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ENZYMATIC RESOLUTION OF (*R,S*)-IBUPROFEN AND (*R,S*)-KETOPROFEN BY MICROBIAL LIPASES FROM NATIVE AND COMMERCIAL SOURCES

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ABSTRACT

The enantioselectivity (E) of native lipases from *Aspergillus niger, Aspergillus terreus, Fusarium oxysporum, Mucor javanicus, Penicillium solitum* and *Rhizopus javanicus* in the resolution of (*R,S*)-ibuprofen and (*R,S*)-ketoprofen enantiomers by esterification reaction with 1-propanol in isooctane was compared with known commercial *Candida rugosa* (Sigma) and *Candida antarctica* (Novozym®435) lipases. In the resolution of (*R,S*)-ibuprofen, *C. rugosa* lipase showed good selectivity (E = 12) while Novozym®435 (E = 6.7) and *A. niger* (E = 4.8) lipases had intermediate selectivities. Other enzymes were much less selective (E around 2.3 and 1.5), under tested conditions. After preliminary optimization of reaction conditions (water content, enzyme concentration and presence of additives) the enantioselectivity of native *A. niger* lipase could be enhanced substantially (E = 15). All tested lipases showed low selectivity in the resolution of (*R,S*)-ketoprofen because poor ester yields and low enantiomeric excess of the acid remaining were achieved.

Key words: lipase, *(R,S)*-ibuprofen, (*R,S*)-ketoprofen, enantioselectivity

INTRODUCTION

The use and preparation of chiral pharmaceutical principles as single enantiomers is one of the most relevant goals in pharmaceutical science. For chiral drugs, opposite enantioforms act with different biological properties and the distomer could give undesirable effects. Ibuprofen and ketoprofen are important members of the class non-steroidal anti-inflammatory drugs (NSAIDs), employed as racemate in pharmaceutical formulations, but have their pharmacological activity mainly in belonging in *S*(+)-enantiomer (25).

Lipases (EC 3.1.1.3) are very suitable enzymes for organic syntheses because they accept a wide range of non-natural substrates, are stable and active in organic solvents, do not require cofactors, and are readily available from several (micro) organisms. In organic solvents, lipases can be more enantioselective and the solubility of hydrophobic substrate can be enhanced (11,15). Possible approaches to the enzymatic resolution of chiral profens include the enantioselective hydrolysis (28,41,46), transesterification (19,38) or the direct esterification in non-aqueous medium using microbial lipase (13,32,39). There are a certain number of lipases produced by yeast, most of them belonging to the *Candida* genus, which have been used for biotechnological purposes. Lipases from *Candida rugosa (cylindracea), Candida antarctica* and *Rhizomucor miehei* have been used to resolve the enantiomers of ibuprofen (29,32,44), naproxen (35,39,43), suprofen (40) and flurbiprofen (5). Some studies have been done exploiting the enantioselectivity from novel microbial lipases (6,7,27). In a preliminary study, we reported that some native lipases provided good results in terms of thermostability and enantioselectivity in the esterification reaction using racemic substrates (8,9).

The objective of this work was to characterize the catalytic performance of six crude native lipase preparations in terms of the enantioselective resolution of (*R,S*)-ibuprofen and (*R,S*)-ketoprofen using 1-propanol as acyl acceptor. We have used lipase-producing microorganisms isolated from natural sources and compared their lipases with commercial lipases from

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Candida rugosa (Sigma) and *Candida antarctica* (Novozym®435). In addition, a preliminary optimization with respect to the influence of the water content, enzyme concentration and the presence of additives in the yield ester (conversion degree) and the enantioselectivity value (E-value) of selected lipases were carried out.

MATERIALS AND METHODS

Chemicals

Crude commercial *Candida rugosa* lipase was obtained from Sigma Chemical Co (St Louis, MO, USA) and Novozym®435 (lipase immobilized on acrylate resin from *Candida antarctica*) was a gift of Novozymes Latin America Ltda (Araucária, PR, Brasil). Isooctane, 1-propanol, (*R,S*)-ibuprofen were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and (*R,S*)-ketoprofen and pures enantiomers were gifts from The Boots Company (Nottingham, UK) and Aldrich (Milwaukee, WI USA). Yeast extract and Bacto peptone were purchased from Difco Laboratories, (Detroit, MI, USA). The culture media, salts, chemical reagents and the other solvents were obtained from Merck (Darmstadt, Germany) and from Sigma-Aldrich Chemical Co. in the highest purity available. Low acidity olive oil (Carbonel) was purchased at a local market.

Microorganisms

All the microorganisms used in this study were isolated from soil and fruit samples and collected around Southest Brazil. The isolation process was performed by serial dilution of the samples according to standard techniques (33) and the taxonomic identification was performed by the Centro de Micologia da Universidade de Lisboa, Portugal.

Lipase production

Fungal lipase was produced in a medium with an initial pH value of 6.0 consisted of 2 % peptone, 0.5% yeast extract, 0.1% $NaNO_3$, 0.1%, KH_2PO_4 0.05% $MgSO_4$. $7H_2O$ and 2 % of olive oil. Cultures were carried out in Erlenmeyer flasks (500 mL) containing 120 mL of the growth medium. The cultures were inoculated with 1 mL of spore suspension (10^5-10^6 spores/mL) and the flasks were shaken at 130 rpm for 72h at 35ºC on a rotary shaker. After this period, the cultures were filtered and clear filtrates were treated with ammonium sulphate (80% saturation). The precipitates were dialyzed overnight against in sodium phosphate buffer, pH 7.0, and lyophilized for use as crude lipase preparation in powder form.

Enzymatic assays

The hydrolytic activity of the solid biocatalysts was determined at 35ºC, pH 6.0 - 7.0 by the titration of free fatty acids with 0.1 N NaOH released from olive oil hydrolysis (42). The enzymatic activity was expressed as µmoles of oleic acid released per minute of reaction (µmol min^{-1}). The amount of protein was determined by the Lowry methodology (31) using egg albumin as standard and measures the absorbance of 280nm.

Esterification reaction

The powder lipases obtained by controlled fermentation and the commercial lipases were tested in the esterification of chiral substrates using 1-propanol as acyl acceptor. The standard reaction mixture was composed of (*R,S*)-ibuprofen (66 mM) or (*R,S*)-ketoprofen, 1-propanol (66 mM) and anhydrous isooctane (10 mL) without addition of water. The reaction was started by adding 5.0 mg of lipases and carried out in sealed Erlenmeyer's on a shaker with orbital magnetic stirring at 180 rpm, at 35ºC. Samples of 100 µL of the solution were withdrawn at different times and diluted in 1.4 mL of isooctane. The amount of ester (conversion degree) formed during the reaction and the enantiomeric excess of the (*S*)-enantiomer were determined by gas chromatography (GC) and by high performance liquid chromatography (HPLC), respectively, as described below.

Chromatographic analysis

Gas chromatography was performed using a CHROMPACK CP 9001 gas chromatographer equipped with flame ionization detector (FID) and a CP-Sil 5 CB column (10 m x 0.25 mm x 0.12 µm). Injector temperature was 300ºC and the detector was 350ºC; oven temperature was maintained at 180ºC. Carrier gas was hydrogen with a flow of 12 mL/min. An external standard method was employed to quantify the formed ester and the remaining acid. The enantiomers of the unreacted substrate were separated by HPLC using a chiral column (Chiralcel OD, Daicel Chemical Industries, Ltd., Japan). The mobile phase was a mixture of n-hexane/Isopropanol/trifluoracetic acid (100/1/0.1 v/v/v) at a flow rate of 1.0 mL/min and detection was by UV at 254 nm.

Enantioselectivity-value (E-value) measurements

The value of enantioselectivity (E-value) was calculated from the enantiomeric excess of the substrate (ee_s) and the conversion degree (c) according to the method described by Chen *et al.* (12) (Equation 1). Free shareware programs for the calculation of the enantiomeric ratio can also be obtained via the Internet (http://www-orgc.tu-graz.ac.at).

$$\text{Equation 1: } E = \frac{[\ln (1 - c) (1 - ees)]}{[\ln (1 - c) (1 + ees)]}$$

Statistical analysis

One–way analysis of variance (ANOVA) and Tukey´s studentized range test were used to determine differences in mean values based on data collected from three replications of each measurement. Significance was established at $p \leq 0.05$.

RESULTS AND DISCUSSION

Hydrolytic and esterication activities of lipases

The hydrolytic activity of olive oil hydrolysis in aqueous medium and the esterification activity from the reaction between (*R,S*)-ibuprofen with 1-propanol in isooctane of the tested lipases are shown in Table 1.

There are no significant differences between the values observed for the hydrolitic activity and specific hydrolitic activity of the native lipases. The protein percentage in the solid lyophilized biocatalysts is slightly greater in *Aspergillus terreus* and Novozym 453. Evidently many other proteins are also present, not only lipases, as all the preparations employed were crude enzymes. Only Novozym® 435 lipase is immobilized on acrylate resin (according to the manufacturer's information).

Of the native lipases tested, *Aspergillus niger* and *Aspergilus terreus* lipases resulted in the highest esterification activity in the synthesis the propilic ester of ibuprofen (20.3 and 15.1%, respectively. *Fusarium oxysporum* (4.9%) and *Mucor javanicus* (3.2%) lipases showed lower esterification activity compared with the *Aspergillus* species. These results did not correlate with the result obtained for the hydrolysis of olive oil where low catalytic activity was observed for *Aspergillus terreus*. Finally, as could be expected, the commercial *Candida rugosa* lipase showed the highest hydrolytic activity and Novozym® 435 lipase the highest esterification activity.

Lipase-catalyzed enantioselective esterification

The native and commercial lipases were tested for their ability to catalyze the enantioselective esterification between racemic ibuprofen and ketoprofen with 1-propanol in isooctane. In Table 2 the summary of the final results obtained during the first 212 hours of reaction are presented.

The native crude lipase from *A. niger* is significantly more enantioselective for (*R,S*)-ibuprofen than other lipases from *A. terreus, Fusarium oxysporum* and *Mucor javanicus*. *Penicillium solitum* and *Rhizopus javanicus* lipases had no selectivity for these enantiomers. Under test conditions, the lipase from *A. niger* has an E-value for ibuprofen of 4.8; when the conversion reaches 25%, the enantiomeric excess of (*S*)-ibuprofen has the maximal value of 19.9% after 162 hours. The values are considerably higher than those previously found for the *Aspergillus niger* lipase (3,13). Mustranta *et al.* (32) reported that *Aspergillus niger* lipase (Biocatalyst, UK) was almost inactive in the ester formation in the resolution of racemic ibuprofen. In a previous paper we reported that lipases from *A. niger* and *A. terreus* were good candidates for biocatalysts in organic media. These lipases were highly thermostable, retaining 60% and 50% activity at 60ºC after 1 hour, respectively; and provided the best results in terms of E-value in the esterification of (*R,S*)-2-octanol with octanoic acid in n-hexane (E = 4.9 and E= 4.5, respectively) (9). As a rule thumb, Enantiomeric Ratios below 15 are unacceptable for practical purposes. They can be considered as moderate to good from 15-30, and above this value they are excellent (17).

It is worthwhile to note that initial esterification rate (conversion degree) for ibuprofen was higher than for ketoprofen for all lipase preparations tested. The E-value of lipases from *C. rugosa* and Novozym® 435 in the resolution of ibuprofen was 2.0-fold higher than those for resolution of ketoprofen. These results have been previously observed and may be due to differences in size and geometry of the substrates. Ketoprofen is referred to as a bad substrate when lipase from *C. rugosa* is used as biocatalyst because poor ester yields and enantiomeric excess were achieved (20,21). Modeling using *Candida rugosa* lipase explained why the rule often fails when the large and branched substituent is used. The large

Table 1. Comparison of hydrolytic and esterification activities of native and commercial lipases.

Sources of lipases	Hydrolitic activity[a] (μmol min^{-1})	Total Protein[b] (mg)	Specific hydrolytic activity (μmol min^{-1} mg^{-1} of total protein)	Esterification activity[c] (%)
Aspergillus niger	4.8 ± 0.5 [a]	18.0 ± 0.8 [a]	0.26 [a]	20.3 ± 1.3 [a]
Aspergillus terreus	3.6 ± 0.3 [a]	22.5 ± 1.0 [b,c]	0.16 [a]	15.1 ± 1.4 [b]
Fusarium oxysporum	5.9 ± 0.5 [a]	17.4 ± 2.1 [a]	0.33 [a]	4.9 ± 1.0 [c]
Mucor javanicus	5.9 ± 0.5 [a]	19.6 ± 0.9 [a,b,c]	0.30 [a]	3.2 ± 0.4 [c]
Penicilium solitum	4.2 ± 0.3 [a]	18.9 ± 0.7 [a,b]	0.22 [a]	ND
CR (Sigma)	108 ± 7.5 [b]	20.4 ± 1.5 [a,b]	5.29 [b]	52.1 ± 1.5 [d]
CA (Novozym® 435)	69 ± 4.2 [c]	22.9 ± 0.8 [c]	3.01 [c]	62.4 ± 1.6 [e]

Commercial lipases: CR: *Candida rugosa* and CA: *Candida antarctica*.
[a]hydrolytic activity was expressed as μmoles of acid released per minute (μmol min^{-1}) in the reaction conditions described. [b]total protein content (mg) of lyophilized lipase preparation [c]esterification activity (or conversion degree) is given as the percentage of (*R,S*)-ibuprofen esterified after 100 h. Means within a column with different roman superscript letters are significantly different ($p \leq 0.05$). ND: not detected.

Table 2. Resolution of (*R,S*)-ibuprofen and (*R,S*)-ketoprofen by the native and commercial lipases.

Producing strain	Reaction time (h)	c [a] (%)	ee [b]	E-value [c]	Stereopreference[d]
		(*R,S*)-ibuprofen			
Aspergillus niger	162	25.0 ± 2.1	19.9 ± 1.6	4.8 [a]	R
Aspergillus terreus	162	22.0 ± 1.9	9.8 ± 0.9	2.3 [b]	R
Fusarium oxysporum	212	16.2 ± 1.6	7.0 ± 0.8	2.2 [b]	S
Mucor javanicus	212	15.1 ± 1.2	4.9 ± 0.8	2.0 [b]	S
Penicillium solitum	212	4.1 ± 1.4	ND	ND	S
Rhizopus javanicus	212	2.7 ± 1.8	ND	ND	S
CR (Sigma)	54	53.0 ± 2.8	77.8 ± 1.5	12.0 [c]	S
CA (Novozym® 435)	54	64.0 ± 2.4	82.6 ± 1.8	6.7 [d]	R
		(*R,S*)-ketoprofen			
Aspergillus niger	212	18.3 ± 0.8	8.9 ± 0.6	2.7 [a,b]	R
Aspergillus terreus	212	14.1 ± 0.9	3.3 ± 0.8	1.5 [a]	R
Fusarium oxysporum	212	15.0 ± 0.7	4.0 ± 0.3	1.6 [a]	S
Mucor javanicus	212	12.2 ± 0.6	3.0 ± 0.4	1.5 [a]	S
Penicillium solitum	212	ND	ND	ND	S
Rhizopus javanicus	212	ND	ND	ND	S
CR (Sigma)	54	42.0 ± 1.8	41.3 ± 2.1	5.4 [c]	S
CA (Novozym® 435)	54	62.0 ± 2.3	55.0 ± 2.5	3.3 [b]	R

Commercial lipases: CR: *Candida rugosa* and CA: *Candida antarctica*.
[a]Conversion is given as the percentage of racemic profens esterified after the reaction time. [b]Enantiomeric excess of the remaining enantiomer (not esterified). [c]Value of enantioselectivity. [d]Configuration of the enantiomer found in the ester.
The data are expressed as mean ± SD. Means within a column with different roman superscript letters are significantly different ($p \leq 0.05$).

substituent cannot fit the enzyme tunnel, and instead lies outside the tunnel in the large hydrophobic pocket, hindering the enzymatic catalysis (4).

Only the lipase from *C. rugosa* produced desirable conversion (approximate 50%) with E-value 12. The *S*-acid was converted to its ester form, leaving the (*R*)-ibuprofen (ee = 78%) after 54 h of reaction time. *C. rugosa* lipase has been widely used in the resolution of racemic acids due to its high enantioselectivity (23,30). Some authors have reported the resolution of ibuprofen using different strategies (such as alcohols and acid concentration, water content, hydrophobicity of solvents and chemically modified lipases) with higher E-value (above 50) with *Candida rugosa* lipase (Sigma) both without immobilization (22,45) or immobilized (2,30).

On the other hand, Novozym®435 displayed poor enantioselectivity, since it catalyzed the esterification of both R- and S-enantiomers (c = 64% for (*R,S*)-ibuprofen and 62% for (*R,S*)-ketoprofen) of these racemic acids. Arroyo and Sinisterra (3) reported that the E-value for the esterification of ibuprofen catalyzed by Novozym®435 in isooctane was 1.2 ~ 1.3.

Most of the tested lipases preferentially esterified the (*S*)-enantiomer; however only Novozym®435, *A. niger* and *A. terreus* lipases were enantioselective in relation to the (*R*)-enantiomer of ibuprofen and ketoprofen. This stereopreference is advantageous as it allows the direct production of the desired active *S*(+)-enantiomer as unreacted substrate from the enzymatic reaction without further chemical manipulation.

Conversion degree (ester yield) in the resolution of (*R,S*)-ibuprofen with 1-propanol catalyzed by lipases without addition of water is presented in Fig. 1.

A high degree of conversion was obtained with Novozym® 435 (64%) and *C. rugosa* (53%) after 54 h of reaction, in accordance with reports by other authors (2,16). Native lipases from *A. niger* and *A. terreus* showed a moderate ester yield after 162 hours of reaction (25% and 22% respectively). We observed that the reversible nature of the esterification reaction caused a worsening of the rate of propilic ester of ibuprofen with increased incubation time, due to the gradual formation of water in the reaction which hydrolyses the product. It is important to point out that crude extracts of lipase from the microorganisms, may be highly heterogeneous, showing different enzymatic activity and selectivity depending on the different operational fermentation conditions (37).

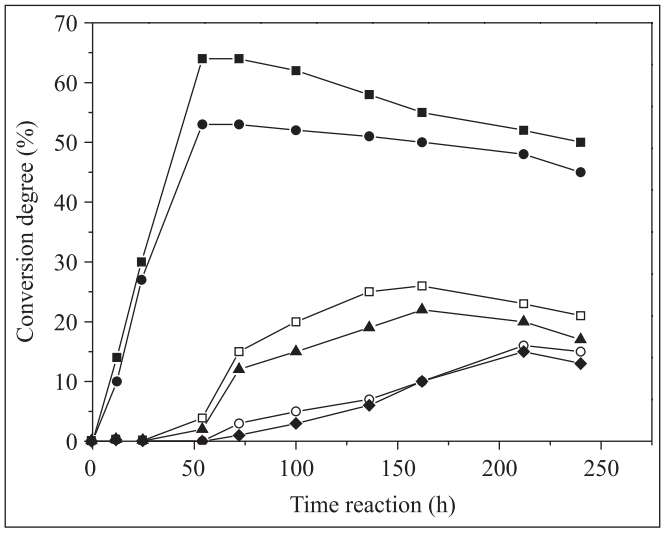

Figure 1. Time course of the enantioselective esterification of (*R,S*)-ibuprofen with 1-propanol in the absence of water catalyzed by several of the selected lipases.
Commercial lipases: ■ *Candida antarctica* (Novozym® 435) and ● *Candida rugosa* (Sigma). Native lipases: □ *Aspergillus niger*, ▲ *Aspergillus terreus*, ○ *Fusarium oxyporum* and ◆ *Mucor javanicus*.

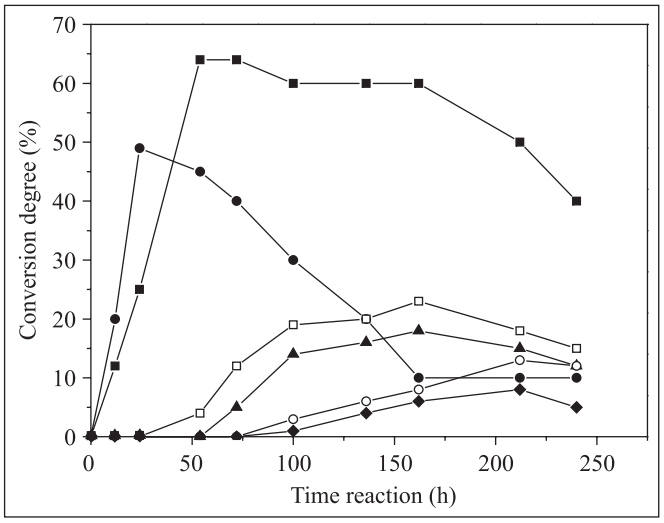

Figure 2. Time course of the enantioselective esterification of (*R,S*)-ibuprofen with 1-propanol in presence of water (0.1 %v/v) catalyzed by several of the selected lipases.
Commercial lipases: ■ *Candida antarctica* (Novozym® 435) and ● *Candida rugosa* (Sigma). Native lipases: □ *Aspergillus niger*, ▲ *Aspergillus terreus*, ○ *Fusarium oxyporum* and ◆ *Mucor javanicus*.

Optimal water content (% p/v)

The amount of water is one of the most influential factors on the activity of lipases in organic media (24,30). Thus, the same set of experiments was preliminarily carried out with the addition of 0.1% v/v of water (10 µL water in 10 mL solvent) to the reaction medium (Fig. 2). This strategy was based on previously published results stating that the addition of small amounts of water resulted in an increase in the E-value of lipases (10,36).

The influence of added water (0.1% v/v) on the synthesis of propilic ester of ibuprofen was not relevant with immobilized lipase from Novozym®435 and with native crude lipases under the conditions tested, whereas it greatly influenced the commercial enzyme from *Candida rugosa*. As can be seen, the conversion degree using lipase from *C. rugosa* increased at first, but later decreased with increasing time reaction, reaching its maximum rate (c = 49.2%) after 24 h of reaction. When the enzyme was used without the addition of any water (the commercial lipase powder had an adsorbed water content of 0.74% w/w determined by mass balance at 105ºC) the reaction rate was low and the ester yield was about half (c = 26.8%) of the theoretically expected value (50%) after 24 h. It is interesting to note how an excess of water in the microenvironment of the enzyme increase the hydrolase activity after 24 hours of reaction. Lipases from *A. niger, A. terreus, F. oxysporum, M. javanicus* and *R. javanicus* did not show appreciable differences in terms of conversion degree (as shown in Fig. 2) and the E-value (data not shown) compared with the reaction in the absence of water (Fig. 1). A possible explanation of this phenomenon could be that most of the water involved in the reaction mixture came from the lipase powder and it was enough for the activity of these enzymes. Although there was a great percentage of water eliminated in the lyophilization (approximately 96 - 98% for 24 h of lyophilization), an important amount of water remained in these crude lipase preparations.

In the next experiment (Table 3), the E-value of the two selected lipases, one native (*A. niger*) and other commercial (*C. rugosa*) were evaluated when different contents of water were added to the reaction mixture (0.1 - 0.4% v/v) for the resolution of (*R,S*)-ibuprofen.

By using *C. rugosa* lipase, the presence of a small water amount (0.1 - 0.2% v/v) in the reaction system increased significantly the E-value (14 and 10) for the resolution of this drug, giving the active (*S*)-ibuprofen (ee = 72 and 65%) with high optical purity after 24 h of reaction. Low hydration of the enzyme is believed to decrease the flexibility of enzymes, impeding their catalytic activity, because of interactions between polar residues, no longer screened by bound water molecules (1, 47). Xie *et al.* (45) observed that a higher racemate conversion (41.9%) and the E-value (112) were obtained when 0.1% (v/v) of water was added at a reaction time of 50 h using 60 mg of lipase from *Candida rugosa* (Sigma) in the esterification of (*R,S*)-ibuprofen with n-butanol in isooctane. However, crude commercial extracts of *Candida rugosa* lipase

Table 3. Effect of the addition of water in the reaction system on the resolution of (*R,S*)-ibuprofen catalyzed by lipases from *Candida rugosa* (24 h of reaction) and *Aspergillus niger* (162 h of reaction).

Addition of water (% v/v)	c [a] (%)	ee [b]	E-value [c]
Lipase from *Candida rugosa* (Sigma)			
0	26.8 ± 2.3	21.1 ± 1.6	4.3 [a]
0.1	49.2 ± 2.2	72.0 ± 1.8	14.0 [b]
0.2	48.0 ± 2.5	64.9 ± 1.8	10.0 [b]
0.3	45.2 ± 2.0	49.6 ± 1.5	6.4 [a]
0.4	38.1 ±2.1	31.0 ± 1.4	4.0 [a]
Lipase from *Aspergillus niger*			
0	25.0 ± 2.1	19.9 ± 1.6	4.8 [a]
0.1	23.2 ± 1.6	17.1 ± 1.1	4.4 [a]
0.2	18.7 ± 2.3	14.2 ± 1.3	4.6 [a]
0.3	20.2 ± 1.6	14.8 ± 1.6	4.3 [a]
0.4	14.6 ± 2.0	8.8 ± 1.4	3.2 [a]

[a]Conversion is given as the percentage of racemic ibuprofen esterified after the reaction time. [b]Enantiomeric excess of the remaining enantiomer (not esterified). [c]Value of enantioselectivity.
The data are expressed as mean ± SD. Means within a column with different roman superscript letters are significantly different ($p \leq 0.05$).

may be highly heterogeneous, showing different enzymatic activity and selectivity depending on the commercial lot (14). On the other hand, the amount of water added in the reaction mixture was not enough to modify significantly the E-value of *A. niger* lipase. These results confirm the previous results, indicating that this reaction mixture already has enough water for the catalysis or might even be in excess, which would justify the low E-values obtained for this lipase. In fact, the addition of 0.4% v/v of water caused a small decrease in the E-value of *A. niger* lipase (E = 3.2). In a reaction system, the amount of water present will depend mainly on the nature of the biocatalyst, the nature of the treatment employed for semi-purification (solvent or ammonium sulphate), the nature of the solvent used in the reaction mixture (because of their different affinity for the water) and others. To control water activity in organic media, several methods have been reported and most of them are based on the use of saturated salt solutions, which give a defined water activity (16,18).

Effect of enzyme loading

The influence of the amount of *C. rugosa* and A. *niger* and lipases in the resolution of (*R,S*)-ibuprofen is shown in Figs. 3 and 4, respectively. The amounts used were 5, 15, 30 and 50 mg and the reactions were examined without addition of water after 24 hours.

For commercial lipase from *Candida rugosa*, using 15 mg, the conversions were 42%, ee 58%, getting an E-value 16. Using 30 mg and 50 mg a slight increase of the conversions and ee values was obtained, these being 65 % and 72%, with E values of 17 and 14, respectively.

On the other hand, increasing the quantity of *Aspergillus niger* lipase in the reaction medium improved significantly the enzyme performance with respect to esterification activity (conversion degree) and enantioselectivity. The optimal amount of enzyme was 30 mg, forming the propilic ester ibuprofen in 30% conversion, ee 26, getting an E-value of 5.1. There was not much difference in the final conversions and ee between amount of 30 mg and 50 mg of the enzyme.

This can be explained by the fact that the excess use of enzyme not only prevents the active sites of the enzyme molecules from exposing to the substrate but also leads to internal diffusion limitations within the heterogeneous catalyst (34). Considering these results, the amount of 30 mg of lipases from *A. niger* and 15 mg of lipases from *C. rugosa* were selected for the subsequent experiments, which furnished the best results.

Effect of presence of additive

The E-value of the lipases from *C. rugosa* and *A. niger* were evaluated when different additives (silica gel, molecular sieves 4ºA and celite) were added and the reactions were examined without addition of water after 24 hours. It should be noted that these solid supports were used as additives, therefore, the lipases were not immobilized onto the supports prior to use.

The results in the Table 4 show that conversion degree of *A. niger* lipase can be effectively enhance in presence of silica gel, but not significantly in presence of molecular sieves and celite.

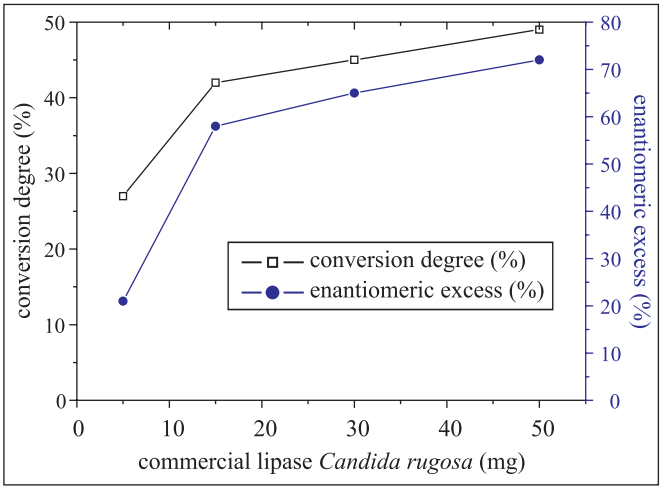

Figure 3. Effect of the amount of commercial *Candida rugosa* lipase on the enantioselective esterification of (*R,S*)-ibuprofen with 1-propanol in the absence of water at 35ºC, 24 h.

After 24 h of reaction, the conversion was 36, ee 46 % and E-value 15, which is considered moderate to good for synthetic approaches (17).

We observed that the silica gel was uniformly dispersed in the reaction medium and seemed to contribute to the homogeneity of the native lipase, as this lipase forms aggregates in the reaction medium and therefore the mass transfer limitations could be impaired. Probably, the high selectivity obtained was due to an improved dispersion of the catalyst in the organic media in the presence of silica gel. The presence of silica (E = 20) and molecular sieves (E = 23) in the reaction medium was found to have positive effect in the E-value *C. rugosa* lipase. When celite was used, low selectivity of this lipase (E= 12) was obtained under the present experimental conditions. Additives already present in the commercial powder, such as polysaccharides, dextrans or other products, showed an important influence on the catalytic performance of the *Candida rugosa* lipase (26,37).

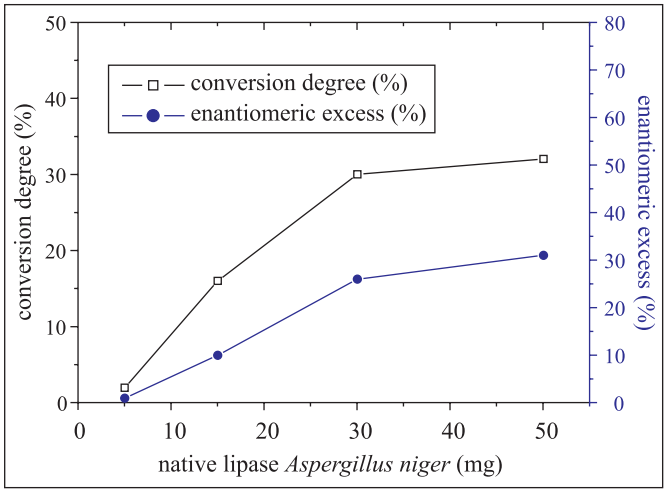

Figure 4. Effect of the amount of native *Aspergillus niger* lipase on the enantioselective esterification of (*R,S*)-ibuprofen with 1-propanol in the absence of water at 35ºC, 24 h.

Table 4. Effect of the additives (0.5% w/v) in the reaction system in the resolution of (*R,S*)-ibuprofen catalyzed by lipases from *Candida rugosa* (15 mg) and *Aspergillus niger* (30 mg) after 24 h.

Additive	c [a] (%)	ee [b]	E-value [c]
Lipase from *Candida rugosa* (Sigma)			
Absence	42.4 ± 1.2	57.6 ± 1.3	16.0 [a]
Celite	40.1 ± 2.7	51.0 ± 3.0	12.0 [b]
Molecular sieves 4ºA	40.2 ± 1.9	56.9 ± 3.6	23.0 [c]
Silica gel	36.8 ± 3.1	49.2 ± 2.8	20.0 [c]
Lipase from *Aspergillus niger*			
Absence	30.0 ± 2.1	25.9 ± 1.6	5.1 [a]
Celite	32.1 ± 2.6	28.4 ± 1.9	5.2 [a]
Molecular sieves 4ºA	32.3 ± 2.4	32.9 ± 2.1	7.8 [a]
Silica gel	36.2 ± 3.0	46.1 ± 2.7	15.0 [b]

[a]Conversion is given as the percentage of racemic ibuprofen esterified after 24h. [b]Enantiomeric excess of the remaining enantiomer (not esterified). [c]Value of enantioselectivity.
The data are expressed as mean ± SD. Means within a column with different roman superscript letters are significantly different ($p \leq 0.05$).

Additives already present in the commercial powder, such as polysaccharides, dextrans or other products, showed an important influence on the catalytic performance of the *Candida rugosa* lipase (26,37).

CONCLUSION

This study contributes to the use of native microbial sources for the expansion of biocatalysis in the resolution of racemic drugs. Considerable variability in the enantioselectivity in the resolution of (*R,S*)-ibuprofen between commercial and native lipases tested was observed. The different hydration degrees of the tested enzymatic preparations and other components present in the commercial powder or produced jointly with the enzyme in the fermentation process are some of the factors that influence the catalytic performance of lipases. When the *Aspergillus niger* lipase was used as a crude lyophilized powder without additives, the reaction rate was very low (especially in the initial phase) and the conversion degree was only about half of the theoretically expected value (50%). Nevertheless, we show that the enantioselective esterification rate of (*R,S*)-ibuprofen by *Aspergillur niger* lipase was markedly enhanced when the concentration of the enzyme was varied, and silica gel (0.5% w/v) was added to the reaction medium. These promising results point out to future applications of these enzymes in the resolution of the (*R,S*)-ibuprofen after semi-purification, as well as immobilization in different supports.

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RESUMO

Resolução enzimática do (*R,S*)-ibuprofeno e (*R,S*)-cetoprofeno por lipases microbianas de fontes nativas e comerciais

A enantioseletividade (E) das lipases nativas de *Aspergillus niger, Aspergillus terreus, Fusarium oxysporum, Mucor javanicus, Penicillium solitum* e *Rhizopus javanicus* na

resolução dos enantiômeros do (*R*,*S*)-ibuprofeno e (*R*,*S*)-cetoprofeno na reação de esterificação com 1-propanol em isoctano foi comparada com as lipases comerciais de *Candida rugosa* (Sigma) e *Candida antarctica* (Novozym®435). A lipase de *C. rugosa* mostrou boa enantioseletividade (E = 12) comparada com as da Novozym®435 (E = 6.7), de *A. niger* (E=4.8) e com as outras lipases que foram muito menos seletivas (E por volta de 2.3 e 1.5) na resolução do (*R*,*S*)-ibuprofeno, dentro das condições testadas. Após uma otimização preliminar das condições da reação (conteúdo de água, concentração da enzima e presença de aditivos) a enantioseletividade da lipase de *A. niger* pôde ser substancialmente aumentada (E = 15). Todas as lipases testadas mostraram baixa seletividade na resolução de (*R*,*S*)-cetoprofeno, resultando baixos rendimentos de éster e de excesso enantiomérico do ácido não esterificado.

Palavras-chave: lipase, (*R*,*S*)-ibuprofeno, (*R*,*S*)-cetoprofeno, enantioseletividade

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