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General Characterization of Noncommercial Microbial Lipases in Hydrolytic and Synthetic Reactions

C. Otero,*[,1] M. A. Berrendero,[1] F. Cardenas,[2] E. Alvarez,[2] and S. W. Elson[2]

[1]*Instituto de Catálisis y Petroleoquímica, C. S. I. C. Campus Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain, E-mail: cotero@icp.csic.es; and* [2]*GlaxoSmithKline, Centro de Investigación Básica, Santiago Grisolía, 4, Parque Tecnológico de Madrid, 28760 Tres Cantos, Madrid, Spain*

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Abstract

Fourteen noncommercial preparations of microbial lipases were investigated with respect to their catalytic activity for hydrolysis and synthesis of ester bonds. Six of the lipases were derived from microorganisms that have not previously been described as lipase producers, and another four were characterized for the first time. The synthetic reactions were carried out in two solvents of different polarities (*n*-heptane and acetone) using a series of fatty acids and primary and secondary alcohols with different chain lengths. Under the culture conditions employed, *Pseudomonas cepacia* produced more active enzyme than the other microorganisms. The lipase preparations produced using *Ovadendron sulphureo-ochraceum*, *Monascus mucoroides*, *Monascus* sp., *Fusarium oxysporum*, *Penicillium chrysogenum, Rhodotorula araucariae*, *Pseudomonas cepacia*, *Streptomyces halstedii*, and *Streptomyces* sp. were the most efficient catalysts for hydrolysis at lipid-water interfaces. Enzyme preparations from *P. cepacia*, *Streptomyces* sp., *S. halstedii*, and *R. araucariae* were good biocatalysts for esterification in the polar medium (acetone). When the lipase preparations with the greatest activity for hydrolytic reactions were excluded, regression analysis of the data for the hydrolytic and synthetic activities of the remaining lipase preparations yielded high multiple correlation coefficients for these reactions in both *n*-heptane and acetone ($R = 0.82$ and 0.91, respectively).

Index Entries: Lipases; esterases; hydrolysis; synthesis; esterification; triacylglycerols; *Pseudomonas*; *Candida*; *Streptomyces*.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

Lipases (glycerol ester hydrolases; EC 3.1.1.3) constitute one of the most studied categories of enzymes because of their great potential for use in a number of applications. They not only mediate for the hydrolysis of naturally occurring triglycerides *(1)*, but also catalyze other hydrolysis reactions, such as esterification, transesterification, polyesterification, amidation, and a variety of other reactions of both natural and synthetic materials *(2)*.

Most lipases are commercialized as crude preparations and contain a variable number of isoenzymes or isoforms (e.g., *Candida antarctica, Candida rugosa*) *(3)*. These crude biocatalysts often also exhibit esterase activity (e.g., *C. rugosa* lipase). Consequently, they may be employed for a variety of biotechnological applications involving both hydrolytic and synthetic reactions.

The commercial potential for lipase-catalyzed reactions is based on the regio-, chemical, and stereoselectivities *(3)* of these enzymes. This range of catalytic properties confers these enzymes with extraordinary potential for a wide variety of applications. These crude lipase preparations can be employed in the production of pharmaceuticals *(4)*, detergents *(5,6)*, cosmetics *(7)*, aroma and flavor compounds *(8)*, fatty acids *(9,10)*, polyesters *(11)*, and biomodified fats *(12)*. To date, important progress has been made in the generation of novel biocatalysts using genetic and biochemical approaches, in immobilization and modification of these biocatalysts with amphiphilic polymers, and in modulation of the stereochemistry of enzymatic reactions *(13)*.

Nonetheless, the number of lipases (commercial and noncommercial) that has been subjected to detailed investigation is rather limited. Even though nature offers many more lipases than those that have been studied to date (they are widely distributed among microorganisms, animals, and plants), considerable effort is required to find new naturally occurring lipases with the properties desired for specific applications in either aqueous or organic media. Several attempts to identify new biologic sources of lipases have been reported, and new techniques for detection of lipase activity have been developed *(14)*.

Several model systems have been designed to be used as references for the identification of enzymes that could be of interest for biotechnological applications. These systems are based on correlations between the different activities exhibited by these enzymes. Thus, correlations between hydrolysis and synthesis activities through the use of multiple correlation coefficients obtained from regression analyses have been employed as a basis for characterizing the performance of lipases as biocatalysts for esterification reactions *(15,16)*.

In this article, we present a general study of the catalytic behavior of 14 noncommercial preparations of microbial lipases in hydrolysis reactions in aqueous media and for synthesis of esters in nonaqueous systems.

Table 1
Enzyme Preparations From the GlaxoSmithKline Collection

Strain	Specie	Country of origin
h1	*Acremonium murorum* (Corda) W. Gams	Spain
h2	*Monascus mucoroides* van Tiegh	Spain
h4	*Fusarium poae* (Peck) Wollenw	Cuba
h5	*Fusarium oxysporum*	Cuba
h6	*Penicillium chrysogenum* Thom	Cuba
h8	*Monascus* sp.	Spain
h11	*Ovadendron sulphureo-ochraceum* (van Beyma) Sigler & Carmichael	Cuba
b1	*Pseudomonas cepacia*	Spain
b2	*Pseudomonas fluorescens*	Spain
b3	*Brevibacterium linens*	Spain
l1	*Rhodotorula araucariae*	Spain
a3	*Streptomyces fradiae*	Spain
a6	*Streptomyces* sp.	Spain
a7	*Streptomyces halstedii*	Nicaragua

Recently, several reports support the concept that solvents should be selected within the context of the nature (polarity) of the substrates and products of particular reactions *(17–21)*. With this goal in mind, we investigated the effects of two organic solvents with different polarities on the abilities of several lipase preparations to catalyze the direct esterification of several alcohols with different fatty acids. The ratio of the specificities of these preparations for esterasic and lipasic substrates in water are compared with their corresponding specificities for fatty acids of different chain lengths in the formation of esters in organic media.

Materials and Methods

Chemicals

All chemicals were of the highest purity available. Lauric acid was purchased from Merck (Darmstadt, Germany). Tributyrin, arabic gum, palmitic acid, butyric acid, and isopropanol were obtained from Sigma (St. Louis, MO). Acetone and *n*-heptane were from Scharlau (Barcelona, Spain). Organic solvents were stored in the presence of 4-Å molecular sieves.

Isolation of Microorganisms and Screening of Lipolytic Activity

All microorganisms were isolated from soil samples collected in various countries (Table 1). Taxonomic identification of the unicellular bacteria, actinomycetes, and yeasts was performed by the Spanish Type Culture Collection (C. E. C. T., Valencia, Spain). The filamentous fungi were identified in-house using mature cultures on suitable media *(22)*.

Preliminary screening of the lipolytic filamentous fungi, yeasts, and bacteria was carried out on BYPO agar plates supplemented with a 10% (v/v) olive oil or tributyrin emulsion prepared in 10% (w/v) arabic gum solution. Culture plates were incubated at 28°C and periodically examined for 4 d. Actinomycetes were screened on plates containing NE medium *(23)* with the same supplement, incubated at 28°C, and examined for 7 d. Colonies showing clear zones around the actinomycetes were picked out and screened for lipase production in liquid media, as described previously *(22)*. A specific substrate for lipolytic enzymes (1,2-*O*-dilauryl-*rac*-glycero-3-glutaric acid resorufinyl ester) was employed. Positive supernatants were dialyzed overnight against 25 m*M* Tris-HCl buffer, pH 7.0, and then lyophilized and stored at 4°C. Positive supernatants were also tested for proteolytic activity (resulting in negative tests in all cases). These lipases from different origins (i.e., fungi, yeast, and bacteria) were identified as *Ovadendron sulphureo-ochraceum, Fusarium oxysporum, Fusarium poae, Penicillium chrysogenum, Acremonium murorum, Monascus mucoroides, Monascus* sp., *Rhodotorula araucariae, Pseudomonas cepacia, Pseudomonas fluorescens, Brevibacterium linens, Streptomyces halstedii, Streptomyces fradiae,* and *Streptomyces* sp.

Determination of Protein

Protein concentrations in the different lipase samples were determined by the method of Bradford *(24)* with bovine serum albumin as a standard.

Reverse-Phase High-Performance Liquid Chromatography

Quantitative analyses of the esters formed in synthetic reactions were carried out in a high-performance liquid chromatography system consisting of an L-7100 isocratic pump connected to a Kromasil RP-18 column (Merck), an RI-71 refractive index detector, and a D-7500 integrator (Hitachi, Japan). Mobile phases were acetonitrile/methanol/water (50/15/35 [v/v/v]; flow rate: 2 mL/min) and acetonitrile/water (20/80 [v/v]; flow rate: 1 mL/min) for butyric acid and butyric acid ethyl ester, respectively; acetonitrile/acetone/water (45/45/10 [v/v/v]; flow rate: 2 mL/min) for lauric acid and lauric acid ethyl ester; acetonitrile/acetone/water (40/42/18 [v/v/v]; flow rate: 2 mL/min) and acetone/acetonitrile (90/10 [v/v]; flow rate: 2 mL/min) for lauric acid and lauric acid lauryl ester, respectively; and acetonitrile/acetone/water (46/47/7 [v/v/v]; flow rate: 2 mL/min) for palmitic acid and its ethyl and isopropyl esters, respectively. Retention times were 7.04, 8.56, 2.74, 5.94, 4.15, 3.26, 4.34, 10.45, and 11.5 min for butyric acid and butyric acid ethyl ester, lauric acid and lauric acid ethyl ester, lauric acid and lauric acid lauryl ester, palmitic acid and palmitic acid ethyl and isopropyl esters, respectively.

Determination of Esterase Activity

Esterase activity was measured spectrophotometrically in a Uvikon 930 spectrophotometer (Kontron, Italy) equipped with cells thermostated

at 30°C and magnetic stirring. The assay mixture (3 mL) consisted of 50 µL of 49 m*M* *p*-nitrophenyl butyrate solution in acetonitrile as cosolvent and 2.95 mL of 0.1 *M* sodium phosphate buffer, pH 7.2. The final acetonitrile concentration in the mixture was 1.7% (v/v). Liberation of *p*-nitrophenol into the solution was measured at 346 nm (isosbestic point of the *p*-nitrophenol/*p*-nitrophenoxide pair) taking the molar extinction coefficient to be 4800 M^{-1} cm^{-1}. In all cases the spontaneous hydrolysis was monitored for 3–5 min, and the contribution of this reaction was then subtracted from the total reaction in the subsequent analysis of the data. The enzymatic activity was expressed in units per milligram of lyophile (1 U = production of 1 µmol of *p*-nitrophenol/min under the aforementioned conditions).

Determination of Lipase Activity

Lipolytic activity was determined in a DL21 pH-Stat (Mettler, Spain) at 30°C in 1 m*M* Tris-HCl, pH 7.2, containing 0.1 *M* NaCl and 0.1 *M* $CaCl_2$. Tributyrin was the substrate of choice. The reaction mixture consisted of 0.9 mL of tributyrin and 1.5 mL of a 10% (w/w) gum arabic solution, with the final volume of the solution made up to 40 mL using Tris-HCl buffer containing 3% (v/v) acetonitrile. The resultant mixture was sonicated for 3 min before each assay. The spontaneous hydrolysis reaction was monitored first and the enzymatic reaction was then started by the addition of the biocatalyst. The fatty acids released were titrated with NaOH. The enzymatic activity was expressed in units per milligram of lyophile (1 U = production of 1 µmol of free acid per minute under the aforementioned conditions).

Esterification Reactions

Enzymatic syntheses of ethyl laurate, lauryl laurate, ethyl butyrate, ethyl palmitate, and isopropyl palmitate were performed using 0.175 mmol of the corresponding fatty acid, and 0.175 mmol of ethanol, dodecanol, or isopropanol. The reaction was initiated by the addition of the corresponding lipase (3% [w/w]). The solutions were incubated at 40°C in an orbital shaker at 200 rpm. The final reaction volume was 1 mL. Molar yields of the esters produced during the reactions were calculated with respect to the concentration of the acid added to the reaction mixture.

Karl Fischer Determinations

Water contents of the lyophilized enzymes, solvents, and substrates were determined using a KF DL18 Karl Fischer apparatus (Mettler).

Results and Discussion

Hydrolytic Activity

It is well known that lipases are the only hydrolases exhibiting both esterase and lipolytic activities. Traditionally, *p*-nitrophenyl esters have been employed as model compounds for determination of esterase activity

and triglycerides such as tributyrin for determining lipase activity *(25)*. In the present study, determinations of hydrolytic activity were carried out in aqueous media to characterize the esterase and lipase activities of selected enzyme preparations (*see* Table 2). The enzyme preparations contained 2–10% (w/w) lipase with respect to the total protein content. The optimum pH for all of the enzyme preparations was about 7.2. Hence, their activities were compared at this pH. An effective comparison was established by considering the ratio of the lipase and esterase activities. The highest ratio (4.5×10^4) was exhibited by the lipase preparation from *O. sulphureo-ochraceum,* followed by the lipase preparations from *M. mucoroides*, *F. oxysporum*, *Monascus* sp., *Streptomyces* sp., *P. cepacia*, *S. halstedii*, *R. araucariae* and *P. chrysogenum* (0.3×10^4). By contrast, the enzyme preparations from *A. murorum*, *B. linens*, *S. fradiae, P. fluorescens*, and *F. poae* demonstrated much higher affinities for esterasic substrates (values ranged from 0.12×10^4 to 5×10^{-5}). The reader should bear in mind that for a sample of a particular enzyme preparation, the units of the esterase and lipase activities are different. Consequently, a value of 10^4 for the ratio of these two activities does not imply that the enzyme preparation functions 10^4 times better as a lipase than as an esterase. However, the use of this ratio permits one to conduct a comparative study of the activities of the different lipase preparations. The enzyme preparations belonging to the first group of microorganisms (those with high ratios of lipase to esterase activity) might be considered more efficient catalysts than the others for reactions occurring at lipid/water interfaces, and the preparations corresponding to the second group of microorganisms function better as biocatalysts for hydrolysis of soluble esters.

In the literature, the corresponding ratios of esterase to lipase activities vary a great deal from one commercial or noncommercial lipase preparation to another. Recent studies on lipase structure have permitted structural factors to be identified with the specificities of lipases and esterases for different substrates. Pleiss et al. *(26)* studied the shape and physicochemical properties of the fatty acid binding site of six lipases and two serine esterases in an effort to elucidate the molecular basis of substrate specificity. They reported that lipases from *Candida antarctica, Pseudomonas* sp., and *Rhizopus* sp. all have large hydrophobic binding sites with different shapes. In addition, Otero et al. *(27)* recently described differences in the nature of the binding (mobility, polarity, binding constants) of fatty acid–lipase complexes and their relation to variations in the substrate specificities of the different isoforms present in the commercial preparation of *C. rugosa* lipase. The mobility of the labeled fatty acid was greater in the lipase with higher esterase activity, in particular, a lipase having cholesterol esterase activity *(27)*.

Synthetic Activity

Direct esterification for production of a standard ester (ethyl laurate) was carried out using each of the 14 lipases as a biocatalyst in both *n*-heptane

Table 2
Hydrolytic and Synthetic Activities of Microbial Lipases Ordered in Terms of Decreasing Ratio of Lipase to Esterase Activity

		Hydrolytic activity			Synthetic activity[a]			
					n-heptane		Acetone	
Lipase	Protein content (% [w/w])	Esterase activity (U/mg × 10^4)[b]	Lipase activity (U/mg)[c]	Lipase/ esterase activity (×10^4)	3 h	24 h	3 h	24 h
O. sulphureo-ochraceum	0.77	0.18	0.81	4.50	14.3	52.2	0.75	3.27
M. mucoroides	10.8	0.03	0.09	2.83	8.40	49.4	3.50	7.57
F. oxysporum	1.33	1.74	2.30	1.32	15.7	85.8	1.00	10.9
Monascus sp.	8.95	0.09	0.12	1.30	23.3	73.3	1.05	5.50
Streptomyces sp.	10.5	0.28	0.36	1.28	43.8	96.7	7.89	44.0
P. cepacia	6.70	53.7	52.3	0.97	68.7	93	9.30	42.7
S. halstedii	5.80	0.51	0.42	0.82	47.3	98.0	9.40	56.4
R. araucariae	4.90	0.77	0.32	0.42	39.5	59.1	32.6	34.3
P. chrysogenum	2.55	24.6	7.34	0.30	11.6	54.0	0.33	1.45
A. murorum	6.90	1.60	0.18	0.12	0.80	2.30	0.32	0.46
B. linens	6.90	0.02	2×10^{-3}	0.09	1.60	3.10	1.56	2.16
S. fradiae	1.46	0.13	2×10^{-3}	1.6×10^{-2}	3.30	4.90	2.97	3.28
P. fluorescens	10.0	0.05	6×10^{-4}	1.3×10^{-2}	0.91	1.70	0.90	1.20
F. poae	0.60	0.06	3×10^{-10}	5×10^{-9}	3.10	15.7	0.63	0.72

[a]Synthetic activity is defined as the molar yield (in percent) of acid converted at the time indicated.
[b]Esterase activity is defined as millimoles of *p*-nitrophenol per minute per milligram of enzyme preparation.
[c]Lipase activity is expressed as millimoles of free acid per minute per milligram of enzyme preparation.

(log P = 4) and acetone (log P = –0.23) as reaction media. These enzyme-catalyzed reactions were carried out without the addition of water. The degree of hydration of the lyophilized lipases was 3.12 ± 1.02% (w/w). The amount of crude lipase used in these experiments was 3% (w/w). Water contents were 2.4×10^{-3}, 0.48, 0.12, and 0.11% (w/w) for *n*-heptane, acetone, ethanol, and lauric acid, respectively.

The results (Fig. 1) indicate that the 14 culture broths contain lipases with different synthetic capabilities in both polar and apolar solvents. Some of these enzymes (those from *P. cepacia, S. halstedii, R. araucariae*, and *Streptomyces* sp.) acted as better biocatalysts in acetone. A review of the literature indicates that only a small number of lipases have been described as catalyzing synthetic reactions in polar organic solvents *(28–34)*. The structure of the enzyme (shape, size, and depth of the binding pocket) and the polarity of the residues present at the surface of the protein may play decisive roles in determining catalytic activity in solvents with different polarities.

Specificities for Acids and Alcohols With Different Chain Lengths: Comparative Studies of Reactivities for Primary and Secondary Alcohols

The studies described next were carried out in order to compare esterase and lipase (hydrolytic) activities of the lipases of interest and their corresponding activities for synthesis of esters of short-, medium-, and long-chain fatty acids. For this purpose, syntheses of ethyl esters of butyric, lauric, and palmitic acids were carried out in both acetone and *n*-heptane.

In both solvents, the enzyme preparations from *P. chrysogenum, F. poae, A. murorum, M. mucoroides, S. fradiae, P. fluorescens*, and *R. araucariae* displayed higher specificities for short-chain fatty acids than for long-chain fatty acids (*see* Table 3; formation of ester linkages). These effects could be related to the high esterase activities of these preparations, but some of these lipases exhibited greater activity than the others for hydrolysis of *p*-nitrophenyl esters in water (*see* Table 2). Moreover, the specificities of the enzymes also depended on the solvent used as the reaction medium. In particular, lipases from *P. cepacia, O. sulphureo-ochraceum, F. oxysporum, S. halstedii, Monascus* sp., and *B. linens* did not display different activities for acids of different chain lengths when assayed in *n*-heptane. Similar behavior was observed in acetone for enzyme preparations from *S. halstedii* and *B. linens*. The other enzyme preparations exhibited variable specificities in acetone: while the *P. cepacia* lipase was more specific for palmitic acid, the lipases from *F. oxysporum* and *Monascus* sp. were better catalysts for the synthesis of ethyl butyrate than for synthesis of ethyl palmitate. In aqueous media, these three enzyme preparations exhibited higher lipase activity than esterase activity.

The results obtained with our selected lipase preparations indicate that for the biocatalysts investigated, one cannot establish a global relation between the activities for hydrolysis of esters and/or lipids in water (Table 2) and the corresponding selectivities for synthesis of esters from acids of

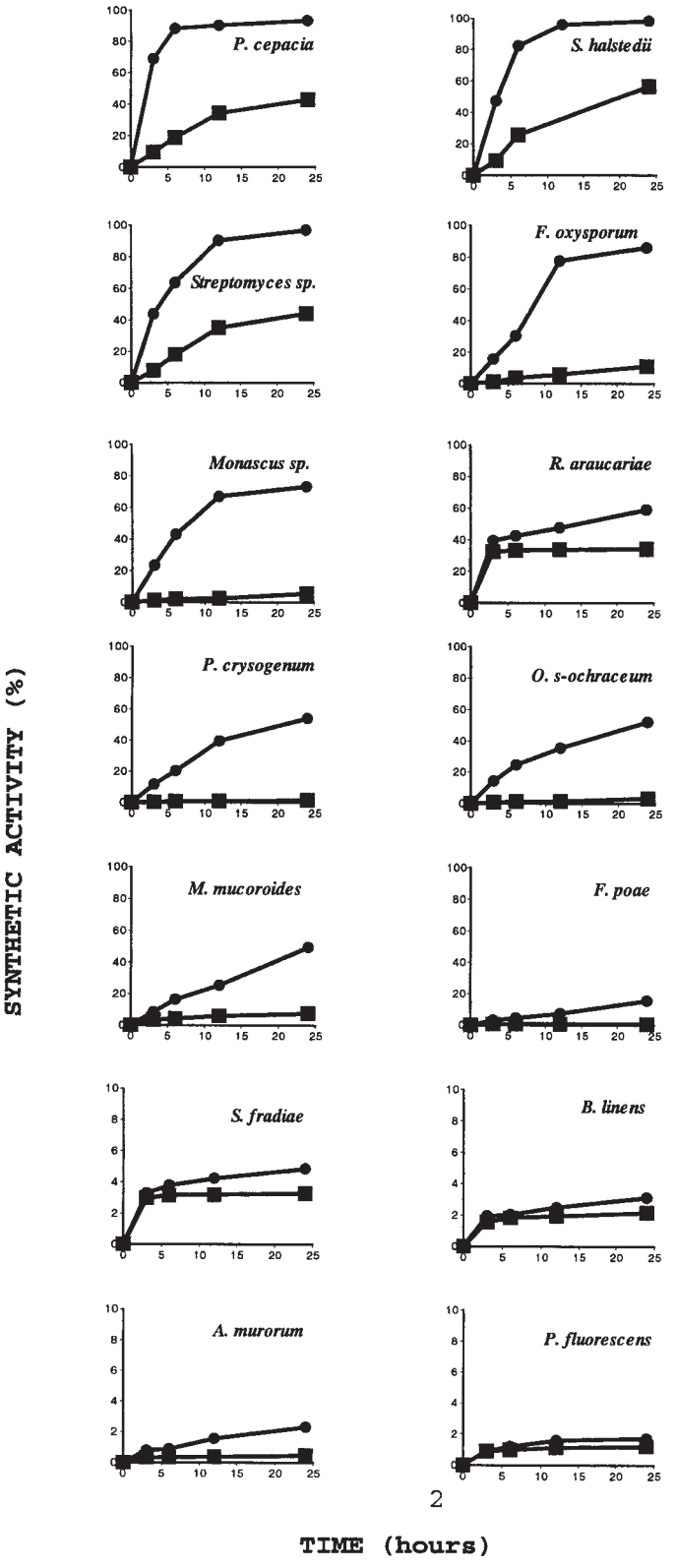

Fig. 1. Time-courses for esterification at 40°C in *n*-heptane (●) and acetone (■) with 14 new lipases as biocatalysts (loading 3% [w/w]).

Table 3
Synthetic Activities of Selected Lipases Based on Use of Acids of Different Chain Lengths for Esterification of Primary and Secondary Alcohols[a]

	Fatty acid						Primary alcohol		Secondary alcohol	
	Heptane (%)			Acetone (%)			Heptane	Acetone	Heptane	Acetone
Lipase	EB	EL	EP	EB	EL	EP	(%) (LL)	(%) (LL)	(%) (IP)	(%) (IP)
P. chrysogenum	56.2	11.6	10.1	2.64	0.33	0.21	43.1	0.20	2.50	0.00
F. poae	9.90	3.10	2.52	13.3	0.63	0.16	1.90	0.03	0.18	0.00
A. murorum	3.10	0.80	0.00	0.74	0.32	0.00	0.00	0.00	—	—
M. mucoroides	22.3	8.40	8.20	5.12	3.50	0.46	4.62	0.10	0.00	0.00
S. fradiae	17.7	3.30	0.00	28.0	2.97	0.00	1.17	0.00	—	—
P. fluorescens	0.75	0.54	0.30	0.67	0.53	0.15	0.05	0.05	0.00	0.00
R. araucariae	65.7	39.5	1.14	93.3	32.6	0.00	6.76	0.00	1.10	0.00
P. cepacia	72.3	68.7	70.4	2.50	9.30	31.7	63.5	13	23.8	2.62
O. sulphureo-ochraceum	7.60	14.3	14.9	4.90	0.80	0.90	11.9	0.05	0.24	0.00
F. oxysporum	15.4	15.6	18.3	5.12	1.00	0.92	4.64	0.50	0.37	0.00
S. halstedii	52.9	47.3	58	12.6	9.40	10.6	62.5	9.54	33.7	4.93
Monascus sp.	19.2	23.4	26.0	24.0	1.10	0.65	19.9	0.35	0.13	0.00
B. linens	0.86	1.70	1.93	1.00	1.56	1.68	0.13	0.00	0.00	0.00
Streptomyces sp.	17.1	43.8	27.1	2.70	7.90	5.50	13.3	3.28	5.51	0.35

[a]Synthetic activity is defined as the molar yield of acid converted in percent at 40°C. EB, ethyl butyrate; EL, ethyl laurate; EP, ethyl palmitate; LL, lauryl laurate; IP, isopropyl palmitate.

different chain lengths in organic media (Table 3). The absence of a correlation between hydrolytic and synthetic activities has been reported for other lipases *(35)*. Moreover, substrate specificities of enzymes functioning in organic media can be considerably different from the corresponding specificities in water *(36)*. Molecular considerations indicate that in organic media, electrostatic interactions of the polar groups of the enzyme with one another and with the substrate may be quite different from those in aqueous solution. Enzymes may undergo significant conformational rearrangements that depend on the nature of the solvent *(37)*. Furthermore, hydrophobic interactions between substrates and enzymes may be reduced in organic solvents *(38)*.

On the other hand, our studies of alcohols with different chain lengths revealed that the enzyme specificities did not depend on the polarity of the solvent. In most cases, the enzymes preferring short- or long-chain alcohols in *n*-heptane also exhibited the same preference in acetone (*see* Table 3). In general, most of the enzymes investigated (*F. poae, A. murorum, M. mucoroides, S. fradiae, P. fluorescens, R. araucariae, F. oxysporum, B. linens, Monascus* sp. and *Streptomyces* sp.) displayed high activities for synthetic reactions involving ethanol in both solvents. This effect is probably a consequence of the effective solvation of the long-chain alcohols in these solvents, resulting in low activity coefficients and low reaction rates *(35)*. However, whereas lipases from *P. chrysogenum* and *S. halstedii* displayed a significant preference for long-chain alcohols only when *n*-heptane was the reaction medium, the lipases from *P. cepacia* and *O. sulphureo-ochraceum* did not exhibit any specificity for alcohols of different chain lengths in either solvent.

Specificities for secondary alcohols were studied only for those enzymes that were efficient biocatalysts for the synthesis of ethyl palmitate. As expected, the rates of esterification of secondary alcohols (Table 3) were lower than those for primary alcohols *(39)*. This difference in catalytic behavior for esterification of primary and secondary alcohols has previously been observed for the *C. antarctica* lipase by both Miller et al. *(40)* and From et al. *(39)*. Use of the lipase from *S. halstedii* as a biocatalyst led to a 34% yield of isopropyl palmitate from its precursors. This yield is about half the yield obtained for ethyl palmitate. Much higher ratios between reactivities for primary and secondary alcohols were found for the lipases from *P. cepacia* (3 times), *F. oxysporum* (4 times), and *Streptomyces* sp. (4.9 times). Differences in esterification rates for primary and secondary alcohols were smaller in acetone than in *n*-heptane. Structural explanations have been proposed for differences in reactivity toward enantiomers of secondary alcohols. Thus, a small, well-defined pocket suitable for accommodation of the secondary alcohol has been suggested for the *C. antarctica* lipase *(41)*.

Comparison of Hydrolytic and Synthetic Activities

The use of lipases to catalyze synthetic reactions has been continuously increasing in recent years. Because lipases are normally marketed on the basis of their hydrolytic activities, there is a clear need for a protocol that

permits one to establish correlations between hydrolytic activity and ability to function as a biocatalyst for particular categories of synthesis reactions. We have explored the feasibility of developing such a correlation based on studies of our lipase preparations that employ the hydrolysis of tributyrin (Table 2) and the syntheses of ethyl laurate in *n*-heptane and acetone (Table 3) as model reactions. Of the lipase preparations investigated in this work, the enzyme from *P. cepacia* displayed the highest hydrolytic activity (52.3 U/mg). This value was 7 times greater than that for the lipase preparation from *P. chrysogenum*, 23 times higher than that from *F. oxysporum*, and almost 90,000 times higher than that from *P. fluorescens*. The synthetic activity of the *P. cepacia* lipase (Fig. 1) was also high in *n*-heptane (88% yield of ethyl laurate after 6 h of reaction) and medium in acetone (42.7% in 24 h). However, other lipase preparations with much lower hydrolytic activities in water displayed synthetic activities similar to those of *P. cepacia* in anhydrous systems. In particular, lipases from *S. halstedii, Streptomyces* sp., *F. oxysporum, R. araucariae, P. chrysogenum*, and *O. sulphureo-ochraceum* displayed intermediate to high hydrolytic activities, and high synthetic activities in *n*-heptane (98, 96.7, 85.8, 59.1, 54, and 52.2% yields of ester in 24 h, respectively). Poor yields of ethyl laurate were obtained with the first three enzymes in acetone (1.4, 10.9, and 3.2%, respectively). These low yields do not correlate well with the relatively good hydrolytic activities of these enzymes. On the other hand, lipases from *F. poae, A. murorum, S. fradiae, P. fluorescens*, and *B. linens* displayed the lowest hydrolytic and synthetic activities in both solvents, thereby indicating a potentially good correlation between these two types of activities.

Similar efforts to develop correlations between activities for hydrolysis and synthesis reactions for other lipases have encountered difficulties *(15,16)*. According to Wu et al. *(15)*, the hydrolytic activities of enzymes may be of little value for predicting their synthetic activities. Furthermore, in extreme cases, an enzyme may exhibit no synthetic activity at all, yet demonstrate high hydrolytic activity. Furutani et al. *(16)* reported a convincing example involving the esterification and transesterification reactions of myristic acid with ethanolamine. A series of commercial lipases (Lipase QL, Lipase PL, Lipase AK20, and Lipase PS) displayed high hydrolytic and synthetic activities. However, in spite of their high hydrolytic activity, enzymes such as polymixin acylase did not exhibit any synthetic activity after 7 d of reaction. Mustranta *(42)* reported that for the *C. rugosa* lipase, a good correlation is obtained between the activities for hydrolysis and synthesis of esters.

Because we have found that hydrolytic and synthetic activities are well correlated for a significant number of the microbial lipases investigated in our study, regression analyses of these activities were carried out. The multiple correlation coefficients obtained from the regression analyses based on the activities of the 14 lipases employed for the synthesis of ethyl laurate and their corresponding hydrolytic activities were $R = 0.36$ in

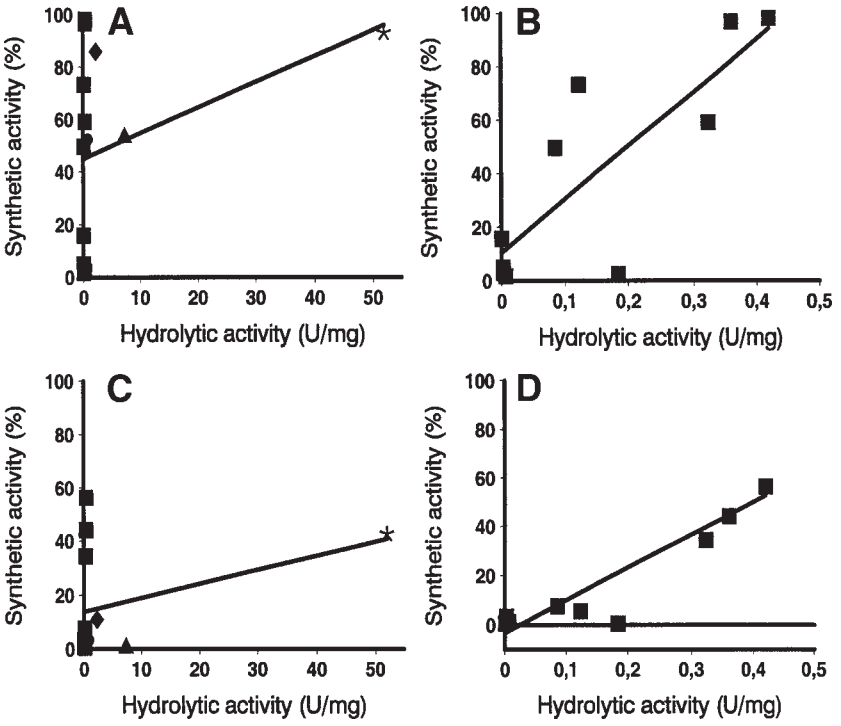

Fig. 2. **(A)** Conversion of lauric acid during esterification in *n*-heptane (R = 0.36) based on all 14 lipases and **(B)** conversion (R = 0.82) without *P. cepacia* (■), *O. sulphureo-ochraceum* (●), *F. oxysporum* (◆), and *P. chrysogenum* (▲) lipases; **(C)** conversion in acetone (R = 0.33) based on all 14 lipases and **(D)** conversion (R = 0.91) without *P. cepacia* (■), *O. sulphureo-ochraceum* (●), *F. oxysporum* (◆), and *P. chrysogenum* (▲) lipases. One hundred percent conversion to the ester corresponds to consumption of 0.133 mol of fatty acid / mg of enzyme preparation during the synthesis of ethyl laurate.

n-heptane (Fig. 2A) and R = 0.33 in acetone (Fig. 2B). In our investigation, we found four lipases (*O. sulphureo-ochraceum*, *F. oxysporum*, *P. cepacia*, and *P. chrysogenum*) that displayed a very good activity for hydrolysis (for both lipasic and esterasic substrates). Omission of these lipases from the regression analysis led to increases in the multiple correlation coefficients to 0.82 and 0.91 for *n*-heptane and acetone, respectively (Fig. 2C,D). This result cannot be attributed to different water contents in these four enzyme preparations because the 14 enzyme preparations all had similar hydration levels (see Materials and Methods). In general terms, correlations for hydrolytic and synthetic activities cannot always be successfully established. However, for the enzyme preparations considered here (except for four lipases), it was possible to generate a satisfactory correlation ($R \sim 0.9$).

Further optimization of the fermentation processes for the production of culture broths containing higher concentrations of these lipases can now be carried out to obtain activity levels comparable with those of commercial lipase preparations.

Conclusion

Fourteen noncommercial microbial lipase preparations were produced using different culture media. In spite of the low concentrations of active proteins in the culture media, all of these preparations displayed significant activities for both hydrolysis and synthesis reactions. Several of the selected lipases were obtained using microorganisms that have not previously been described in the literature as lipase producers (*A. murorum, M. mucoroides, F. poae, O. sulphureo-ochraceum, R. araucariae*, and *S. halstedii*) or that have been described as producers but whose enzymes have not been characterized (*F. oxysporum, P. chrysogenum, B. linens*, and *S. fradiae*). Fungi provide a huge potential source of enzymes; a recent estimate *(14)* of the number of fungal species is 1.5 million, and it is believed that only about 1 to 2% of the existing fungal species have been detected and characterized.

Most of the enzymes of interest are active in apolar solvents. Four of these enzyme preparations could be suitable biocatalysts for the synthesis of esters in polar media (lipases from *P. cepacia, S. halstedii, Streptomyces* sp., and *R. araucariae*). Hence, although most of the aforementioned lipases would be appropriate for use in the modification of fats (e.g., via interesterification and acidolysis reactions), these specific four enzyme preparations should be more appropriate than the others for the synthesis of biosurfactants in anhydrous polar media.

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