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A systematic analysis of the variables that control a highly stereoselective resolution of racemic non-steroidal antiinflammatory drugs using immobilized lipase from *Candida cylindracea*

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Abstract

An enzymatic method for the production of *S*(+) 2-arylpropionic acids has been developed. The process consists of the stereoselective hydrolysis of the racemic ethyl esters catalyzed by covalent immobilized *Candida cylindracea* lipase. The performance and yield of the reaction were evaluated as a function of the critical reaction parameters such as temperature, substrate concentration, pH, ionic strength and stirring speed. An increase in the stirring speed, a diminution of the substrate concentration and pH = 7.0 favors the hydrolysis of esters. The influence of the alkyl chain and the alcoholic residue of the ester have been studied. The ethyl and butyl esters are the most interesting esters for carrying out hydrolysis. High enantioselective hydrolysis of the racemates (yielding *S*(+) isomer; ee $\geq$ 95%) can be achieved using the immobilized derivatives. The different yields of *S*(+) 2-arylpropionic acids produced with the several immobilized derivatives are related to structural parameters of the substrate, which were studied by molecular mechanics, and to the relation between the hydrophilicity of the supports and the lipophilicity of the substrates. A model of the active site is proposed in relation to substrates used and reaction yields. The immobilized derivatives were re-used in three cycles and showed an 80% residual activity after 336 h of operating time.

Keywords: Antiinflammatory drugs; *Candida cylindracea*; Enantioselective hydrolysis; Hydrolysis; Immobilized lipase

1. Introduction

There is a growing interest in the use of optically pure enantiomers for drugs, because they are more target-specific and have fewer side-effects than racemic mixtures. Of the 1800 drugs currently available, about half are chiral mixtures [1]. 2-Arylpropionic acids are an important class of non-steroidal antiinflammatory drugs. They are widely used as racemic mixtures to control the symptoms of arthritis and related connective tissue diseases [2]. However, it is well documented that only the *S*(+)-form is active (in the case of the Naproxen (2-(6-methoxy-2-naphthyl) propionic acid) the *S*-enantiomer is 28 times more active than the corresponding *R*-enantiomer [3], while only a certain portion of the *R*(−)-form could be transformed to the *S*(+)-isomer by metabolic inversion via stereoselective formation of the coenzyme A ester of the *R*(−)2-arylpropionic acid [4]. This last compound can replace the natural fatty acids in triacylglycerols to form 'hybrid'

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triglycerides that may produce toxic effects, such as disrupted normal lipid metabolism and membrane function [5].

In recent years, the use of biological systems for production of optically enriched compounds has become an alternative to classical methodologies of chemical synthesis. Different types of hydrolytic enzymes have been used for the resolution of racemic alcohols and carboxylic acids through stereoselective hydrolysis of the corresponding esters [6,7]. In organic media, this approach has been extended to asymmetric esterification and transesterification [8,9]. However, the reactions carried out in aqueous media are less expensive from an industrial point of view.

The enzymatic enantioselective resolution of 2-substituted propionic acids has been the subject of intense investigation. Much of this effort has centred on the synthesis of *R*-2-chloropropionic acid, due to its high value as an intermediate in the production of herbicides [10,11].

Recently, the preparation of optically active Naproxen has been accomplished by using microorganisms [12] and enzymes [13]. Partial resolution has been obtained through microbial hydrolysis of the racemic methyl ester of Naproxen [12]. A stereospecific oxidative degradation has been carried out by using a *Rhodococcus sp.* on α-methyl long-chain alkyl derivatives of 6-methoxynaphthalene [14]. However, the *R*-enantiomer was obtained as free acid.

Stereoselective hydrolysis of several esters of 2-arylpropionic acid has been successfully developed with lipases from different organisms [13,15]. However, only the lipase from *C. cylindracea* gave selectively the *S*-form of the acid. The enzyme was used either free [16] or immobilized by adsorption [17]. Using the soluble enzyme the costs of the process are elevated and the enantiomeric excess is lower in comparison when the biocatalyst is an immobilized enzyme. On the other hand, the immobilized derivatives of lipase from *Candida cylindracea* obtained by adsorption on inorganic or organic supports present lower values of thermal stability due to the weak enzyme–support interactions produced in this methodology [18].

For industrial use, the immobilization of lipase on solid support by covalent attachment can offer several advantages, such as high concentration and even distribution of the enzyme, enhancing operational stability, continuous operation, repeated usage of derivative and retention of the biocatalyst in the reaction vessel [19]. However, when the immobilization was carried out by covalent attachment the values of activity retained were considerably lower than the residual activities of other types of immobilized enzymes [20] (between 1 and 30 percent). This problem has been avoided using as support for the immobilization agarose activated by tosylation methodology [21] and alumina and silica activated by trichlorotriazine methodology [22]. We have thus been able to develop very active and stable immobilized derivatives.

The aim of the present study is the development of an enzymatic method for the production of the *S*-enantiomer of 2-arylpropionic acids through stereoselective hydrolysis of their racemic esters, using as biocatalysts active and stable covalent immobilized derivatives of lipase from *C. cylindracea*. In the present paper we show what are the main technical variables to be controlled in order to obtain good yields, enantiomeric excess and repetitive results.

2. Experimental

Materials. Lipase from *Candida cylindracea* (EC 3.1.1.3) (type VII, containing 1,010 U/mg solid, using olive oil as substrate) and lipase substrate (stabilized olive oil emulsion 50% (v/v)) were from Sigma Chemicals Co. (St. Louis, MO, USA). Agarose gel beads (Bio-gel A-150 m, 100–200 mesh) were from Biorad Lab. (Richmond, USA). Alumina-60 (70 Å pore size) and Silica-60 (120 Å pore size) were from Merck (Germany). (±) 2-Phenylpropionic acid, (±) 2-phenylbutyric acid and the alcohols used were from Fluka Chemika (Switzerland).

(±) 2-(4-Isobutylphenyl)propionic acid (Ibuprofen) was supplied by Boots Pharmaceuticals (Nottingham, UK), (±) 2-(6-methoxy-2-naphthyl)propionic acid (Naproxen) was by Syntex Research (Palo Alto, CA) and (±) 2-(3-benzoylphenyl)propionic acid (Ketoprofen) was by Meranini (Badalona, Spain). All other chemicals were of reagent grade.

Ester synthesis. The esterification of the 2-arylpropionic acids used here was carried out using anhydrous alcohols and $ClSO_2$ as catalyst. The esterification time was 24 h (with the exception of the Ketoprofen where the reaction time was 4 h). The ester was extracted with 100 ml of diethyl ether and purified by column chromatography using silica gel and dichloromethane as eluent (with the exception of the Naproxen which was purified by recrystallization in n-hexane).

^{1}H-NMR spectra were taken with a Varian VXR-300 NMR spectrometer using $CDCl_3$ with TMS as internal standard. IR spectra were obtained with Buck Scientific 500 spectrophotometer.

(a) (±) *Methyl 2-phenylpropionate.* Elemental analysis. Found: C, 73.06%; H, 7.36%. Calc for $C_{10}H_{12}O_2$: C, 73.00%; H, 7.36%. ^{1}H-NMR ($CDCl_3$, δ): 7.3–7.0 (m, 5H), 3.8 (q, 1H), 3.65 (s, 3H), 1.5 (d, 3H). IR (ν_{max} cm^{-1}): 3080, 3040, 2970, 1743.

(b) (±) *Ethyl 2-phenylpropionate.* Elemental analysis. Found: C, 74.02%; H, 7.99%. Calc for $C_{11}H_{14}O_2$: C, 74.13%; H, 7.92%. ^{1}H-NMR ($CDCl_3$, δ): 7.3–6.9 (m, 5H), 3.9 (q, 2H), 3.4 (q, 1H), 1.25 (d, 3H), 0.9 (t, 3H). IR (ν_{max} cm^{-1}): 3100, 3060, 2990, 1736.

(c) (±) *Butyl 2-phenylpropionate.* Elemental analysis. Found: C, 75.47%; H, 8.73%. Calc for $C_{13}H_{18}O_2$: C, 75.60%; H, 8.79%. ^{1}H-NMR ($CDCl_3$, δ): 7.4–7.2 (m, 5H), 4.05 (t, 2H), 3.7 (q, 1H), 1.5 (d, 3H), 0.9 (t, 3H). IR (ν_{max} cm^{-1}): 3070, 3040, 2970, 1740.

(d) (±) *t-Butyl 2-phenylpropionate.* Elemental analysis. Found: C, 75.17%; H, 8.65%. Calc for $C_{13}H_{18}O_2$: C, 75.60%; H, 8.79%. ^{1}H-NMR ($CDCl_3$, δ): 7.4–7.2 (m, 5H), 3.61 (q, 1H), 1.4 (d, 3H), 1.3 (s, 9H). IR (ν_{max} cm^{-1}): 3070, 3040, 2990, 1730.

(e) (±) *Cyclohexyl 2-phenylpropionate.* Elemental analysis. Found: C, 77.55%; H, 8.73%. Calc for $C_{15}H_{20}O_2$: C, 77.50%; H, 8.67%. ^{1}H-NMR ($CDCl_3$, δ): 7.4–7.2 (m, 5H), 4.86–4.7 (m, 1H), 3.70 (q, 1H), 1.5 (d, 3H), 1.3 (d, 10H). IR (ν_{max} cm^{-1}): 3070, 3040, 2995, 1729.

(f) (±) *Octyl 2-phenylpropionate.* Elemental analysis. Found: C, 77.50%; H, 9.80%. Calc for $C_{17}H_{26}O_2$: C, 77.81%; H, 9.98%. ^{1}H-NMR ($CDCl_3$, δ): 7.4–7.2 (m, 5H), 4.08 (t, 2H), 3.72 (q, 1H), 1.33–1.15 (m, 12H), 0.9 (t, 3H). IR (ν_{max} cm^{-1}): 3060, 3030, 2993, 1738.

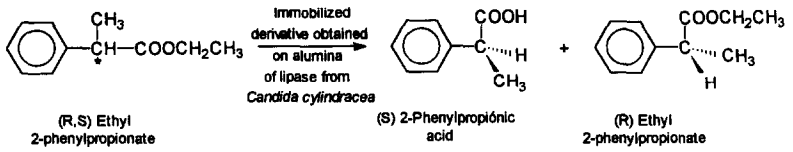
(g) (±) *Cetyl 2-phenylpropionate.* Elemental analysis. Found: C, 79.94%; H, 11.06%. Calc for $C_{25}H_{42}O_2$: C, 80.15%; H, 11.30%. ^{1}H-NMR ($CDCl_3$, δ): 7.4–7.2 (m, 5H), 4.02 (t, 2H), 3.70 (q, 1H), 1.5 (d 3 H), 1.20–1.13 (m, 28H), 0.8 (t, 3H). IR (ν_{max} cm^{-1}): 3070, 3030, 2993, 1744.

(h) (±) *Ethyl 2-phenylbutyrate.* Elemental analysis. Found: C, 73.3%; H, 8.23%. Calc for $C_{12}H_{16}O_2$: C, 75%; H, 8.32%. ^{1}H-NMR ($CDCl_3$, δ): 7.31–7.25 (m, 5H), 4.18–4.02 (m, 2H), 3.43 (t, 1H), 2.16–2.03 (m, 2H), 1.86–1.68 (m, 2H), 1.20 (t, 3H), 0.89 (t, 3H). IR (ν_{max} cm^{-1}): 3060, 3030, 2960, 1730.

(i) (±) *Ethyl 2-(4-isobutylphenyl) propionate.* Elemental analysis. Found: C, 76.98%; H, 9.5%. Calc for $C_{15}H_{22}O_2$: C, 76.8%; H, 9.46%. ^{1}H-NMR ($CDCl_3$, δ): 7.35–7.0.5 (m, 4H); 4.15 (q, 2H); 3.6 (q, 1H); 2.05 (d, 3H); 1.8 (m, 1H); 1.1 (t, 3H); 0.8 (t, 6H). IR (ν_{max} cm^{-1}): 3090, 3040, 2780, 1730.

(j) (±) *Ethyl 2-(6-methoxy-2-naphthyl) propionate.* Elemental analysis. Found: C, 74.29%; H, 7.12%. Calc for $C_{16}H_{18}O_3$: C, 74.4%; H, 7.02%. ^{1}H-NMR ($CDCl_3$, δ): 7.75–7.1 (m, 6H); 4.20 (q, 2H); 3.9 (s, 3H); 3.8 (q, 1H); 1.56 (d, 3H); 1.2 (t, 3H). IR (ν_{max} cm^{-1}): 3100–2800, 1740.

(k) (±) *Ethyl 2-(3-benzoylphenyl) propionate.* Elemental analysis. Found: C, 75.5%; H, 6.73%. Calc for $C_{18}H_{18}O_3$: C, 76.6%; H, 6.38%. ^{1}H-NMR ($CDCl_3$, δ): 7.54–7.12 (m, 9H);



Scheme 1.

4.15 (t, 2H); 3.65 (q, 1H); 1.4 (d, 3H); 1.0 (t, 3H). IR (ν_{max} cm^{-1}): 3070–2970, 1740.

Immobilization process. The activation of agarose was carried out according to the tosylation method previously described [21]. The inorganic supports have been activated by treatment with 2,4,6-trichloro-1,3,5-triazine as we described previously [22].

The immobilization of lipase was carried out at 4°C, for 3 hours, with slow stirring in 0.1 M Tris/HCl buffer, pH = 8.0. One gram of each activated support was added to the enzyme solution (25 mg/ml in the case of agarose and 40 mg/ml in the case of the inorganic supports). After the desired contact time, the insoluble enzyme derivative was filtered and washed with the same buffer and then with bidistilled water.

Enzymatic hydrolysis. The reactions were carried out in a batch reactor, in which 2 or 1 g of the immobilized catalysts on alumina/silica or agarose respectively, were mixed with a 15 ml of Tris/HCl buffer 0.1 M containing as substrate the (*R,S*) esters emulsified by ultrasound (2 min at 20 W) using a Branson model sonifier 450. The reaction mixture was incubated at different temperatures and samples (100 μl) were withdrawn at 24, 48, 72, 144 and 168 h, and added to 1.4 ml of acetonitrile. Ester and acid content was assayed using a HPLC with Tracer analytical C_8 column (Nucleosil 120, 5 μm), a LDC analytical pump, and a LDC analytical 3100 UV detector. Elution was carried out by acetonitrile/H_2O (60/40), at 1 ml/min flow rate and was monitored at 254 nm.

Enantiomeric excess determination. The reactions were stopped with the addition of 20 μl of H_2SO_4 and then were filtered to eliminate the immobilized derivative. The resultant was extracted with diethyl ether (3×5 ml). The organic phase was transferred to a clean glass and a second extraction was carried out with NaOH 0.1 M (3×5 ml). The aqueous phase was acidified with HCl and then a new extraction was performed with diethyl ether (3×5 ml). The remaining organic phase was evaporated to dryness in a Buchi concentrator–evaporator.

10 mg of residue in the case of Ketoprofen was mixed with 5 mg of (*R,R*) 1,2-diphenyl-1,2-diaminomethane in $DCCl_3$ to form diastereoisomeric salt complexes allowing the direct ^{1}H-NMR determination of the enantiomeric purity as described in the literature [23].

The enantiomeric excess of the Ibuprofen, Naproxen and 2-phenylpropionic acid residues was determined in Water HPLC with CHIRACEL column model OD, using as eluent n-hexane/isopropanol/trifluoroacetic acid (1000/10/1) (v/v/v) in the case of the Ibuprofen, n-hexane/isopropanol/formic acid (98/2/1) (v/v/v) for 2-phenylpropionic acid and n-hexane/isopropanol/acetic acid (97/3/1) for Naproxen. The flow rate was 1 ml/min and the UV detector was fixed at 254 nm.

3. Results and discussion

In order to study the influence of several effectors in the hydrolysis of 2-arylpropionic acid, we carried out the hydrolysis of the (*R,S*) ethyl 2-phenylpropionate using the immobilized derivative obtained on alumina as standard biocatalyst (Scheme 1).

Effect of temperature: The effect of temperature on the hydrolysis of (*R,S*) ethyl 2-phenylpropionate was tested at temperatures ranging from 25 to 55°C. The results of this assay are shown in Fig. 1. In this Figure we observed that the initial velocity of the reaction increases when the temperature is greater (8% of yield at 25°C vs. 28% at 55°C). However, the immobilized derivative that works

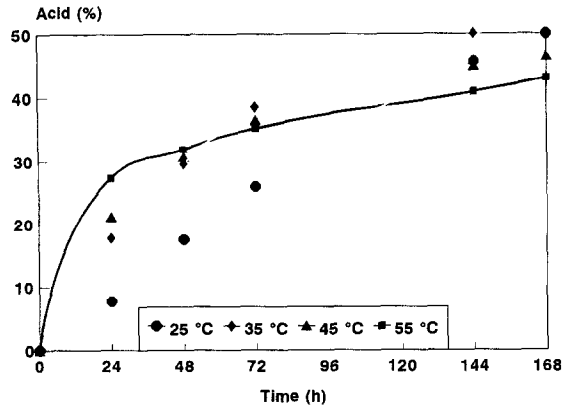


Fig. 1. Influence of temperature in the hydrolysis of (*R,S*) ethyl 2-phenylpropionate with immobilized derivative obtained on alumina. Experimental conditions: 2 g of immobilized catalysts; 15 ml of Tris/HCl buffer, pH = 7, 0.1 M; [Substrate] = 0.05 M; 700 rpm.

at 50°C, suffers thermal deactivation. Therefore, the reaction yield obtained at 168 h is lower than that obtained working at lower temperatures.

On the strength of these results we selected 35°C as optimum value of temperature. The same temperature is reported in the literature in the hydrolysis of Naproxen esters with an immobilized derivative obtained by adsorption of lipase from *C. cylindracea* on Amberlite [17].

2^3 *Factorial design*: Using the conventional approach, all the reaction parameters are maintained constant except the one under analysis. Operating in this way, the true maximum could not be reached at times because only a part of the experimental domain is explored. This situation can be avoided by using multivariable methods, such as factorial analysis. In this methodology, all the reaction parameters are simultaneously changed in a suitable, programmed manner, allowing an efficient and rational scan of the experimental domain for all the variables. By following this approach, optimization is reached with fewer experiments [24]. The dependence of the variable (Y) which describes the system, upon the experimental variables x_i, can be approximated with a polynomial Eq. 1.

$$Y = b_0 + \Sigma_i b_i x_i + \Sigma_{ii} b_{ii} x_i^2 + \Sigma_{i<j} b_{ij} x_i x_j + \ldots \quad (1)$$

All x_i variables must be continuous. The variation of the reaction yield (Y) was expressed by functions of four experimental variables (Table 1). Diffusional problems were eliminated using 2 g of immobilized enzymatic derivative on alumina in all cases. The selection of independent variables was made considering the chemistry of the system and the practical use of factorial design. Selection of the levels was carried out on the basis of results obtained in a previous study described in the literature (the range of working pH for similar reactions reported in the literature fluctuates between 7.2 [25] and 8.0 [15]. The influence of substrate concentration [24a] and ionic strength [26] has been also described). The maximum (+1) and minimum (−1) levels of the variables are shown in Table 1.

The variation of the percentage of conversion in relation to the reaction time was evaluated by measurement of the response, Y (reaction yield), at different reaction times. The experiments were randomly performed according to a factorial design 2^3. The results obtained in the experiments and the central point (entry 9–11) are shown in Table 2.

Estimate coefficients of the polynomial equation for the reaction yield, obtained using the STATGRAF [27] program, are given in Table 3. These coefficients were compared with the experimental error obtained from the standard deviation of the three replicated runs of the central point ⟨⟨O⟩⟩ of the factorial design (Table 2), because, generally, large coefficient values indicate a strong influence of the variable(s) on the response [24b]. Daniel's method [28] was applied to analyze the effects of the variables on the yield. The results of this method are presented in Fig. 2 for

Table 1
Variables and conditions in factorial design

Variables	−1	0	+1
x_1 = stirring speed (rpm)	300	500	700
x_2 = pH of the medium	7.0	7.5	8.0
x_3 = ionic strength (M)	0.1 [b]	0.35 [c]	0.7 [d]
x_4 = [S] [a]	0.05	0.075	0.1

[a] [S] = (±) ethyl 2-phenylpropionate (M).
[b] Tris/HCl buffer.
[c] 0.1 M $CaCl_2$ and 0.1 M NaCl.
[d] 0.2 M $CaCl_2$ and 0.2 M NaCl.

Table 2
Factorial design matrix 2^3

Entry	x_1	x_2	x_3	x_4	Yield (%) after different times (h)				
					24	48	72	144	168
1	−1	−1	−1	−1	13.8	23.4	37.0	41.2	49.9
2	1	−1	−1	1	6.2	16.8	28.4	31.0	34.6
3	−1	1	−1	1	3.6	10.6	14.2	17.9	21.4
4	1	1	−1	−1	11.8	20.3	35.8	39.0	42.6
5	−1	−1	1	1	17.7	27.7	43.9	48.4	53.6
6	1	−1	1	−1	27.9	38.6	50.0	56.6	60.1
7	−1	1	1	−1	12.5	27.1	35.4	47.6	50.9
8	1	1	1	1	14.3	22.5	38.8	48.6	51.7
9	0	0	0	0	14.1	23.2	36.9	42.1	48.3
10	0	0	0	0	11.9	21.2	35.8	41.0	44.6
11	0	0	0	0	12.8	22.6	34.2	41.5	46.5
Standard deviation central point				0	1.11	1.03	1.36	0.55	1.85

Table 3
Main and interaction effects upon reaction yield.

Time (h)	b_0	b_1	b_2	b_3	b_4	b_1b_2	b_1b_3	b_1b_4
24	13.5	9.2	−5.9	3.1	−6.1	2.8	−3.5	1.8
48	23.4	11.2	−6.5	2.3	−7.9	0.8	−1.8	0.2
72	34.4	11.1	−6.1	7.7	−6.1	1.2	−3.1	8.9
144	41.2	18.0	−9.6	5.0	−9.6	−0.5	1.6	6.0
168	45.6	16.9	−7.9	3.3	−10	0.3	2.3	7.7

b_1 = stirring speed (rpm); b_2 = pH; b_3 = ionic strength (M); b_4 = [Substrate].

the effects at 144 h. Similar behavior was observed for the other reaction times. In this method, the points not fitted to the statistical probability model are the effects that have some influence on the process [24a].

Table 3 and Fig. 2 show that the main variables are the stirring speed (x_1), the pH (x_2) and [Substrate] (x_4) because b_1, b_2 and b_4 are not on the straight line (Fig. 2). The most important variable is the stirring speed (x_1) and its positive effect must be related to the stabilization of the microemulsion produced by the increase of this variable in our experimental conditions – in absence of tensioactives. This effect has been reported by Wang et al. [29] in the hydrolysis of tributyrin using the same enzyme (native) in a batch reactor. Due to the fact that the enzyme remains active if adsorbed on the interface, the enzymatic activity is mainly determined by the scale of interface area that provides the anchorage site for the enzyme molecule. The interface area increases as the stirring speed increases, because the size of the oil microdrops diminishes. The increase of this effect with the reaction time (Table 3 coeff. b_1) can be explained by the fact that the greater the reaction time, the greater the percentage of hydrolysis and the smaller number of ester molecules in the medium and thus, the possibility of forming microdrops is diminished. Therefore, it is neces-

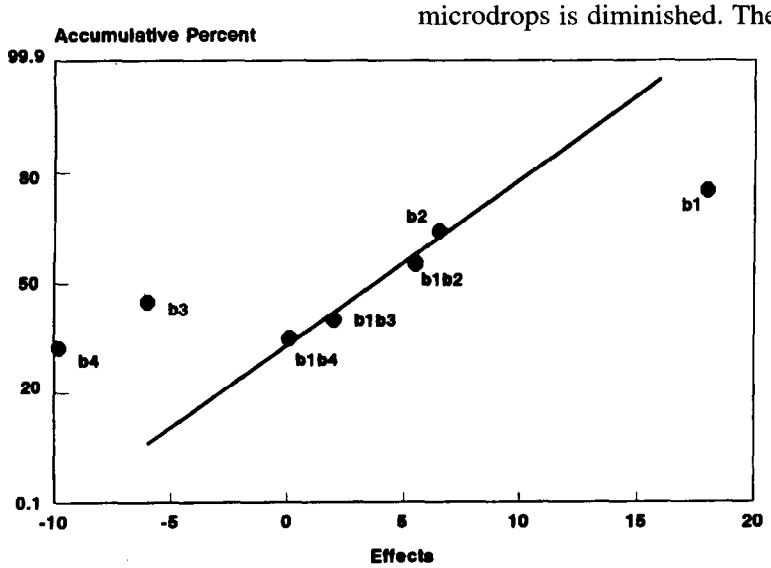


Fig. 2. Error estimation for the coefficients at 144 h (Daniel's method).

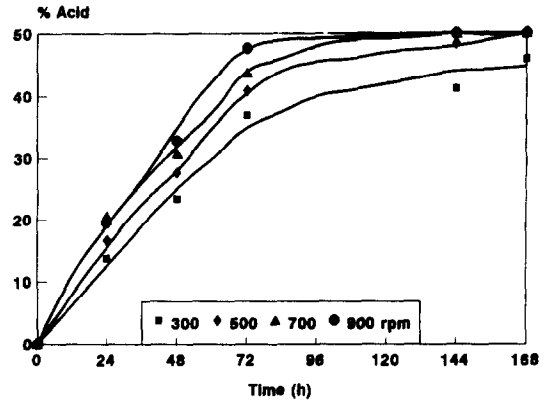


Fig. 3. Influence of stirring speed in the hydrolysis of (*R,S*) ethyl 2-phenylpropionate with immobilized derivative obtained on alumina. Experimental conditions: 2 g of immobilized catalysts; 15 ml of Tris/HCl buffer, pH = 7, 0.1 M; temperature = 35°C; [Substrate] = 0.05 M.

sary to increase the stirring speed to diminish the diffusional problems that would decrease the reaction yield [30]. To confirm these results, the effect of stirring speed was studied between 300 and 900 rpm using a conventional O.V.A.T. (on variable at time) approach. The results are shown in Fig. 3. In this Figure we can observe that when the stirring speed increases the initial slope of the curves increases too.

The second variable [Substrate] (x_4) exerts a negative effect on the hydrolysis process (Table 3 coeff. b_4). A similar effect was observed by Garcia et al. [24a] in the synthesis of an analogue of jojoba oil with immobilized *Mucor miehei* lipase. This fact can be due to two different explanations:

(i) Inhibition of the enzyme by the presence of the substrate or of the acid (reaction product). This effect has been reported by Ebert et al. [24c] in the case of mandelic esters.

(ii) Increase in the size of the microdrops of oil phase, which diminishes the water/oil interface and, thus, the enzymatic activity [30]. Probably, due to the importance of the interface area, the second explanation would be more adequate.

These results were confirmed by conventional approach where we studied the influence of substrate concentration. In Table 4 we can observe that when the substrate concentration diminishes, the velocity of the reaction increases.

The negative effect observed when the pH (x_2) increases from 7 to 8.0 has been described in the literature in the case of Naproxen [31] and olive oil [32]. Finally, a small positive effect is observed in the case of ionic strength (x_3). A positive effect has been reported by other workers in the case of native enzyme [32,33] and has been related to the fact that Na(I) favors the emulsification of lipids giving micelles [34] (increasing the interfacial water/lipid surface) and Ca(II) removes the fatty acids produced during the hydrolysis [35] from the interfaces.

Nevertheless, the increase in ionic strength (x_3) exerts a negligible positive effect on the hydrolysis of ethyl 2-phenylpropionate using lipase from *Candida cylindracea* immobilized on alumina (Table 3 and Fig. 2). The immobilized derivative was not very sensitive to the variation of the ionic strength due to the fact that the electrostatic field of the alumina surface exerts the same stabilizing effect on the water/oil interface as the ions Na(I) and/or Ca(II) in the case of native enzyme. Therefore, the addition of external ions does not exert a positive effect in this case.

The increase of the effect of each variable observed when the reaction time increases (Table 3) could be related to the deactivation of the enzymatic derivative (Fig. 4) that progressively diminishes the activity of biocatalyst. However, the immobilized derivatives were between 50 and 75 times more stable than the native enzyme stored at the same conditions (50°C), as can be deduced from the results shown in Fig. 4.

Table 4
Influence of substrate concentration on the reaction yield

[Substrate] (M)	Yields (%) after different times (h)		
	24	72	144
0.0125	27	50	50
0.025	25	46	50
0.05	20	45	50
0.075	19	43	50
0.1	17	42	49

Experimental conditions: 2 g of the immobilized catalysts on alumina; 15 ml of Tris/HCl buffer, pH = 7, 0.1 M; temperature = 35°C; 700 rpm.

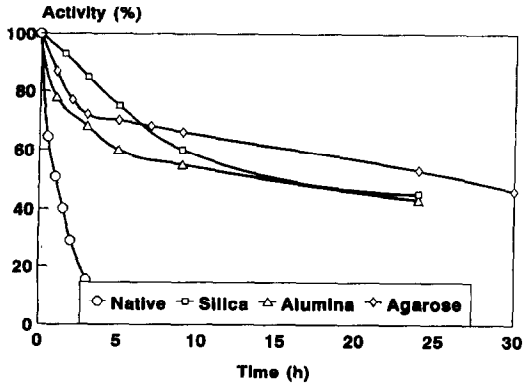


Fig. 4. Stability of the native enzyme and immobilized derivatives stored at 50°C.

Finally, the interaction effects are not significant, as can be deduced from Daniel's representation method (Fig. 2). Therefore we can conclude that the response surface is Eq. 2. The b_i values at each reaction time are shown in Table 3:

$$Y\,(\text{yield}) = b_0 + b_1x_1 - b_2x_2 - b_4x_4 \qquad (2)$$

The influence of the technical variables on the enantiomeric excess (ee) was analyzed using lipase from *Candida cylindracea* immobilized on alumina as enzymatic derivative and the results are shown in Table 5. In all cases, the enzymatic derivative stereoselectively hydrolyzes the *S*(+) ester. This result agrees with that reported by other workers [17,31].

We can observe high enantioselectivity values when the reaction yield is lower than 50% (entries 1–4). This value diminishes when the reaction yield increases (entries 5,6), as described in the literature [36]. Therefore, we can state that these experimental variables – which are achiral parameters – do not affect the enantiomeric excess.

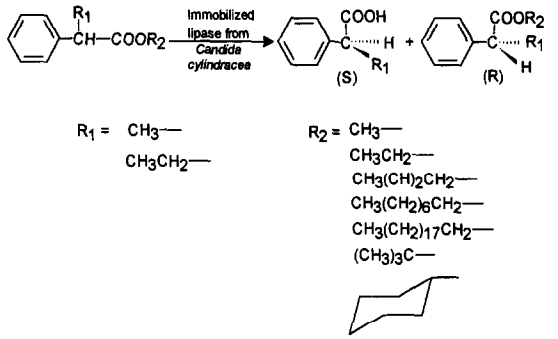
Influence of alcoholic residue of the ester and size of the alkyl chain: It is well documented in the case of native lipases that the alcoholic residue of the ester and of the alkyl chain in C_α of the acid moiety of the ester molecule influences hydrolysis yield and enantiomeric excess [36]. To analyze these effects in the case of immobilized enzymes, the hydrolysis of some synthetic esters was carried out as shown in Scheme 2.

The results obtained in these studies are shown in Table 6. Cyclohexyl and t-butyl esters were not

Table 5
Hydrolysis of (*R,S*) ethyl 2-phenylpropionate with 2 g of immobilized derivative on Al_2O_3

Entry	pH	*I* (M) [a]	[Substrate] (M)	rpm [b]	Yield [c] (%)	ee [d] (%)
1	7.0	0.0	0.05	300	50	<98
2	8.5	0.7	0.05	300	43	<98
3	8.5	0.0	0.05	700	48	<98
4	8.5	0.7	0.1	700	50	<98
5	7.0	0.7	0.1	700	54	60
6	7.0	0.0	0.05	700	60	44

At 35°C, $V = 15$ ml.
[a] Ionic strength.
[b] Stirring speed.
[c] Yield in acid (HPLC) at 168 h (experimental error ±3.7 %).
[d] Enantiomeric excess of *S* acid determined by HPLC (maximum experimental error ±2.0 %).



Scheme 2.

Table 6
Reaction yields at 144 h in the hydrolysis of (*R,S*) X 2-phenyl-Y

Derivative	X	Y	Yield (%)
Alumina	methyl	propionate	39
	ethyl		50
	butyl		45
	octyl		38
	cetyl		39
	ethyl	butyrate	3
Agarose	methyl	propionate	32
	ethyl		48
	butyl		45
	octyl		33
	cetyl		28
	ethyl	butyrate	7

Experimental conditions: 2 g of the immobilized catalysts on alumina and 1 g of immobilized derivative obtained on agarose; 15 ml of Tris/HCl buffer, pH = 7, 0.1 M; temperature = 35°C; 700 rpm; [Substrate] = 0.05 M.

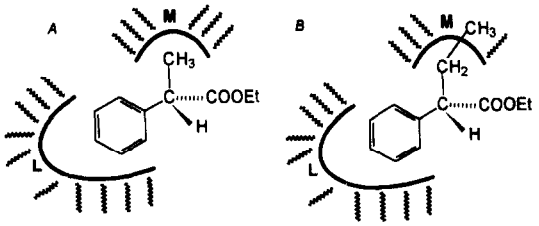
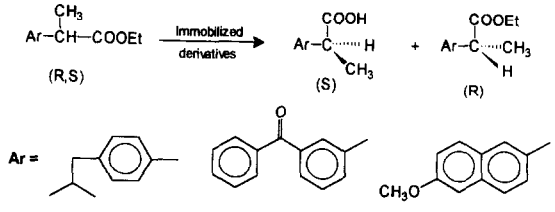


Fig. 5. Steric hindrance produced in the subsite M due to the enhancement of the alkyl chain ((*S*) 2-phenylpropionic acid (a) and (*S*) 2-phenylbutyric acid (b)).

hydrolyzed by the native or the immobilized enzyme on agarose and alumina. Therefore, the immobilization methodology (tosyl chloride or trichlorotriazine activation methods) and the nature of the support do not alter the chemoselectivity of lipase from *Candida cylindracea* with respect to the substrate, because esters from secondary and tertiary alcohols are not hydrolyzed [37].

The best substrates for hydrolysis are the ethyl and n-butyl esters, as described by Monot et al. [37] using *Mucor miehei* lipase. The influence of the support is important in the case of unstable microemulsion systems (methyl, octyl and cetyl esters). In these cases, alumina is a better support than a hydrophilic support such as agarose (using the same amount of active enzyme in all the experiments). This finding must be related to the positive effect of the ionic support (alumina) in maintaining the water/oil microemulsion around the immobilized enzyme molecules as has been indicated in the case of the influence of ionic strength. This explanation is better than the one based on the steric hindrance associated with the alkyl chain. On the basis of experiments carried out with chiral alcohols [38], a model of the active site of *C. cylindracea* lipase has been proposed by Kazlaukas [39] where the large group of the alcohol is located near the interphase. Therefore no



Scheme 3.

steric hindrance would be observed with our alcoholic chains, similar yields would be observed, and any difference in the yields would be related to the different stability of the microemulsion.

When (±) ethyl 2-phenylbutyrate is used as substrate a diminution in the yield is observed (Table 6) compared to that observed with 2-phenylpropionic esters with the same immobilized derivatives. In order to explain these results, the conformers of the substrates were analyzed by a molecular mechanics methodology [40], using the MMX88 program [41]. The PCmodel program [42] was used to draw starting structures and to measure spatial distances between groups in the minimized conformations. According to this methodology, the diminution of the reaction yields leads to us to assume the presence of steric hindrance problems in M subsite in the active site, because the volume of the alkyl chain in the case of the ethyl 2-phenylbutyrate is greater than the other substrate (Fig. 5).

Influence of the aryl group: The racemic mixtures of different ethyl esters were hydrolyzed with the immobilized derivatives obtained on agarose, alumina and silica in optimum conditions (Scheme 3). The results are shown in Fig. 6.

The conversion pattern at 168 h (this time was considered as the limit time of the reaction) is similar in the two immobilized derivatives obtained on inorganic supports (2-phenylpropionic
acid > Naproxen > Ibuprofen > Ketoprofen). The derivative obtained on silica is less sensitive to the substrate structure, since the derivative obtained on alumina produced minor yields with Ketoprofen. In order to explain these results, the conformers of the substrates were analyzed by a molecular mechanics methodology. According to this methodology, this pattern of reactivity can be explained based on the structure and the π value of substrate [43] (Table 7). In the case of Ketoprofen, the angle between the C=O group and the aromatic ring is greater in comparison with the other molecules and its different geometry could explain the diminution in reaction yields (Fig. 6). In the case of Naproxen and Ibuprofen, the differences can

be explained by their different lipidic characteristics, due to the fact that the rest of the parameters present in Table 7 are very similar.

The derivative obtained on the most hydrophilic support (agarose) hydrolyzed the substrate with minor π value with higher reaction yields. Therefore, the reactivity pattern was 2-phenylpropionic acid > Ketoprofen > Naproxen > Ibuprofen, which is equivalent to decreased order of π value (Table 7). This effect is due to the high hydrophilicity of the support, which acts as limiter of the substrate diffusion through the aqueous mantle.

The immobilized derivative obtained on silica is the most useful in the hydrolysis of the different substrates. This fact may be due to the addition of its hydrophobicity, higher than in the case of agarose, and its retained activity (data not shown), higher than in the case of alumina.

Table 7
Characteristics of non-steroidal antiinflammatories

Substrate	π [a]	d [b] (Å)	a [c] (°)
(*S*) 2-phenylpropionic acid	1.18	6.114	−56.0
(*S*) Ibuprofen	4.29	9.045	−65.1
(*S*) Ketoprofen	2.71	10.216	−19.5
(*S*) Naproxen	3.08	10.502	−41.6

[a] Lyophilicity. This parameter has been calculated from the published data using propionic acid as reference compound [43].
[b] Distance between C=O group and the extreme of *p*-substituent of aromatic ring calculated by molecular mechanics (Fig. 7).
[c] Angle formed between C=O group and the aromatic ring calculated by molecular mechanics (Fig. 7).

Finally, we can observe that the reaction yields of the hydrolysis of (*R*,*S*) ethyl 2-phenylpropionate are higher than in the other substrates (Fig. 6) using any immobilized derivatives. This effect may be related to the size of the molecules (Table 7), so there may be problems of steric hindrance

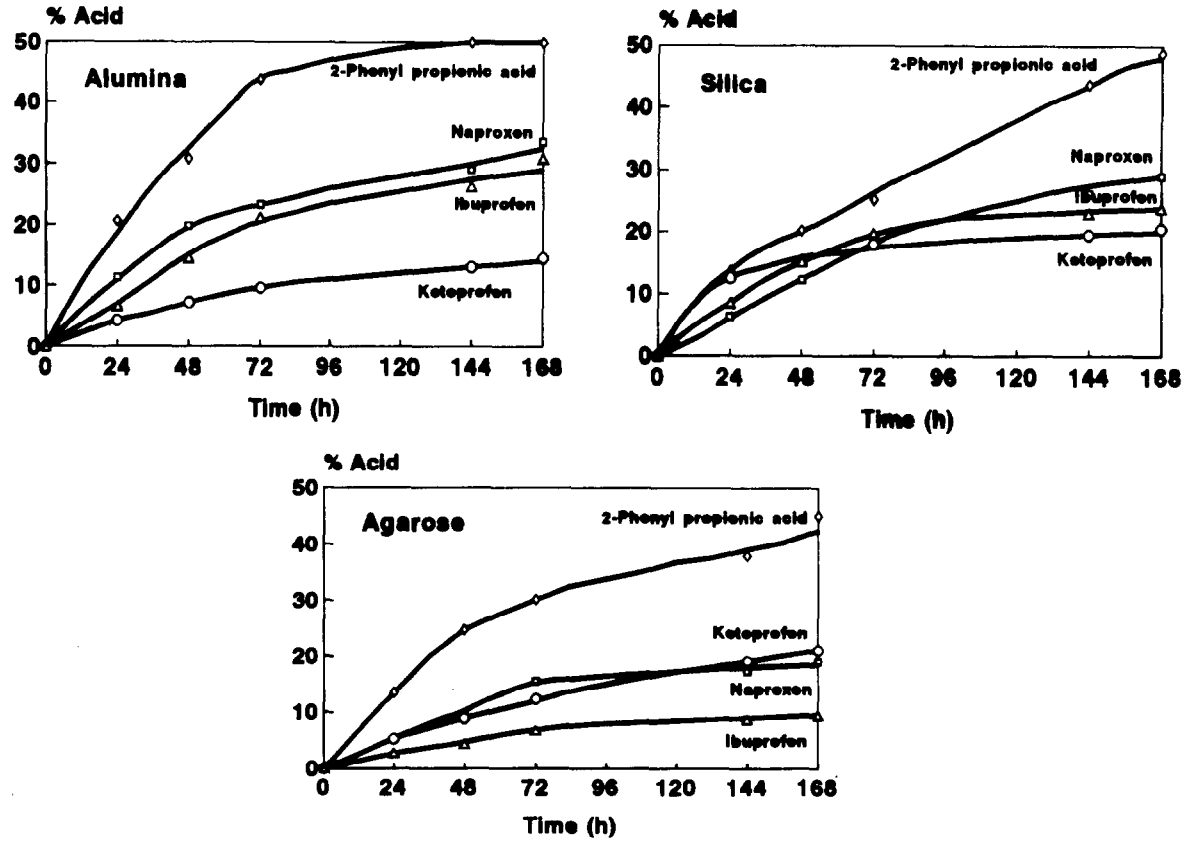


Fig. 6. Results of the hydrolysis of the racemic mixtures of different ethyl esters of NSAIDS with the immobilized derivatives. Experimental conditions: 2 g of immobilized catalysts; 15 ml of Tris/HCl buffer, pH = 7, 0.1 M; temperature = 35°C; [Substrate] = 0.05 M; 700 rpm.

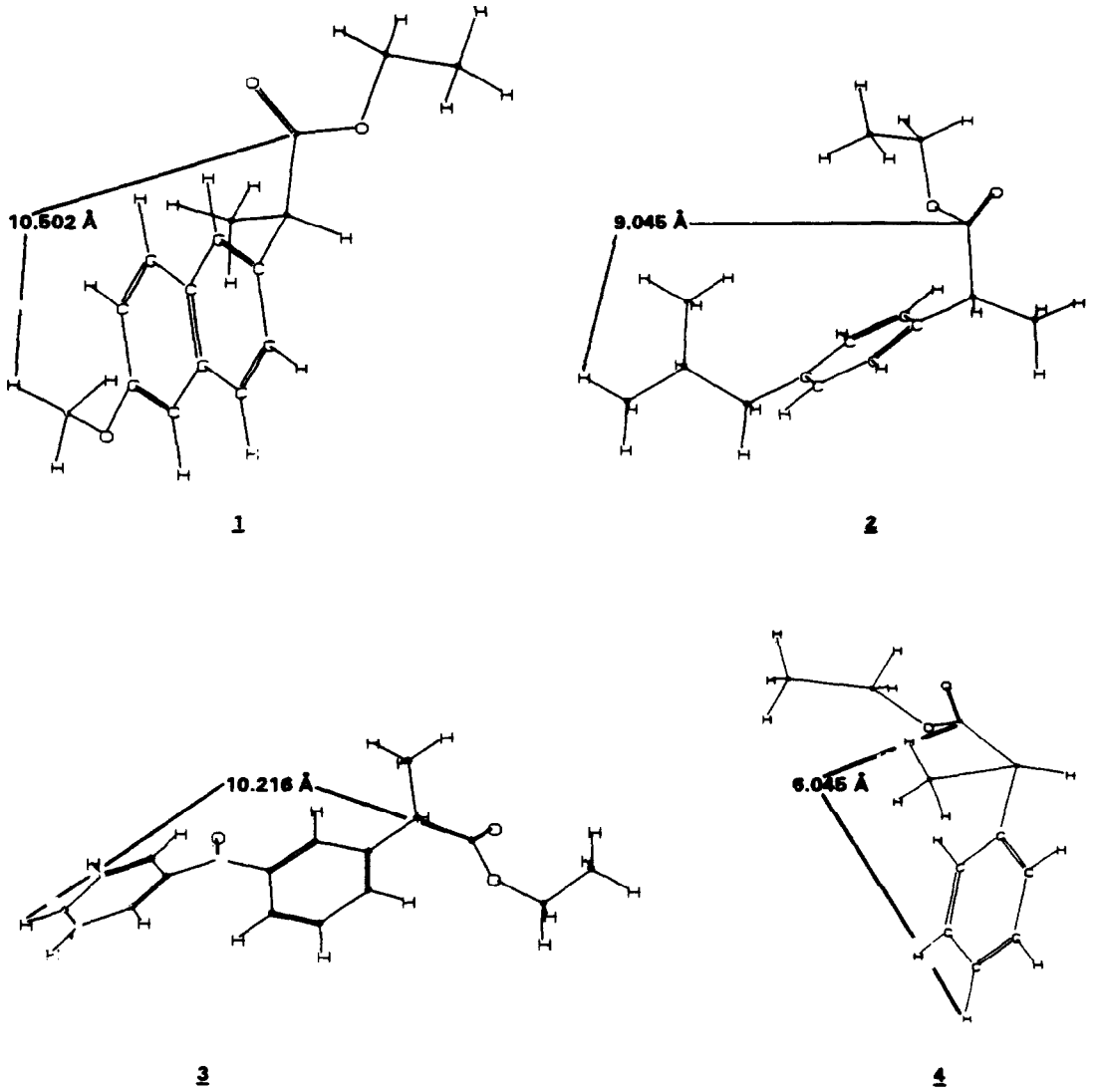


Fig. 7. Minimum energy conformer of *S*(+) 2-arylpropionic acids. 1: Naproxen; 2: Ibuprofen; 3: Ketoprofen; 4: 2-phenylpropionic acid.

in the case of the higher substrates (Fig. 7), that are a negative influence in the process. We have also analyzed the stability of the emulsion (a parameter with a great influence on hydrolysis). In the case of the 2-phenylpropionic ester, the emulsion is stable without stirring during 3 days, while in the case of Ketoprofen ester the emulsion is stable in the same conditions during 9 h only.

The structure of the substrates, the yields obtained in the reactions and the results described by other authors [39] permit us to describe – at a qualitative level – the active site of the enzyme (Fig. 8). The M subsite can accept methyl and

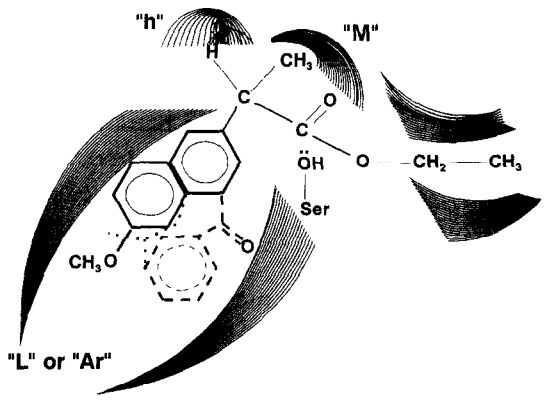


Fig. 8. Model for the active site of the immobilized enzyme.

Table 8
Enantioselectivity of the hydrolysis of (*R,S*) 2-arylpropionic esters

Derivative	Substrate	% Acid (168 h)	ee (%)
Alumina	2-phenylpropionic acid	50.0	95
	Ibuprofen	30.9	100
	Ketoprofen	14.5	95
	Naproxen	33.6	98
Silica	2-phenylpropionic acid	49.0	100
	Ibuprofen	23.8	98
	Ketoprofen	20.5	97
	Naproxen	29.0	100
Agarose	2-phenylpropionic acid	45.5	100
	Ibuprofen	9.5	100
	Ketoprofen	21.1	94
	Naproxen	19.9	95

Experimental conditions: 2 g of the immobilized catalysts on inorganic supports and 1 g of immobilized derivative obtained on agarose; 15 ml of Tris/HCl buffer, pH = 7, 0.1 M; temperature = 35°C; 700 rpm; [Substrate] = 0.05 M.

minor type structures; however, it is a steric restriction zone to greater structures due to the diminution in the yield observed in the hydrolysis of the ethyl 2-phenylbutyrate. The 'tunnel' of the subsite L accepts naphthalene ring (Naproxen) 4-isobutylphenyl (Ibuprofen) and diphenyl ketones (Ketoprofen) type structures, which presents 10 Å as ratio distance between the extreme of the molecule and the C=O group. In addition, the zone of the alcoholic residue accepts primary alcohols up to 16 C atoms in length. However, its width is limited because the enzyme cannot hydrolyze secondary and tertiary alcohols.

In Table 8 we show the results of enantiomeric excess of the different reactions. We can observe that immobilized derivatives stereoselectively hydrolyze the *S*(+)-isomer. These results are similar to those reported in the literature in the hydrolysis of Naproxen ester with an immobilized derivative obtained on Amberlite by adsorption [17]. However, in comparison with the results obtained with the native enzyme, an increase in the enantioselectivity in the case of immobilized enzyme is produced. In this case, 70% *S*(+) and 30% *R*(−) of enantiomers are obtained in the hydrolysis of racemic ester of 2-phenylpropionic acid using the native enzyme [25,31]. Nevertheless, in the case of hydrolysis of this racemate using the immobilized enzyme on agarose, silica or alumina only one isomer is obtained.

The observed increase in stereoselectivity by the effect of the immobilization must be related to the alteration of the microenvironment of the enzyme produced by the presence of the support. Therefore, our results could be considered in agreement with those reported by Sih et al. [44] that show how treatment with deoxycholate and diethyl ether/ethanol mixtures 1/1 (v/v) produces an increase in the enantioselectivity of lipase in the hydrolysis of (±) 2-phenylpropionic esters. This effect is explained by non-covalent modification of the enzyme which makes the H and M subsites more rigid in the new protein than in the crude one. The immobilization of the enzyme may produce the same rigidification in the active site of the protein.

Re-use of immobilized derivatives: In Table 9 we show the results obtained in the study of the operational stability of the immobilized derivatives. After the first assay (168 h), each solid was recovered, washed and re-assayed with fresh substrate mixture in the same conditions as the first experiment, and then the process was repeated three times. The immobilized derivatives retained 80% of their initial activity after 336 h; however, the immobilized lipase had lost 50% of this orig-

Table 9
Results of the re-use of immobilized derivatives

Derivative	Time of use (h)	Activity (%)	Cycle no.
Alumina	168	100	1
	336	80	2
	504	50	3
Silica	168	100	1
	336	85	2
	504	45	3
Agarose	168	100	1
	336	75	2
	504	48	3

Experimental conditions: 2 g of the immobilized catalysts on inorganic supports and 1 g of immobilized derivative obtained on agarose; 15 ml of Tris/HCl buffer, pH = 7, 0.1 M; temperature = 35°C; 700 rpm; [Ethyl 2-phenylpropionate] = 0.05 M.

inal activity after the third re-use, but enantiomeric excesses > 90% remain after re-use. This deactivation is similar to that described in the literature in a derivative obtained on titanium chloride-activated cellulosa of lipase from *Candida cylindracea* [45]. On the other hand, the immobilized derivatives present greater operational stability when the reaction is carried out in a packed-bed reactor (90% at 400 h) where an apparent stabilization occurs due to the presence of the substrate [17]. This kind of reactor was not tested in our laboratories.

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