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Covalent immobilization of organic solvent tolerant lipase on aluminum oxide pellets and its potential application in esterification reaction

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ABSTRACT

This study was carried out to covalently immobilize the partially purified lipase from *Bacillus* sp. DVL2 on glutaraldehyde-activated aluminum oxide pellets and subsequently use the immobilized enzyme for esterification of oleic acid and ethanol. The immobilization process parameters were optimized through response surface methodology. Under optimized conditions, maximum immobilization yield of the enzyme was 78.20%. The immobilized lipase could be reused for 5 consecutive cycles without any loss of enzyme activity. However, the enzyme showed 75% residual activity after 10th cycle. The stability of the immobilized enzyme towards pH, temperature and organic solvents was enhanced as compared with its soluble counterpart. Higher values of half-lives, *D*-values, enthalpy and free energy change after covalent immobilization implied an improvement in enzyme stability. The K_m and V_{max} values of the enzyme were enhanced after immobilization. The immobilized lipase was found to be most stable in DMSO followed by toluene, hexane and xylene, exhibiting more than 90% residual activity in these solvents after 24 h of incubation. The immobilized lipase was more efficient in catalyzing the esterification between oleic acid and ethanol in hexane. The formation of ethyl oleate was confirmed by TLC and ^{1}H NMR spectroscopy.

1. Introduction

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) belongs to a group of enzymes whose biological function is to catalyze the hydrolysis of triacylglycerols into diacylglycerols, monoacylglycerols, free fatty acids (FFA) and glycerol at an oil–water interface [1]. In addition to their natural function of hydrolyzing carboxylic ester bonds, lipases can catalyze esterification, interesterification, and transesterification reactions in non-aqueous media [2–4]. These reactions usually proceed with high chemo-, regio- and/or enantioselectivity, making lipases an important group of biocatalysts [5]. Lipases are highly diversified in enzymatic properties and substrate specificity. Lipases have emerged as key enzymes which find usage in food, paper, textile and detergent industries, waste water treatment, production of fine chemicals, pharmaceuticals, cosmetics and so on [6–10]. Though lipases are widely distributed among microorganisms, animals and plants yet, microbial lipases are receiving much more attention in industry mainly because of the availability of a wide range of hydrolytic and synthetic activities, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and easy cultivation of microbes on inexpensive media [11–13].

Lipases have been used as biocatalyst in esterification/transesterification reactions [14]. The biosynthesis of esters is of commercial interest because of the increasing popularity and demand for natural products amongst consumer. A range of fatty acid esters is now being produced commercially using the immobilized lipase in non-aqueous solvents. Ethyl oleate esters have been reported to possess applications in several industrial fields such as food, aromatics, cosmetics, detergents, flavors and pharmaceuticals. They may also be used as plasticizers and lubricants, biological additives and hydraulic fluids [15].

The application of lipase has been deterred by the high cost of enzymes, contamination of products with residual protein and their low storage, operational, and thermal stabilities. Immobilization of lipase might be the best solution to overcome these limitations. Immobilization of lipases on water insoluble carriers, helps in their economic reuse and in the development of continuous bioprocesses. Immobilization also facilitates the separation of products and improves lipase properties such as thermostability and activity in non-aqueous media. The choice of appropriate support as well as method of immobilization is essential to make

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industrially important biocatalyst. Accordingly, various immobilization methods for lipase have been developed including physical adsorption, ionic binding, covalent attachment, entrapment and microencapsulation [16–23]. Although physical adsorption on solid supports is simple, inexpensive and easy to perform yet it suffers from the limitation of enzyme leaching from the support. Encapsulation is a mild and especially useful method for enzymes which are easily deactivated, for instance by covalent immobilization. Covalent immobilization, which involves multipoint covalent attachment of an enzyme to a carrier either directly or through a spacer, has the advantage of preventing enzyme leaching. The immobilization of lipase has been carried out on different supports including both hydrophobic (e.g. octyl-agarose, sol–gels and octyl-sephabeads) and hydrophilic (e.g. celite, alumina, silica, agarose and nylon-6) [17,24]. Nanostructured materials such as carbon nano tubes [25] and electrospun nanofibres [26] were also used as supports for immobilization. Recently, lipases have been immobilized on poly (allyl glycidyl ether-co-ethylene glycol dimethacrylate) polymer microsphere [27], hydrophobic/strong cation-exchange functional silica particles [28] and magnetic silica nanocomposite particles [29,30].

The yield of the immobilized enzyme may be enhanced through process optimization by using statistical approach, which is easy, less laborious, quick and more accurate. Response surface methodology (RSM), a collection of mathematical and statistical techniques, is widely used to determine the effect of several variables at a time to optimize the biotechnological processes [31].

In this study, the strain *Bacillus* sp. DVL2 used for lipase production was isolated in our laboratory. The lipase produced by this strain was optimally active at 37 °C and pH 8.0. It showed stability in various organic solvents. This lipase was covalently immobilized on glutaraldehyde-activated aluminum oxide pellets and the immobilized enzyme was examined for its properties. The above support was selected for immobilization due to its stability at high pH and temperature. As per our knowledge, no report is available for the immobilization of lipase on aluminum oxide pellets through covalent attachment. The immobilization process parameters were statistically optimized to maximize the immobilization yield. The potential of the immobilized lipase in esterification of oleic acid and ethanol to ethyl oleate was also investigated.

2. Materials and methods

Aluminum oxide pellets (3 mm) and p-Nitrophenyl palmitate (p-NPP) were purchased from Sigma–Aldrich Chemical Company (St. Louis, USA) and all the other chemicals were of analytical grade.

2.1. Microorganism

The microorganism *Bacillus* sp. DVL2 was isolated from dairy industry waste collected from Karnal district of Haryana (India) and identified by morphological, biochemical and16 S rRNA sequencing. The 16S rDNA sequence of 1406 bp was generated and submitted to NCBI Gene Bank depository under the Accession No. JX846495.

2.2. Lipase production

The culture (isolated *Bacillus* sp. DVL2) was grown under optimal conditions for lipase production. The basal production medium containing (g/L) peptone 5.0, yeast extract 5.0 and olive oil 1.0 was autoclaved at 1.05 kg/cm^2 for 20 min, inoculated with 3.0% (v/v) of 18 h old inoculum and incubated in an Orbital shaker at 30 °C under agitation of 200 rpm for 24 h. The contents of the flasks were then centrifuged at 10,000 × *g* for 20 min at 4 °C to remove the cells. The clear supernatant containing the lipase was used for its partial purification and subsequent immobilization.

2.3. Assay of free lipase

The activity of free lipase was determined spectrophotometrically using p-nitrophenyl palmitate (p-NPP) as substrate according to the method of Nawani et al. [32] with some modifications. The reaction mixture containing 0.3 mL of 0.05 M phosphate buffer (pH 8.0), 0.1 mL of 0.8 mM p-NPP and 0.1 mL of lipase was incubated at 37 °C for 10 min. The reaction was then terminated by adding 1 mL ethanol. A control was run simultaneously, which contained the same contents but the reaction was terminated prior to addition of the enzyme. Absorbance of the resulting yellow colored product was measured at 410 nm in a spectrophotometer. One International Unit (IU) of lipase activity was defined as the amount of enzyme catalyzing the release of 1 μmol of p-nitrophenol per min from p-NPP under the standard assay conditions.

2.4. Estimation of protein content

Protein content in different enzyme preparations was estimated by Lowry's method [33].

2.5. Partial purification of enzyme

All the steps of enzyme purification were performed at 4 °C.

2.5.1. Ammonium sulphate precipitation

To 500 ml of culture supernatant, ammonium sulphate was added (20–70% saturation) and the protein was allowed to precipitate by keeping it overnight in a refrigerator. The protein precipitates were collected by centrifugation at 12,000 × *g* for 20 min at 4 °C and dissolved in a small volume of 0.05 M phosphate buffer (pH 8.0).

2.5.2. Hydrophobic interaction chromatography

The concentrated ammonium sulphate fraction was applied on to a column of Phenyl-sepharose CL-4B (Sigma) pre-equilibrated with 1.0 M ammonium sulphate dissolved in 0.05 M phosphate buffer (pH 7.0). The bound lipase was eluted by applying a negative linear gradient of 1.0–0 M ammonium sulphate in 0.05 M phosphate buffer (pH 7.0). The fractions were collected and analyzed for lipase activity and protein content. The fractions containing high activity of lipase were pooled and concentrated using a 10 kDa cut-off membrane.

2.6. Immobilization of lipase on aluminum oxide pellets

Aluminum oxide pellets were employed as support for immobilization of partially purified lipase through covalent attachment. The pellets were activated by dipping in 2% aqueous glutaraldehyde solution for 1 h at room temperature followed by addition of the enzyme. The coupling of enzyme with the glutaraldehyde-activated pellets was allowed to occur at room temperature for 90 min. The pellets were washed with distilled water at each step to remove the excess of glutaraldehyde and the unbound enzyme. The enzyme activity was determined in the supernatant as well as in the enzyme bound pellets. Immobilization yield (IY %) was calculated in the following manner:

$$\text{Immobilization yield}(\%) = \frac{\text{Total activity recovered on pellets}}{\text{Total activity offered for immobilization}} \times 100$$

The observed activity of the immobilized enzyme refers to the activity of the enzyme immobilized on aluminum oxide pellets whereas total activity immobilized on pellets refers to

Table 1
Experimental range of each selected variable for lipase immobilization.

Factor	Name	Unit	Low level (−)	High level (+)	Mean (0)	SD
A	No. of beads	numeric	4.0	8.0	6.0	1.79
B	Glutaraldehyde	(%, v/v)	2.0	6.0	4.0	1.79
C	Enzyme dose	mL	0.02	0.06	0.04	0.02
D	Coupling period	min	30.0	120.0	75.5	39.21

the difference in enzyme activity offered for immobilization and activity recovered in the supernatant.

2.7. Assay of the immobilized lipase

Activity of the immobilized lipase was assayed by adding 0.1 mL of 0.8 mM p-NPP and 0.4 mL of 0.05 M phosphate buffer (pH 8.0) to the enzyme bound aluminum oxide pellets in an eppendorf tube. After incubating the reaction mixture for 10 min at 37 °C, the pellets were removed by transferring the contents of the reaction mixture to another eppendorf tube. The reaction was then stopped by adding 1 mL ethanol and intensity of the resulting yellow colored product was read at 410 nm. The pellets were washed with phosphate buffer and reused.

2.8. Statistical optimization of immobilization

To examine the cumulative effect of immobilization parameters response surface methodology was employed using a statistical software package Design Expert 7.1.2, Stat-Ease, Inc. A 2^4 full factorial central composite design (CCD) with 16 trials for factorial design, 8 trials for axial point and 6 replicate trials at the central point, leading to a set of 30 experiments was designed. The range and levels of variables ($-\alpha$, -1, 0, 1, $+\alpha$) are presented in Table 1 and the experimental design is shown in Table 2. All the variables were taken at a central value represented by "0". The response value from each experiment of CCD was the average of triplicates.

2.9. Operational stability of the immobilized lipase

To evaluate the recycling stability of the immobilized lipase, enzyme bound pellets were incubated with buffer and p-NPP for 10 min at 37 °C followed by termination of the reaction according to standard assay conditions. The bound enzyme was repeatedly used to hydrolyze p-NPP up to 15 batch reactions. The residual activity (%) was determined as:

$$\text{Residual activity} = \frac{\text{enzyme activity in } n\text{th cycle}}{\text{enzyme activity in 1st cycle}} \times 100$$

2.10. Effect of pH on free and the immobilized lipase

The effect of pH on soluble and the immobilized lipase was studied by determining the enzyme activity using different buffers (0.05 M) viz. sodium citrate (pH 4.0–6.0), sodium phosphate (pH 7.0–9.0) and glycine–NaOH (pH 10.0–11.0). The residual activity (%) at each pH was calculated with reference to the enzyme activity at the optimum pH (taken as 100%).

The pH stability of soluble as well as the immobilized lipase was investigated by pre-incubating the enzyme in the above-mentioned buffers of different pH for 2 h followed by measurement of its activity under standard assay conditions. The residual activity (%) at each pre-incubating pH was calculated relative to the maximum enzyme activity (taken as 100%). The results of the effect of pH on the immobilized enzyme were compared with those of the free enzyme.

2.11. Effect of temperature on free and the immobilized lipase

The temperature optima of soluble and the immobilized lipase were investigated by determining the enzyme activity at different temperatures (25–45 °C). The relative activity (%) at each temperature was calculated with reference to the enzyme activity at the optimum temperature (which was taken as 100%). The activation energy (E_a) of catalysis for free and the immobilized lipase was determined from the slope of the Arrhenius plot [log *V* (logarithm of % relative activity) versus reciprocal of absolute temperature in Kelvin (1000/T)], using the following equation:

$$\text{Slope} = \frac{E_a}{R}$$

Thermal stability of soluble and the immobilized lipase was studied by pre-incubating the enzyme at various temperatures (25–60 °C) for 1 h followed by determination of activity using standard assay at the optimum temperature. The residual activity (%) was calculated relative to the maximum enzyme activity taken as 100%. Thermal stability of the immobilized lipase was also investigated by pre-incubating it at temperatures ranging from 30 to 60 °C for different periods (30, 60, 90 and 120 min). The residual activity was calculated by taking the enzyme activity at 0 min incubation as 100%.

The first order thermal deactivation rate constants (k_d) were determined from the regression plot of log relative activity (%) versus time (min). The activation energy (E_d) for lipase denaturation was determined by a plot of log denaturation rate constants ($\ln k_d$) versus reciprocal of the absolute temperature (*K*) using the following equation:

$$\text{Slope} = \frac{E_d}{R}$$

The half-lives ($t_{1/2}$) and *D*-values (decimal reduction time or time required to pre-incubate the enzyme at a given temperature to maintain 10% residual activity) of the immobilized lipase at each temperature were determined from the following relationships:

$$t_{1/2} = \frac{\ln\ 2}{k_d}$$

$$D\text{-value} = \frac{\ln\ 10}{k_d}$$

The changes in enthalpy (ΔH°, kJ mol^{-1}), free energy (ΔG°, kJ mol^{-1}) and entropy (ΔS°, J mol^{-1} K^{-1}) for thermal denaturation of lipase were determined using the following equations:

$$\Delta H^\circ = E_d - RT$$

$$\Delta G^\circ = -RT\ \ \ln\left(\frac{k_d h}{k_B T}\right)$$

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T}$$

where *T* is the corresponding absolute temperature (*K*), *R* is the gas constant (8.314 J mol^{-1} K^{-1}), *h* is the Planck's constant (11.04×10^{-36} J min), and k_B is the Boltzmann constant (1.38×10^{-23} J K^{-1}).

The *z*-value (temperature rise necessary to reduce *D*-value by one logarithmic cycle) was calculated from the slope of graph between log *D* versus temperature (°C) using the following equation:

$$\text{Slope} = \frac{1}{z}$$

Table 2
Experimental design (CCD) for lipase immobilization using RSM.

Sr. No.	*A*: no. of pellets	*B*: glutaraldehyde (%, v/v)	*C*: enzyme dose (mL)	*D*: coupling period (min)	% immobilization
1	4	2	0.02	30	26.60
2	8	2	0.02	30	36.32
3	4	6	0.02	30	32.30
4	8	6	0.02	30	40.17
5	4	2	0.06	30	18.58
6	8	2	0.06	30	40.86
7	4	6	0.06	30	21.30
8	8	6	0.06	30	46.01
9	4	2	0.02	120	39.70
10	8	2	0.02	120	55.02
11	4	6	0.02	120	51.44
12	8	6	0.02	120	62.30
13	4	2	0.06	120	28.10
14	8	2	0.06	120	46.10
15	4	6	0.06	120	30.20
16	8	6	0.06	120	59.20
17	2	4	0.04	75	16.20
18	10	4	0.04	75	78.20
19	6	0	0.04	75	19.30
20	6	8	0.04	75	31.20
21	6	4	0	75	15.40
22	6	4	0.08	75	29.14
23	6	4	0.04	0	18.10
24	6	4	0.04	165	40.79
25–30	6	4	0.04	75	60.72

2.12. Effect of organic solvents on the activity of the immobilized enzyme

To study the effect of organic solvents on the immobilized lipase, the enzyme bound aluminum oxide beads were treated with organic solvents viz. isopropanol, xylene, methanol, ethanol, dimethylsulphoxide (DMSO), toluene, hexane and acetonitrile to a final concentration of 25% (v/v) for 12, 24 and 36 h. The residual activity of the immobilized lipase was then determined under standard assay conditions. The enzyme activity of the sample without adding any organic solvent was taken as control (100%). The stability of the immobilized lipase in various organic solvents was also compared with that of free enzyme after an incubation of 24 h.

2.13. Determination of kinetic parameters

The apparent Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) of free and the immobilized enzyme were calculated using the Lineweaver–Burk plot. The substrate concentration used for enzyme assay ranged from 0.17 to 2.6 mM.

2.14. Application of the immobilized lipase in esterification of ethanol with oleic acid

Partially purified lipase immobilized on aluminum oxide pellets was used as biocatalyst for the esterification of oleic acid and ethanol in 1:1 (v/v) ratio in hexane. The reaction was carried out at 37 °C with shaking at 100 rpm for 4, 8, 12, 16, 20 and 24 h with heat inactivated free enzyme as control. The ester content was quantified by using the alkalimetric method of titrating unreacted acid with 0.1 N NaOH using phenolphthalein as an indicator. The percentage conversion in ester synthesis was based on the amount of acid consumed [34]. Reusability of the immobilized lipase in the above esterification reaction was also tested.

2.15. TLC and ^{1}H NMR

Analytical TLC was performed using pre-coated silica gel 60 F_{254} MERCK TLC plates (20 cm × 20 cm). The spots were visualized by immersing the plates in 10% H_2SO_4 in ethanol followed by heating on hot plate. ^{1}H NMR spectra were recorded with BUCKER 500 MHz NMR instrument. Chemical data for protons are reported in parts per million (ppm, scale) downfield from tetramethylsilane (TMS) and are referenced to the residual proton in the NMR solvent (CDCl3: δ 7.26).

3. Results and discussion

The lipase produced by the isolate *Bacillus* sp. DVL2 under submerged fermentation was partially purified through ammonium sulphate fractionation (20–70%) and Hydrophobic Interaction Chromatography on Phenyl-sepharose CL-4B column. The partially purified enzyme (specific activity 1.5 IU/mg protein) was covalently immobilized on glutaraldehyde-activated aluminum oxide pellets. This support is preferred for immobilization as these pellets are mechanically tough and resistant to high pH and temperature. In addition, the conformation of pellets was maintained during moderate shaking for esterification reaction. The parameters of the immobilization process were statistically optimized using RSM to enhance yield of the immobilized enzyme.

3.1. Optimization of lipase immobilization through RSM

Four process parameters, i.e. the number of pellets, glutaraldehyde concentration, volume of lipase and coupling time were optimized using RSM. Tables 1 and 2 represent the range of components and experimental design for RSM, respectively. Analysis of the results obtained from RSM showed that immobilization yield (IY) varied greatly from 15.4 to 78.2% (Table 2). These results encouraged the use of statistical optimization of process parameters. The responses of the CCD design were fitted with a polynomial quadratic equation (Eq. (1)). The overall polynomial equation for the immobilization (Y) of lipase was

$$Y = +60.19 + 10.91 \times A + 3.14 \times B - 1.08 \times C + 7.25 \times D + 0.45 \times A \times B + 3.14 \times A \times C + 0.54 \times A \times D - 0.34 \times B \times C + 1.05 \times B \times D - 2.26 \times C \times D - 1.65 \times A^2 - 7.14 \times B^2 - 7.88 \times C^2 - 7.08 \times D^2 \quad (1)$$

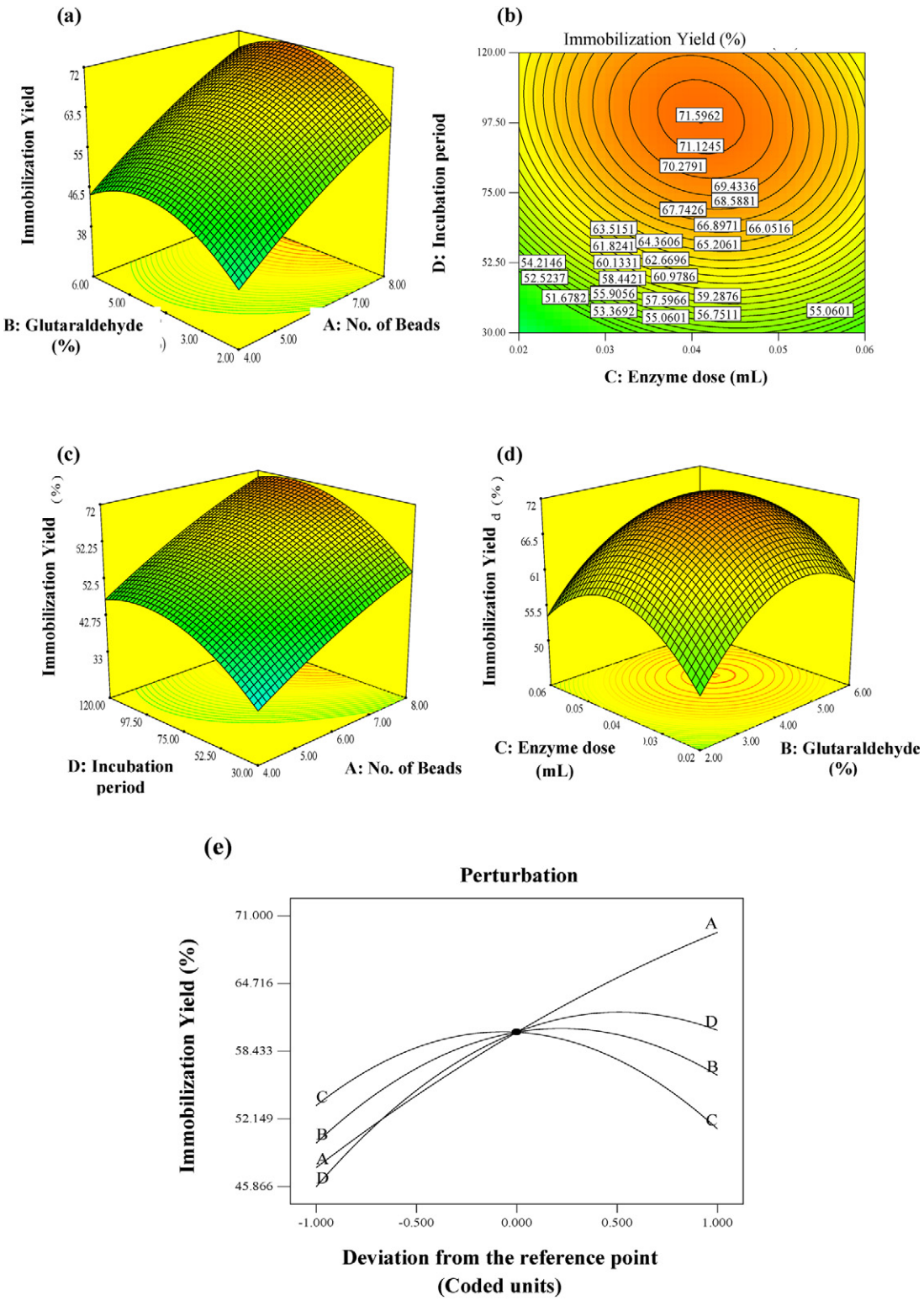

Fig. 1. Response surface plots showing cumulative effect of two variables on the immobilization of lipase while keeping other variables at "0" level; (a) interaction between factors *A* and *B*, (b) between factor *C* and *A*, (c) between factors *C* and *B*, (d) between factors *D* and *B* and (e) perturbation graph showing the effect of variables on immobilization yield (where *A*: number of beads; *B*: glutaraldehyde concentration; *C*: enzyme dose and *D*: incubation period).

where *A* is the number of pellets, *B* is glutaraldehyde concentration, *C* is enzyme dose and *D* is coupling time.

Statistical significance of the model equation was evaluated by the *F*-test for analysis of variance (ANOVA), which showed that the regression was statistically significant (Table 3). The model *F* value for IY was 7.62 as shown by Fisher's test, which indicated that the model was significant. The value of "Prob > *F*" less than 0.05 was desirable for a significant model. The *P* > *F* value of the model was <0.0002; therefore, the model terms were also significant. It implied that the model was statistically significant for the lipase immobilization. There was only a 0.01% chance that a "Model *F*-Value" was not significant which could occur due to noise. The determination

Table 3
Analysis of variance (ANOVA) for response surface quadratic model for lipase immobilization.

Term	*F*-value	*P*-value (Prob > *F*)	Value	
Model	7.62	0.0002	–	Significant
A: no. of beads	39.34	<0.0001	–	
B: glutaraldehyde	3.27	0.0907	–	
C: enzyme dose	0.39	0.5423	–	
D: incubation period	16.18	0.0011	–	
R^2	–	–	0.88	
Adjusted R^2	–	–	0.76	
Predicted R^2	–	–	0.30	
Adequate precision	–	–	9.93	
Standard deviation	–	–	8.52	
Mean	–	–	41.56	
Coefficient of variation %	–	–	20.50	
Predicted residual sum of squares (PRESS)	–	–	6172.09	

coefficient (R^2) of the model was 0.88 which showed the aptness of the model. The "Adeq Precision" measures the signal to noise ratio (a ratio of greater than 4 was desirable) and ratio of 9.93 of the model indicated an adequate signal. Thus, the model was significant for the process. A lower value of coefficient of variation (CV 20.50%) showed that the experiments conducted were precise and reliable.

The response plots (3D and contour curves) were analyzed to determine interaction among the variables and the optimum value of each component for maximum IY. Fig. 1a shows the cumulative effect of two factors viz. number of beads and glutaraldehyde concentration (%) on IY at central level of the remaining two factors. It showed clearly that IY increased proportionately when number of beads was increased from −1 to +1 level at 0 level of glutaraldehyde. The IY declined when glutaraldehyde concentration was increased or decreased beyond the central level. Fig. 1b reveals the variation of IY with incubation period and enzyme dose. The IY enhanced with increase in incubation period and enzyme dose initially. Maximum IY was obtained close to mid of 0 and +1 level of incubation period at 0 level of enzyme dose. The effect of incubation period and number of beads on IY is shown in Fig. 1c. Incubation period at mid of 0 and +1 level resulted in maximum IY. However, number of beads resulted in maximum IY at +1 level. Both the factors viz. incubation period and number of beads showed strong interaction effect as indicated by a more elliptical contour plot. Fig. 1d analysis reveals that the maximum immobilization yield was obtained when both enzyme dose and glutaraldehyde concentration were taken near '0′ level. Umbrella shaped curve indicates strong interaction between factors close to '0′ level. Any change in concentration of both the factors from the central level leads to decrease in IY. As glutaraldehyde is the cross linking agent, so its concentration is directly proportional to the cross linking of enzyme. The simultaneous effect of all the variables on the lipase immobilization yield can be seen in the perturbation graph (Fig. 1e). The plot reveals that the immobilization process is most sensitive to the number of pellets while least influenced by the enzyme dose.

All the RSM graphs were analyzed in the similar way to determine the optimum value of each factor for maximum IY. The maximum IY of 78.20% was obtained when 10 aluminum oxide pellets were treated with 4% glutaraldehyde and subsequent incubation with 0.04 mL of enzyme for 75 min.

3.2. Operational stability of the immobilized enzyme

Immobilized enzymes are preferred as they can be recycled, resulting in lower production costs. The operational stability of the lipase immobilized on glutaraldehyde-activated aluminum oxide pellets was investigated for 15 consecutive cycles. There was no loss in enzyme activity up to 5 consecutive cycles but thereafter, a decrease was observed (Fig. 2). After 10th and 15th cycles, the enzyme retained nearly 75% and 25% of its activity. So, the immobilized enzyme could apparently be reused for 10 cycles without substantial loss of activity. The decrease in enzyme activity during repeated use might be due to inactivation of enzyme as suggested earlier [35].

3.3. Effect of pH

The pH optima of free and the immobilized lipase are shown in Fig. 3. It was evident from the data that the free enzyme showed a broad pH optimum (6.0–9.0) with maximum at pH 8.0. The immobilized lipase also showed broad pH optimum in the range of 7.0–9.0 but it exhibited slightly higher activity at acidic and alkaline pH values as compared to its soluble counterpart. It could be due to effect of the microenvironment of the immobilized enzyme.

The pH stability data shown in Fig. 4 revealed that both free and the immobilized lipase exhibited substantial stability in the pH range of 6.0–10.0 upon pre-incubation for 2 h but the immobilized form showed better stability at extreme pH values. The soluble enzyme was completely inactive at pH 4.0 and exhibited 12.4% activity at pH 11.0 while the immobilized enzyme showed 25% and 46.2% activity at pH 4.0 and 11.0, respectively. Ortega et al. [36] also reported enhanced stability of lipase at acidic and alkaline pH upon immobilization. The increase in stability upon immobilization might be due to multipoint covalent linking between lipase and support, which prevents lipase denaturation in acid or alkaline environments as suggested by Yang et al. [37]. Similar results were obtained with the immobilized *Candida rugosa* lipase on chitosan by Hung et al. [38].

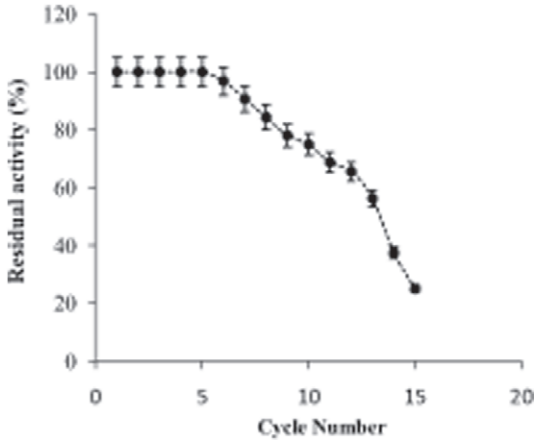

Fig. 2. The operational stability of covalently the immobilized lipase on glutaraldehyde-activated aluminum oxide pellets for 15 cycles.

Fig. 3. Effect of pH on the activity of free (*solid circle*) and the immobilized (*hollow circle*) lipase. The lipase activity was measured at different pH values using buffers viz. sodium citrate (pH 4.0–6.0), sodium phosphate (pH 7.0–9.0) and glycine–NaOH (pH 10.0–11.0) at 0.05 M each under standard assay conditions.

3.4. Effect of temperature on free and the immobilized lipase

The effect of varying temperature from 25 to 45 °C revealed that the activity of free lipase increased up to 30 °C and declined on further raising the temperature while the immobilized lipase displayed maximum activity at 37 °C (Fig. 5a). It was inferred from these results that the temperature optima for catalysis of the reaction by free and the immobilized enzymes were 30 and 37 °C, respectively. So, there was displacement of optimum temperature of the enzyme from 30 to 37 °C after immobilization. Nawani et al. [32] also observed a shift in the optimum temperature after the immobilization of lipase.

Activation energy for the immobilized lipase was calculated from the slope of the Arrhenius plot (Fig. 5a inset). The regression equation for Arrhenius plot of free and the immobilized lipase was $y = -0.600x + 0.751$ and $y = -0.758x + 1.138$, respectively. The activation energy calculated for the immobilized lipase was 6.3 kJ mol^{-1}, which was higher than that of free enzyme (5.0 kJ mol^{-1}). The strong affinity between the lipase and aluminum oxide pellets might cause a higher energy barrier of the first deactivation step. Similar results were reported during the immobilization of lipase by other researchers [7,39].

On comparing the thermal stability of free and the immobilized lipase it was found that free lipase was completely inactive after 1 h incubation at 60 °C while the immobilized lipase retained 25% of its activity (Fig. 5b). So, the immobilized lipase was found to have better thermo-stability as compared to its soluble form. It could be due to the formation of intermolecular covalent linkages between the enzyme and support which impart rigidity to the structure of the enzyme molecule [26,32]. Similar to our finding, Dosanjh and Kaur [40], observed that temperature stability of the immobilized enzyme was enhanced from 50 to 60 °C in comparison with the soluble enzyme from *Bacillus* sp. Increased thermo-stability of lipase upon immobilization was also reported by other researchers [26,36]. The restricted interaction between the immobilized enzyme molecules could also be responsible for retaining the enzyme activity at higher temperatures [41].

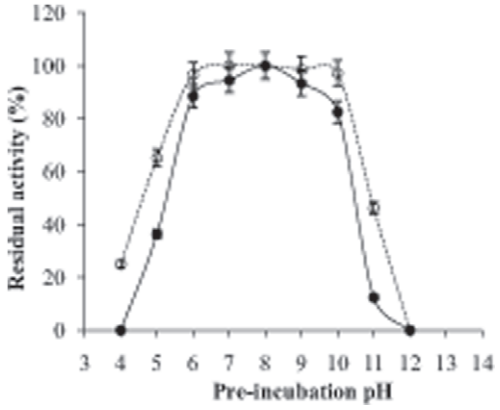

Fig. 4. Investigation of pH stability of free (*solid circle*) and the immobilized (*hollow circle*) lipase. Free and the immobilized lipases were pre-incubated separately in the buffers (mentioned in Fig. 3) for 2 h followed by determination of residual activity at the optimum temperature under standard assay conditions.

The investigation of thermal stability of the immobilized enzyme at different temperatures (30–60 °C) with respect to time period revealed that the enzyme was nearly fully stable up to 40 °C for 60 min but gradually lost its activity upon incubation at higher temperatures (Fig. 5c). The immobilized enzyme showed 45.2, 22.1 and 12.0% residual activity on incubation for 120 min at 45, 50 and 55 °C, respectively.

The plots of log (% residual activity) versus time were linear indicating the first order kinetics of immobilized enzyme (Fig. 5d). To determine the thermodynamic parameters for thermal stability, the activation energy (E_d) for thermal denaturation was determined from slope of the plot between $\ln k_d$ and reciprocal of the absolute temperature (Fig. 5d inset). The E_d for the immobilized enzyme (178.67 kJ mol^{-1}) was higher in comparison to free enzyme (166.08 kJ mol^{-1}). The higher value of E_d meant more energy was required to denature the bound enzyme. These observations implied that the immobilized enzyme was more stable and resistant to heat denaturation. At higher temperatures, the rate of enzyme inactivation was greater as indicated by lower *D*-values [42].

The thermo stability parameters of free and the immobilized lipase are summarized in Table 4. The half-lives and *D*-values of lipase prolonged remarkably at all temperatures after covalent attachment indicating better thermo stability of the immobilized lipase. The values of ΔH° were higher for the immobilized lipase as compared to free lipase (Table 4). At 30 °C, ΔH° of free lipase was 163.54 kJ mol^{-1} while that of the immobilized enzyme was 176.12 kJ mol^{-1} indicating the requirement of more energy for thermal denaturation of the immobilized enzyme. The thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with concomitant increase in the enthalpy of activation [43]. The ΔG° for thermal unfolding at 30 °C was 110.16 kJ mol^{-1} for free enzyme while after immobilization it was 114.85 kJ mol^{-1}. A higher value of free energy of thermal denaturation indicated the resistance of the immobilized enzyme towards thermal unfolding at higher temperatures which meant more stability of the immobilized enzyme. The ΔS° values were also enhanced after immobilization (Table 4). This could result from thermal agitations and conformational changes. In accordance with the results of this study, immobilization caused significant increase in both entropy and enthalpy changes compared to the native enzyme.

The *z*-values of free and the immobilized lipases were 12.2 and 11.1 °C, respectively (Fig. 5e). The higher *z*-value of the immobilized enzyme implied that it was more sensitive to increase in temperature rather than its duration as compared to its soluble counterpart.

3.5. Effect of organic solvents

The immobilized lipase showed better stability than free enzyme in organic solvents (Fig. 6a). However, the extent of

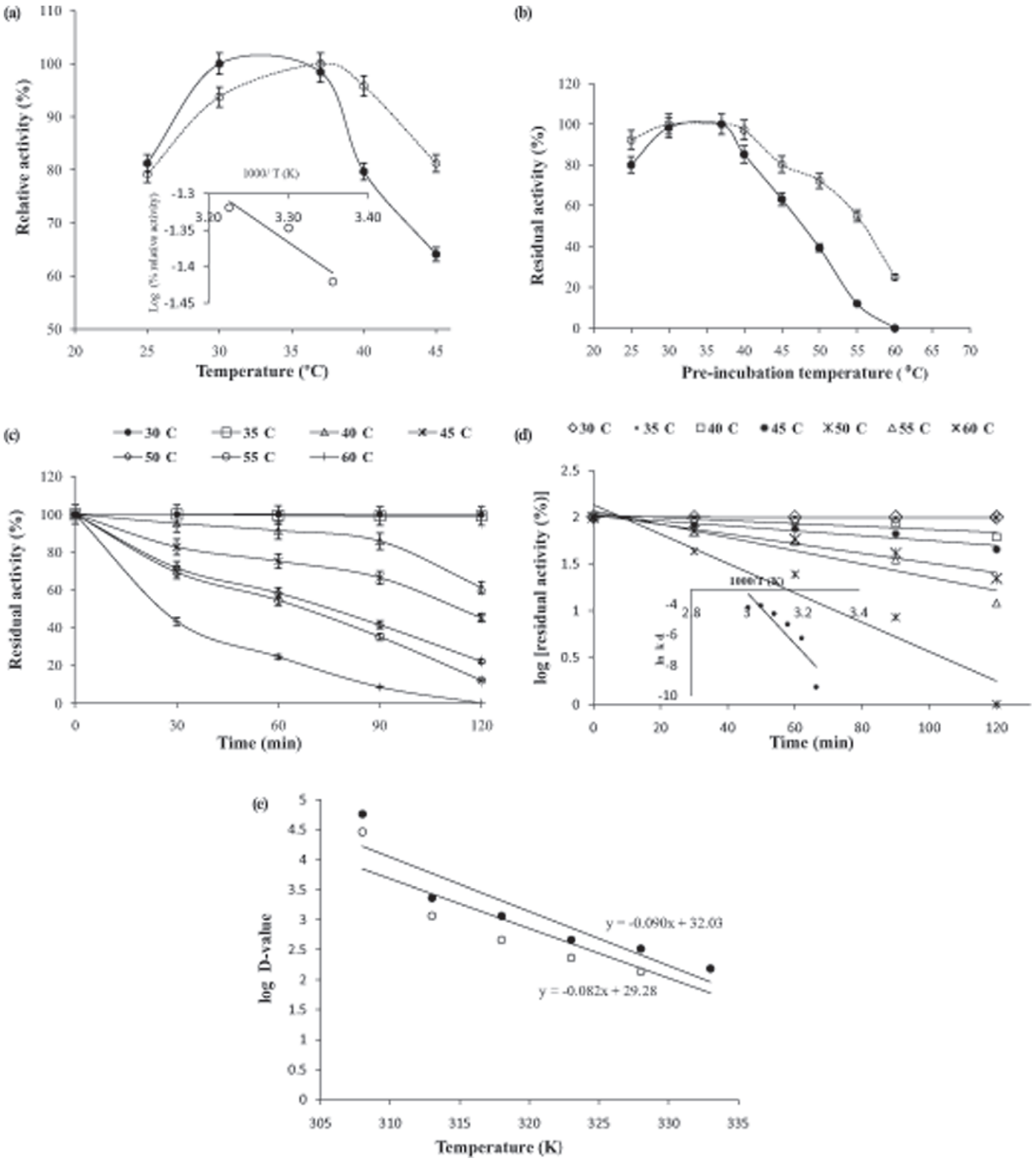

Fig. 5. Effect of temperature on free (solid circle) and the immobilized (hollow circle) lipase. (a) Temperature optima, Inset is the Arrhenius plot to calculate activation energy (E_a) of catalysis; (b) comparison of thermal stability of free and the immobilized lipase; (c) thermal stability of the immobilized lipase with respect to time; (d) first order thermal deactivation of the immobilized lipase, Inset is the Arrhenius plot to calculate activation energy (E_d) for denaturation and (e) temperature dependence of the decimal reduction of free and the immobilized lipase to calculate *z*-values.

stability varied from solvent to solvent. The enhancement in the stability of the immobilized lipase might be due to the increase in rigidity of the enzyme upon covalent attachment of the enzyme on aluminum oxide pellets. Increase in solvent stability after the immobilization on synthetic beaded macroporous copolymers has earlier been reported [44].

The effect of organic solvent on the activity of the immobilized lipase with respect to time of incubation (12, 24 and 36 h)

Table 4
Kinetic and thermodynamic parameters for thermal inactivation of free and immobilized lipase.

Thermodynamic entity		Temperature						
		30 °C	35 °C	40 °C	45 °C	50 °C	55 °C	60 °C
k_d (min^{-1})	F	0.00005	0.00008	0.00200	0.00500	0.01000	0.01700	0.01500
	I	0.00001	0.00004	0.00100	0.00200	0.00500	0.00700	0.01500
$t_{1/2}$ (min)	F	13,862.94	8664.34	346.57	138.63	69.32	40.77	46.21
	I	115,524.53	17,328.68	693.15	346.57	138.63	99.02	46.21
D-value (min)	F	46,051.70	28,782.31	1151.29	460.52	230.26	135.45	153.51
	I	383,764.18	57,564.63	2302.59	1151.29	460.52	328.94	153.51
ΔH°(kJ mol^{-1})	F	166.48	166.46	166.44	166.43	166.42	166.42	166.42
	I	179.06	179.04	179.02	179.01	179.00	179.00	179.00
ΔG°(kJ mol^{-1})	F	109.51	110.16	103.61	102.88	102.68	102.87	102.82
	I	114.85	111.93	105.41	105.31	104.54	104.29	103.62
ΔS°(J mol^{-1} K^{-1})	F	188.02	182.82	200.73	199.83	197.34	193.76	184.96
	I	211.91	217.90	235.15	231.77	230.53	224.74	222.74

F and I represent free and the immobilized lipase, respectively.

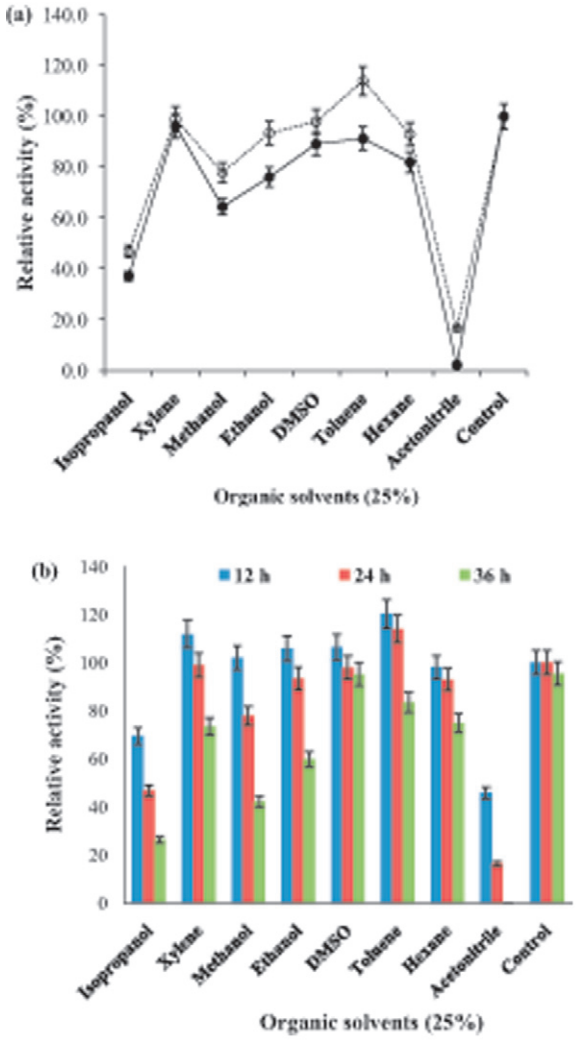

Fig. 6. (a) Comparison of stability of free (solid circle) and the immobilized (hollow circle) lipase in different organic solvents each viz. isopropanol, xylene, methanol, ethanol, dimethylsulphoxide (DMSO), toluene, hexane and acetonitrile at a final concentration of 25% (v/v) after 24 h incubation. (b) Effect of the above mentioned organic solvents on the activity of the covalently immobilized lipase with respect to time of incubation.

was also investigated and the results are shown in Fig. 6b. The immobilized lipase was found to be 100% stable in xylene, ethanol, methanol, toluene and hexane after 12 h of incubation. The stability in these solvents though declined after 24 h incubation as compared to 12 h yet the relative activity was greater than 90%, except methanol (80%). After 36 h of incubation, the bound enzyme was most stable in DMSO followed by toluene, hexane and xylene with residual activity of 93.1, 81.8, 73.4 and 71.9%, respectively. Further, isopropanol and acetonitrile inactivated the immobilized lipase to some extent as indicated by low residual activity, i.e. 69.5% and 45.8% after 12 h, respectively. The greater stability of *Bacillus* sp. DVL 2 lipase in water immiscible solvents is in accordance with earlier findings [18,21]. Most lipases exhibit a lid of hydrophobic amino acids over the active site. In the presence of hydrophobic organic solvents such as toluene, the lid is opened up and more substrate gets accessible to active site resulting in higher enzyme activity than the control [45,46]. The effect of organic solvents may also vary among different lipases [47,48].

3.6. Determination of kinetic parameters

The Kinetic parameters were calculated using the Lineweaver–Burk plot. The K_m was 0.94 and 1.3 mM while V_{max} was 99 and 108 μmol/min for free and the immobilized lipase, respectively (Fig. 7). An increase in K_m upon immobilization might be due to limited diffusion of the substrate to the active site of the enzyme. The increase in V_{max} upon immobilization might be due to enhanced stability of the covalently bound enzyme.

3.7. Esterification

Enzymatic methods of ester synthesis are more effective in non-aqueous media. In this study, the potential of free and the immobilized lipases were compared in esterification of oleic acid with ethanol to form ethyl oleate in hexane (Fig. 8a). The immobilized lipase showed maximum 63% conversion of oleic acid into ethyl oleate in 16 h whereas free lipase showed maximum 60% conversion of oleic acid into ethyl oleate in 24 h. The ester formation by the immobilized enzyme was also confirmed by TLC chromatogram and ^{1}H NMR spectra. Analysis of ^{1}H NMR spectrum of ethyl oleate is shown below:

^{1}H NMR (500 MHz, $CDCl_3$): δ 5.38–5.30 (m, 2H), 4.14–4.10 (q, 2H, J = 7.1 Hz), 2.30–2.27 (t, 2H, J = 7.5 Hz), 2.05–2.00 (m, 4H), 1.62–1.58 (m, 6H), 1.35–1.24 (m, 19H), 0.89–0.86 (t, 3H, J = 7.0 Hz).

Since the immobilized lipase enhanced the conversion of acid into ester by 3% and reduced esterification time by 8 h, it was more

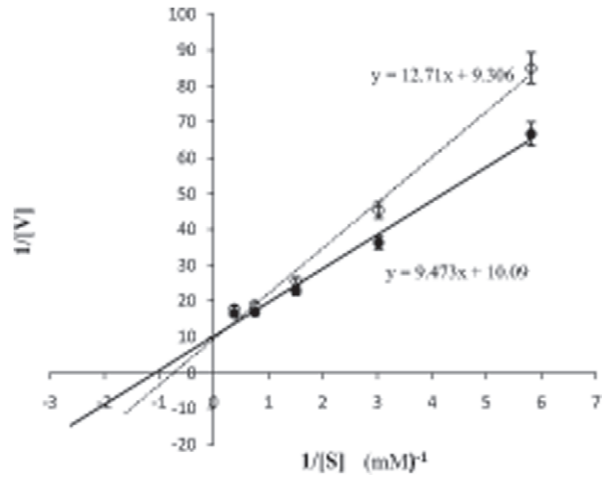

Fig. 7. Lineweaver–Burk plot for estimation of kinetic parameters for free (solid circle) and the immobilized (hollow circle) lipase. The substrate concentration used to generate this plot ranged from 0.17 to 2.6 mM.

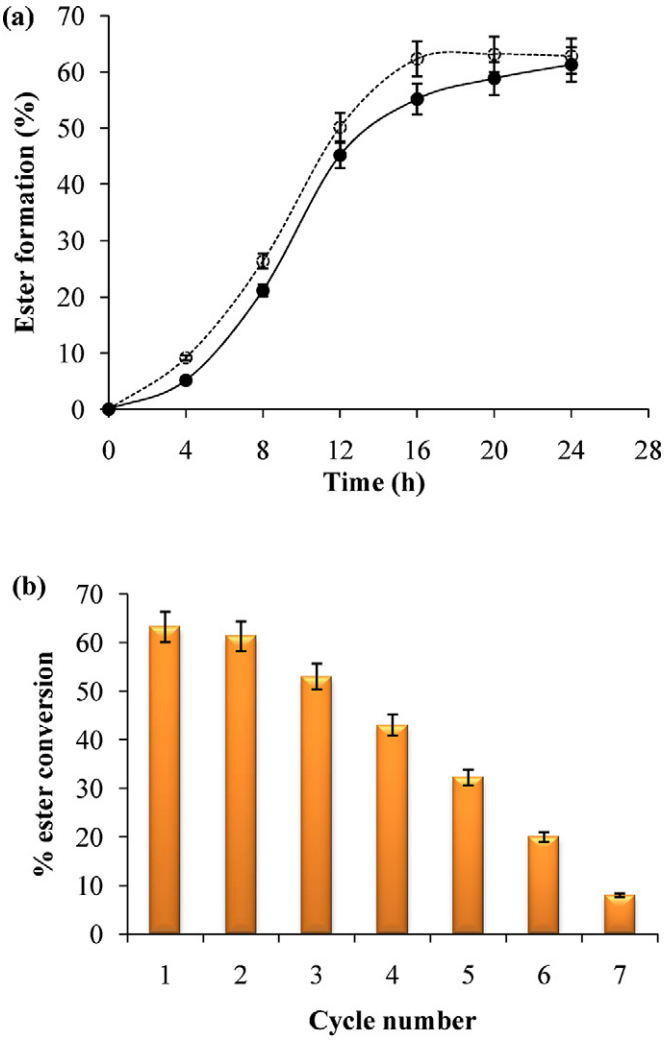

Fig. 8. (a) Esterification of oleic acid and ethanol in hexane to produce ethyl oleate by the free (solid circle) and the immobilized lipase (hollow circle). (b) Reusability of the immobilized enzyme in esterification reaction of oleic acid and ethanol to produce ethyl oleate.

efficient in esterification than free lipase. This might be due to greater stability of the covalently immobilized enzyme in organic solvents. These results are similar to those of Kanwar et al. [49] who observed 58% conversion of oleic acid and ethanol into ethyl oleate in n-nonane using synthetic hydrogel-bound lipase of *Bacillus coagulans*.

The investigation of reusability of the immobilized lipase in the above esterification reaction for seven cycles revealed a gradual decrease in esterification efficiency after 2 cycles, retaining about 50, 31 and 12% of the initial activity after 5th, 6th and 7th cycle respectively (Fig. 8b) which could be due to several reasons including the inhibitory effect of water released during esterification [50] and prolonged exposure to the organic solvent. In agreement with these findings, repeated use of *C. rugosa* lipase covalently immobilized on chitin for butyl butyrate synthesis lost almost 83% of its initial activity in just 5 s [51]. Similarly, reuse of immobilized *Candida antarctica* lipase-B by adsorption on Lewatit for three consecutive esterification of lauric acid and isopropanol showed 40% reduction in percent relative conversion after each cycle [52]. They attributed it to enzyme deactivation and protein desorption. Another report [53] revealed linear decay in percentage of residual activity of cross linked *C. antarctica* lipase-B on chitosan in ethyl oleate synthesis and the enzyme was completely deactivated after four cycles. They related this deactivation to a combined effect of deactivation/desorption of lipase and repeated manipulation operations. It is likely that recyclability of the immobilized lipase depends on either the nature of support or the enzyme source as suggested earlier [52].

4. Conclusion

In this study *Bacillus* sp. DVL2 lipase was covalently immobilized on glutaraldehyde activated aluminum oxide pellets, which are mechanically tough and resistant to high pH and temperature. This is apparently the first report for the immobilization of lipase on aluminum oxide pellets through covalent attachment. The immobilized lipase showed maximum immobilization yield of 78.20% under RSM optimized conditions and exhibited operational stability for 5 consecutive cycles without any loss of enzyme activity. The stability of the immobilized enzyme to pH, temperature and organic solvents was enhanced as compared to free lipase. Thermodynamic studies also indicated an improvement in enzyme stability after immobilization. The efficiency of the immobilized lipase in esterification of oleic acid and ethanol was better as it increased the conversion of acid to ester by 3% and reduced the time of esterification by 8 h as compared to free enzyme, which showed 60% conversion of acid to ester in 24 h. Reuse of the immobilized enzyme for seven cycles in esterification reaction showed a gradual decrease in esterification efficiency after 2 cycles, retaining about 50, 31 and 12% of the initial activity after 5th, 6th and 7th cycle respectively.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2012.10.002.

References

[1] A.P. Kempka, N.R. Lipke, T.L.F. Pinheiro, S. Menoncin, H. Treichel, D.M.G. Freire, Bioprocess Biosyst. Eng. 31 (2008) 119–125.
[2] C. Hedfors, K. HultK, M. Martinelle, J. Mol. Catal. B: Enzyme 66 (1–2) (2010) 120–123.
[3] C. Schmidt-Dannert, Bioorg. Med. Chem. 7 (1999) 2123–2130.
[4] K.P. Zhang, J.Q. Lai, Z.L. Huang, Z. Yang, Bioresour. Technol. 102 (2011) 2767–2772.

[5] M.T. Reetz, Curr. Opin. Chem. Biol. 6 (2002) 145–150.
[6] K.E. Jaeger, T.M. Reetz, Trends Biotechnol. 16 (1998) 396–403.
[7] S. Sharma, M.N. Gupta, Biotechnol. Appl. Biochem. 33 (2001) 161–165.
[8] R. Gupta, N. Gupta, P. Rathi, Appl. Microbiol. Biotechnol. 64 (2004) 763–781.
[9] G.D. Haki, S.K. Rakshit, Bioresour. Technol. 89 (2003) 17–34.
[10] R. Sangeetha, I. Arulpandi, A. Geeta, Res. J. Microbiol. 6 (2011) 1–24.
[11] R.K. Saxena, P.K. Gosh, R. Gupta, W.S. Davidson, S. Bradoo, R. Gulati, Curr. Sci. 77 (1) (1999) 101–115.
[12] F. Hasan, A.A. Shah, A. Hameed, Enzyme Microb. Technol. 39 (2006) 235–251.
[13] J. Vakhlu, A. Kour, Electr. J. Biotechnol. 9 (2006) 69–85.
[14] M. Iso, B. Chen, M.T. Eguchi, T. Kudo, S. Shrestha, J. Mol. Catal. B: Enzyme 16 (2001) 53–58.
[15] S. Hazarika, N. Goswami, N. Dutta, A. Hazarika, Chem. Eng. J. 85 (1) (2002) 61–68.
[16] J.M. Palomo, C. Ortiz, M. Fuentes, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, J. Chromat. A 1038 (2004) 267–273.
[17] Z.D. Knezevic, S.S. Siler-Marinkovic, L.V. Mojovic, Acta Period. Technol. 35 (2004) 151–164.
[18] J.A. Silva, G.P. Macedo, D.S. Rodrigues, R.L.C. Giordano, L.R.B. Goncalves, Biochem. Eng. J. 60 (2012) 16–24.
[19] D.S. Rodrigues, A.A. Mendes, W.S. Adriano, L.R.B. Goncalves, R.L.C. Giordano, J. Mol. Catal. B: Enzyme 51 (2008) 100–109.
[20] J. Brem, M.C. Turcu, C. Paizs, K. Lundell, M.I. Tosa, F.-D. Irimie, L.T. Kanerva, Process Biochem. 47 (2012) 119–126.
[21] A. Tomin, D. Weiser, G. Hellner, Z. Bata, L. Corici, F. Peter, B. Koczka, L. Poppe, Process Biochem. 46 (2011) 52–58.
[22] C. Zarcula, L. Corici, R. Croitoru, A. Ursoiu, F. Peter, J. Mol. Catal. B: Enzyme 65 (2010) 79–86.
[23] A.A. Mendes, R.C. Giordano, L.C. De, R. Giordano, H.F.D. Castro, J. Mol. Catal. B: Enzyme 68 (2011) 109–115.
[24] R. Fernandez-Lafuente, P. Armisen, P. Sabuquillo, G. Fernandez-Lorente, J.M. Guisan, Chem. Phys. Lipids 93 (1998) 185–197.
[25] H.K. Lee, J.K. Lee, M.J. Kim, C.J. Lee, Bull. Korean Chem. Soc. 31 (2010) 650–652.
[26] S. Sakai, Y. Liu, T. Yamaguchi, R. Watanabe, M. Kawabe, K. Kawakami, Biotechnol. Lett. 32 (2010) 1059–1062.
[27] M.-M. Zheng, L. Dong, Y. Lu, P.-M. Guo, Q.-C. Deng, W.-L. Li, Y.-Q. Feng, F.-H. Huang, J. Mol. Catal. B: Enzyme 74 (2012) 16–23.
[28] M.-M. Zheng, Y. Lu, L. Dong, P.-M. Guo, Q.-C. Deng, W.-L. Li, Y.-Q. Feng, F.-H. Huang, Bioresour. Technol. 115 (2012) 141–146.
[29] M. Kalantari, M. Kazemeini, F. Tabandeh, A. Arpanaei, J. Mater. Chem. 22 (2012) 8385.
[30] Y. Kuwahara, T. Yamanishi, T. Kamegawa, K. Mori, M. Che, H. Yamashita, Chem. Commun. 48 (2012) 2882–2884.
[31] J.F.M. Burkert, F. Maugeri, M.I. Rodrigues, Bioresour. Technol. 91 (2004) 77–84.
[32] N. Nawani, J. Khurana, J. Kaur, Mol. Cell. Biochem. 290 (2006) 17–22.
[33] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1) (1951) 265–275.
[34] R. Bovara, G. Carrea, G. Ottolina, S. Riva, Biotechnol. Lett. 15 (9) (1993) 937–942.
[35] G. Fernandez-Lorente, M. Terreni, C. Mateo, A. Bastida, R. Fernandez-Lafuente, P. Dalmases, J. Huguet, J.M. Guisan, Enzyme Microb. Technol. 28 (2001) 389–396.
[36] N. Ortega, M.P. Mateos, M.C. Pilar, M.D. Busto, J. Agric. Food Chem. 57 (2009) 109–115.
[37] Y. Yang, Y.X. Bai, Y.F. Li, L. Lei, Y.J. Chui, C.G. Xia, Process Biochem. 43 (2008) 1179–1185.
[38] T.C. Hung, R. Giridhar, S.H. Chiou, W.T. Wu, J. Mol. Catal. B: Enzyme 26 (2003) 69–78.
[39] T. Matsumoto, M. Ito, H. Fukuda, A. Kondo, Appl. Microbiol. Biotechnol. 64 (4) (2004) 481–485.
[40] N.S. Dosanjh, J. Kaur, Biotechnol. Appl. Biochem. 36 (1) (2002) 7–11.
[41] W. Ping, D. Sheng, S.D. Waezsada, A.Y. Tsao, B.H. Davinson, Biotechnol. Bioeng. 74 (2001) 249–255.
[42] M. Singh, R.S. Singh, U.C. Banerjee, Process Biochem. 45 (2010) 25–29.
[43] Y. Nosoh, T. Sekiguchi, Trends Biotechnol. 8 (1990) 16–20.
[44] I. Bhushan, R. Parshad, G.N. Qazi, G. Ingavle, C.R. Rajan, S. Ponrathnam, V.K. Gupta, Process Biochem. 43 (2008) 321–330.
[45] H. Ogino, S. Nakagawa, K. Shinya, J. Biosci. Bioeng. 9 (2008) 451–457.
[46] N. Doukyu, H. Ogino, Biochem. Eng. J. 48 (2008) 270–282.
[47] J.L. Ramos, E. Duque, M.T. Gallegos, P. Godoy, M.I. Ramos-Gonzalez, A. Rojas, W. Teran, A. Segura, Annu. Rev. Microbial. 56 (2002) 743–768.
[48] C.J. Hun, R.N. Zaliha, A. Rahman, A.B. Salleh, M.A. Basri, Biochem. Eng. J. 15 (2003) 147–151.
[49] S.S. Kanwar, R.K. Kaushal, M.L. Verma, Y. Kumar, W. Azmi, R. Gupta, S.S. Chimni, G.S. Chauhan, Indian J. Biotechnol. 6 (1) (2007) 68–73.
[50] S. Chand, P. Adlercreutz, B. Mattiasson, Enzyme Microb. Technol. 20 (2) (1997) 102–106.
[51] F.M. Gomes, E.B. Pereira, H.F. Castro, Biomacromolecules 5 (2004) 17–23.
[52] A. Vaidya, G. Gera, S. Ramakrishna, World J. Microbiol. Biotechnol. 24 (2008) 2987–2995.
[53] M.L. Foresti, M.L. Ferreira, Enzyme Microb. Technol. 40 (2007) 769–777.