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Immobilization of *Candida rugosa* lipase on glutaraldehyde-activated polyester fiber and its application for hydrolysis of some vegetable oils

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ABSTRACT

Candida rugosa lipase was effectively immobilized on the poly(ethylene terephthalate) grafted acrylamide (PET-g-AAm) fiber which was prepared through Hofmann reaction. The activities of the immobilized enzyme and the free enzyme were investigated in the hydrolysis reaction of olive oil in isooctane–water biphasic medium. The activities of the free and the immobilized lipases were measured at different pH values, and temperatures. The thermal stability and storage stability of them were also determined. The kinetic parameters of the free and the immobilized lipases, K_m and V_{max} were calculated, as well. Moreover, the application of immobilized lipase to the hydrolysis of different vegetable oils was realized. The effect of organic solvents on olive oil hydrolysis was examined and time course of the oil hydrolysis was studied. The optimum pH was shifted from 6 to 7 by immobilization of the enzyme. The maximum activity of the free and the immobilized enzymes occurred at 40 °C. It was found that the immobilized lipase stored at 4 °C retained 90% of its original activity after 60 days, whereas the free lipase stored at 4 °C retained 75% of its activity after the same period. In addition, the immobilized lipase exhibited as 0.06 U of the remaining activity even after 10 times reuses. Kinetics studies show that the corresponding values of K_m and V_{max} were 47.2 mg ml^{-1} and 48.1 U mg^{-1} protein for free lipase and 151.6 mg ml^{-1} and 10.9 U mg^{-1} protein for immobilized lipase. The immobilized and free enzymes showed similar behavior with respect to the different oil hydrolysis. The time course of the immobilized lipase on canola and olive oils hydrolysis used as substrate was much better than that of the free lipase. A high rate of oil hydrolysis was obtained when isooctane was used as solvent.

1. Introduction

Lipases, triacylglycerol hydrolases, are an important group of biotechnologically relevant enzymes and they find immense applications in food, dairy, detergent and pharmaceutical industries. Lipases are by and large produced from microbes specifically bacterial lipases play a vital role in commercial ventures. Lipases are also defined as glycerol ester hydrolases that catalyze the hydrolysis of triglycerides to free fatty acids and glycerol. Lipases catalyze esterification, interesterification, acidolysis, alcoholysis and aminolysis in addition to the hydrolytic activity on triglycerides. Novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers and biodiesel, the production of enantiopure pharmaceuticals, agrochemicals, and flavor compounds [1]. The production of fatty acids by the hydrolysis of natural oils and fats is a very important component in the economic exploitation of these naturally produced renewable raw materials. A significant number of high-value products require fatty acids in their manufactures. These include coatings, adhesives, specially lubricating oil, shampoos and other personal care products [2,3]. The traditional method of oil hydrolysis is carried out by using a chemical catalyst at high temperature and pressure. Enzymatic hydrolysis is an advantageous approach because it can be performed at lower temperature to save energy, and it exhibits high selectivity, leading to products with high purity and fewer side products [4,5]. The industrial application is often hampered by a lack of long-term operational stability and difficult recovery and reuse of the enzyme. These drawbacks can often be overcome by immobilization. There are several reasons for using an enzyme in an immobilized form. In addition to more convenient handling of the enzyme, it provides for its facile separation from the product, thereby minimizing or eliminating protein contamination of the product. Immobilization also facilitates the efficient recovery and reuse of costly enzymes, and enables their use in continuous, fixed-bed operation [6]. Lipase has been immobilized by several methods, namely adsorption [7,8], covalent binding [9] and entrapment [10]. Support material, which plays an important role in the utility of an immobilized enzyme, should be readily available and non-toxic, and also should provide a large surface area suitable for enzyme reaction, and sub-

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strate and product transport with the least-diffusional restriction [11].

Poly(ethylene terephthalate) (PET) fiber is one of the most important synthetic fibers used in the textile industry. PET fiber has good resistance to weak mineral acids, most strong acids, oxidizing agents, sunlight and micro-organisms [12]. However, it is hydrophobic in nature and do not contain chemically reactive groups. Certain desirable functional groups can be imparted to the PET fiber by grafting with different monomers [13]. Also it is non-toxic and readily available and can be obtained in a number of forms.

In the present study, *Candida rugosa* lipase has been immobilized on the poly(ethylene terephthalate) grafted acrylamide (PET-g-AAm) fiber. The biochemical properties of the immobilized lipase and the free lipase, such as effect of pH, temperature, concentration of substrate and the recycled use of the biocatalyst on activity were investigated. Moreover, the catalytic behaviors of the free lipase and immobilized lipase were evaluated with respect to different vegetable oils hydrolysis (e.g. sunflower oil, olive oil, canola oil and corn oil), effect of organic solvents on olive oil hydrolysis (e.g. isooctane, toluene and n-heptane) and time course of canola and olive oil hydrolysis.

2. Experimental

2.1. Chemicals

The poly(ethylene terephthalate) fiber (126 denier, 28 filaments) was kindly provided by SASA Co. (Adana, Turkey). Lipase from *C. rugosa* (EC 3.1.1.3), and glutaraldehyde were obtained from Sigma. Oleic acid and Folin-Ciocalteu's phenol reagent were purchased from Fluka. Olive oil, corn oil, sunflower oil, canola oil and soybean oil were purchased in a local market. Acrylamide, acetic acid, sodium hydroxide, cupric acetate, bovine serum albumin and benzoyl peroxide were purchased from Merck. Isooctane, toluene, n-heptane, n-hexane sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Riedel-deHaen. Solutions were prepared with deionized water (Millipore, Elix 3 water purification system).

2.2. Apparatus

Pharmacia Biotech Ultrospec 2000 model UV–vis spectrophotometer was used for the determination of optical density of solutions in the visible region. The infrared spectra were obtained by Thermo-Nicolet 6700 FT-IR spectrometer attached to an attenuated total reflection (ATR) apparatus, using diamond prism with an incident angle of 45°. All pH measurements were performed with HANNA 221 model digital pH meter.

2.3. Preparing the support material

The fiber samples (0.30 ± 0.01 g) were swollen in dichloroethane for 2 h at 90 °C, and then the solvent on the fiber was removed by blotting in a filter paper. As described by Coşkun and Soykan [14], acrylamide monomer was grafted onto the PET fiber by using benzoyl peroxide as an initiator. The swelled PET fiber samples were placed in a 100 ml polymerization tube. In addition, appropriate amount of acrylamide monomer dissolved in 18 ml aqueous and Bz_2O_2 dissolved in 2 ml acetone at suitable concentration was added. The polymerization tube was placed into the water bath at 85 ± 1 °C. The fiber samples taken at the end of the 2 h of polymerization were removed from homopolymer by washing with water at 50 °C for 5 h by changing the washing water at five times. The PET-g-AAm fiber was dried at 50 °C and weighed. The graft yield (GY) was calculated from the weight increase in the grafted fiber as follows:

$$\mathrm{GY}(\%) = \left[\frac{W_g - W_i}{W_i}\right] \times 100 \quad (1)$$

where W_i and W_g denote the weights of the original and the grafted PET fiber, respectively. The amide groups of poly(acrylamide) were converted to the amine groups by Hofmann degradation reaction [15,16]. The PET-g-AAm fiber was chemically modified as described in previous research [17]. The PET-g-AAm fiber (0.03 g) was immersed in 15 ml of suitable concentration of NaOCl and NaOH aqueous solution at 20 °C and after continuous shaking for 30 min, the PET fiber was removed from the mixture and washed with 10 ml of deionized water four times. The PET fiber was shaken in 5 ml of 2% glutaraldehyde solution at 100 rpm for 18 h [18]. Activated PET fiber was washed with deionized water four times to remove the unreacted glutaraldehyde.

2.4. Immobilization of lipase on the activated fiber

Activated PET fiber and lipase solution (0.2 mg ml^{-1} protein in buffer solution, pH 4) was shaken at 100 rpm for 18 h. PET fiber was washed with phosphate buffer solution for 10 min by shaking at 100 rpm four times. Immobilized enzyme was stored in buffer solution at 4 °C. The amount of immobilized protein on the fiber was determined by measuring the initial and final concentrations of protein within the enzyme solutions and washings using Folin-Ciocalteu's phenol reagent [19]. Bovine serum albumin was used as standard to construct a calibration curve.

2.5. Assay of the lipase activity

The substrate was prepared by dissolving oil in isooctane. The reaction mixture containing 2 ml of the oil solution and 2 ml of buffer solution was incubated with the free lipase (0.1 ml, 0.5 mg ml^{-1}) and the immobilized lipase in shaking water bath at 150 rpm for 30 min. The organic phase was then transferred to a test tube and cupric acetate pyridine reagent (0.5 ml) was added. The sample was vigorously mixed on vortex for 2 min. The optical density of green solution in organic phase was determined spectrophotometrically at 715 nm [20]. One unit (U) of enzyme activity was defined as the amount of enzyme which liberated 1 μmol free fatty acid per min under assay conditions. The relative activity was calculated as follows:

$$\text{Relative activity}(\%) = \left(\frac{\text{Activity}}{\text{Maximum activity}}\right) \times 100 \quad (2)$$

Thus the highest activity is regarded as 100% for free and immobilized studies separately.

3. Results and discussion

The PET-g-AAm fiber was used for the immobilization of the lipase. The amide groups of poly(acrylamide) were converted to the amine groups through Hofmann degradation reaction. As a first attempt in the study, the enzyme was immobilized on the matrix without glutaraldehyde, but it proved no activity for reuse. Thus the amine groups were activated by glutaraldehyde. *C. rugosa* lipase was immobilized to the activated PET fiber.

The infrared spectra of PET (spectrum 1a), the PET-g-AAm (40.1% graft yield) (spectrum 1b), the grafted PET after Hofmann degradation reaction (spectrum 1c), the glutaraldehyde coupling PET (spectrum 1d) and lipase binding PET fiber (spectrum 1e) are shown in Fig. 1. The examination of these spectra (a and b) revealed that there appeared a characteristic peak at 1651 cm^{-1} due to the carbonyl group (C=O stretching vibration) of AAm. This data

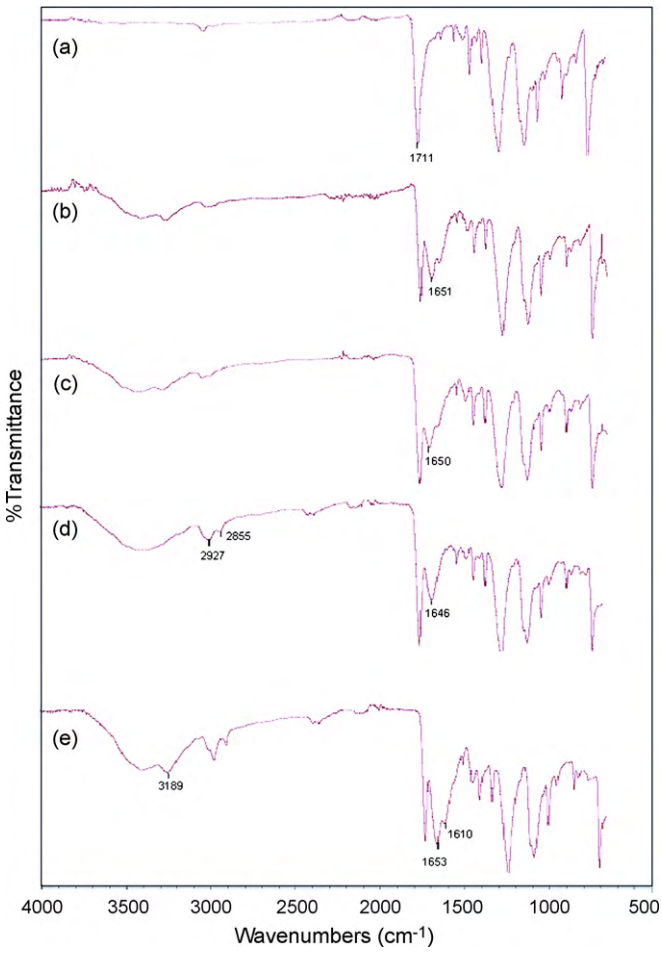

Fig. 1. FT-IR spectra of PET (a), PET-g-AAm (40.1% graft yield) (b), the grafted PET after Hofmann degradation reaction (c), the glutaraldehyde coupling PET (d) and lipase binding PET fiber (e).

showed that amide groups were attached into the fiber structure. The comparison of these spectra (b and c) revealed that there was decreased intensity of carbonyl peak at 1650 cm^{-1} of AAm. This result is attributed that amide groups were partially converted to amine groups on PET-g-AAm fiber by Hofmann reaction. In the spectrum 1d, the characteristic peak of glutaraldehyde appeared at 2855 cm^{-1} (C–H stretching vibration in –CHO) and intensity of stretching vibration C–H at 2927 cm^{-1} (in $-CH_2-$) increased. Furthermore, the peak broadened at 1646 cm^{-1} and the intensity increased due to the carbonyl group of glutaraldehyde and imine bound (C=N stretching vibration). These data showed that the glutaraldehyde was attached to grafted PET fiber. In addition, the intensity of some peaks, at 3189 cm^{-1}, 1653 cm^{-1} and 1610 cm^{-1} increased as a result of the immobilization of lipase (in spectrum 1e).

3.1. Effect of pH on hydrolytic activity

The behavior of an enzyme molecule may be modified by its immediate microenvironment. An enzyme in solution may have a different pH optimum from that of the same enzyme immobilized on a solid matrix, depending on the surface and residual charges on the solid matrix and the pH value in the immediate vicinity of the active site. A change in the optimum pH normally accompanies the immobilization of the enzymes, depending upon the polymer used as matrix. Since the enzyme activity is markedly influenced by environmental conditions, especially pH the change in behavior caused by enzyme immobilization is useful for understanding the structure–function relationships of enzyme. Therefore, it is very useful to compare the activity of soluble and immobilized enzyme as a function of pH [21]. The effect of pH of the reaction medium on hydrolytic activity of the free lipase and the immobilized lipase was evaluated by adjusting the pH in the range of 4–9 at 35 °C. A pH of 6 for the free lipase and 7 for the immobilized lipase was found to be optimum for achieving efficient hydrolysis of olive oil (Fig. 2). As expected, the immobilization procedure shifted optimum pH for lipase to neutral value. This means that the hydrogen and hydroxyl ions were distributed differently between the area close to the surface and the remainder of the solution, with negative charges clustering close to the immobilized enzyme [22]. A similar shift in pH optimum of immobilized lipase has been reported in the literature [23,24].

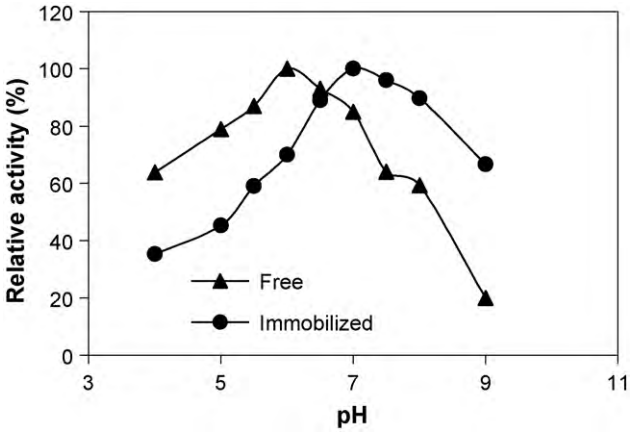

Fig. 2. The effect of pH on hydrolytic activity (T = 35 °C, substrate concentration = 0.1 g ml^{-1})

3.2. Effect of temperature on hydrolytic activity

The reaction mixture was incubated at temperatures varying in the range of 30–60 °C with the free lipase and the immobilized lipase. As shown in Fig. 3, it was observed that the optimum temperature for hydrolysis reaction for the free and the immobilized lipases remained the same at 40 °C. As the temperature was increased above the optimum value, hydrolytic activity drastically decreased. This result is due to the denaturation of the enzyme at higher temperature. A similar result was reported in literature

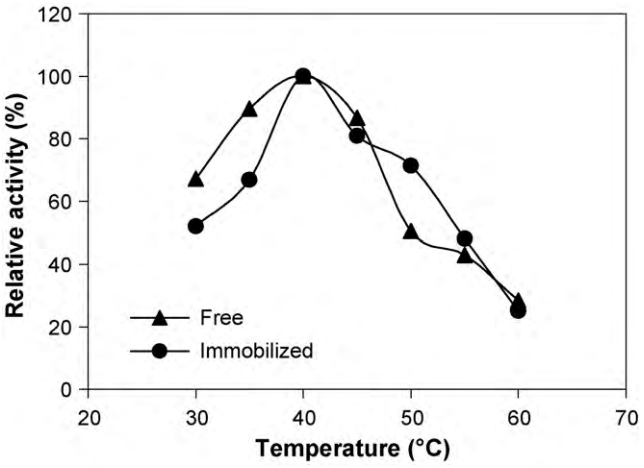

Fig. 3. The effect of temperature on hydrolytic activity (substrate concentration = 0.1 g ml^{-1}).

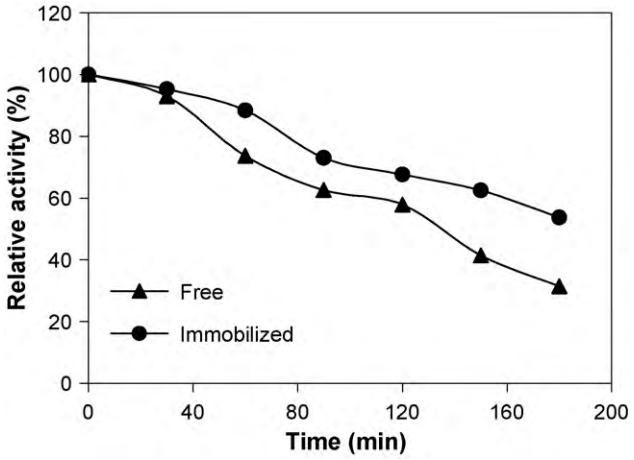

Fig. 4. The thermal stability of the free and the immobilized lipases (T = 35 °C, substrate concentration = 0.1 g ml^{-1})

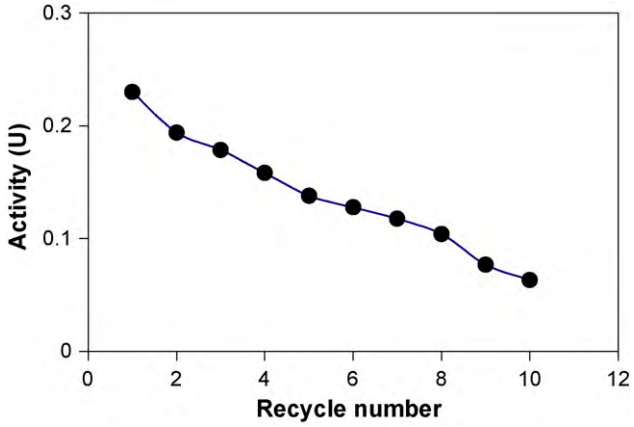

Fig. 5. The effect of repeated use of the immobilized lipase on hydrolytic activity (T = 35 °C, substrate concentration = 0.1 g ml^{-1})

[21,25]. However, Oliveira et al. [26] have reported that the temperature optima of immobilized lipase activity shifted towards higher temperature from 40 °C to 50 °C. A similar increase in temperature optima had been reported by Kanwar et al. [23], Santos et al. [24] and Paula et al. [27]. Additionally, Mateo et al. [28] reported that the immobilized enzyme (if multipoint covalent immobilization is achieved) is more stable towards harsh conditions like high temperatures and extreme pH values.

3.3. Kinetic parameters

To determine the Michaelis–Menten kinetics parameters (K_m and V_{max}) for the free and immobilized lipase, the concentration of olive oil was varied over the range of 0.025–0.30 g ml^{-1}. These two parameters can be determined from Lineweaver–Burk method [29]. The Michaelis–Menten constants K_m and V_{max} were calculated as 47.2 mg ml^{-1} and 48.1 U mg^{-1} protein for the free lipase and 151.6 mg ml^{-1} and 10.9 U mg^{-1} protein for the immobilized lipase by Lineweaver–Burk equation. This increase in K_m value might be either due to structural changes in the enzyme induced by the applied immobilization procedure or due to the lower accessibility of the substrate to the active sites of the immobilized enzyme by the increased diffusion limitation [30]. A similar result involving increase in the K_m value of lipase after immobilization has been reported in the literature [22,31].

3.4. Thermal stability

The thermal stabilities of the free and the immobilized lipases were assayed by immersing them in buffer solution (pH 7) at 50 °C for 180 min and periodically determining their activity. Fig. 4 shows that the immobilized lipase retained an activity of about 54% whereas the activity retained by the free lipase was only 30%. These results indicate that the thermal stability of immobilized lipase is much better than that of the free one owing to the covalent bond between the enzyme and support, which prevents the conformation transition of the enzyme at high temperature. It was reported that the thermal stability enhancement was one of the general advantages of the immobilized enzymes [30,31].

3.5. Reuse stability of the immobilized lipase

When comparing the performance of immobilized biocatalysts intended for preparative or industrial use, characterization of their operational stabilities is very important. To evaluate reuse stability, the immobilized lipase was washed with buffer solution (pH 7) after any run and reintroduced into fresh solution. The effect of repeated use on the activities of the immobilized lipase is shown in Fig. 5. After 10 reuses, the immobilized lipase activity decreased from 0.23 U to 0.06 U. The similar behavior of immobilized enzyme was reported by Ye et al., Nakane et al. and Dandavate et al. [31–33]. This result is due to the inactivation of the enzyme caused by the denaturation of protein [31].

3.6. Storage stability

One of the most important parameters to be considered in enzyme immobilization is storage stability. Immobilized and free enzymes were stored in buffer solutions (pH 4 and pH 7) at 4 °C. Enzyme activity was tested at different times for 60 days and obtained results were given as relative activity in Fig. 6. The immobilized lipase exhibited good performance throughout 60 days at pH 4. It was observed that the immobilized lipase stored at 4 °C retained 90% of its original activity after 60 days, whereas the free lipase stored at 4 °C retained 75% of its activity after the same period. When stored in pH 4, the immobilized lipase showed a better stability than the free lipase. The immobilized lipase provided significant advantage in stability over the free enzyme, especially at a longer storage time.

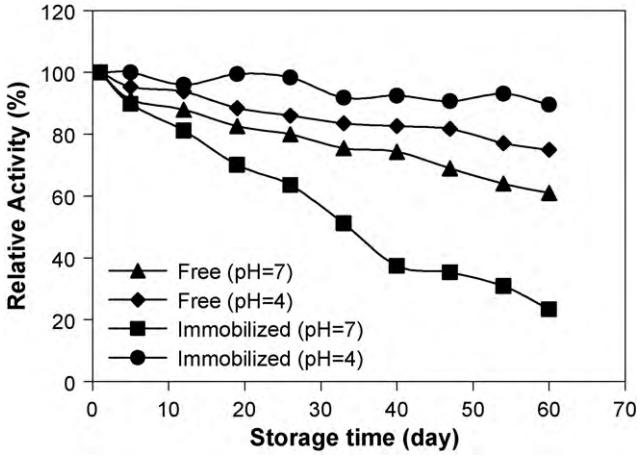

Fig. 6. The storage stability of the free and the immobilized lipases (T = 35 °C, substrate concentration = 0.1 g ml^{-1})

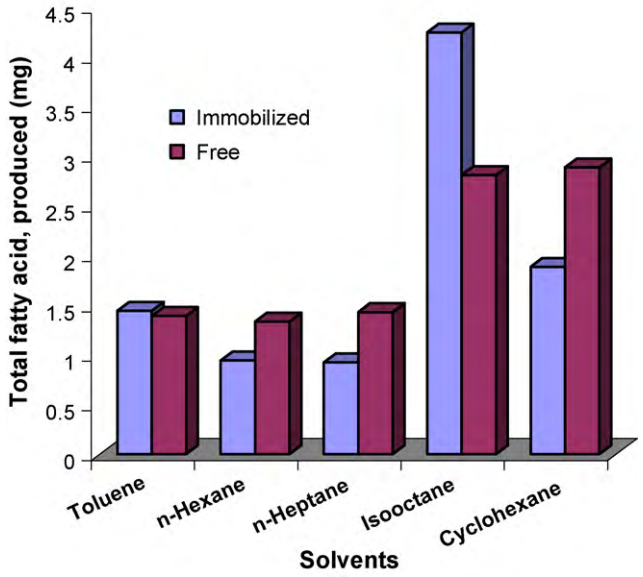

Fig. 7. The effect of various solvents on olive oil hydrolysis by immobilized and free lipases (T = 35 °C, substrate concentration = 0.1 g ml^{-1})

3.7. Effect of solvent on hydrolysis of olive oil

Lipase has shown improved activity when used with different organic solvents. Solvents such as, short chain alcohols, isooctane and n-hexane have been successfully used in enzymatic reactions to get high yields. These solvents also improve the rates of the reactions by dissolving the oil and reducing its viscosity, thus improving interfacial area and emulsification of the reaction [34]. The effect of various solvents used on the hydrolysis of olive oil is shown in Fig. 7. It is clear from the figure that except isooctane all other solvent used inhibited the immobilized lipase activity. Better result was obtained with isooctane as compared to n-hexane, n-heptane, toluene and cyclohexane in oil–water type of emulsion. As shown in figure, a high activity of free lipase was obtained for olive oil hydrolysis in the presence of isooctane and cyclohexane. Kim et al. [35] reported that lipase shows superior activity for olive oil hydrolysis in the presence of isooctane and cyclohexane to the other solvents examined. Rathod and Pandit [36] also have reported that lipase shows higher activity for castor oil hydrolysis in presence of isooctane. The influence of the solvent on catalytic activity may be explained by its ability to penetrate into the essential layer of water that stabilizes the enzyme. Water is not displaced from the enzyme by the solvent because water saturated solvents also influence catalytic activity [37].

3.8. Hydrolysis of the vegetable oils

The capacity of the immobilized lipase as well as of the free ones to hydrolyze olive oil, corn oil, sunflower oil, canola oil and soybean oil in isooctane–water biphasic medium was investigated. As shown in Fig. 8, a higher amount of fatty acid was obtained with the canola oil; however, a lower activity was obtained with the other oils for both immobilized and free lipases. This may be due to the dissolution effect, reducing the effective viscosity of the canola oil resulting into better emulsification. In addition, it has been reported that the difference in the yield of hydrolysis for the different oils is due to oil impurities in the oil or due to the physical structure of oil [36]. Furthermore, as can be seen from the figure, the immobilized enzyme shows a better hydrolyzing performance with olive oil, canola oil and corn oil than the free enzyme.

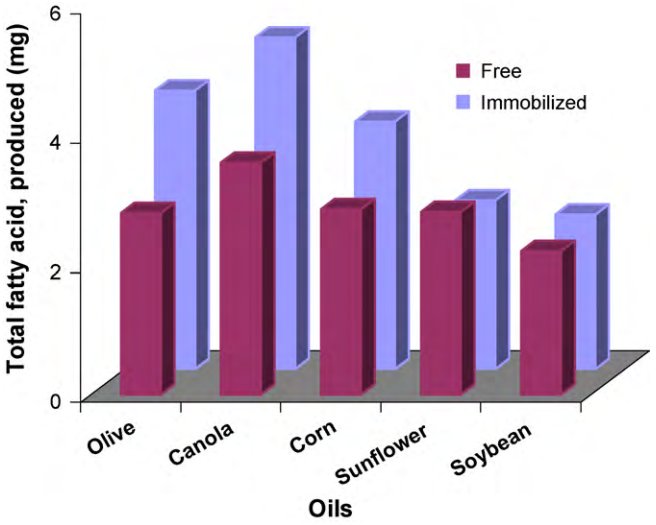

Fig. 8. Hydrolysis of the vegetable oils by immobilized and free lipases (T = 35 °C, substrate concentration = 0.1 g ml^{-1})

3.9. Time course of olive and canola oils hydrolysis

The problem associated with hydrolysis reaction is the fast decrease in the reaction rate as the conversion of oil to fatty acid increases [34]. In order to understand the pattern of the decreased hydrolysis reaction rates in isooctane–water type of emulsion, a set of time study was carried out. Hydrolysis of canola and olive oils was carried out for various time intervals ranging from 30 min up to 5 h. Fig. 9 shows that, for the immobilized lipase, the production of fatty acid from canola and olive oils has increased with increasing time; the rate has not been decreased significantly with the progression of reaction. As shown in figure, the rate of the production of the fatty acid using free lipase tends to decline after 2 h for olive oil and after 3 h for canola oil. This indicates that the immobilized lipase on the PET-g-AAm fiber catalyzes canola and olive oils hydrolysis well. Moreover, the immobilized lipase provided a prominent advantage in stability over the free lipase, especially at a longer duration.

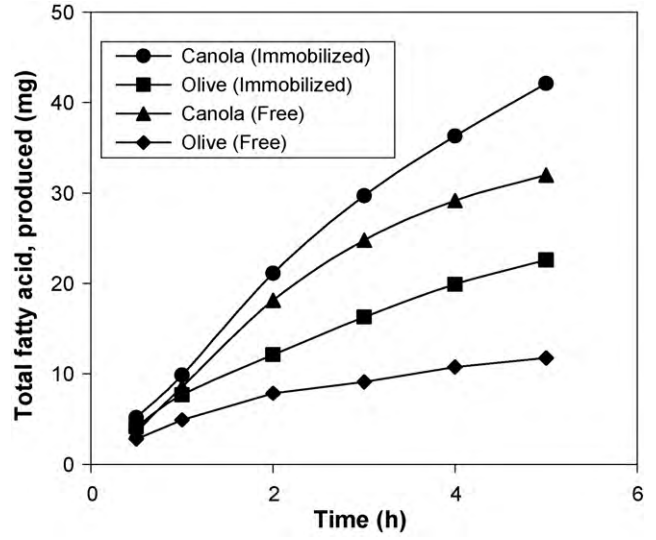

Fig. 9. Time course of olive and canola oils hydrolysis by immobilized and free lipases (T = 35 °C, substrate concentration = 0.1 g ml^{-1})

4. Conclusion

The present study demonstrated the successful immobilization of *C. rugosa* lipase onto functionalized PET fiber. The covalent binding process consists of four steps: grafting of AAm on PET backbone, conversion of amide groups to amine groups, glutaraldehyde activation and enzyme coupling. The immobilization of lipase increased its thermal stability, storage stability and reusability. Solvents i.e. isooctane has no effect on the rate of hydrolysis of olive oil while n-hexane, n-heptane and toluene deactivate enzyme, reducing. Canola oil gives higher yield as compared to olive, corn, sunflower and soybean oils. From the time course study of immobilized lipase it was observed that the variation in canola and olive oils hydrolysis was approximately linear with time, whereas there was a tendency for the olive oil and canola oil hydrolysis to decrease with the free lipase as time passed. These results suggest that the proposed method for immobilization of *C. rugosa* lipase has potential in industrial applications for oil hydrolysis, especially biodiesel process.

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