Journal of Molecular Catalysis B: Enzymatic 61 (2009) 136–142

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Effects of organic solvents on activity and stability of lipases produced by thermotolerant fungi in solid-state fermentation

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ARTICLE INFO

Article history:
Received 24 September 2008
Received in revised form 22 May 2009
Accepted 2 June 2009
Available online 10 June 2009

Keywords:
Lipase stability
Organic solvents
Thermotolerant fungus
Lipase production
Solid-state fermentation

ABSTRACT

Dried solid-state fermented solids (biocatalysts) produced by seven thermotolerant fungal strains were tested for lipase activity and stability in organic solvents. Two strains of *Rhizopus* sp. (19 and 43a) produced biocatalysts (L-19 and L-43a) that showed high lipase activities (74 and 72 U/g of dry matter, respectively) comparable to Lipozyme® RM IM (118 U/g DM). The use of the dipole moment of the organic solvents along with their classification based on the functional groups (non-polar, protic polar, aprotic polar) allowed the establishment of four different relative activity profiles for the seven biocatalysts evaluated. Compared to a biocatalyst not exposed to the organic solvent (100% relative activity), all biocatalysts showed a high relative activity (greater than 90%) in aprotic polar solvents (acetonitrile, acetone and ethyl acetate), whereas in protic polar solvents (ethanol and *i*-propanol) activity was reduced (lower than 40%). In addition, the incubation of biocatalysts L-19 and L-43a in *i*-amyl alcohol increased lipase activity in the synthesis of ethyl oleate 3.36 and 1.46 times, respectively. L-19 activity also increased after incubation in toluene (2.0 times), *i*-propanol (1.5 times) and acetonitrile (1.3 times) at temperatures from 30 to 50 °C. The results suggest that these biocatalysts can be used for a broad range of lipase reactions.

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are serine hydrolases that catalyze the hydrolysis and the synthesis of esters from glycerol and long-chain fatty acids. These enzymes are of great interest for many reasons: they do not require cofactors, they remain active in organic solvents, they can be used with a broad range of substrates, and they often exhibit high enantio- and regio-selectivity [1]. Before the mid-1980s, lipases were used most frequently in laundry applications and in the modification of triglycerides [2]. However, they are also very effective biocatalysts in nearly anhydrous organic solvents [3]. Since lipases can be used in a variety of synthesis reactions (e.g., esterification, transesterification, alcoholysis, acidolysis, aminolysis, acylation and resolution of racemic mixtures), they have become potentially important for biotechnology and industry applications [4].

Biocatalysis in organic solvents has several potential advantages over biocatalysis in aqueous media: (i) increased solubility of hydrophobic compounds; (ii) ability to carry out new reactions that are kinetically or thermodynamically restricted in water; (iii) suppression of undesirable side reactions; (iv) control or modification of enzyme selectivity (substrate, enantio-, regio-, prochiral); (v) the possibility of recovery of some products by the use of low-boiling-point organic solvents; and (vi) an increased enzyme thermostability [5]. In spite of these advantages, enzymes do not always meet desired levels of activity, productivity and, most importantly, stability in organic solvents [6]. Hydrophobic solvents typically lead to higher enzymatic activity and stability than their hydrophilic counterparts [5], which tend to strip some of the water required for enzymatic function, thereby lowering the catalytic activity [7]. The availability of lipases that were stable in polar solvents would favour new applications in biotechnological processes with polar substrates. For example, Slotema et al. [8] reported oleamide production via direct amidation of carboxylic acid with ammonia. For this reaction, the use of hydrophilic organic solvents with increased polarity shifted the thermodynamic equilibrium, leading to: (i) higher enzymatic activity; (ii) higher dissolution of water generated as reaction product while the water activity remains close to the optimum for the enzyme; (iii) higher solubility of substrates and products; and (iv) higher conversions of substrates into products. Thus, it is still necessary to find lipases that are stable in polar solvents. It has been reported that the structural properties that confer enzyme thermostability also confer stability in organic solvents [9,10]. Therefore, thermophilic or thermotolerant

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doi:10.1016/j.molcatb.2009.06.004

organisms can be used as suitable resources to obtain enzymes that are stable in organic solvents, since enzymes from these organisms possess intrinsic thermal stability [11].

Fungi are one of the most important sources of lipases for industrial applications. Their enzymes are usually produced extracellularly and can be obtained either by submerged (SmF) or by solid-state (SSF) fermentations [12]. Compared to SmF, SSF is preferable because of its higher productivity, reduced catabolic repression and lower water requirements. In addition, in processes with fast growing or osmotolerant microorganisms and the use of heavy inocula, sterilization of culture medium might be unnecessary due to the low water activity in the solid medium, which limits the growth of undesirable microorganisms. Overall, this leads to a lower capital investment and reduced operating costs [13,14]. Furthermore, the produced enzymes are retained in the fermented solids without expensive extraction, purification and immobilization processing. Hence, dried SSF preparations can be considered as economical biocatalysts for synthetic reactions in organic solvents. Recently, Nagy et al. [15], Fernandes et al. [16] and Martínez-Ruíz et al. [17] have shown the feasibility of using dried fermented solids, containing lipases, in biocatalytic reactions using organic solvents. However, comprehensive stability patterns of those lipase preparations are still unknown in solvents commonly used in different biocatalytic systems; the degree of enzyme stability will be important in determining the economic feasibility of such systems. In the present study, the objective was to produce lipases by SSF on a inert support from six strains of thermotolerant fungi (*Rhizopus* sp.) and one strain of thermophilic fungus (*Rhizomucor* sp.), and then to characterize their stability as a function of both organic solvent polarity and temperature.

2. Materials and methods

2.1. Microorganisms and maintenance of cultures

The fungal strains employed in this work were *Rhizopus microsporus* var. *rhizopodiformis* (9a), *R. microsporus* var. *oligosporus* (13b), *R. microsporus* var. *tuberosus* (19), *Rhizomucor pusillus* (23a), *R. microsporus* var. *chinensis* (43a) and *R. microsporus* (56c). These strains were isolated [18] and deposited by Jesús Córdova in the Universidad Autónoma Metropolitana microbial collection (Mexico). *Rhizopus homothallicus* var. *rhizopodiformis* (13a) was kindly donated by S. Roussos from the IRD culture collection, Marseille, France. Fungal strains were cultured for 7 days at 45 °C on potato dextrose agar (DIFCO) slants and stored at 4 °C. The commercial enzyme preparation Lipozyme® RM IM (Novozymes) was kindly donated by Novozymes (Mexico).

2.2. Inocula preparation

In order to obtain the inocula, each fungal strain was propagated in 250 mL Erlenmeyer flasks containing 60 mL of potato dextrose agar at 45 °C for 7 days. The spores were harvested in 0.01% (v/v) Tween-80 solution and counted in a Neubauer chamber.

2.3. Culture medium composition

The fermentation medium for lipase production contained (g/L) [19]: urea (6.0); olive oil (60.0); lactose (7.5); K_2HPO_4 (7.5); $MgSO_4{\cdot}7H_2O$ (1.5); polyvinyl alcohol (2.4); plus 6 mL/L of an oligo-elements solution containing (g/L): EDTA (10); $MnCl_2{\cdot}4H_2O$ (1.98); $CoSO_4{\cdot}7H_2O$ (2.81); $CaCl_2{\cdot}2H_2O$ (1.47); $CuCl_2{\cdot}2H_2O$ (0.17); $ZnSO_4{\cdot}7H_2O$ (0.29). The pH of the medium was adjusted to 6.5; olive oil was incorporated and then emulsified using an Ultra-Turrax homogenizer (IKA-WERK) at 10 000 rpm for 10 min.

2.4. Production of lipases by SSF

Rhizopus homothallicus var. *rhizopodiformis* (13a) was used to establish the culture conditions in solid-state fermentation. Polyurethane foam (PUF) and expanded perlite were tested as inert solid supports. Both materials were sieved (to obtain particles between 0.8 and 2.0 mm), washed three times with distilled water and dried at 60 °C for 24 h. Supports were impregnated with the culture medium and then inoculated with a spore suspension (3×10^7 spores per gram of dry solid support). In all cases, inoculum volume was lower than 5% of the culture medium volume. Column reactors (2.5 cm × 20 cm) were then packed either with 8 g of impregnated PUF or 20 g of impregnated perlite. Culture conditions: temperature, 45 °C; aeration rate, 40 mL/min; initial moisture content, 55%; initial pH, 6.5. Two columns were sacrificed at each sampling time (20, 24 and 28 h) during the fermentation. Culture media, column reactors and input air were not sterilized for lipase production. For enzyme recovery, the fermented material obtained was mixed with distilled water (1:10 w/v) and vortexed for 1 min. Solid particles were filtered (Whatman 41) and the clear filtrate assayed for lipase activity. Specific growth rate (μ) and lag phase were estimated through respiratory activity by CO_2 measurements during the course of culture. For CO_2 measurements, an on-line gas monitoring system was connected to the outlet air streams from column reactors that were connected to a gas chromatograph linked to a personal computer, as described by Saucedo-Castañeda et al. [20].

2.5. Preparation of naturally immobilized lipases from fermented solids (biocatalysts)

Once SSF conditions were established, production of lipases by six thermotolerant strains (*Rhizopus* sp.) and one thermophilic strain (*Rhizomucor* sp.) was carried out at 45 °C for 20 h. The fermented solids were lyophilized for 12 h at −50 °C in a LYPH LOCK 6 Lyophilizer (LABCONCO®) and stored at 4 °C. Enzymatic activity losses were 17 and 18% when the fermented solids were air-dried or lyophilized, respectively. These lyophilized solids (biocatalysts) were used to evaluate the effect of organic solvent polarity and temperature on lipase stability.

2.6. Assays of hydrolytic lipase activity

Depending on the nature of the lipase (liquid enzymatic extract or dried powder), one of two analytical methods was used to estimate the lipase activity.

2.6.1. Hydrolysis of p-nitrophenyl palmitate

Lipase activity in liquid enzymatic extracts was assayed according to the protocol of Pencreac'h and Baratti [21] with some modifications. The assay solution was prepared by creating an emulsion from 1 volume of a 13.4 mM *p*-nitrophenyl palmitate (*p*NPP) solution (in *i*-propanol) to 9 volumes of a 100 mM Tris–HCl buffer, pH 8.0 (at 45 °C), containing 20 mM $CaCl_2$ and 0.25% (w/v) polyvinyl alcohol (PVA). The reaction mixture contained 2.7 mL of the emulsion and 0.3 mL of an enzyme solution properly diluted in 100 mM Tris–HCl buffer, pH 8.0 (at 45 °C). Once the reaction was started, absorbance (410 nm against a blank without enzyme) was monitored for 3 min using a spectrophotometer (SHIMADZU® UV-160). The reaction rate was calculated from the slope of the absorbance versus time curve using an apparent molar extinction coefficient of 15.783 $cm^2\ \mu mol^{-1}$ for *p*-nitrophenol (*p*NP). One unit of activity (IU) was defined as the amount of enzyme that liberated 1 μmol of *p*NP per min at 45 °C and pH 8.0 under the assay conditions. All assays were done in triplicate; the results are presented as mean ± standard deviation.

2.6.2. Hydrolysis of olive oil

Lipase activity in biocatalysts (dried solids) was measured using a colorimetric method based on the formation of copper soaps of free fatty acids (Kwon and Rhee [22] with some modifications [19]). The reaction mixture consisted of 20 g olive oil and 100 mL of 100 mM Tris–HCl buffer, pH 8.0 (at 45 °C), containing 10 mM $CaCl_2$, 0.25% (w/v) polyvinyl alcohol and 0.3% (w/v) arabic gum. This system was emulsified for 10 min at 10 000 rpm (45 °C) using an Ultra-Turrax (IKA-WERK®). The reaction was started by adding 3 mL of emulsion per 40 mg of biocatalyst. Incubation was performed at 45 °C for 15 min, and enzymatic activity was stopped with 0.6 mL of 6 M HCl. Fatty acids released were extracted in 3 mL of *n*-heptane, vortexed for 30 s. The tubes were centrifuged (9800 × *g*, 10 min, 4 °C) and 0.5 mL of a copper acetate–pyridine solution (50 g/L, pH 6.1) was added to 2.5 mL of recovered organic phase and vortexed for 30 s. Absorbance was measured at 715 nm in a spectrophotometer (SHIMADZU® UV-160). A calibration curve was constructed using oleic acid concentrations from 0 to 10 mM. A blank was prepared running a parallel assay with a biocatalyst previously deactivated with 0.6 mL of 6 M HCl. One unit of activity (IU) was defined as the amount of enzyme able to release 1 μmol of fatty acid per min at 45 °C and pH 8.0 under the above conditions. All assays were done in triplicate and the results are presented as mean ± standard deviation.

For both lipase assays, activity (IU) was referred to the dried fermented solids, expressed as dry matter (DM). For Lipozyme® RM IM the activity was expressed in units per gram of dry enzymatic commercial preparation, which includes the inert solid carrier and enzymatic protein.

2.7. Assay of synthetic lipase activity

Synthesis of ethyl-oleate was followed by measuring the residual free fatty acids using the protocol of Lowry–Tinsley [23], which has proved to be reliable by different authors [16,17,24]. Lipase mediated synthesis of ethyl-oleate was performed in 3 mL screw-capped glass vials using a molar ratio of oleic acid:ethanol of 1:5, in which 0.1 g biocatalyst was added to 2 mL 50 mM oleic acid solution in *n*-heptane. The reaction was started by addition of concentrated ethanol (99.9% purity) and incubated at 45 °C for 2 h. Capped glass vials were stirred in an orbital shaker at 150 rpm for 1 min every 5 min. The reaction was stopped by adding 0.5 mL 6 M HCl. Aliquots (0.3 mL) of the organic phase were diluted with 1.2 mL *n*-heptane followed by addition of 1 mL copper acetate–pyridine solution (50 g/L, pH 6.1) and were then vortexed for 30 s. Absorbance was measured at 715 nm using a spectrophotometer (SHIMADZU® UV-160). A standard curve was constructed using oleic acid. A blank was run under the assay conditions with the biocatalyst previously deactivated with 0.5 mL 6 M HCl. All assays were done in triplicate; the results are presented as mean ± standard deviation.

2.8. Lipase stability in organic solvents

The effect of organic solvents on lipase stability (including the stability of commercial enzyme Lipozyme® RM IM) was evaluated. The following organic solvents were selected and classified according to their values of Log *P* and dipole moment (in Debye units, D) respectively [25,26]: acetonitrile (−0.33, 3.2), acetone (−0.23, 2,88), ethyl acetate (0.68, 1.78), ethanol (−0.24, 1.69), *i*-propanol (0.28, 1.66), toluene (2.5, 0.36), *n*-hexane (3.5, 0) and *n*-decane (5.6, 0). For subsequent analysis, *i*-amyl alcohol (1.16, 1.8) and tetrahydrofuran (0.49, 1.75) were also included. All solvents were dried with molecular sieves (nominal pore diameter 4 Å) before use. Biocatalysts (0.15 g) were incubated with 1 mL solvent for 1 h at 25 °C. The solvent was then removed through a forced air current with a flow-rate of 22 L/min for 2 min. Residual lipase activity was measured by hydrolysis of olive oil (see Section 2.6.2). The relative enzymatic activity (% REA) was reported. Biocatalyst not exposed to the organic solvent was used as reference. Duncan tests ($\alpha < 0.05$) were used to establish significant differences between treatments for each biocatalyst.

2.9. Effect of organic solvents and temperature on lipase stability

In order to determine the effect of temperature on the stability of the enzyme, 0.5 g biocatalyst was incubated at 30, 40 and 50 °C in 1.5 mL of various organic solvents with different polarity (non-polar = toluene; protic polar = *i*-propanol; aprotic polar = acetonitrile) for 1 h. The solvent was then removed through an air current with a flow of 22 L/min for 2 min. Lipase activity was measured as a function of the residual oleic acid concentration during the synthesis of ethyl-oleate at 45 °C in *n*-heptane. The relative enzymatic activity (% REA) was reported using a non-treated biocatalyst as a reference.

3. Results and discussion

3.1. Evaluation of two inert solid supports for growth and lipase production of R. homothallicus (strain 13a) cultured via solid-state fermentation (SSF)

Polyurethane foam (PUF) and expanded perlite were evaluated as inert solid supports for SSF. As Table 1 shows, no significant differences ($\alpha < 0.05$) were found for specific growth rate (μ) and lag phase between both supports. For both solid supports, maximum lipase activity (Table 1) and CO_2 production rate were observed at 20 h of culture (profiles not shown). Lipase production was higher (40%) in perlite than in PUF. Thus, SSF for lipase production was conducted using perlite as an inert solid support.

3.2. Preparation of naturally immobilized lipases from fermented solids (biocatalysts)

Table 2 shows specific growth rate (μ), lag time and lipase activity values obtained for each fungal strain after 20 h of culture at 45 °C. Among the different tested strains, significant differences ($\alpha < 0.05$) were found for μ and for lipase activity, while no significant differences in lag time were detected. Specific growth rates were between 0.4 and 0.5 h^{-1}. Strains 19 and 43a produced the highest values of lipase activity using *p*NPP (15.5 and 11.1 U/g DM, respectively) and olive oil (74.0 and 72.4 U/g DM, respectively). These activity values were similar to those obtained with a commercial enzyme from *Rhizomucor miehei* (Lipozyme® RM IM) (118.1 U/g DM, using olive oil). The ratio of enzymatic activity between both substrates (*R*) was calculated (Table 2). For all the assayed lipases, a preference toward natural substrates (triglycerides) was evident ($R > 1$). The differences in the *R*-values among *Rhizopus* strains suggested the presence of different lipases; this is in agreement with previous reports describing differences in N-terminal sequences of several lipases produced by *R. homothallicus* (13a), *Rhizopus niveus*, *Rhizopus oryzae* and *Rhizopus delemar* [27,28].

It is worth noting that the lipase activities on olive oil that were obtained in this short process of 20 h were similar to or higher than those obtained by other workers using wild or mutant fungal strains with improved lipase production (Table 3). Another advantage of this SSF system is the use of an inert support (perlite) as a simple matrix for enzyme immobilization, avoiding the possibility of taking undesirable compounds into the biocatalysis process, which might occur with complex substrates. This would facilitate the product purification steps. To date there has been relatively little use of inert supports for the production of enzymes, although the polymeric resins Amberlite IRA-900 [35] and perlite [17] have

Table 1
Fermentation parameters and lipase activity during solid-state cultures of *Rhizopus homothallicus* (strain 13a) with two different inert solid supports.

Inert support	$\mu_{CO_2}(h-1)$[b]	Lag phase (h)[b]	Lipase activity (U g^{-1} dry matter)[c] *p*NPP		
			20 h	24 h	28 h
PUF[a]	0.32 ± 0.04	9.9 ± 2.0	4.6 ± 0.2	0.6 ± 0.3	0.6 ± 0.1
Perlite	0.42 ± 0.06	10.8 ± 1.1	6.4 ± 0.1	2.2 ± 1.3	1.9 ± 0.8

[a] PUF, polyurethane foam.
[b] No significant differences were found between inert supports (ANOVA test, $\alpha < 0.05$).
[c] Significant differences were found between inert supports (ANOVA test, $\alpha < 0.05$).

Table 2
Fermentation parameters and lipase activity of seven fungal strains during solid-state cultures using expanded perlite as an inert support.

Strain	μ (h^{-1})		Lag phase (h)	Lipase activity (U g^{-1} dry matter)[a,b]				
				*p*NPP		Olive oil		*R*[d]
9a	0.53 ± 0.020	A	11.0 ± 0.9	2.7 ± 0.6	D	36.7 ± 4.0	C	13.5
13a	0.38 ± 0.001	C	11.3 ± 1.4	8.4 ± 0.5	C	30.0 ± 1.8	C	3.5
13b	0.50 ± 0.003	B	9.1 ± 1.2	1.9 ± 0.7	D	22.5 ± 0.1	D	10.7
19	0.41 ± 0.006	C	8.5 ± 0.2	15.2 ± 1.6	A	74.0 ± 7.0	B	4.8
23a	0.41 ± 0.004	C	8.8 ± 0.8	n.d[c]		3.2 ± 0.3	E	–
43a	0.40 ± 0.005	C	8.0 ± 0.5	11.1 ± 0.6	B	72.4 ± 5.7	B	6.4
56c	0.36 ± 0.034	D	8.1 ± 1.1	7.1 ± 0.9	C	16.8 ± 0.3	D	2.4
Lipozyme® RM IM						118.12 ± 1.0	A	–

ABCDE: The same letter indicates no significant difference according to Duncan's test ($\alpha < 0.05$).
[a] Lipase production corresponds to that obtained at 20 h of culture.
[b] Lipase activity (IU) of fermented dry matter, in units of activity per gram of dry matter (U/g DM).
[c] n.d: not detected.
[d] *R*: ratio of lipase activity on olive oil/lipase activity on *p*NPP.

Table 3
Lipase production by several fungal strains in SSF systems.

Microorganism	Type	Lipase activity (U g^{-1} dry matter)[a,b]	Time (h)	Solid substrate	Reference
Rhizopus rhizopodiformis	Wild	80	12	Olive oil cake- Bagasse	[29]
Penicillium restrictum	Wild	30	24	Babassu cake	[30]
Penicillium simplicissimum	Wild	30	36	Babassu cake	[31]
Penicillium simplicissimum	Wild	26.4	n.r[c]	Babassu cake	[32]
Rhizopus oligosporus T^{UV}-31	Mutant	76.6	48	Almond meal	[33]
Rhizopus oligosporus ISU^{UV}-16	Mutant	81.2	48	Almond meal	[34]

[a] Lipase activity was determined using olive oil as substrate.
[b] Lipase activity (IU) in units of activity per gram of dry matter (U/g DM).
[c] n.r: not reported.

been used for lipase production by SSF. In the work by Martínez-Ruíz et al. [17], the fermented material (biocatalyst) was directly used to synthesize ethyl-oleate with high esterification rates and substrate conversion; this biocatalyst retained more than 80% of its catalytic activity after 7 months of storage at 4 °C.

3.3. Lipase stability and relative activity in organic solvents

In addition to activity, the stability of lipases in organic solvents is an important parameter for industrial applications. Therefore, the effect of several organic solvents on the stability of the lipase was determined, not only for the biocatalysts produced by each fungal strain but also for the commercially available enzyme Lipozyme® RM IM. The Log *P*-value is generally used to correlate solvent polarity with enzyme activity and stability in non-aqueous phases [36,37,38]. However, a correlation between lipase stability and Log *P*-value was not found in this work (Fig. 1). A similar lack of correlation was reported for lipases produced from *Acinetobacter* sp. SY-01 [39] and *Acinetobacter* sp. RAG-1 [40].

In order to establish a correlation between the type of solvent and the enzyme stability, the organic solvents were ordered according to their dipole moment and their functional groups [41] as follows: non-polar (*n*-decane, *n*-hexane, toluene), protic polar (*i*-propanol, ethanol), and aprotic polar (ethyl acetate, acetone, acetonitrile) (Fig. 2). In order to determine lipase stability, each biocatalyst was incubated for 1 h in different organic solvents at 25 °C; then the residual hydrolytic activity was measured with olive oil as the substrate (see Section 2.6.2). The obtained results suggest that the activity values are influenced by several phenomena, including not only inhibition and activation, but also denaturation.

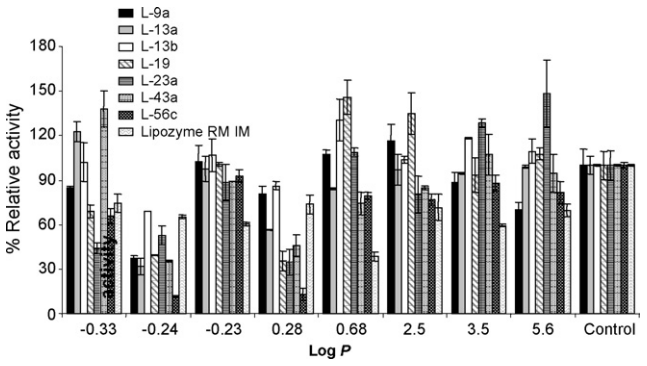

Fig. 1. The effect of organic solvents in function of their Log *P*-value on lipase activity. Lipases used in this study were produced by six strains of thermotolerant fungi and one strain of thermophilic fungus. The solvents and their Log *P*-values were: acetonitrile, −0.33; ethanol, −0.24; acetone, −0.23; *i*-propanol, 0.28; ethyl acetate, 0.68; toluene, 2.5; *n*-hexane, 3.5; *n*-decane, 5.6. The activity was measured as described in Section 2.6.2.

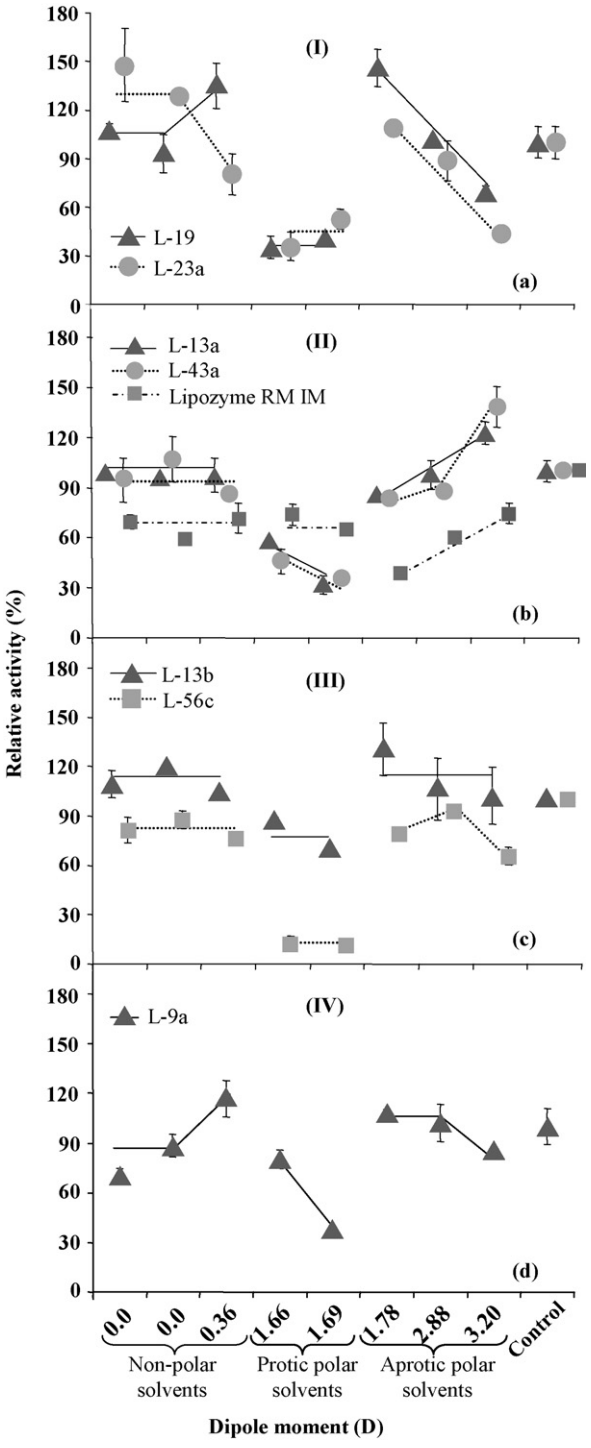

Fig. 2. Stability profiles of lipases after exposure (1 h) to different organic solvents. Lipases used in this study were produced by six strains of thermotolerant fungi and one strain of thermophilic fungus. L-19, L-23a (Profile I) (a); L-13a, Lipozyme® RM IM (Profile II) (b); L-13b, L-43a, L-56c (Profile III) (c); L-9a (Profile IV) (d). The solvents and their dipole moment values were: *n*-decane, 0.0; *n*-hexane, 0.0; toluene, 0.36; *i*-propanol, 1.66; ethanol, 1.69; ethyl acetate, 1.78; acetone, 2.88; acetonitrile, 3.2. The activity was measured as described in Section 2.6.2.

Some reports have tried to correlate enzyme function with properties related to the polarity of organic solvents, such as: dielectric constant (ε) [42], polarizability, hydrogen bond donor ability (α), hydrogen bond acceptor ability (β) [43], polarity index [44] and Log *P* [26,38,45]. Although the Log *P*-value has been commonly used as a quantitative measure of polarity, this property denotes hydrophobicity that is related to polarity but is not synonymous with it [26]. Therefore, in this work the dipole moment was considered as a polarity parameter; this value depends on chemical and structural properties of the organic solvent, such as: number and type of chemical bonds, presence of delocated electron pairs, electronegativity of atoms, molecular size and symmetry, and electrostatic potential which determine the stabilization of neighbouring molecules [41]. In addition to this polarity parameter, the use of categories related to functional groups of the organic solvents (non-polar, protic polar, aprotic polar) allowed us to establish four different profiles of relative activity as shown in Fig. 2. Attempts to establish a correlation with either Log *P* or the dipole moment were not successful. This is in accordance with Valivety et al. [46], who stated that "there is probably no single parameter for solvent polarity that will predict the enzyme activity in organic media".

Lipase activity after incubation of the biocatalysts in non-polar solvents (toluene, *n*-hexane, *n*-decane) was from 70 to 148% compared to that of the control without solvent (100%). These biocatalysts could be used in systems with reaction media based on non-polar solvents. In similar reports for free and immobilized lipase from Basidiomycete *Bjerkandera adusta* R59, residual activities from 70 to 120% were reported after 24 h of incubation in cyclohexane, *n*-hexane and *n*-heptane at room temperature [47]; this behaviour was attributed to the fact that non-polar organic solvents do not strip off the water layer from the surface of enzyme. In contrast, Sulong et al. [48] found that the relative activity of the lipase, produced by the bacterium tolerant to organic solvents *Bacillus sphaericus* 205y, was as low as 40% after 30 min of incubation in toluene at 37 °C. These results show that it is not possible to establish a general rule on the behaviour of activity and stability in non-polar solvents.

Currently, suitable performance of enzymes in non-polar solvents has been established in different biocatalytic processes [3,26]. However, the availability of lipases that are active and stable in polar solvents would open new opportunities in biocatalysis with polar substrates. Incubation in the presence of protic polar solvents (*i*-propanol and ethanol) showed a negative effect in the biocatalysts assayed in this work; a relative activity value as low as 12% was obtained for the biocatalyst L-56 in the presence of protic polar solvents. Similarly, low relative activity values have been reported after 1 h of exposition in protic polar solvents for lipases from different organisms such as *Penicillium simplicissimum*, 33% in ethanol [49], *Penicillium aurantiogriseum*, less than 2% in methanol, ethanol and *i*-propanol at 29 °C [38] and *Bacillus megaterium*, 45% in ethanol at 28 °C [45]. In contrast to this behaviour, it is noteworthy that the lipases from biocatalysts L-9a and L-13b retained a hydrolytic relative activity higher than 80% in *i*-propanol. These results are comparable to those reported for lipases from *P. simplicissimum*, 79% after 1 h in propanol [49], *B. megaterium*, 97% after 1 h in *i*-propanol at 28 °C [45] and *B. sphaericus* 205y, 96% after 30 min at 37 °C in ethanol [48]. This high relative activity might be due to the moderate competence of alcohols for amide hydrogen bonds [50] in addition to the substitution of water with the hydroxyl groups of the alcohol [44].

Studies dealing with the effect of aprotic polar solvents (ethyl acetate, acetone, acetonitrile) showed that most of the biocatalysts assayed (L-9a, L-13a, L-13b, L-19, L-23a and L-43a) had high relative activities (80–146%) after 1 h of incubation at 25 °C (Fig. 1). Instability of lipases in aprotic polar solvents is frequently associated with the stripping of water from the protein surface [51], along with solvent penetration into the enzyme, leading to protein unfolding and subsequent denaturation [52]. There are only few reports showing the stability of lipases in aprotic polar solvents. A lipase produced by *Pseudomonas* sp. had relative activity values from 100 to 110% after 15 h at room temperature in acetone, tetrahydrofuran and ethyl acetate [53]. In addition, Wu et al. [54], using a lipase from

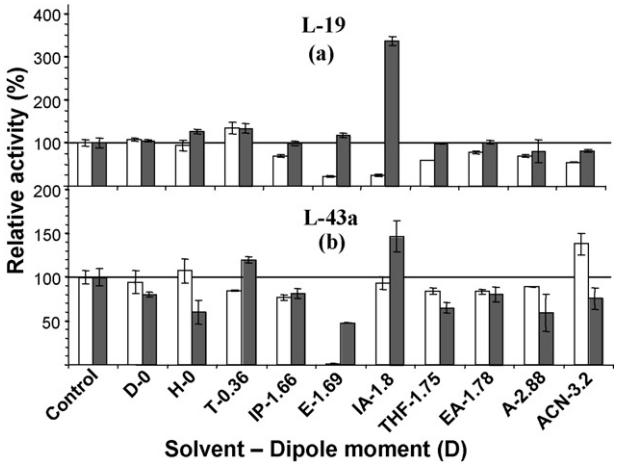

Fig. 3. Residual activity of lipases after exposure (1 h) to organic solvents, grouped by their dipole moment: (a) L-19 and (b) L-43a. Lipase activity was evaluated as described in Section 2.6.2 for (□) hydrolysis of olive oil and Section 2.7 for (■) synthesis of ethyl-oleate. The solvents and their dipole moment values were: *n*-decane, D-0; *n*-hexane, H-0; toluene, T-0.36; *i*-propanol, IP-1.66; ethanol, E-1.69; *i*-amyl alcohol, IA-1.8; tetrahydrofuran, THF-1.75; ethyl acetate, EA-1.78; acetone, A-2.88; acetonitrile, ACN-3.2.

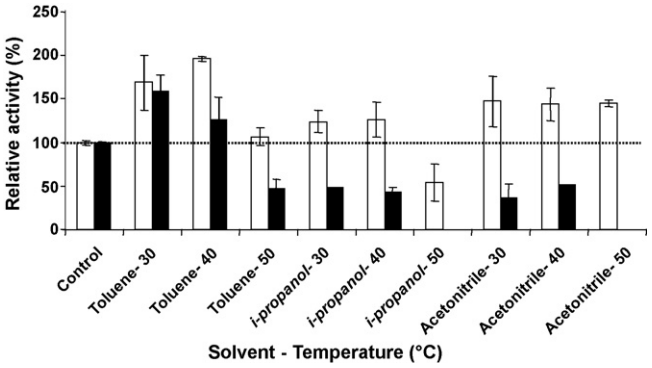

Fig. 4. Effect of organic solvent (toluene, *i*-propanol and acetonitrile) and temperature (30, 40 and 50 °C) on residual activity of (□) L-19 and (■) L-43a. Lipase activity was evaluated by synthesis of ethyl-oleate as described in Section 2.7.

Mucor javanicus, observed high stability and an increased activity after 2 h of incubation at 25 °C in acetonitrile (85%), ethyl acetate (168%) and acetone (246%).

3.4. Relative activity of lipases in both hydrolysis and synthesis reactions

Fig. 3 shows the relative activity of the lipases from biocatalysts L-19 and L-43a as a function of the dipole moment, in both hydrolysis and synthesis reactions. A relative activity over 80% was observed in non-polar solvents (*n*-decane, *n*-hexane and toluene) and higher than 70% in *i*-propanol. However, ethanol reduced hydrolysis activity (L-19 and L-43a) more than 80% and lowered synthesis activity (L-43a) by 50% (Fig. 3). This suggests that ethanol molecules might cause significant structural changes in the enzyme, as was observed for the *Penaeus penicillatus* acid phosphatase [55]. On the other hand, aprotic polar solvents (tetrahydrofuran, acetone, ethyl acetate and acetonitrile) did not affect the stability of L-19 for the synthesis of ethyl-oleate nor L-43a for hydrolysis of olive oil. These results suggest that the interaction between the aprotic polar solvents and the enzyme did not affect the hydration level or the conformation required for enzymatic activity [8,56].

An increase in lipase activity (synthesis and hydrolysis) after incubation in organic solvents was observed as follows: L-19 activity for synthesis increased 1.26, 1.18, 1.34 and 3.36 times in *n*-hexane, ethanol, toluene, and *i*-amyl alcohol, respectively; similarly, the rate of the hydrolysis reaction increased 1.35 times in toluene. For L-43a, activity increased 1.20 and 1.46 times in toluene and *i*-amyl alcohol, respectively (for the synthesis reaction) and 1.38 times in acetonitrile (for the hydrolysis reaction). As suggested by Wu et al. [54], the increase in enzymatic activity could be explained by the structural transition from the inactive "closed" form of lipase to the active "open" form, as a result of the treatment with organic solvents. These authors observed an enhanced activity (up to 193%) for the immobilized lipase of *M. javanicus* after treatment for 2 h at 25 °C with both protic and aprotic polar solvents (*i*-propanol, ethyl acetate and acetone). The same effect was reported for *P. aurantiogriseum* lipase and *B. megaterium* lipase after 1 h of incubation in *n*-heptane (non-polar solvent) with relative activities of 113 and 120%, respectively [38,45].

Up to now, several lipase-based processes have been established using non-polar solvents [57]. Therefore, as showed above for the biocatalysts L-19 and L-43a, their stability and increased activity in polar solvents (*i*-propanol, ethanol, *i*-amyl alcohol, tetrahydrofuran, ethyl acetate, acetone, acetonitrile) will allow the development of reactions using polar substrates (e.g., amides, flavonoids, lactones, carbohydrates, hydroxycinnamic acids). In addition, polar solvents have technological advantages compared to non-polar solvents (e.g., lower toxicity, lower boiling points and lower costs). Finally, a further advantage of the system is that the direct use of fermented solids in the biocatalytic process improves process economics in relation to systems in which the biocatalyst must be extracted, purified and immobilized.

3.5. Effect of the temperature on lipase activity of biocatalysts incubated in different organic solvents

The effect of temperature and type of organic solvent on lipases stability was determined for biocatalysts L-19 and L-43a, which were incubated in toluene (non-polar), *i*-propanol (protic polar) and acetonitrile (aprotic polar) at 30, 40 and 50 °C. After 1 h of incubation, synthesis activity was evaluated (Fig. 4). Lipase activity of biocatalyst L-19 was higher (from 10 to 100%) than the control activity in most of the conditions tested; activity was only reduced (50%) after incubation in *i*-propanol at 50 °C. In contrast, lipase activity from biocatalyst L-43a was only increased after incubation in toluene at 30 and 40 °C. An important loss of activity was observed after incubation in *i*-propanol and acetonitrile at 30–50 °C, in fact lipases were totally inactivated at 50 °C. Secondary and tertiary structural changes have been reported for a *R. oryzae* lipase after treatment with *i*-propanol, which was accompanied by lipase activation [58].

4. Conclusions

Solid-state fermentation was used to produce lipases from six strains of thermotolerant fungi (*Rhizopus* sp.) and one strain of thermophilic fungus (*Rhizomucor* sp.) using perlite as an inert solid support. Biocatalysts L-19 and L-43a showed comparable lipase activities to the commercial enzyme preparation Lipozyme® RM IM (Novozymes); in both cases enzymatic activity was quantified per gram of dry matter. The dry fermented solids could be used as economical naturally immobilized biocatalysts in synthesis reactions.

In this work, organic solvents were classified according to both dipole moment and functional groups (non-polar, protic polar and aprotic polar), then effect of each organic solvent was evaluated on the activity and stability of the seven biocatalysts produced by SSF.

The dipole moment was chosen as polarity parameter and alternative to Log *P*. To our knowledge, this is the first report where the dipole moment of solvents and their functional groups are used to characterize lipase reactions in organic solvents. By using this approach, four relative activity profiles of lipases as a function of the solvent were obtained, suggesting structural differences among the seven assayed lipases. Biocatalysts L-9a, L-13a, L-13b, L-19, L-23a and L-43a showed high stability in several polar solvents (protic and aprotic). In addition, L-19 was thermostable in non-polar and polar solvents. Remarkably, incubation in polar organic solvents increased lipase activity for L-19 and L-43a. These results suggest that naturally immobilized biocatalysts have great potential in biotechnological processes. In particular, the produced biocatalysts show potential with polar solvents, which have technological advantages such as low toxicity, low boiling points, low costs and the possibility to use polar substrates for novel reactions. Further studies will reinforce these results, especially related to the kinetics of activity changes during incubation time under these various solvent/temperature regimes.

Acknowledgements

B. Hernández-Rodríguez acknowledges a scholarship from CONACyT. The authors thank Novozymes (Mexico) for their kind donation of lipase (Lipozyme® RM IM). The suggestions from Ph.D. Arely Prado and Ph.D. Octavio Loera are gratefully acknowledged.

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