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Enantioselective resolution of racemic ibuprofen esters using different lipases immobilized on octyl sepharose

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ABSTRACT

Here we report the stereoselective hydrolysis of racemic esters catalyzed by *Candida rugosa* lipase (CRL) and *Rhizopus oryzae* lipase (ROL) immobilized on octyl-sepharose via physical adsorption. Hydrophobic immobilization caused to almost six fold hyperactivation with 229.2 and 81.3 U/mg enzyme for immobilized CRL and ROL, respectively (13.2 and 48.75 U/mg for corresponding free enzyme). Based on the preliminary results, CRL was chosen for further investigation. The performance and yield of the reaction were evaluated as a function of the critical reaction parameters such as temperature, enzyme to substrate ratio and organic co-solvent. An increase in the temperature resulted to decrease in enantioselectivity of hydrolysis reaction. The hydrolysis reactions were carried out in presence of two organic solvents; *n*-hexane and isooctane. Generally *n*-hexane was a better co-solvent compared to isooctane. High enantioselective hydrolysis of the racemic esters (yielding *S*(+) ibuprofen; ee $\geq$ 95%) can be achieved using the immobilized CRL. Among various esters the kinetic resolution of ibuprofen butyl ester yielded the best results (*E* value 70 and 74; conversion 14.6 and 8.9 in *n*-hexane and isooctane, respectively). The immobilized derivatives were re-used in four cycles and showed little decrease in enantiomeric excess of (*S*)-ibuprofen. 96.7 ee_p and conversion 14.6 in first cycle reached to 90.5 ee_p and conversion 11.3 in the forth cycle.

1. Introduction

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) have the natural catalytic function of hydrolysis of triglycerides with subsequent release of free fatty acids. They are also most commonly used enzymes in organic synthesis in various reactions such as esterification, transesterification and aminolysis [1]. Therefore, lipases are nowadays extensively studied for their potential industrial applications. In this point of view, they are mainly used as biological catalysts to manufacture other products (such as food ingredients) and by their application in making fine chemicals [2]. Furthermore, because of high enantioselectivity of lipases, lipase catalyzed resolution of racemate drugs (i.e. ibuprofen) is increasingly used among all methods such as asymmetric synthesis [3].

Ibuprofen as a non-steroidal anti-inflammatory drug (NSAID) is known as one of the most commercially successful and important classes of analgesic anti-inflammatory drugs used in the treatment of headache, rheumatoid arthritis, cephalgia and muscular strain [4]. All profens have a stereogenic centers which is the carbon bearing carboxyl group. The (*S*)-enantiomer of ibuprofen has the desired therapeutic effect (160 times more active than its (*R*)-enantiomer) in the in vitro inhibition of prostaglandin synthesis, while the (*R*)-ibuprofen is inactive and can cause side effects affecting to the gastrointestinal tract, normal lipids metabolism and membrane function [5,6].

In spite of interesting performance of lipases, their application in industrial level is limited most importantly because of their prohibitive cost and requires in many instances, recovery and reuse of the enzyme to make an economically feasible process [7]. Therefore, there is a great interest in methods trying to develop useful biocatalysts for industrial applications by improvement of their catalytic properties such as activity, stability and recycling capacity. Such improvements can be carried out by immobilization of lipases on insoluble supports which enabling easy control of the reaction, avoiding contamination of the product by enzymes, permitting their reuse over many reaction cycles and simplifying the overall design and performance control of the bioreactors [8,9].

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Considering this requirement as an opportunity, many researchers have tried to convert this requirement into a powerful tool to greatly improve enzyme performance [10–14]. For example multipoint covalent immobilization of a monomeric enzyme resulted to generate a favorable environments surrounding the enzyme thus improving its stability [15]. Also multimeric enzymes have been stabilized by immobilizing all enzyme subunits, thus preventing subunit dissociation [16]. In both cases, immobilization is compatible with other strategies (i.e. chemical modification) to yield a more stable biocatalyst [17].

Among all immobilization types, physical adsorption of lipases on hydrophobic supports in low ionic strength is an efficient and simple method for immobilization due to particular physicochemical character of these enzymes [18]. A characteristic feature of lipases is their activation in the presence of hydrophobic interfaces [19]. This lipase activation at interfaces was first reported by Sarda and Desnuelle [20] and now through X-ray structural studies of a number of lipases, it is recognized as a common feature [21–29]. Lipases present a specific catalytic mechanism of action, existing in two structural forms, the closed one; where a polypeptide chain (lid or flat) isolates the active center from the medium and the open form; where this lid moves and the active center is exposed [30,31]. In the hydrophobic interfaces important conformational changes take place yielding the open structure of lipases resulting in a significant increase in activity. In this way, lipases seem to become strongly adsorbed to hydrophobic interfaces through a large hydrophobic surface which surrounds the internal face of the lid [23]. The complexity of the interfacial activation mechanisms of lipases may cause some difficulties in understanding and controlling the behavior of lipases in organic synthesis at both laboratory and industrial scales. However this mechanism could also be used as a 'tool' to develop a simple method for lipase immobilization [32].

Among different types of lipases, *Candida rugosa* lipase (CRL) *Rhizopus oryzae* lipase (ROL) and *Rhizomucor miehei* lipase (RML) have been used frequently in biotransformation [33,34]. The molecular sizes of CRL, ROL and RML are 57, 32 and 31.6 kDa, respectively [26,35]. RML is probably the most used lipase obtained from fungi, even being used as a model for the determination of the structure of some other lipases due to the deep knowledge of its three dimensional structure [36]. It is commercially available enzyme in both soluble and immobilized form with very high activity and good stability under diverse conditions (anhydrous organic solvents, supercritical fluids, etc.) [37]. Among these lipases, ROL is a high cost lipase which needs to bring the cost down for its industrial applications. In this case, improving the lipase performance using immobilization techniques and increasing the expression level of ROL can be the powerful ways to lower operating costs of the enzyme [38].

In our previous work we have reported immobilization of *R. miehei* lipase (RML) via different protocols such as physical adsorption and covalent attachment to catalyze hydrolysis of various ibuprofen esters. The results showed that RML immobilized on octyl-sepharose had high activity and selectivity and butyl ester was the most interesting ester for carrying out hydrolysis [39].

The aim of the present investigation is development of an enzymatic method for the production of the *S*-enantiomer of ibuprofen. For this purpose CRL and ROL were immobilized on octyl-sepharose via physical adsorption and their application was examined in two distinct reactions; (1) esterification of (R,S)-ibuprofen by *n*-propanol in presence of two ionic liquid and isooctane and (2) resolution of various (R,S)-ibuprofen esters by hydrolysis. The optimization of hydrolysis reaction was performed regarding to the amount and activity of the immobilized lipase. Enantioselectivity of reused immobilized lipases has also been studied.

2. Materials and methods

2.1. Materials

Ibuprofen was extracted from the readily marketed tablets according to literature procedure [40]. *p*-Nitrophenyl butyrate (*p*-NPB), Octyl-sepharose TM, molecular sieves (4 Å, 4–8 mesh), (*S*)-(+)-Ibuprofen (purity 99%), Lipase from *C. rugosa* and lipase from *R. oryzae* were obtained from Sigma (Steinhiem, Germany). Other reagents and solvents were of analytical or HPLC grade.

2.2. Immobilization of the lipases on octyl-sepharose

One gram of o ctyl-sepharose was suspended in 10 mL of enzyme solution of ROL, and CRL (0.5 mg/ml) in 10 mM sodium phosphate buffer at pH 7.0, and the mixture was shaken at 25 °C and 250 rpm for 3 h. Thereafter, the immobilized enzyme was washed with distilled water 20 mL (three times) and stored at 4 °C. Suspension sample and the supernatants were withdrawn periodically, and the hydrolytic activity was measured using *p*-NPB as substrate.

2.3. Synthesis

2.3.1. General method for the chemical synthesis of ibuprofen esters (**1**–**5**)

To a solution of 0.1 mol of the racemic acid in 100 ml toluene, 0.5 mol of corresponding alcohol (methanol, ethanol, propanol, *n*-butanol and *iso*-butanol) was added followed by few drops of sulphuric acid (98%) [41]. The mixture was stirred under reflux over night and the solvent was evaporated under vacuum and the residue was neutralized with 10% sodium hydrogen carbonate. The ester was extracted twice with 50 ml chloroform and then dried over anhydrous sodium sulphate. After filtration, the solvent was evaporated under vacuum to afford the racemic ibuprofen esters.

*2.3.1.1. Ibuprofen methyl ester (**1**).* Pale yellowish oil (100%): ^{1}H NMR ($CDCl_3$) δ 7.1–7.3 (dd, 4H, aromatic), 3.7 (q, 1H, **CH**CH_3), 3.6 (s, 3H, **OCH$_3$**), 2.4 (d, 2H, **CH$_2$**$CH(CH_3)_2$), 1.9 (m, 1H, **CH**$(CH_3)_2$), 1.5 (d, 3H, CH**CH$_3$**), 0.9 (d, 6H, CH(**CH$_3$**)$_2$).

*2.3.1.2. Ibuprofen ethyl ester (**2**).* Pale yellowish oil (100%): ^{1}H NMR ($CDCl_3$) δ 7.1–7.2 (dd, 4H, aromatic), 4.1 (q, 2H, **OCH$_2$** CH_3), 3.7 (q, 1H, **CH**CH_3), 2.4 (d, 2H, **CH$_2$**$CH(CH_3)_2$), 1.8 (m, 1H, **CH**$(CH_3)_2$), 1.5 (d, 3H, CH**CH$_3$**), 1.2 (t, 3H, OCH_2**CH$_3$**), 0.9 (d, 6H, CH(**CH$_3$**)$_2$).

*2.3.1.3. Ibuprofen propyl ester (**3**).* Pale yellowish oil (100%): ^{1}H NMR ($CDCl_3$) δ 7.1–7.2 (dd, 4H, aromatic), 4.0 (q, 2H, **OCH$_2$** CH_3), 3.7 (q, 1H, **CH**CH_3), 2.5 (d, 2H, **CH$_2$**$CH(CH_3)_2$), 1.8 (m, 1H, **CH**$(CH_3)_2$), 1.6 (m, 2H, OCH_2**CH$_2$**CH_3), 1.5 (d, 3H, CH**CH$_3$**), 0.9 (t, 3H, OCH_2CH_2**CH$_3$**), 0.8 (d, 6H, CH(**CH$_3$**)$_2$).

*2.3.1.4. Ibuprofen butyl ester (**4**).* Pale yellowish oil (100%): ^{1}H NMR ($CDCl_3$) δ 7.1–7.3 (dd, 4H, aromatic), 4.0 (t, 2H, **OCH$_2$**$CH_2CH_2CH_3$), 3.7 (q, 1H, **CH**CH_3), 2.4 (d, 2H, **CH$_2$**$CH(CH_3)_2$), 1.8 (m, 1H, **CH**$(CH_3)_2$), 1.5 (m, 2H, OCH_2**CH$_2$**CH_2CH_3), 1.4 (d, 3H, CH**CH$_3$**), 0.9 (m, 2H, OCH_2CH_2**CH$_2$**CH_3), 0.8 (d, 6H, CH(**CH$_3$**)$_2$), 0.8 (t, 3H, $OCH_2CH_2CH_2$**CH$_3$**).

*2.3.1.5. Ibuprofen isobutyl ester (**5**).* Pale yellowish oil (100%): ^{1}H NMR ($CDCl_3$) δ 7.1–7.3 (dd, 4H, aromatic), 3.9 (t, 2H, **OCH$_2$** $CH(CH_3)_2$), 3.7 (q, 1H, **CH**CH_3), 2.4 (d, 2H, **CH$_2$**$CH(CH_3)_2$), 1.9 (m, 1H, **CH**$(CH_3)_2$), 1.5 (m, 2H, OCH_2 **CH**$(CH_3)_2$ and CH_2**CH**$(CH_3)_2$), 1.4 (d, 1H, 3H, CH**CH$_3$**), 0.9 (d, 6H, CH(**CH$_3$**)$_2$), 0.8 (d, 6H, OCH_2 CH(**CH$_3$**)$_2$).

2.3.2. General procedures for enantioselective esterification of ibuprofen

In a 15 ml reaction vial, the appropriate racemic acid (0.05 mmol) and *n*-propanol (0.15 mmol), were dissolved in 5 ml organic solvent (isooctane) or ionic liquids ([BMIM]BF_4 or [BMIM]PF_6) in presence of molecular sieves (Scheme 1). The mixture was stirred and heated at 40 °C. Thereafter, 50 μL of the samples were withdrawn and injected at zero time (control). The immobilized enzymes (20 mg) were added and 50 μL sample of the supernatant was withdrawn and directly injected to GC without dilution or workup at several time intervals.

2.3.3. General procedure for enzymatic hydrolysis of ibuprofen esters

The hydrolysis of racemic esters was carried out as follows: in a 15 ml reaction vial, the appropriate racemic ester (0.02 μmol) was dissolved in 2 ml organic solvent (*n*-hexane or isooctane) followed by addition of 2 ml of 0.1 M sodium phosphate buffer, pH 7.0 (Scheme 2). The reaction mixture was stirred at 30, 40, 50 and 60 °C and then 50 μL sample of the organic solvent aliquot was withdrawn and injected at zero time (control). The immobilized derivatives (10 and 20 mg) were added to the reaction vessel and the reaction mixture was shaken at 100 rpm for 72 h. 50 μL of the organic solvent aliquot was withdrawn directly at several time intervals from the organic layer. The samples used directly without dilution for GC analysis. The amount of ibuprofen (the conversion degree) formed during the reaction and the enantiomeric excess of the (*S*)-enantiomer was determined by GC.

2.4. Analysis

2.4.1. Enzymatic activity assay

The activities of the soluble lipase and its immobilized preparations were analyzed spectrophotometrically measuring the increment in absorbance at 348 nm ($\epsilon = 5150\ M^{-1}\ cm^{-1}$). The increase in absorbance produced by the release of *p*-nitrophenol (*p*NP) in the hydrolysis of 0.4 mM *p*NPB in 25 mM sodium phosphate buffer at pH 7.0 and 25 °C. To initialize the reaction, 0.05–0.2 ml of the lipase solution (blank or supernatant) or suspension was added to 2.5 ml of substrate solution under magnetic stirring [38].

2.4.2. Chromatographic analysis

Gas chromatography was performed using a Thermoquest-Finnigan (USA) gas chromatograph equipped with flame ionization detector (FID) and a HP-CHIRAL-20B column (30 m × 0.32 mm × 0.25 μm). Injector temperature was 260 °C and the detector was 300 °C; oven temperature was maintained at 178 °C. Carrier gas was helium with a flow rate of 0.7 ml/min. (*S*)-(+)-Ibuprofen (purity 99%) at above condition had retention time 12.8 min and retention time of (*R*)-(+)-Ibuprofen in racemic mixture was 13.5 min. An external standard method was employed to quantify the amount of ester and the remaining acid [42].

2.4.3. Enantioselectivity-value (E-value) measurements

The value of enantioselectivity (*E*-value) was calculated from the enantiomeric excess of the substrate (ee_s) and the conversion degree (c) according to the equation described by Chen et al. [42].

$$c = \frac{ee_s}{ee_s + ee_p} \quad (1)$$

$$E = \frac{\ln[(1-c)(1-ee_s)]}{\ln[(1-c)(1+ee_s)]} \quad (2)$$

Table 1
Immobilization of the lipases on octyl-sepharose.[a]

Enzyme derivative	Immobilization yield[b] (%)	Activity[c] (U/g support)	Specific activity[d] (U/mg enzyme)
Free enzyme ROL	–	–	13.2
Free enzyme RML[e]	–	–	2.01
Free enzyme CRL	–	–	48.75
Octyl-RML	>98	2.8	13.6
Octyl-ROL	>98	16.5	81.3
Octyl-CRL	>98	46.7	229.2

[a] Immobilizations were performed as described in Section 2.
[b] The activity was measured at 25 °C toward a solution of *p*-nitrophenyl butyrate (4 ml in 16 ml of 100 mM phosphate buffer pH 7). The activity is expressed as U/g of support. U = μmol of substrate hydrolysed per minute.
[c] The specific activity is expressed in U/g of support.
[d] The activity is expressed in U/mg enzyme.
[e] *Rhizomucor miehei* lipase.

3. Result and discussion

3.1. Immobilization on octyl-sepharose

From the comparison of the specific activities, it seems that the enzymes immobilized on octyl-sepharose derivatives are hyperactivated. This over activation can be attributed to the fixation of the open structure of the enzyme on the hydrophobic surface of the support [43]. As can be seen in Table 1, excellent immobilization yield was achieved in each case. The immobilization on octyl-sepharose afforded the derivatives with a high specific activity (seven-fold higher than that of the free enzyme) which is same as Guisan's group report. They reported immobilization of different lipases by interfacial activation on four different hydrophobic supports (hexyl- and butyl-toyopearl and butyl- and octyl-agarose). The results showed that different supports resulted in different activity, ranging from a seven fold hyperactivation to almost fully inactive biocatalysts. Octyl-agarose was the most specific support on the lipase adsorption [44].

3.2. Lipase catalyzed enantioselective esterification of ibuprofen

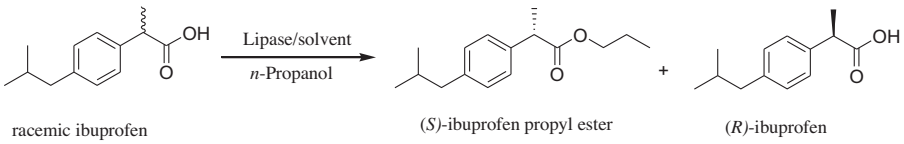
Solvent selection for enzymatic reactions is of great importance due to the inherent ability of solvents to deactivate enzymes, as is the case with hydrophilic solvents. The use of ionic liquids to replace organic solvents in enzymatic processes has recently gained much attention [45]. There have been many reports of enzymatic catalysis in ionic liquids and also the effect of ionic liquid media on the enantioselectivities of these transformations [46–49]. Good activities and in many cases, improved enantioselectivities were observed compared with the same reaction in organic solvents [50].

The immobilized lipases were tested for their ability to catalyze the enantioselective esterification of racemic ibuprofen with 1-propanol in isooctane and two ionic liquids [BMIM]BF_4, [BMIM]PF_6. In Table 2 the results obtained during 120 h of reaction are presented. Conversion degrees increase in two ILs compared to

Table 2
Enzymatic parameters of the lipase-catalyzed enantioselective esterification.

	Isooctane			[BMIM]BF_4			[BMIM]PF_6		
	c[a]	ee_s[b]	E[b]	c[a]	ee_s[b]	E[b]	c[a]	ee_s[b]	E[b]
Octyl-CRL	15.5	1.06	1.13	27.1	3.43	1.24	31.0	3.47	1.21
Octyl-ROL	9.5	1.21	1.27	20.4	1.00	1.09	21.1	2.40	1.22
Octyl-RML[c]	11.6	1.36	1.24	23.1	1.95	1.16	28.2	3.34	1.22

[a] The lipase activity was defined as the conversion of ibuprofen after five days.
[b] *E* was calculated as Eq. (2); where c is substrate conversion and ee_s is enantiomeric excess of the remaining substrates.
[c] The date for octyl-RML has been reported in our previous paper and only given for comparison [35].



Scheme 1. Lipase-catalyzed enantioselective esterification of racemic ibuprofen.

isooctane but the enantioselectivities are almost the same for both solvents implying that ionic liquid does not improve enantioselectivity of lipases immobilized on octyl-sepharose. No further investigations were carried out because of poor conversions and enantioselectivities during the reaction in all solvents.

3.3. Enantioselectivity of lipases immobilized on octyl-sepharose toward different ibuprofen esters

As mentioned above, due to the poor results for enzymatic esterification of ibuprofen, various racemic ibuprofen esters were synthesized (**1**–**5**) chemically and lipase-catalyzed enantioselective hydrolysis was carried out as a model reaction to investigate the performance of these biocatalysts. In order to obtain the corresponding *S*-ibuprofen in optically pure form, ibuprofen butyl ester was selected for further investigation to find the higher conversion and enantioselectivity. Moreover regarding to the high enantioselectivity of octyl-CRL (Table 3), it was subjected to further investigation in hydrolysis reaction.

3.4. Lipase-catalyzed enantioselective hydrolysis of ibuprofen esters

In fact, lipases are capable of catalyzing the hydrolysis of both of the enantiomers of their substrates, but at different rates [51]. The difference in the enantioselectivity values between various esters reflects the individual susceptibility of the substrates to undergo different conformational changes and interfacial binding efficiency with the enzyme [52]. In this regard racemic methyl, ethyl, propyl, butyl and iso-butyl ibuprofen esters were synthesized chemically to study the effect of alkyl chain on reaction parameters such as conversion and enantioselectivity. Since the synthesized racemic esters are insoluble in water, the reactions were performed in a biphasic system containing of sodium phosphate buffer (100 mM) pH 7.0/organic solvent (1:1). Despite that generally interfacial activation occurs before lipases take part in biochemical reactions [53], some of the reported lipase-catalyzed hydrolysis reactions were carried out in aqueous phase. In a previous report, the ester was suspended (if solid) or emulsified (if oil) in the aqueous phase (buffer) forming an interface by which activation of the lipase occurs [54]. However production of water insoluble product may cause inhibition of the enzyme. Therefore to increase the conversion and reduce the reaction time, co-solvent was added in the system. The substrate dissolved in the co-solvent led to increase in the interfacial area available for the reaction. Organic solvents which were used in reactions included isooctane (log *P* 4.5) and *n*-hexane (log *P* 3.5). Log *P* is a quantitative measure of solvent polarity. In general, biocatalytic processes in organic solvents having log *P* < 2 are low and in polar solvents with log *P* between 2 and 4 are moderate [55]. The hydrolysis reaction in various amounts of immobilized enzymes (5, 10 and 20 mg) in four temperatures 30, 40, 50 and 60 °C was carried out. As the conversion and enantioselectivity values were low at 30 °C and 60 °C, the corresponding results are not reported. It seems that the immobilized preparations lose their activities at 60 °C. Analysis of results was performed using GC equipped with the HP-CHIRAL-20B column. The results for kinetic resolution of five ibuprofen esters (**1**–**5**) are shown in Tables 4–8 and some of GC chromatograms are shown in Figs. 1 and 2.

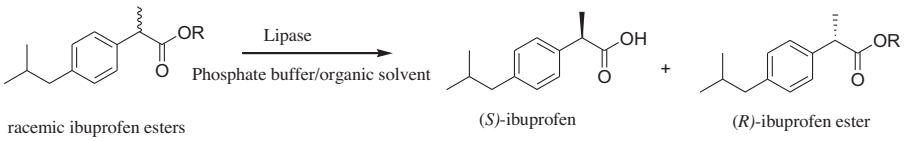
3.4.1. Ibuprofen methyl ester

As can be seen in Table 4, for ibuprofen methyl ester the *E*-values in *n*-hexane are higher than isooctane, all enantioselectivities have remarkable decrease at 50 °C compared to 40 °C most probably because of decrease in enzyme activity. The highest conversion at 30 °C was achieved for 20 mg of the immobilized enzyme (conversion 10.2%, *E* 55.5). Increasing the operating temperature may lead to increase in solubility of the substrates which causes to simple diffusion of substrate to immobilized enzyme where the reaction takes place. However, further increase in temperature up to 50 °C resulted in enzyme deactivation thereby decreasing the kinetic resolution of the substrate. There is a certain temperature range for lipase at which the conformation of the enzyme is optimal [56].

The highest enantioselectivity for hydrolysis of ibuprofen methyl ester was observed at 40 °C by using 5 mg of the immobilized enzyme in *n*-hexane with ee_P 96.3%, c 6.4 and *E* value of 56.1. By increasing the amount of enzyme (20 mg) conversion improves to 47.1% with slight decrease in *E* value. This shows that the lipase enantioselectivity is influenced by the amount of immobilized enzyme. By considering both conversion and enantioselectivity the best condition is obtained by using 20 mg of octyl-sepharose at 40 °C. The highest optical purity of the product in isooctane was observed at 40 °C and 20 mg of octyl-CRL which gave 93.3% of enantiomeric excess, conversion 38.9% and E 38.9 (Table 4).

3.4.2. Ibuprofen ethyl ester

In ethyl ester hydrolysis generally *E* values are lower than for methyl ester and by increasing the immobilized enzyme, the conversion of hydrolysis reaction is also increased. It can be concluded from Table 5 that the hydrolysis of this substrate in 50 °C resulted in decreasing enantiopreferences compared to 40 °C but enantioselectivities in 50 °C are higher than 30 °C. As for methyl ester *n*-hexane is a better co-solvent than isooctane. By considering all parameters such as conversion degree the best condition in *n*-hexane is achieved by 20 mg of octyl-CRL which gives conversion 45.1% and *E* value 32.1. 92.3% (ee_P) of enantioselectivity was observed for 5 mg of the immobilized enzyme at 40 °C with conversion 8.1% and 27.3 *E* value.



Scheme 2. Lipase-catalyzed enantioselective hydrolysis of racemic esters.

Table 3
Enzymatic parameters of the lipase-catalyzed enantioselective hydrolysis for different preparations of immobilized lipases.[a]

	n-Hexane/phosphate buffer pH 7 (1:1)				Isooctane/phosphate buffer pH 7 (1:1)			
	c	ee_s	ee_p	*E*	*c*	ee_s	ee_p	*E*
Octyl-CRL	15.12	16.87	94.70	43.41	8.93	9.30	94.84	41.46
Octyl-ROL	3.76	0.10	2.66	1.05	0.97	0.51	5.15	1.10
Octyl-RML[b]	77.12	96.85	28.73	6.18	9.73	8.12	75.33	7.69

[a] Experimental condition: temperature 40 °C; reaction time 72 h, 0.02 μmol ibuprofen butyl ester in biphasic system containing 2 ml organic solvent/2 ml phosphate buffer; 10 mg immobilized enzyme.
[b] *Rhizomucor miehei* lipase.

Table 4
Hydrolysis of ibuprofen methyl ester by octyl-CRL.[a]

Enzyme (mg) – temperature (°C)	*n*-Hexane				Isooctane			
	c	ee_s	ee_p	*E*	*c*	ee_s	ee_p	*E*
5–30	6.4	4.9	71.8	6.39	4.9	2.9	56.9	3.7
5–40	6.4	6.6	96.3	56.1	9.8	9.7	89.4	19.7
5–50	9.4	9.7	93.4	32.3	22.1	24.2	85.5	16.2
10–30	7.7	6.0	71.94	6.50	8.3	7.9	87.6	16.4
10–40	35.3	51.2	93.7	51.8	41.2	58.9	84.3	21.3
10–50	40.9	60.7	87.7	28.5	24.7	28.8	87.8	20.5
20–30	10.2	11.09	97.7	55.5	9.7	8.27	76.8	8.2
20–40	47.1	80.6	90.6	50.1	38.9	30.0	93.3	38.9
20–50	48.0	74.6	80.8	20.9	37.4	50.0	83.9	18.8

[a] Experimental condition: reaction time 72 h, 0.02 μmol ibuprofen methyl ester in biphasic system containing 2 ml organic solvent/2 ml sodium phosphate buffer at pH 7.0

Table 5
Hydrolysis of ibuprofen ethyl ester by octyl-CRL.[a]

Enzyme (mg) – temperature (°C)	*n*-Hexane				Isooctane			
	c	ee_s	ee_p	*E*	*c*	ee_s	ee_p	*E*
5–30	3.6	3.5	94.4	36.3	2.5	0.9	37.0	2.2
5–40	12.7	13.6	93.5	32.7	8.1	8.1	92.3	27.3
5–50	11.7	12.1	91.9	26.8	7.2	6.7	86.1	14.3
10–30	6.9	6.6	88.3	17.2	6.2	6.4	90.6	21.5
10–40	24.3	30.0	93.3	38.9	19.3	21.6	90.2	23.9
10–50	20.9	24.6	93.0	25.1	17.8	18.5	85.4	15.2
20–30	7.5	5.8	71.3	6.3	8.2	7.9	87.6	16.3
20–40	45.1	71.9	87.4	32.1	21.6	24.2	87.9	19.8
20–50	41.9	60.0	83.3	20.1	33.0	41.5	84.2	17.5

[a] Experimental condition: reaction time 72 h, 0.02 μmol ibuprofen ethyl ester in biphasic system containing 2 ml organic solvent/2 ml sodium phosphate buffer at pH 7.0.

3.4.3. *Ibuprofen propyl ester*

As in the case of octyl-RML in our previous report [38]. The lowest enantiopreferences among all examined esters were observed for ibuprofen propyl ester (2.1–8.8). From results in Table 6 it can be concluded that the highest conversion, ee_P and E in *n*-hexane belongs to 20 mg of immobilized CRL at 40 °C. Also in isooctane highest reaction parameters were afforded at 40 °C and 20 mg of enzyme. Similar to two previous ibuprofen esters *n*-hexane resulted in better performance of enzyme and enantioselectivities were decreased in 50 °C likely due to starting denaturation process of enzyme. Like two previous esters two important parameters, conversion (1.3–13.4%) and enantioselectivity (1.2–8.6) are very low at 30 °C.

3.4.4. *Ibuprofen butyl ester*

There are some reports that butyl ester is among the best esters in hydrolysis reactions [38,41,57,58]. In this investigation also butyl ester showed higher optical purity in terms of the desired pure

Table 6
Hydrolysis of ibuprofen propyl ester by octyl-CRL.[a]

Enzyme (mg) – temperature (°C)	*n*-Hexane				Isooctane			
	c	ee_s	ee_p	*E*	*c*	ee_s	ee_p	*E*
5–30	1.3	0.56	40.4	2.4	1.1	0.103	9.0	1.2
5–40	15.2	13.4	75.0	7.9	8.3	4.4	48.5	3.0
5–50	15.8	9.0	48.1	3.1	11.6	4.5	34.5	2.1
10–30	8.9	7.6	77.8	8.6	9.7	8.2	76.8	8.2
10–40	29.1	26.5	64.5	5.9	8.0	3.7	42.5	2.5
10–50	25.6	13.4	39.0	2.6	11.7	4.4	32.3	2.0
20–30	9.6	5.8	54.5	3.6	13.4	6.3	40.6	2.5
20–40	35.2	38.8	71.5	8.6	31.7	33.8	72.8	8.8
20–50	43.8	48.0	61.6	6.7	35.0	24.9	46.3	3.4

[a] Experimental condition: reaction time 72 h, 0.02 μmol ibuprofen propyl ester in biphasic system containing 2 ml organic solvent/2 ml sodium phosphate buffer at pH 7.0.

Table 7
Hydrolysis of ibuprofen butyl ester by octyl-CRL.[a]

Enzyme (mg) – temperature (°C)	*n*-Hexane				Isooctane			
	c	ee_s	ee_p	*E*	*c*	ee_s	ee_p	*E*
5–30	5.2	5.2	94.2	35.5	4.9	4.9	95.9	50.4
5–40	8.4	8.9	96.7	64.7	6.8	7.1	96.8	65.6
5–50	10.3	11.2	96.5	63.2	8.9	9.5	97.1	74.3
10–30	6.6	6.6	90.5	21.9	8.1	9.9	77.2	8.6
10–40	21.6	26.2	95.0	50.4	13.6	15.0	95.4	49.7
10–50	14.6	16.5	96.7	70.4	14.2	15.7	95.6	52.1
20–30	8.1	8.2	93.3	31.5	11.4	9.9	77.2	8.6
20–40	30.5	35.8	81.8	14.2	26.5	29.8	82.6	14.0
20–50	31.7	41.7	90.0	28.7	39.5	54.9	84.1	20.0

[a] Experimental condition: reaction time 72 h, 0.02 μmol ibuprofen butyl ester in biphasic system containing 2 ml organic solvent/2 ml sodium phosphate buffer at pH 7.0.

Table 8
Hydrolysis of ibuprofen isobutyl ester by octyl-CRL.[a]

Enzyme (mg) – temperature (°C)	*n*-Hexane				Isooctane			
	c	ee_s	ee_p	*E*	*c*	ee_s	ee_p	*E*
5–30	5.6	0.42	75.0	7.02	5.3	4.9	87.6	16.0
5–40	6.5	5.7	81.3	10.3	8.6	3.7	90.9	20.0
5–50	7.9	7.04	82.0	10.9	7.3	7.0	89.8	19.9
10–30	7.6	7.4	89.5	19.4	7.7	7.2	86.8	15.2
10–40	36.0	46.2	82.1	16.0	13.7	15.2	95.7	53.0
10–50	14.7	14.6	84.9	14.0	10.7	11.3	94.9	43.0
20–30	6.8	6.6	90.5	21.4	4.8	4.7	92.2	25.9
20–40	22.0	23.8	71.3	7.5	1.4	1.2	84.4	11.9
20–50	27.2	29.4	78.7	11.1	4.5	3.9	83.9	11.9

[a] Experimental condition: reaction time 72 h, 0.02 μmol ibuprofen isobutyl ester in biphasic system containing 2 ml organic solvent/2 ml sodium phosphate buffer at pH 7.0.

product (ee_P) and the remaining less reactive substrate (ee_S). In contrast to other esters, 10 °C increase in temperature resulted in improvement of conversion and enantioselectivities (Table 7). Relatively high operating temperatures may reduce the viscosity of the substrate phase and thus assist the substrate diffusion [59,60].

The hydrolysis of ibuprofen butyl ester in isooctane with 5 mg of immobilized enzyme at 50 °C gives the highest *E*-value 74.3, conversion 8.9% and 97.1% ee_P. The same results obtained with 10 mg of immobilized enzyme at 50 °C in *n*-hexane with *E*-value 70.4, conversion 14.6% and 96.7% ee_P. By considering the obtained results it can be concluded that the ester group of butyl-ibuprofen ester may fit in more specific way within the molecular recognition site than that possessed by other esters.

3.4.5. Ibuprofen isobutyl ester

Enantioselectivities for isobutyl ibuprofen ester as for butyl ester in *n*-hexane are higher than isooctane. Acceptable results were

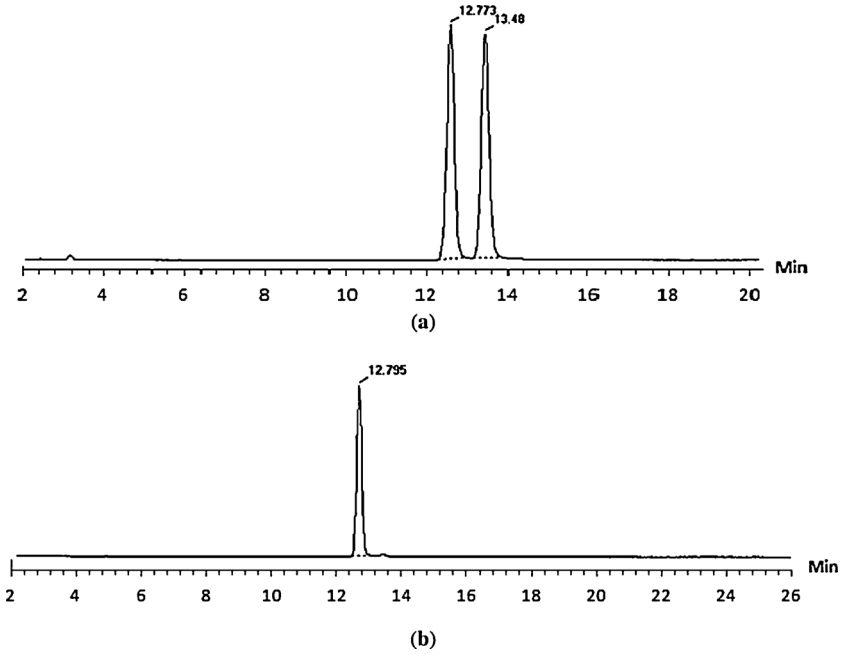


Fig. 1. GC chromatograms of (*R*,*S*-ibuprofen) (a) and (*S*)-ibuprofen (b).

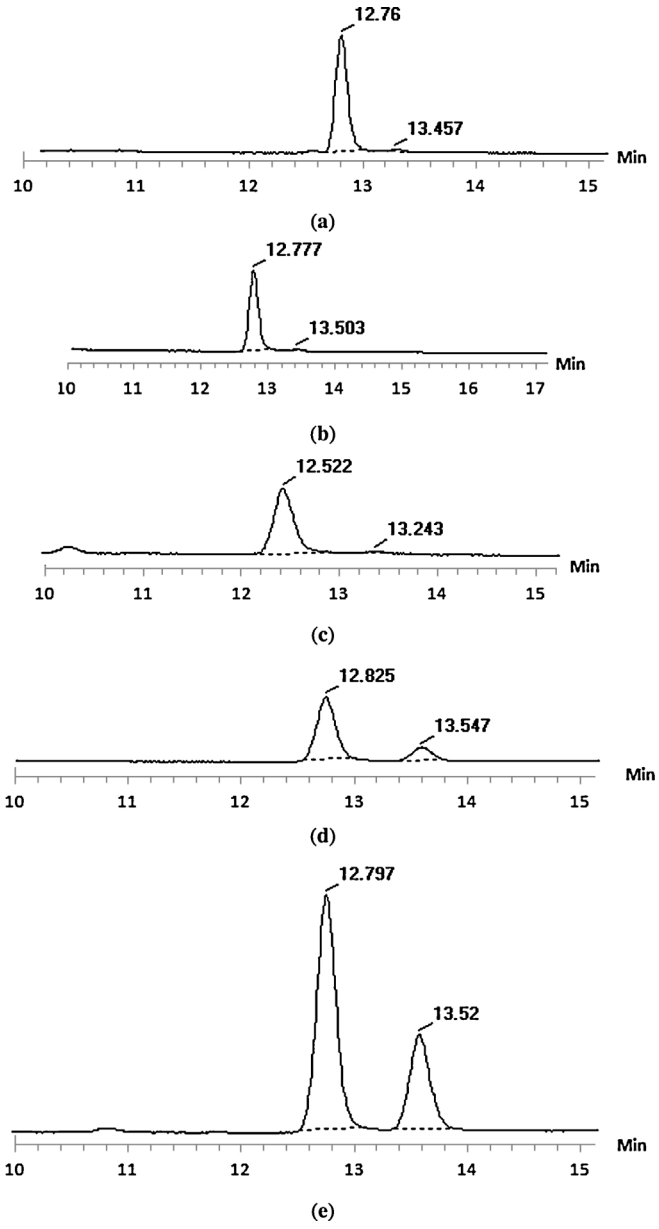


Fig. 2. GC chromatograms of some reactions (ibuprofen moiety). (a) Hydrolysis of butyl ester in isooctane (5 mg enzyme; 40 °C), conversion 6.8; ee_p 96.8; *E* 65.68; (b) hydrolysis of butyl ester in *n*-hexane (10 mg enzyme; 50 °C), conversion 14.6; ee_p 96.7; *E* 70.4; (c) hydrolysis of methyl ester in *n*-hexane (10 mg enzyme; 50 °C), conversion 24.7; ee_p 87.8; *E* 20.4; (d) hydrolysis of propyl ester in *n*-hexane (10 mg enzyme; 40 °C), conversion 29.1; ee_p 64.5; *E* 5.9; (e) hydrolysis of propyl ester in *n*-hexane (20 mg enzyme; 50 °C), conversion 43.8; ee_p 61.6; *E* 6.7.

obtained for 10 mg of immobilized enzyme at 40 °C for both *n*-hexane and isooctane which yielded 16.0 and 53.0 *E*-values.

3.5. Reuse of immobilized lipase in the kinetic resolution of racemic ibuprofen esters

The reusability of the immobilized lipase is very important from economic point of view. In order to test the efficiency of immobilized CRL, after each run (72 h) the immobilized derivative was recovered by filtration and after that washed by *n*-hexane 5 mL (three times). Then it was re-used with new substrate mixture containing ibuprofen butyl ester and octyl-CRL in *n*-hexane (50 °C). The reaction was repeated up to four cycles with the same enzyme derivative to determine the enantioselectivity of the esterification

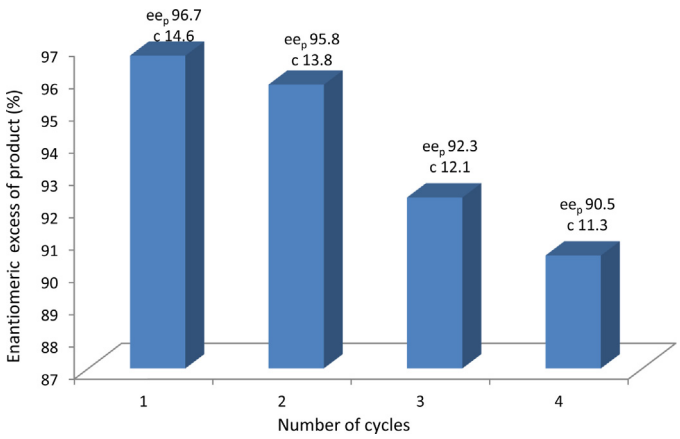


Fig. 3. Effect of repeated use of immobilized CRL onto octyl-sepharose on the enantioselectivity of the hydrolysis of (*R*,*S*)-ibuprofen butyl ester. Reaction conditions: (*R*,*S*)-ibuprofen butyl ester (0.02 μmol), immobilized CRL (10 mg), *n*-hexane(2 ml), sodium phosphate buffer, pH 7.0, 100 mM (2 ml), temp. 50 °C, after 72 h.

reaction (Fig. 3). During the four cycles of the kinetic resolution of ibuprofen butyl ester little changes of the enantioselectivity of the reaction (expressed as enantiomeric excess of product) were observed.

4. Conclusion

In this study the immobilized preparations of CRL and ROL on octyl-sepharose were used in the kinetic resolution of (*R*,*S*)-ibuprofen by enantioselective hydrolysis of its racemic esters. CRL immobilized on the octyl-sepharose support demonstrated high catalytic activity and allowed to obtain products of (*S*)-ibuprofen with high enantioselectivity. Among various esters, ibuprofen butyl ester had the best results regarding conversion degree, enantiomeric excess of products (ee_P > 95%) and enantioselectivity (*E* value 70 and74; conversion 14.6 and 8.9 in *n*-hexane and isooctane, respectively). It is clear from the results that the (*S*)-ester conversion and *E* values reported in the present investigation are very promising for kinetic resolution of racemic esters catalyzed by *C. rugosa* immobilized on octyl-sepharose.

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