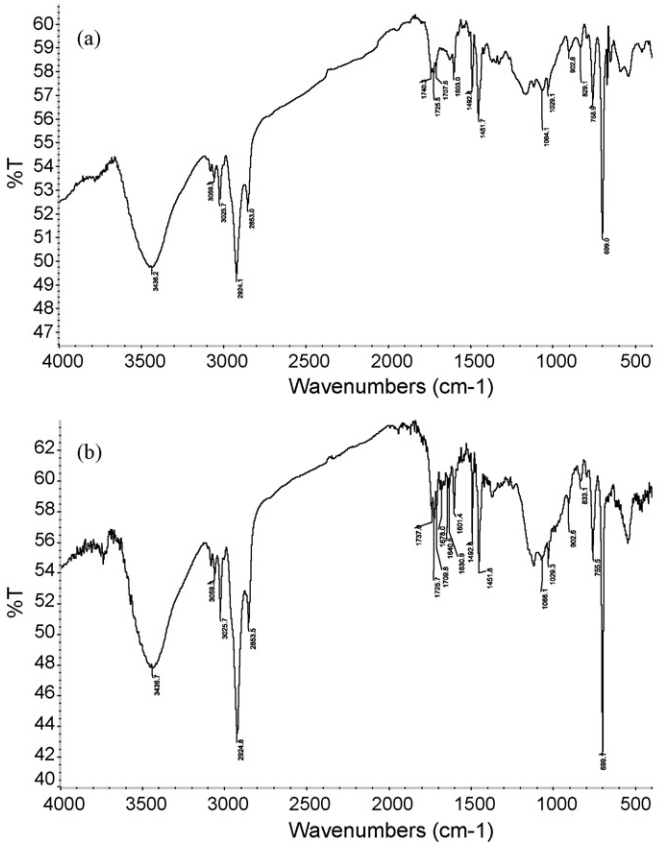

Fig. 2. FTIR spectra of STY–DVB–PGA beads (a) before and (b) after immobilization of lipase enzyme.

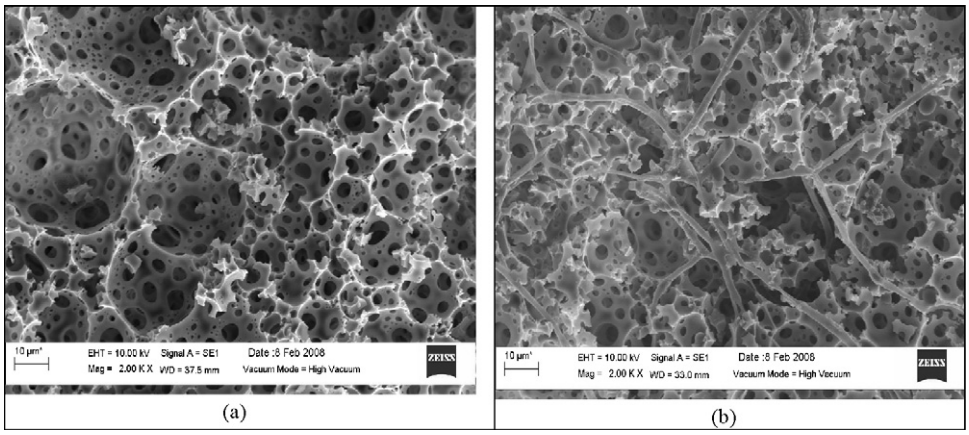

Fig. 3. SEM picture of STY–DVB–PGA beads (a) before and (b) after immobilization of lipase enzyme.

Scanning electron microscopy (SEM) allowed the verification of morphological differences of STY–DVB–PGA beads in the non-immobilized and immobilized states of the enzyme. A typical polyHIPE (high internal phase emulsion) structure, which is the open cellular architecture consisting of voids and connecting pores through the walls of the polymerized continuous phase of the emulsion, can be seen on the SEM pictures, which was used to examine the surface morphology of STY–DVB–PGA beads before and after immobilization of lipase from *T. lanuginosus* (Fig. 3). The enzyme immobilization by means of polyglutaraldehyde leads to notable surface morphology changes as illustrated in Fig. 3b. The changes of the surface morphology of copolymer indicate the presence of the enzyme attached on the STY–DVB–PGA beads.

3.2. Optimization of immobilization parameters

The selection of suitable immobilization conditions is critical to maximize the multipoint covalent attachment. Immobilization conditions should favor the enzyme–support reaction [26]. The main objective of this work is the development and evaluation of a statistical approach to better understand the relationship between the parameters of the lipase immobilization. Optimization of immobilization parameters was performed using central composite design procedure. The coded values, experimental specific activity and predicted specific activity are given in Table 1 and the experimental domain is shown in Table 2. Among the 30 experiments including 5 replicates, experiment 17 (enzyme concentration 4%, pH 7, buffer concentration 60 mM and immobilization time 24 h) had the greatest specific activity (7.93 μmol *p*-NP/mg enzyme min) and experiment 21 (enzyme concentration 10%, pH 7, buffer concentration 20 mM and immobilization time 24 h) had the smallest specific activity (2.33 μmol *p*-NP/mg enzyme min). The effects of each factor and its interactions were calculated using a Design Expert program (version 7.1.4). Fitting of the data with various models and the subsequent analysis of variance (ANOVA) showed that immobilization of lipase was most suitably described with quadratic polynomial model. From the Design Expert, the quadratic polynomial equation (2) with significant terms was given below:

$$y = 4.39 - 0.32x_1 + 0.55x_2 + 0.49x_3 - 0.02x_4 + 0.63x_1^2 - 0.33x_3^2 - 0.26x_4^2 \quad (2)$$

where x_1 is the enzyme concentration, x_2 is the pH, x_3 is the buffer concentration, and x_4 is the immobilization time.

The quadratic polynomial model was highly significant and sufficient to represent the actual relationship between the response and significant parameters with very low *p*-value (0.0001) from the ANOVA (Table 3). The computed model *F*-value of 16.52 was higher than the tabular value of $F_{0.05(14,15)} = 2.42$, implying the model is significant at 95% confidence level. The model also showed statistically insignificant lack of fit, as is evident from the computed *F*-value of 2.67 which is lower than the tabular value of $F_{0.05(10,5)} = 4.74$ at 95% confidence level. Furthermore, the value of pure error (0.35) is low which indicates good reproducibility of the data obtained with a small *p*-value (0.0001) from the ANOVA and a satisfactory coefficient of determination ($R^2 = 0.9391$). The coefficient of determination also revealed that there are excellent correlations between the independent variables.

The effects of immobilization parameters such as enzyme concentration, pH, buffer concentration, and immobilization time were investigated on the specific activity. The *p*-values mark the significance of coefficients and are also important for understanding the pattern of the mutual interactions between the parameters. A value of Prob > *F* less than 0.05 indicates that the model terms are significant. x_1 (enzyme concentration), x_2 (pH), x_3 (buffer concentration), x_1^2, x_3^2, and x_4^2 are the most significant parameters (Prob > *F* less than 0.05). However, x_4 (immobilization time), x_1x_2, x_1x_3, x_1x_4, x_2x_3, x_2x_4, x_3x_4 and x_2^2 have less effect (Prob > *F* more than 0.05) on the specific activity of the immobilized lipase (Table 3). The relationship between predicted and experimental specific activities is shown in Fig. 4. It can be seen that there is a high correlation ($R^2 = 0.9226$) between the predicted and experimental specific activities.

The relationship between immobilization parameters and specific activity was investigated by contour plots. Fig. 5 shows the

Table 2
Range of coded and actual values for central composite design.

Coded value	Enzyme concentration (%, v/v)	pH	Buffer concentration (mM)	Immobilization time (h)
−2	4	6.0	20	8
−1	7	6.5	40	16
0	10	7.0	60	24
1	13	7.5	80	32
2	16	8.0	100	40

Table 3
Analysis of variance for the fitted quadratic polynomial model for optimization of immobilization parameters.

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*-value (Prob > *F*)
Model	34.14	14	2.44	16.52	<0.0001[a]
Enzyme (x_1)	2.40	1	2.40	16.25	0.0011[a]
pH (x_2)	7.14	1	7.14	48.35	<0.0001[a]
Buffer concentration (x_3)	5.77	1	5.77	39.06	<0.0001[a]
Immobilization time (x_4)	0.01	1	0.01	0.04	0.8354[b]
x_1x_2	0.00	1	0.00	0.00	0.9490[b]
x_1x_3	0.05	1	0.05	0.33	0.5745[b]
x_1x_4	0.07	1	0.07	0.46	0.5097[b]
x_2x_3	0.11	1	0.11	0.74	0.4039[b]
x_2x_4	0.03	1	0.03	0.23	0.6389[b]
x_3x_4	0.24	1	0.24	1.62	0.2220[b]
x_1^2	10.79	1	10.79	73.09	<0.0001[a]
x_2^2	0.10	1	0.10	0.65	0.4324[b]
x_3^2	2.96	1	2.96	20.03	0.0004[a]
x_4^2	1.87	1	1.87	12.68	0.0028[a]
Residual	2.21	15	0.15		
Lack of fit	1.86	10	0.19	2.67	0.1453[b]
Pure error	0.35	5	0.07		
Cor total	36.35	29			
R^2 = 0.9391					

[a] Significant at "Prob > *F*" less than 0.05.
[b] Insignificant at "Prob > *F*" more than 0.05.

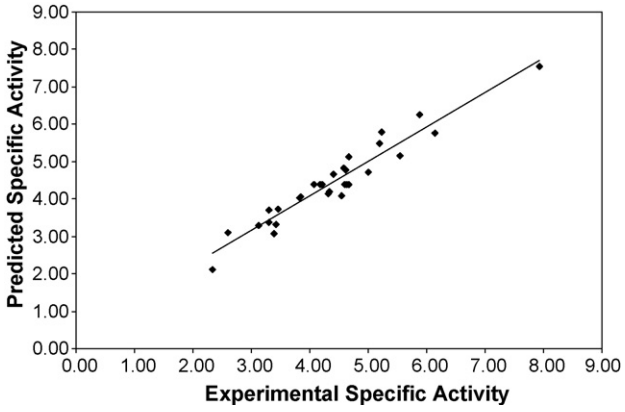

Fig. 4. Predicted specific activity versus experimental specific activity.

effect of enzyme concentration, pH and their mutual interaction on specific activity of immobilized lipase. Enzyme concentration of 4% (v/v) and pH of 7.5 led to the maximum specific activity (7.47 μmol *p*-NP/mg enzyme min). An enzyme concentration of 16% (v/v) also yields high specific activity (6.58 μmol *p*-NP/mg enzyme min) at pH 7.5. The effect of buffer concentration, enzyme concentration and their mutual interaction on the specific activity of immobilized lipase is illustrated in Fig. 6. Apparently, low enzyme concentration (5%, v/v) increased the specific activity (6.85 μmol *p*-NP/mg enzyme min) at a higher buffer concentration (75 mM). Fig. 7 represents the effects of varying buffer concentrations and pHs on specific activity. An increase in specific activity was observed with the increasing of buffer concentration at first, then the trend was stable when the buffer concentration reached to 75 mM at pH 7.0. There is no significant interaction between the immobilization parameters. However, higher interaction was observed between enzyme concentration and pH than between other factors. Although immobilization may be performed at neutral pH in many cases, incubation at alkaline pH values, where the reactivity of the nucleophiles of the protein may be improved, is convenient to reach a high enzyme–support reaction.

The optimum specific activity of lipase enzyme from the model was determined as 4% (v/v) for enzyme concentration, pH 8.0 for buffer, 75 mM for buffer concentration and 24 h for immobilization time within the experimental region. The pH and buffer

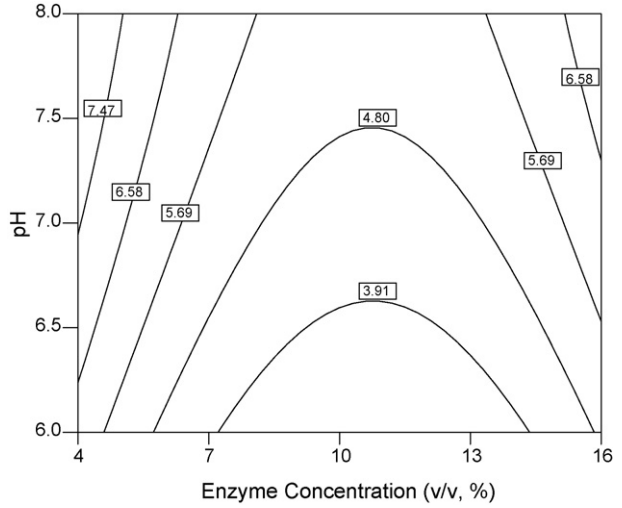

Fig. 5. Contour plot of the combined effects of enzyme concentration and pH on the specific activity.

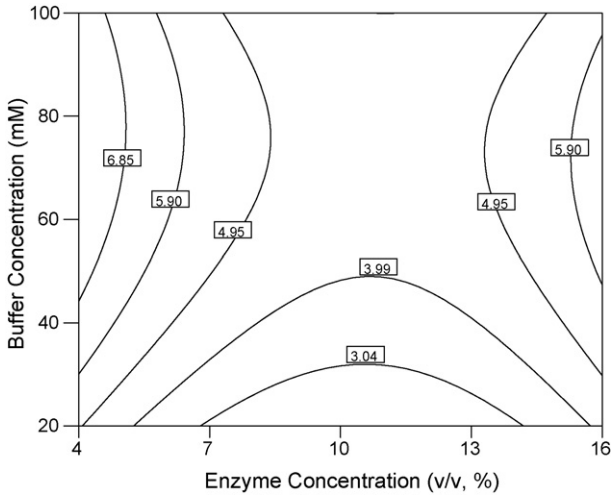

Fig. 6. Contour plot of the combined effects of enzyme concentration and buffer concentration on the specific activity.

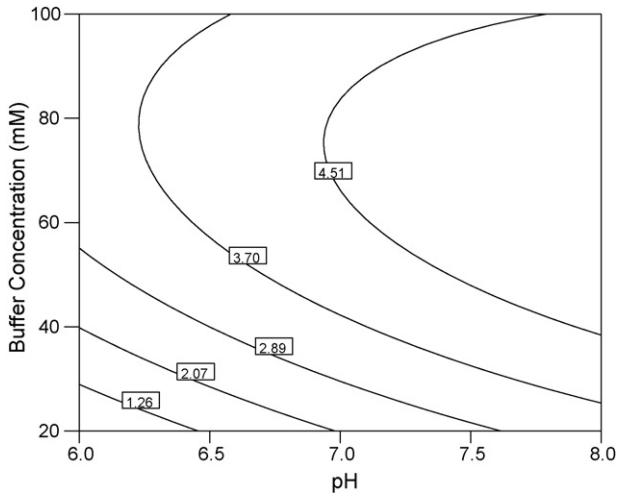

Fig. 7. Contour plot of the combined effects of pH and buffer concentration on the specific activity.

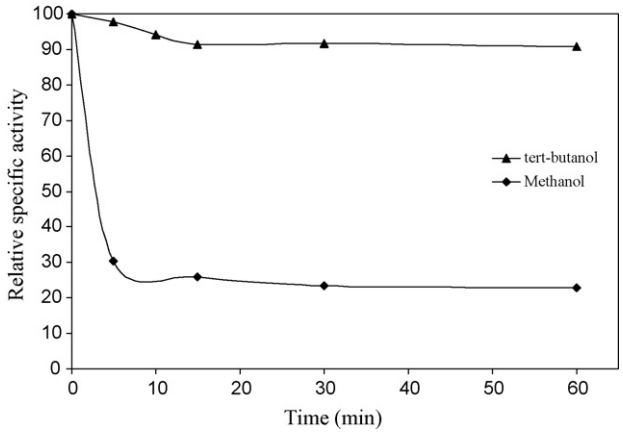

Fig. 8. Stability of immobilized *Thermomyces lanuginosus* lipase as a function of incubation time in methanol and *tert*-butanol at 25 °C.

concentration have an effect on the ionic state of the lipase molecules. At pH 8.0 and high buffer concentration, the enzymatic polarity might be weakened, which could enhance the lipase binding onto the hydrophobic surface. A similar study was shown by Huang and Cheng [41] who reported the effect of pH on the lipase from *Penicillium expansum* immobilization process. The activity of immobilized enzyme on a bimodal ceramic foam was changed with the increase of the immobilization pH value (6–10), and lipase immobilized at pH 8.0 showed maximum activity. Lower enzymatic activity was obtained at both lower and higher pH values. Similar optimum pH was obtained with our study although different types of lipase enzyme and support were used.

The accuracy of the model was validated under the optimal conditions obtained from central composite design. Theoretical specific activity was calculated as 8.78 μmol *p*-NP/mg enzyme min from the model according to the limit criterion of specific activity maximization. The immobilization was repeated at the optimum conditions and experimental specific activity was found as 8.41 μmol *p*-NP/mg enzyme min. The specific activity of the free lipase enzyme was determined as 10.15 μmol *p*-NP/mg enzyme min. Verification experiments confirmed the validity of the predicted model. As a result, the model from central composite design was considered to be accurate and reliable for predicting the specific activity for immobilization of *T. lanuginosus* lipase by covalent binding on the STY–DVB–PGA. The lipase loading capacity and immobilization yield were determined as 5.71 mg/g support and 59%, respectively. The recovered activity was obtained as 83% after immobilization on the polymeric beads at optimum conditions. Similar results were obtained with literature [38]. The authors compared the loading capacity, immobilization yield and recovered activity of the immobilized lipase on the STY–DVB–PGA and STY–DVB. In addition, under different experimental conditions, the amount of protein immobilized onto STY–DVB–PGA beads (11.81 mg/g support) was higher than that of protein immobilized onto STY–DVB (10.79 mg/g support). The results indicate that STY–DVB–PGA beads are more efficient than STY–DVB in terms of these parameters and covalent attachment has some real advantages on lipase immobilization.

The activities of both the immobilized lipase on the STY–DVB–PGA and the commercial immobilized lipase (lipozyme TL IM) were measured with the same procedure as described above. The lipase slightly increased its activity (48 μmol *p*-NP/g support min) when it was immobilized on the STY–DVB–PGA while the commercial immobilized lipase displayed slightly less activity (45 μmol *p*-NP/g support min) that is immobilized on porous silica granulates. Different immobilization preparations of the same lipase, when acting at different experiment conditions, may exhibit a very different activity. *T. lanuginosus* lipase was immobilized on different hydrophobic supports and their properties were compared with those found in literature [32,42]. The results suggested that selection of different supports yielded very different results in terms of activity and stability. On the other hand, the type of carbonyl group together with the immobilization method has an important effect on the catalytic properties of *T. lanuginosus* lipase.

Microbial lipases have been found to exhibit a tendency to form bimolecular aggregates in the solution at low concentrations [43,44]. Detergents have been suggested to be able to shift the open/close equilibrium of lipase toward the open conformation, by coating the hydrophobic areas of the lipase that surround the active center of the enzyme. In this paper, we focused on the evaluation of a statistical approach to better understand the relationship between the immobilization parameters in order to obtain the highest lipase activity. The mechanism of aggregation of lipase, stabilization of the open form of lipase by covalent linking with STY–DVB–PGA and the effect of detergent need to be studied in detail with this particular support and lipase.

3.3. Enzyme stability in organic solvents

The objective of studying the effect of organic solvents on the specific activity of lipase immobilized on the STY–DVB–PGA was its potential application in biodiesel production. Studies have shown that the stability of commercial immobilized lipase from *T. lanuginosus* (lipozyme TL IM) in biodiesel production could be significantly enhanced in the *tert*-butanol system [30,31]. In the *tert*-butanol system, both methanol and by-product glycerol are soluble, so the negative effect caused by methanol and glycerol could be eliminated totally. The effect of methanol and *tert*-butanol on the specific activity of lipase immobilized on the STY–DVB–PGA is shown in Fig. 8. The immobilized enzyme lost 80% of its activity in 15 min in the presence of methanol. The reason is that the supports might trap and prevent the disruption of the enzyme-bound water essential to maintain the three-dimensional structure of the enzyme for catalysis as the polar solvents tend to strip water from the enzyme molecule. Therefore, the enzyme may not be stable in the presence of excessive methanol in the enzymatic reaction medium. On the other hand, the specific activity of lipase was almost stable in *tert*-butanol (Fig. 8). It can be seen that the immobilized enzyme was stable with 90% of its relative specific activity after 15 min incubation in the presence of *tert*-butanol. Therefore, the incubation of the immobilized lipase in *tert*-butanol helped

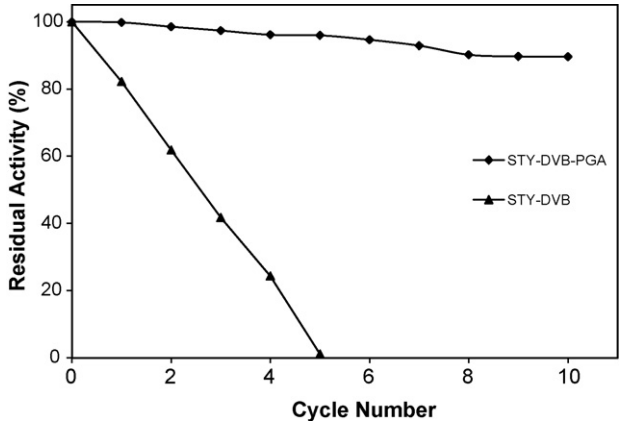

Fig. 9. Stability of *Thermomyces lanuginosus* lipase immobilized by covalent attachment on the STY–DVB–PGA and physical adsorption on the STY–DVB beads with repeated use for transesterification of canola oil. The reaction conditions: 20% enzyme based on oil weight (100 g); oil/alcohol molar ratio 1:4; reaction temperature 40 °C and reaction time 24 h.

improve the enzyme activity and stability. Immersion of lipases in *tert*-butanol was claimed as a pretreatment method to increase lipase activity in the synthesis of methyl ester [31]. The activity of the lipase immersed in the *tert*-butanol was found to be higher than that of the enzyme immersed in methyl ester [34]. When the enzyme was immersed in *tert*-butanol, the yield of methyl ester was about 7–10 times higher than that of the immobilized enzyme without pretreatment. The reason is that the methanol adsorbed on the immobilized enzyme can be dissolved in *tert*-butanol. Hence, it is possible to propose that washing with *tert*-butanol is an efficient way to regenerate the deactivated immobilized lipase and remove the contaminant of glycerol in lipase-catalyzed biodiesel production.

3.4. Stability of immobilized lipase on reuse

The stability of the immobilized enzyme is of great importance for industrial applications [15,26,45]. The *T. lanuginosus* lipase immobilized on the STY–DVB–PGA was stable for 10 cycles of canola oil transesterification retaining more than 88% residual activity (Fig. 9), thereby indicating its robust biocatalytic potential. The enzymatic activity of lipase immobilized onto STY–DVB–PGA was compared with celite 545, silica gel, and STY–DVB supports. The immobilized lipase lost its whole activity after five repeated reuses with these supports. Decrease in the enzyme activity of the lipase immobilized on the STY–DVB could be due to leaching of enzyme by washing the solvent with *tert*-butanol and transesterification reaction mixture from support pores or due to inactivation of enzyme. The high reusability of immobilized lipase has been reported for cross-linked enzyme aggregates (CLEAs) [15] and cross-linked lipase on silica gel [46]. On the other hand, commercial lipase (lipozyme TL IM) displayed 86.2% activity after being used for 10 batches when waste oil was used for transesterification reaction [37]. The lipase immobilized on the electrospun fibrous membranes retained 30% residual activity after reuses [18]. Our results suggest that the polyglutaraldehyde enzyme–support cross-link is able to produce an intense enzyme–support reaction that leads to a great increase in the enzyme stability.

4. Conclusion

Lipase from *T. lanuginosus* was successfully immobilized on polyglutaraldehyde activated styrene–divinylbenzene by covalent binding. RSM proved to be a powerful tool for the optimization of immobilization parameters. A second-order model was obtained to describe the relationship between the specific activity and the parameters of enzyme concentration, pH, buffer concentration and immobilization time. The results indicated that enzyme concentration, pH and buffer concentration were the significant factors on the specific activity of immobilized lipase. The optimum conditions of immobilized lipase were enzyme concentration 4% (v/v), pH 8.0, buffer concentration 75 mM and immobilization time 24 h. The predicted specific activity was 8.78 μmol *p*-NP/mg enzyme min under the optimal conditions and the subsequent verification experiment with the specific activity of 8.41 μmol *p*-NP/mg enzyme min confirmed the validity of the predicted model. Operational stability was determined with immobilized lipase and it indicated that a small enzyme deactivation occurred after being used repeatedly for 10 consecutive batches with each of 24 h. Lipase stability as a function of incubation time in organic solvent indicated that the enzyme lost most of its activity in a very short time in methanol, whereas lipase could maintain relatively high activity in *tert*-butanol and there was no obvious loss in lipase activity even after being incubated for 2 h.

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