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Lipase-Catalyzed Synthesis of Chiral Amides. A Systematic Study of the Variables that Control the Synthesis

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Abstract: A systematic study of the aminolysis of esters catalyzed by different lipases from different origins was carried out. A factorial analysis showed that the main variables that control the amide synthesis are: temperature, hydrophobicity of the solvent, reaction volume and amount of water added to the reactor medium. Besides, several undescribed interactions of variables are significative in the control of the process, too. The resolution of racemic esters or amines was analyzed. Lipases from *Rhizopus niveus,Candida antarctica* B and PPL gave the best enantioselectivities in the resolution of chiral esters while *C.rugosa* and *P.cepacia* lipases were the less interesting lipases. α-Chymotrypsin shows lower enantioselectivity and yield than *Rhizopus niveus, C. antarctica* B and PPL lipases in the resolution of racemic esters. This protease needs a large excess of acyl donor in respect to the amine and works at a lower temperature than lipases due to its low thermostability. All the tested lipases showed R-enantiopreference in the aminolysis of esters using (R,S) 1-phenyl-ethylamine. In this reaction, the lipase A from *C. antarctica,* (SP526) and *Rhizopus niveus* lipase are good catalysts for the synthesis. On the other, PS and PPL are less interesting biocatalysts. Therefore, the optimum biocatalyst is different if we want to resolve (R,S) esters or (R,S) amines. The aminolysis is interesting for the resolution of racemic amines but not for the resolution of racemic esters. The immobilization does not alter the enantiopreference of the lipases.

INTRODUCTION

Lipases have been widely used for the preparation of chiral alcohols, esters and carboxylic acids through the corresponding asymmetric esterification and transesterification reactions[1,2]. Recently, these enzymes have been used in the preparation of some achiral or chiral amides by aminolysis of esters [3-5].

The use of lipases to catalyse amide bond formation is an interesting alternative to conventional methods using proteases because lipases can act as catalysts in low hydrated organic solvents [3], showing low substrate preference, high enantioselectivities and nule or very low amidase activity. *Candida antarctica* lipase is the most useful lipase for this reaction [5-7] but lipases from *Candida rugosa* (formely *Candida cylindracea*) [8,9] and porcine pancreatic [10] lipases have been used in some cases. Nevertheless very few articles concerning a systematic screening of the commercial lipases for the resolution of racemic esters or racemic amines have been carried out [11]. In this paper we investigate the catalytic potential of some commercial lipases, from different origin, in the amidation of racemic esters or racemic amines analyzing the influence in the yield and in the enatioselectivity of some biotechnological properties of the biocatalyts such as: origin of the lipase, purification degree and the nature of the support, all variables that control the amide yield.

The lipases, we have used:

i)Fungal lipases: native lyophilized ***Rhizomucor miehei*** lipase (**SP524**) or immobilized by adsorption on anionic resin (Doulite A568) (**IM20**), to explore the influence of the support in the enzymatic activity.

-Native lyophilized lipase from ***Rhizopus niveus*** (Newlase F).

-Native lyophilized ***Humicola lanuginosa*** lipase (**SP523**).

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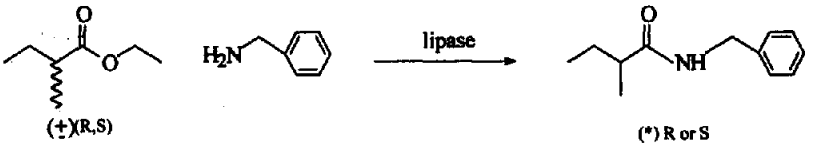
ii)Yeast lipases: native lyophilized ***Candida antarctica*** lipase B (**SP525**) or adsorbed on Lewatit E (**Novozym 435**), to explore the influence of the support in the process.
Native lyophilized ***Candida antarctica*** lipase A (**SP526**), to explore the different activity of both isoenzymes of the lipase of C. antarctica
Native lyophilized ***Candida rugosa*** lipase (**CRL**)
iii)Bacterial lipase: Native lyophilized ***Pseudomonas cepacia*** lipase (**PS**).
iv)Mammal lipase:Lyophilized **porcine pancreatic lipase (PPL).**

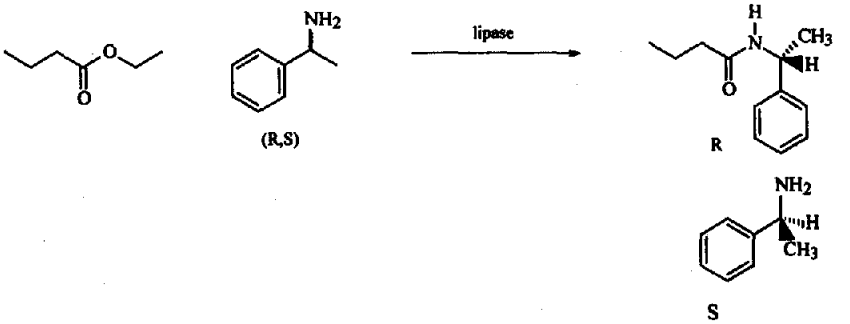
RESULTS AND DISCUSSION

Two test reactions were used to explore if the described enantiopreference of the lipases for acids and alcohols in the esterification reaction is altered in the aminolysis of the esters.

Resolution of racemic esters.



Resolution of racemic amines



The protease α-chymotrypsin was used as the reference, due to the presence of aromatic rings in both amines used in the paper. This protease mainly works on aromatic aminoacids (Phe,Tyr,Trp) [12] to produce the new amide bond. The synthesis of amide bonds using this protease was carried out in the best experimental conditions described in the literature for the synthesis of peptide bond [13].

1.-Factorial experimental design

The first step in the optimization of a catalyzed reaction is to determine the influence of the main variables that control the synthetic process. This point is generally rejected and so, several erroneous conclusions are obtained in the screening of the application of enzymes to one determinated reaction. In the current paper we

have used the aminolysis of ethyl butanoate (0.1 mM) with benzyl amine (0.1mM) as the reaction standard and Novozym 435 as the biocatalyst. This enzyme is a widely used biocatalyst in the literature. The study was undertaken by factorial analysis, a multivariant method in which all the parameters are simultaneously changed in a suitable programmed manner [14]. The selected response was the ester yield (Y (%)) using a polynomial function of seven experimental variables [1].

$$Y = b_0 + \Sigma b_i x_i + \Sigma b_{ij} x_i x_j \quad [1]$$

The selected variables were: x_1 = temperature (°C), x_2 = stirring speed (rpm), x_3 = catalyst weight (g), x_4 = reaction volume (ml), x_5 = solvent hydrophobicity (log P), x_6 = water amount added to the reaction mixture (μl), x_7 = reaction time (days).

Selection of the levels was carried out considering working condition limits of the lipase. The maximum (+) and the minimum (-) levels of each factor are shown in Table 1. In all cases 0.1mM of ester and amine were used as reference concentration of reagents. The experiments were done at random and the results (ester yield) obtained in the standard reactions with different combinations of a maximum and minimum level of each variable (entries 1-16). The center points values (entries 17 and 18) are shown in Table 2. The statistical analysis and the significative influences of this factorial design - using Statgraf program - is summarized in Table 3.

Table 1.- Variables and maximum and minimum levels used in the factorial design.

x_i	Variable	(-)	0	(+)
x	Temperature(°C)	4	32	60
x_2	Agitation(r.p.m.)	1	4	7
x_3	Catalyst (g)	0,1	0.25	0.5
x_4	Volumen (ml)	15	30	45
x_5	solvent (logP)[15]	1.5[a]	2.0[b]	3.5[c]
x_6	μl water	0	100	200
x_7	time (days)	1	3	5

[a] 4-methyl-2-pentanone (MIBK). [b] diisopropyl ether. [c] hexane

Daniels method [15] was used as the significant test to select the main variables. The most significant variables were:

x_1 = temperature (b_1 = -5.12)

x_4 = reaction volume (b_4 = -11.6)

x_5 = Hydrophobicity of the solvent (b_5 = 17.6)

x_6 = amount of water added (b_6 = -15.8).

Table II.- Factorial Design. Experimental matrix.

Experiment	x_1	x_2	x_3	x_4	x_5	x_6	x_7	Yield (%)
1	-	-	-	-	-	-	-	87
2	+	-	-	-	+	-	+	62
3	-	+	-	-	+	+	-	56
4	+	+	-	-	-	+	+	10
5	-	-	+	-	+	+	+	45
6	+	-	+	-	-	+	-	33
7	-	+	+	-	-	-	+	54
8	+	+	+	-	+	-	-	80
9	-	-	-	+	-	+	+	99
10	+	-	-	+	+	+	-	39
11	-	+	-	+	+	-	+	60
12	+	+	-	+	-	-	-	79
13	-	-	+	+	+	-	-	37
14	+	-	+	+	-	-	+	82
15	-	+	+	+	-	+	-	80
16	+	+	+	+	+	+	+	72
17	0	0	0	0	0	0	0	90
18	0	0	0	0	0	0	0	92

Table III.-Factorial design:statistical analysis. Number of experiments =16. Freedom degress=15

Influence of the variables

Single variables

$b_0 = 59.7$ $b_4 = -11.6$

$b_1 = -5.12$ $b_5 = 17.6$

$b_2 = -1.62$ $b_6 = -15.8$

$b_3 = 1.37$ $b_7 = 1.62$

Significative interactions

$b_4b_5 = -21.4$

$b_4b_6 = 23.9$

$b_5b_7 = -21.4$

$b_3b_4b_5b_6 = 17.9$

Furthermore, four variable interactions must be considered as significant:

(Reaction volume) x (amount of water) ($b_4b_6 = 23.9$)

(Hydrophobicity of the solvent) x (reaction volume) ($b_4b_5 = -21.4$)

(Hydrophobicity of the solvent) x (reaction time) ($b_5b_7 = 10.1$)

(Catalyst weight) x (hydrophobicity of the solvent) x (reaction volume) x (amount of water) ($b_3b_4b_5b_6 = 17.9$)

So, the reaction yield (%) may be described , at 95% confidence level, as a polynomial equation with the main significant variables [2]:

$$y(\%) = 59.7-5.12x_1-11.6x_4+17.6x_5+15.8x_6+23.9x_4x_6+10.1\,x_5x_7-21.4x_4x_5+17.9x_3x_4x_5x_6 \quad [2]$$

where x_i have the values (+1)-maximum- or (-1)-minimum according to each experiment (Table II).

The small negative effect of the temperature ($x_1, b_1 = -5.12$) (Table III) may be explained taking into account the two opposite effects that the temperature exerts in the enzyme-catalyzed reactions. At 4°C the reaction is slow (low yield) and at 60°C, the enzymatic reaction is faster but the enzyme is deactivating during the reaction (low yield). So the temperature may exerts a global negative effect on the yield. So, from our statistic study we can deduce that we should work near the medium value (32°C Table I) to improve the yield using this enzymatic derivative. Nevertheless this is not a general conclusion because the influence of this variable is strongly related to the nature and the origin of the lipase and it must be explored again to obtain the optimum value with each concrete lipase and each reaction.

The positive effect of the hydrophobicity of the solvent (x_5, $b_5 = 17.6$) (Table III) has been widely described in the literature for biotransformations in slightly hydrated organic solvents [16-19] where solvents with logP>2 are recommended.

The negative effect of the reaction volume ($x_4, b_4 = -11.6$ Table III), may be explained by the negative effect that causes the dilution in the intramolecular reactions reducing the number of effective collisions between the immobilized biocatalyst (Novozym 435) and the reagents. This topic is well known in heterogeneous catalysis.

Finally the negative effect in the process of the addition of water to the reaction medium (x_6, $b_6 = -15.8$ Table III), is explained because a lot of water deactivates the adsorbed lipase molecules as we proved by using water sorption isotherm methodology [20,21] or by Valivety *et al.*[22]. Adsorbed lyophilized enzymes such as Novozym 435 need some amount of water to be activated but by adding a large amount of water the deactivation of the biocatalyst is caused. The deactivation is produced by removal of the weakly bonded enzyme molecules from the solid support, done by the water molecules. In our case, the solid biocatalyst was not previously dehydrated. So, some water is present in the solid (<10%on weight). This fact explains why the addition of 100 or 200µl of water to the solvent deactivates the biocatalyst.

The positive effect of the interaction between the reaction volume (x_4) (Table III), and the amount of water (x_6), $b_4b_6 = 23.9$, may be deduced from Figure 1 where we only have analyzed the effect of these variables on the yield according to the equation [3], obtained from the factorial analysis:

$$y(\%)=59.7-11.6x_4-15.8x_6+23.9x_4x_6 \quad [3]$$

We can observe that the best yields are obtained with small reaction volume (x_4 = -1; 15ml) and nule addition of water (x_6 =-1; 0.0 μl) because Novozyme 435 is hydrated due to the hydrophilic characteristics of the anionic resin (Lewatit E). If we added more and more water with only 15ml of the solvent (x_4=-1), the solvent is saturated sending the water to the adsorbed catalyst that is deactivated and so, the yield decreases (from 100% to 32%) (Figure 1). On the other hand, with large reaction volume (x_4 = +1; 30ml) the addition of water increases the reaction yield because the solvent adsorbs the excess water added avoiding the deactivation of the biocatalyst.To sump up of Figure 1 we can deduce that the best yields are obtained with the small amount of solvent and without addition of water because commercial Novozym 435 has enough water to be active in this reaction.

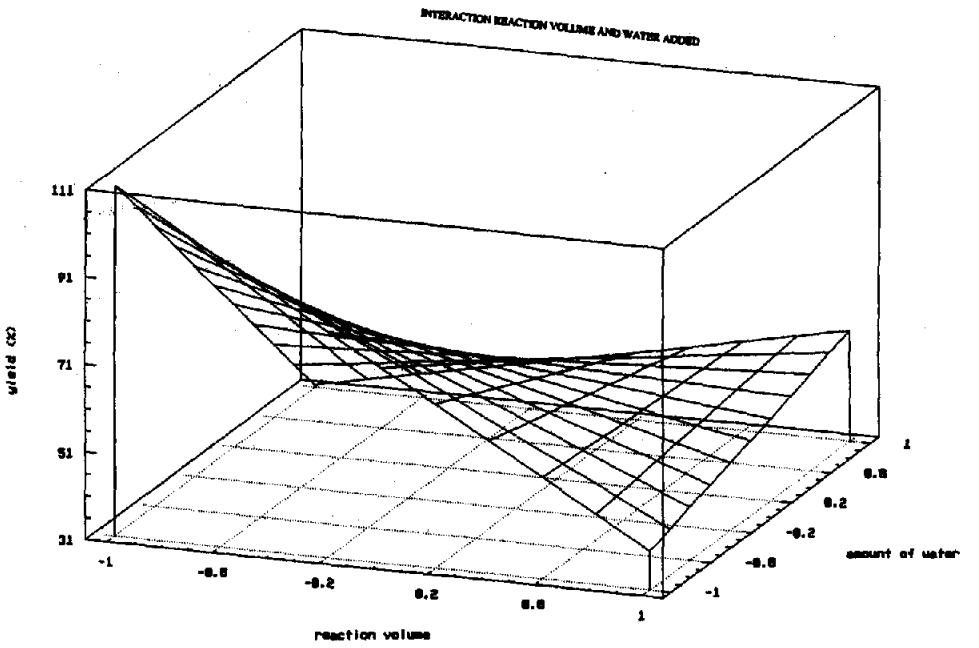


Figure 1.- Influence of the reaction volume (x_4) and water amount (x_6) in the aminolysis yield.

The positive effect of the interaction of the hydrophobicity of the solvent (log P) and the reaction time (b_5b_7=10.1) is evident and must be related more to the stabilization of the adsorbed enzyme, by the hydrophobic organic solvent[16], than to the increase in the reaction time (see influence of these variables Table III).

The negative effect of the interaction between the hydrophobicity of the solvent (x_5) and the reaction volume (x_4) (Table III) is described in Figure 2 where only the influence of these variables have been analyzed [3]

$$y(\%)=59.7+17.6x_5-11.6x_4-21.4x_4x_5 \quad [3]$$

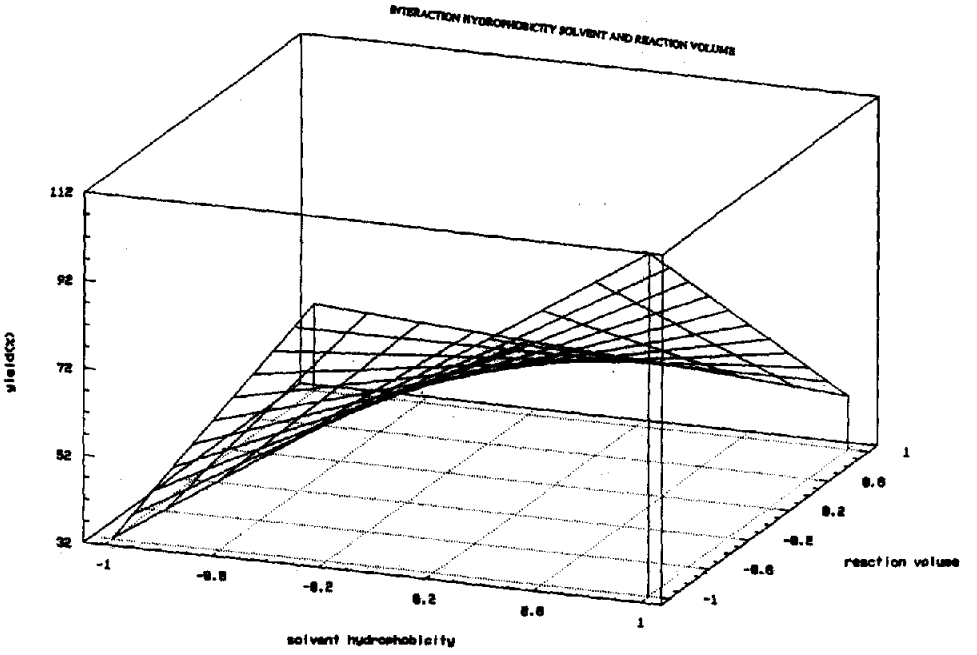


Figure 2.- Influence of the reaction volume (x_4) and hydrophobicity (x_5) in the aminolysis yield.

We can observe in Figure 2 that the best results should be obtained with hydrophile solvents($x_5 = +1$, hexane: logP = 6.5) and 15 ml of reaction volume ($x_4 = -1$). Hydrophilic solvents such as 4-methyl-2-pentanone ($x_5 = -1$; log P = 1.5) or large amounts of solvent give lower yield. This finding may be explained due to the hydrophobic reagents (ester and non protoned amine) they are retained by the hydrophobic solvent avoiding the interaction with the biocatalyst. Therefore, we will not have to use a high volume of the hydrophobic solvent because the yield is diminished (negative interaction x_4x_5).

Finally the multiple interaction between the catalyst weight (x_3), the hydrophobicity of the solvent (x_5), the volume of the reaction (x_4), and the water amount added (x_6) can easily be explained taking into the account the previously discussed interactions.

It is surprising that the amount of catalyst does not strongly influence the yield (b_3=1.37; Table III). Nevertheless this result is easily explained taking into the account that very large amounts of solid immobilized biocatalyst in the reaction mixture, could increase the conversion, but dramatically increases the interparticular diffusional problems, diminishing the yield. Due to these opposite effects, the influence of the catalyst weight is small in the catalyst weight interval considered (Table I).

2.- Amide Synthesis

2.1-Resolution of racemic esters

To explore the influence of: i)the origin of the lipase (fungal, yeast, bacterial or mammal); ii)the immobilization-stabilization of the lipase using the adsorption methodology and iii) the different activity of the isoenzymes, the reaction was carried out with different lipases in the same experimental conditions and at the same reaction time.

The aminolysis of racemic esters using the information deduced from the statistic analysis of the variables described before was performed. The obtained yields, after 3 days of reaction time, are shown in Tables IV and V. The enantiomeric excess of the amides were determined by ^{1}H-NMR (see experimental).

Table IV.- Amides obtained form ethyl (±) 2-methyl butyrate and benzylamine using different lipases.

LIPASE	% YIELD [a]	% ee [b]	Configuration of the amide
SP525	76	86	R
SP526	80	46	R
NOV-435	88	77	R
CRL	58	15	R
Newlase F	23	84	R
IM20	41	50	R
SP524	75	84	R
SP523	31	67	S
PS	56	12	R
PPL	15	87	S
α-CT	42	61	S
α-CT	22	30	R

[a] Yield in amide at 3 days, calculated by HPLC. [b] e.e. determinated by ^{1}H-RMN.[c] Medium 97/3(v/v) AcOEt/0.1M Tris/HCl buffer pH=9.0. [d] Medium 99/1 (v/v) Cl_3C-CH_3/0.1M Tris/HCl buffer pH=9.0

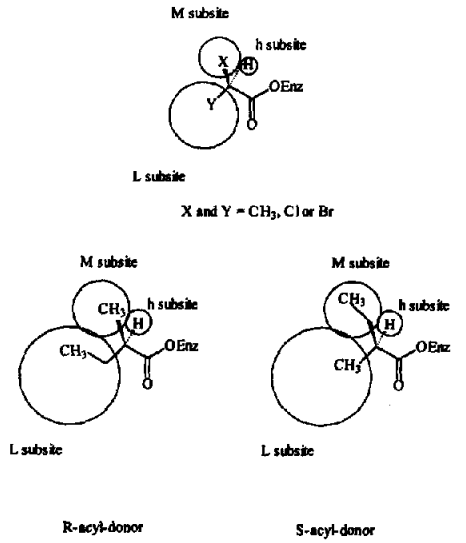
From the results of Table IV we can deduce that both isoforms of pure lipase from *Candida antarctica* A (CALA (SP526)) and the isoform B (CALB (SP525)) show the same R-enantiopreference in the aminolysis and in the alcoholysis [23] of the acyl donor (R > S). CALB is more stereoselective than CALA for the resolution of the racemic esters because at similar reaction yield, greater enantiomeric purity is obtained in the amide with CALB (SP525) than with CALA (SP526).

There are differences in the experimental conditions between both lipase catalyzed reactions:

i)esterification 66mM ester= 66mM alcohol; 300 mg biocatalyst in isooctane [23]. ii)aminolysis 0.16 mM ester; 0.1mM amine; 100 mg of biocatalyst in hexane (Table IV). Therefore, we need a molar excess of ester and greater amount of biocatalyst in the aminolysis compared to the esterification reaction. So, the aminolysis seems to be more disfavorable than the esterification-catalyzed lipase. Nevertheless, the enantiopreference observed with *Candida antarctica* lipase, is the same as observed in the case of the esterification of the (R,S) 2-arylpropionic acids [23] where the ester is mainly formed from the R-acyldonor. So, we may conclude that the nature of the nucleophile does not change the stereocontrol of the reaction that is determined, in this case, in the formation of the acyl-enzyme complex by the enzyme specificity ((R)-acyl-Enz > (S)-acyl-Enz). The stereochemistry we obtained is the same that reported by Quiros *et al* [24] using the same substrates and immobilized CALB (SP435), but these workers obtained a lower yield and lower e.e.(25% 3 days, e.e.=78%; T=30°C in hexane) than those obtained by us (Table IV), probably because they worked at 30°C and used more catalyst than us.

We would like to point out that CALA is active in the aminolysis of esters but not in the esterification reaction [25]. On the other hand the immobilization of CALB on Lewatit E (Novozym 435) does not change the (R)-enantiopreference of CRLB[23] in the resolution of racemic acyl-donor.

Candida rugosa lipase (CRL) shows lower activity and stereoselectivity (Table IV), than *Candida antarctica* lipases. These results agree with results previously reported in the literature for this enzyme where low enantioselectivities were observed when the size of the substituent in the stereogenic center of the acyl donor have a similar size: aminolysis of (R,S) 2-bromopropionate (Br and CH_3, e.e.<5%[24]) and hydrolysis of (R,S) 2-chloropropionate (Cl and CH_3) e.e.=6.4%[26](Scheme 1). In our case, the low e.e. obtained can be explained assuming that CH_3 and CH_3CH_2 - from 2-methylbutyrate - are interchangeable in the active subsites that recognize these groups in the CRL active site (Scheme 1).



Scheme 1

The enantiopreference observed with CRL (R≥S) is opposite to that described for the hydrolysis and the esterification of (R,S) 2-arylpropionates ((S)-enantiopreference [27]) but analogous to that described by Quiros et al [24] in the aminolysis of (R,S) 2-chloropropionate or to that described by Gotor et al [28] in the aminolysis of (R,S) 2-methylbutyrate. These authors describe different enantiopreferences for CRL depending on the nucleophile structure and/or the organic solvent as can be expected from a low enantioselective enzyme (yield 58% and e.e.=15%) with these substrates. CRL can only be used from the organic synthesis point of view if the substituents of the chiral carbon of the carboxylic acid are very different in molecular size as described [8,29]. Only in this case, good e.e. are observed in the resolution of the racemic acyl-donors [30].

Fungal lipases: *Rhizopus niveus*,(Newlase F) and *Humicola lanuginosa* (SP523) lipases gave low yields and low enantiopreference. *Rhizomucor miehei* lipase native and lyophilized (SP524) and immobilized by adsorption on Duolite A568 (IM20) show the same (R)-enantiopreference. So, the immobilization of the lipase by adsorption methodology does not alter the enantiopreference and so not the active site. The lower reaction yield attained with the immobilized enzyme (IM20) compared to the native enzyme (SP524), Table IV, may be explained by diffusional problems in the case of immobilized enzyme.

Lyophilized *Pseudomonas cepacia* lipase (PS), shows very low e.e. and porcine pancreatic lipase (PPL), shows low activity but high enantioselctivity (e.e.=87%) with opposite enantiopreference.

Finally α-chymotrypsin shows lower enzymatic activity and enantiopreference than the best lipases. When this protease is used as a biocatalyst, a large ester excess 0.3mM in respect to the amine 0.1mM is necessary and the reaction must be carried out at a low temperature (25°C) due to the high thermolability of α-chymotrypsin in the biphasic conditions. This point favors lipases in respect to α-chymotrypsin in the synthesis of amides. The alteration in the enantioprefrence observed with α-chymotrypsin, in a different media, is well documented in the literature [29].

2.2.-Resolution of racemic amines

The resolution of the racemic (R,S) 1-phenyl-ethylamine using the aminolysis of ethyl butyrate (Table V) was carried out in the same experimental conditions than Table IV. This reaction allows us to explore the enantioselectivity of the lipases from different origins in respect to the nucleophile.

In all cases the (R)-enantiomer of the amine was preferred as described [4b,31]. This enantiopreference is the opposite to that described for Subtilisin Carlsberg in this reaction [29], that must be considered the alternative to lipases in the preparation of chiral amides.

The most interesting lipase is the isoenzyme A from *Candida antarctica* that givesa yield near 50% and 99% e.e. This is the first time that this enzyme shows better enzymatic activity than the isoenzyme-B in the resolution of racemic mixtures.

As in the previous reaction, the immobilization of the lipase (IM20 or Novozym-435) does not alter the enantiopreference of the lipase.

The obtained yields after three days show us that PPL and *Humicola lanuginosa* (SP523) lipases can be rejected from synthetic point of view due to the low yields obtained and *Rhizomucor miehei* lipase (SP524) due to the low enantioselectivity. The other enzymes can be used because high e.e. are obtained. Therefore we can conclude that aminolysis reaction catalyzed by lipases is interesting in the resolution of racemic amines (Table V) but it is not very efficient in the resolution of racemic esters (Table IV), where the alcoholysis catalyzed by lipases seems to be more effective.

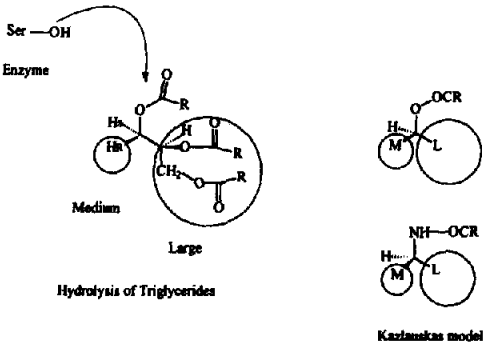
Table V.- Amides obtained from ethyl butyrate and (±) 1-phenyl ethyl amine using different lipases.

LIPASE	YIELD	ee % [b]	Configuration of the
SP525	23	99	R
SP526	58	99	R
NOV-435	66	95	R
CRL	20	99	R
Newlase	23	99	R
IM20	30	99	R
SP524	41	62	R
SP523	10	99	R
PS	22	80	R
PPL	9	92	R

[a] Calculated by HPLC. [b] Determinated by ^{1}H-RMN.

3. Lipase-alcoholysis versus lipase-aminolysis reaction.

The R-enantiopreference observed is the same than when one alcohol molecule is used as nucleophile in accordance with the affirmation that the nucleophile recognition subsite of the lipases is very rigid, accepting only the (R)-enantiomer, as described by Kazlaukas et al [32]. This rigid R-enantiopreference is related to the *in vivo* activity of lipases that hydrolyzes triglycerides as described in Scheme 6. The substitution of pro-(R)-hydrogen of the glycerol by the medium group give us the empirical model described by Kazlaukas et al [32] (Scheme 2).



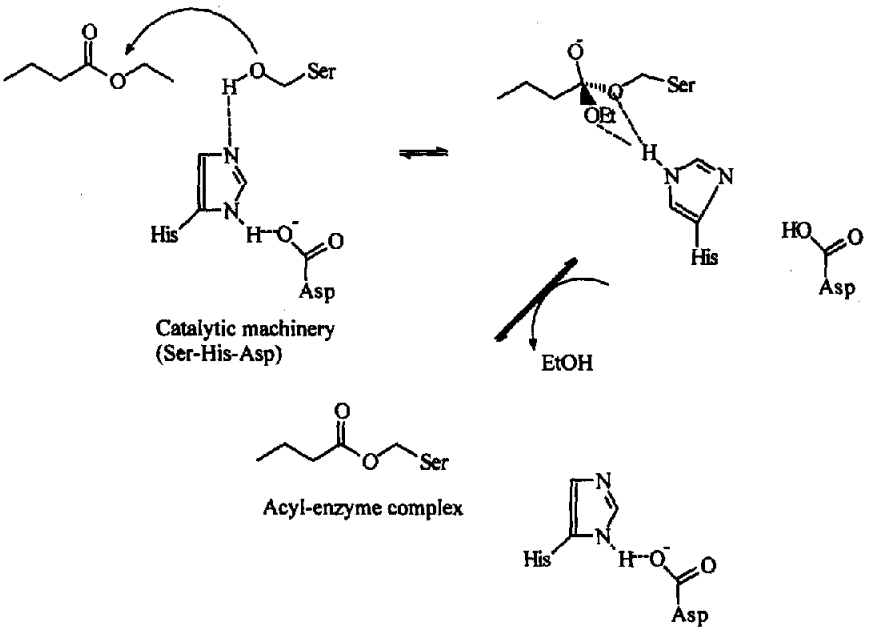
Scheme 3

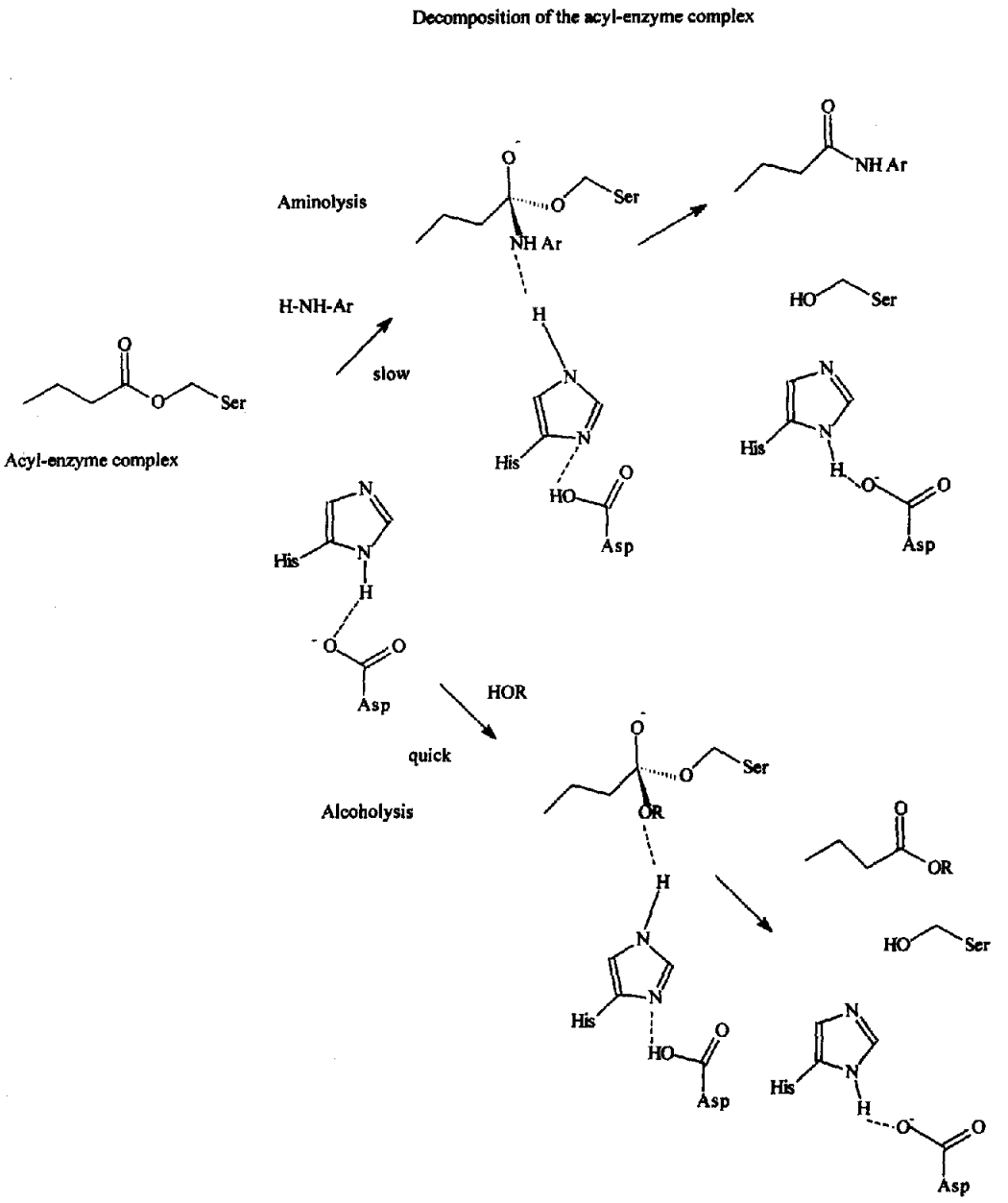
Therefore the (R)-alcohol molecule can be substituted by the (R)-amine molecule explaining the enantiopreference observed in the aminolysis. The aminolysis of esters need a greater catalyst amount and longer reaction times than the alcoholysis of esters [21,23]. These observed differences in the reaction rate are opposite of what one would expect from the corresponding nonenzymatic reaction because amine is more nucleophile than alcohol. It may be explained looking closer at the enzymatic reaction mechanism. (Scheme 3).

Two well defined steps may be described in these reactions i)formation of acylenzyme complex, identical in both reactions and ii) decomposition of the acyl-enzyme complex that is different in aminolysis and in alcoholysis reactions (Scheme 3). The rate of the proton transfer from the acyl acceptor to the His residue of the catalytic triad [33] in the active site during the nucleophile attack on the acyl-enzyme complex is likely to be lower for a neutral amine which has a significant higher acid dissociation constant than the corresponding alcohol. If the rate-limiting step of the acyl transfer reaction is the proton transfer, it would support the low reaction rates observed for the amines compared to the alcohols.

The higher e.e. values obtained can be explained taking into account the low reactivity of the formed amides as substrates for lipases compared to the esters. So, in general, high enantioselectivities are observed in the aminolysis than in the alcoholysis reactions, but more severe experimental conditions are necessary.

Formation of the acyl-enzyme complex




Decomposition of the acyl-enzyme complex
Aminolysis
H-NH-Ar
slow
Acyl-enzyme complex
Ser
NH Ar
His
Asp
HO
HOR
quick
Alcoholysis
OR

Scheme 3

EXPERIMENTAL

Candida cylindracea Type VII crude (CRL), *Porcine pancreatic* lipase Type II crude (PPL) and α-Chymotrypsin were purchased from Sigma Chemical Co. *Candida antarctica* (SP525, SP526, NOV-435), native *Rhizomucor miehei* (SP524) and *Lipozyme IM20* lipases were obtained from Novo Nordisk. *Rhizomucor niveus* lipase (NEWLASE F) and Lipase PS (*Pseudomonas cepacia)* were purchased from Amano Pharmaceutical Co.

All the reagents were of commercial quality and were purchased from Aldrich Chemie. For column chromatography, Merck silica gel 7-230 mesh was used. Optical rotations were measured using a Perkin-Elmer 241 polarimeter. IR spectra were recorded on a Perkin-Elmer Paragon 1000 spectrophotometer. ^1H and ^{13}C were obtained with TMS (tetramethylsilane) as internal standard, using a Bruker AC-250 (^1H- 250 MHz and ^{13}C- 62,89 MHz) spectrometer. Analytical HPLC was performed on a LDC chromatograph using a Nucleosil C8 120 (20 x 0.4 cm 10μm) column with MeOH/ HCl pH=3 (70:30) as eluent, flow rate 0.5 ml/min with UV detection λ=254nm.

Determination of enantiomeric excess was calculated by ^1H-RMN spectroscopy using the chiral shift reagent tris-[3-heptafluoropropylhydroxy-methylene)-(+)-camphorate)europium (III) derivative. The molar ratios amide/Eu-derivative were: 1/0.5 (benzylamide) and 1/0.3(phenyl-ethylamide). The absolute configuration of the amides were assigned by comparing of their optical rotation with authentic chiral amides[24].

Lipase reactions conditions:

To a solution of 5mmol of ester and 3,5 mmol of amine in 30 ml of hexane ((+1) x_4), was added 100mg of catalyst (x_2=-1). The suspension was stirred at 60°C for 3 days. Water was not added according to the negative effect of this variable described in the factorial analysis. The enzyme was removed by filtration and the solvent evaporated. The conversion was determinated by HPLC. The chromatographic separation on neutral silica of the resulting residue yield the amide (eluent hexane- ethyl acetate 1:1(v/v)) and the enantiomeric excess was determined by ^1H-NMR.

Protease reaction conditions:

To a solution of 10 mmol of ester and 3,5 mmol of amine in 30 ml of solvent (97/3 (v/v) Ethyl acetate-Tris/HCl 0.1M pH=9 or 99/1 (v/v) Trichloroethane- Tris/HCl 0.1M pH=9) a solution of α-chymotrypsin (4.8 mg/ml) was added. The suspension was stirred at 25°C for 3 days. The enzyme was removed by filtration and the solvent evaporated. The conversion was determinated by HPLC. The chromatographic separation on neutral silica of the resulting residue yield the amide (eluent hexane-ethyl acetate 1:1 (v/v)).

N-benzyl-2-methylbutyramide: I.R (KBr) ν_{max}: 1640 (C=O) cm^{-1}; ^1H-RMN ($CDCl_3$)δ (ppm): 0,89 (t, 3H, CH_3), 1,10 (d,3H,CH_3), 1,40 (m, 1H, C*H*H), 1,62 (m, 1H, CH*H*),2,13 (m, 1H, CH), 4,36 (d,2H,CH_2), 5,74 (bs, 1H, NH), 7,17-7,30 (m,5H, arom).^{13}C ($CDCl_3$) δ (ppm): 11,7 (CH_3), 17,61 (CH_3), 27,4 (CH_2), 42,16 (CH), 43,2 (CH_2), 127,6 (CH), 127,7 (CH), 128,7 (CH), 138,67 (C), 176,74 (CO). Anal. Calcd. For $C_{12}H_{17}ON$: C, 75,32; H, 8.96; N, 7,32. Found: C,75.4; H, 8.97; N, 7.37.

N-1'-phenylethyl butyramide: I.R (KBr) ν_{max}: 1640 (C=O) cm^{-1}; ^1H-RMN ($CDCl_3$)δ (ppm): 0,84 (t,3H,CH_3), 1,40 (d,3H,CH_3), 1,53 (m,2H, $\underline{CH_2}CH_3$), 2,1 (t, 1H, C*H*HCO), 2,27(t,1H,CH*H*CO),5,06

(q,1H,CH), 5,91(bs,1H, NH), 7,07-7,28 (m,5H, arom).^{13}C ($CDCl_3$) δ (ppm):13,7 (CH_3),18,67 (CH_3), 21,91 (CH_2), 35,52 (CH_2), 47,69 (CH), 125,84 (CH), 127,52 (CH), 128,27 (CH), 143,62 (C), 177,15 (CO). Anal. Calcd. for $C_{12}H_{17}ON$: C, 75,32; H, 8.96; N, 7.32. Found: C, 75.15; H, 8.95; N, 7.29.

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