Korean J. Chem. Eng., **31**(7), 1225-1232 (2014)
DOI: 10.1007/s11814-014-0020-8

pISSN: 0256-1115
eISSN: 1975-7220

Immobilization of *Candida antarctica* lipase onto cellulose acetate-coated Fe_2O_3 nanoparticles for glycerolysis of olive oil

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(*Received 19 May 2013 • accepted 14 January 2014*)

Abstract−*Candida antarctica* lipase was covalently immobilized onto the surface of cellulose acetate-coated Fe_2O_3 nanoparticles. The characterizations of immobilized lipase were examined by Fourier transform infrared spectrophotometer (FTIR) and field emission gun-scanning electron microscopy (FEG-SEM). The immobilized lipase was assayed for production of monoglycerides (MG) and diglycerides (DG) by glycerolysis of olive oil in a solvent medium. The effect of various reaction conditions on the MG and DG production such as reaction time, temperature, the molar ratio of glycerol to oil and amount of immobilized lipase was investigated. The optimum condition for MG and DG production was found at 50 ºC temperature and 0.025 g of lipase with the molar ratio of glycerol to oil 1.5 : 1 in 5 h of reaction time. The effect of substrate concentration on enzymatic activity of the free and immobilized lipase showed the best fits to the Lineweaver-Burk plots. The K_m and V_{max} values of immobilized lipase were found to be 25 mM and 0.58 mM/min, whereas that for free lipase was 52.63 mM and 1.75 mM/min, respectively. The activation and deactivation energy was found to decrease for immobilization of lipase on cellulose acetate-coated Fe_2O_3 nanoparticles.

Keywords: Immobilization, *Candida antarctica* Lipase, Glycerolysis, Monoglycerides, Diglycerides

INTRODUCTION

Immobilization of enzymes is important for improving their activity and stability, easy separation from the reaction mixture, easy recovery and reusability for industrial purposes [1]. The support materials used for enzyme immobilization should possess thermostability, mechanical strength, hydrophilicity, regenerability, microbial resistance, low-cost and a high capacity of enzyme [2]. Several approaches have been reported for the immobilization of enzyme on the different supports either by covalent binding [3], entrapment [4], or physical adsorption [5].

Lipase is a water-soluble enzyme that catalyzes the hydrolysis of fats and oils to free fatty acids, diglycerides, monoglycerides and glycerol over an oil-water interface [6]. Mono- and diglycerides are nonionic emulsifier molecules with both hydrophilic and hydrophobic parts. They are the most widely used for the food, cosmetic, and pharmaceutical industries [7]. However, the industrial applications of lipase have been limited due to the high costs and inability in their separation, poor stability under operational conditions, and impossibility of multiple reuses [8]. Enzyme immobilization is the most commonly used strategy to overcome these limitations.

Lipases have been immobilized on various support materials, including chitosan beads, agarose, alginate [9], celite [10], bentonite [11], sucrose [12], polypropylene [13], $CaCO_3$ [14] and glass bead [15]. In recent years, nanoparticles have emerged as an alternative support material for lipase immobilization because of their high specific surface area to favor the binding capacity, lower transfer resistance to solve diffusion problem, easy preparation, versatile surface chemistry and lower operational cost [16]. Therefore, a variety of nanoparticles have been used for lipase immobilization such as magnetite [17], silica [18], tin dioxide [19], Zirconia [20], chitosan [21] and cerium oxide [22]. Fe_2O_3 nanoparticles are considered suitable for immobilization of lipase because of their multifunctional characteristics, such as super paramagnetism, high specific surface area, low toxicity, biocompatibility, easy separation by magnetic field, assembling behavior and reduced operating cost [23]. However, the problem of aggregation and biocompatibility of Fe_2O_3 nanoparticles can delay applications in industry [24]. It is an efficient method to modify Fe_2O_3 nanoparticles using polymer by preparing nano-composite particles. Polymer-coated nanoparticles have more potent magnetic susceptibility and large surface area of polymer surface for enzyme immobilization. For therapeutic applications, the polymer coating for the immobilization of enzyme used must be biocompatible, eco-friendly, non-toxic and of low processing cost [25]. Cellulose acetate (CA) is a derivative of cellulose that has been widely applied for the enzyme immobilization due to its good biocompatibility and high stability [26]. Thus, there are many reports available on the use of various synthetic polymeric agents as a supporting material for the immobilization of enzyme, but few reports have been focused upon the use of magnetic composite materials based on polymer coated Fe_2O_3 nanoparticles for lipase immobilization.

Recently, Wu et al. [27] used chitosan-coated magnetic nanoparticles for lipase immobilization. Lee and co-workers [28] developed SDS-bound nanosized magnetite particles to immobilize porcine pancreas lipase. Yong et al. [29] reported polymer-grafted modified magnetic nanoparticles for immobilizing *Candida rugosa* lipase. Liu et al. [24] also developed magnetic nanoparticles grafted polymer for immobilization of *Candida rugosa* lipase. Hu et al. [30] employed magnetic nanoparticles Fe_3O_4 coated with 3-aminopropyltriethoxysilane (APTES) as carriers to immobilize lipase from *Serratia marcescens* with glutaraldehyde as the coupling agent. Chen et al. [31] reported the covalent immobilization of *Candida antarctica*

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lipase on epoxy-activated carriers such as macroporous poly (methyl methacrylate) Amberzyme beads and nanoparticles (nanoPSG) with a poly (glycidyl methacrylate) outer region. The high activity of nanoparticles (nanoPSG) immobilized *Candida antarctica* lipase relative to the other immobilized lipase was largely attributed to its small size and nonporosity that circumvents diffusion constraints encountered for Lewatit VPOC 1600 and Amberzyme-immobilized *Candida antarctica* lipase.

Our objective was to immobilize *Candida antarctica* lipase onto cellulose acetate coated Fe_2O_3 nanoparticles, and prepare biocatalyst used for the synthesis of MG and DG by glycerolysis of olive oil. The immobilized lipase was characterized using FEG-SEM and FTIR spectroscopy techniques. The production of monoglycerides (MG) and diglycerides (DG), by immobilized lipase catalysed glycerolysis of olive oil with and without solvent systems was investigated. The optimum glycerolysis reaction condition, thermal stability, kinetic properties and reusability were also studied.

EXPERIMENTAL

1. Materials

Commercial lipase from *Candida antarctica*, glycerol, Fe_2O_3 nanoparticle and cellulose acetate, bovine serum albumin (BSA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Olive oil was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), 875.8 g/mol of mean molecular weight [32]. All aqueous solutions were prepared with deionized water from the Millipore (India) water purification system. Standards (pure) of MG, DG and TG of major components of olive oil (palmitate, olein, sterine, linoleic and linolenic) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All other chemicals and solvents were either of analytical or HPLC grades.

2. Preparation of Cellulose Acetate-coated Fe_2O_3 Nanoparticles

Fe_2O_3 nanoparticles were coated with cellulose acetate by dispersing 5 g of Fe_2O_3 nanoparticles in a 5 ml ethanol/acetone solvent mixture. To the well dispersed nanoparticle solution, 10 g cellulose acetate/acetone solution was added under constant stirring to ensure homogeneous mixing for 1 h at room temperature. Then, the product cellulose acetate coated Fe_2O_3 nanoparticles were collected, washed thoroughly with deionized water and then dried at 40 °C.

The cellulose acetate coated Fe_2O_3 nanoparticles were redispersed in periodic acid (0.03 M) and shaken gently in a water bath at 80 °C for 12 h. After that, Fe_2O_3-CA nanoparticles were then filtered and washed by deionized water for five times and dried in a vacuum oven at 40 °C.

3. Lipase Immobilization on Fe_2O_3-CA Nanoparticles

The immobilization experiment was carried out in a conical flask containing 0.5 g of Fe_2O_3-CA nanoparticles and 0.2 g of *Candida antarctica* lipase in 10 ml of phosphate buffer (0.1 M, pH 7.0). The resulting mixture of nanoparticles and lipase was then incubated in water bath at 30 °C with shaking at 150 rpm for 2 h. The lipase immobilized on Fe_2O_3-CA nanoparticles was subsequently separated by magnetic decantation and washed with same buffer (0.1 M, pH 7.0) two times to remove the unreacted lipase. The immobilized lipase was freeze dried at −40 °C under vacuum using a lyophilizer and stored at −4 °C prior to being used. The amount of immobilized lipase on Fe_2O_3-CA nanoparticles was determined by measuring the protein concentration (unbound) in the supernatant by the standard method [33]. All analyses were replicated at least three times.

4. Determination of Lipase Activity

The activity of free and immobilized lipase was assayed by titration with the olive oil and 0.01 mol/*l* NaOH, according to Yamada and Machida [34] with some modifications. The hydrolytic reaction was carried out in 100 ml closed flasks containing olive oil and n-octane (10 ml). The olive oil concentration was varied from 0.4 to 2 mg/ml. The reaction was then initiated by adding 0.01 g of lipase (free lipase or immobilized lipase) in oil reaction mixture. This was the actual amount of immobilized lipase without support. Reaction mixture was incubated for 20 min with agitation at the reaction temperature of 40 °C. After warming-up to 40 °C, 1 ml of phosphate buffer (0.1 mol/*l*, pH 7.0) solution was added. The reaction mixture was allowed to be magnetically stirred for 30 min at the same temperature. After 30 min, the mixture was centrifuged at 6,000 rpm for 10 min to separate the three phases. Both oil and organic phase were taken out and diluted with deionized water and ethanol-ether mixture (1 : 1 by vol.) solution, respectively. The amount of free fatty acids (FFA) released during hydrolysis was measured by titration with 0.01 mol/*l* NaOH solution using phenolphthalein as the indicator. One unit (IU) of lipase activity is defined as the amount of enzymes required for liberation of 1 μmol fatty acid from olive oil per minute under the assay conditions. The immobilization yield (%) was calculated as follows [35],

$$\text{Immobilization yield } (\%) = \frac{A_{imm}}{A_{free}} \times 100 \quad (1)$$

where, A_{imm}: the activity of immobilized enzyme (U/g) and A_{free}: the activity of free enzyme (U/g).

5. Thermal Stability

Thermal stability was determined by incubating free and immobilized lipase in phosphate buffer solution (0.1 M, pH 7.0) at 50 °C waterbath for 7 h. Samples were taken at periodic intervals (every 1 h for 7 h). The enzymatic activity of lipase was measured by the titration of fatty acid, which comes from hydrolysis of olive oil [34]. The percentage residual activity of lipase was calculated by the following [36]:

$$\text{Residual activity } (\%) = \left(\frac{A_t}{A_o}\right) \times 100 \quad (2)$$

where, A_o is the activity (U/g) of untreated lipase and A_t is the activity (U/g) of treated lipase.

6. Characterization

The size and morphology of Fe_2O_3-CA nanoparticles before and after lipase immobilizations were assessed by field emission gun-scanning electron microscopy (FEG-SEM) and energy dispersive X-ray microanalysis (EDX) using a JEOL model JSM-7600F at 5 kV. The KBr pellet technique was used for determining the Fourier transform infrared spectrophotometer (FTIR) spectra of free lipase, immobilized lipase, and Fe_2O_3-CA nanoparticles. Spectra were recorded on a Nicole MAGNA 550 spectrometer.

7. Glycerolysis Reaction

Glycerolysis reaction between olive oil and glycerol was performed in the presence of immobilized lipase, with or without organic solvents. The reaction mixture was stirred in a water bath with a magnetic stirrer (IKA RCT Basic S22, Bangalore, India) at 600 rpm. The reaction time, temperature, the molar ratio of glycerol to oil

and amounts of immobilized lipase were changed to study their effect on MG and DG conversion. Aliquots (0.10 ml) of the reaction mixture were taken at a different time, the immobilized lipase was removed by centrifugation at 2,900 ×g for 15 min, and the supernatant was used for quantitative or qualitative analysis. The experiments were performed at least three times.

8. Reusability

The immobilized *Candida antarctica* lipase was used in the repeated glycerolysis experiments at 50 ºC. The experimental conditions were the same as described above for glycerolysis reaction. After each cycle, the immobilized lipase was filtered and then washed with the phosphate buffer (0.01 mol/L, pH 7.0) and dried for 4 h at room temperature. The immobilized lipase was reused for next cycle of reaction. This procedure was repeated for 15 cycles.

9. Analytical Methods

Mono-, di-, and triglycerides were analyzed by HPLC system using an Agilent 1100 model equipped with refractive index detector and with a Zorbax C18 column (4.6 m×250 mm, 5 μm). The following conditions were used: flow rate of 1.0 mL/min, column temperature of 35 ºC; detector temperature 45 ºC; the mobile phase n-hexane and isopropyl alcohol (4 : 5 v/v). n-hexane and isopropyl alcohol was used as a sample dissolving solvent with injection volume of 25 μL. Standards for MG, DG, and TG were used to establish the calibration charts. In using these calibration charts, all of the integration results were corrected for mole percentages of the individual components.

10. Determination of Kinetic Parameters

Kinetic parameters of Lineweaver-Burk equation (K_m and V_{max}) for the free and immobilized lipase were determined by measuring the initial reaction rates of hydrolytic reaction with various initial concentration of olive oil as the substrate. The kinetic expressions were as in Eq. (3).

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \tag{3}$$

where [S] is the substrate concentration, K_m is the Michaelis-Menten constant (mM), V and V_{max} represents the initial and maximum rate in the Michaelis-Menten equation, respectively (mM/min). The substrate concentration [S] was based on the organic phase. Kinetic parameters (K_m and V_{max}) were determined by measuring the initial reaction rates of hydrolytic reaction with various concentration of olive oil (0.4 to 2 mg/ml).

The influence of free and immobilized lipase on the activation energy for the hydrolytic reaction of olive oil was calculated from Arrhenius equation as in Eq. (4) [34]:

$$AT = A\exp\left(-\frac{E_a}{RT}\right) \tag{4}$$

where, AT is enzyme activity (mM/min), E_a is the activation energy (kJ/mol), A is the pre-exponential factor, T is the absolute temperature (ºK) and R is the universal gas constant. The dependence of lipase activity on temperature was observed to follow an Arrhenius equation for both free and immobilized *Candida antarctica* lipase from 20 to 40 ºC.

The thermal deactivation of free and immobilized lipase was studied at temperature ranges from 40 to 70 ºC. The thermal deactivation of free and immobilized lipase is described by the pseudo first-order equation as in Eq. (5) [37,38].

$$\ln\frac{a}{a_0} = -k_d t + C \tag{5}$$

where, a: enzyme activity at time t, a_o: the initial activity of enzyme, k_d: the first order rate constant, and C: constant.

The rate constant depends on the reaction temperature according to the Arrhenius's law:

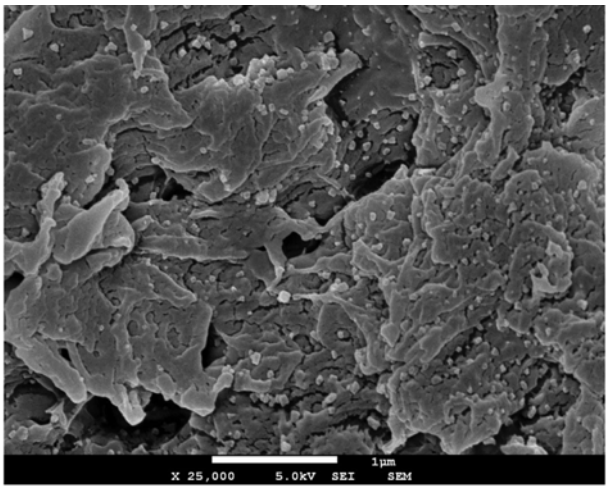
$$\frac{d(\ln k_d)}{d(1/T)} = -\frac{E_d}{R} \tag{6}$$

where E_d: the activation energy (kJ/mol) for the deactivation of the enzyme, T: the absolute temperature (K) and R: the universal gas constant.

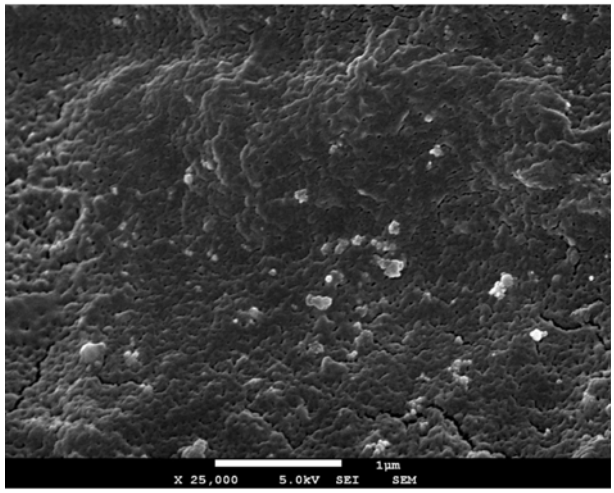
RESULTS AND DISCUSSION

1. Characterization of Immobilized Lipase

The immobilization of *Candida antarctica* lipase on Fe_2O_3-CA



(a)



(b)

Fig. 1. Field emission gun-scanning electron micrograph of (a) Fe_2O_3-CA nanoparticles (b) Fe_2O_3-CA nanoparticles containing immobilized *Candida antarctica* lipase.

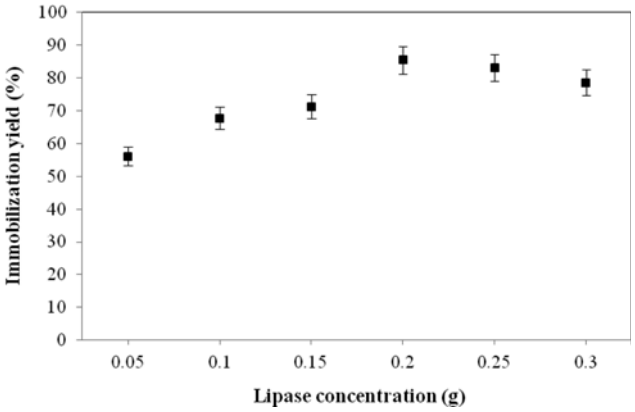


Fig. 2. Effect of lipase concentration on immobilization yield.

nanoparticles was investigated by field emission gun-scanning electron microscopy (FEG-SEM). Fig. 1(a) and (b) show the FEG-SEM images of cellulose acetate coated Fe_2O_3 nanoparticles without and with bound *Candida antarctica* lipase, respectively. Before lipase immobilization, the nanoparticles remained spherical nanostructure, in which the average size is about 100 nm. After the lipase immobilization onto Fe_2O_3-CA, the support surface area was filled with irregular structures, which were likely to be protein aggregates (Fig. 1(b)). EDX spectra (Fig. 1, refer supporting information) showed the presence of signal elements Ca, P, S of lipase. This was also detected in the Fe_2O_3-CA immobilized lipase, which confirms the presence of *Candida antarctica* lipase onto Fe_2O_3-CA nanoparticle support.

The FTIR spectra (Fig. 2, refer supporting information) confirmed the binding of lipase onto Fe_2O_3-CA nanoparticles. For the cellulose acetate coated magnetite, the absorption peak at around 600 cm^{-1} belonged to the stretching vibration of Fe-O bonds. After lipase immobilization on Fe_2O_3-CA support (Fig. 2(b), refer supporting information), the strong bond at 1,745 cm^{-1} corresponds to C=O stretching mode of an acetate group, while in case of pure cellulose acetate, peak appeared at 1,750 cm^{-1} [39]. The band of proteins at 1,642 cm^{-1} (C=O), 2,945 cm^{-1} (C-H) and 3,481 cm^{-1} (N-H) was observed in lipase bound Fe_2O_3-CA nanoparticles, which verified the binding of *Candida antarctica* lipase to the surface of Fe_2O_3-CA nanoparticles [40].

2. Optimization of the Lipase Amount

The immobilization yield was determined with different concentrations of *Candida antarctica* lipase 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 g. At different lipase concentrations (0.05, 0.1, 0.15, 0.2, 0.25, 0.3 g), 10 ml phosphate buffer solution (0.1 M, pH 7.0) and 0.5 g of Fe_2O_3-CA nanoparticles were added. Fig. 2 shows that the immobilization yield depends upon the lipase concentration used. 0.2 g lipase concentration was the most suitable concentration to activate the support (Fe_2O_3-CA nanoparticles) with 85% immobilised yield observed. As the lipase concentration was increased, a multilayer of lipase filled the pores of the Fe_2O_3-CA nanoparticles, and thus the surface layer prevented contact between the substrate and enzymes in lower layers.

3. Optimization of Glycerolysis in a Solvent or Solvent-free System

In general, organic solvents are used to improve the stability and homogenization of substrates as well as to influence the mass transfer by reducing the viscosity of the reaction system. In the present study, seven organic solvents were screened for their suitability in the immobilized *Candida antarctica* lipase-catalyzed glycerolysis of olive oil. These organic solvents were selected with different log *P* values from −0.24 to 4. All our trials were at 40 °C based on our previous results. Table 1 illustrates the effect of different organic solvents on glycerolysis reaction of olive oil catalyzed by immobilized *Candida antarctica* lipase. The highest MG content 38.2 wt% was observed with *tert*-butanol (log *P*-value of 0.35) solvent. The highest DG content 23.7 wt% was obtained with n-hexane (log *P*-values of 4.0) solvent (Table 1). The hydrophilic solvents showed higher MG yield compared to hydrophobic solvents.

Table 1. MG and DG content after a glycerolysis reaction in solvent or solvent-free systems. Reaction conditions: molar ratio of glycerol to oil, 1.5 : 1; time, 4 h; temperature, 40 °C; enzyme dosage, 0.02 g; water content 3.5% (w/w) in glycerol; and solvent, 4 ml/1 g olive oil

Solvent	Log P value	MG content (wt%)	DG content (wt%)
Ethanol	−0.3	10.7	9.5
Acetone	−0.24	14.2	11.3
Isopropanol	0.28	28.6	10.5
tert-Butanol	0.35	38.2	12.2
Chloroform	1.97	18.6	17.8
Toluene	2.5	19.8	18.5
n-Hexane	4	16.4	23.7
Solvent-free	-	10.8	13.1

The lower log *P* value (0.35) of tert-butanol indicated both hydrophilic and hydrophobic characteristics, with dominantly hydrophilic characteristics. *tert*-butanol is a suitable solvent for glycerolysis reaction with high MG yields [41].

4. Optimization of MG and DG Production by Immobilized *Candida antarctica* Lipase-catalyzed Glycerolysis

4-1. Effect of Reaction Time

The effect of the reaction time on the glycerolysis reaction was evaluated by using an immobilized *Candida antarctica*. The glycerolysis was conducted in *tert*-butanol (4 ml) for different time periods (1, 2, 3, 4, 5, 6 and 7 h) with 0.015 g of immobilized lipase at 40 °C. Our optimal water concentration in glycerol was 3.5% (w/w),

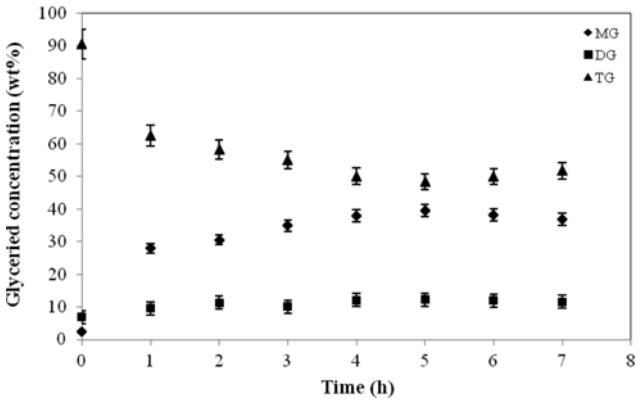


Fig. 3. Effect of time on olive oil glycerolysis at a 1.5: l molar ratio glycerol/oil, 1 g olive oil, water content in glycerol of 3.5% (w/w), 0.02 g lipase, 40 °C temperature, and 4 ml *tert*-butanol. (◆) Monaglycerides; (■) Diglycerides; (▲) Triglycerides.

based on our previous results [42]. The MG and DG yield increased when the reaction time was increased from 1 to 5 h but equilibrium was reached after 5 h (Fig. 3). The total amounts of MG (2.5 wt%), DG (6.9 wt%) and TG (90.6 wt%) at a specific time (initial reaction time) are as shown in Fig. 3. Since the maximum conversion of TG (90.6 wt%) to MG (39.5 wt%) and DG (12.2 wt%) occurred at 5 h, the reaction time 5 h was then used for the glycerolysis reaction. Zhong et al. [43] obtained that the optimum time for the highest yield of MG and DG during glycerolysis of soybean oil in *tert*-butanol and isopropanol catalyzed by immobilized *Thermomyces lanuginosus* lipase was at 4 h. Valério et al. [44] found that the optimum reaction time was 3 h at 70 °C for the glycerolysis of olive oil in Triton X-100 by immobilized lipase.

4-2. Effect of Temperature

Temperature plays a very important role in the enzymatic reacting system. Glycerol has a low miscibility with fats and oils. At a high temperature, the mixture viscosity was reduced, and thus the substrate diffusion or solubility increased. This reduced the mass transfer limitations and interaction between enzyme, and substrate was improved. However, if the temperature is set too high, lipase denaturation can occur. Therefore, an optimal working temperature value should be selected. The effect of temperature on performance of immobilized *Candida antarctica* lipase towards the glycerolysis reaction in *tert*-butanol is shown in Fig. 4. The yield of MG and DG increased when the reaction temperature was increased. The yield of MG and DG reached maximum at the value of 44.5 and 14.2 wt%, respectively at 50 °C. Above 50 °C, MG and DG yield decreased dramatically. A reaction temperature of 50 °C was selected as an optimum temperature for the glycerolysis reaction. McNeill and Yamane [45] obtained maximum MG yield (43 wt%) for the glycerolysis of palm oil catalyzed by *Humicola lanuginosa* lipase at 40 °C. On the other hand, Stevenson et al. [46] reported the 35 wt% MG from the glycerolysis of tallow with immobilized *Mucor meihei* lipase at 50 °C.

4-3. Effect of a Molar Ratio of Glycerol/Oil

The effect of glycerol to oil molar ratio at 1 : 1, 1.5 : 1, 2 : 1, 2.5 : 1, and 3 : 1on MG and DG production was evaluated at 50 °C with 0.02 g of lipase concentration in 4 ml *tert*-butyl alcohol. The results, shown in Fig. 5, indicate that the excess of glycerol reduced the MG and DG production. The MG and DG production yield achieved using glycerol to oil molar ratio of 1.5 : 1 was 44.2 and 14.2 wt%, respectively, while higher molar ratios, 2 : 1 and 3 : 1, and caused a decrease on the MG and DG yield. The decrease in reaction yields was presumably due to increased viscosity as the glycerol content increases. The other work reported by Tuter and Aksoy [47] showed the molar ratio of glycerol to palm kernel oil by *Humicola lanuginose* lipase at 2 : 1 was the optimum for the yield of MG (31 wt%) and DG (42 wt%). Yamane et al. [47] reported that at a low molar ratio of glycerol to palm olein (1 : 2), the main product of glycerolysis was MG and DG. This means that the enhancement of MG production from DG glycerolysis reaction needs excess glycerol compared to DG production from TG. Furthermore, Brady et al. [49] and Yang and Rhee [50] have suggested that glycerol acts as an effective stabilizer against thermal and solvent deactivation.

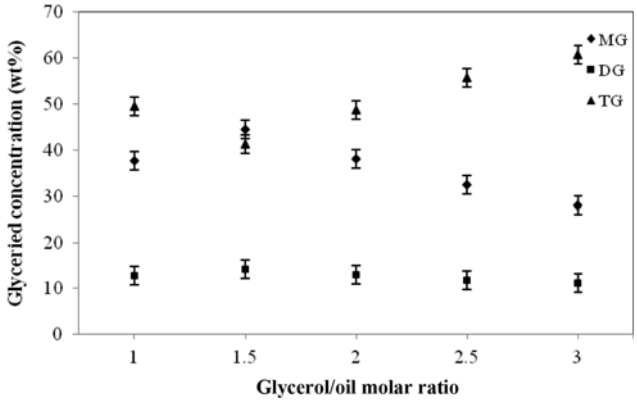


Fig. 5. Effect of glycerol/oil molar ratio on product formation in the glycerolysis of olive oil at 50 °C, 0.02 g lipase, water content in glycerol of 3.5% (w/w), 1 g olive oil, 5 h reaction time, and 4 ml *tert*-butanol. (◆) Monaglycerides; (■) Diglycerides; (▲) Triglycerides.

4-4. Effect of Lipase Loading

The experiments to evaluate the effect of lipase concentration

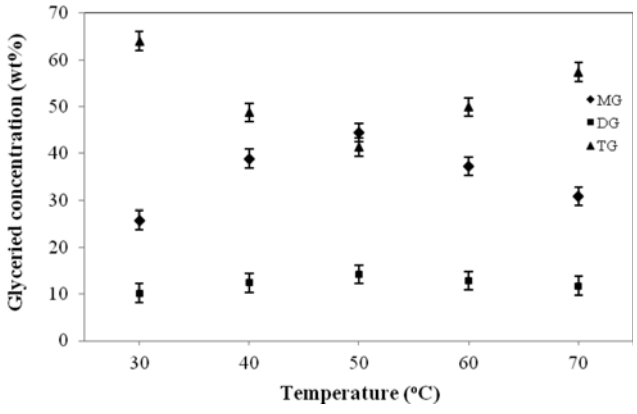


Fig. 4. Effect of temperature on olive oil glycerolysis at a 1.5: 1 molar ratio glycerol/oil, 1 g olive oil, 0.02 g lipase, water content in glycerol of 3.5% (w/w), 5 h reaction time and 4 ml *tert*-butanol. (◆) Monaglycerides; (■) Diglycerides; (▲) Triglycerides.

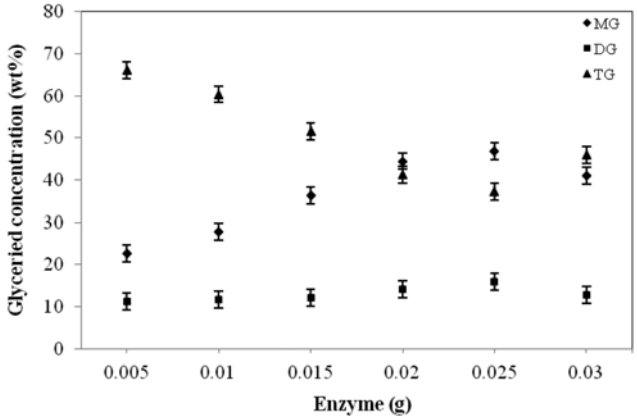


Fig. 6. Effect of lipase loading on product formation in the glycerolysis of olive oil at 50 °C temperature, 1.5: 1 molar ratio of glycerol/oil, 1 g olive oil, water content in glycerol of 3.5% (w/w), 5 h reaction time, and 4 ml *tert*-butanol. (◆) Monaglycerides; (■) Diglycerides; (▲) Triglycerides.

on MG and DG production were performed at 50 °C, in the 4 ml of *tert*-butanol using the different amount of lipase (0.005, 0.01, 0.015, 0.02, 0.025, and 0.03 g), while keeping fixed the molar ratio of glycerol to oil (1.5 : 1) and reaction time of 5 h. When increasing the amount of lipase in the reaction mixture, the MG and DG production was also increased as shown in Fig. 6. Increase in the lipase amount from 0.025 g to 0.03 g led to insignificant increase in MG and DG yield, probably due to the poorer mixing in the reaction. This result is in agreement with the reported work [42]. High lipase amount may lead to the formation of protein aggregates, and thus the lipase active sites are not available for the substrates for the further reaction. 0.025 g of immobilized lipase was the most suitable for MG (46.8 wt%) and DG (15.9 wt%) production. Pawongrat et al. [51] reported the effect of enzyme loading on the glycerolysis of tuna oil catalyzed by immobilized *Pseudomonas fluorescence* lipase with *tert*-butyl methyl ether. They obtained that in the enzyme loading of 0.3 g, MG and DG yield were 25 and 42 wt%, respectively, after 24 h at 45 °C.

5. Kinetic Parameters

The kinetics of the hydrolytic activity of free and immobilized lipase was studied by taking olive oil (875.8 g/mol) of varying concentrations (0.4-2.0 mg/ml). The kinetic constants (K_m and V_{max}) were determined from the equations of Lineweaver-Burk (Eq. (3)). The values of K_m and V_{max} for free and immobilized lipase are shown in Table 2 (refer to supporting information). The K_m value of immobilized lipase (0.3 mM) was 2.1 times lower than that of free lipase. The concentration of lipase immobilized onto support material (Fe_2O_3-CA) was determined by measuring the protein concentration by the Lowry method. Analysis was done in triplicate, and the values were of average with ±5% error. The value of K_m of immobilized lipase was lower compared to that of the free lipase. Thus, the affinity of the lipase towards the substrate upon covalent immobilization on the surface of Fe_2O_3-CA nanoparticles was increased. The lower K_m value for the immobilized lipase was also due to mass transfer limitations. The presence of the hydrophobic groups in the Fe_2O_3-CA surface layer enhanced the adsorption of hydrophobic substrate molecules on the support surface and hence influenced the kinetic parameter K_m. In addition, the V_{max} value for free lipase was 1.75 mM/min. The V_{max} value for immobilized lipase was 0.58 mM/min, which was three-times lower than that of free lipase.

The activation energy (E_a) of lipase binding to cellulose acetate-coated Fe_2O_3 was determined by the Arrhenius equation (Eq. (4)) and is shown in Table 2. The E_a of immobilized lipase (5.82 kJ/mol) was lower than that of the free lipase (10.26 kJ/mol), which indicated that the lipase has significantly higher affinity for the Fe_2O_3-CA nanoparticles active sites and lower sensitivity to temperature. Furthermore, the deactivation energy (E_d) for free and immobilized lipase was also studied (Eq. (5)) and is given in Table 2. The E_d for the free and immobilized lipase was found to be 7.49 and 4.1 kJ/mol, respectively. The E_d of immobilized lipase was lower than that of the free lipase, suggesting that the lipase has a lower sensitivity to temperature.

Table 2. Kinetic parameters of the free and immobilized lipases

Types	E_a (kJ/mol)	E_d (kJ/mol)	V_{max} (mM/min)	K_m (mM)
Immobilized lipase	5.82	4.1	0.58	25.00
Free lipase	10.26	7.49	1.75	52.63

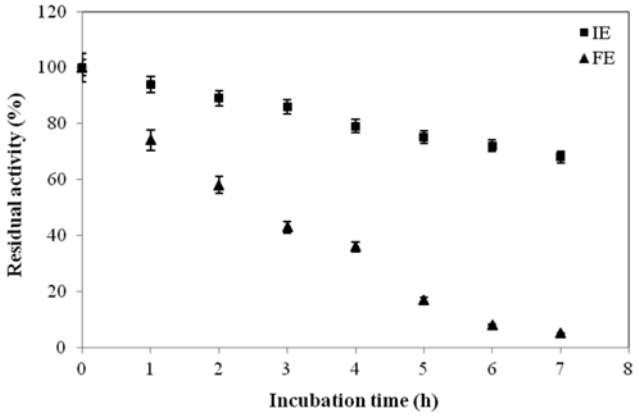


Fig. 7. Thermal stability of free and immobilized lipase at 50 °C. (▲) Free lipase; and (■) immobilized lipase.

6. Thermal Stability

Thermal stability of free and immobilized lipase was determined by measuring the residual activity at the specified temperature (50 °C) for 7 h. As shown in Fig. 7 the residual activity of immobilized lipase and free lipase was decreased. The rate of residual activity was slow for the immobilized lipase compared to the free lipase. The immobilized lipase retained about 72% of its catalytic activity after 6 h, whereas the residual activity of free lipase rapidly decreased to about 9%. The immobilized *Candida antarctica* lipase showed higher thermal stability compared to the free lipase. This was due to the formation of covalent bonds between the lipase and the surface of Fe_2O_3-CA. Immobilization of lipase on modified nanoparticles increased the lipase rigidity and thus protected it from protein unfolding [52]. Gomes et al. [53] reported that the immobilized *Candida antarctica* lipase retained about 40% of its initial activity after 1 h of incubation at 40-60 °C. Tumturk et al. [54] reported that the activity retained about 29% of its initial activity after an incubation of 25 min at 45 °C, when lipase was covalently immobilized on polymeric matrix.

7. Reusability of Enzyme

The reusability of immobilized lipase is a very important aspect in application, especially in industrial applications. In the reusability studies, the immobilized *Candida antarctica* lipase and free lipase were used in 15 subsequent cycles in the glycerolysis reaction of olive oil with *tert*-butanol at 50 °C (5 h for each batch). The experimental results showed higher production of MG with immobilized lipase compared to that of free lipase. The immobilized lipase retained 94% of its initial yield up to 11 cycles of reuse (Fig. 8(a) and (b)). After the 15th cycle, the MG yield in immobilized and free lipases declined to about 42 and 10 wt%, which was approximately 90 and 35% of the initial yield, respectively. This result confirmed that the immobilized lipase on Fe_2O_3-CA nanoparticles has good stability and reusability. The decrease of MG yield was due to the inactivation of the lipase caused by the denaturation of protein and the leakage of protein from the supports during the process of use [55]. The amino-functionalized (immobilized) lipase retained about 59% of its initial activity after 11th reuse [30]. Kim et al. [56] found that the silica-coated magnetic nanoparticle immobilized lipase preserved about

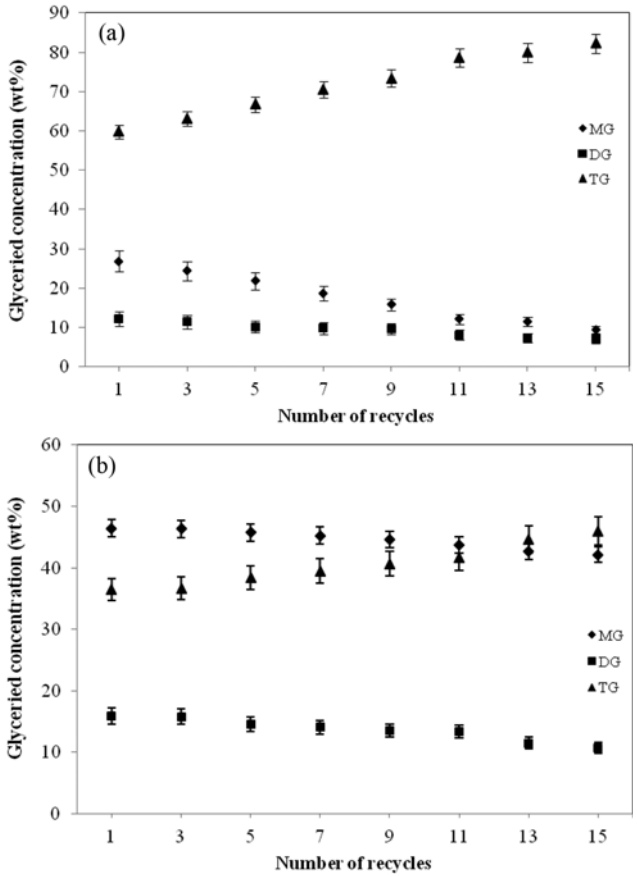


Fig. 8. Operational stabilities of (a) free and (b) immobilized lipase during the glycerolysis of olive oil. Reaction conditions: 1.5 : 1 glycerol/oil molar ratio, 1 g olive oil, 0.025 g lipase load, 50 °C temperature, water content in glycerol of 3.5% (w/w), 5 h reaction time and 4 ml *tert*-butanol. (◆) Monaglycerides; (■) Diglycerides; (▲) Triglycerides.

70% of the initial activity after five times reuse. Yong et al. [28] reported that the *Candida rugosa* lipase immobilized on polymer-grafted modified magnetic nanoparticles lost about 30% of the initial activity after being reused five times. Lee et al. [28] reported immobilization of lipase by SDS-bound nanosized magnetite particles with adsorption capacity of 52 mg/g, but the lipase retained only about 50% of its initial activity after the third reuse. The Fe_2O_3-CA immobilized lipase in this work showed higher TG conversion, thermal stability and high reusability compared to the previous reported results.

CONCLUSIONS

Fe_2O_3 nanoparticles coated with cellulose acetate were prepared and used to immobilize the lipase from *Candida antarctica*. The lipase binding was confirmed by FEG-SEM, FTIR and showed catalytic activity. This immobilized lipase was applied successfully in the glycerolysis of olive oil and the synthesis of MG and DG. The effect of organic solvent was evaluated, and best conversions of MG and DG in *tert*-butanol were obtained. The results showed that the highest yield was obtained at 50 °C with a molar ratio of glycerol to oil 1.5 : 1 and 0.025 g lipase in 5 h time. Furthermore, the immobilized lipase held high activity and better applicability than the free lipase. K_m values of the immobilized lipase and the free lipase were found to be 25.0 mM and 52.63 mM by the Lineweaver-Burk plots. K_m value of the immobilized lipase was lower than that of the free lipase, which indicated that the immobilized lipase had more affinity for its substrate than that of the free lipase. The activation energy of free and immobilized lipase was 10.26 and 5.82 kJ/mol, respectively. The deactivation energy of immobilized lipase was 4.1 kJ/mol, lower than that of free lipase (7.49 kJ/mol). The immobilized lipase had higher thermal stability compared with that of the free lipase and high operation reusability in repeated batch reactions.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Sanjay M. Mahajani, Chemical Engineering Department, IIT Bombay for using HPLC for this research work. The authors are grateful to the Sophisticated Analytical Instrument Facility (SAIF), IIT Bombay, for providing the research facility for characterizations of samples.

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Supporting Information

Immobilization of *Candida antarctica* lipase onto cellulose acetate-coated Fe_2O_3 nanoparticles for glycerolysis of olive oil

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(*Received 19 May 2013 • accepted 14 January 2014*)

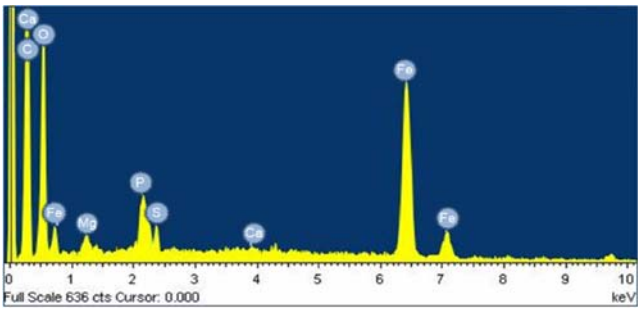

Fig. 1. EDX analysis of a sample of lipase from *Candida antarctica* immobilized on cellulose acetate coated Fe_2O_3 nanoparticles.

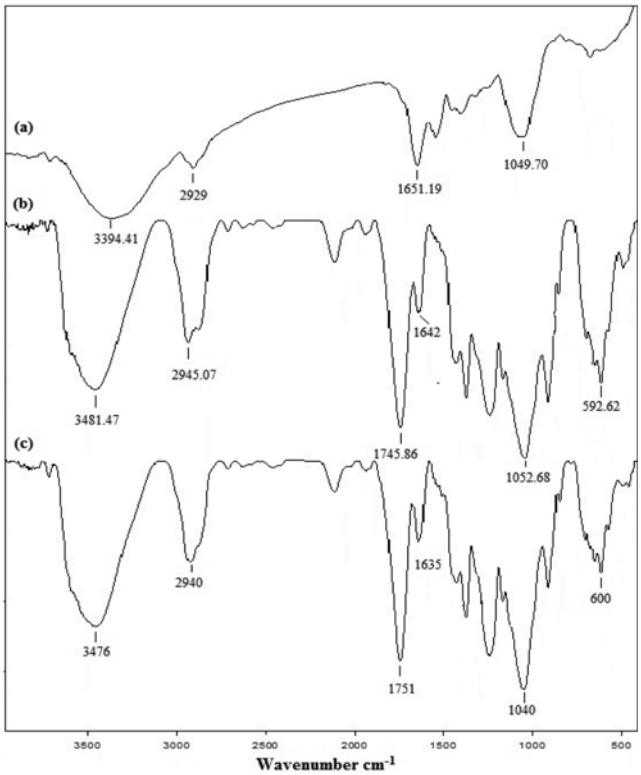

Fig. 2. FTIR spectra of Fe_2O_3-CA nanoparticles with and without bound lipase and free lipase ((a) free lipase; (b) Fe_2O_3 nanoparticles+cellulose acetate+lipase; (c) Fe_2O_3 nanoparticles+cellulose acetate).

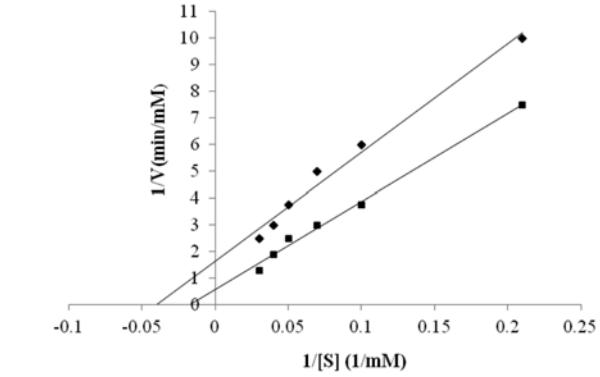

Fig. 3. Lineweaver-Burk plot for free and immobilized lipase. (■) Free lipase; (◆) immobilized lipase.

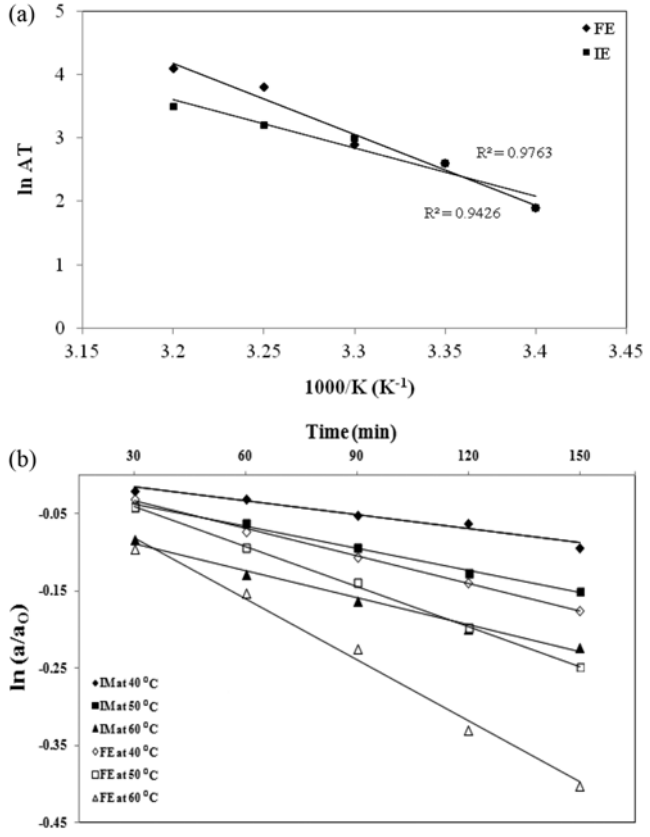

Fig. 4. Thermal activation of free and immobilized lipases. (a) Activation energy and (b) deactivation energy of free and immobilized lipase. (◆) Free lipase; (■) immobilized lipase.