UDC 541.632:547.26:577.152.311
ISSN 1330-9862 *original scientific paper*

(FTB-1440)

Resolution of Racemic Acids, Esters and Amines by *Candida rugosa* Lipase in Slightly Hydrated Organic Media

*Andrés R. Alcántara, Pablo Domínguez de María, María Fernández, María José Hernaíz, José María Sánchez-Montero and José Vincente Sinisterra**

Biotransformations group, Department of Organic & Pharmaceutical Chemistry, Faculty of Pharmacy, Universidad Complutense, Plza. Ramón y Cajal s/n, Ciudad Universitaria, E-28040 Madrid, Spain

Received: July 19, 2004
Revised version: September 23, 2004
Accepted: November 22, 2004

Summary

Commercial crude lipase from *Candida rugosa* is widely used as a biocatalyst in the resolution of racemic mixtures and in organic synthesis in slightly hydrated organic solvents. In many cases, reproducible results are not obtained when the same crude lipase is used, but from different suppliers of lots, this being due to the presence of different isoenzymes. The current work addresses this problem and strategies to overcome it. The yeast *Candida rugosa* ATCC 14380 was cultivated in a minimal culture medium, using different substances as inducers and carbon sources. The percentage of inducer that gave the optimum productivity of extracellular lipases was determined. Lyophilized extracellular enzymes were characterized by SDS-PAGE electrophoresis and isoelectric focusing (IEF). Depending on the nature of the carbon source, different isoenzymes were produced in various proportions. These samples were partially purified by different methodologies, including dialysis, adsorption chromatography and precipitation with ammonium sulfate or organic solvents. These characterizations allowed us to explain the relative catalytic activity of different samples, showing that in biocatalysis enzymes should not be treated simply as a »white magic powder« that can solve all the challenges in organic synthesis. Heptyl oleate synthesis, alcoxycarbonylation of amines and hydrolysis of the ester of ketoprofen are excellent reaction tests for the evaluation of lipase samples as biocatalysts.

Key words: lipase, isoenzymes, biocatalysis, purification of lipases, resolution of racemics

Introduction

Lipases (triacylglycerol-acylhydrolases, E.C. 3.1.1.3) catalyse, *in vivo*, the hydrolysis of triacylglycerols in a heterogeneous reaction. They are widely distributed in microorganisms, plants and animals. The rapid cell growth of microorganisms makes the microbial lipases more useful than animal or plant lipases. A great number of yeasts, fungi and bacteria have been described as lipase producers (*1–5*). The physiological function of lipases in fat digestion and in mobilization of stored triacylglycerols is well known. In addition, lipases are of high practical relevance in several fields of industry, such as the manufacturing of foods, leather, pharmaceuticals and cosmetics (*6–8*).

Lipases remain active in slightly hydrated organic solvents, enabling them to be used as biocatalysts in organic synthesis (*9*). In organic media, restricted amounts

* Corresponding author; E-mail: jvsgago@farm.ucm.es; Website: *www.biotransformaciones.com*

of water reduce the hydrolytic activity of lipases and the enzymes can be used as effective biocatalysts in a wide range of stereoselective reactions: including hydrolysis, esterification, transesterification and aminolysis (*10–13*).

Commercial lipase from *Candida rugosa* is probably the most traditional biocatalyst. Nevertheless, preparative organic chemists and chemical engineers are often discouraged by the poor reproducibility in either or both the yield and the enantiomeric excess (e.e.) of some *C. rugosa* lipase-catalysed reactions. This poor reproducibility occurs even when they use »the same commercial enzyme« from different lots or suppliers. In the present paper we undertake some fundamental studies to explain the source of these problems. These studies allowed us to select the best biocatalyst for a particular reaction.

Materials and Methods

Commercial lipases from *C. rugosa* (Type VII) were obtained from Sigma Chemical Co. (Alcobendas, Spain) (Lot 78H1147) and from Roche, L-3 lyophilized Chyrazyme® (Lot 83934628-04). *(R)*- and *(S)*-2-(3-benzoyl)--phenylpropionic acid (ketoprofen) and 2-(4-isobutylphenyl)propionic acid (ibuprofen) were gifts from Menarini Laboratories (Barcelona, Spain). Arabic gum, *p*-nitrophenyl acetate (*p*NPA), *(R,S)*-1-phenylethylamine, vinylchloroformiate, acetone, 1-dodecanol, *n*-butanol, *n*-octanol, *n*-propanol, tributyrin, NaCl, glycerol, isooctane and vinyl acetate were obtained from Sigma-Aldrich (Alcobendas, Spain). Oleic acid and *n*-heptanol were provided from Merck.

Microorganism, medium and growth conditions

Candida rugosa ATCC 14830 was maintained on YED (Yeast Extract Dextrose) plates at 4 °C. YED medium contains: yeast extract 10 g/L, glucose 20 g/L, and agar 15 g/L. The minimum mineral solution of medium contained: KH_2PO_4 15 g/L, K_2HPO_4 5.5 g/L, $(NH_4)_2SO_4$ 4 g/L, $MgSO_4 \cdot 7H_2O$ 1 g/L, and micronutrients: $FeCl_3$ 10 mg/L, biotin 0.008 mg/L, thiamin 0.2 mg/L and inositol 0.004 mg/L. Preliminary studies were carried out in 250-mL flasks, with 50 mL of culture medium and the appropriate carbon source. The flasks were incubated for 72 h at 28 °C in an orbital shaker (Rotabit, Selecta S.A., Spain).

Biomass

Samples were diluted in double-distilled water, and their absorbance at A_{600nm} was used to measure the cell concentration C, expressed in 10^6 cells/mL. The linear relationship was: A_{600nm}=0.0883 C (10^6 cells/mL) + 0.0183. (r=0.998). The pellets obtained after centrifugation of the samples were dried at 120 °C until constant weight, and the dry cell weight (d.c.w.) was determined.

Fermentation conditions

Fermentation experiments were carried out in a 6-L Braun fermentor Biostat E (4 L of working volume). Different inducers were used as the carbon source at various percentages: olive oil, sunflower oil, oleic acid, 1-dodecanol and glycerol, according to preliminary assays (*14*). Stirring speed was associated with the oxygen demand, in order to maintain the same oxygen concentration in the fermentor. Extracellular crude lipase was obtained from the fermentor broth after centrifugation to remove the cells. The crude broth was concentrated, ultrafiltered, using a Prep/Scale – TFF concentrator from Millipore with a 30-kDa membrane CDUFC001LL, and lyophilized using a LabConco lyophilizer of 12 liters. The crude lipase was obtained after lyophilisation for 72 h.

Esterase activity

The hydrolysis of *p*-nitrophenyl acetate (*p*NPA) was spectrophotometrically followed (λ=400 nm) at 25 °C, monitoring the absorbance increase due to the *p*-nitrophenol release. The assay mixture (2.7 mL) consisted of 20 μL of *p*NPA (50 mM) in acetonitrile, 2.5 mL of buffer solution (0.1 M Tris/HCl, pH=7.5) and 50 μL of lipase solution. One unit of esterase activity was defined as the amount of enzyme that liberates 1 μmol of *p*-nitrophenol per minute, in these conditions. All enzymatic reactions were duplicated. A maximum standard deviation of 8 % of the degree of conversion was observed.

Lipase activity

The titrimetric assay was used to determine the lipolytic activity using tributyrin. This activity was monitored in a Metrohm pH-Stat (Impulsomat 614, Dosimat 665 (with microstirrer), pH-meter (Herisau, Switzerland)). A NaOH solution (0.025 M) was used as the titrating agent. The reaction mixture consisted of different volumes of substrate emulsion (varying from 2 to 6 mL), several volumes of lipase solution (which ranged from 0.25 to 1 mL) and 0.1 M Tris/HCl buffer (pH=7), up to a total volume of 10 mL. In a typical procedure, Tris-HCl buffer (pH=7) and enzyme solution were placed in the thermostatically controlled cuvette of the pH-Stat and maintained at 30 °C. The mixture was shaken vigorously for 10 min. Stirring rate was adjusted to approximately 400 rpm and the substrate emulsion was added. In all cases, lipase activity was quantified as the initial reaction rate to avoid possible inhibition that might take place due to the appearance of reaction products. The slopes of the initial linear stretch of the kinetic curves were graphically determined. Experiments were performed in duplicate. Maximum deviations from the mean were less than 5 %. A unit of specific lipase activity was defined as the amount of enzyme that releases 1 μmol of free fatty acid per min and per mL of enzymatic extract.

SDS PAGE electrophoresis and isoelectric focusing

SDS-PAGE was performed in 15 % polyacrylamide gels, run on a Mini Protean II cell (Biorad Richmond, CA), and proteins were visualized by staining with Coomassie blue according to standard procedures. Molecular weight standards were supplied from Bio-Rad. Isoelectric focusing (IEF) was performed according to the Bio-Rad instructions for the MINI IEF cell. IEF standards were from Bio-Rad: broad range pI=4.45–9.60 and narrow range pI=4.45–6.10. Ampholines (pH=10–3 and 6–4) were also from Bio-Rad. Sample protein concentration was determined according to the Bradford method, using bovine serum albumin as the standard, and equal amounts of protein were loaded.

Synthesis of heptyl oleate

The reaction mixture was composed of isooctane 3 mL, 1-heptanol 20 mM, oleic acid 20 mM and 0.3 mL of bi-distilled water. The reaction was carried out at 25 °C in 10-mL flasks for 24 h. The samples were analyzed by gas chromatography using a Shimadzu GC-14 A with FID detector and split factor ½ and a SPB™ column (15 m × 0.32 mm and 0.25 μm film), Supelco Inc. Bellafonte P.A., U.S. Nitrogen was used as the carrier gas at a constant flow of 60 mL/min. Both injector and detector temperatures were 250 °C. An initial temperature of 120 °C was applied for 5 min, followed by a gradient of 15 °C/min to reach 240 °C. This temperature was maintained for 10 min. 1-Octanol was used as internal standard for calibration.

Enantioselective esterification of (R)- or (S)--2-(3-benzoylphenyl) propionic acid (ketoprofen) and other 2-arylpropionic acids

Standard reaction mixture was composed of isooctane (10 mL), *(R)*- or *(S)*-ketoprofen (66 mM) and 1-propanol (264 mM). The reaction was carried out at 30 °C with magnetic stirring in 25-mL flasks. The addition of the same weight of lipase extracts (0.2 g) started the reaction. Ester conversion was analyzed by HPLC (*15*). The conversion and the enantiomeric excess (e.e.) values were determined using a Chiracel-OD column (Daicel Chemical Ind. Japan). The mobile phase was: hexane/2--propanol/acetic acid (volume ratio 90:10:1). The flow rate was 0.5 mL/min.

Enantioselective hydrolysis of the esters of (R)- or (S)--2-(3-benzoylphenyl) propionic acid (ketoprofen) and other 2-arylpropionic acids

The enantioselective hydrolysis of these esters was performed in organic solvents saturated with 0.1 M Tris/HCl buffer (pH=7). The reaction profile was followed by HPLC, as described above.

Enzymatic alkoxycarbonylation

The octyl and butylvinylcarbonates, which were used as acyl donors, were synthesized according to a previously described methodology (*16*). The enzymatic synthesis of carbamates was performed as follows. To a solution of 1.2 mmol of vinyl carbonate and 2 mmol of *(R,S)*-1-phenylethyl amine in 15 mL of hexane, 0.2 g of catalyst was added. Temperature was 25 °C. After 72 h, the enzyme was removed by filtration. The conversion was determined by HPLC. Chromatographic separation on neutral silica of the resulting residue yielded the carbamate (eluent hexane-ethyl acetate 7:3), and the e.e. was determined by ^{1}H-NMR (*16*). For this purpose we used the chiral shift reagent Tris-[3-(heptafluoropropylhydroxy-methylene)-(+)-camphorate] europium (III) derivative (*16*). The absolute configuration of the carbamate was assigned by comparing its optical rotation value with those of pure homochiral carbamates (*17*). HPLC was performed using a Nucleosil C_{18} 120 (25 × 0.46 cm, 5 μm) column with methanol/H_2O as eluent (50:50 for butyl carbamate and 40:60 in the case of octyl carbamate); flow rate 0.3 mL/min. The compounds were detected with an UV detector at 254 nm.

Results and Discussion

Effect of inducers on the production of Candida rugosa isoenzymes

Candida rugosa (formerly *Candida cylindracea*) is a non-sporogenic, pseudofilamentous, unicellular and non--pathogenic yeast that synthesizes and secretes a mixture of lipase isoenzymes (*18*) in different proportions, depending on both the fermentation procedure and the inducer (*14*). Pure fermentation control conditions (*19,20*) or pure physiological control of cell growth (*18,21*) have been used to explain this phenomenon. These papers show the isolation, after purification, of several isoenzymes, which have been given different names by different authors: CRLA and CRLB (*22*), or L1 and L2 (*20,21*), or lip 1, lip 2, lip 3, lip 4 and lip 5 (*23,24*).

In our laboratories we have proved that both the percentage and the nature of the isoenzymes excreted by *C. rugosa* ATCC 14380 change depending on the inducer and its concentration. In the first experiment, we tested a culture medium in which the only carbon source was the inducer (*25*). In this case cell proliferation was not observed in the minimum medium, in the absence of inducers.

Table 1 shows the specific lipase and esterase activities obtained at the optimum concentration of each inducer. Note that the optimum percentage of each inducer is different and that the specific lipase activity is always lower than the specific esterase activity. This fact could be related to the presence of esterases and proteases in the cell broth, as described by Sánchez *et al.* (*21*), as will be shown later. We can consider olive and sunflower oils as quite similar inducers of esterase production. Contrarily, sunflower oil gives lower lipase activity than olive oil. This result could be explained by the lower percentage of oleic acid in sunflower oil compared to that in olive oil. Oleic acid is the best inducer for lipase production, as shown by a comparative study of the activity of different carboxylic acids as inducers, using additional carbon sources (*26*). This result is confirmed in the case of oleic and palmitic acids (Table 1). Oleic acid leads to the production of a lot of biomass, so the esterase productivity is lower than in the case of palmitic acid. Nevertheless, the use of palmitic acid leads to a poorer specific lipase activity.

Several alcohols (geraniol, farnesol, nerol, dodecanol) were tested as inducers, but only 1-dodecanol yielded good results. The inducer activity of this alcohol had been previously reported by Ushio *et al.* (*27*) for the production of lipase by *Pseudomonas* sp. Surfactants such as Tween 80 and Span 85 (oleic acid esters) led to good biomass production although the lipase activity was low (Table 1).

The total number of lipase units present in the cell culture broth, corresponding to the three highest specific activities, was 6.86 U/mL cell culture broth for 0.2 % olive oil, 3.94 U/mL cell culture broth for 0.5 % oleic acid and 1.04 U/mL cell culture broth for 0.2 % 1-dodecanol (0.2 %). We can say that the secretion of lipases by *Candida rugosa* ATCC 14830 is not related only to the oleic acid structure or to the presence of oil drops in the medium as indicated by Dalmau *et al.* (*28*) because 1-dode-

Table 1. Evaluation of the best inducers and optimum percentage in the production of biomass and lipase

φ(inducer) / %	d.c.w.[a] (g/50 mL)	Biomass (10^6 cell/mL extract)	Specific esterase activity (U/10^6 cell)	Specific lipase activity (U/10^6 cell)
None	–	–	–	–
Olive oil (0.2 %)	0.15 ± 0.01	49 ± 15	2.0 ± 0.6	0.14 ± 0.04
Sunflower oil (0.2 %)	0.17 ± 0.03	36 ± 10	2.1 ± 0.6	$<10^{-5}$
Oleic acid (0.5 %)	0.35 ± 0.02	219 ± 10	0.56 ± 0.03	0.018 ± 0.001
Palmitic acid (1 %)	0.18 ± 0.02	61 ± 1	2.5 ± 0.1	$<10^{-5}$
Dodecanol (0.2 %)	0.08 ± 0.02	40 ± 5	2.0 ± 0.1	0.026 ± 0.007
Tween 80 (0.5 %)	0.12 ± 0.01	51 ± 3	2.8 ± 0.2	$<10^{-5}$
Span 85 (0.2 %)	0.26 ± 0.02	85 ± 15	2.2 ± 0.5	$<10^{-5}$
Glycerol (0.2 %)	0.04 ± 0.01	15 ± 5	0.25 ± 0.02	$<10^{-5}$

[a] dry weight cell obtained from 50 mL of culture broth

canol is solid at the fermentor temperature and this alcohol induces the production of lipase. A somehow more complex mechanism, such as that postulated by Lotti *et al*. (*18*), must be involved in the secretion of lipases by the yeast.

The higher specific lipase activity obtained with 1-dodecanol compared to that using oleic acid (see Table 1) is explained by the fact that the first compound supports only low production of biomass. Nevertheless, the absolute lipase units are higher with oleic acid than with 1-dodecanol, as we reported previously (*14*). This finding indicates that lipase production is not simply proportional to the cell growth (*18*,*28*,*29*). Similar conclusions were reported by Montesinos *et al.* (*30*). These authors showed that the substrate/biomass ratio is the key variable for the induction of lipase using oleic acid as inducer. Besides, Lotti *et al.* (*18*) have shown that the transport to the periplasmic zone may be the limiting secretion step. Therefore we must conclude that the physical properties and the molecular structure of the inducer are important but not determinant for the enzyme production.

Fermentation of C. rugosa in the best conditions

The fermentation was scaled up to a 6-liter fermentor using variable stirring speed to maintain the saturation level of oxygen in the cell broth. Minimum culture medium and the same percentages of inducer according to Table 1 were selected.

The fermentation curves obtained with oleic acid (Fig. 1A) are qualitatively similar to those obtained using vegetable oils (data not shown). There is a maximum stirring speed zone where the cells exponentially grow, due to a high oxygen demand ($0<t<12$ h). However, both biomass production and lipase secretion take place earlier with oleic acid than with olive oil. In fact, with olive oil there is a slight delay between the secretion of enzymes and the cell growth. This was previously described by del Río *et al.* for olive oil (*31*) and is related to an inhibitory effect of the glycerol produced from the previously hydrolyzed triglycerides (vegetable oils). Therefore, the observed delay in the secretion of lipase using olive oil as the inducer, compared to oleic

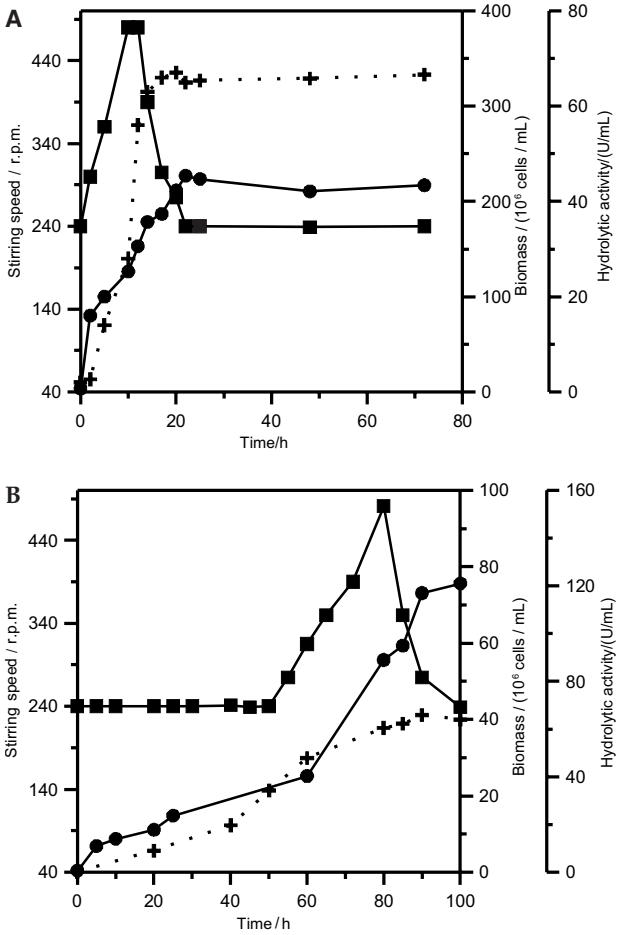


Fig. 1. Fermentation profiles of *Candida rugosa* using (A) oleic acid (0.5 %) and (B) 1-dodecanol (0.2 %), in minimum medium. The parameters studied were: biomass (10^6 cell/mL) (+), stirring speed (r.p.m.) (■), and esterase activity (U/mL) (●), measured in the hydrolysis of PNPA

acid, agrees with literature data (*14*,*18*,*27*), which also reports oleic acid as the best inducer (*26*).

The fermentation process with dodecanol (0.2 %) as inducer is shown in Fig. 1B. The stirring speed peak associated with exponential cell growth is delayed com-

pared to Fig. 1A. Further, the biomass and enzyme profiles are different from those observed with the other inducers because the maximum stirring speed is obtained at long fermentation times. Lipase secretion is observed in spite of the small cell growth. One hypothesis that might explain the differences is that it is necessary for the alcohol to be oxidized to lauric acid and that it is lauric acid that induces the production of biomass and the secretion of lipases. The relative proportion of the lipase isoenzymes is very different from the other cases, as will be shown later.

From these results, we suggest that the long chain carboxylic acids (*30*) are the true inducers of enzyme production and that the transport across the cell membrane is the rate-controlling step in the secretion of isoenzymes of *Candida rugosa* lipase (*18*). On the other hand, oleic acid is the best inducer tested, because of the small delay observed in the enzyme secretion. Nevertheless, an alcohol that is solid at 28 °C, such as 1-dodecanol, may be used as carbon source and inducer.

SDS-PAGE electrophoresis and isoelectric focusing of the extracts

SDS-PAGE of two commercial lipases and those obtained in our experimental conditions was performed. The results obtained in the densitometric assay are presented in Figs. 2 and 3. As we can see, both commercial preparations present the same quality and quantity of enzymes (Fig. 2). Two lipase peaks, which we will refer to as L1 (60.1 kDa) and L2 (58.4 kDa), were detected. The ratio of peak areas L1:L2 was approximately 80:20. The differences in molecular weights between the literature data and our results could be related to different glycosylation percentages, as described by several authors (*20*,*21*). If we compare our peaks with the literature, we can deduce that the L1 band should be CRLA and the L2 band should be CRLB (*22*).

We observed 4 extra bands. One of them, at approximately 53.9 kDa, could be the enzyme referred to in previous literature as a protein with an N-terminal composition not found in the Protein Data Bank (*21*). The other bands, at approximately 45.9 kDa, should be a carboxyesterase (*21*,*32*,*33*). Between the band of 53.9 kDa and the carboxyesterase of 45.9 kDa, we found a protein of 48.6 kDa, the nature of which is still unknown. We can conclude that the two commercial samples have the same qualitative composition but that the lipase lots have different percentages of protein. This result agrees with Bradford assays (1.2 % of protein, referred to mass, in the case of Sigma lipase and 1.8 % in the case of Roche lipase).

Fig. 3 shows the densitometry profiles obtained after SDS-PAGE of the crude extracts obtained in the fermentation experiments reported in the previous section. The profile for the commercial CRL from Sigma is shown in order to facilitate comparisons. In the case of the extract from the fermentation in which oleic acid was used as the inducer (Fig. 3A), the sample presents the same two peaks of lipases as the commercial preparation, but with the proportion of L2 being greater than that of L1. According to previous literature (*21*), when *C. rugosa* was cultured in this medium with oleic acid as

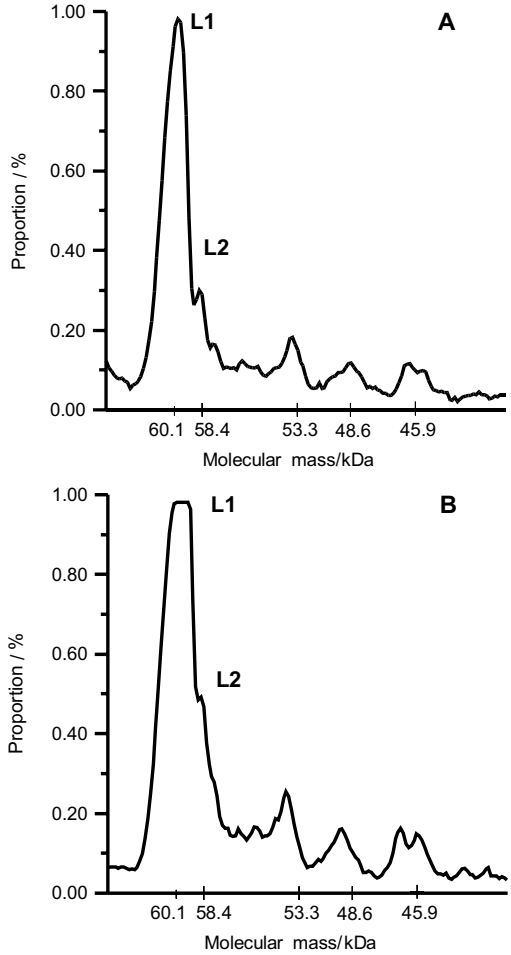


Fig. 2. Densitometry analysis of (A) commercial lipase from Sigma, and (B) commercial lipase from Roche

the only carbon source, only Lip-2 (57.7 kDa) and Lip-3 (62 kDa) (as named by the authors) were secreted. It is possible that our peaks correspond to these enzymes (*i.e.* that L1 is Lip-3 and L2 is Lip-1) but this cannot be affirmed unequivocally. On the other hand, all the esterases and proteins found in commercial samples are present in our crude biocatalysts, although in higher amounts than in the commercial samples.

In the case of the lipase obtained using olive oil as the inducer in the fermentation (Fig. 3B), the densitometric profile is qualitatively analogous to that obtained when oleic acid was used as the inducer (Fig. 3A). Nevertheless, the proportion of lipase relative to the other enzymes is higher in this case than in Fig. 3A. As a consequence, we postulate that the bands at 53.3, 48.6 and 45.9 kDa are induced by oleic acid. The higher enzymatic activity observed in the hydrolysis of *p*NPA by the crude lipase produced using olive oil as the inducer, compared to that obtained with the crude enzyme produced using oleic acid as the inducer (Table 1), could be related to the higher proportion of other hydrolytic enzymes, different from lipases.

In the case when sunflower oil is used as the inducer (Fig. 3C), the lipase peaks are, once again, L1 (60.1 kDa) and L2 (58.4 kDa), with L2 being present in a higher proportion. The bands at 48.4 and 45.9 kDa are

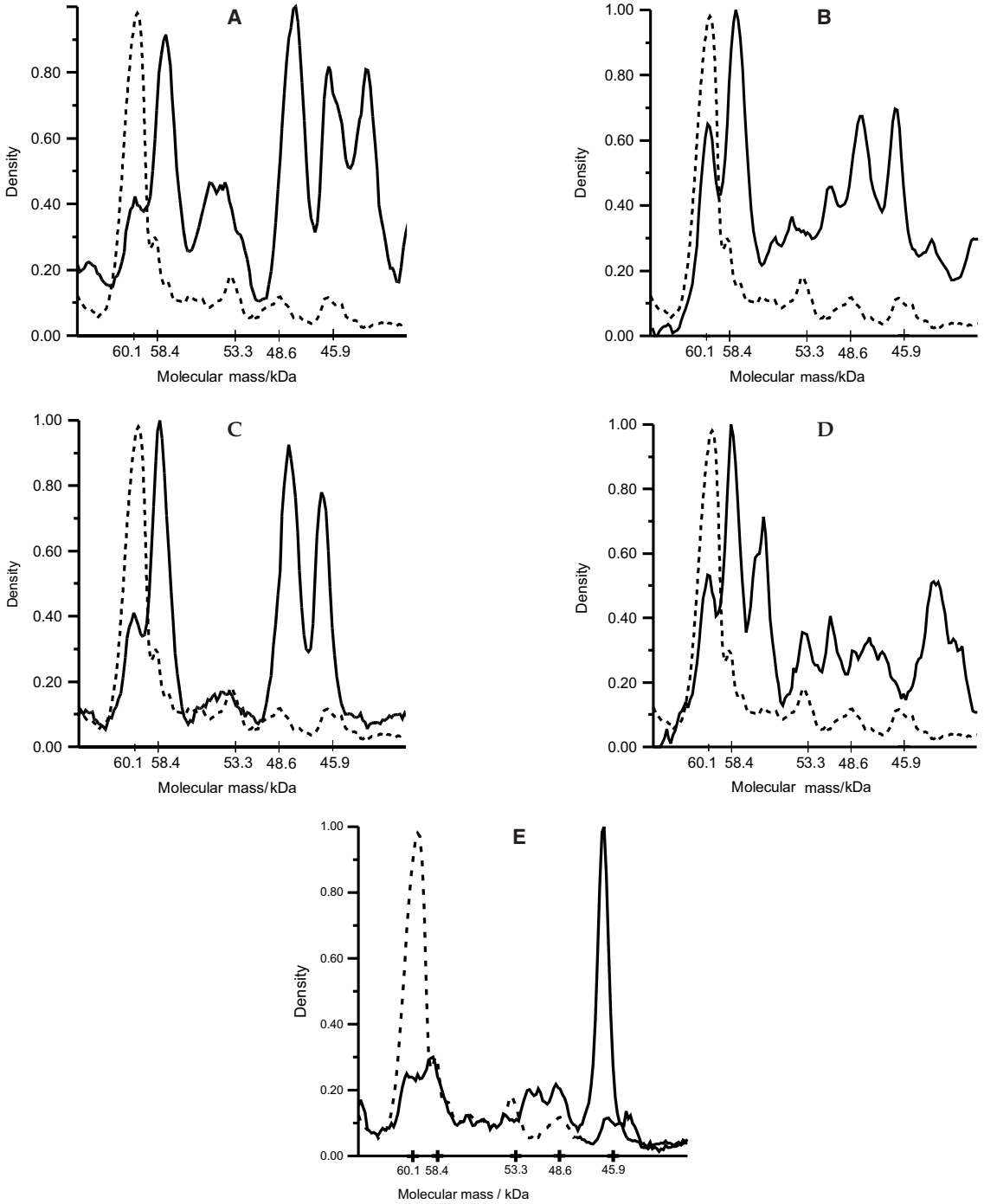


Fig. 3. Densitometry analysis of crude lipases of *C. rugosa* obtained with different inducers (%) in minimum medium (—), and comparison with commercial lipase from Sigma (- - -). (A) Oleic acid (0.5 %), (B) olive oil (0.2 %), (C) sunflower oil (0.2 %), (D) 1-dodecanol (0.2 %) and (E) glycerol (0.2 %)

also present at high levels compared to the commercial samples. These enzymes must be esterases that lack lipase activity since, as shown in Table 1, the lipase activity is very low.

Finally, the densitometry pattern of the crude enzyme obtained with 1-dodecanol (Fig. 3D) is completely different from the other cases, showing the importance of the chemical structure of the inducers in the production of extracellular enzymes. Peaks similar to peaks L1 and L2 of the commercial sample are present, but in the opposite relative proportion (*i.e.* L1<L2). Further, proteins with molecular mass below 44 kDa are the main components of the crude extract. In the extract of the fermentation in which 1-dodecanol was the inducer, there was a new peak, at approximately 55.4 kDa, which corresponds to a protein that has not yet been identified.

In summary, we can conclude that under the conditions in our fermentations L1 is the minor component

(L1<L2), which contrasts with the commercial samples (Fig. 2). Other extracellular proteins with different molecular mass or the same proteins in different relative proportions are produced, depending on the carbon source and inducer used. The percentage of inducer only influences the amount of total protein secreted and not the densitometry profile (data not shown).

To confirm the hypothesis of del Rio *et al.* (*31*) about the inhibitory effect of glycerol on lipase production, we carried out the fermentation using glycerol as the only carbon source. Biomass production was observed (Table 1). The densitometry analysis of crude extracellular enzymes and of the commercial lipase is shown in Fig. 3E. From this graph, we can see that L1 and L2 peaks are very small compared to commercial sample and to the crude lipases obtained by us. This finding indicates that glycerol is not an inducer of lipases. While the secretion of lipases seems to be reduced by glycerol, one of the carboxyesterases of 45.9 kDa (*21*,*30*,*33*) is over-expressed, while the other peaks appear in a very low concentration.

Characterization of the enzymes of C. rugosa obtained in different degrees of purification

Dialysis and adsorption column purification methods

Dialysis purification of crude *Candida rugosa* commercial sample from Sigma (LCRC) was carried out using a membrane with a 20 kDa cut-off (*34*). The preparation obtained was named LCRS and shows a lower enzymatic activity than the commercial preparation CRL that is described in the literature (*34*).

In addition, this commercial lipase was purified to a higher level than in CRSL, according to the procedure described by Rúa *et al.* (*22*). The results in Table 2 show that highly purified samples L1 and L2 are more active than commercial lipase LCRC. L2 is more active than L1 in both hydrolytic reactions. Unfortunately L1 and L2 are less stable (half lives of 0.28 and 0.23 h) than crude (LCRC) and semi-purified lipase (LCRS). This result can be explained because the lactose monohydrate (*14*) present in the crude lipase (LCRC) is 34.8 % of the biomass and it is removed in the purification. This monosaccharide acts as a shield against the deactivation associated with the conformational change of the protein, induced by collision between protein and water molecules (*35*,*36*).

Table 2. Half life, esterase and lipase specific activities of the lipase of *C. rugosa* in preparations of different degrees of purification

Enzyme	Half life / h	Stability factor[a]/%	Lipase activity[b]	Esterase activity[c]
LCRC	0.5	100	9.0 ± 0.5[d]	236 ± 12[d]
LCRS	0.38	76	5.0 ± 0.3[d]	207 ± 10[d]
L1	0.28	56	253 ± 12[e]	14.000 ± 700[e]
L2	0.23	46	290 ± 14[e]	19.100 ± 0.100[e]

[a] (half life of biocatalysts/half life of LCRC) · 100
[b] using triolein as the substrate
[c] using *p*-nitrophenyl butyrate
[d] U/mg solid (crude or semi-purified lipase)
[e] U/mg protein

Semi-purification by precipitation with ammonium sulfate or with organic solvents

Due to the increase in the biocatalyst costs associated with purification and because of the fact that the productivity is very low (25 g of commercial lipase gave 100 mg of L2 and 40 mg of L1), we tried to develop other semi-purification methods that would allow us to obtain a biocatalyst with acceptable price and higher catalytic activity compared to LCRC (*37*,*38*).

These purification methods gave us samples with higher specific lipase activity compared to specific esterase activity (Table 3). Therefore a good purification level of lipase was achieved. Precipitation with ammonium sulfate gave a good catalyst in the hydrolysis of small esters (esterase activity).

In the cases of precipitation with organic solvents, the specific activity increased in the purified fractions. The best biocatalysts were E-CRL and A-CRL (specific lipase activity, Table 3). Three possible mechanisms could

Table 3. Partial purification of *C. rugosa* lipase using ammonium sulfate or organic solvent precipitation procedures

Enzyme	log P[a]	Protein /mg[b]	Lipase			Esterase		
			Activity U	Specific activity (U/mg)	Purif. (fold)	Activity U	Specific activity (U/mg)	Purif. (fold)
CRL (lot54)	–	89.6	11.9	133	1	88.7	1	1
SA-CRL[c]	–	4.3	3.4	783	5.7	6.03	1.4	1.4
2P-CRL[d]	0.16	8.9	2.8	319	2.4	5.4	0.6	0.6
1P-CRL[e]	0.34	8.6	1.9	223	1.7	2.0	0.2	0.2
E-CRL[f]	0.19	4.5	1.4	317	2.4	20.5	4.6	4.6
CRL (lot85)	–	93.8	11.6	124	1	112.6	1.2	1
A-CRL[g]	0.16	10.8	3.7	337	2.7	28.6	2.6	2.2
M-CRL[h]	0.72	12.8	3.4	286	2.3	3.1	0.2	0.2
2B-CRL[i]	0.69	24.3	6.8	279	2.2	6.6	0.3	0.2
1B-CRL[j]	0.88	24.7	4.6	188	1.5	3.3	0.1	0.1

[a] log P values of the solvents (*39*); [b] total amount of protein; [c] 60 % saturation; [d] 40 % 2-propanol/Mes buffer (50 mM, pH=6) on CRL; [e] 30 % 1-propanol/Mes buffer (50 mM, pH=6) on CRL; [f] 40 % ethanol/Mes buffer (50 mM, pH=6) on CRL; [g] 30 % acetone/Mes buffer (50 mM, pH=6) on CRL; [h] 30 % methanol/Mes buffer (50 mM, pH=6) on CRL; [i] 30 % 2-butanol/Mes buffer (50 mM, pH=6) on CRL; [j] 30 % 1-butanol/Mes buffer (50 mM, pH=6) on CRL

be postulated to explain why these treatments increase the specific activity: (*i*) by removing other hydrolytic enzymes (esterases, proteases, *etc.*) with lower enzymatic activities; (*ii*) by removing non-protein contaminants that inhibit the lipase and decrease its catalytic activity (SDS-PAGE shows that the treatment removes small proteins with M_r<45 kDa); and (*iii*) by changing the conformation of the native lipase and, as a consequence, activating the biocatalysts. The opening of the lid requires a *cis-trans* isomerization of a prolyl amide link at residues Ser-91-Pro-92 (*40*). Organic solvents that accelerate the *cis-trans* isomerization of prolyl amide residues have been described (*41*).

Thermal stability assays of these biocatalysts were performed at 50 °C. The activity values were measured using tributyrin as substrate and were fitted using the EXFIT program from the package SIMFIT v.4.0 to a double exponential decay according to the Henley and Sadana model (*42*). Except for SA-CRL and 1B-CRL, our derivatives were more stable than CRL.

Stabilization of enzymes

Immobilization by attachment of the protein to a solid support, which increases the rigidity of the protein, is the most useful methodology to stabilize biocatalysts against thermal deactivation and deactivation by organic solvents. In Table 4 we show the results obtained for covalent immobilization on agarose or silica.

Table 4. Stability of several lipases of *C. rugosa* stored at 50 °C in wet conditions

Lipase	Support	Half life/h	Stability factor
LCRC		0.5	1
LCRS		0.38	0.76
L1		0.28	0.56
L2		0.23	0.46
LCRC	Agarose	27	5.4
LCRS	Agarose	16	32
LCRA	Agarose	5	10
LCRB	Agarose	4	8
LCRC	Silica	19	38
LCRS	Silica	10	20
LCRA	Silica	5	10
LCRB	Silica	4	8

The immobilization was performed by the tosyl method in the case of agarose (*43*) and by the cyanuric chloride method in the case of silica (*44*). The stability at 50 °C in wet conditions is improved in all cases as indicated by the values of the stability factors (ratio between the half life of the immobilised lipases and of the commercial lipase (LCRC)). Relative thermal stability is strongly related to the stability of the native protein. Indeed in both supports (agarose and silica) relative stability is:

LCRC > LCRS > L1 and L2

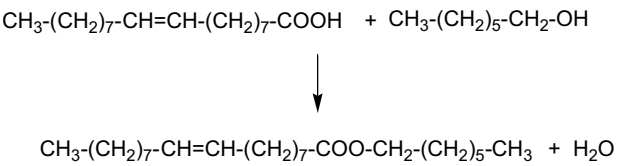
Therefore, a high purification degree of commercial *C. rugosa* lipase is not interesting for large-scale synthesis, because purified enzymes are costly and have low thermal stability, even when immobilized on different supports.

Catalytic activity of various catalyst preparations

We have shown that many different biocatalysts can be prepared from the classic *Candida rugosa* lipase. These catalysts differ with respect to their isoenzyme composition, thermal stability, water content in the microenvironment and hydrolytic activity. In this section we will compare the catalytic activities of our various preparations in various lipase-catalyzed reactions in organic media and in various hydrolytic reactions in aqueous media. The knowledge of the biocatalyst obtained in the previous sections allows us to explain the relative catalytic activity of the different preparations, showing that enzymes should not simply be considered as a »white magic powder« that will resolve our problems in chemical synthesis. We need a multidisciplinary study of different aspects of the biocatalysts in order to understand their catalytic action and to obtain an appropriate biocatalyst for each particular application that we might wish to scale up.

Influence of the isoenzyme composition on heptyl oleate synthesis

We propose the synthesis of heptyl oleate, which occurs according to Scheme 1,



Scheme 1

as a test to confirm the presence of lipases in solid crude biocatalysts, due to the irreproducible nature of the results obtained when these biocatalysts are used in hydrolysis reactions. The problem is that any esterases or proteases present in the crude biocatalyst can contribute to the hydrolytic activities observed (*45*). Our results for heptyl oleate synthesis by several of our preparations are shown in Table 5.

Table 5. Yield of heptyl oleate at 24 h of reaction using crude lyophilised enzymes produced in a fermentation involving minimum medium and the specified concentration of inducer

Inducer/%	Ester yield (24 h)/%
Commercial (Roche)	92
Commercial (Sigma)	90
Oleic acid (0.5 %)	87
Olive oil (0.2 %)	78
Sunflower oil (0.2 %)	75
1-Dodecanol (0.2 %)	86
Glycerol (0.2 %)	13

Two commercial lipases from different suppliers were also tested. Both gave a similar yield at 24 h (Table 5). This result is consistent with the similar densitometry patterns obtained in Fig. 2, in which both preparations have two main peaks of lipase ($M_r \approx 60$ kDa). The preparation obtained using glycerol as the inducer (0.2 %), which gave very low lipolytic activity (Fig. 3E), gave a crude biocatalyst with a very low synthetic activity (13 % at 24 h). This result confirms the specificity of this assay as a test for catalytic activity of lipases.

The other crude biocatalysts gave similar ester yields at 24 h (Fig. 3). The values obtained are similar to those obtained with the commercial biocatalysts, in spite of the fact that the relative proportions of L1 and L2 in the commercial crude samples (Fig. 2) and the preparations produced in the present work are opposite. Therefore, this assay is not useful to distinguish between isoenzymes. Besides, these results show that the relative lipase activity measured in hydrolytic reactions (Table 1) is not useful to evaluate the enzymatic activity of crude biocatalysts in reactions catalysed in organic media (see Table 5). This is not surprising, since the two reactions take place under different physical conditions: the hydrolysis reaction is catalysed by a soluble enzyme at an oil/water interface, while the synthesis reaction is catalysed by a solid lyophilised enzyme in a homogeneous organic medium with a water activity value (a_w) near 1.0 (*46*).

Influence of the isoenzyme composition on enantioselective esterification of (*R*)- or (*S*)-ketoprofen

The esterification of *(R)*- or *(S)*-ketoprofen with *n*-propanol (Scheme 2) was performed using our enzymes as biocatalysts to explore the ability of the crude lyophilised biocatalysts to recognize large chiral aromatic carboxylic acids. The yields (Table 6) were, in general, low, possibly due to the low water activity value of the medium (a_w<0.5) (*19*). The crude biocatalyst obtained from the fermentation that had glycerol as the carbon source was not active, as expected. Very low yields (<5 %) were obtained with the crude lipase produced with sunflower oil or palmitic acid (data not shown).

The commercial lipase was moderately (*S*)-stereoselective in these conditions (*S/R* ratio=8.5), while our lipases were not very stereoselective at all, producing *S/R* ratios close to 1. This fact could be related to the different compositions of the mixture of extracellular lipases that we observed for production under different conditions. Commercial lipases (Fig. 2) show near 80 % of L1 and 20 % of L2. Contrarily, crude biocatalysts obtained using oleic acid or olive oil as inducers have L2>L1 (Fig. 3). Therefore we can hypothesise that L2 is *R*-stereoselective while L1 is *S*-stereoselective.

Table 6. Enzymatic esterification of (*R*)- or (*S*)-ketoprofen using crude lipases produced in minimum medium

Inducer/%	Yield (*R*)-ester (500 h)/(%)	Yield (*S*)-ester (500 h)/(%)	e.e./% (*S*/*R*)
Commercial lipase[a]	2	17	79
Oleic acid (0.5 %)	10.8	13.2	10
Olive oil (0.2 %)	8.5	11.6	15
1-Dodecanol (0.2 %)	12.8	11	7.5 (*R*>*S*)

[a] commercial lipase from Roche

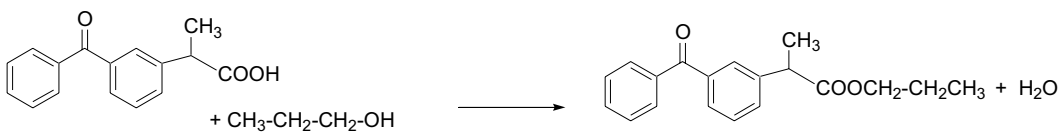
Crude lyophilised lipase obtained using 1-dodecanol as inducer and carbon source shows opposite enantioselectivity. This result indicates that changing the structure of the inducer from a fatty acid to a fatty alcohol not only changes the fermentation curves (Figs. 1A and 1B) but also the relative proportion and composition of the isoenzymes in the bands L1 and L2. Nevertheless, this affirmation is only qualitative because 5 lipase genes have been cloned and expressed (*23*,*24*). As a consequence, we should have to accept that our L1 and L2 are mixtures of isoenzymes.

Influence of the isoenzyme composition on enzymatic alkoxycarbonylation of 1-phenylethylamine

We explored the alkoxycarbonylation of *(R,S)*-1-phenylethylamine with *n*-butyl or *n*-octyl vinyl carbonate to give carbamates (Scheme 3).

Usually, a moderate e.e. is achieved (*16*) with commercial lipases. As expected, carbamate was not obtained with the enzyme produced with glycerol as inducer (Table 7). These results support the presence of esterases in this crude biocatalyst.

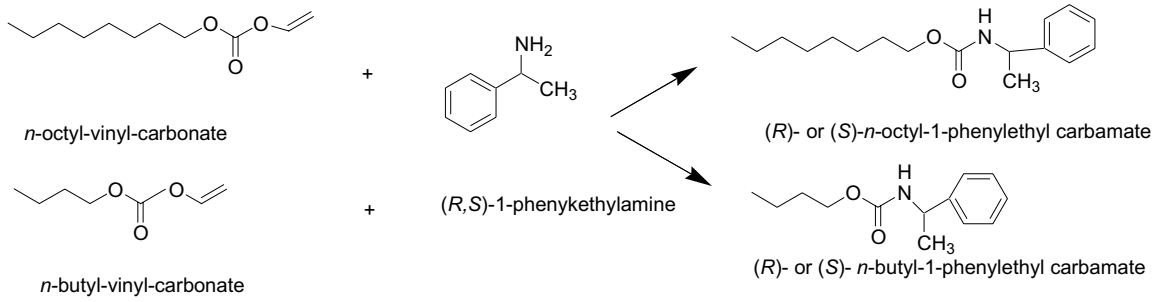
Generally, better yields were achieved using *n*-butyl than *n*-octyl vinylcarbonate as acyl donor, probably due to steric reasons associated with the acyl donor reagent. The process was *(R)*-stereoselective, as described for several commercial lipases (*16*). Nevertheless, the yield and the enantiomeric excess are different for each crude biocatalyst. These differences must be related to the different amount of lipase and to the different percentages of isoenzymes in the lyophilised crude biocatalysts. The result achieved with the lipase obtained using olive oil (2 %) as the inducer is very interesting. In order to interpret these results, it would be necessary to determine



(*R*)- *or* (*S*)-ketoprofen

(*R*)- *or* (*S*)-ketoprofen *n*-propyl ester

Scheme 2



Scheme 3

Table 7. Enzymatic alkoxycarbonylation of (*R,S*)-1-phenyl-ethyl-amine with *n*-butyl or *n*-octyl-vinyl-carbonate

Inducer/%	with *n*-butyl-vinyl-carbonate		with *n*-octyl-vinyl-carbonate	
	Yield (72 h)/%	e.e./% (*R*>*S*)	Yield (72 h)/%	e.e./% (*R*>*S*)
Commercial lipase[a]	77	30	41	30
Oleic acid (0.5 %)	34	19	41	20
Olive oil (0.2 %)	57	28	10	99
1-Dodecanol (0.2 %)	65	48	1	n.d.
Sunflower oil (0.2 %)	24	44	6	n.d.

[a] commercial lipase from Roche
n.d. not determined

the stereoselectivity of each isoenzyme: lip 1 to lip 5 (*23*,*24*). Unfortunately, a large amount of biocatalyst would be necessary to perform such a study.

Influence of the purification degree on catalytic activity of purified lipases L1 and L2

The hydrolysis of ethyl esters of different (*R,S*)-2--arylpropionic acids was performed with lipases of different purification degrees in order to determine the catalytic activity of these enzymes against unnatural substrates.

In all cases the amount of protein was 1.72 mg, but the amount of biocatalyst was different, due to the different purification degree of the enzymes. The greater the purification degree the greater the yield. This behaviour is associated with an increase in the specificity constant ($k_s=k_{cat}/K_m$) as described by Gu and Sih (*47*). This situation is general for all the substrates tested (*48*).

LCRS is more active than LCRC. This behaviour is opposite to that observed in the hydrolysis of olive oil or of *p*-nitro-phenylacetate (Table 2). This could be related to the different structure of the carboxylic acids (oleic acid is linear and aliphatic, while the 2-arylpropionic acids are branched and have an aromatic ring). In Fig. 4 we show that, depending on the structure of the substrate, the yield achieved is different for each biocatalyst. Therefore, the hydrolysis of esters is a good reaction to compare the biocatalysts, better than alkoxycarbonylation of amines because a low amount of biocatalyst is necessary. In all cases, high e.e. values are achieved. Probably these good results are associated with the value of a_w, which was maintained near 1.0 (Fig. 4) (*35*,*36*).

Relative reactivity of the ethyl esters is: (*R,S*)-2-(4--isobutyl phenyl)-propionic acid (ibuprofen) ≥ (*R,S*)-2--(6'-methoxy-2-naphthyl)propionic acid ≥ (*R,S*)-2-(3-benzoyl-phenyl)-propionic acid (ketoprofen). This behaviour is related to the steric hindrance associated with the aromatic ring. In all cases the hydrolysed ester was the (*S*)--isomer with 98 % e.e. and yields near 50 %.

Influence of the support on the hydrolysis of (*R,S*)-2-arylpropionate ethyl ester

Since the hydrolysis of esters of 2'-arylpropionic acids is the most interesting reaction to compare the new biocatalysts, we performed the reaction using immobilised biocatalysts. LCRS was immobilised because it is purer, more active in hydrolysis and as stable as LCRC (Table 4). Such immobilized biocatalysts are interesting for the resolution of non-steroidal anti-inflammatory drugs with a 2-arylpropionic acid structure, as is shown in Table 8. Good e.e. values are obtained and there is not a dramatic effect of the support in the process.

Re-use of the biocatalysts

After the first assay (168 h) each solid biocatalyst was recovered, washed and re-assayed with fresh substrate mixture in the same experimental conditions as the first experiment. This process was repeated three times. The immobilized derivatives retained 80 % of their initial activity after 336 h. However, the immobilized lipase lost 50 % of this original activity after the fourth re-use. The immobilized derivatives presented greater operational stability when the reactions were carried out in a packed bed reactor (95 % of activity retained after 400 h) (*49*).

Conclusion

The widely used crude lipase from *C. rugosa* is a very complex biocatalyst because of the fact that there are at least 5 lipase genes present in the yeast. Each gene is expressed under different micro-environmental conditions. As a result, the composition of the lipase preparation, in terms of the relative proportions of the various isoenzymes, depends on the experimental conditions. Conse-

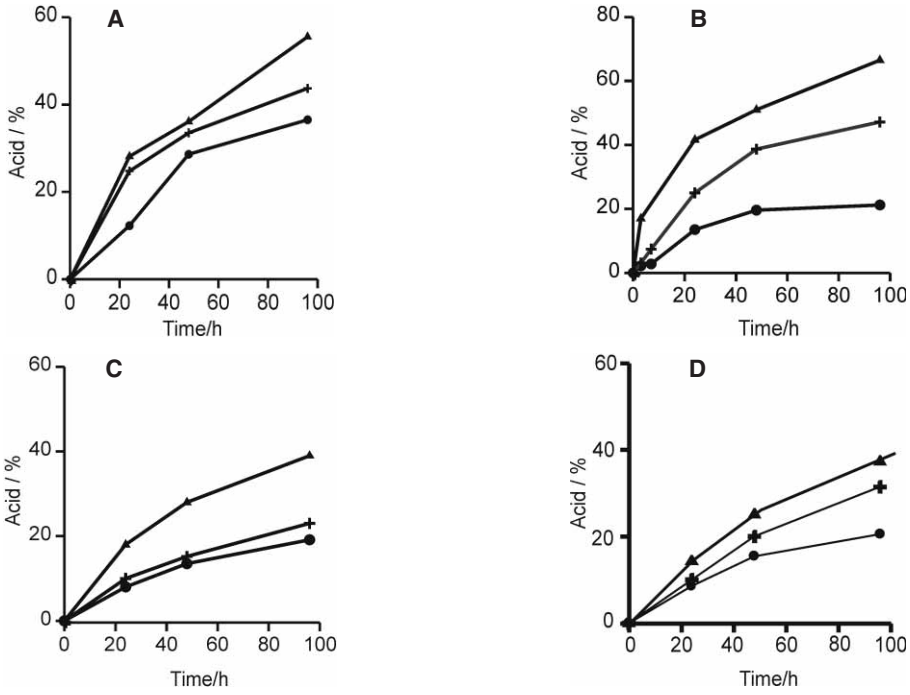


Fig. 4. Hydrolysis of different racemic esters of (*R,S*)-2-arylpropionic acid using *Candida rugosa* lipase, in different purification degrees: commercial lipase from Sigma (LCRC (●)); semi-purified lipase (LCRS (✚)); purified lipase (L2 (▲)). (A) ethyl ester of (*R,S*)-2-phenylpropionic acid; (B) ethyl ester of (*R,S*)-2-(4-isobutylphenyl) propionic acid; (C) ethyl ester of (*R,S*)-2-(6′-methoxy-2-naphthyl) propionic acid; (D) ethyl ester of 2-(3′-benzoylphenyl) propionic acid

Table 8. Enantioselective hydrolysis of esters of racemic steroidal anti-inflammatory drugs using immobilized biocatalysts. Lipase LCRS immobilized on different supports

Support	Substrate	Yield in acid (168 h)/%	e.e./% (*S*>*R*)
Alumina	Ibuprofen	31	100
	Ketoprofen	14.5	95
	Naproxen	34	98
Silica	Ibuprofen	24	98
	Ketoprofen	20.5	97
	Naproxen	29	100
Agarose	Ibuprofen	9.5	100
	Ketoprofen	21	94
	Naproxen	20	95

quently, the catalytic activity and enantioselectivity are not reproducible when different lots from the same or different suppliers are used. Strategies to obtain reproducible biocatalysts include controlling the conditions in the fermentor and purifying or semi-purifying the lipolytic product. Heptyl oleate synthesis, alcoxycarbonylation of amines and hydrolysis of the esters of ketoprofen are excellent test reactions for lipase samples.

References

1. K. H. Jaeger, S. Ransac, B. W. Dickstra, C. Colsoc, M. van Henvel, O. Misset, *FEMS Microb.Rev. 15* (1984) 29–63.
2. A. Kovac, H. Scheib, J. Pleiss, R. D. Schmidt, F. Paltauf, *Eur. J. Lipid. Sci. Technol. 102* (2000) 61–77.
3. F. Cárdenas, E. Álvarez, M. S. de Castro-Álvarez, J. M. Sánchez-Montero, S. Elson, J. V. Sinisterra, *Biocatal. Biotransform. 19* (2001) 315–329.
4. F. Cárdenas, E. Álvarez, M. S. de Castro-Álvarez, J. M. Sánchez-Montero, M. Balmaceda, S. Elson, J. V. Sinisterra, *J. Mol. Catal. B-Enzym. 14* (2001) 111–123.
5. F. Martínez-Alzamora, A. R. Alcántara, J. V. Sinisterra, J. M. Sánchez-Montero: Characterisation of Two New Lipases from *Pseudomonas cepacia* and *Fusarium oxysporum*. In: *Recent Research Development in Biotechnology and Bioengineering, Vol. 5*, Research Signpost, Trivandrum (2003) pp. 55–64.
6. M. Arroyo, J. V. Sinisterra, *J. Org. Chem. 59* (1994) 4410–4417.
7. D. R. Duarte, E. Castillo, E. Bárzana, A. López-Mungía, *Biotechnol. Lett. 22* (2000) 1811–1814.
8. F. J. Plou, M. A. Cruces, M. Ferrer, G. Fuertes, E. Pastor, M. Beranbé, M. Christensen, F. Comelles, J. L. Parra, A. Ballesteros, *J. Biotechnol. 96* (2002) 55–66.
9. S. M. Roberts: *Biocatalysis for Fine Chemical Synthesis*, Wiley & Sons, New York (1999).
10. H. Stamatis, P. Cristakopoulos, D. Kekos, B. J. Macris, F. N. Kolisis, *J. Mol. Catal. B-Enzym. 4* (1999) 229–236.
11. A. Kamal, M. Sandbhor, K. Ahmed, S. F. Adil, A. A. Shaik, *Tetrahedron: Asymmetry, 14* (2003) 3861–3866.
12. M. J. Sorgedrager, R. Malpique, F. van Rantwijk, R. Sheldon, *Tetrahedron: Asymmetry, 15* (2004) 1295–1299.
13. M. S. de Castro, J. V. Sinisterra, *Tetrahedron, 54* (1998) 2877–2892.
14. P. Domínguez de Maria, J. V. Sinisterra, *Tetrahedron, 55* (1999) 8555–8566
15. J. M. Moreno, M. J. Hernáiz, J. M. Sánchez-Montero, J. V. Sinisterra, M. T. Bustos, M. E. Sánchez, J. F. Bello, *J. Mol. Catal. B-Enzym. 2* (1997) 177–184.
16. M. S. de Castro, P. Domínguez, J. V. Sinisterra, *Tetrahedron, 56* (2000) 1387–1391.
17. M. Pozo, V. Gotor, *Tetrahedron, 49* (1993) 4321–4326.

18. M. Lotti, S. Monticelli, J. L. Montesinos, S. Brocca, F. Valero, L. Lafuente, *Chem. Phys. Lipids*, *93* (1998) 143–148.
19. A. Sánchez, R. M. de la Casa, J. V. Sinisterra, F. Valero, J. M. Sánchez-Montero, *Appl. Biochem. Biotechnol. 80* (1999) 65–75.
20. A. Sánchez, P. Ferrer, A. Serrano, F. Valero, C. Solà, M. Pernas, M. L. Rúa, R. Fernández-Lafuente, J. M. Guisán, R. M. de la Casa, J. V. Sinisterra, J. M. Sánchez-Montero, *J. Biotechnol. 69* (1999) 169–182.
21. A. Sánchez, P. Ferrer, A. Serrano, M. A. Pernas, F. Valero, M. L. Rúa, C. Casas, C. Solà, *Enzyme Microb. Technol. 25* (1999) 214–223.
22. M. Rúa, T. Díaz-Mauriño, V. M. Fernández, C. Otero, A. Ballesteros, *Biochim. Biophys. Acta. 1156* (1993) 181–191.
23. S. Benjamin, A. Pandey, *Yeast*, *14* (1998) 1069–1087.
24. M. Lotti, A. Tramontano, S. Longhi, *Protein Eng. 7* (1994) 531–535.
25. J. L.del Río, P. Serra, F. Valero, M. Poch, C. Solà, *Biotechnol. Lett. 12* (1990) 835–838.
26. N. Obradors, J. L. Montesinos, F. Valero, J. Lafuente, C. Sola, *Biotechnol. Lett. 15* (1993) 357–360.
27. K. Ushio, T. Hirata, K. Yoshida, M. Sakaue, C. Hirose, T. Suzuki, T. M. Ishizuka, *Biotechnol. Tech. 10* (1996) 267–272.
28. E. Dalmau, A. Sanchez, J. L. Montesinos, F. Valero, J. Lafuente, C. Casas, *J. Biotechnol. 59* (1998) 183–192.
29. M. A. Gordillo, J. L. Montesinos, C. Casas, F. Valero, J. Lafuente, C. Solà, *Chem. Phys. Lipids*, *93* (1998) 131–142.
30. J. L. Montesinos, J. Lafuente, M. A. Gordillo, F. Valero, C. Solà, S. Charbornnier, A. Cheruy, *Biotechnol. Bioeng. 48* (1995) 573–584.
31. L. del Río, P. Serra, V. Valero, M. Poch, C. Solà, *Biotechnol. Lett. 12* (1990) 835–838.
32. M. A. Diczfalusy, S. E. H. Alexon, *Arch. Biochem. Biophys. 334* (1996) 104–112.
33. M. A. Diczfalusy, U. Hellman, S. E. H. Alexon, *Arch. Biochem. Biophys. 348* (1997) 1–8.
34. J. M. Sánchez-Montero, V. Hamon, D. Thomas, M. D. Legoy, *Biochim. Biophys. Acta*, *1078* (1991) 345–350.
35. R. M. de la Casa, J. M. Sanchez-Montero, J. V. Sinisterra, *Biotechnol. Lett. 21* (1999) 123–128.
36. R. M. de la Casa, J. M. Guisan, J. M. Sanchez-Montero, J. V. Sinisterra, *Enzyme Microb. Technol. 30* (2002) 30–40.
37. S. Chamorro, A. R. Alcántara, J. M. Sanchez-Montero, J. V. Sinisterra, *Stability and Stabilization of Biocatalysts*, A. Ballesteros, F. Plou, J. L. Iborra, P. Halling (Eds.), Elsevier Science BV (1998) pp. 741–746.
38. S. Chamorro, J. M. Sánchez-Montero, A. R. Alcántara, J. V. Sinisterra, *Biotechnol. Lett. 20* (1999) 499–505.
39. C. Hansch, A. Leo, D. Hoeckman: *Exploring QSAR. Hydrophobic, Electronic and Steric Constants*, ACS Professional Reference Book, American Chemical Society, Washington DC (1995).
40. P. Grochulsky, L. Young, F. Scharg, F. Bouthiller, P. Smith, D. Harrison, B. Rubin, M. Cygler, *J. Biol. Chem. 268* (1993) 12843–12849.
41. A. Radzcika, S. A. Acheson, R. Wolfender, *Bioorg. Chem. 20* (1992) 282–290.
42. J. P. Henley, A. Sadana, *Enzyme Microb. Technol. 7* (1985) 50–61.
43. M. Arroyo, J. M. Moreno, J. V. Sinisterra, *J. Mol. Catal. 83* (1993) 261–271.
44. J. M. Moreno, J. V. Sinisterra, *J. Mol. Catal. 83* (1994) 357–369.
45. P. Domínguez de Maria, F. Martínez-Alzamora, M. Rua, J. M. Sánchez-Montero, J. V. Sinisterra, A. R. Alcántara, *Enzyme Microb. Technol. 31* (2002) 283–288.
46. J. M. Sánchez-Montero, R. M. de la Casa, J. V. Sinisterra, In: *Stability and Stabilization of Biocatalysts*, A. Ballesteros, F. J. Plou, J. L. Iborra, P. Halling (Eds.), Elsevier Science (1998) pp. 4353–458.
47. Q. M. Gu, Ch. J. Sih, *Biocatalysis*, *6* (1992) 115–26.
48. M. J. Hernáiz, J. M. Sánchez-Montero, J. V. Sinisterra, *J. Mol. Catal. 96* (1995) 317–327.
49. J. M. Moreno, J. V. Sinisterra, *J. Mol. Catal. A-Chem. 98* (1995) 171–184.

Rezolucija racemata kiselina, estera i amina pomoću lipaze iz *Candida rugosa* u slabo hidratiziranim organskim otapalima

Sažetak

Komercijalna kruta lipaza iz *Candida rugosa* uvelike se koristi kao biokatalizator pri rezoluciji racemijskih smjesa te u organskoj sintezi u slabo hidratiziranim organskim otapalima. Kada se koristi sirova lipaza, dobivena iz različitih šarža dobavljača, često se ne dobivaju reproducibilni rezultati zbog prisutnosti različitih izoenzima. U radu je taj problem osobito istaknut, a i načini njegova rješavanja. Kvasac *Candida rugosa* ATCC 14380 uzgajan je u minimalnoj podlozi koristeći razne spojeve kao induktore i izvore ugljika. Određen je i postotak induktora koji osiguravaju optimalnu proizvodnju ekstracelularnih lipaza. Liofilizirani ekstracelularni enzimi karakterizirani su SDS-PAGE-elektroforezom i izoelektričnim fokusiranjem (IEF). Ovisno o prirodi izvora ugljika dobivena je različita količina izoenzima. Ti su uzorci djelomično pročišćeni različitim postupcima, i to dijalizom, adsorpcijskom kromatografijom i taloženjem s amonijevim sulfatom ili organskim otapalima. Time se objasnila relativna katalitička aktivnost različitih uzoraka, pokazujući da se enzimi u biokatalizi ne mogu smatrati »bijelim magičnim prahom« koji može riješiti sve poteškoće u organskoj sintezi. Sinteza heptiloleata, alkoksikarbonilacija amina i hidroliza estera ketoprofena odlične su test-reakcije za procjenu uzoraka lipaze kao biokatalizatora.