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Chitosan-immobilized lipases for the catalysis of fatty acid esterifications

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Abstract

Lipases from *Candida rugosa, Pseudomonas fluorescens* and *Candida antarctica* B were immobilized onto chitosan and glutaraldehyde-pretreated chitosan powders. The prepared biocatalysts were assayed in the direct esterification of oleic acid and ethanol to produce the ethyl oleate. In order to maximize ester production and avoid solvent-related costs (the solvent itself, solvent recovery and solvent recycle), the synthesis of ethyl oleate was performed in a solvent-free system.

The different structures of the lipases chosen for the catalysis led to very different activity levels, with *C. antarctica* B derivatives being the most active ones. The parametric study performed revealed that the best operation conditions for ester synthesis are found at mild temperatures (35–45 °C), and in "biphasic systems" (two liquid phases), generated upon addition of relatively high quantities of water to the mixture of substrates. The reduction of the concentration of water in the organic reactive phase of biphasic systems favored ester synthesis, with higher ester yields in the first hours of reaction than those measured in systems with no added water.

In the optimum conditions mentioned the biocatalyst resulting from the immobilization of lipase from *C. antarctica* B onto untreated chitosan powder led to 75% conversion of the fatty acid in 24 h of reaction. The stability of this catalyst also proved to be very attractive with five consecutive 24 h uses with a residual activity of 90–95%.

Keywords: Esterification; Lipase; Immobilization; Chitosan

1. Introduction

Lipases (EC 3.1.1.3) are a family of enzymes that in their natural environment catalyze the hydrolysis of fats. However, under appropriate operation conditions lipases have shown to be very active catalysts in transesterification, alcoholysis and esterification reactions. Moreover, immobilization has become a widely used technique to overcome practical problems in the use of crude lipases. Fixation of lipases to solid materials avoids product contamination and allows biocatalyst recovery, reuse and continuous operation. Immobilization onto an insoluble matrix may also enhance the operational lifetime and stability of the biocatalysts. In particular, the solid matrix of the support can confer a more rigid structure to the biocatalyst leading to enhanced thermal stability. Thermal deactivation of lipases can be significantly reduced by immobilization on non-soluble supports [1,2]. On the other hand, lipase immobilization may also lead to reduced stability (if the support exerts negative interactions with the protein); and also to reduced catalytic activity of the biocatalyst, if the enzyme conformation is negatively altered during the immobilization process some of the aminoacids from the active site or from the access to the active site are involved in the bonds established with the support, or steric restrictions occur [3].

Although there is still no universal support that it is suitable for all enzymes and all their applications, any material that is to be considered as enzyme support must fulfill some requirements: high affinity for proteins, availability of reactive functional groups (for direct reactions with enzymes, or for chemical modification of the support) mechanical stability, rigidity, feasibility of regeneration and high loading capacity. Depending on its application, non-toxicity and biodegradability of the support material could be required. Moreover, the capability of some supports to interfacially activate the immobilized lipase is a desirable characteristic of the support material. Interfacial activation of lipases adsorbed onto hydrophobic materials is well recognized [4–7]. The phenomenon of the interfacial activation of lipases was first described by Sarda and Desnuelle [8]. The capability of hydrophobic supports to interfacially activate adsorbed lipases was later discussed in detail by Sugira and Isobe [9]; and by Bastida et al. [10]. In reference to the lipases studied

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in this contribution, in some previous works of our group lipases from *Candida rugosa* and *Pseudomonas fluorescens* exhibited important activation upon adsorption onto polypropylene powder [11–13].

Another desirable characteristic of lipase supports is that the matrix does not significantly increase the cost of the biocatalyst. Materials commonly used in commercial immobilized lipases, like Novozyme 435 and Lipozyme RM IM (acrylic resins and exchange resins, respectively), have exhibited a number of the mentioned requirements, conferring high activity and stability to the immobilized lipase, and allowing several biocatalyst reuses. However, studies devoted to the fixation of lipases onto cheaper support materials that also exhibit high activity and stability are widely found in current literature. Some non-expensive materials have been used as support substitutes such as wood cellulignin [14], rice husk and rice straw [15]; chitin [16]; Amberlite [17]; and chitosan [18–23].

In this work, lipases from *C. rugosa* (CRL), *P. fluorescens* (PFL) and *Candida antarctica* B (CALB), have been immobilized on chitosan powder previously treated with aqueous glutaraldehyde solutions of different concentration. The chitosan used for this purpose fulfills the requisite of being a cheap support material since it was obtained from prawn's shells, a waste of the seafood processing industry. Moreover, this polyaminosaccharide is recognized by its excellent properties for lipase support, such as biocompatibility, biodegradability to harmless products, nontoxicity, physological inertness, and great affinity for proteins [18]. Also, reactive amino and hydroxyl groups of chitosan chains are amenable to chemical modifications and activation treatments, such as the glutaraldehyde pretreatment assayed in this contribution. Native amino groups of chitosan first bond to the aldehyde groups of glutaraldehyde. Then, pretreated chitosan is contacted with the enzyme solution, and covalent bonds between lipase aminoacids and free carbonyl groups of the dialdehyde occur. The methodology is very simple and allows improving enzyme stability by multipoint or multisubunit immobilization. The possibility of ionic adsorption of glutaraldehyde in the case of aminated supports, and the introduction of the character of ionic exchanger because of this ionic adsorption cannot either be ruled out [24].

Immobilization of enzymes on glutaraldehyde-pretreated supports has been described in literature. In particular, immobilization of invertase on support materials activated with the dialdehyde has received great attention [25–28]. In reference to the support material studied in this contribution, glutaraldehyde has been used by a number of authors as a coupling agent in the immobilization of enzymes onto chitosan powders [23,29,30]. The dialdehyde has also been frequently used to introduce intermolecular crosslinking in proteins or to modify adsorbed proteins on aminated supports. The exact structure of glutaraldehyde on the support is under discussion, but polymers, monomers and dimers of glutaraldehyde onto different surfaces have been proposed to be present [31]. The control of support activation with glutaraldehyde is very important. It has been reported that monomers and dimers of glutaraldehyde have different reactivity: while the dimer is able to rapidly immobilize proteins via a direct covalent attachment, the monomer yields a very low immobilization rate [32]. Activity/stability properties of enzymes immobilized on glutaraldehyde activated supports depend on the exact immobilization protocol employed. Due to the existence of one or two ionic groups (amino groups) under the glutaraldehyde, which provide a certain anionic exchanger nature to the support, altering the ionic strength during the immobilization can modify the immobilization rate and also the region of the protein that is implied in the interaction with the support [32]. Since in this contribution immobilization was performed in neutral medium, reaction should have involved the most reactive amino groups in the protein.

In reference to the immobilization of CRL, PFL and CALB onto chitosan supports, although there are several reports about *C. rugosa* derivatives [20,21,23,33–36], to the best of our knowledge this is the first study devoted to the immobilization of lipases from *P. fluorescens* and *C. antarctica* B on chitosan materials. In reference to CRL immobilized on chitosans, they have been mainly used for hydrolytic reactions with just two contributions devoted to synthetic activity [21,34].

The immobilized biocatalysts prepared in this contribution were assayed in the solvent-free synthesis of ethyl oleate by direct esterification of oleic acid and ethanol. Solvent-free systems (SFS) are highly concentrated media, economically and operationally interesting for industrial processes. In this kind of systems not only the cost of the solvent itself is avoided, but also its separation from un-reacted substrates and products, and the cost of recycle as well. Many authors have chosen this kind of systems for their enzymatically-catalyzed esterifications [37–39].

In order to determine the operation conditions that maximize ester yield, for each prepared catalyst we performed a parametric study that included the analysis of the influence of water content, reaction temperature, and the mass of catalyst added to reaction medium. In particular, the generation of a second liquid phase upon addition of unusually high amounts of water to the mixture of substrates, led to interesting results related to the extraction of water from the reactive organic phase. Migration of water to the aqueous phase formed reduces its content in the reactive oily phase, allowing higher esterification yields in the first hours of reaction. Together with biocatalyst hydration and shifting of equilibrium position, formation of a biphasic system showed the huge importance of studying the effect of water on enzymatic esterifications.

2. Experimental

2.1. Materials

C. rugosa AY lipase (64,000 g/mol to 30,000 U/g) and *P. fluorescens* AK lipase (33,000 g/mol–25,800 U/g) were kindly donated by Amano Enzyme. Native lipase B from *C. antarctica* B (5000 U/ml) was supplied by Novozyme. Oleic acid (99%) was purchased from J.T. Baker. Absolute ethanol (99%) and sulphuric ether (99%) were both purchased from Dorwil. Buffer solution of pH 7 (di-sodium hydrogenophosphate) and potassium hydroxide were both from Merck. Chitosan powder (batch number: TM 369, 60–100 mesh, MW: 70,000–80,000 g/mol, BET surface area: 3–5 m^2/g) was obtained from Primex Ingredients, ASA Norwegian. The material was obtained from prawn's shells with a degree of deacetylation of 85.2%. Triolein (glycerine trioleate), Tritón X-100 and pH 7 buffer Tris–HCl 1 M, were bought to Sigma–Aldrich.

2.2. Preparation of glutaraldehyde-pretreated chitosans

Glutaraldehyde-modified supports were prepared by suspending 375 mg of chitosan powder in 50 ml of 0.025% or 0.25% (v/v) glutaraldehyde/phosphate buffer pH 7.0 solutions. The suspension was kept under 800 rpm stirring at room temperature for 30 min. After that time the supports were recovered by filtration and washed with distilled water. The modified supports (CHIT/GLU 0.025 and CHIT/GLU 0.25) were stored at 50 °C for 12 h.

2.3. Lipase immobilization

The immobilization of lipases was performed at room temperature during 7 h with 350 rpm stirring. Phosphate buffer kept pH at 7, and the ionic strength of the immobilization medium equal to 0.014 M. In the case of CRL and PFL (solid powders), 150 mg of these lipases were added to 50 ml of phosphate buffer solutions, and subjected to strong stirring during 30 min in order to solubilize lipase. A filtering step was then performed in order to retain carbohydrates and other insoluble compounds. In the case of CALB (liquid ambar solution), 0.9 ml of the enzyme commercial solution were diluted up to 50 ml with phosphate buffer.

Each lipase solution was then contacted with 375 mg of chitosan powder and glutaraldehyde-pretreated chitosans (room temperature, 350 rpm). After the desired contact time (7–8 h) solids were recovered by filtration, washed with distilled water and dried at 50 °C for 12 h. From this procedure we obtained nine immobilized catalysts namely CR/CHIT, CR/CHIT/GLU 0.025, CR/CHIT/GLU 0.25, PF/CHIT, PF/CHIT/GLU 0.025, PF/CHIT/GLU 0.25, CA/CHIT, CA/CHIT/GLU 0.025, CA/CHIT/GLU 0.25.

2.4. Determination of immobilized biocatalysts activity and biocatalysts characterization

The hydrolytic activity of both immobilized and native lipases was measured following the procedure described by Peled and Kenz [40], consisting of the hydrolysis of triolein in pre-established conditions [40]. Synthetic activity was assayed in the solvent-free synthesis of ethyl oleate under definite conditions (see Section 2.5). Characterization of the immobilized biocatalysts included SEM, EDX and FT-IR techniques. Scanning electron images (SEM) were obtained using a JEOL 35CF microscope (operated at 15 kV), equipped with a secondary electron detector and energy dispersive X-ray microanalysis (EDX). FT-IR spectra were obtained using a Nicolet 150 spectrometer.

2.5. Esterification reaction

The prepared biocatalysts were all assayed in the direct esterification of oleic acid and ethanol. The products of this reversible reaction are the ethyl oleate ester and water. The synthesis was carried out in an isothermal batch type reactor of 10 ml at 350 rpm stirring. In all the experiments performed, the initial reaction medium consisted of the stoichiometric mixture of substrates (10.6 mmol), and different percentages of added water (W = initial mass of water/initial mass of oleic acid, %). To assay the effect of temperature, the reaction vial was alternatively kept at temperatures in the range of 35–75 °C. Different masses of biocatalyst were also tested.

The progress of the esterification was monitored by determination of the residual acid content by titration with a basic solution of potassium hydroxide. Phenolphthalein was used as the end-point indicator and a mixture of ethanol–sulphuric ether 50/50% (v/v) was used as quenching agent. The withdrawing of samples from the emulsioned reaction mixture was performed following an optimized method that showed to be accurate for sampling from two-liquid phases systems [41]. The accumulated conversion of oleic acid at a given time, was determined by the relative reduction of the acidity index of the samples. Error in conversion determinations was in all cases lower than 1%.

3. Results and discussion

3.1. Characterization of chitosan-immobilized biocatalysts

Fig. 1 shows four scanning electron microphotographs of (a) chitosan powder, (b) PF/QUIT/GLU 0.025, (c) CA/CHIT/GLU 0.025, and (d) CA/CHIT. While lipase presence shown by the

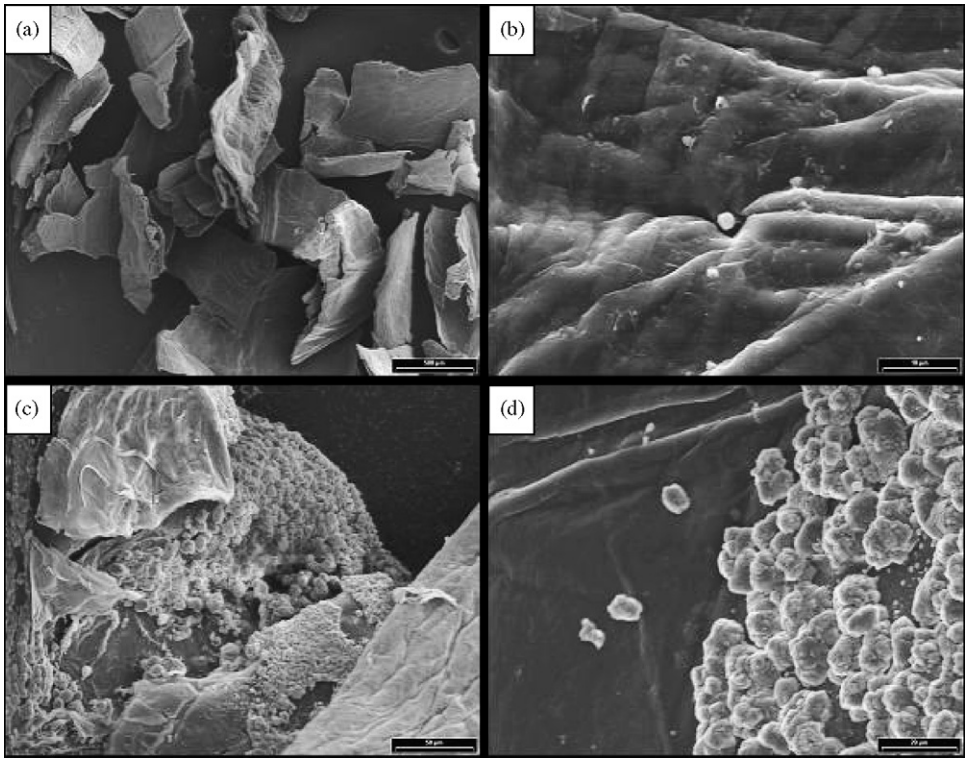

Fig. 1. (a) Chitosan powder (40×), (b) PF/QUIT/GLU 0.025 (2000×), (c) CA/CHIT/GLU 0.025 (400×), and (d) CA/CHIT (1000×).

Table 1
Hydrolytic and synthetic activity of crude and chitosan-immobilized lipases

Biocatalyst	Hydrolytic activity (U/g of total catalyst[a])	Conversion achieved in linear period (time, h)	Initial synthetic activity (mmol/h)	Ultimate perceptual conversion measured (time, h)
CRL	3963	9% (1)	0.477	11% (5)
CR/CHIT	522	8% (1)	0.848	8% (5)
CR/CHIT/GLU 0.025	445	10% (1)	1.060	10% (5)
CR/CHIT/GLU 0.25	186	6% (1)	0.636	6% (5)
PFL	4583	6% (1)	0.636	13% (30)
PF/CHIT	773	10% (2)	0.530	33% (30)
PF/CHIT/GLU 0.025	591	6% (1)	0.636	32% (30)
PF/CHIT/GLU 0.25	635	5% (1)	0.530	19% (30)
CALB	303[b]	55% (2)	2.915	77% (24)
CA/CHIT	189	19% (2)	1.007	72% (24)
CA/CHIT/GLU 0.025	177	8% (1)	0.848	55% (24)
CA/CHIT/GLU 0.25	234	6% (1)	0.636	47% (24)

Hydrolytic activity was measured in the triolein hydrolysis following Peled et al. [30]. Synthetic activity was measured in the ethyl oleate synthesis ($W = 20\%$, 45 °C, 10 mg of crude lipases, 50 mg of immobilized lipases, 350 rpm).

[a] For free catalysts "g of total catalyst" refers to the crude enzyme preparation. For immobilized catalysts "g of total catalyst" refers to support + immobilized enzyme.

[b] A densitiy of 1 g/ml was assumed for the aqueous solution of CALB.

microphotograph of PF/QUIT/GLU 0.025 is scarce, agglomerates of *C. antarctica* B lipase in photos (c) and (d) are clearly seen.

In EDX spectra, lipase presence was detected by the signals of elements like Ca and Si which were also present in the EDX spectra of crude lipase preparations. Bands of elements like C, N and O (main components of lipases) did not give evidence of lipase presence since those elements are also the most abundant components of chitosan molecules. FT-IR spectra were not useful for immobilized lipase determination either, since chitosan absorption bands mask lipase ones (bands of chitosan at 1049, 1125, 1587 and 1625 cm^{-1}, for example cover lipase carbonyl bands detectable in that range).

3.2. Hydrolytic and synthetic activity of crude and chitosan-immobilized biocatalysts

Native lipases were crude preparations with variable hydrolytic activity. Based on the method of triolein hydrolysis previously mentioned [40], Table 1 presents the hydrolytic activity determined for both crude and immobilized biocatalysts. Results included in the second column of Table 1 demonstrate that in reference to hydrolytic activity PFL derivatives showed the highest activities, whereas the hydrolytic activities of CALB derivatives were the poorest ones. Although not representative of the synthetic activity of lipases, hydrolytic activity characterizes the potential of the prepared biocatalysts.

The synthetic activity of the biocatalysts was assayed in the solvent-free synthesis of ethyl oleate in the conditions described in Section 2.5. Table 1 presents not only *initial* synthetic activity data, but also the final ester yield achieved ("final" refers to the constant yield measured when no further conversion increment was detected). In this way Table 1 shows the initial catalytic activity of the biocatalysts (relatively high for all catalysts assayed), and also illustrates the important inhibition–deactivation effects that affect some of the biocatalysts, which lead to short linearity periods and low final ester yields. This is the case of CRL derivatives which, in spite of exhibiting high initial synthetic activities, they show a drastic reduction of activity in relatively short periods of reaction, with almost no further conversion increase after 1 h of synthesis. Comparison of the yields achieved in 1 and 5 h of reaction (third and fifth columns of Table 1) show similar values for CRL derivatives. On the other hand, PFL and CALB derivatives show not only attractive initial synthetic rates, but also high final ester yields. Lower inhibition–deactivation phenomena experienced by these catalysts allow further conversion of fatty acid after the first minutes of reaction, with 24 or 30-h ester yields significantly higher than the ones achieved in the linear period.

The importance of the point raised is due to the fact that although *initial* synthetic data obtained in the initial linear period of reaction may be of use (in fact those are the values more frequently reported in published literature), inhibition–deactivation effects that may occur after the first minutes of reaction might drastically decrease the catalytic activity of the biocatalyst, leading to low long-term conversions (just the ester yields obtained in the first minutes of reaction!). With the purpose of showing short-term and long-term effects, in the following sections complete kinetics are presented.

3.3. Screening of the best chitosan support for each lipase

As mentioned above, the synthetic activity of the nine biocatalysts prepared was assayed in the esterification of oleic acid with ethanol in selected conditions (45 °C, 50 mg of catalyst, water percentage: $W = 20\%$ or 0%). Complete kinetic data was obtained in order to infer for each immobilized lipase the chitosan support that leads to greatest activity. As a first conclusion of this screening study, results revealed the very low activity of CRL derivatives towards oleic acid esterification. As we anticipated in the previous section, conversion of oleic acid using CR/CHIT, CR/CHIT/GLU 0.025 and CR/CHIT/GLU 0.25

stopped after 1–2 h of reaction with values in the range of 6–10% (45 °C, $W = 20\%$, 50 mg of catalyst). CRL derivatives showed the lowest synthetic activity, even if their hydrolytic activity was very high, especially if compared with the hydrolytic activities of CALB derivatives (Table 1). Measured kinetics were similar to that found with the crude lipase [11].

In the case of PF/CHIT, PF/CHIT/GLU 0.025 and PF/CHIT/GLU 0.25, we followed the fatty acid conversion during 30 h, measuring ester yields in the range of 19–33% (45 °C, $W = 20\%$, 50 mg of catalyst). In reference to CA/CHIT, CA/CHIT/GLU 0.025 and CA/CHIT/GLU 0.25, 24 h yields followed in the range of 47–72%, slightly lower than values measured with crude CALB [12].

In reference to the different chitosan supports assayed, the initial study revealed that PFL and CALB derivatives showed highest activity when immobilized onto non-pretreated chitosan (CHIT) or – slightly lower – when fixed onto CHIT/GLU 0.025. Then, in order to choose just one PFL derivative and one CALB derivative that combine high activity and operational stability, reuse experiments of the correspondent four biocatalysts (PF/CHIT, PF/CHIT/GLU 0.025, CA/CHIT, CA/CHIT/GLU 0.025) were performed. The four biocatalysts chosen were repeatedly used in the catalysis of oleic acid esterification in definite conditions ($W = 20\%$, 45 °C, 50 mg of biocatalyst, 350 rpm, 24 h). After each reaction interval the biocatalysts were recovered by filtration, washed, dried, and added to a new reaction mixture.

In the case of PFL derivatives, reuse data revealed that although PF/CHIT was slightly more active than PF/CHIT/GLU 0.025, the first one deactivated more easily than PF/CHIT/GLU 0.025, with a decrease in 24-h conversion in the second use of 65% and 49%, respectively. On the other hand, reuse of CA/CHIT and CA/CHIT/GLU 0.025 demonstrated the strength of the physical bonds established in CA/CHIT, which showed to be resistant enough to allow several reuses. Important differences between lipases structures are responsible for the diversity in the number, type, and strength of the bonds established during immobilization, which have intimate relation with the stability and reusability of the biocatalyst prepared [42]. PF/CHIT/GLU 0.025 and CA/CHIT were the biocatalysts selected for the following parametric study.

3.4. Parametric study

In order to maximize the yield of ethyl oleate, the activities of PF/CHIT/GLU 0.025 and CA/CHIT were assayed in several reactions, in which temperature of reaction, water content and mass of catalyst, were alternatively varied.

3.4.1. Effect of water content (W) on fatty acid conversion

Conversion of oleic acid in systems with no water added ($W = 0\%$), and in systems with 10% and 20% of total water added (initial mass of water/initial mass of oleic acid × 100), was measured during 24 h for reactions catalyzed by 50 mg of PF/CHIT/GLU 0.025 and CA/CHIT. Fig. 2 shows the evolution of oleic acid conversion in the synthesis catalyzed by CA/CHIT in systems with $W = 0\%$, 10% and 20%.

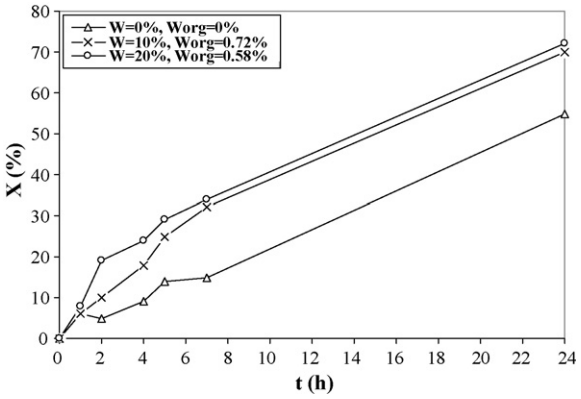

Fig. 2. Evolution of oleic acid esterification. Influence of overall water content, W; 45 °C, 50 mg of CA/CHIT, 350 rpm. Error in measured values lower than 1%.

From a kinetic point of view, it is widely reported that lipases need water to develop activity. On the other hand, addition of water has an adverse effect on esterification reactions, by shifting equilibrium to reactants (thermodynamic effect). Considering these two consequences – of different origin – of the addition of water to reaction mixtures, publications dealing with enzymatic esterifications agree on the fact that a minimum amount of water – namely 0.1–3% (w/w) – should be added to the mixture of substrates [43–46]. In this way, by hydrating lipase while not shifting equilibrium to reactants, ester yield would be maximized.

However, Fig. 2 shows that in the solvent-free synthesis of ethyl oleate very high amounts of water (far greater than 0.1–3%), led to the best results in the first hours of reaction. In fact, data presented in Fig. 2 reveal that during the first 24 h of reaction *oleic acid conversion increases with the amount of water added to the mixture*. In this period, the high amounts of water added ($W = 10\%$ and 20%) – far superior to the contents needed to hydrate the lipase – do not seem to have an adverse effect on the esterification rate. To explain the observation, we propose the existence of a third effect of water, this one related to the instability of the monophasic system formed upon addition of such high amounts of water.

In the experiments with $W = 10\%$ and 20%, addition of water led to the formation of biphasic systems (two-liquid phases), with a light organic phase (at zero time, mainly oleic acid), and a heavy phase with a very high concentration of water. In a previous work of our group we demonstrated that for reactions with initial $W = 10\%$ and 20%, phase equilibrium software (UNIFAC group contribution method with geometric and interaction parameters based on liquid–liquid equilibria), predicts the instability of the monophasic system and the formation of a liquid–liquid biphasic system with very low concentration of water in the organic phase [47,48]. Monot et al. [49] reported the formation of a biphasic system in the lipase-catalyzed synthesis of butyl butyrate. During the progress of the solvent-free synthesis, the authors detected the formation of a discrete aqueous phase. Measurement of the water content of the organic phase revealed a notorious reduction which the authors partially attributed to migration to a second phase [49]. The same occurred

in the synthesis of ethyl oleate when high overall water contents were added to substrate mixture. Systems with a global water content (W) of 20% lead to a water content in the organic phase (W^{org}) of 0.58%, while $W = 10\%$ lead to a W^{org} of 0.72%. Thermodynamic calculations also showed that upon formation of the biphasic system, the presence of oleic acid in the aqueous phase is minimal restricting the esterification reaction to the organic phase. In reference to ethanol partition, liquid–liquid equilibrium thermodynamic calculations for the initially biphasic mixtures revealed that nearly 50–65% of the ethanol added migrated to the aqueous phase (for $W = 10\%$ and 20%, respectively). Low concentration of water in the reactive organic phase justifies the absence of important effects of the reversal reaction even for systems with very high overall water products ($W = 20\%$) [47,48].

Moreover, in the experiments performed, the thermodynamic software revealed that higher total water contents extracted more alcohol mols to the aqueous phase, reducing alcohol's presence in the organic phase. Being the ethanol the compound more "similar" to water, reduction of ethanol in the organic phase also reduces water presence: ethanol reduction makes the organic phase "less hydrophilic" and more water is transferred to the aqueous phase, reducing its concentration in the reactive phase (see the values of W^{org} obtained for $W = 20\%$ and 10%: 0.58% and 0.72%, respectively).

To sum up, the combination of biocatalyst hydration and product extraction from the reactive phase may justify the greatest yields shown in Fig. 2 for the system with the highest global water content: higher global water contents reduce water presence in the organic reactive phase, diminishing the contribution of hydrolysis to the net reaction rate. In fact, the second aqueous phase acts as a drain phase which extracts water generated in reaction, favoring ester synthesis during the first hours of reaction [47,48]. Furthermore, the presence of two immiscible phases is also beneficial for shifting reaction to products in the aqueous phase. Although oleic acid concentration in the aqueous phase has been shown to be very low, the ester produced by esterification of the solubilized fatty acid is continuously extracted into the organic phase, also contributing to driving esterification to products.

Tendencies illustrated in Fig. 2 have also been found for native lipases of CRL, PFL and CALB, and their polypropylene immobilized derivatives [11,12]. In reference to the other chitosan-immobilized lipases prepared in this contribution and listed in Section 2.3, all of them showed the same behavior with respect to water addition to reaction medium. In all cases (see the screening study performed for all catalysts in systems with $W = 0\%$ and 20% (Section 3.3)), the highest fatty acid conversion was found in the system with the greatest water content assayed.

3.4.2. Effect of temperature (T) on fatty acid conversion

Enzymatic reactions show temperature optimums. The reason for this common pattern of enzyme-catalyzed reactions is explained by the combination of two consequences of the temperature increment. The initial rate of enzymatic reactions increases with temperature, in the usual Arrhenius fashion. However, the stability of the enzyme will decline with temperature, and at high enough temperature catalytic activity will be lost rapidly before significant conversion is reached. As a consequence, temperature optimums as the ones depicted in Fig. 3 are found. Reactions illustrated in Fig. 3 were performed in biphasic systems ($W = 20\%$), and 50 mg of CA/CHIT and PF/CHIT/GLU 0.025 were used in the catalysis of the synthesis. Reaction temperature was alternatively set at 35, 45, 65 and 75 °C.

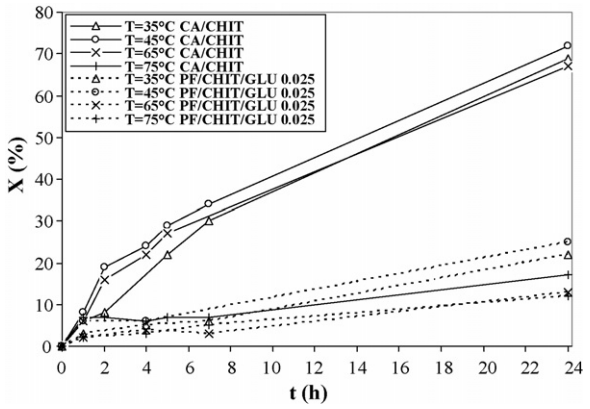

Fig. 3. Evolution of oleic acid esterification. Influence of reaction temperature, T. $W = 20\%$, 50 mg of CA/CHIT (full lines) and 50 mg of PF/CHIT/GLU 0.025 (dashed lines), 350 rpm. Error in measured values lower than 1%.

Data shown in Fig. 3 demonstrates that CA/CHIT develops highest activity in the range of 45–65 °C. As it is shown by conversion data collected at 35 °C, lower reaction temperatures reduce reaction rate. On the other hand, consideration of reaction performed at 65 °C reveals incipient denaturation effects, which are notably enhanced in the reaction carried out at 75 °C. At 75 °C high temperature selected induces lipase unfolding and biocatalyst denaturation, leading to a 24 h conversion more than 75% lower than the one found at 45 °C. In reference to the response to temperature of PF/CHIT/GLU 0.025 (dashed lines of Fig. 3), the catalyst developed highest activity at 45 °C. This biocatalyst, less active than CA/CHIT, was also less stable with respect to temperature, showing a drastic reduction in its catalytic activity at 65 °C.

3.4.3. Effect of mass of catalyst (m) on fatty acid conversion

The effect of increasing the mass of catalyst used in reaction was studied in a system with $W = 20\%$ and kept at 45 °C. Fig. 4 shows the evolution of the fatty acid conversion in the reactions catalyzed by increasing amounts of CA/CHIT (full lines) and by PF/CHIT/GLU 0.025 (dashed lines). The lower activity of the PFL derivative is also shown in this figure.

In the case of CA/CHIT, the conversion profiles reveal that 24 h conversion is similar in all cases, with approximately 75% of fatty acid conversion. The high activity of CA/CHIT allowed 24 h conversions very close to equilibrium conversions (80% for systems with $W = 20\%$ [50]), with only 50 mg of the prepared biocatalyst. If 150 mg of CA/CHIT are added almost constant conversion is achieved in just 7 h of reaction. However, increasing the mass of biocatalyst added to 200 mg does not lead to significant conversion increments with respect to 150 mg. Data included in Fig. 4 allows a rapid selection of an amount of bio-

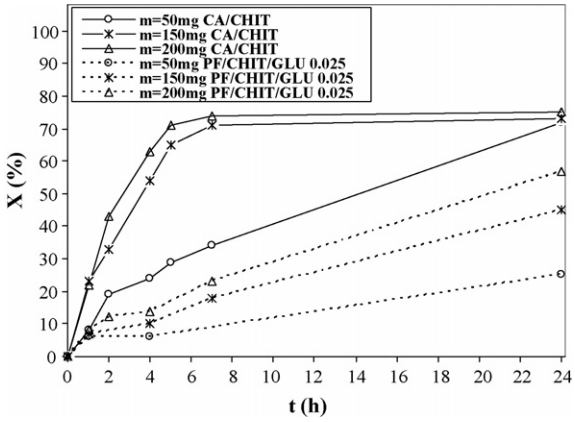

Fig. 4. Evolution of oleic acid esterification. Influence of the amount of CA/CHIT (full lines) and PF/CHIT/GLU 0.025 (dashed lines) added, $W = 20\%$, 45 °C, 350 rpm. Error in measured values lower than 1%.

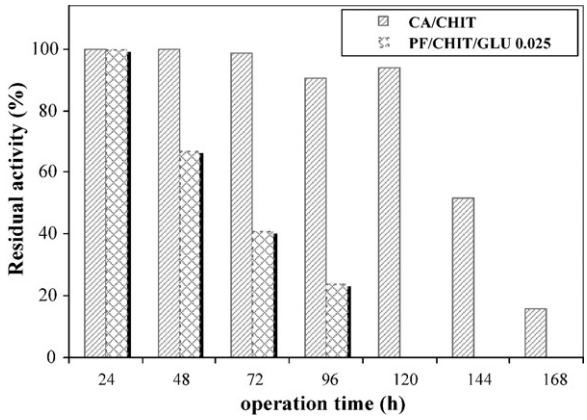

Fig. 6. Residual activity of several 24 h uses of 200 mg of CA/CHIT and 200 mg of PF/CHIT/GLU 0.025. $W = 20\%$, 45 °C, 350 rpm. Error in measured values lower than 1%.

catalyst to add to reaction medium which looks convenient in terms of ester yield, time used to achieve it, and mass of catalytic material involved.

In reference to PF/CHIT/GLU 0.025 (dashed lines), the lower activity of this catalyst leads to 24-h conversions far from equilibrium. Then, ester yields measured at definite times increase with the mass of catalyst added to reaction medium.

Fig. 5 presents data in terms of specific activities. Plotted data reveal that for both CA/CHIT and PF/CHIT/GLU 0.025, the unit of mass of catalytic material is used more effectively when lower masses of catalyst are used. Agglomeration effects may justify this observation. Agglomeration using free and immobilized lipases in solvent-free systems has been reported [51]. The phenomenon of clumping leads to aggregate formation and inhomogeneous enzyme distribution. The enzymes molecules on the outer surface of such particles are exposed to high substrate concentrations (especially high when solvent free media is used), but mass transport into a particle of clumped catalyst (free or immobilized) can severely limit the concentration of substrate inside the particle. Lower activity of a fraction of biocatalyst reduces global yield, decreasing the efficiency per the mass unit of biocatalyst. Agglomeration phenomena in oleic acid

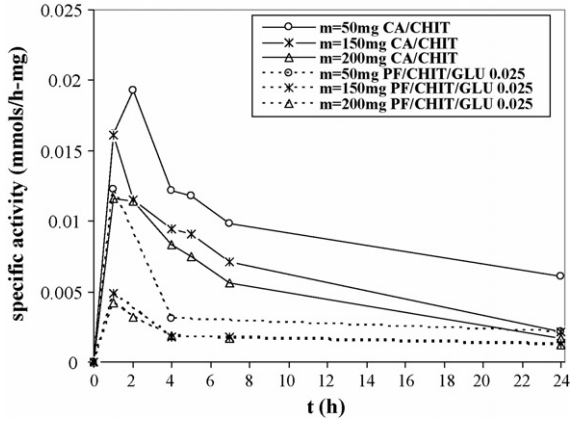

Fig. 5. Specific activity developed by 50, 150 and 200 mg of CA/CHIT (full lines) and PF/CHIT/GLU 0.025 (dashed lines), $W = 20\%$, 45 °C, 350 rpm. Error in measured values lower than 1%.

esterification was also found in the synthesis catalyzed by free CRL, PFL and CALB, and also in the polypropylene-adsorbed derivatives [11,12].

3.5. Biocatalyst reuse

The parametric study performed demonstrated the great activity of CA/CHIT with conversions very near to equilibrium conversions (80% for $W = 20\%$, 45 °C [50]) in just 6–7 h of reaction (150–200 mg). However, the development of an attractive biocatalyst requires not only high activity but also high stability that allows repeated use of the catalytic material. The operational stability of CA/CHIT and PF/CHIT/GLU 0.025 was assayed by using 200 mg of the immobilized biocatalysts in successive batches of ethyl oleate synthesis ($W = 20\%$, 45 °C, 350 rpm). At the end of each batch (24 h), the immobilized lipases were removed from the reaction medium and washed with ethanol to remove any substrate or product retained in the matrix. After 1 h of drying at 50 °C, the immobilized lipase were introduced into fresh medium.

Fig. 6 shows the residual activity measured for 200 mg of CA/CHIT and for 200 mg of PF/CHIT/GLU 0.025 after each use of 24 h. Data included in Fig. 6 (full lines) show not only the high activity of CA/CHIT (very close to equilibrium conversion) but also the stability of this biocatalyst. After five uses (120 h) at 45 °C, the remaining activity of CA/CHIT accounts for more than 90% of the activity measured on its first use. Taking into account the high concentrations of the solvent-free medium employed, the number of mmols of ethyl oleate produced after those five uses of CA/CHIT is higher than 38 mmol. In reference to the reduction in the conversion measured after the fifth use of CA/CHIT, the phenomenon is a consequence of a combined effect of deactivation/desorption of lipase, and the loss of biocatalyst material due to high stirring speeds and repeated manipulation operations (filtration/drying/addition to a new substrate mixture). In the case of PF/CHIT/GLU 0.025 (Fig. 6, dashed lines), the reuse of the biocatalyst was limited by lipase deactivation, which led to a linear-type decay in biocatalyst activity.

4. Conclusion

Crude lipases from *C. rugosa*, *P. fluorescens* and *C. antarctica* B have been immobilized onto chitosan and glutaraldehyde-pretreated chitosan powders. Lipase immobilization was confirmed by SEM-EDX techniques and by the hydrolytic and synthetic activity developed by the prepared catalysts. The synthetic activity of the nine immobilized lipases was assayed in a model esterification reaction: the solvent-free synthesis of ethyl oleate. The screening study performed allowed us to select among the prepared catalysts two materials which combined high activity and selectivity: CA/CHIT and PF/CHIT/GLU 0.025. With those biocatalysts we performed a parametric analysis in order to determine the operating conditions (water content, reaction temperature, mass of catalyst), which promoted the highest yield of ester.

The parametric study demonstrated the beneficial effect of the addition of water contents relatively high for enzymatic esterifications which led to the formation of a second liquid phase. In these systems reaction mainly occurred in the organic where almost all oleic acid remained. On the other hand, the second aqueous phase generated acted as an extractive phase by draining the water generated by reaction. The reduction of the concentration of water in the organic reactive phase favored ester synthesis, with higher ester yields in the first hours of reaction.

The optimized experimental conditions found for the synthesis of ethyl oleate catalyzed by the chitosan-immobilized lipases were the following: 20% of water, 45 °C, 150 mg of CA/CHIT. Under these conditions 75% of fatty acid conversion was achieved after 7 h of reaction. Reuse of CA/CHIT (the most active biocatalyst prepared) was also achieved. Up to five 24 h uses were possible until significant activity loss was detected. Considering the high substrate concentration of the solvent free systems, the activity and stability of the catalyst allowed the production of 38 mmol of ester in a total time of reaction of 120 h.

In reference to the comparison of the performance of the biocatalysts prepared in this contribution with other chitosan immobilized lipases reported in literature, published information showed to be very scarce and also incomplete (time used in achieving the reported yield, time used in the calculus of activities and specific activities, etc. were not reported), making comparisons of catalytic activity very difficult. The absence of the additional data necessary to establish comparisons of catalytic activity has been previously pointed out [41]. With respect to native lipases, immobilization on chitosan allowed easy recovery and reuse of the catalytic material, with no activity decrease. In some derivatives, immobilization also induced higher temperature resistance.

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