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Immobilization of lipase in organic solvent in the presence of fatty acid additives

Taylan K. Ozturk, Ali Kilinc*

Department of Biochemistry, Faculty of Science, Ege University, 35100 Bornova-İzmir, Turkey

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ABSTRACT

In this study porcine pancreatic lipase (PPL) was covalently immobilized on cross-linked polyvinyl alcohol (PVA) in organic media in the presence of fatty acid additives in order to improve its immobilized activity. The effects of fatty acid additions to the immobilization media were investigated choosing tributyrin hydrolysis in water and ester synthesis by immobilized PPL in *n*-hexane. Various fatty acids which are also the substrates of lipases in esterification reactions were used as active site protecting agents during the immobilization process in an organic solvent. The obtained results showed that covalent immobilization carried out in the presence of fatty acids as protective ligands improved the hydrolytic and esterification activity of immobilized enzyme. A remarkable increase in activity of the immobilized PPL was obtained when octanoic acid was used as an additive and the hydrolytic activity was increased from 5.2 to 19.2 μmol min^{-1} mg^{-1} as compared to the non-additive immobilization method. With the increase of hydrolytic activity of immobilized lipase in the presence of octanoic acid, in an analogous manner, the rate of esterification for the synthesis of butyl octanoate was also increased from 7.3 to 26.3 μmol min^{-1} g^{-1} immobilized protein using controlled thermodynamic water activities with saturated salt solutions. In addition, the immobilized PPL activity was maintained at levels representing 63% of its original activity value after 5 repeated uses. The proposed method could be adopted for a wide variety of other enzymes which have highly soluble substrates in organic solvent such as other lipases and esterases.

1. Introduction

The use of organic solvents as reaction media for biocatalytic reactions instead of conventional aqueous media has proven to be an extremely useful approach for expanding the range and efficiency of practical applications of lipases and it has been well documented [1,2]. However, the activity of the reported native enzymes in anhydrous organic media is lower than in water. To counter this limitation many efforts have been made to improve the catalytic activity and stability in non-aqueous media. In many cases immobilization of lipases has proved to be a useful technique for enhancing enzyme activities in organic solvents [3].

One of the main drawbacks associated with the use of immobilization methods is the loss of catalytic activity [4]. On the other hand, immobilization of lipases when used in anhydrous media warrants the dispersion of the biocatalyst and, in addition, prevents the aggregation of hydrophilic protein particles [3]. Nevertheless, the loss of activity of lipases due to the restriction of conformational changes and formation of rigid structures upon immobilization remains a great challenge in covalent fixation [5].

By using additives such as ligands, substrates, substrate analogues or other components during the enzyme immobilization process the enzyme's active center can be protected. In this way, the active center occupied by a substrate molecule often prevents a conformational change of the enzyme during covalent binding, thus preserving its high catalytic activity [6]. In addition, it has been reported that lipases pretreated with organic solvents yield an enhancement of both enzyme activity and stability [2,7].

Recently several papers regarding the immobilization of lipases by adsorption in organic solvents have been published demonstrating that lipase immobilization carried out in organic solvents enhanced the enzyme activity and stability as compared to immobilization in aqueous phases [8–11].

In this study porcine pancreatic lipase (PPL) was covalently immobilized onto cross-linked PVA in an organic solvent in the presence of a fatty acid substrate. Using an organic solvent provides effective solubility of fatty acid additives thereby enhancing the interaction of these compounds with the active center of the enzyme. Their presence often counteracts conformational changes of the biocatalyst during covalent immobilization, thus retention of the activity can be secured. The main idea of this approach using fatty acids as protective additives is the avoidance of harmful denaturation effects during covalent immobilization thereby improving its efficiency. Various fatty acids can be tested as protective candi-

* Corresponding author. Tel.: +90 232 311 1775; fax: +90 232 311 1775.
E-mail address: ali.kilinc@ege.edu.tr (A. Kilinc).

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dates for improved activity recovery. Their effect on the hydrolytic activity of PPL in water as well as the reverse reaction (esterification) in *n*-hexane with controlled water activity has been studied in this contribution. In addition, the optimal solvent, alcohol and fatty acid type as well as the water activity and the temperature effect on the esterification activity of PPL were also determined.

2. Experimental

2.1. Materials

Porcine pancreatic lipase (EC 3.1.1.3, Type II, Crude; PPL) was obtained from Sigma Chem. Co. (St. Louise, USA). Polyvinyl alcohol (PVA) and adipoyldichloride were purchased from E. Merck (Darmstad, Germany). Tributyrin as lipase substrate was obtained from Sigma Chem. Co. Alcohols (methanol, ethanol, *n*-propanol, *n*-butanol, *n*-octanol, ±2-octanol, iso-propanol) and organic acids (Butyric (C4), Octanoic (C8), Capric (C10), Lauric (C12), Myristic (C14) and Palmitic (C16) acids) were purchased from Aldrich and all were of GC grade. All solvents were likewise of GC grade and all inorganic salts were of analytical grade.

2.2. Immobilization procedure

Preparation of cross-linked PVA as an immobilization matrix with low solubility in water and organic solvents has been reported by Kilinc and Önal [12]. In a typical immobilization reaction one gram of cross-linked PVA matrix and 200 mg of crude PPL with 45.3 U specific activity (hydrolytic activity) were suspended in 5 ml of anhydrous cyclohexane as immobilization media and then 20 μl of adipoyldichloride were added. In order to investigate the effect of fatty acid additives, these compounds (substrates of the esterification reaction catalyzed by lipases) were added to the immobilization media containing the enzyme prior to covalent binding to the support. Thereafter the substrate-primed enzyme molecules interact with carriers bearing functional hydroxyl groups. Following this, multipoint attachment occurs during immobilization by cross-linking the polymer–enzyme conjugate by the means of adipoyldichloride. For this purpose saturated fatty acids including C4, C8, C10, C12, C14 and C16 were added to the immobilization media at 0.3 M concentrations 5 min prior to addition of the covalent linker. The reaction mixture was incubated at room temperature for 30 min with continuous stirring. The solid support was filtered off by suction and successively washed with acetone, cyclohexane and water, respectively, to remove impurities and unbound protein. The immobilized enzyme was lyophilized and stored at 4 °C until use.

2.3. Protein determination

Protein concentrations were determined by the method of Lowry et al. [13] by using bovine serum albumin as standard. The amount of bound protein was calculated from the difference between the amount of protein introduced into the coupling reaction mixture and the amount of protein present in the filtrate and washing solutions after immobilization.

2.4. Hydrolytic and esterification activities of lipase

The hydrolytic activities of free and immobilized lipase were measured titrimetrically at pH 8.0 at 37 °C with a pH-stat (718 Stat Titrino, Metrohm Ltd., Switzerland), under the standard assay conditions described previously [12], using tributyrin (250 μL) as the substrate in 25 mL, 0.1 M NaCl. The released free fatty acids from tributyrin were titrated with 0.05 M NaOH and one unit of lipase activity was defined as 1 μmol of fatty acid released per minute.

Esterification activities of enzymes were performed in screw-capped test tubes with a working volume of 1 ml. Unless otherwise stated, the typical reaction mixture consisted of 0.1 g immobilized enzyme and 1.0 ml of *n*-hexane containing octanoic acid and *n*-butanol as substrates at 300 mM concentrations. The initial water activity (a_w) of substrates and enzyme samples was pre-equilibrated to $a_w = 0.75$ with a saturated salt solution of NaCl. The reaction mixture was incubated at 40 °C under constant agitation at 200 rpm in an incubator. Initial reaction rates for esterification have been calculated using the time-dependent results and expressed in μmol ester released per minute by gram immobilized protein ($\mu mol\ min^{-1}\ g^{-1}$).

2.5. The effect of initial water activity

In order to determine the optimal initial water activity for esterification reactions of free and immobilized lipase all solvents and substrates of the esterification reaction were pre-equilibrated at 25 °C for 24 h in closed vessels over appropriate saturated salt solutions at different water activities (a_w) including NaOH ($a_w = 0.0$) LiCl ($a_w = 0.11$), $MgCl_2$ ($a_w = 0.33$), $Mg(NO_3)_2$ ($a_w = 0.53$), NaCl ($a_w = 0.75$) and K_2SO_4 ($a_w = 0.97$) [14]. The effect of the initial a_w on esterification rates was investigated by using equimolar amounts of *n*-butanol and octanoic acid (300 mM) for the synthesis of butyl octanoate at 40 °C in *n*-hexane.

2.6. GC analysis of fatty acid esters

At the end of the incubation period the reaction mixtures were cooled and diluted with *n*-hexane and a 1 μL aliquot was injected in a split mode into a Thermo Finnigan Trace GC Ultra® gas chromatograph equipped with a flame-ionization detector. A ZB-WAX (Zebron®) fused silica capillary column (30 m × 0.32 mm i.d.; film thickness 0.25 μm) was used. Nitrogen was used as carrier gas and both injector and detector temperatures were set at 250 °C. The initial column temperature of 100 °C was held for 2 min and then raised to 220 °C at a rate of 40 °C min^{-1} and then held at 220 °C. The extent of synthesis was calculated based on alcohol injected and quantified by comparison with standard curves of alcohol. All analyses were performed in triplicates.

2.7. Determination of molar ratios of substrates for esterification

The effect of substrates concentrations was investigated by varying the concentration of *n*-butanol and octanoic acid in the reaction system from 50 to 500 mM. Esterification reactions were carried out for a period of 4 h using the optimized assay conditions.

2.8. The effect of temperature on esterification activity

The effect of reaction temperature was investigated through incubation of reaction mixtures (*n*-hexane as solvent, octanoic acid and *n*-butanol as substrates at 300 mM concentrations) at various temperatures ranging from 30 to 80 °C for 4 h.

2.9. Reusability of immobilized PPL

The immobilized enzyme was used in the standard ester synthesis repeatedly. After each run the reaction media was centrifuged (1000 rpm, 5 min) and the immobilized PPL was washed with 1 ml of pre-equilibrated *n*-hexane ($a_w = 0.75$, NaCl) several times. Then fresh reaction medium containing 300 mM octaoic acid and *n*-butanol in 1 ml *n*-hexane was added and initial reaction rates were determined from the product produced after 2 h of reaction.

Table 1
Effect of different fatty acid additives (300 mM) on hydrolytic activities and initial reaction rates of esterification reaction of butanol and octanoic acid.

Type of additive	Protein immobilized[a] (mg g^{-1} support)	Hydrolytic activity (μmol min^{-1} mg^{-1})	Esterification activity[b] (μmol min^{-1} g^{-1})
Free PPL	–	45.3	36.8
Non-additive	39	5.2	7.3
C4	36	11.1	13.6
C8	36	19.2	26.2
C10	37	17.2	21.4
C12	37	14.1	17.6
C14	36	13.9	17.5
C16	37	14.0	18.6

[a] Total protein offered to 1 g support for immobilization: 64 mg. The yield of immobilized protein for non-additive immobilization is 61%.
[b] Esterification activities determined in hexane at 40 °C and initial water activities of free and immobilized enzymes pre-equilibrated to a_w = 0.75.

3. Results and discussion

3.1. Immobilization of PPL in organic solvents

In the present work multipoint covalent immobilization of PPL on cross-linked PVA was carried out in cyclohexane in the presence of different types of fatty acids which are also substrates of lipases in esterification reactions. This method is the so-called imprinting enzyme immobilization in which the active site is protected during the immobilization process in the presence of a ligand [6]. Most of the studies reported before, however, involve using soluble detergents or non-soluble additives such as oils before immobilization or during immobilization carried out in an aqueous environment [15–18].

Moreover, de Castro et al. immobilized PPL by adsorption on Celite comparing buffer or *n*-hexane as an immobilization medium without any additives. It was also found that the esterification activity of organic-solvent immobilized PPL was seven times higher than in aqueous immobilization (the specific activity was 0.22 μmol min^{-1} g^{-1} for the synthesis of butyl butanoat in hexane) [10]. de Oliveira et al. showed that CRL immobilization in an organic solvent not only had higher activity for both hydrolysis and esterification but also had higher stability [11].

The successful introduction of organic solvents as immobilization media prompted our studies using fatty acid substrates as effective protective additives because of their high solubility in organic solvents. Consequently, to identify the most effective fatty acid additive resulting in the highest activity retention, various fatty acids at different concentrations were assayed. The highest hydrolytic activities were obtained when the fatty acid concentration was 300 mM with octanoic acid being the most effective one (Table 1).

Table 1 shows that all the fatty acid additives improved the hydrolytic activity of immobilized enzyme in comparison to the non-additive immobilization method. The enhancement of hydrolytic activity ranged from 5.2 to 19.2 μmol min^{-1} mg^{-1} when octanoic acid was used as an additive. Improvement of the activity can be explained with the affinity of lipase for the fatty acid additive which is also a substrate. Hence, the active cavity of PPL was filled with a substrate molecule and as a result the adipoyldichloride bifunctional covalent linker could not reach and react with the catalytically important amino acids.

Furthermore, esterification activities of immobilized lipases were also investigated. In a similar manner enhanced esterification activities were observed when fatty acids were added to the immobilization media. The highest esterification activity was likewise obtained when octanoic acid was used as an additive and the esterification activity increased from 7.3 to 26.3 μmol min^{-1} g^{-1} for the synthesis of butyl octanoate (Table 1).

Using fatty acids during immobilization in organic solvents led to a pronounced increase both of hydrolytic and esterification activity. There was, however, no significant effect of fatty acid addition on the amount of protein bound to the carrier. Bound protein yields were in the case of non-additive immobilization about 61% and in the presence of fatty acids 57–58% (Table 1).

3.2. Effect of solvent type on esterification activities of lipases

The effect of solvent polarity on the esterification activity of lipase was investigated. For this purpose initial reaction rates and esterification yields of immobilized lipases were measured with various monophasic solvents as reaction media including tetrahydrofuran, benzene, *n*-hexane, iso-octane and acetonitrile.

The initial water activity of reaction conditions was pre-equilibrated to a_w = 0.75. Higher initial specific activities and overall reaction yields were obtained when *n*-hexane and iso-octane were used as solvents with water activities set to 0.75. Initial rates, log *P*-values and overall yields after 96 h are represented in Table 2.

The initial reaction rate and over all yield for butyl octanoate synthesis was higher in iso-octane and *n*-hexane when compared to polar solvents. A similar trend correlating enzymatic activity with log *P*-values of solvents was also reported by other lipase-catalyzed esterification reactions [19,20]. Lower enzymatic activities and reaction yields were obtained in polar solvents due to their ability to strip off essential water molecules from the enzyme microenvironment [21–23]. Therefore *n*-hexane was used for further esterification reactions.

Table 2
Effect of solvent polarity on the activity and yield after a 96 h lipase-catalyzed esterification of octanoic acid and *n*-buthanol; initial water activity was set to a_w = 0.75.

Solvent	Log *P*-value[a]	Initial reaction rates (μmol min^{-1} g^{-1})	Yield[b] %
Iso-octane	4.50	20.19	95
n-Hexane	3.50	19.30	90
Benzene	2.00	9.68	67
Tetrahydrofuran	0.52	3.41	13
Acetonitrile	−0.35	1.92	0.5

[a] Log*P*-value was defined as the partition co-efficient of the individual solvent between water and *n*-octanol.
[b] Yield (%) after a 96 h reaction was calculated as the concentration of the ester product.

3.3. Effect of a_w on esterification reactions

The effects of water activity on initial reaction rates were also investigated. The type of organic solvent and the water content of the microenvironment on the enzyme surface are essential parameters for the synthetic activity of lipases. Critical amounts of water around the enzyme maintains the enzyme in an active conformation. In addition, the active site polarity and the thermodynamic equilibrium of the esterification reaction are directly related to the water activity [24]. Additional water content of the support and mass transfer from the reaction mixture are affected by the support

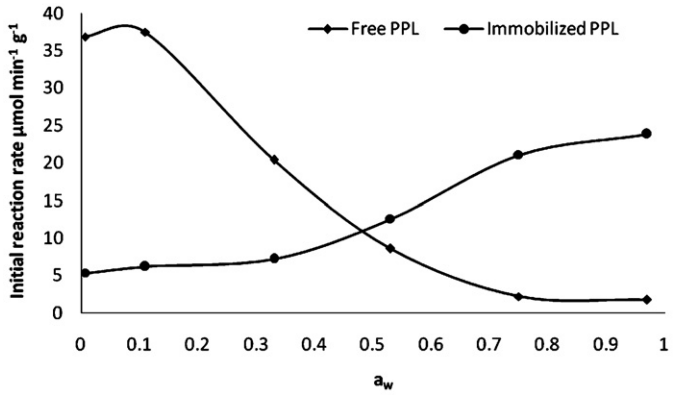

Fig. 1. Effect of water activity on initial esterification rates of free and immobilized PPL in the presence of octanoic acid. Initial rates were determined from the results of 2 h reactions.

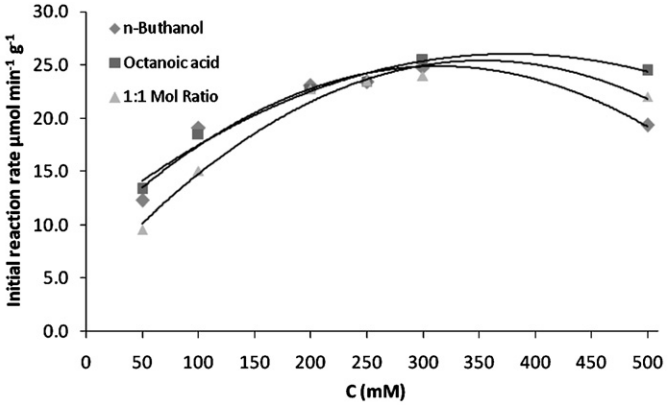

Fig. 2. Effect of substrate concentrations on initial reaction rates of esterification (40 °C; a_w = 0.75; *n*-hexane) (♦) 300 mM *n*-butanol was kept constant; (■) 300 mM octanoic acid kept constant; (▲) molar ratio 1:1.

properties [25]. Low water activities favor in general ester synthesis over the hydrolysis reaction [26].

The effect of initial a_w on the esterification rate for the synthesis of butyl octanoate is shown in Fig. 1. With an increase of the initial a_w the rate of enzyme activity for non-immobilized PPL decreased. Higher values of a_w caused aggregation of the enzyme and inhibited the esterification reaction. In addition, the inhibitory effect of the aggregation for crude PPL was augmented when the reactions were carried out as well with lower a_w values because of the formation of water product from the esterification reaction. As a consequence of aggregation, the reaction yield (data not shown) was lower than with immobilized PPL despite the high initial reaction rates (Fig. 1). However, the optimal water activity of immobilized PPL was higher than a_w = 0.75. As is obvious from Fig. 1, the measured initial reaction rates indicate a change of the inhibitory effect of water. High polarity of the support material is apparently responsible for this change by binding the water molecules which are essential for the enzyme's synthetic activity. Moreover, the esterification yield of immobilized PPL after 96 h reactions with a starting value of a_w = 0.75 was more than 90% (Fig. 3 and Table 3). This water activity was used for the determinations of esterification activities of immobilized PPL throughout this work.

3.4. Effect of substrate concentrations on esterification

The substrate concentration has a significant influence on the initial esterification rates of immobilized lipase [10]. Initial reaction rates of immobilized PPL were determined by using equimolar amounts of *n*-butanol and octanoic acid from 50 mM to 500 mM for a reaction period of 2 h. In Fig. 2 the increase of the initial reaction rates with increasing substrate concentrations with a saturation maximum at 300 mM are shown. Higher levels of acidic or alcoholic substrates can change the enzyme microenvironment removing essential water molecules or acidifying the enzyme's aqueous phase.

Table 3
Effect of chain length of fatty acids on esterification; a_w = 0.75.

Fatty acid	Initial reaction rate (µmol min^{-1} g^{-1})	Yield (%)		
		24-h	48-h	96-h
C8	22.5	37.7	66.5	93.7
C10	15.6	35.2	57.2	93.0
C12	16.4	34.5	61.0	92.1
C14	23.3	36.6	66.9	93.7
C16	31.4	47.5	75.2	94.3

3.5. Effect of various alcoholic substrates on esterification

The effect of the primary alcohol chain length was investigated in the synthesis of octanoic acid esters. Low reaction rates and yields obtained with methanol, ethanol and *n*-propanol when compared to *n*-butanol and *n*-octanol (Fig. 3). The inhibition effect of highly polar short chain alcohols was directly related to their ability to strip water molecules from the enzyme's surrounding microenvironment. Immobilized PPL did not catalyze the reaction with secondary alcohols as examined (2-propanol and 2-butanol).

3.6. Influence of fatty acid chain length on esterification

The effect of the chain length of acyl donors on the initial reaction rates of esterification and their yield with butanol was investigated. Table 3 displays the molar conversion of butanol as a function of the fatty acid chain length. The different fatty acids (C8–C16) resulted in similar molar butanol conversions (93%) after a reaction period of 96 h. It is well known that crude preparations of PPL represent a composition of various lipolytic enzymes with different properties in terms of activity and selectivity [27]. Therefore we could not detect any selectivity improvement for immobilized PPL in the presence of octanoic acid.

3.7. Effect of temperature on the esterification reaction

The temperature has a significant effect on the equilibrium of the reaction and on the activity and stability of immobilized lipase.

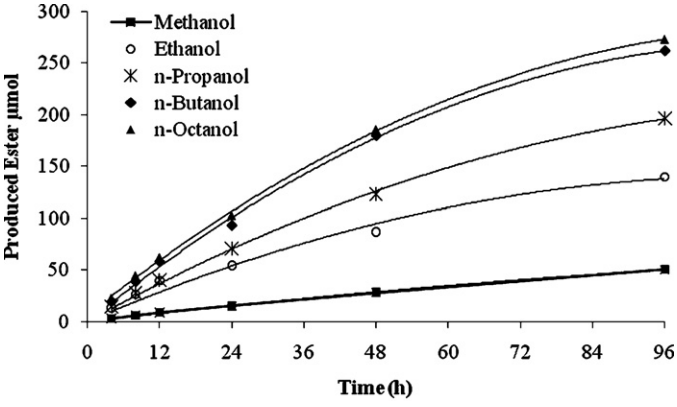

Fig. 3. Effect of different chain length alcohols on initial reaction rates (3.5, 15.6, 16.9, 22.9, and 26.2 µmol min^{-1} mg^{-1}, respectively); 40 °C; a_w = 0.75.

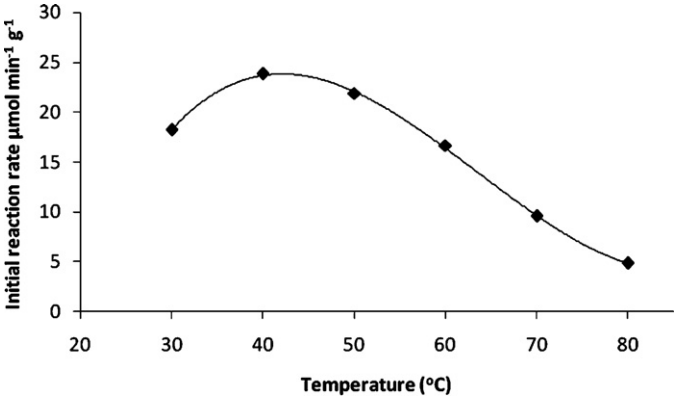

Fig. 4. Effect of temperature on initial reaction rates of esterification reaction.

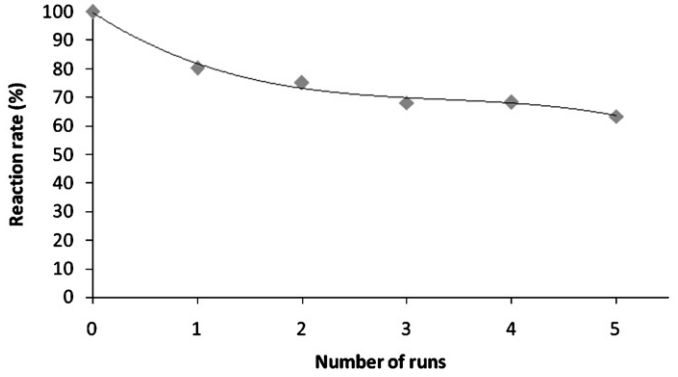

Fig. 5. Reusability of immobilized PPL.

As shown in Fig. 4, the optimal reaction temperature was 40 °C for the initial rate. The reaction rate decreased slightly at temperatures higher than 50 °C probably as a consequence of thermal deactivation of the enzyme.

3.8. Reusability of immobilized PPL

Reusability of an immobilized enzyme without loss of activity is important for its economic viability. The reusability of the immobilized enzyme was tested by its repeated use for butyl octanoate synthesis for several 2 h runs (Fig. 5). There was no significant change of its reusability due to the immobilization in the presence of octanoic acid if compared to the non-additive immobilization mode. Immobilized PPL kept its nearly 63% activity after five separate runs without any additional water activity control. The observed loss of enzyme activity caused by the change of the microenvironment of the enzyme during each run probably may be due to partial denaturation of the enzyme or losses of critical amounts of water when the reaction mixture was replaced.

4. Conclusion

Organic-solvent immobilization of enzymes is a rather rare application in enzymology. With lipase as an example we studied for the first time its immobilization in non-conventional solvents in the presence of fatty acids for substrate imprinting. From the viewpoint of using organic solvents as an immobilization medium fatty acid substrates possessing high solubility in organic solvents appeared to us as effective protective additives for the preparation of an immobilized biocatalyst with improved activity. In this study we present a simple protocol to immobilize PPL in an organic solvent with retention of high activity.

Our results suggest that using fatty acids during immobilization in organic solvents lead to a pronounced increase both in hydrolytic and also esterification activity.

The synthesis of various aliphatic esters was successfully performed with immobilized PPL. There is obviously a great potential in using fatty acid additives as a means to improve the activity of the immobilized biocatalyst. The proposed method could also be applicable for a wide variety of other lipases and esterases acting on non-soluble substrates.

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