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Organic reactions catalyzed by immobilized lipases. Part I. Hydrolysis of 2-aryl propionic and 2-aryl butyric esters with immobilized *Candida cylindracea* lipase

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Abstract

The alteration of the selectivity of enzymes due to the immobilization methodology is discussed. The hydrolysis of esters of (*R,S*) 2-phenyl propionic and 2-phenyl butyric acids has been used as a reaction test. Lipases from *Candida cylindracea* immobilized on agarose and alumina have been used as enzymatic derivatives. The stirring speed, [Substrate]/[Enzyme] ratio and pH are the main variables that control the process. An increase in the stirring speed, a diminution of the [Substrate]/[Enzyme] ratio and pH = 7.0 favours the hydrolysis of esters. The effect of the support on the enzymatic activity is discussed. Inorganic supports such as Al_2O_3 or SiO_2 stabilize the oil/water interface acting in the same way as Na(I) or Ca(II) in the case of native enzyme. Enzymatic derivative on Al_2O_3 is the most interesting biocatalyst. The effect of the alkyl chain of the ester is not related to the steric hindrance but to the stability of the microemulsion. The butyl ester is the most interesting ester for carrying out the hydrolysis. A model of the ester–active site interaction is proposed to explain the increase observed in the stereoselectivity of the hydrolysis of (*R,S*)-ester. High enantioselective hydrolysis of the racemates (yielding *S*(+) isomer; ee $\geq$ 98%) can be achieved using the immobilized derivatives.

1. Introduction

There are many commercially available lipases from different sources microbial, fungal, mammalian, etc, that catalyze in vivo the hydrolysis of triacylglycerols. This process takes place in heterogeneous conditions since it involves a water/oil interface. Depending on the source of the lipases they have different enzymatic activity and estereoselectivity with respect to standard substrates such as tributyrin and *R* or *S* methyl 2-chloropropionate [1]. The influence of small percentages of water on the reaction rate [2,3] and of the nature of the organic solvent on the yield [4] is well documented in the literature. In addition the enantioselectivity is altered by the effect of no covalent chemical modification, as has been reported [5] for lipase from *Candida cylindracea*. In spite of the great amount of work accomplished in the field of the application of lipases to the resolution of racemates, little work has been done in the case of covalently bonded lipases and in the systematic analysis of the experimental variables that control the process and the stereoselectivity. In some cases they mask the true influence of the structural variables on the mechanism of the biocatalytic process. Therefore a fun-

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damental study from the mechanistic point of view was necessary.

The hydrolysis of the racemates of the esters of 2-aryl propionic acids has been chosen as a reaction test. These compounds exhibit antiinflammatory properties [6] by inhibition of arachidonate cyclooxygenase [7]. They are used as racemic mixtures, with the exception of (+)-(*S*)-naproxen (2(2′(6′-methoxynaphthyl)) propionic acid) and (+)-(*S*)-flurbiprofen (2(3′-fluor, 4′-phenyl)phenyl propionic acid). However the pharmacological activity of these drugs is mainly due to the *S*-enantiomer. The *R*-antipode is essentially inactive as inhibitor of the arachidonate cyclooxygenase or is potentially toxic [8]. Different synthetic methodologies have been described for the synthesis of these acids with enantiomeric excess: alkylation of chiral enolates [9], asymmetric hydrogenation of unsaturated carboxylic acids [10], asymmetric carbonylation of benzyl halides [11], etc. One alternative to these complex methodologies is the synthesis of the racemic esters and the stereospecific resolution using lipases [12], esterases [13] or microorganisms [14,15].

In the present paper we analyze what the main variables are that control the hydrolysis reaction by a multivariate approach. In addition we have analyzed how these variables and other structural variables such as immobilization methodology, the nature of the aryl group or the size of the alkyl chain and of the alcoholic residue of the ester can affect the stereoselectivity of the process.

2. Experimental

2.1. 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-phenyl propionic and (±) 2-phenyl butyric acids were from Aldrich Chem. (Germany). All other chemicals were of reagent grade.

2.2. Methods

2.2.1. Ester synthesis

The 2-(4-nitrophenyl) butyric acid was obtained from 2-phenyl butyric acid according to the methods previously described in the literature [16]. The 2-(4-aminophenyl) butyric acid was obtained by catalytic reduction of the nitro counterpart using a hydrogenator Parr type at 30 p.s.i. and Pd/C at 4% for 4 days at room temperature. The esterification of the 2-aryl propionic and 2-aryl butyric acids was carried out using anhydrous alcohols and $ClSO_2$ as catalyst. The esterification time was 24 h. The ester was extracted with 100 ml of diethyl ether and purified by column chromatography using silica gel and dichloromethane as eluent.

^{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.

(±) *Methyl 2-phenyl propionate.* 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.

(±) *Ethyl 2-phenyl propionate.* 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.

(±) *Butyl 2-phenyl propionate.* 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.

(±) *t-Butyl 2-phenyl propionate.* 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.

(±) *Cyclohexyl 2-phenyl propionate.* 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.

(±) *Octyl 2-phenyl propionate.* 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.

(±) *Cetyl 2-phenyl propionate.* Elemental analysis; Found: C, 79.94%; H, 11.06%. Calc for $C_{25}H42_{26}O_2$: 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.

(±) *Ethyl 2-phenyl butyrate.* Elemental analysis; Found: C, 73.3%; H, 8.23%. Calc for $C_{12}H_{16}O_2$: C, 73%; 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.

(±) *Ethyl 2-(4-nitrophenyl) butyrate.* Elemental analysis; Found: C, 60.75%; H, 6.32%; N, 5.9%. Calc for $C_{12}H_{15}NO_4$: C, 60.91%; H, 6.61%; N, 5.70%. ^{1}H-NMR ($CDCl_3$, δ): 8.18 (d, 2H), 7.49 (d, 2H), 4.20–4.08 (m, 2H), 3.56 (t, 1H), 2.19–2.09 (m, 2H), 1.86–1.72 (m, 2H), 1.22 (t, 3H), 0.91 (t, 3H). IR (ν_{max} cm^{-1}): 3100, 3070, 2975, 1740.

(±) *Ethyl 2-(4-aminophenyl) butyrate.* Elemental analysis; Found: C, 64.36%; H, 8.15%; N, 6.7%. Calc for $C_{12}H_{17}NO_2$: C, 69.5%; H, 8.20%; N, 6.70%. ^{1}H-NMR ($CDCl_3$, δ): 7.10–7.07 (d, 2H), 6.64–6.61 (d, 2H), 4.16–4.03 (m, 2H), 3.62 (broad singlet, 2H), 3.31 (t, 1H), 2.08–1.98 (m, 2H), 1.77–1.68 (m, 2H), 1,20 (t, 3H), 0.87 (t, 3H). IR (ν_{max} cm^{-1}): 3100, 3070, 2980, 1730.

2.2.2. Activation of supports

The activation of agarose was carried out according to the tosylation method previously described [17]. The inorganic supports (1 g) have been activated by treatment with 0.15 g of 2,4,6-trichloro-1,3,5-triazine in 10 ml of toluene in presence of 0.2 g of triethylamine at 50°C under gentle stirring. The reaction was stopped at 4 h and the activated support was filtered and washed with 10 ml of toluene and 50 ml of anhydrous acetone, then was desiccated at room temperature.

2.2.3. Immobilization process

The immobilization of lipase was carried out at 4°C, for 3 h, 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. The catalytic efficiency of immobilized derivatives was determined by the hydrolysis of olive oil emulsion in 1 mM Tris/HCl buffer pH = 7.5 at 35°C. The acid released was continuously titrated with several NaOH solutions (1 to 10 mM) in a pH-stat Crison model microTT 2022. The stirring speed was 500 rpm and the total reactor volume was 5 ml. From this assay we can deduced that one gram of the immobilized derivative on agarose, alumina and silica is equivalent respectively to 70, 37 and 36 mg of native enzyme.

2.2.4. Enzymatic hydrolysis

The reactions were carried out in a batch reactor, in which 2 or 1 g of the immobilized catalysts on alumina or agarose respectively, or 75 mg of native enzyme were mixed with a determined volume of Tris/HCl buffer 0.1 M containing as substrate the (*R,S*) esters emulsified by ultrasound (2 min at 20 watt) using a Branson model sonifier 450 (specific reaction conditions in each case in

Tables and Figures). The reaction mixture was incubated at 35°C 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 (3×25 ml). The organic phase was transferred to a clean glass and a second extraction was carried out with NaOH 0.1 M (3×25 ml). The aqueous phase was acidified with HCl and then a new extraction was performed with diethyl ether (3×25 ml). The remaining organic phase was evaporated to dryness in a Buchi concentrator–evaporator.

10 mg of residue 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 [18].

3. Results and discussion

3.1. 2^3 Factorial design

Using the conventional approach, all the reaction parameters are maintained constant but the one under analysis. Operating in this way, the true maximum at times could not be reached because only a part of the experimental domain is explored. This situation can be avoided by using multivariate 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 [19–22]. The dependence of the variable (Y) which describes the system, upon the experimental variables x_i, can be approximated with a polynomial, Eq. 1.

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

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 lit-

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)	0.0	0.35[b]	0.7[c]
x_4 = [S]/[Enz][a]	3.46	5.19	6.92

[a] [S] = (±) ethyl 2-phenyl propionate (g/ml); [Enz] = enzymatic derivative amount (g/ml).
[b] 0.1 M $CaCl_2$ and 0.1 M NaCl.
[c] 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 (%) for different durations (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					1.11	1.03	1.36	0.55	1.85

Table 3
Main and interaction effects [a] 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

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

erature (the range of working pH for similar reactions reported in the literature fluctuates between 7.2 [23] and 8.0 [12]a, the influence of catalyst concentration [19] and ionic strength [24] has been also described) and considering the experimental installation limits (stirring speed). 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, obtained using the STATGRAF [25] program, for the reaction yield 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 «0» of the factorial design (Table 2) because, generally, large coefficient values indicate a strong influence of the variable(s) on the response [20]. Daniel's method [26] was applied to analyze the effects of the variables on the yield. The results of this method are presented in Fig. 1 for the effects at 144 h. Similar behaviour 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 [19].

Table 3 and Fig. 1 show that the main variables are the stirring speed (X_1), [Substrate]/[Enzyme] (x_4), and the pH (x_2) because b_1, b_2 and b_4 are not on the straight line (Fig. 1). 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. [27] in the

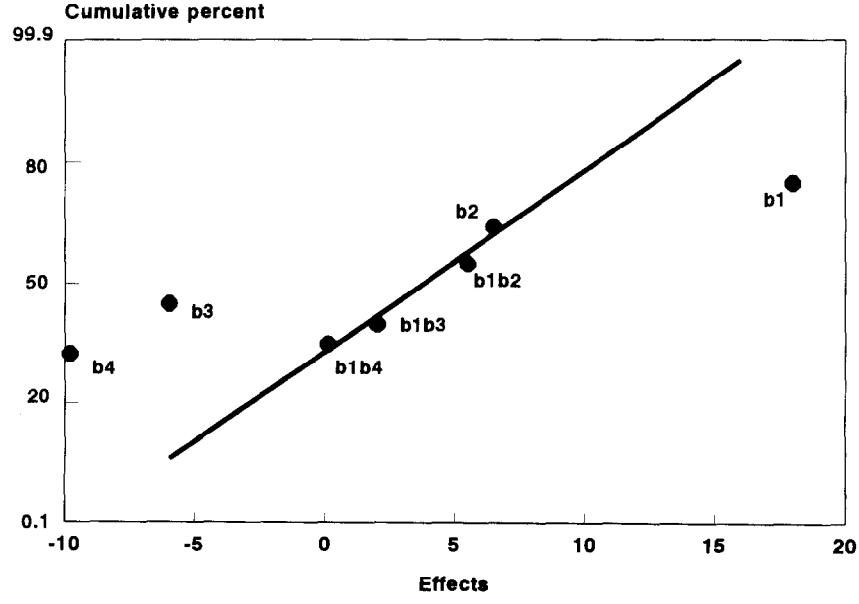


Fig. 1. Daniel's plot for the analysis of the effects at 144 h.

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 [27], 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 because the greater the reaction time, the greater the percentage of hydrolysis and the smaller number of ester molecules in the medium and so, the possibility to form microdrops diminishes. Therefore, it is necessary to increase the stirring speed to diminish the diffusional problems that would decrease the reaction yield [28].

The second variable [S]/[Enz] (x_4) exerts a negative effect on the hydrolysis process (Table 3 coeff. b_4). A similar effect was observed by Garcia et al. [19] in the synthesis of an analogue of jojoba oil with immobilized *Mucor miehei* lipase. Two different explanations could be postulated:

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. [23] 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 [28]. Probably, due to the importance of the interface area, the second explanation would be more adequate than the first one.

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 and olive oil [12e] [12,29]. 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 [30,31] and has been related to the fact that Na(I) favours the emulsification of lipids giving micelles [32] (increasing the interfacial water/lipid surface) and Ca(II) removes from the interfaces the fatty acids produced during the hydrolysis [33,34].

Nevertheless, the increase in the ionic strength (x_3) exerts a negligible positive effect on the hydrolysis of ethyl 2-phenyl propionate using lipase *Candida cylindracea* immobilized on alumina (Table 3 and Fig. 1). In order to analyze this topic several experiments were carried out at different ionic strengths, increasing with the addition of NaCl and $CaCl_2$. The results obtained are shown in Fig. 2. We can observe that the native enzyme is very sensitive to the increase of the ionic strength, while the immobilized derivatives are not very sensitive to this variation. The derivative obtained on the most hydrophilic support (agarose) is more sensitive to the increase of ionic strength than the immobilized enzyme on alumina. To confirm this result we carried out the immobilization of the enzyme in the same conditions of alumina on a support of similar characteristics (silica) and we observed the same behaviour as in the case of alumina when the ionic strength was increased. Therefore we can conclude that the influence of the ionic strength is very important when the enzyme is in a hydrophilic microenvironment but not in the case of a hydrophobic microenvironment. These results can be explained because the electrostatic field of the silica or alumina surface exerts the same stabilizing effect on the interface water/oil 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. 3) that progressively diminishes the activity of biocatalyst.

Finally the interaction effects are not significant as can be deduced from Daniel's representation method (Fig. 1). 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 and the results are shown in Table 4 using lipase from

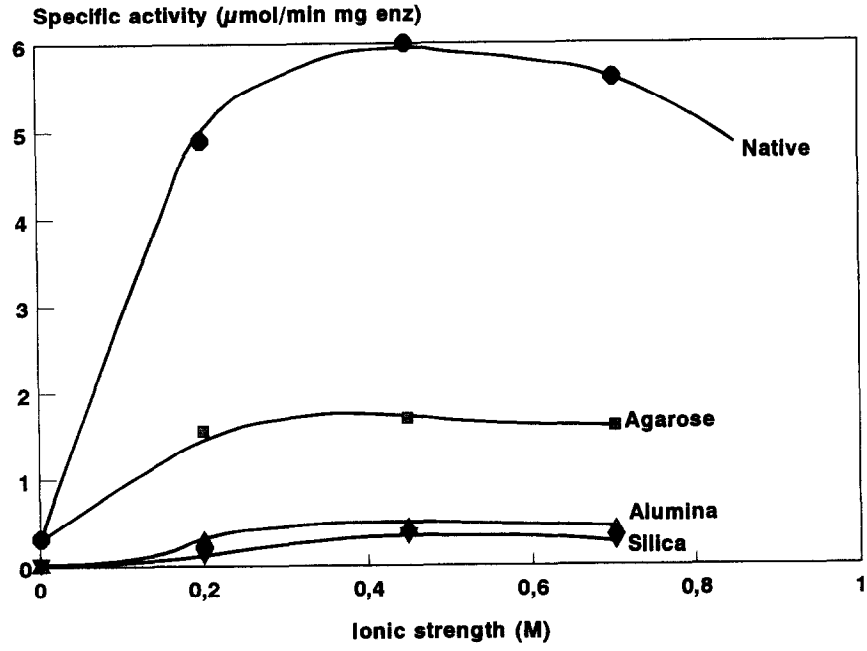


Fig. 2. Influence of the ionic strength in the lipase activity versus olive oil emulsion of the native (●) and immobilized derivatives: on agarose (■), on alumina (▲) and on silica (▼). Experimental points: 1 mM = Tris/HCl buffer; 0.2 M = Tris/HCl buffer + 0.057 M NaCl + 0.057 M $CaCl_2$; 0.45 M = Tris/HCl buffer + 0.128 M NaCl + 0.128 M $CaCl_2$; 0.7 M = Tris/HCl buffer + 0.2 M NaCl + 0.2 M $CaCl_2$.

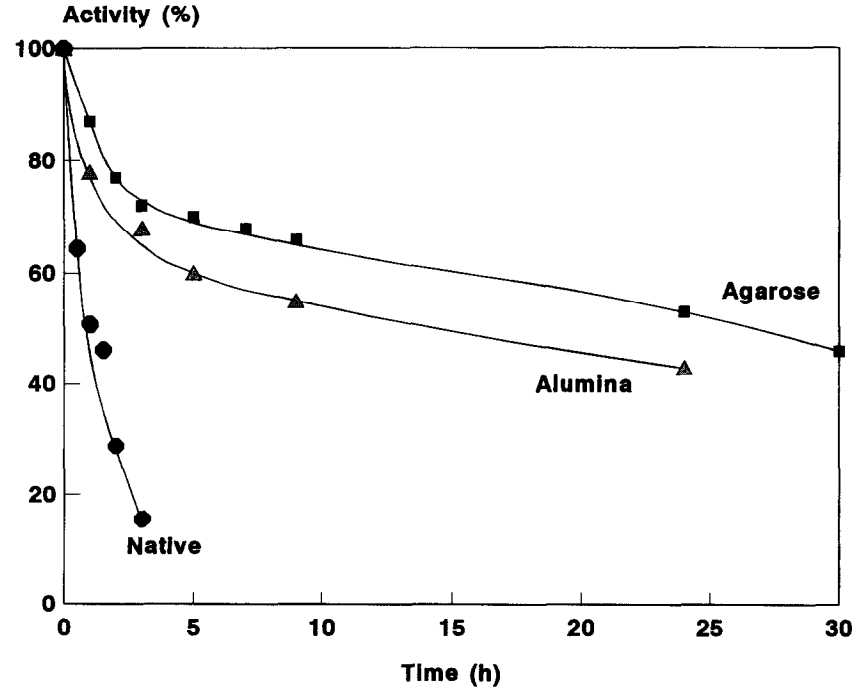


Fig. 3. Stability of the native and immobilized derivative storage at 50°C.

Table 4
Hydrolysis of (*R,S*) ethyl 2-phenyl propionate with 2 g of immobilized derivative on Al_2O_3 at 35°C [a], $V = 15$ ml

Entry	pH	*I* (M) [a]	[Subs.] (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

[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 ^{1}H-NMR in the presence of (*R,R*) 1,2-diphenylamino ethane (maximum experimental error ±2.0%).

Candida cylindracea immobilized on alumina as enzymatic derivative. The ee values were obtained using (*R,R*)-1,2-diphenyl-1,2-diaminoethane as chiral agent as described in the experimental part. In all cases, the enzymatic derivative stereoselectively hydrolyzes the *S*(+) ester as was observed by polarimetric measures and ^{1}H-NMR spectra of the acid produced in the presence of the chiral agent. This result agrees that the reported by other workers [35].

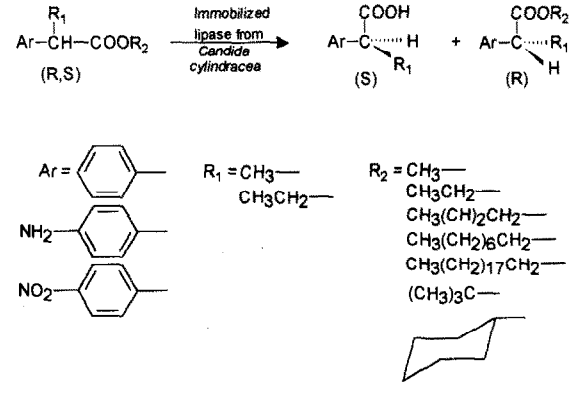
We can observe high enantioselectivity values when the reaction yield is lower than 50% (entries 1–4). This value diminishes when the reaction yield increases (entries 5,6), as described in the literature [36]. Therefore, we can say that these experimental variables – which are achiral parameters – do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

3.2. *Influence of alcoholic residue of the ester*

It is well documented in the case of native lipases that the nature of the aromatic ring (Ar) and of the alkyl group influences on the hydrolysis yield and on the enantiomeric excess [38]. To analyze these effects in the case of immobilized enzymes, the hydrolysis of some synthetic esters was carried out as shown in Scheme 1.

Cyclohexyl and t-butyl esters were not 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 of *Candida cylindracea* with respect to the substrate because esters from secondary and tertiary alcohols are not hydrolyzed [38]. The results obtained with the primary alcohol esters are shown in Fig. 4.

We can observe that the enzymes are esteroselective – in all cases – because hydrolysis yields greater than 50% could be obtained in extreme conditions. These results agree with those reported in the literature [40]. The best substrate for the native enzyme is the n-butyl ester as described by Monot et al. [38] using *Mucor miehei* lipase. Alkyl chains larger than butyl produce long induction periods with near null conversions at 24 h (octyl and cetyl ester). The same behaviour was observed with methyl ester. These results could be related to the instability of the microemulsion of these substrates at the stirring speed used (700 rpm), which was the same in all cases in order to compare the results. We have shown that this technical variable is the main variable that affects the yield in the case of the hydrolysis of esters. When the reaction is under way, the carboxylate and/or the large alcohol molecules act as a tensioactive favouring the stability of the microemulsion and this, the reaction yield increases. Therefore, the hydrolysis of esters must be carried out at the optimum stirring speed for each ester, which depends on the tensioactive



Scheme 1.

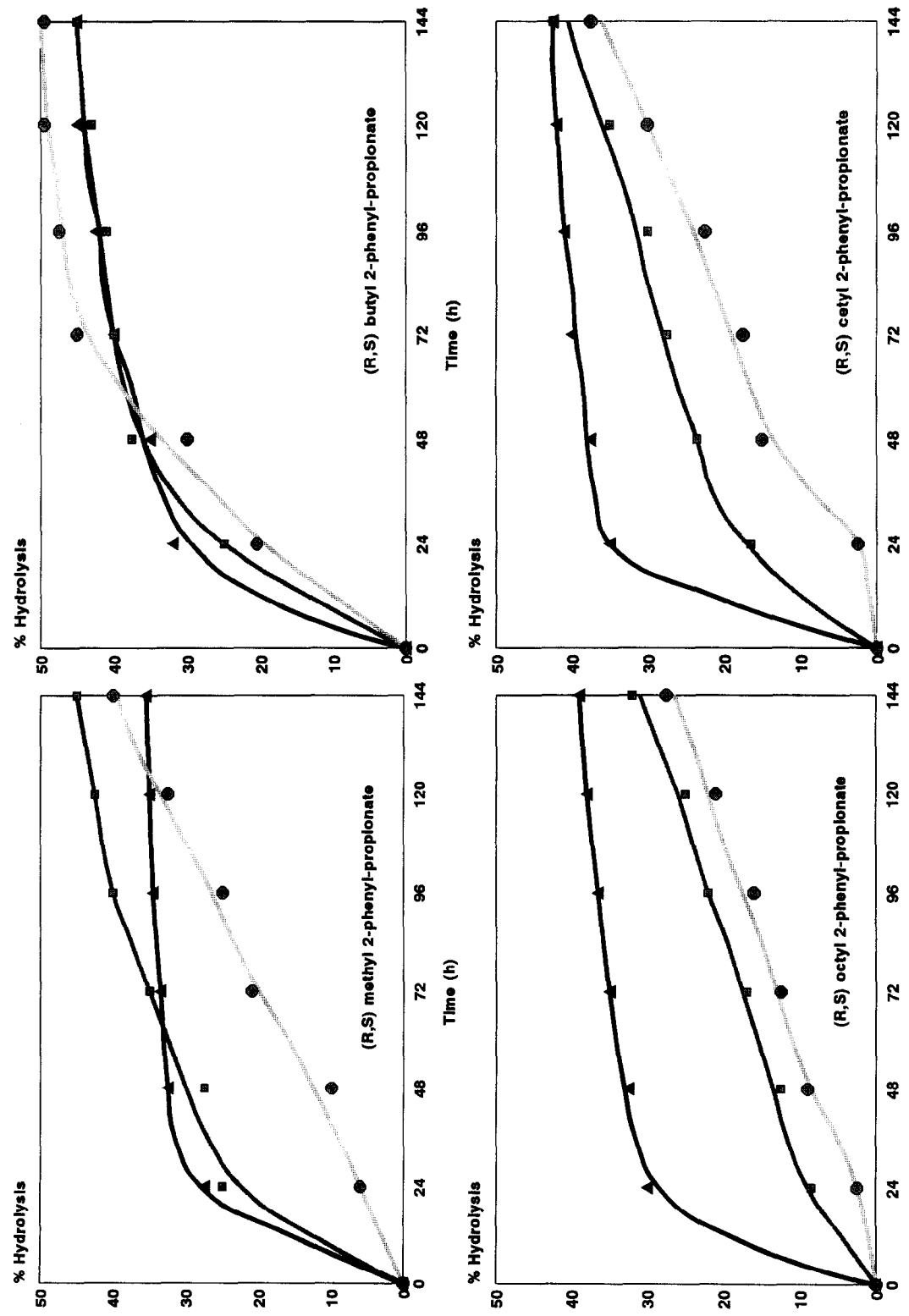
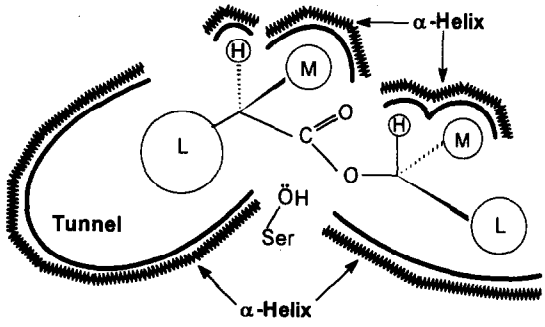


Fig. 4. Hydrolysis of 2-phenyl-propionic esters with native (●) and immobilized lipase from *Candida cylindracea* on agarose (■) and alumina (▲). pH = 7.5; Stirring speed = 700 r.p.m.; $V = 5$ ml; [S] = 0.04 M; [Enz] = 15 mg/ml; $T = 35°C$.



Scheme 2.

properties of the substrate and reaction product. The experiments carried out at the same stirring speed can give us erroneous information because the low yields could be due to the stability of the emulsion.

This effect is less important in the case of the immobilized enzyme than in the case of the native one, probably due to the positive effect of the support in order to maintain the microemulsion around the immobilized lipase. The same explanation was done in the case of the influence of ionic strength on the enzymatic activity (Fig. 2). Differences between the native and the immobilized enzyme are not observed in the hydrolysis of butyl ester because the emulsion is stable and the native enzyme can easily work.

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 Fig. 2. In this figure we show that the derivative immobilized on non ionic support (agarose) and the native enzyme need ions to work. This explanation is better than one based on the steric hindrance associated with the alkyl chain. On the basis of experiments carried out with chiral alcohols [39–41] a model of the active site of *C. cylindracea* lipase has been proposed by Kazlaukas [42] (Scheme 2) where the large group of the alcohol is located near the interphase. Therefore no steric hindrance would be observed with our alkyl chains, similar yields should be observed, and any difference in the yields should be related to the different stability of the microemulsion.

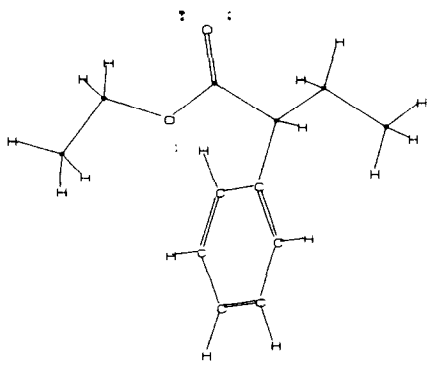
3.3. Influence of the aryl group and size of the alkyl chain

When (±) ethyl 2-aryl butyrates are used as substrates a diminution in the yield is observed (Table 5, yields at 144 h) respect to that observed with 2-phenyl propionic esters with the same native and immobilized enzymes (Table 2 and Fig. 4). The presence of polar groups (NH_2 or NO_2) in para position diminishes the reaction yield in all cases. In order to explain these results the conformers of the substrates were analyzed by a molecular mechanics methodology [43], using the MMX88 program [44]. The PCmodel program [45] 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 cannot be related to steric reasons, because the distance between the CO–OEt and the end of the aromatic ring is larger in the case of the ibuprofen (9.779 Å) than those obtained for the other substrates (6.516 Å $p(NO_2)$ and 6.240 Å $p(NH_2)$) (Scheme 3) and ibuprofen was hydro-

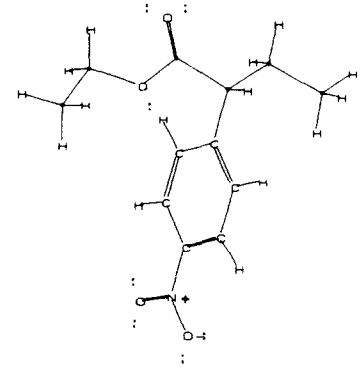
Table 5
Hydrolysis yield of (±) ethyl 2(4-X-phenyl)butyrate with native/immobilized lipase [a]

Enzyme	X	Yield (%) at 144 h
Native	H	6.5
Immob. on alumina	H	3.2
Immob. on agarose	H	7.3
Native	NH_2	7.5
Immob. on alumina	NH_2	0.3
Immob. on agarose	NH_2	7.9
Native	NO_2	0
Immob. on alumina	NO_2	2.4
Immob. on agarose	NO_2	1.8

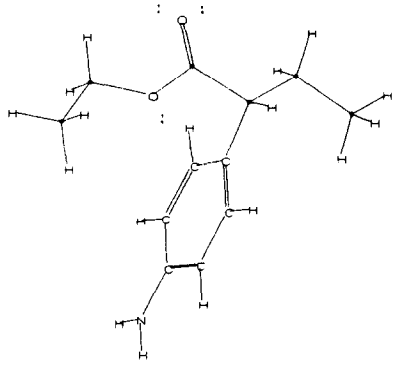
[a] pH = 7.5, Stirring speed = 700 rpm, V = 5 ml, [S] = 0.04 M, [Enz] = 15 mg/ml, T = 35°C.



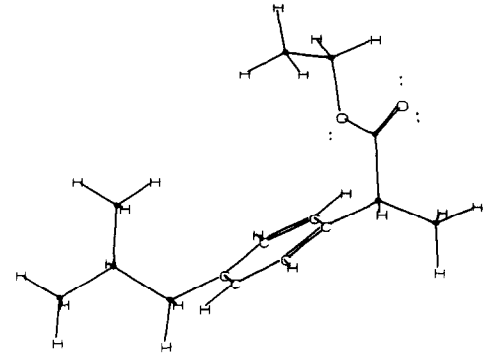
ethyl 2-phenyl butyrate



ethyl 2-(4-nitrophenyl) butyrate



ethyl 2-(4-aminophenyl) butyrate



2-(4-isobuthylphenyl) propionate

Scheme 3.

lyzed with the native enzyme in our group's work [46] (35% at 144 h).

Therefore we may deduce strong lipidic characteristics for the subsite tunnel L_1 (Scheme 2) which rejects polar substrates such as NH_2 or NO_2. New experiments are in progress to analyze this point. This affirmation is not surprising because lipases hydrolyze neutral lipids in the oil/water interface, while phospholipids do not. In addition, the lower yields obtained – in all cases – with 2-phenyl butyrates than with 2-phenyl propionates lead to us to assume the presence of steric hindrance problems in M subsite (Scheme 2).

3.4. Influence of immobilization in the enantioselectivity

Finally we have observed an increase in the enantioselectivity in the case of immobilized enzyme with respect to that in the case of native enzyme. In Fig. 5 we show the ^{1}H-NMR spectra (CH_3–CH– zone) of the complex of (*R,R*) 1,2-diphenyl-1,2-diamino ethane with the 2-phenyl propionic acid produced in the hydrolysis of its ethyl ester with immobilized enzyme on agarose (A) and with native enzyme (B). We can see, in the case of native enzyme (Fig. 5B), two doublets that can be resolved using a deconvolution program. In this case 70% *S*(+) and 30% *R*(−) of enantiomers are obtained in the hydrolysis of the

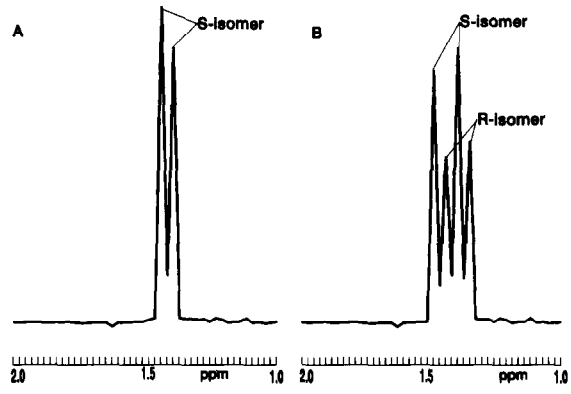
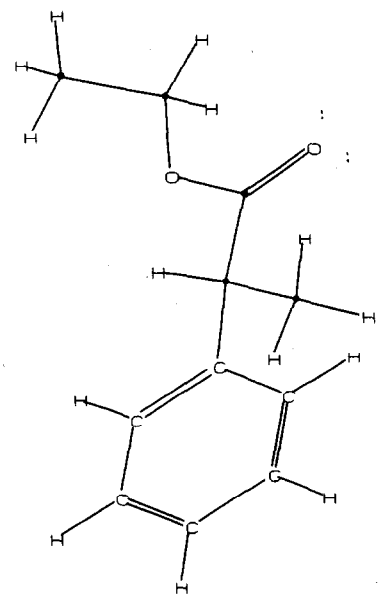


Fig. 5. Increase in the enantioselectivity in the hydrolysis of ethyl 2-phenyl-propionate with immobilized derivative on (A) agarose (ee = 100%) and (B) native enzyme (ee = 40%).

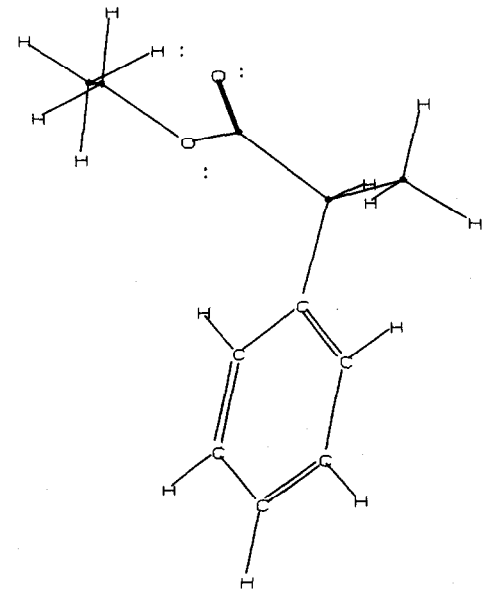
racemic ester using the native enzyme. This enantiomeric excess is similar to that described by us in the hydrolysis of methyl (*R,S*)-2-chloropropionate (1) (30%) and that described for other workers with these substrates, e.g. Sih et al. [12a,12c) (ee = 30–40%), and Bloch et al. [23]. Nevertheless, in the case of the hydrolysis of this racemate using the immobilized enzyme on agarose only a narrow doublet is obtained. The position of this doublet ($\delta = 1.4$ ppm) agrees with that of the doublet (CH_3–CH) of the *S*(+) enantiomer.

The observed increase in the stereoselectivity by the effect of the immobilization should 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. [5] that show how the 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-phenyl propionic esters. This effect is explained by non-covalent modification of the enzyme which makes the H_1 and M_1 subsites more rigid (Scheme 2) in the new protein than in the crude one. The immobilization of the enzyme can produce the same rigidification in the active site of the protein.

This rigidification does not permit the enzyme works on *R*-isomer which the angle O*C*–C–C=C– = 4° while on *S*-isomer the angle O*C*–C–C=C– < −56° (determined by molecular mechanics methodology) (Scheme 4).



R(−) ethyl 2-phenylpropionate



S(+) ethyl 2-phenylpropionate

Scheme 4.

4. Conclusion

From the experimental results of the present paper we can conclude that the hydrolysis of esters catalyzed by immobilized lipases are controlled by the stirring speed, [ester]/[enzyme] ratio and pH. The enzymatic activity is controlled by the stability of the water/oil interface. The immobilized enzyme on inorganic support makes it less sensible to the presence of ions than native enzyme or the lipase immobilized on agarose, probably due to the fact that the electrostatic field of the inorganic solid surface stabilizes the water–enzyme–support/oil interface in the same way as Na(I) or Ca(II) in the case of native enzyme.

The different yields observed in the hydrolysis of methyl, butyl, octyl and cetyl esters of (±) 2-phenyl propionic acid – at the same stirring speed – with the native and with the immobilized enzymes are related to the different stability of the microemulsion in the microenvironment of the enzyme and not to the steric hindrance of the alkyl chain. The native enzyme presents higher differences in the reactivity with respect to the different esters than the immobilized enzymes – especially on Al_2O_3 – due to the additional stabilization of the interface by the effect of the support. Butyl ester – which produces a stable microemulsion – is the most interesting compound for carrying out the hydrolysis with the native enzyme. Methyl, butyl or octyl ester can be used with the immobilized enzyme, giving excellent yields.

The immobilization of lipase from *Candida cylindracea* increases the enantioselectivity of the biocatalyst with respect to the native enzyme, probably by a rigidification of the active site of the enzyme.

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