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# Screening and catalytic activity in organic synthesis of novel fungal and yeast lipases

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**Abstract**

A total of 969 microbial strains were isolated from soil samples and tested to determine their lipolytic activity by employing screening techniques on solid and in liquid media. Ten lipase-producing microorganisms were selected and their taxonomic identification was carried out. From these strains *Achremonium murorum*, *Monascus mucoroides*, *Arthroderma ciferri*, *Fusarium poae*, *Ovadendron sulphureo-ochraceum* and *Rhodotorula araucariae* are described as lipase-producers for the first time. Hydrolysis activity of the crude lipases against both tributyrin and olive oil was measured. Heptyl oleate synthesis was carried out to test the activity of the selected lipases as biocatalysts in organic medium. All the selected lipases were tested as biocatalysts in several organic reactions using unnatural substrates. Lipases from the fungi *Fusarium. oxysporum* and *O. sulphureo-ochraceum* gave the best yields and enantioselectivities in the esterification of carboxylic acids. *F. oxysporum* and *Penicillium chrysogenum* lipases were the most active ones for the acylation of alcohols without steric hindrance. *A. murorum* lipase is very useful for the esterification of menthol. *F. oxysporum* and *Fusarium. solani* lipases were very stereoselective in the synthesis of carbamates. 

*Keywords:* Screening of microbial lipases; Biotransformations; Fungal lipases; Yeast lipases; Resolution of racemic alcohols; Resolution of chiral carboxylic acids

## 1. Introduction

Lipases (triacylglycerol hydrolases EC 3.1.1.3) are enzymes that catalyse the hydrolysis of triglycerides in the oil–water interface. Lipases are produced by animals, plants and microorganisms [1–3]. Microbial lipases have a great potential for commercial applications due to their stability, selectivity and broad substrate specificity because many unnatural acids, alcohols or amines can be used as the substrates [4]. To date, a large number of lipases from filamentous fungi have been extensively studied, both from the biochemical and from the genetic point of view. The most productive species belong to the genera *Geotrichum*, *Penicillium*, *Aspergillus* and *Rhizomucor* [5,6]. There are also a certain number of lipases produced by yeasts, most of them belonging to the *Candida* genus, that have been used for biotechnological purpose [7,8].

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Among the high number of lipases described in the literature, only the enzymes belonging to a few species have been demonstrated to have adequate stability and biosynthetic capabilities to allow routine use in organic laboratories, and hence their consideration as industrially relevant enzymes [9,10]. The application of different techniques of enzyme immobilisation and of chemical modification of enzymes has introduced considerable improvements in the efficiency of certain processes [11,12]. A more detailed study on the nature of the reaction medium, involving parameters such as water activity and $\log P$ [13,14], and the use of optically active solvents has improved the knowledge about the catalytic process [15–17]. However, despite the above-mentioned advances in modification and improvement of the enzyme activity and in the use in triglyceride industry, there still remain many important problems to be solved in the application of lipases to organic synthesis using chiral unnatural substrates. Therefore, access to a wider range of lipase types would be beneficial [18]. In this study we describe the selection by screening techniques of 10 lipase-producing microorganisms and the preliminary characterisation of the lipases in reactions involving triglyceride hydrolysis and in heptyl oleate synthesis. Then, these new crude enzymes were used in some reactions in organic media with unnatural substrates.

## 2. Experimental

### 2.1. Chemicals

Crude commercial lipase (EC 3.1.1.3) from *C. rugosa* (type VII) was obtained from Sigma (St. Louis, MO, USA). Merck Chemical (Darmstadt, Germany) supplied olive oil emulsion (50/50 (v/v)) and silica gel for chromatography.

The lipase chromogenic substrate, 1,2-*O*-dilauryl-*rac*-glycero-3-glutaric acid resorufinyl ester, was purchased from Boehringer Mannheim Biochemicals (Ingelheim, Germany). (*R*,*S*)-2-(4-isobutylphenyl) propionic acid (ibuprofen) **1a**, was obtained from Boots Pharmaceuticals (Spain). Yeast extract and bacto-peptone were purchased from Difco Products (Sparks, MD, USA). The other components of the culture media and the chemical reagents were obtained from Merck Chemical and from Sigma in the highest purity available.

### 2.2. Isolation of microorganisms and screening of lipolytic activity

All the microorganisms used in this study were isolated from soil samples collected from around the world. The isolation process was performed by serial dilution of the samples according to standard techniques [19]. Taxonomic identification of yeasts was performed by the Spanish Type Culture Collection (C.E.C.T., Valencia, Spain). The filamentous fungi were identified in-house by using mature cultures on suitable media (normally standard potato dextrose agar and/or oat meal agar) in order to ensure a good development of taxonomically relevant features, and following the identification keys provided by von Arx [20], Domsch et al. [21] and the more specific methodology of by Singler and Carmichael [22] in the case of *O. sulphureo-ochraceum*.

Preliminary screening of lipolytic filamentous fungi, yeasts and bacteria was carried out on BYPO [21], agar plates supplemented with a 10% (v/v) olive oil or tributyrin emulsion prepared in 10% (w/v) gum Arabic solution. Culture plates were incubated at 28°C and periodically examined for 4 days. Colonies showing clear zones around them were picked out and screened for lipase production in liquid medium.

Two different liquid media (H1 and H2) were employed for filamentous fungi. Medium H1 contained 0.5% yeast extract, 3% bacto-peptone, 0.1% $KH_2PO_4$, 0.1% $NaNO_3$, 0.05% $MgSO_4$ and 1% (v/v) olive oil. Medium H2 contained 7.5% soybean flour, 2% glucose, 0.1% $KH_2PO_4$, 0.1% $NaNO_3$, 0.05% $MgSO_4$ and 1% (v/v) olive oil. The final pH was 7 for both media. Yeasts were cultured in liquid YED medium and unicellular bacteria were cultured in liquid BYPO medium [23]. All the cultures were grown in 250 ml flasks containing 50 ml of medium and in 2 l flasks containing 250 ml of medium. Filamentous fungi and yeasts were incubated at 28°C for 3 days. Flasks were incubated on a reciprocal shaker at 250 rpm. After this time, culture broths were collected, centrifuged and assayed for lipase activity. Positive supernatants were dialysed overnight against 25 mM Tris–HCl buffer, pH 7, and further lyophilised and stored at 4°C.

### 2.3. Assay of lipase activity

Lipase activity present in culture broths was initially monitored using the chromogenic substrate 1,2-*O*-dilauryl-*rac*-glycero-3-glutaric acid resorufinyl ester. This reaction was performed at 37°C for 15 min in 96-well microtiter plates using the conditions indicated by Boehringer Mannheim Biochemicals. The appearance of a red colour due to free resorufinol indicated the presence of lipase activity in the culture broth.

Supernatants that hydrolysed the chromogenic substrate were selected in order to quantify their lipase activity by using olive oil and tributyrin as substrates. This titrimetric assay was carried out essentially as described by Tietz and Fiereck [24]. One unit of lipase activity (U) is defined as the amount of enzyme that releases 1 μmol of fatty acid from triglyceride, per minute of reaction at 37°C.

### 2.4. Heptyl oleate synthesis

Heptyl oleate synthesis was carried out to verify the catalytic activity of the lipases in the synthesis of esters in organic medium. The reaction mixture was composed of 20 mM oleic acid, 20 mM 1-heptanol, 3 ml of isooctane, 0.3 ml of water and lyophilised crude enzyme. The amount of enzyme used per reaction was that corresponding to 50 ml of the centrifuged culture supernatant. The reaction was carried out at 30°C with vigorous magnetic stirring. Samples of 50 μl were withdrawn at different reaction times and diluted in 500 l of isooctane. The amount of ester formed during the reaction was quantified by gas chromatography. The analysis was performed in a Shimadzu GC-14 using a SPB-1 (15 m × 0.32 mm i.d.) column (Supelco Inc., Bellafonte, PA, USA). The initial temperature was 120°C (54 min), end temperature 250°C (10 min), heating rate (13°C/min), flux 13 ml/min, split 1:2.

### 2.5. Resolution of (R,S)-ibuprofen **1*a***

The resolution of (*R*,*S*)-2-(4-isobutylphenyl) propionic acid (ibuprofen) **1a**, was performed by stereoselective esterification using 1-propanol as acyl acceptor. The reaction mixture was composed of 66 mM (*R*,*S*)-ibuprofen **1a**, 66 mM 1-propanol, 2 ml of isooctane, 0.2 ml of water and lyophilised enzyme. The amount of enzyme used per reaction was that corresponding to 50 ml of the centrifuged culture supernatant and then lyophilised. The reaction was carried out at 30°C with vigorous magnetic stirring. Samples of 30 μl were withdrawn at different times and diluted in 500 μl of isooctane. The amount of ester formed during the reaction and the enantiomeric excess of the remaining acid were determined by HPLC, using a chiral column (Chiracel-OD Daicel Chemical Industries GmbH, Germany), with hexane/iPrOH/ trifluoracetic acid (100/1/0.1 (v/v/v)) as mobile phase, $\lambda = 254$ nm, retention times of (*R*) acid = 22 min and of (*S*) acid = 27 min.

### 2.6. Acylation of cyclopropylmethanol **2*a***

Cyclopropylmethanol (100 mM), vinyl acetate (100 mM), solvent (4 ml) diisopropyl ether (iPrOiPr), 200 mg of lyophilised enzyme obtained by precipitation of cell culture medium with 90% saturated solution of ammonium sulphate. The reaction yield was measured by GLC in a Shimadzu 14 A gas chromatograph. TR-wax 15 m, 0.25 mm i.d. column (Supelco Inc., Bellafonte, PA, USA). $T = 90$°C, flux of $N_2 = 13$ ml/min cyclopropylmethanol ($t_r = 2.1$ min), cyclopropylmethyl acetate ($t_r = 3.2$ min).

### 2.7. Acylation of R or S glycidol **3*a***

Alcohol (100 mM), vinyl acetate (100 mM), solvent (4 ml) iPrOiPr, 200 mg of lyophilised enzyme obtained by precipitation of cell culture medium with 90% saturated solution of ammonium sulphate. The reaction yield was measured by GLC in a Shimadzu 14 A gas chromatograph. TR-wax 15 m, 0.25 mm i.d. column (Supelco Inc., Bellafonte, PA, USA). $T = 110$°C, flux of $N_2 = 13$ ml/min (*R*) or (*S*) glycyl acetate ($t_r = 2.7$).

### 2.8. Acylation of cyclohexanol **4*a***

Cyclohexanol (250 mM), vinyl acetate (250 mM), solvent (4 ml) iPrOiPr, 200 mg of lyophilised enzyme obtained by precipitation of cell culture medium with 90% saturated solution of ammonium sulphate. The reaction yield was measured by GLC in a Shimadzu 14 A gas chromatograph. TR-wax 15 m, 0.25 mm i.d. column. $T = 110$°C, flux of $N_2 = 20$ ml/min

cyclohexanol ($t_r$ = 1.8 min), cyclohexyl acetate ($t_r$ = 4.3 min).

### 2.9. Acylation of menthol **5a**

Menthol (100 mM), vinyl acetate (100 mM), solvent (4 ml) iPrOiPr, 200 mg of lyophilised enzyme obtained by precipitation of cell culture medium with 90% saturated solution of ammonium sulphate. The reaction yield was measured using a GLC in a Shimadzu 14 A gas chromatograph. TR-wax 15 m, 0.25 mm i.d. column. $T$ = 120°C, flux of $N_2$ = 20 ml/min, menthol ($t_r$ = 6.45 min), methyl acetate ($t_r$ = 12.3 min).

The acylation of (+) neomenthol **6a**, and (+) isoneomenthol **7a**, were performed in the same experimental conditions but no reaction was observed.

### 2.10. Stereoselective acylation of (R,S)-1-phenylethanol **8a**

The resolution of (*R*,*S*)-1-phenylethanol **8a**, was performed with 100 mM (*R*,*S*)-1-phenylethanol, 100 mM of vinyl acetate, 5 ml of isooctane and the lyophilised crude enzyme corresponding to 50 ml of the centrifuged culture supernatant and then lyophilised. The reaction was carried out at 30°C with vigorous magnetic stirring. Samples of 100 μl were withdrawn at different times and diluted in 900 μl of isooctane. Detection of the1-phenylethyl acetate formed during the transesterification reaction and the enantiomeric excess of the remaining alcohol were also determined using a Chiracel-OD column. The mobile phase was a mixture of *n*-hexane:isopropanol (97:3 (v/v)) at a flow rate of 0.7 ml/min, λ = 254 nm. Retention times of (*S*) **8a** = 15 min, (*R*) **8a** = 17 min and (*R*,*S*) **8b** = 6.7 min.

### 2.11. Synthesis of butyl carbamate **9b**

#### 2.11.1. Synthesis of vinyl carbonate

Vinyl chloroformate (50 mmol) was slowly added to a solution of *n*-butanol (35 mmol) in dry pyridine (4 ml). The solution was stirred for 2 h and then was acidified with HCl 3N and extracted with dichloromethane, the organic layer was dried over $Na_2SO_4$ and submitted to adsorption chromatography on neutral silica using hexane–ethyl acetate (9:1 (v/v)). IR (KBr) ($\nu$=: 1763 $cm^{-1}$; $^1$H-RMN ($CDCl_3$) ($\delta$ ppm): 7.10 (dd, 1H, CH), 4.90 (dd, 1H, CH), 4.60 (dd, 1H, CH), 4.20 (t, 2H, CH), 1.70 (m, 2H, $CH_2$), 1.45 (m, 2H, $CH_2$), 1 (t, 3H, $CH_3$); $^{13}$C-RMN ($CDCl_3$) ($\delta$ ppm): 152.68 (C=O), 142.52 (CH), 97.39 ($CH_2$), 68.63 ($CH_2$), 36.40 ($CH_2$), 18.73 ($CH_2$), 13.47 ($CH_3$). Analysis: calculated for $C_7H_{12}O_3$, 58.30% C, 8.39% H. Found: 58.17% C, 8.41% H.

#### 2.11.2. Synthesis of racemic carbamates

To a solution of *(R,S)*-1-phenylethylamine (5.2 mmol) and triethylamine (5.2 mmol) in 20 ml of dichloromethane was slowly added to the correspondent vinyl chloroformate (5.2 mmol) at 0°C. The solution was stirred for 12 h and then was acidified with HCl 3N. The organic layer was dried over $MgSO_4$ and the solvent was evaporated at vacuum. IR (KBr) ($\nu$=: 1692 $cm^{-1}$; $^1$H-RMN ($CDCl_3$) ($\delta$ ppm): 7.40 (m, 5H, arom), 5.20 (bs, 1H, NH), 480 (bs, 1H, CH), 4 (m, 2H, $CH_2$), 1.70–1.20 (m, 7H) 0.90 (t, 3H, $CH_3$); $^{13}$C-RMN ($CDCl_3$) ($\delta$ ppm): 154.84 (C=O), 143.62©, 128.36 (2× CH), 126.98 (2× CH), 125.73 (CH), 64.51 ($CH_2$), 50.34 (CH), 30.86 ($CH_2$), 22.27 ($CH_3$), 18.85 ($CH_2$), 13.53 ($CH_3$). Analysis: calculated for $C_{13}H_{19}NO_2$, 70.54% C, 8.66% H, 6.33% N. Found: 70.45% C, 8.67% H, 6.34% N.

#### 2.11.3. Lipase reaction conditions

To a solution of 1.2 mmol of vinyl carbonate and 2 mmol of amine in 15 ml of hexane, was added 200 mg of catalyst. The temperature was 25°C. After 72 h, the enzyme was removed by filtration and the solvent was evaporated. The conversion was determined by HPLC. The chromatographic separation on neutral silica 60 mesh (Merck) of the resulting residue yield the carbamate (hexane–ethyl acetate (7:3 (v/v)) and the enantiomeric excess was determined by $^1$H-NMR.

#### 2.11.4. Analytical HPLC

It was performed on a LDC chromatograph using a Nucleosil $C_{18}$ 120 (25 cm × 0.46 cm × 5 m) column using MeOH/$H_2O$ (60/20 (v/v)), flow rate 0.3 ml/min with UV detector λ = 254 nm.

#### 2.11.5. Determination of enantiomeric excess

It was calculated by $^1$H-NMR spectroscopy using the chiral shift reagent tris-[3-(heptafluoropropylhydroxy-methylene)-(+)-camphorate] europium(III)

derivative. The molar ratios carbamate/Eu derivatives were 1/0.4 (butyl carbamate) and 1/0.3 (octyl carbamate). The absolute configuration of the carbamate was assigned by comparing of their optical rotation with authentically chiral carbamates, $\delta$ CH-Eu ppm: $(R) = 5.6$; $(S) = 6.3$.

## 3. Results and discussion

### 3.1. Selection of lipase-producer microorganisms

The use of solid media supplemented with emulsified triglycerides is a standard methodology for the selection of lipase-producing microorganisms [25,26]. A total of 960 microorganisms isolated from soil samples, including yeasts (100 strains) and filamentous fungi (860 strains), were employed in this work. All the microbial strains were screened for lipase activity in solid medium plates containing olive oil or tributyrin as indicator. A total of 440 microorganisms produced a clear halo around them in plates containing tributyrin. Of these, only 92 microorganisms also showed hydrolysis on the olive oil plates, thus, indicating a greater presence of lipases acting on long chain triglycerides.

The 440 microorganisms selected in the first step were cultured in liquid medium by using olive oil as lipase inducer. After centrifugation of culture broths, supernatants were tested for the hydrolysis of 1,2-*O*-dilauryl-*rac*-glycero-3-glutaric acid resorufinyl ester, a chromogenic substrate specific for lipases. The appearance of a red colour due to the release of resorufinol indicated that the supernatant had lipase activity, 118 culture broths were positive in this qualitative assay and were used to further quantify their lipase activity in liquid medium by using tributyrin and olive oil as substrates.

From this analysis, 10 strains were selected showing the best lipase yields (lipase activity higher than 1 U/ml for one or for both triglycerides) and the best reproducibility in liquid medium cultures (Table 1) [27]. The producing strains were taxonomically identified as follows: nine filamentous fungi *Achremonium murorum* (Corda) W. Gams, *Monascus mucoroides* (van Tiegh), *Monascus* sp., *Arthroderma ciferrii* (Vars and Ajello), *Fusarium poae* (Pec.) Wollenw, *Fusarium solani* (Mart.) Sacc., *Fusarium oxysporum*, *Penicillium chrysogenum* (Thom), *Ovadendron sulphureo-ochraceum* (van Beyma) Singler and Carmichael and one yeast (*Rhodotorula araucariae*). As far as we know, *A. murorum*, *M. mucoroides*, *A. ciferrii*, *F. poae*, *O. sulphureo-ochraceum*, *Rhodotorula araucariae* and *S. halstedii* are reported here for the first time as lipase-producers. *F. solani* has been reported to produce a very active cutinase that also displays triacylglycerol lipase activity [28]. *F. oxysporum*, *P. chrysogenum* have also been previously found to have lipolytic activity in solid and in liquid media [1,29,30]. In addition, other strains belonging to *Monascus* [1,6] *Fusarium* [31] and *Rhodotorula* [32] genus have been reported to show lipolytic activity. Nevertheless the use of these new lipases as biocatalysts in organic synthesis has not been described till our knowledge.

Table 1
Lipase activity (U/ml of supernatant) measured in culture broths from 14 microorganisms selected in this study

| Producing strain | Culture medium | Lipase activity (U/ml) | |
|---|---|---|---|
| | | Olive oil | Tributyrin |
| *A. murorum* | H1 | 0.72 | 5.18 |
| *M. mucoroides* | H1 | 3.02 | 4.31 |
| *Monascus* sp. | H1 | 2.35 | 2.37 |
| *A. ciferrii* | H2 | 1.43 | 1.89 |
| *F. poae* | H2 | 0.93 | 1.47 |
| *F. solani* | H2 | 0.60 | 1.25 |
| *F. oxysporum* | H2 | 2.83 | 5.70 |
| *P. chrysogenum* | H2 | 0.64 | 4.68 |
| *O. sulphureo-ochraceum* | H2 | 4.33 | 6.10 |
| *R. araucariae* | YED | 0.75 | 3.64 |

As shown in Table 1, all the selected lipases displayed better hydrolytic activity against tributyrin than against olive oil. This is a general characteristic previously observed during the screening in solid medium. This result has been observed with other lipase microorganisms producers such as *Candida rugosa* [33], where extracellular esterases and proteases (actives with short chain triglycerides) have been detected with extracellular lipases (actives with long chain triglycerides). The most active crude enzymes in the hydrolysis of olive oil were

$$O.\,sulphureo\text{-}ochraceum > M.\,mucoroides > F.\,Oxysporum > Manascus\,\text{sp.} > A.\,ciferrii$$

These crude enzymes could be considered as the biocatalysts with the highest lipase concentration, because olive oil hydrolysis is the standard lipase activity test.

In general, lipase yields obtained for microorganisms cultured in 250 ml flasks were similar to those obtained in 2 l flasks (data not shown). When stored at 4°C for 2 months as lyophilised extracts, all the lipases showed a 90–100% recovery of the enzyme activity previously measured in fresh culture broths.

### 3.2. Esterification of carboxylic acids

#### 3.2.1. Heptyl oleate synthesis

The main interest in lipases in organic synthesis is their ability to work in organic media and their low substrate specificity, as it can be demonstrated with many unnatural substrates [34]. Esterification of oleic acid with 1-heptanol may be considered as an standard reaction to initially verify the activity of lipases in organic medium [35]. This reaction let us to discriminate between the lipases (active as biocatalyst), esterases (low active biocatalysts) and proteases (inactive biocatalysts). The reactions were done using the crude biocatalyst obtained after centrifugation and lyophilisation of 50 ml of culture cell broth. Addition of a small amount of water (0.3 ml per reaction flask) was found to be necessary for enzyme activity. In the absence of added water, catalytic activity was undetectable. It is well documented in the literature that lyophilised lipases need the presence of some amount of water in the reaction medium to achieve the active conformation [36]. As it can be seen in Table 2, all the crude enzymes used in this study gave an almost quantitative esterification of the oleic acid (85–100% yield) after 96 h of reaction. The only exception to this was the lipase from *A. murorum*, with a 77% conversion. In four cases (*M. mucoroides*, *F. poae*, *F. solani* and *O. sulphureo-ochraceum*) the maximum yield was attained after short reaction times (4–8 h), that could be considered qualitatively as the crude biocatalyst with the highest percentages in lipase active in organic medium. Contrarily, *A. murorun* and *P. chrysogenum* must be considered as the less active biocatalysts in this hydrophobic medium. These strains showed low hydrolytic activity in the hydrolysis of olive oil (Table 1).

Table 2
Time course of heptyl oleate synthesis performed by the lipases selected in this study[a]

| Producing strain | Yield[b] (%) | | | |
|---|---|---|---|---|
| | 4 h | 8 h | 24 h | 96 h |
| *A. murorum* | 6 | 29 | 44 | 77 |
| *M. mucoroides* | 95 | 96 | 96 | 96 |
| *Monascus* sp. | 34 | 49 | 70 | 87 |
| *A. ciferrii* | 41 | 77 | 89 | 90 |
| *F. poae* | 77 | 89 | 91 | 91 |
| *F. solani* | 79 | 83 | 88 | 88 |
| *F. oxysporum* | 28 | 52 | 81 | 87 |
| *P. chrysogenum* | 11 | 39 | 78 | 89 |
| *O. sulphureo-ochraceum* | 71 | 84 | 88 | 88 |
| *R. araucariae* | 23 | 35 | 66 | 86 |

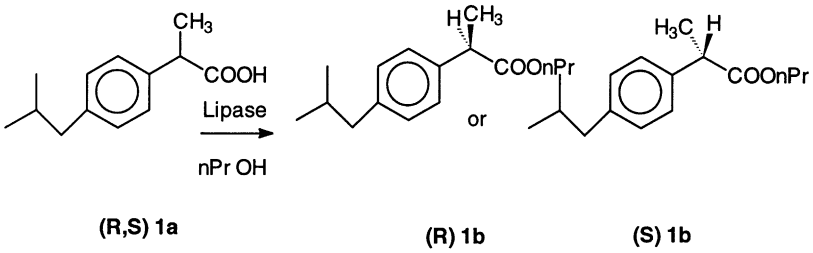
[a] Oleic acid = heptanol = 20 mM, $V_T$ = 3 ml of isooctane, 0.3 ml $H_2O$.
[b] Yield is given as the percentage of the initial oleic acid esterified at each time.

Although, all the biocatalysts tested by us were very active in both hydrolytic and synthetic assays, the most active lipases in the hydrolytic assay were not necessarily the most active ones in the synthetic one (compare Tables 1 and 2). Thus, lipases from *F. poae* and from *F. solani*, which displayed a low hydrolytic activity, were very active in the synthesis of heptyl oleate. On the contrary, lipase from *F. oxysporum*, which showed a very good activity against both tributyrin and olive oil substrates displayed a moderate activity in the early times of heptyl oleate reaction. The rest of the enzymes showed similar level of activity in both types of assay. The relative catalytic activity was

$$M.\,mucoroides > F.\,solani \geq F.\,Poae \geq O.\,sulphureo\text{-}ochraceum$$

different to that obtained in the hydrolysis of olive oil (Table 1). This different behaviour may be related to the different resistance of each crude lipase to the deactivation effect produced by the organic solvents. This topic is well documented in the literature in the case of other lipases [37–39]. From this, we can conclude the absence of a general correlation between hydrolytic and synthetic activities, as it has been reported by other authors [40].

In the same experimental conditions, the commercial crude lipase from *Candida rugosa* gave a 80% of ester at 15 min and 100% esterification yield at 1 h. So,




Scheme 1.

this commercial enzyme, used as the reference, was more active in this reaction, indicating a high number of synthetic units per gram of lyophilized powder.

### 3.2.2. Enantioselective esterification of (R,S)-ibuprofen **1a**

One of the most interesting process catalysed by lipases in organic media is the resolution of racemic mixtures. Resolution of (*R*,*S*)-4-isobutylphenyl propionic acid (ibuprofen) **1a** (see Scheme 1) by stereoselective esterification using lipases as biocatalysts has been extensively studied and demonstrated to be implemented as an interesting process from the industrial point of view [41,42]. Taking this into account we have employed this reaction as a reference test to analyse the behaviour of the selected lipases with respect to a chiral carboxylic acid (Table 3).

As in the case of the heptyl oleate, all the lipases tested were active as biocatalysts in the esterification but the ester yield, the enantiomeric excess and the enantiopreference were rather heterogeneous among the enzymes. As it can be seen in Table 3, the highest ester yields were obtained with the lipases from two filamentous fungi, *F. oxysporum* and *O. sulphureo-ochraceum* (84 and 70%, respectively). The lowest yields were obtained with lipases from *A. murorum*, *P. chrysogenum* that showed the lowest hydrolytic activity versus olive oil (Table 1) and *Monascus* sp. These crude enzymes were slightly actives in the heptyl oleate synthesis.

Nevertheless there is not a strong relationship between the heptyl oleate yields (Table 2) and the esterification of this unnatural carboxylic acid as we can see for *F. oxysporum* crude lipase. These results can be related to different sub-sites for the carboxylic acids. Probably, *F. oxysporum* and *O. sulphureo-ochraceum* lipases have an adequate sub-site for this chiral acid. Contrarily, *M. mucoroides*, *F. solani* and *F. poae*

Table 3
Resolution of (*R*,*S*)-ibuprofen **1a**, by the new crude lipases[a]

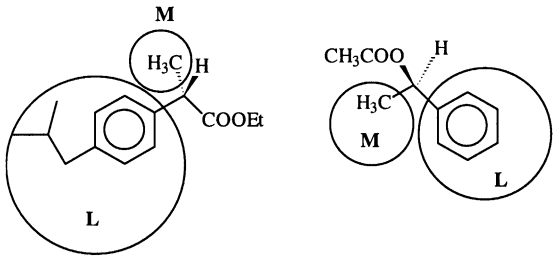
| Producing strain | U/mg solid[b] $10^3$ | Reaction time (h) | Yield in **1b**[c] (%) | ee of **1a** (%) | ee of **1b** (%) | Stereopreference[d] |
|---|---|---|---|---|---|---|
| *A. murorum* | 9 | 264 | 5.5 | 2.6 | 44 | *S* |
| *M. mucoroides* | 142 | 264 | 28 | 10 | 26 | *S* |
| *Monascus* sp. | 50 | 264 | 7.5 | 7 | 86 | *S* |
| *A. ciferrii* | 62.5 | 336 | 16 | 15 | 76 | *S* |
| *F. poae* | 115 | 336 | 44 | 14.5 | 18 | *R* |
| *F. solani* | 117 | 336 | 11 | 0.7 | 4 | *R* |
| *F. oxysporum* | 42.5 | 264 | 84 | 100 | 18 | *S* |
| *P. chrysogenum* | 27.5 | 336 | 3.7 | 0.2 | 4.8 | *R* |
| *O. sulphureo-ochraceum* | 107.5 | 336 | 70 | 65 | 27 | *S* |
| *R. araucariae* | 35 | 336 | 21 | 10.5 | 40 | *S* |
| *C. rugosa* | 3200 | 144 | 49.5 | 62 | 61 | *S* |

[a] **1a** = *n*-propanol = 66 mM, $V_T$ = 4 ml isooctane, 0.2 ml $H_2O$.
[b] U/mg of lyophilised biocatalyst 1 unit = amount of enzyme that produces 1 mmol heptyl oleate/h.
[c] Ester yield is given as the percentage of initial ibuprofen esterified after the reaction time.
[d] Configuration of the main enantiomer found in the ester.

lipases will have a narrow and long acid recognition sub-site, useful for oleic acid (Table 2) but not for this branched acid **1a**. These differences could only be confirmed after the isolation and structural X-ray analysis of pure proteins. Pleiss et al. [43], described a short and narrow recognition active sub-site for the cutinase of *F. solani* probably similar to our lipase from our strain of *F. solani* and a broad one for *C. rugosa* lipase. These approximations are in accordance with the better yield achieved with the commercial lipase of *C. rugosa* than with the lipase of our strain of *F. solani* (Table 3). Only the crude biocatalysts: *F. oxysporum* and *O. sulphureo-ochraceum* crude lipases overcomed the 50% yield. The results obtained with these two enzymes are similar to those previously found for the *C. antarctica* lipase [41], but better than those obtained with crude commercial *C. rugosa* lipase (Table 3). In all cases, lower amount of heptyl oleate units were added to the reaction catalysed by our lipases than in the case of commercial Sigma lipase. Therefore, our lipases could be interesting as biocatalysts after a partial purification or cloning and expression.

The crude biocatalysts are (*S*)-stereoselective as *C. rugosa* lipase (Table 3), except for *F. poae* lipase that shows high activity and the opposite stereoselectivity to that proposed by Kazlauskas et al. [44] (Scheme 2).

The ee of the ester **1b**, is low or moderate compared to that obtained with the commercial *C. rugosa* lipase that seems to be better than our lipases from this point of view.




Scheme 2. Stereopreference predicted by Kazlauskas et al. [44].

### 3.3. Acylation of primary alcohols

Lipases have been successfully employed for the resolution of racemic alcohols to explore this topic with our lipases, the resolution of several alcohols have been performed. The acylation reactions were done in iPrOiPr as the solvent and not in a more hydrophobic solvent such as isooctane, according to a factorial experimental design carried out for this kind of reactions [45]. This change in the solvent is justified because the alcohols used are less hydrophobic than oleic acid, heptanol or **1a**. So, we need a less hydrophobic solvent to avoid the difussional problems in the medium.

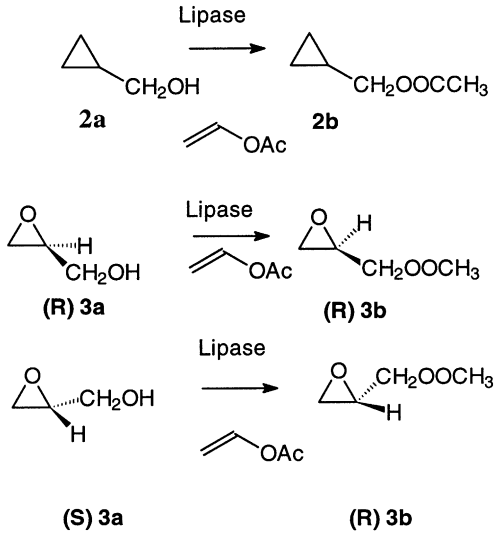
#### 3.3.1. Acylation of cyclopropylmethanol **2a**

The acylation of cyclopropylmethanol **2a**, as a small primary alcohol, has been studied using 200 mg of lyophilised biocatalyst (Section 2). The obtained results are shown in Table 4. All the lipases were active, as can be expected for primary alcohol acylation. The

Table 4
Acylation of cyclopropylmethanol **2a**, using as biocatalysts the selected crude lipases[a]

| Lipase | U/mg solid[b] $10^3$ | Yield in **2b** (%); 96 h | Yield in **2b** (%); 168 h |
|---|---|---|---|
| *F. oxysporun* | 42.5 | 37 | 84 |
| *O. sulphureo-ochraceum* | 107.5 | 10 | 26 |
| *F. poae* | 115 | 6 | 22 |
| *F. solani* | 117 | 8 | 20 |
| *P. chrysogenum* | 27.5 | 32 | 95 |
| *A. ciferrii* | 62.5 | 35 | 82 |
| *Monascus* sp. | 50 | 23 | 65 |
| *A. murorum* | 9 | 12 | 33 |
| *M. mucoroides* | 142 | 5 | 18 |
| *R. araucariae* | 35 | 39 | 84 |
| *C. rugosa* | 3200 | 42 | 81 |

[a] Cyclopropylmethanol = vinyl acetate = 100 mM, solvent iPrOiPr = 4 ml. Crude enzyme = 200 mg of lyophilised biocatalyst.
[b] U/mg of lyophilised biocatalyst 1 unit = amount of enzyme that produces 1 mmol heptyl ester/h.




Scheme 3.

lipases of *F. oxysporum*, *P. chrysogenum*, *A. ciferri* and *R. araucariae* are the most active biocatalysts at short reaction times, giving yields greater than 80% at 168 h (Table 4). These enzymes let us to obtain similar or greater yields than commercial *C. rugosa* lipase. Now, we can see that the relative catalytic activity depends on the reaction. Only *F. oxysporum* lipase is active in all the reaction tested till now (Scheme 3).

The good yields obtained with the lipases from *P. chrysogenum*, *A. ciferrii* and *R. araucariae* may be related to the good activity observed in the hydrolysis of small triglyceride (tributyrin Table 1), but this affirmation is not general because *A. murorum* lipase shows low catalytic activity (Table 4) but high hydrolytic activity respect to tributyrin (Table 1). Another time, the most plausible explanation could be the influence of the solvent in the stability of the crude enzymes at long reaction times.

### 3.3.2. Acylation of (R) or (S) glycidol **3a**

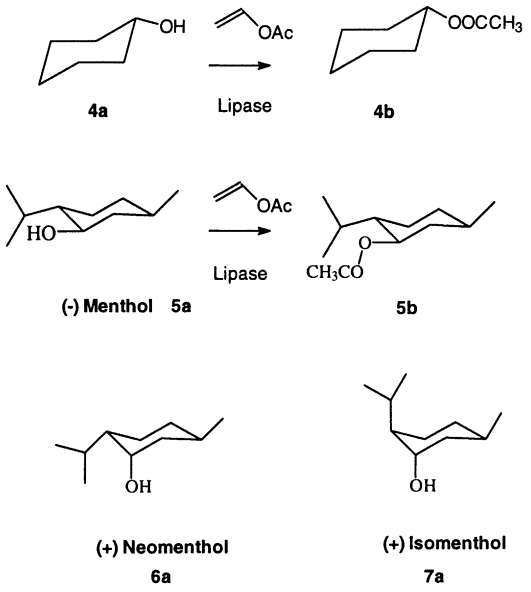
The acylation of (*R*) or (*S*) **3a**, was performed to study the influence of the substitution of one $CH_2$ bridge **2a**, by an oxygen **3a**. The obtained results are shown in Table 5. We can observe in Tables 4 and 5 a similar relative behaviour in both acylations. The most active lipases are those from *F. oxysporum*, *P. chrysogenum* and *A. ciferrii*. These crude enzymes are more active than *C. rugosa* lipases assuming that different number of units were added to the reaction flask in each reaction. Only *F. solani* lipase shows good catalytic activity in the acylation of (*R*) or (*S*) **3a** (Table 5) but low activity for **2a**. The lipases less active were the same for both substrates (see Tables 4 and 5).

The enantioselectivity, expressed as the ratio (*R*) **3b**/(*S*) **3b**, was very low, probably due to the small molecular size of these reagents. It is interesting to point out that *F. oxysporum* lipase showed higher *S*-enantiopreference than *C. rugosa* lipase and that *F. solani* lipase showed the opposite enantioselectivity. These promising results point out to future applications of these enzymes after semi-purification or cloning and overexpression.

Table 5
Acylation *R* or *S* glycidol **3a**, using the selected crude lipases as biocatalysts[a]

| Lipase | U/mg solid[b] $10^3$ | (*R*) **3b** (%) | (*S*) **3b** (%) | (*R*) **3b**/(*S*) **3b**[c] |
|---|---|---|---|---|
| *F. oxysporum* | 42.5 | 34 | 41 | 0.8 |
| *O. sulphureo-ochraceum* | 107.5 | 8 | 9 | 0.8 |
| *F. poae* | 115 | 6.5 | 16 | 0.4 |
| *F. solani* | 117 | 21 | 15 | 1.4 |
| *P. chrysogenum* | 27.5 | 28 | 29 | 0.95 |
| *A. ciferrii* | 62.5 | 14 | 15 | 0.9 |
| *Monascus* sp. | 50 | 8 | 8 | 1.1 |
| *A. murorum* | 9 | 5 | 4 | 1.1 |
| *M. mucoroides* | 142 | 2 | 1.5 | 1.3 |
| *R. araucariae* | 35 | 9.5 | 9 | 1.1 |
| *C. rugosa* | 3200 | 30 | 34 | 0.9 |

[a] Reaction time = 96 h, glycidol = vinyl acetate = 100 mM, solvent iPrOiPr = 4 ml. Enzyme 200 mg of lyophilised powder.
[b] U/mg of lyophilised biocatalyst 1 unit = amount of enzyme that produces 1 mmol heptyl oleate/h.
[c] Ratio between the yields obtained.




Scheme 4.

### 3.4. Acylation of secondary cycloalkanols

Cyclohexanol **4a**, (−) menthol **5a**, (+) neomenthol **6a** and (+) isoneomenthol **7a** were selected as cycloalkanols with different steric hindrance around the OH group, to test our new lipases (Scheme 4).

In Table 6 we can observe that all the lipases are active in the esterification of **4a** but not in the case of (−) **5a** (Table 7). In this case the lipase from *A. murorum* let us to achieve the best results, similar to that obtained with *C. rugosa* lipase. Therefore, we can conclude that this lipase must be the enzyme with the largest alcohol recognition sub-site **6a** and **7a** were not esterified according to the Kazlauskas postulates [42].

Table 6
Acylation of cyclohexanol **4a**, using the selected crude lipases (200 mg lyophilised powder)[a]

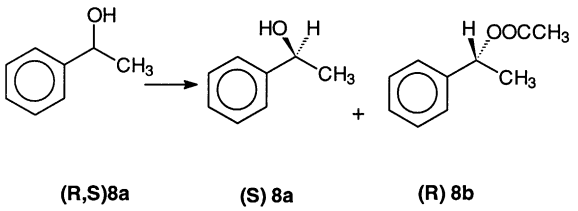
| Lipase | U/mg solid[b] $10^3$ | Time (h) | Yield in **4b** (%) |
|---|---|---|---|
| *F. oxysporum* | 42.5 | 168 | 26 |
| *O. sulphureo-ochraceum* | 107.5 | 504 | 4 |
| *F. poae* | 115 | 240 | 21 |
| *F. solani* | 117 | 504 | 1.5 |
| *P. chrysogenum* | 27.5 | 120 | 16 |
| *A. ciferrii* | 62.5 | 120 | 16 |
| *Monascus* sp. | 50 | 240 | 8 |
| *A. murorum* | 9 | 168 | 26 |
| *M. mucoroides* | 142 | 504 | 1 |
| *R. araucariae* | 35 | 120 | 15 |
| *C. rugosa* | 3200 | 120 | 42 |

[a] Cyclohexanol = vinyl acetate = 250 mM, solvent iPrOiPr = 4 ml.
[b] U/mg of lyophilised biocatalyst 1 unit = amount of enzyme that produces 1 mmol heptyl oleate/h.

Table 7
Acylation of (−) menthol **5a**, using the selected lipases as biocatalysts (200 mg of lyophilised powder)[a]

| Lipase | U/mg solid[b] $10^3$ | Yield in **5b** (%) |
|---|---|---|
| *F. oxysporum* | 42.5 | 4 |
| *P. chrysogenum* | 27.5 | 3.5 |
| *A. ciferrii* | 62.5 | 4 |
| *A. murorum* | 9 | 22 |
| *R. araucariae* | 35 | 7 |
| *C. rugosa* | 3200 | 25 |

[a] Reaction time = 21 days, menthol = vinyl acetate = 100 mM, solvent iPrOiPr = 4 ml.
[b] U/mg of lyophilised biocatalyst 1 unit = amount of enzyme that produces 1 mmol heptyl oleate/h.




Scheme 5.

### 3.5. Acylation of (R,S)-1-phenylethanol **8a**

The resolution of (*R*,*S*)-1-phenylethanol **8a** (see Scheme 5) by stereoselective transesterification with vinyl acetate was performed as a test reaction to analyse the steric restrictions of the active sub-site in the recognition of the acyclic secondary alcohols. *F. oxysporum* and *O. sulphureo-ochraceum* were the most active biocatalysts (Fig. 1). In the cases where reaction proceeded, the *R*-1-phenylethanol was esterified much faster than the *S*-antipode and high stereoselectivities were achieved.

| Lipase | ee at 240 h reaction time (%) |
|---|---|
| *F. oxysporum* | 89.6 |
| *O. sulphureo-ochraceum* | 100 |

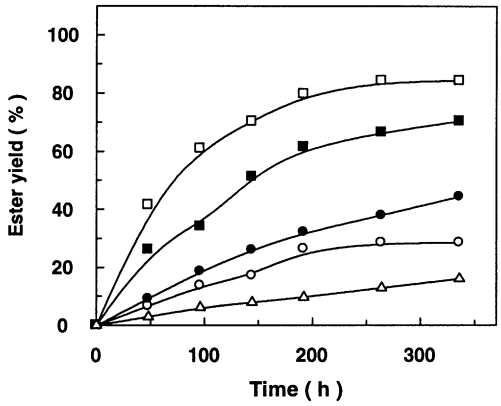



Fig. 1. Time course of the esterification of (*R*,*S*)-ibuprofen with 1-propanol catalysed by several of the selected lipases. Yield is given as the percentage of the racemic ibuprofen esterified at each reaction time. Lipases from: (■), *O. sulphureo-ochraceum*; (□), *F. oxysporum*; (○), *R. araucariae*; (●), *M. mucoroides*; (△), *F. poae* were used.

Therefore, these lipases showed the same enantiopreference that has been described for commercial enzymes [44,46,47]. It should be noted that in the same experimental conditions *C. rugosa* lipase only gave 9% yield, and the same *R*-enantioprefernce.

### 3.6. Carbamates synthesis **9b**

The synthesis of butyl carbamate **9b** (see Scheme 6) was selected as C–N bond formation reaction catalysed by our lipases (Table 8).

From the results of Table 8 we can see that, except *P. chrysogenum* lipase, all the new lipases are active in the alkoxy-carbonylation of the amine **9a**, but the concentrations of the amine and of the carbonate are lower than that used in the acylation of alcohols (Section 2). These more severe experimental conditions are general for all the lipase catalysed

Table 8
Synthesis of the carbamate **9b** using the selected lipases as biocatalysts[a]

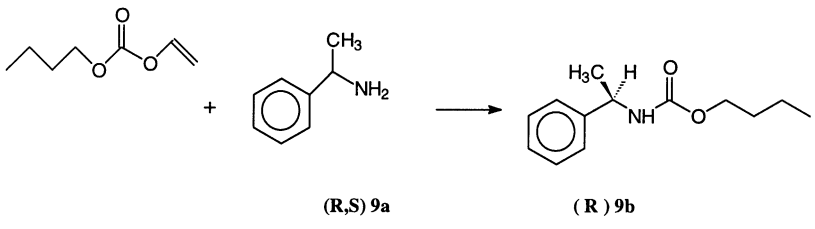
| Lipase | U/mg solid[b] $10^3$ | Yield in **9b** (%) | ee of **9b** (%) |
|---|---|---|---|
| *F. oxysporum* | 42.5 | 58 | 95 |
| *F. poae* | 115 | 47 | 70 |
| *F. solani* | 117 | 46 | 99 |
| *O. sulphureo-ochraceum* | 107.5 | 6 | 60 |
| *A. ciferrii* | 62.5 | 25 | 10 |
| *M. mucoroides* | 142 | 85 | 46 |
| *Monascus* sp. | 50 | 80 | 38 |
| *R. araucariae* | 35 | 21 | 47 |
| *C. rugosa* | 3200 | 61 | 16 |

[a] Enzyme = 200 mg of lyophilised powder, **9a** = 2 mmol, vinyl carbonate = 1.2 mmol, $T = 25^\circ$C, $t = 72$ h.
[b] U/mg of lyophilised biocatalyst 1 unit = amount of enzyme that produces 1 mmol heptyl oleate/h.

reactions where the nitrogen is the nucleophile [48,49]. As in the case of the acylation of alcohols, the *R*-enantioprefrence remains according to the Kazlauskas law for the nucleophile recognition sub-site [44]. The high ee values obtained (>95%) with lipases from *F. oxysporum* and *F. solani* make these enzymes interesting for these kind of reactions.

## 4. Conclusions

From the results of our paper we can conclude that from our screening of lipase-producer microorganisms, we have isolated new crude lipases that, after a future development of semi-purification or cloning and overexpresion process, may be interesting as biocatalysts for organic synthesis. In this way: (1) *F. oxysporum* and *O. sulphureo-ochraceum* lipases are very interesting in the enantioselective esterification of carboxylic acids; (2) *F. oxysporum*,




Scheme 6.

*F. solani*, *R. araucariae*, *P. chrysogenum* and *A. ciferrii* are useful for the acylation of primary or secondary alcohols with low steric hindrance around the OH; (3) *A. murorum* and *F. oxysporum* lipases are useful in the acylation of cycloalkanols; (4) *F. oxysorum* and *O. sulphureo-ochraceum* are the most interesting biocatalysts for the enantioselective acylation of (*R*,*S*)-1-phenylethanol; (5) *F. oxysporum* and *F. solani* lipases are useful for synthesis of carbamates.

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