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Effects of alcohol and solvent on the performance of lipase from *Candida* sp. in enantioselective esterification of racemic ibuprofen

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ABSTRACT

The *Candida* sp. lipase prepared in our lab was used for the resolution of racemic ibuprofen. In order to study the effects of alcohol and solvent on the performance of *Candida* sp. lipase in enantioselective esterification of racemic ibuprofen, different alcohols were chosen as acyl acceptors in the same solvent, and identical substrates were used in different solvents. The reactions were performed under controlled water activity, thereby permitting the influences of the alcohols and the solvents to be separated from their ability to strip water from the solid enzyme. The results showed that alcohols and solvents had great effects on the performance of *Candida* sp. lipase.

1. Introduction

Ibuprofen, 2-(4-isobutylphenyl) propionic acid, is a widely used nonsteroidal anti-inflammatory drug which belongs to the family of 2-arylpropionic acid derivatives with an asymmetric carbon in the second position. It is mainly marketed as a racemate. However, it has been reported that (*S*)-ibuprofen is 160-fold more active than its antipode in the synthesis of prostaglandin 'in vitro' [1]. Especially in the pharmaceutical industry, the need for enantiomerically pure compounds has grown since legislation required investigation on the pharmacological effect of each enantiomer.

Obtaining optically pure enantiomers has attracted much interest. Since classical ways to obtain pure enantiomers, that is, chemical asymmetric synthesis, stereoselective crystallization and chiral chromatography, are often expensive, enzymatic procedures have become attractive and promising due to the excellent stereoselectivity of enzymes and mild reaction conditions. Lipases are frequently used because of their high stability, their non-requirement of cofactors, synthetic activity in organic solvents and mainly in the wide range of substrate specificities. In this field, lipases from *Candida rugosa* [2,3], *Candida cylindracea* [4,5], *Candida antarctica* [6], *Rhizomucor miehei* [7,8], *Aspergillus niger* [9], *Thermomyces lanuginosa* [3], *Porcine pancreas*, *Pseudomonas cepacia*, and *Pseudomonas fluorescens* [10] have been used to resolve the enantiomers of ibuprofen.

Our laboratory previously isolated a stable and high lipase producing mutant strain *Candida* sp. which was very effective in catalyzing methyl esters synthesis from triacylglycerols or free fatty acids [11] and catalyzing isooctyl palmitate synthesis from palmitic acid and 2-ethyl hexanol [12]. However, there is no report about this lipase for the resolution of (*R*, *S*)-ibuprofen. Herein, lipase from *Candida* sp. was used to enantioselective esterification of racemic ibuprofen.

It is well known that enzyme-catalyzed esterification reactions in biphasic systems (water–water immiscible organic solvent) are reversible [13], and the equilibrium constants of the reactions was greatly affected by the water content or water activity. Therefore, all the experiments were conducted under controlled water activity. Furthermore, enzymes are strongly affected by the choice of solvent they used in. It has been reported that even reversal of substrate specificity [14] and enantiopreference [15] due to solvent changes. Even if enzymes were used in the same solvent, they usually showed different enantioselectivity because of the different substrates used in reactions [5]. In this paper, we systemically investigated the effects of alcohol and reaction medium on the performance of lipase from *Candida* sp. in enantioselective esterification of racemic ibuprofen.

2. Experimental

2.1. Materials

The lipase from *Candida* sp. was obtained in our laboratory. Racemic and optically pure ibuprofen was purchased from

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Sigma–Aldrich (USA). All other chemicals used were of analytical grade and were obtained commercially from Yili Chemical Co. Ltd. (Beijing, China). All solvents used were dehydrated by activated 4 Å molecular sieves.

2.2. Preparation of immobilized lipase

Lipase culture was obtained from our previous work [16] operating at 400 rpm, 1 vvm in 30 L bioreactor.

10 g of textile was pre-socked for 1 h in 20 mL of coimmobilization solution, consisting of 5% (w/v) glutin, 2% (w/v) lecithin, 2% (w/v) polyethylene glycol-6000 and 1% (w/v) magnesium chloride. The textile was then dried at room temperature and was used as the support in the immobilization of the lipase. The support (about 1 g) and 2 mL of the supernatant of the fermentation broth were mixed and following dried to constant weight at room temperature. The resulting textile was cut into small pieces (0.16 cm^2) and the immobilized lipase was stored at 4 °C in refrigerator [17].

2.3. Typical procedure for enantioselective esterification

Before use, both the organic solvent and the alcohol were dried over 4 Å molecular sieves. A salt hydrate pair ($Na_2SO_4/Na_2SO_4{\cdot}10H_2O$) (totally 500 mg, with molar ratio of 1:1) was added to 10 mL organic solvent containing (*R*, *S*)-ibuprofen (0.3 mmol) and alcohol (0.3 mmol) and the resultant mixture was shaken for 1 h at 30 °C, 180 rpm. After the addition of 200 mg immobilized lipase (1 g immobilized lipase containing 50 mg enzyme protein), the mixture was incubated on a shaker at 30 °C and 180 rpm. Samples were withdrawn at certain time intervals. The salt hydrate pairs $Na_2SO_4/Na_2SO_4{\cdot}10H_2O$ could maintain the water activities (a_w) of the reaction system effectively at 0.83 at 30 °C [18].

2.4. Determination of E and K

The equilibrium constant *K* was determined by allowing the reaction to proceed until the fast-reacting substrate attained equilibrium. The value of *K* could then be estimated from the concentrations of the remaining substrate and product of the fast-reacting substrate. From the experimentally determined data of ee_s, ee_p, *c*, and *K*, the *E* value was calculated with use of Eq. (1) or (2) [13].

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

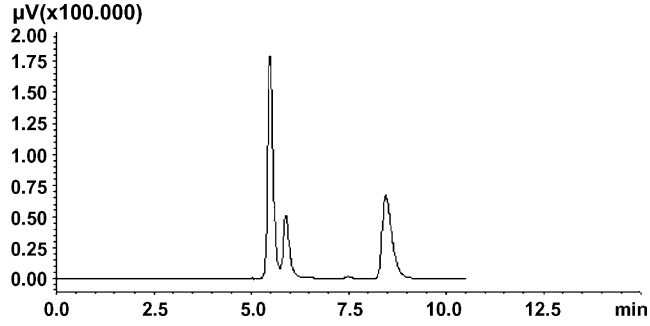

Fig. 1. HPLC figures of ibuprofen in reaction medium after 72 h reaction. Reaction conditions: 0.3 mmol racemic ibuprofen, 0.3 mmol 1-propanol, 0.2 g *Candida* sp. lipase, 10 mL isooctane, 0.5 g $Na_2SO_4/Na_2SO_4{\cdot}10H_2O$ (with molar ratio of 1:1) 30 °C, 180 rpm.

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

where

$$c = 1 - \frac{A+B}{A_0+B_0} \text{ or } \frac{ee_s}{ee_s+ee_p}\,[19]; \quad ee_s = \frac{B-A}{A+B}; \quad ee_p = \frac{P-Q}{P+Q};$$

$$K = \frac{A}{P} = \frac{B}{Q}; \quad A_0 = A+P; \quad B_0 = B+Q$$

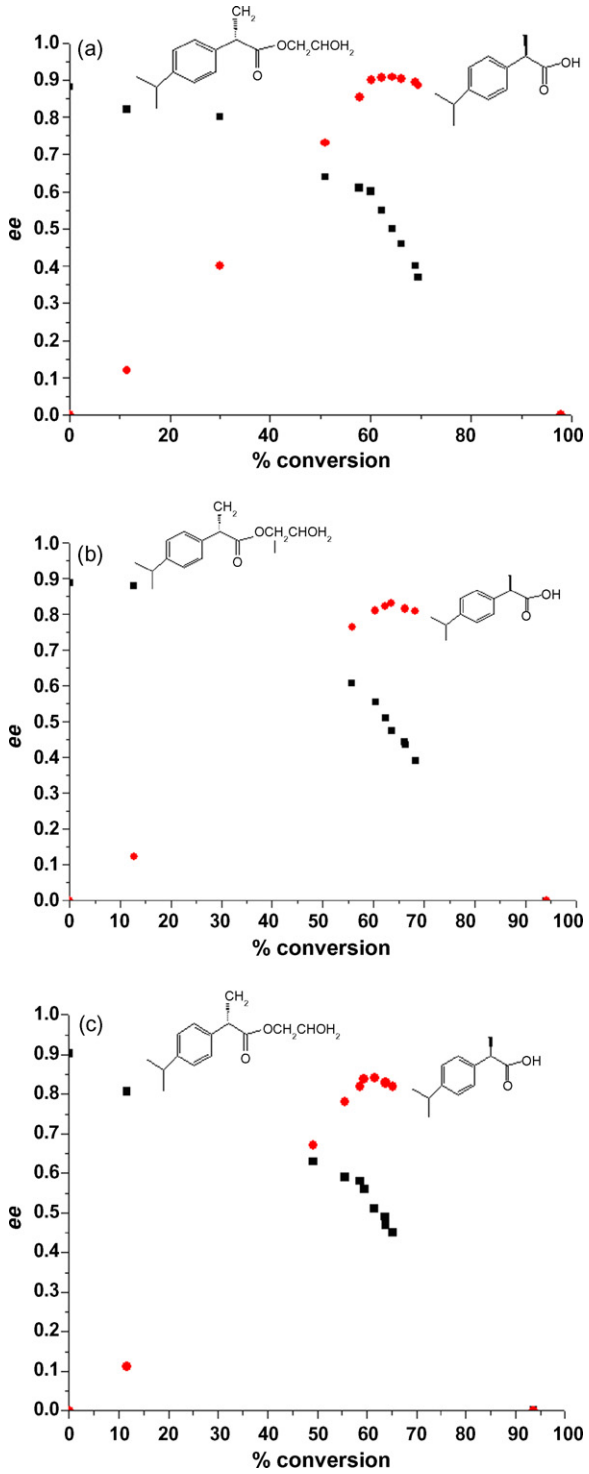

Fig. 2. Dependence of enantiomeric excess (ee) of product and substrate on the percent conversion. (a) Using 1-propanol as acyl acceptor; (b) using isobutanol as acyl acceptor; (c) using 1-butanol as acyl acceptor.

Table 1
Effect of alcohol on the lipase-catalyzed esterification of (*R*, *S*)-ibuprofen

Entry	Alcohol	Initial rate (μ mol h^{-1} immobilized lipase g^{-1})	*K* (the equilibrium constant)	Time[a] (h)	*E* (enantioselectivity)
1	Methanol	93.7	0.181	333	5.4
2	Ethanol	111.7	0.155	333	6.1
3	1-Propanol	173.9	0.021	120	16.0
4	Allyl alcohol	129.9	0.224	333	/
5	1-Butanol	171.7	0.067	192	17.1
6	1-Pentanol	131.3	0.018	312	5.8
7	1-Hexanol	116.07	0.029	312	9.1
8	1-Heptanol	105.33	0.049	312	9.2
9	1-Octanol	116.91	0.044	312	9.6
10	1-Nonanol	59.5	0.935	333	/
11	1-Decanol	128.5	0.032	312	9.2
12	1-Dodecanol	117.8	0.100	333	11.4
13	1-Tetradecanol	106.2	0.125	375	11.5
14	1-Hexadecanol	98.3	0.149	375	10.2
15	1-Heptadecanol	87.5	0.136	375	12.0
16	1-Octadecanol	97.3	0.106	375	9.3
17	Isobutanol	191.4	0.063	216	19.4
18	Isoamylol	104.3	/	/	/
19	Isooctanol	24.5	/	/	/
20	Benzyl alcohol	22.5	/	/	/
21	Isopropyl alcohol	0	/	/	/
22	Cyclohexanol	0	/	/	/
23	Terpilenol	0	/	/	/
24	*tert*-Butanol	0	/	/	/
25	*tert*-Amyl alcohol	0	/	/	/
26	Glycol	0	/	/	/
27	1,3-Propanediol	0	/	/	/
28	1,4-Butanediol	0	/	/	/
29	1,6-Hexanediol	0	/	/	/
30	Glycerol	0	/	/	/

Reaction conditions: 0.3 mmol ibuprofen, 0.3 mmol alcohol, 0.2 g lipase, and 10 mL solvent of hexane, 0.5 g $Na_2SO_4/Na_2SO_4{\cdot}10H_2O$ (with molar ratio of 1:1) 30 °C, 180 rpm (/: not determined).

[a] When the equilibrium of the fast-reacting enantiomer was established.

(Here *A*, *B* and *P*, *Q* are pairs of enantiomers. That is, if *A* is the fast-reacting enantiomer in the forward reaction, *P* must be the fast-reacting enantiomer in the reverse reaction.)

2.5. HPLC analysis

The enantiomeric excess of ibuprofen enantiomers, and lipase catalyzed esterification conversion were analyzed respectively by HPLC with a chiral column ((*R*, *R*)-Whelk-o 1 25 cm × 4.6 mm, Regis Technologies Inc., USA) capable of separating the (*S*)-ibuprofen ester, (*R*)-ibuprofen ester, (*S*)-ibuprofen, and (*R*)-ibuprofen. The mobile phase was a mixture of *n*-hexane:isopropanol:acetic acid (98:2:0.5, v/v/v) at a flow rate of 1.0 mL min^{-1}. UV detection at 260 nm was for quantification at ambient temperature.

The activity of enzyme was calculated from the initial rate of reaction (0–5% esterification of racemic ibuprofen).

3. Results and discussion

3.1. Enantiopreference

To study the *Candida* sp. lipase enantiopreference towards racemic ibuprofen and compare that obtained with this lipase to those obtained with the widely used lipases in the same solvent already described in the literature, we designed an experiment wherein *Candida* sp. lipase was added to catalyze enantioselective esterification of racemic ibuprofen. This experiment was carried out using 1-propanol as the alcohol in isooctane. As shown in Fig. 1, the retention times of (*S*)-ibuprofen ester, (*R*)-ibuprofen ester, (*S*)-ibuprofen, and (*R*)-ibuprofen were 5.421, 5.935, 7.514, and 8.503 min, respectively. This indicated that this lipase enantioselectively catalyzed the esterification of racemic ibuprofen to (*S*)-ibuprofen ester; whereas no (*R*)- or (*S*)-ibuprofen ester was detected in the control experiments (data not shown here).

Obviously, *Candida* sp. lipase gives an opposite enantiopreference to the lipase from *C. antarctica* which prefers (*R*)-enantiomer [3], and gives an identical enantiopreference to the lipase from *R. miehei* which prefers the (*S*)-enantiomer [3–8]. This may be the result of the differences in the structure of their active sites. Lipase from *C. antarctica* does not possess a lid, whereas the active site of lipase from *R. miehei* is at the surface of the protein protected by a lid. The three dimensional structure of *Candida* sp. lipase is unknown, but the primary structure of the *Candida* sp. lipase has been determined in our lab (see http://www.ncbi.nlm.nih.gov/; Genebank accession No. CAB91111). Blasting amino acid sequences showed that there was no significant similarity between the sequence of *Candida* sp. lipase and that of *C. antarctica* lipase. However, the result of comparison of the sequence of *Candida* sp. lipase and that of *R. miehei* lipase was that Identities and Positives were 79/296 (26%), 128/296 (43%), respectively. It can be assumed that the active site of *Candida* sp. lipase is different from that of lipase from *C. antarctica*, but similar to that of lipase from *R. miehei*, thus explaining the different and same enantiopreference.

3.2. Effect of alcohol

It is assumed that the formation of an acyl–enzyme intermediate as the crucial step during esterification, and the final reaction yield will depend on the accessibility of the alcohol to the acyl–enzyme complex, which is unique for each acylation reagent. This fact would create a specific geometry around the active site, which would

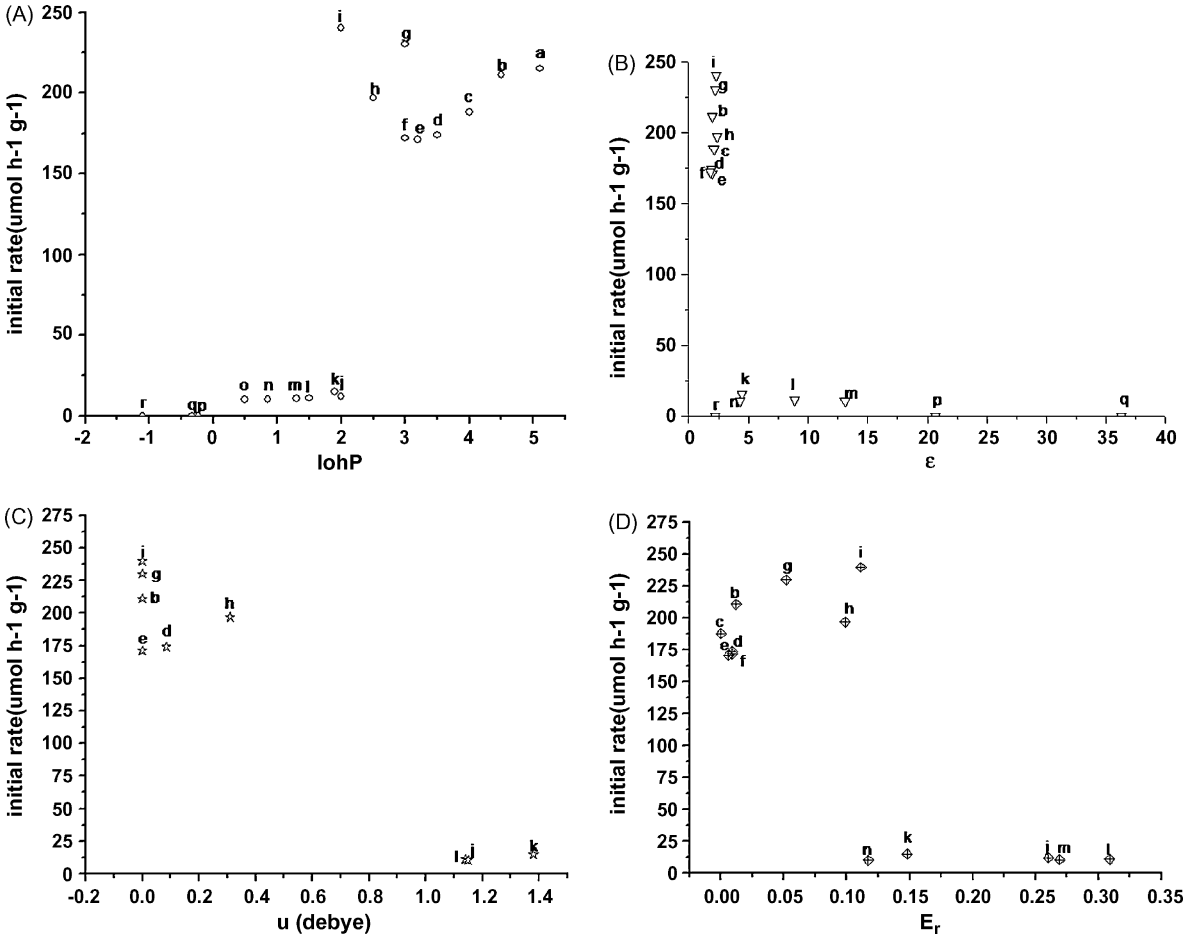

Fig. 3. Correlations between initial rate and the properties of the solvents. (A) log *P*; (B) ε; (C) μ; (D) E_T. Here initial rate was for total (*R*, *S*)-ibuprofen. The properties of the solvents were taken from reference (6). Solvents: a, *n*-nonane; b, isooctane; c, *n*-heptane; d, *n*-hexane; e, cyclohexane; f, *n*-pentane; g, carbon tetrachloride; h, toluene; i, benzene; j, trichloromethane; k, isopropyl ether; l, dichloromethane; m, methy-1-isobutyl ketone; n, ethyl ether; o, tetrahydrofuran; p, acetone; q, acetonitrile; r, 1,4-dioxane. Reaction conditions: 0.3 mmol ibuprofen, 0.3 mmol 1-propanol, 0.2 g lipase, and 10 mL solvent, 0.5 g $Na_2SO_4/Na_2SO_4{\cdot}10H_2O$ (with molar ratio of 1:1) 30 °C, 180 rpm.

determine the nature of the best recognized alcohol. Because of all these factors the nature of alcohol moiety must play an important role in the development of esterification reactions.

With the aim of studying the effect of alcohol nature on our esterification reaction, we employed different alcohols in the esterification of racemic ibuprofen. The results were shown in Table 1. *Candida* sp. lipase did not act on any of the studied polyols (entry 26–30). The studied secondary (entry 21–22) or tertiary alcohols (entry 23–25) were not esterified by the lipase, either. However, all monohydric primary alcohols (entry 1–20) brought about esterification of ibuprofen.

The reaction initial rate was influenced by the length of the alcohols. Among linear primary alcohols (entry 1–16), the fast reaction was obtained with the studied alcohols possessing three or four atoms of carbon. The initial rates were 173.9 and 171.7 $\mu mol\,h^{-1}\,g^{-1}$, respectively. The lipase expressed low activity towards 1-nonanol (59.5 $\mu mol\,h^{-1}\,g^{-1}$). Long-chained ones (1-hexadecanol, 1-heptadecanol, 1-octadecanol) exhibited moderate activities (98.3, 87.5, 97.3 $\mu mol\,h^{-1}\,g^{-1}$, respectively).

Interestingly, branched primary alcohols (entry 17–20) showed different effect on the activity of lipase from *Candida* sp. Isobutanol is shorter than 1-butanol, with three atoms of carbon in chain, but the activity was highest (191.4 $\mu mol\,h^{-1}\,g^{-1}$) because the methyl radical in position 2 may facilitate this alcohol to the acyl–enzyme complex. On the contrary, Isooctanol and benzyl alcohol exhibited low activity (24.5, 22.5 $\mu mol\,h^{-1}\,g^{-1}$, respectively) because the ethyl group in position 2 and aryl group in position 1, respectively, may impede the approach of these alcohols to the acyl–enzyme complexes.

The equilibrium constant, *K* (*i.e.*, the final theoretical ester yield, the *K* for the pair of enantiomers in achiral solvent media being equal without taking enzyme inactivation into account, and the final ester yield = $1/(K+1)$) depended markedly on species of alcohol. Using long-chained alcohols (atom of carbon, $C > 10$) as substrates, the *K* was medium ($0.1 < K < 0.15$), and the time when the fast-reacting enantiomer reached the equilibrium was long ($t > 333$ h); using alcohols ($2 < C \leq 10$, except allyl alcohol and 1-nonanol) as substrates, the *K* was low ($0.018 \leq K \leq 0.067$), and the time of equilibrium was short ($t < 312$ h); using the alcohols ($C \leq 2$) as substrates, the *K* was large ($K > 0.15$), and the time was moderate ($t = 333$ h). It was reported that the low ester conversion was obtained with shorter polar alcohols, like methanol or ethanol, which were able to dehydrate the enzyme [20]. Our study indicated that shorter polar alcohols were detrimental to the activity of the enzyme in nature and not just because they removed water molecules from the solid enzyme because of the fact that these reactions were all performed at a fixed water activity, maintaining a constant water content of the solid enzyme. Due to the hydrophobic nature of the active site of the lipase, hydrophobic alcohols would also be the most appropriate for enzyme recognition. Nevertheless, not only the lipophilicity but also the geometry and the electron acceptance of the alcohols must be considered. Although allyl alco-

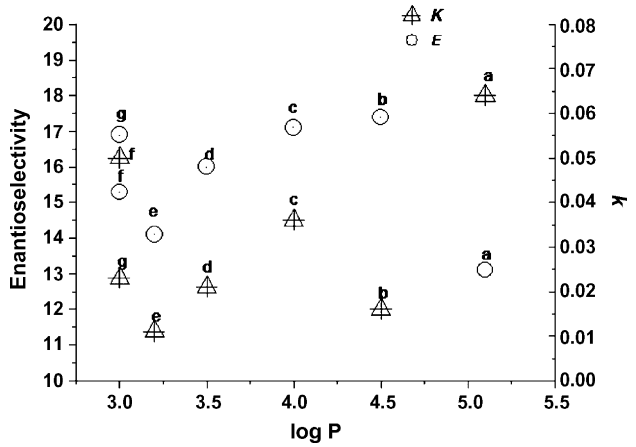

Fig. 4. Enantioselectivity and the equilibrium constant as a function of the log *P* of the solvents. Solvents are described as in Fig. 3.

hol is longer than ethanol, the *K* (0.224) was more than that (0.155) of ethanol.

The influence of alcohol moiety on the enantioselectivity of the esterification was also studied. The results were also shown in Table 1, where we can observe high enantioselectivity for isobutanol (*E* = 19.4). However, both *E* and *K* have significant effects on the enantiomer excess of product (or substrate) and yield. Fig. 2 is the figure of dependence of enantiomeric excess (ee) on the percent conversion. Using 1-propanol as substrate, the ester with ee = 0.59 was obtained when the conversion attained 60%. When 1-butanol or isobutanol was used as substrate, the ester with ee = 0.55 or 0.56 was observed at 60% conversion. Therefore, 1-propanol was recommended for the good substrate of *Candida* sp. lipase in the enantioselective esterification of racemic ibuprofen at constant water activity of 0.83 at 30 °C in hexane. On the other hand, the enantiopreference was the same for all the alcohols tested.

3.3. Effect of solvent

The effect of different organic solvents on the esterification of racemic ibuprofen with 1-propanol catalyzed by *Candida* sp. lipase was investigated. The reactions were performed in controlled water activity, thereby permitting the influence of the solvents to be separated from their ability to strip water from the solid enzyme. Different properties were used to describe the solvents, namely the hydrophobicity, quantified by log *P*; ε, the dielectric constant; μ, the dipole moment; and E_T, a normalized electron acceptance index. Fig. 3 is the figure of initial rate as a function of the hydrophobicity (A), the dielectric constant (B), the dipole moment (C), and a normalized electron acceptance index (D) of the solvents. As it was seen, there was no a fair linear correlation between any of the properties of the solvents and initial rate. At this constant water activity, *Candida* sp. lipase was active only with organic solvents with $\log P \geq 0.49$, and inactive with acetone ($\log P = -0.23$), acetonitrile ($\log P = -0.33$), and 1,4-dioxane ($\log P = -1.1$). When no salt hydrate pair ($Na_2SO_4/Na_2SO_4 \cdot 10H_2O$) was used to control water activity, *Candida* sp. lipase presented activity only with organic solvents with $\log P \geq 3$ (results not shown here) for the same substrates. This study showed that the amount of enzyme-bound water has great effect on the activity of the enzyme, and the hydrophilic solvents probably can remove water from the enzyme. However, the hydrophilic solvents impede enzyme activity in essence and not just because they strip water from the enzyme.

The enantioselectivity and the equilibrium constant were also affected by the solvent. The results were shown in Fig. 4, where we saw that there was no reasonably good correlation between the log *P* of the solvent and *E* or *K*, either. Similar correlations were found with ε, μ, and E_T (results not shown here). In the absence of a universal and comprehensive predictive model, for a certain system, one should at least undertake a solvent screening. Here we chose isooctane as the good organic solvent for the enantioselective esterification of racemic ibuprofen catalyzed by *Candida* sp. lipase because of the high enantioselectivity (*E* = 17.4) and low *K* (0.016).

4. Conclusions

It was reported that *Candida* sp. lipase was able to resolve racemic ibuprofen. In all case, the (*S*)-enantiomer was preferred by the lipase. Alcohols and solvents had great effects on the performance of the lipase. The study demonstrated that shorter polar alcohols and the hydrophilic solvents impede enzyme activity in nature and not just because they remove water from the enzyme due to the fact that these reactions were all performed at a fixed water activity, maintaining a constant water content of the solid enzyme. Under operational conditions 1-propanol and isooctane were recommended for the good substrate and reaction medium respectively, because of relatively high enantioselectivity and low *K*.

Esterification with studied secondary or tertiary alcohols was not observed. *Candida* sp. lipase had high specificity for monohydric primary alcohols in the esterification of racemic ibuprofen. The equilibrium constant and enantioselectivity were profoundly affected by the chain length, but there was no linear correlation between them, indicating that not only the lipophilicity but also the geometry and the electron acceptance of the alcohols must be considered.

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