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Biocatalytic characterization of a naturally immobilized lipase found in *Araujia sericifera* Brot. (Apocynaceae) latex

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Lipase activity found in the insoluble fraction of *Araujia sericifera* Brot. (Apocynaceae) (ASL) latex, a native South American milkweed, was characterized by use of different test reactions and under different reaction conditions. In this context, hydrolytic lipase activity towards both natural and synthetic substrates and towards fatty acid esterifications was assayed at different temperatures, pH values, and biocatalyst loads. In the case of natural substrates (cottonseed oil), highest lipase activity was found at pH 8.5 and 60 °C. In the hydrolysis of synthetic substrates (*p*-nitrophenyl esters) the lipase showed preference for the lowest molecular-weight *p*-nitrophenyl ester assayed (butyrate). Results of the direct esterification of fatty acids of different chain length in organic media showed that esterification levels of up to 45% could be obtained in one hour of reaction. Activity results were compared with the activity shown by the commercial immobilized lipase Novozym 435 under defined reaction conditions. The high activity exhibited by ASL in the hydrolysis of natural substrates and particularly in the direct esterification of different fatty acids in organic medium, together with its high storage stability, suggests that this plant lipase is a promising biocatalyst for various biotechnological applications.

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Introduction

Lipases (EC 3.1.1.3) are a family of enzymes which in their natural environment catalyze the hydrolysis of triacylglycerols producing free fatty acids, diacylglycerols, monoacylglycerols and glycerol. In living organisms lipases play a key role in the catabolism of fats or oils as well as in their storage and release when used as energy reservoirs.[1] In general terms, lipases are defined as carboxylesterases which catalyze the hydrolysis (and synthesis) of long chain acylglycerols (glycerol esters of acyl groups consisting of ten or more carbons). Besides, under the proper conditions, lipases are also capable of catalyzing esterification, transesterification, aminolysis and lactonization reactions.[2] The balance between hydrolysis and synthesis reactions is controlled by the water activity of the reaction mixture.[1]

Currently, lipases are widely used as ingredients in detergents and medicine formulations, as catalysts for the resolution of chiral compounds, as food additives, in special oils and fats production, in wood acylglycerols degradation for paper production, and in the removal of fats in leather tanning, among others.[3] Likewise, lipases of microbial origin have been used in the obtention by esterification of monoacylglycerols (widely used emulsifiers in cosmetics, food and pharmaceutical industries) and phytosterol esters (food additives for reducing blood cholesterol levels).[4] Transesterification products such as fragrances (*e.g.* geraniol acetate) are also obtained by reactions catalyzed by lipases with high yield.[5] Other lipase-catalyzed transesterification products include substitutes of human milk fat[6] as well as biodiesel from edible and inedible oils.[7] All these applications and many others make lipases the enzymes most widely used in organic synthesis.[8]

Lipases are ubiquitous in nature and are produced by plants, animals and microorganisms. Lipases of microbial origin (fungal and bacterial) are the most commonly used enzymes in biotechnological applications and in organic chemistry.[9] In general, extracellular lipases are the most commercially important because their mass production is much simpler. For this reason, most lipases currently used in industry are of microbial origin.[3] By the way, plant lipases seem very attractive due to their low cost, ease of purification, and the fact that they are widely available from natural sources without the need for molecular genetics technology to produce them. Lipases of plant origin are often found in seeds (as in the case of castor plant, rapeseed, peanuts, flax, soybean, oats, *etc.*) and also in latex-producing plant species such as Asclepiadaceae, Sapotaceae, Euphorbiaceae,

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Moraceae, Papaveraceae and Asteraceae.[10] Recently, much research has been devoted to the characterization and use of plant lipases obtained from latex, with a view to provide an alternative to the microbial lipases produced using genetic engineering methods.[11–13] Given the ease of their obtention, their high selectivity and specificity, these lipases are more acceptable for their application—for example in the food industry—than animal or microbial lipases.[6] In particular, the plant lipase from *Carica papaya* (CPL) latex has been studied extensively in applications such as resolution of various racemates,[14,15] structured lipids synthesis,[1,16] synthesis of terpene esters,[17] *etc.* Lipases obtained from the latex of other plant families have been characterized in terms of diverse biocatalytic features, but they have been less studied in terms of their potential biotechnological applications.[18,19] On the other hand, oilseed plant lipases are usually present in very low concentration levels, which represents a drawback for their use in large scale or pilot scale and justifies why, until now, their current applications are mainly on a laboratory scale. Plant lipases from sources other than oil seeds can be considered as promising catalysts for higher production levels.[10]

In the current contribution, the hydrolytic and synthetic activities of lipase from the latex of fruits of *Araujia sericifera* Brot. (synonym: *Araujia hortorum* E. Fourn.) (Apocynaceae)—from here on ASL—are characterized by use of target substrates. *Araujia sericifera* is a native South American milkweed (Argentinian folk names: "*tasi*", "*doca*") that can also be found in subtropical regions of the world as an invading species that affects the growth and development of the native flora and valuable crops.[20–24] Proteases present in the latex of *Araujia sericifera* have been widely studied, including their purification and determination of biochemical features, as well as their application in different fields, especially those related to their performance as biocatalysts in peptide bond formation and the engineering to improve their ability in such reactions.[5,25–32] However, to the authors' knowledge, the presence of lipolytic activity in the latex of ASL has not been characterized in detail before. A previous work by Giordani *et al.*[33] reported the presence of lipolytic activity in the latex belonging to an Asclepiadaceae plant species, *Araujia serilofera*, which could not be found in the currently available world plant databases.[34,35] The authors of the present paper work under the assumption that both species are the same and that the name *Araujia serilofera* is a deviation from *Araujia sericifera*. The fact that we considered this species as belonging to the Apocynaceae family is because the Asclepiadaceae family has been recently subsumed with the Apocynaceae, being the former Asclepiadaceae, a subfamily of the Apocynaceae.[36]

Experimental

Materials

Sodium salts of the following Good's buffers, AMPSO (3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid), CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), MES (2-[*N*-morpholino]ethanesulfonic acid), MOPS (3-[*N*-morpholino]propanesulfonic acid) and TAPS (*N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid), as well as cottonseed oil (0.7% 14:0, 21.6% 16:0, 2.3% 18:0, 18.9% 18:1, 54.4% 18:2, 0.7% 18:3, 0.3% 20:0, 0.2% 22:0), oleic acid, *p*-nitrophenyl butyrate, *p*-nitrophenyl laurate, *p*-nitrophenyl palmitate, butyric acid, caproic acid and lauric acid were all purchased from Sigma-Aldrich. Other chemicals used in this work were of analytical grade. Novozym 435 (*Candida antarctica* B lipase (CALB) adsorbed onto a polymethacrylate support) was also bought from Sigma-Aldrich.

Araujia sericifera lipase (ASL) extraction

4.92 kg of unripe fruits were harvested in late summer from different individuals of a wild population of *A. sericifera* mature plants grown in La Plata, Province of Buenos Aires, Argentina (34°54′38″S, 57°59′47″W). The latex was obtained by removal of the petioles of the fruit and by collection in a solution containing 5 mM EDTA kept in an ice bath. The homogenate was centrifuged (9600 × g, 4 °C, 30 min) to separate the insoluble fraction (gums) from the soluble part. The insoluble fraction obtained was lyophilised, ground with mortar and pestle to reduce the particle size of the desiccated gums. The final powder (4.26 g) was fractionated at different temperatures to test storage stability. Biocatalyst particles showed varying shapes with a medium equivalent diameter of 44 ± 24 μm. The fraction used for ASL characterization was stored at −20 °C.

Determination of the hydrolytic activity of ASL

Hydrolytic activity of ASL towards a natural substrate. The natural substrate chosen to assay the lipolytic activity of ASL was cottonseed oil (Sigma). 0.5 ml of cottonseed oil was contacted with 1 ml of aqueous buffer and 0.4 ml of $CaCl_2$ solution (see details on the buffers used and their pH below). The reaction system contained isooctane (9.5 ml) as the organic solvent and two liquid phases could be identified. The amount of catalyst used was 5 mg. The hydrolytic reaction was carried out for 2 min at 40 °C with constant magnetic stirring (300 rpm). At the target reaction time, reaction was stopped by addition of 1 ml of 6 N HCl and the liquid phases were left to settle. Then, 1 ml of the organic phase (upper phase) was carefully extracted and ASL's lipolytic activity was determined by use of an adapted version of the method of Kwon and Rhee[37] which relies on the formation of a Cu^{2+} between the colorimetric reactant ($CuAc_2$) and the fatty acids released from the substrate due to the action of lipases. 0.2 ml of a solution of $CuAc_2$ (5%, w/v) previously brought to pH 6.1 with pyridine was thus added to the extracted organic phase. The mixture obtained was mixed by vigorous vortex agitation and the absorbance of the resulting complex was measured spectrophotometrically at 715 nm. A calibration curve with oleic acid was performed, in order to report activity values in terms of nominal micromoles of oleic acid produced per minute of reaction (μmol min^{-1}). Blank reactions without enzyme (to determine

the contribution of the non-catalyzed reaction to activity determinations) and without substrate (to assay the contribution to activity values of lipids and fatty acids that the biocatalyst may contain) were performed.

Characterization of the lipolytic activity of ASL towards cottonseed oil included the study of the effect of reaction temperature and the pH of the aqueous buffer used. The effect of pH was studied using solutions of sodium salts of the following Good's buffers: MES, CAPS, TAPS, AMPSO and MOPS.[38] The salts were dissolved in distilled water and brought to the target pH with HCl or NaOH. The concentration of the solutions was 50 mM, and EDTA and Triton X-100 at final concentrations of 5 mM and 1% (v/v), respectively, were added to each of them. The pH values at which ASL activity was assessed (40 °C) were 4, 6, 7, 8, 8.5, 10, 12 and 13.4. The effect of temperature was evaluated in the range of 17–80 °C using Tris–HCl buffer, pH 8.5. In order to evaluate the relative performance of ASL, its activity under definite reaction conditions was compared with that exhibited by Novozym 435.

Hydrolytic activity of ASL on synthetic substrates. The hydrolytic activity of ASL towards chromogenic substrates (*p*-nitrophenyl esters) was assessed by adapting the protocol described by Rashid *et al.*[39] The esters chosen for the analysis were butyrate, laurate, and palmitate (Sigma). An aliquot (300 μl) of substrate stock solution (1 mM) prepared in a mixture of acetonitrile/isopropanol (1 : 4) was added to the reaction medium (final concentration: 0.05 mM) which contained Tris–HCl buffer (0, 1 M, pH 8.0), $CaCl_2$ (5 mM in the reaction medium) and the biocatalyst (2.5 mg). The reaction was conducted with magnetic stirring using a heating jacket (37 °C). Samples were collected every 2 min and filtered through a nylon membrane (Osmonics) to remove the biocatalyst, and the absorbance of the filtrate was measured at 405 nm. The reactions were carried out in the presence/absence of Triton X-100 at a final concentration of 0.0075% (v/v). Blank reactions were performed to consider the appearance of the product due to the spontaneous hydrolysis of the substrate. The effect of the biocatalyst loading (2.5, 5 and 10 mg) on the rate of *p*-nitrophenyl butyrate and *p*-nitrophenyl laurate hydrolysis was studied. In all cases, the enzyme activity was calculated as international units (IU, 1 IU being the amount of enzyme that liberates 1 micromole of *p*-nitrophenol per min under the assay conditions). To this end, a calibration curve with *p*-nitrophenol in the presence and absence of Triton X-100 was performed. Blank reactions without enzyme were carried out. ASL activity was contrasted with that of Novozym 435.

Determination of synthetic activity of ASL

To assess its synthetic activity in esterification reactions, ASL was tested as catalyst of the direct esterification of three different carboxylic acids using a procedure adapted from Vacek *et al.*[40] The carboxylic acids assayed were butyric, caproic and lauric acids, and the alcohol used in all cases was *n*-butanol. The reaction was performed in organic solvent medium. Two organic solvents were assayed: *n*-hexane and *n*-heptane. A solution of substrates in each solvent (10 ml) was prepared (20 mM alcohol and 40 mM carboxylic acid), 10 mg of biocatalyst was added and reaction began. The reactions were incubated at 40 °C with magnetic stirring (300 rpm) for 1 h. At the target time, the reaction was stopped by the addition of absolute ethanol. The remaining acid was titrated with ethanolic 0.01 N KOH solution and phenolphthalein was used as the end-point indicator. Titration of the mixture of substrates without addition of enzyme was included for data correction. Fatty acid conversion (X, %) was calculated as indicated in eqn (1):

$$X\,(\%) = N_{KOH} \times EW_{KOH} \times V_{KOH,\,t=0}\,(1 - V_{KOH,\,t=t}/V_{KOH,\,t=0}) \times 100/W \quad (1)$$

where N_{KOH} accounts for KOH normality, $V_{KOH,\,t=0}$ represents the volume of KOH solution consumed during titration of the initial mixture of substrates, $V_{KOH,\,t=t}$ accounts for the volume of KOH solution consumed during titration of the sample at the target reaction time, EW_{KOH} is the equivalent weight of KOH (56 g eq^{-1}), and W is the mass of sample titrated.

To further characterize the synthetic activity of ASL in the described esterifications, the effect of reaction temperature (25–50/70 °C) and catalyst load (2.5–20 mg) was evaluated under selected conditions, using hexane and heptane as solvents. As in the previous reaction tests described, blank reactions without enzyme and without substrate and comparisons with Novozym 435 were carried out.

Storage stability

The storage stability of ASL at different temperatures (25 °C (room temperature), 4 °C, and −20 °C) was determined in terms of the evolution of its retained lipolytic activity in cottonseed oil hydrolysis during 60 weeks.

Results and discussion

Hydrolytic activity of ASL over cottonseed oil

The spectrophotometric method used to determine the release of fatty acids from cottonseed oil hydrolysis catalyzed by ASL revealed the lipolytic activity of the plant lipase towards the oil chosen. Commercial oils are frequently used to determine the hydrolytic activity of plant lipases, as illustrated in the contributions of Abdelkafi *et al.* (olive oil)[13] and Cambon *et al.* (sunflower oil).[19] Fig. 1 shows the evolution of the enzymatic activity registered for increasing pH values of the buffer used in cottonseed oil hydrolysis. The selection of Good's buffers was aimed at avoiding the influence of ionic strength on the activity of the enzyme. Results showed that for different Good's buffers assayed, the optimum pH is between pH 8.0 and pH 9.0. Alkaline optima are frequent when the hydrolytic activity of plant lipases towards natural substrates is assayed.[13,14,41,42] In the hydrolysis of olive oil catalyzed by

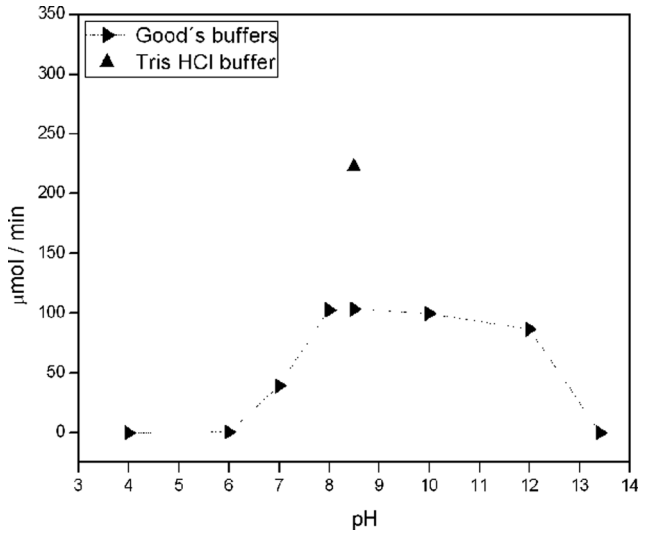

Fig. 1 Effect of pH on the enzymatic activity of ASL. Hydrolysis of cottonseed oil in different Good's buffers (40 °C, 5 mg of ASL, 2 minutes).

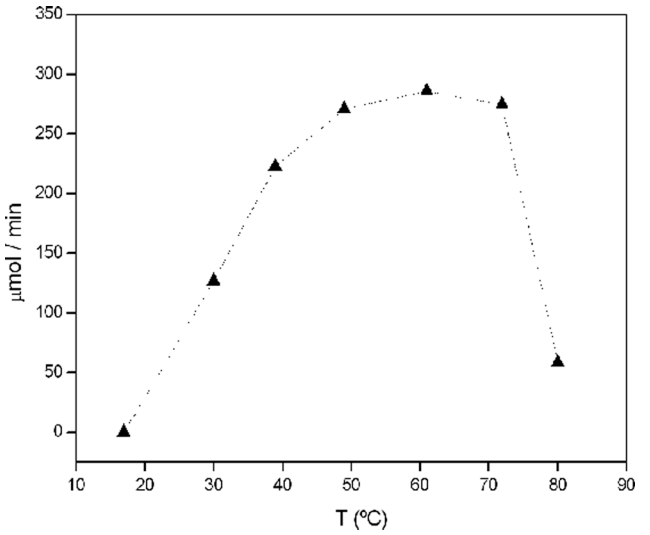

Fig. 2 Effect of temperature on the enzymatic activity of ASL. Hydrolysis of cottonseed oil (pH 8.5, 0.1 M Tris–HCl, 5 mg of ASL, 2 minutes).

Carica papaya lipase (CPL), pH optima in the range of 8.5–9.5 have been reported.[13,14]

The effect of a different type of buffer, but with a pH in the optimum pH range found for Good's buffers analysis, was assayed. Hydrolysis was then performed with 0.1 M Tris–HCl buffer of pH 8.5. The lipolytic activity found for ASL in this case was 87% higher than the one found at the same pH when a Good's buffer was used (highest point in Fig. 1). The behaviour could be attributed to the effect of the ionic strength of the Tris–HCl buffer (μ = 0.163 M) or the ion type over enzymatic activity. The activity value registered using 0.1 M Tris–HCl buffer of pH 8.5 accounts for a nominal conversion of cottonseed oil to oleic acid of 27%.

The effect of reaction temperature on the hydrolytic activity of *Araijia sercifera* lipase was studied at pH 8.5 (0.1 M Tris–HCl). Results are included in Fig. 2. Increasing activity was observed in the 17–80 °C range. Highest activity values were recorded in the 50–70 °C interval, with a notorious decrease of lipase activity at 80 °C due to enzyme denaturation. In the 50–70 °C interval, activity values registered account for nominal conversions of the oil to oleic acid in the range of 33–35%. Review of other temperature optima shown by plant lipases in oil hydrolysis generally evidence values slightly lower than the one found for ASL in the current system, as in the case of lipases present in the oil of the fruit of oil palm (*Elaeis guineensis*, Arecaceae) (temperature optimum in the hydrolysis of olive oil = 45 °C)[41] and lipases present in the latex of *Plumeria rubra* and *Vasconcellea heilbornii* (temperature optima in the hydrolysis of sunflower oil = 55 °C and 50 °C, respectively).[19] The high thermal stability of ASL could be attributed to its auto-immobilized condition, which may contribute to preventing protein unfolding. The hydrolytic activity of ASL towards cottonseed oil was compared with that shown by the commercial immobilized biocatalyst Novozym 435 (40 °C, pH 8.5, 2 min reaction, 5 mg of biocatalyst). Differently from ASL, under the current conditions the commercial biocatalyst showed no detectable hydrolytic activity. This might be ascribed to the moderate/low lipase activity normally shown by CALB in hydrolytic reactions of high molecular weight substrates, caused by the shape, size and hydrophobicity of its active site which may hinder the access of voluminous substrates. Blank assays allowed ruling out the contribution to measured activity of the uncatalyzed reaction as well as that from fatty acids remaining in the biocatalyst.

Hydrolytic activity of ASL towards *p*-nitrophenyl esters

Initial assays on the activity of ASL towards *p*-nitrophenyl esters were aimed at determining the need of using a surfactant to disperse the biocatalyst in the reaction medium as well as its impact on the hydrolytic activity. The presence of surfactants in the reaction medium is known to improve the dispersion of substrates by stabilization of the emulsion. Surfactants generally favor the activity of the lipase provided that their concentration is not too high, usually below their critical micelle concentration (cmc).[42] Surfactants also interact with the "lid" of the lipase, discovering its active site to the medium and favoring the access of the substrates.[43] In the current system, when the *p*-nitrophenyl ester substrate was *p*-nitrophenyl butyrate, the addition of Triton X-100 (nonionic detergent with cmc = 0.015% (v/v)) at a concentration of 0.0075% (v/v) led to an increase in the hydrolysis activity of ASL of 75%, giving a measured hydrolytic activity of 0.0028 IU mg^{-1}. The effect coincides with that found for lipases from prokaryote and eukaryote organisms, whose activity towards *p*-nitrophenyl butyrate increased with the addition of Triton X-100 at concentrations lower than 0.01% (v/v).[44]

Fig. 3 shows the hydrolytic activity of ASL towards higher molecular weight *p*-nitrophenyl esters. In all cases Triton X-100 was added at a concentration of 0.0075% (v/v). The higher specificity of ASL towards *p*-nitrophenyl butyrate could be attributed to the size of the substrate to be hydrolyzed.

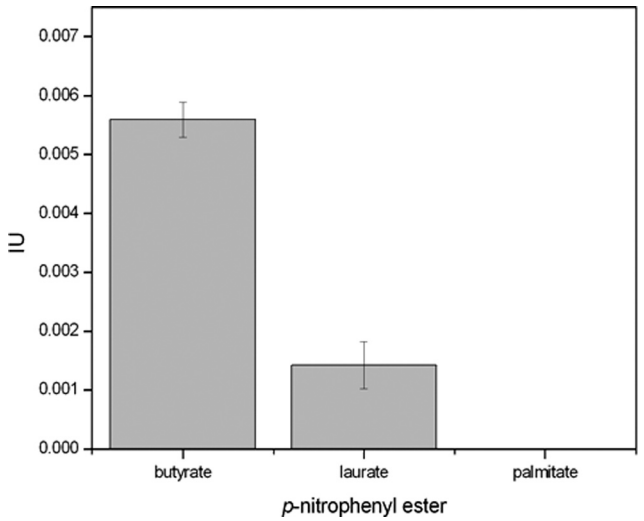

Fig. 3 Specificity of ASL towards the hydrolysis of different *p*-nitrophenyl esters (pH 8.0, 0.1 M Tris–HCl, 0.0075% (v/v) Triton X-100, 37 °C, 2.5 mg of ASL).

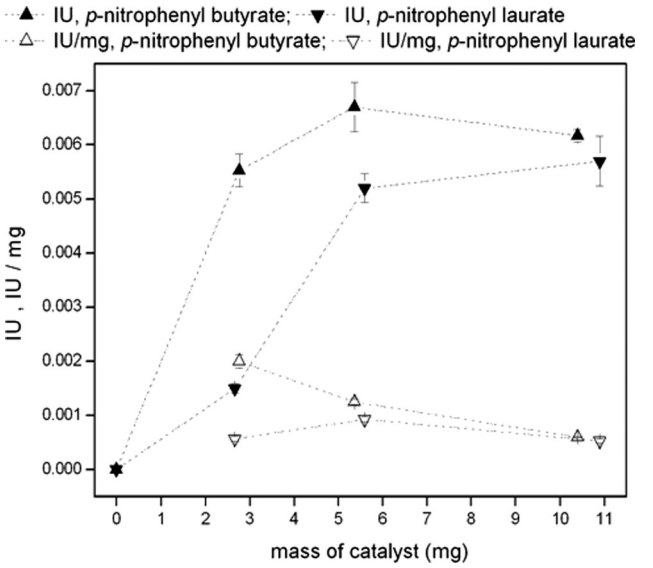

Fig. 4 Effect of biocatalyst load (mg of ASL) on the initial rate and the initial specific rate of hydrolysis of *p*-nitrophenyl butyrate and *p*-nitrophenyl laurate (pH 8.0, 0.1 M Tris–HCl, 0.0075% (v/v) Triton X-100, 37 °C).

The higher the size of the ester, the higher the steric hindrance for the substrate to access and be accommodated in the active site of the lipase. In the case of *p*-nitrophenyl palmitate (16C) no hydrolytic activity could be detected. A similar pattern was observed for the hydrolysis of triacylglycerols of increasing carbon length catalyzed by lipase from *Carica papaya* (CPL). Among triacylglycerols with acyl carbon chains in the range of two to eighteen, CPL showed its highest hydrolytic activity towards tributyrin. For triacylglycerols with higher molecular weight than tributyrin, the higher the carbon number of the acyl group of the triacylglycerol, the lower the hydrolytic activity shown by CPL.[34] To the authors' knowledge, CPL has not still been assayed in the hydrolysis of *p*-nitrophenyl esters.

For the *p*-nitrophenyl esters towards which ASL showed hydrolytic activity, the effect of adding higher masses of biocatalyst was studied. Fig. 4 shows the effect of the mass of biocatalyst used in the hydrolysis of *p*-nitrophenyl butyrate and *p*-nitrophenyl laurate. Data are given in terms of IU and IU mg^{-1} of catalyst.

Fig. 4 shows that in the hydrolysis of *p*-nitrophenyl butyrate the use of lipase loads higher than 2.5 mg does not lead to a significant increase in the initial hydrolysis rate (full upward-pointing triangles), and thus, specific activity progressively drops with the addition of higher biocatalyst masses (empty upward-pointing triangles). Plateau values observed for international units (IU) might probably be due to biocatalyst agglomeration at high lipase loadings. Intrinsic tryptophan fluorescence, surface hydrophobicity and dynamic light scattering (DLS) have been used to demonstrate that lipase aggregation in aqueous media dramatically increases with enhanced protein concentration.[45,46] Formation of aggregates reduces the real concentration of enzyme available for contacting substrates. The enzyme molecules on the outer surface of the aggregates are exposed to high substrate concentrations, but mass transport into a particle of clumped catalysts can severely limit the concentration of substrates inside the particles. Then, just the fraction of the catalyst that remains on the outer surface of the agglomerates is truly available for catalysis, being the measured specific activity seriously reduced. In the case of the hydrolysis of *p*-nitrophenyl laurate the addition of increasing lipase loadings leads to initial IU increments (full downward-pointing triangles). However, the use of 11 mg of biocatalyst does not lead to a proportional increase in lipase activity towards *p*-nitrophenyl laurate hydrolysis, which in turn leads to a reduction of the corresponding specific activity value (empty downward-pointing triangles).

Differently from the hydrolysis of cottonseed oil, in which under the assay conditions ASL had better performance than Novozym 435, in the hydrolysis of *p*-nitrophenyl butyrate (pH 8.0, 0.05 M Tris–HCl, 0.0075% (v/v) Triton X-100, 2.5 mg of biocatalyst, 37 °C), the activity of ASL was much lower than that exhibited by the commercial biocatalyst (0.0028 IU mg^{-1} for ASL *versus* 1.61 IU mg^{-1} for Novozym 435).

Synthetic activity of ASL towards fatty acid esterification

Although once lipase action was considered to be restricted to aqueous media, today lipases are employed not only in traditional water-based systems but also in non-aqueous systems with dissolved substrates.[47] In this context, in the last decades the study of lipase activity in synthetic reactions performed in organic media has been an issue of much research and review.[48,49] In particular, esterification of fatty acids in organic medium has been commonly used for testing the synthetic activity of native and immobilized lipases in these media.[44,51–53]

Esterification of fatty acids is traditionally performed by use of acid or basic catalysis, but these reactions generate undesired by-products. Alternatively, lipases can perform the same function but under milder reaction conditions and with much higher specificity.[19] Bearing in mind that butyl esters of fatty acids can be used as lubricants, biodiesel additives and plasticizers among other products,[54] the synthetic activity of ASL was assayed in the direct esterification of three saturated fatty acids with *n*-butanol in organic media (*n*-hexane and *n*-heptane). A short-chain fatty acid (butyric acid, C4:0) and two medium-chain fatty acids (caproic acid, C6:0 and lauric acid, C12:0) were assayed. Results are included in Fig. 5.

In both solvents assayed, ASL showed higher affinity for butyric and lauric acids, whereas esterification of caproic acid was less favored. In reference to the effect of the solvent used, the activity of ASL was found to be higher in *n*-heptane than in *n*-hexane. Since the presence of an organic solvent is known to alter the performance of an enzyme, correlation of the activity and selectivity of enzymes with various physicochemical parameters of the solvent has been attempted.[55,56] Particularly, the correlation with solvent polarity quantified on the basis of log *P* values is one of the most accepted.[56] The log *P* value of a solvent is defined as the logarithm of the partition coefficient of the solvent in an *n*-octanol/water two-phase system.[56] It is generally observed that biocatalysis in organic solvents is low in polar solvents having a log $P < 2$, moderate in solvents having a log *P* between 2 and 4, and high in non-polar solvents having a log $P > 4$, since as log *P* increases the relative ability of organic solvents to distort the essential water layer that stabilizes the biocatalysts is progressively diminished. In this context, and considering that the log *P* of *n*-hexane is 3.764 whereas the log *P* value of *n*-heptane is 4.274, the slight differences observed herein for fatty acid esterifications catalyzed by ASL may be attributed to the relatively lower tendency of heptane to strip the essential water of the biocatalyst and thus distort its active conformation.

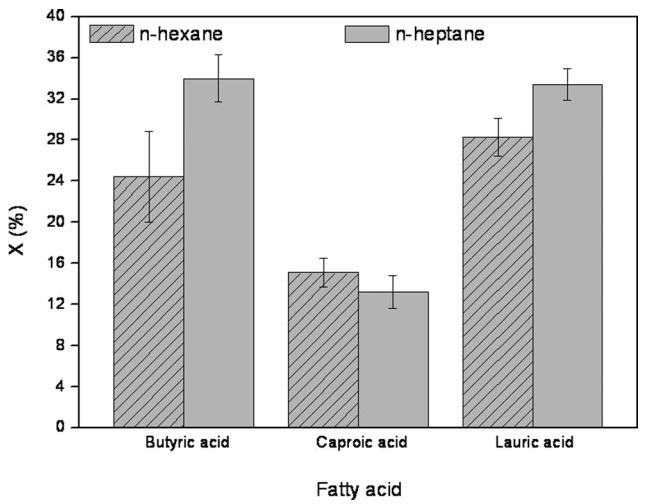

Fig. 5 Fatty acid conversion in esterifications with *n*-butanol catalyzed with ASL in *n*-hexane and *n*-heptane media. Fatty acid (40 mM), alcohol (20 mM), 10 mg of ASL, 40 °C, 1 h.

To further study the effect of reaction conditions on the synthetic activity of ASL, the effect of reaction temperature on the level of esterification achieved for butyric and lauric acids was evaluated in both organic solvents (Fig. 6). The ranges of temperature values assayed were chosen considering the boiling points of the solvents (*i.e.* 25–50 °C for *n*-hexane whose boiling point is 68–69 °C, and 25–70 °C for reactions carried out in *n*-heptane which has a boiling point of 98–99 °C). Fig. 6a shows the results for *n*-hexane. As it is shown, in the range assayed lauric acid conversion continuously increases with temperature. On the other hand, butyric acid esterification exhibits relatively lower values (confirming data included in Fig. 5) and the presence of an optimum at 40 °C.

In Fig. 6b the effect of temperature on the conversion of butyric acid and lauric acid in *n*-heptane is illustrated. As it is shown, in the 25–50 °C interval lauric acid conversion is similar to the one found in *n*-hexane. Conversion determinations performed at higher temperatures (60–70 °C) evidence a drastic reduction of lipase activity. In the case of butyric acid esterification, and similarly to esterifications carried out in *n*-hexane, conversion shows an optimum, in this case at higher temperature (*i.e.* 50–60 °C). The optima observed result from the effect of temperature on both the initial reaction rate and the enzyme deactivation rate. In *n*-heptane at 70 °C both fatty acid esterifications evidence an important reduction of lipase activity with fatty acid conversion values equal/lower than those found at 25 and 30 °C. The previous results suggest that at 70 °C the deactivation rate is severely enhanced and a drastic reduction of biocatalyst activity due to lipase unfolding and biocatalyst denaturation takes place in short periods of time. Similar behaviours have been found for a number of native and immobilized lipases when tested in the catalysis of fatty acid esterifications in solvent and solventless media.[50,51,57]

The effect of using increasing ASL loadings on esterification yield was assayed on the synthesis of butyl butyrate in *n*-heptane at 40 °C. Results are shown in Fig. 7 for biocatalyst masses in the 2.5–20 mg interval. Data show that conversion increases with the mass of ASL used as catalyst up to 10 mg. The addition of higher ASL masses (*i.e.* 20 mg) does not lead to any further significant increment in fatty acid conversion, suggesting that at high lipase loadings—and maybe due to enzyme agglomeration effects—the biocatalyst is used less efficiently. Anyway, the potential biocatalyst aggregation effects described seem less significant in the organic solvent medium used herein than in hydrolysis reactions detailed in the previous sections (*i.e.*, reduction of specific activity is observed at higher biocatalyst load). These results could be explained by the hydrophobicity of the biocatalyst, which might promote higher agglomeration in hydrophilic media.

The synthetic activity of ASL was compared with that shown by the commercial immobilized biocatalyst Novozym 435 (40 °C, 1 h, 10 mg of biocatalyst). Results showed that in the esterification of butyric acid with butanol in heptane at 40 °C a 1 h conversion of 93 ± 4% was observed for Novozym 435, whereas for ASL the conversion reached after

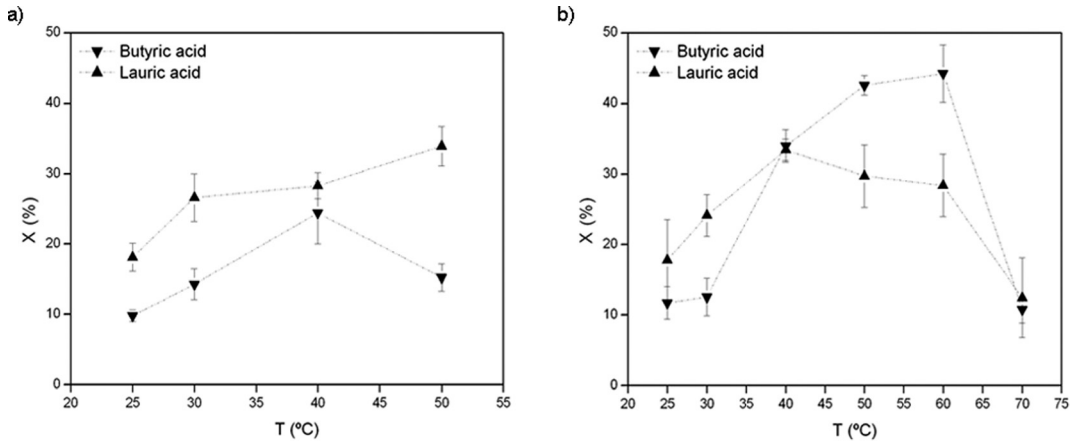

Fig. 6 Effect of reaction temperature on fatty acid (butyric and lauric acids) conversion in organic medium catalyzed by ASL. a) *n*-Hexane; b) *n*-heptane. Fatty acid (40 mM), alcohol (20 mM), 10 mg of ASL, 1 h.

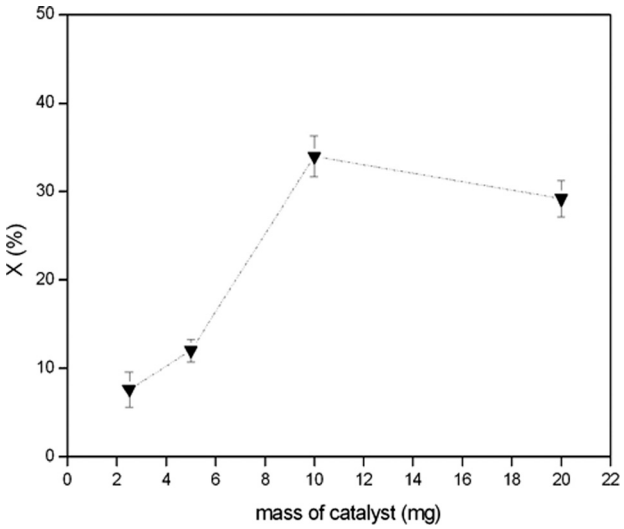

Fig. 7 Effect of ASL mass on butyric acid esterification with butanol in *n*-heptane. Butyric acid (40 mM), *n*-butanol (20 mM), 40 °C, 1 h.

1 hour of reaction was 33 ± 3%. In the case of the esterification of lauric acid with butanol in heptane at 40 °C, the conversion achieved with Novozym 435 was 89 ± 5% whereas the one attained with ASL was 33 ± 2%. These results are very encouraging given the well-known high performance of Novozym 435 in fatty acid esterifications. Blank assays allowed the contribution to measured activity from the uncatalyzed reaction as well as from fatty acids liberated from the biocatalyst to be ruled out.

Storage stability of ASL

The stability of ASL under different storage conditions (−20 °C, 4 °C and 25 °C) was studied during 15 months. The storage stability of enzymes is one of the main parameters which need to be taken into account when scheduling its application to a particular reaction.[13] Fig. 8 shows the evolution of the residual lipolytic activity of ASL—determined in the hydrolysis of cottonseed oil as described in the experimental section—as a function of storage time (weeks) and temperature.

Results demonstrated that when ASL is stored at 25 °C, the reduction of its lipolytic activity after 64 weeks (448 days) reaches 39%. On the other hand, when the lipase is stored at −4 °C the reduction of activity is much lower, reaching 14.5% after 64 weeks. In the case of the conservation of ASL at −20 °C, no significant reduction of lipase activity was detected after 64 weeks of storage. Abdelkafi *et al.*[13] studied the storage stability of *Carica papaya* lipase at temperatures of 4 and 20 °C. Results determined in the hydrolysis of olive oil showed that CPL lost about 50% of its activity after 20 weeks of storage at 20 °C, whereas the retained lipolytic activity of CPL stored at 4 °C after more than 40 weeks was greater than 90%. Considering these results, results reported herein suggest that the storage stability of ASL appears to be very promising.

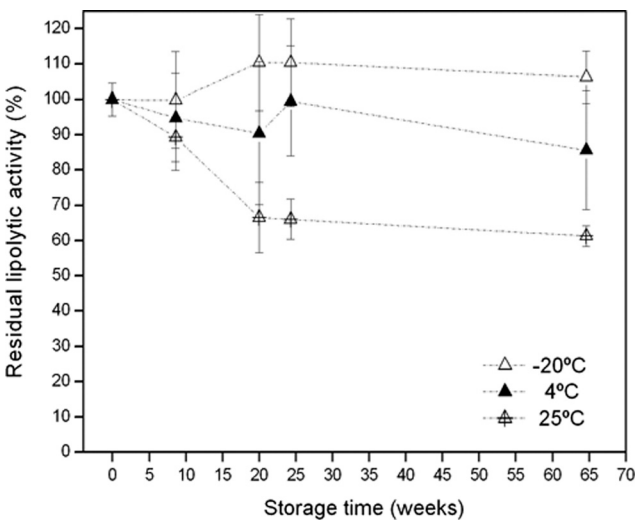

Fig. 8 Stability of ASL stored at different temperatures expressed as residual lipolytic activity. Hydrolysis of cottonseed oil (pH 8.5, 0.1 M Tris–HCl, 5 mg of ASL, 40 °C, 2 minutes).

In general terms, the lipase present in the insoluble fraction of the latex of fruits of *Araujia sericifera* Brot. (synonym: *Araujia hortorum* Fourn.) (Apocynaceae) appears as an interesting biocatalyst to continue studying, for reasons such as i) the relative easiness of its extraction, ii) the high observed ability of ASL for catalyzing target oil hydrolysis and fatty acid esterifications (activity results were compared with those attained with the immobilized commercial lipase Novozym 435), and iii) the high storage stability demonstrated by ASL. ASL catalytic properties suggest that this enzyme may be a promising tool, for example, for the synthesis of structured lipids as food ingredients, especially those containing short chain length carboxylic acids (low calorie fats or other nutraceuticals[58]), as well as in cosmetic or pharmaceutical formulations using eco-friendly technologies.[59] Considering that the aqueous soluble fraction of *Araujia sericifera* is rich in proteolytic activity with high biotechnological potential,[25–33] the presence of lipase activity in the insoluble fraction turns this milkweed—considered as a plague in many parts of the world[20–24]—into an attractive cheap natural resource for the obtention of biocatalysts to be used in the preparation of specific high added value products.

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