Multiple effects of water on solvent-free enzymatic esterifications

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Abstract

Enhancement of the catalytic activity of lipases has commonly been performed by biocatalyst modification (immobilization on hydrophobic supports, activatiFon with interfaces, pretreatment with amphiphiles, etc.). In this work, the enhancement of the reaction rates obtained in lipase-catalyzed esterifications is achieved through modification of the number of liquid phases present in the medium in which the reaction takes place. The liquid medium may be constituted by either just one liquid phase or two liquid phases. The split of the liquid phase can be controlled by manipulating the water initially added to the system.

As a result of the study of the role of water on the solvent-free enzymatic esterification of oleic acid with ethanol, the addition of relatively high amounts of water showed an unexpected beneficial effect: ester yields increased in systems with high amounts of water initially added. The addition of high amounts of water to the mixture of substrates led to the generation of two-liquid phases systems, in which the fatty acid and the produced ester remained in the organic phase, the added and reaction-generated water migrated to the aqueous phase, and the ethanol partitioned between both phases. The existence of a second aqueous phase from the beginning of the reaction favored the extraction of the water generated in the reaction, with an important reduction of the water content in the organic reactive phase. As a consequence, the increase in the global water content of the reaction medium not only did not favor hydrolysis, but also increased the fatty acid conversion in the first hours of reaction. Together with the lipase hydration and equilibrium shift, effects commonly considered in the literature of lipase-catalyzed esterifications, this manuscript emphasizes another effect of water related to the formation of a two-liquid phases system. Experimental and modeling data from reactions catalyzed with up to 15 biocatalysts (native and immobilized lipases) are presented to analyze the role of water on the rate of solvent-free enzymatic esterifications.
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1. Introduction

Lipases (EC 3.1.1.3) are a family of enzymes that catalyze the hydrolysis of fats (their natural function), transesterification, alcoholysis and esterification among other reactions. Due to their high activity, selectivity, and the moderate conditions in which they operate, lipases find wide application in several processes usually found in the food, pulp and paper, textile, and leather industries. The high selectivity of lipases is a key feature of these biocatalysts which are used in the resolution of racemic mixtures for preparation of optically pure compounds for the pharmaceutical and agrochemical industries.

Although lipase action was initially 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 and immobilized enzymes [1]. Some non-conventional media that are currently being used include organic solvents, ionic salts and liquids, supercritic fluids, and solvent-free systems. Lower costs, higher substrate concentration, and greater volumetric production are some of the advantages of solvent-free systems (SFS).

Water plays multiple roles on lipase-catalyzed esterifications performed in non-conventional media. First of all, it is widely known that water is absolutely necessary for the catalytic function of enzymes because it participates, directly or indirectly, in all non-covalent interactions that maintain the conformation of the catalytic site of enzymes [2,3]. On the other hand, in esterification/hydrolysis reactions it is well-known that the water content affects the equilibrium conversion of the reactions as well as the distribution of products in the media [4,5]. Particularly for esterifications, as the water content increases, lower equilibrium conversions are achieved.

Although the proper amount of water for a given enzymatic reaction depends on many factors (the selected enzyme,

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support, solvent, co-solvent, and polarity and quantities of substrates), based on the two effects of water mentioned above, some authors agree on the existence of optimum water contents, generally in the range of 0.2–3% [6–9]. As Svensson et al. reported, for these reactions the activating effect of water dominates at water contents below the optimum, while at higher water contents the net esterification rates decrease, which may be a result of water acting as a substrate in hydrolysis of the acyl-enzyme intermediate [10].

In this contribution, several data of the enzymatic esterification of oleic acid and ethanol performed in a solvent-free medium and catalyzed by a variety of native and immobilized lipases are presented. For every catalyst assayed the effect of the water added to the reaction mixture on the kinetics and also on the reaction equilibrium was analyzed, detecting another consequence of the presence of water. In the studied esterification, the addition of high amounts of water to the initial mixture of substrates promoted the formation of a two-liquid phase reaction system that significantly enhanced the ethyl oleate yield measured on the first hours of the reaction, for the fifteen biocatalysts assayed. Although the following might be considered in disagreement with the known concepts of reversible reactions with product addition, for every biocatalyst assayed in this reaction (within the tested range of added water percentages: 0–20% weight of water/weight of oleic acid) it was found that the higher the amount of water added the greater the conversion achieved during the first hours of reaction.

Since the feasibility to carry out a reversible reaction can not only be based on equilibrium conversions (in fact the rate at which a reaction approaches the equilibrium can be even more important than the final conversion value), the water initially added to the solvent-free enzymatic esterifications is a key parameter to optimize the reactor operation. Based on experimental and reactor simulation data, the aim of this work is to analyze the different effects of water on the kinetic rate and equilibrium of lipase-catalyzed esterifications, with particular emphasis on the benefits of the generation of a second aqueous phase.

2. Experimental

2.1. Materials

Candida rugosa AY lipase (64,000 g/mol – 30,000 U/g) and *Pseudomonas fluorescens* AK lipase (33,000 g/mol – 25,800 U/g) were kindly donated by Amano Enzyme. Native lipase B from *Candida antarctica* (5000 U/ml) and the commercial immobilized biocatalyst Novozyme 435 were kindly 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 potasium hydroxide were both from Merck. Low-molecular-weight polypropylene powder (30,000 g/mol, BET area: 23 m^2/g) was obtained by polymerization using metallocenes [11] and used for enzyme immobilization. Chitosan powder (origin: prawns shells, batch number: TM 369, 60–100 mesh, MW: 70,000–80,000 g/mol, BET surface area: 3–5 m^2/g, degree of deacetylation of 85.2%) was obtained from Primex Ingredients, ASA Norwegian. This material was also used for lipase immobilization.

2.2. Supports pretreatments and lipase immobilization

Polypropylene (PP) particles used for lipase immobilization were pretreated with ethanol as previously described [12]. In the case of the biocatalysts immo-

bilized onto chitosan powder (CHIT), a fraction of the chitosan was pretreated with glutaraldehyde (GLU) which acted as a coupling agent between the chitosan and the lipases. 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, and following the procedure previously described [13]. The modified supports obtained were CHIT/GLU 0.025 and CHIT/GLU 0.25, respectively.

The immobilization of the lipases on polypropylene and chitosan powders was performed at room temperature during 7 h in stirred media (350 rpm). The phosphate buffer kept pH at 7 and the ionic strength of the immobilization medium equal to 0.014 M. In the case of the lipase from *Candida rugosa* (CR) and lipase from *Pseudomonas fluorescens* (PF), 150 mg of the solid powders were added to 50 ml of phosphate buffer solutions, and subjected to strong stirring during 30 min in order to solubilize the lipase. A filtering step was then performed to retain carbohydrates and other insoluble compounds. In the case of the lipase from *Candida antarctica B* (CA), 0.9 ml of the commercial enzyme solution were diluted up to 50 ml with phosphate buffer.

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

2.3. Esterification reaction

All the prepared biocatalysts were assayed in the direct esterification of oleic acid with ethanol, being the products of reaction the ethyl oleate ester and water. Eq. (1) describes the reaction.

$$\text{Oleic acid} + \text{Ethanol} \Leftrightarrow \text{Ethyl oleate} + \text{Water} \tag{1}$$
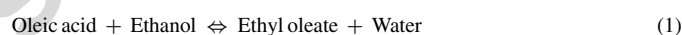
The synthesis was carried out in an isothermal (45 °C) batch type reactor of 10 ml at 350 rpm stirring. In all the experiments performed, the initial reaction medium consisted of the stoichiometric mixture of substrates (10.6 mmol) and different percentages of added water, W (being W the mass of added water/initial mass of oleic acid, wt.%). Added water percentages in the range of 0–20% were assayed. Addition of even higher water quantities made the withdrawal of representative samples extremely difficult.

The reaction began with the addition of 10 mg of the crude lipases or 50 mg of the immobilized biocatalysts. 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. Samples were withdrawn from the reaction mixture following an optimized method that showed to be accurate for sampling from two-liquid phase systems [14]. The accumulated conversion of oleic acid at a given time was determined by the relative reduction of the acidity index of the samples. Conversion determinations were run in duplicate with an average error lower than 1%. In kinetics experiments the total variation of the volume of the reaction mixture was less than 10%. Experiments performed at higher scales demonstrated the feasibility of the 10 ml-vial reactor to obtain kinetic data [15,16].

3. Reactor simulation

According to the amount of water initially added to the mixture of reactants and to the level of conversion achieved, the solvent-free oleic acid esterification may proceed either in a single liquid phase (no initial water added and very low conversion), or in a two-liquid phase system (relatively high initial water contents, or nearly anhydrous initial reactants mixture but with high conversion). The number of the phases depends on the relative amount of water (either added or produced) with respect to the mass of the other compounds. The presence of a second aqueous

phase can be either experimentally identified or predicted by thermodynamic models if the global concentrations are known.

In a previous work of our group, the biphasic nature of the system (two liquid phase) was accurately represented by the development of a mathematical model that simultaneously considered reaction and mass transfer between the organic and aqueous phases, once the phase split is detected [17,18]. In the mentioned model, the reactor is idealized as a series of batch operations with a given duration (Δt) which is a small fraction of the final operating time. After this Δt is achieved, the formation or existence of a second phase is verified by using the UNIFAC group contribution method [19], with geometric and interaction parameters based on liquid-liquid equilibria [20]; and the new composition of both phases is established. After that, the enzymatic reaction is allowed to proceed again for another Δt, and the composition of the phases is recalculated. The same procedure is followed until the final operation time is reached. The developed model assumes isothermal operation (45 °C), the presence of one or two liquid phases, no external or internal mass transfer limitations (similar conversion profiles for different stirring speeds were found, and the supports are non-porous), instantaneous mass transfer of reactants and products between liquid phases, that the reaction is restricted to the organic phase (due to the extremely low solubility of oleic acid in the aqueous phase), and the mass of catalyst in each phase as proportional to the volume of the phases [17,18]. The fitting of the kinetic parameters of the rate equation adopted to represent the enzymatic esterification kinetics (Ping Pong Bi Bi equation) was achieved with an optimization routine that minimizes the difference between calculated and experimental conversions [17,18].

In this contribution, the mathematical model with the fitted kinetic parameters previously reported is employed as an additional tool which, in combination with several original experimental data, is used to explain some effects of water on lipase-catalyzed esterifications.

4. Results

4.1. Influence of water on the rate of esterification of oleic acid catalyzed by native and immobilized lipases

Native, polypropylene-immobilized and chitosan-immobilized lipases from *Candida rugosa, Pseudomonas fluorescens* and *Candida antarctica B* were assayed in the solvent-free esterification of oleic acid and ethanol under the conditions detailed in Section 2.3. Fatty acid conversion (X_{Ac}) was analyzed in systems with added water percentages (W) of 10% and 20%, and also in mixtures with no added water ($W = 0\%$).

In the case of the native lipases from *Candida rugosa* and *Pseudomonas fluorescens*, their poor synthetic activity led to low fatty acid conversion for every added water percentage assayed: 0%, 5%, 10%, 15% and 20% w/w. However, for both crude lipases two-hour conversions of systems with the highest added water percentage assayed almost doubled that found for the medium without added water (two-hour conversions indicate 7–9% for $W = 20\%$, versus 4–5% for $W = 0\%$) [21].
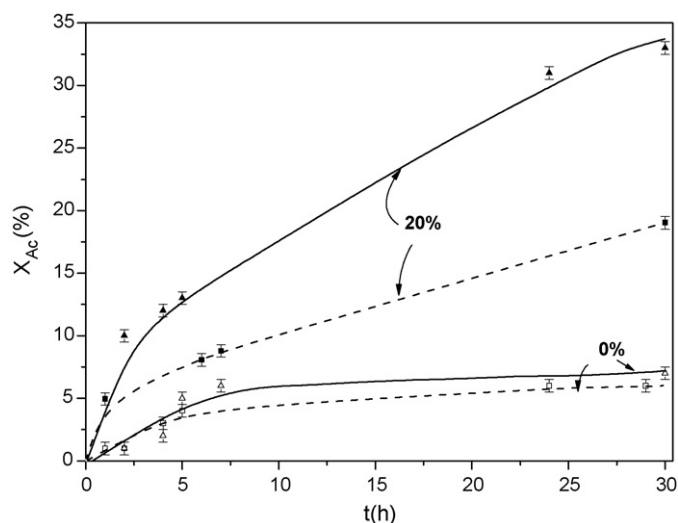

Fig. 1. Oleic acid conversion as a function of time. Reactions performed at 45 °C in systems with $W = 20\%$ catalyzed by 50 mg of PF immobilized on non-pretreated chitosan (full triangles) and on CHIT/GLU/0.25 (full squares). Reactions performed in systems with $W = 0\%$ catalyzed by 50 mg of PF immobilized on non-pretreated chitosan (empty triangles) and on CHIT/GLU/0.25 (empty squares). Curves are trend lines.

Adsorption of lipases from *Candida rugosa* and *Pseudomonas fluorescens* onto polypropylene powder produced two interfacially activated biocatalysts, namely CR/PP and PF/PP. The results of the synthesis of ethyl oleate catalyzed by CR/PP and PF/PP in systems with increasing added water percentages followed the same pattern observed for native lipases. In this case, two-hour conversions also showed greater ester yield for the system with the highest added water percentage assayed ($W = 20\%$) [21].

Immobilization of lipases from *Candida rugosa* and *Pseudomonas fluorescens* on chitosan and glutaraldehyde-activated chitosans also showed the same behavior. In the case of *Candida rugosa* lipase derivatives, the oleic acid conversions measured after two hours of reaction in systems with $W = 20\%$ almost two-folded those found in systems with no water initially added. Fig. 1 shows oleic acid conversions measured at 45 °C for the esterifications catalyzed by the chitosan derivatives of lipase from *Pseudomonas fluorescens*; PF/CHIT and PF/CHIT/GLU 0.25. For both catalysts assayed, conversions measured at $W = 20\%$ are higher than those achieved in systems with no added water. Reactions catalyzed by PF/CHIT/GLU 0.025 showed the same behavior (data not shown). In reference to the relative activity of PF derivatives, for all added water percentages assayed the biocatalysts immobilized on pretreated chitosans showed lower activity than the ones immobilized onto non-treated chitosan. It is probable that chemical bonds established between lipase and chemically modified chitosans during the immobilization process involved aminoacids from the active site or from the access to the active site of the lipase. Then, steric restrictions or improper conformational changes could have taken place, leading to reduced enzymatic activity for glutaraldehyde-pretreated chitosan derivatives.
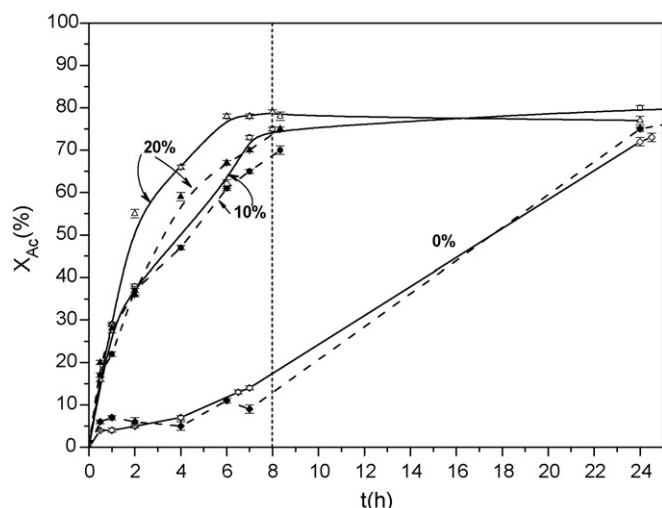
Fig. 2. Oleic acid conversion as a function of time. Reactions performed at 45 °C catalyzed by 50 mg of CA/PP in systems with $W = 20\%$ (full triangles), $W = 10\%$ (full circles), and $W = 0\%$ (full rhombuses). Reactions performed at 45 °C catalyzed by 300 U of native CA in systems with $W = 20\%$ (empty triangles), $W = 10\%$ (empty circles), and $W = 0\%$ (empty rhombuses). Curves are trend lines.

Fig. 3. Oleic acid conversion as a function of time. Reactions performed at 45 °C in systems with $W = 20\%$ catalyzed by 50 mg of CA immobilized on non-pretreated chitosan (full triangles) and on CHIT/GLU/0.25 (full squares). Reactions performed in systems with $W = 0\%$ catalyzed by 50 mg of CA immobilized on non-pretreated chitosan (empty triangles) and on CHIT/GLU/0.25 (empty squares). Curves are trend lines.

Lipase from *Candida antarctica B* showed higher activity in the synthesis of ethyl oleate than CR and PF lipases. Solvent-free oleic acid esterifications catalyzed by native lipase from *Candida antarctica B* (300U, 45 °C, 350 rpm, substrate molar ratio = 1) revealed high ester yields with seven-hour oleic acid conversions up to 70–80%. Immobilization of lipase of *Candida antarctica B* on polypropylene powder (CA/PP) also led to a very active catalyst for the synthesis of ethyl oleate [22]. Fig. 2 shows kinetic data of ethyl oleate synthesis measured for native CA and CA/PP. Reactions were performed in systems with $W = 0\%$, $W = 10\%$, $W = 20\%$. Conversions measured during the first eight hours confirmed the trend observed at 2 h of reaction using native and immobilized forms of CR and PF lipases. During the first hours of the synthesis (when the reaction is still far from achieving equilibrium), and contrarily to the widely-known concepts of reversible reactions with product addition, the addition of water to the initial substrate mixture strongly enhances the rates of ester synthesis.

It is a known fact that water is necessary for the catalytic activity of lipases [2,3]. The low activity observed for native CA and CA/PP in almost anhydrous media ($W = 0\%$) during the first hours of reaction could be explained by insufficient hydration of the biocatalyst. However, the ester yield enhancement achieved when the added water percentage of the system was increased from 10% to 20%, can not be justified with the same explanation: considering that the amount of water added at $W = 10\%$ is absolutely enough for complete lipase hydration, insufficient hydration of the biocatalyst can not explain the ester yield enhancement achieved by increasing the added water percentage from 10% to 20%.

Fig. 3 shows oleic acid conversion measured at 45 °C in the esterifications catalyzed by chitosan-immobilized derivatives of lipase from *Candida antarctica B*, denoted as CA/CHIT and
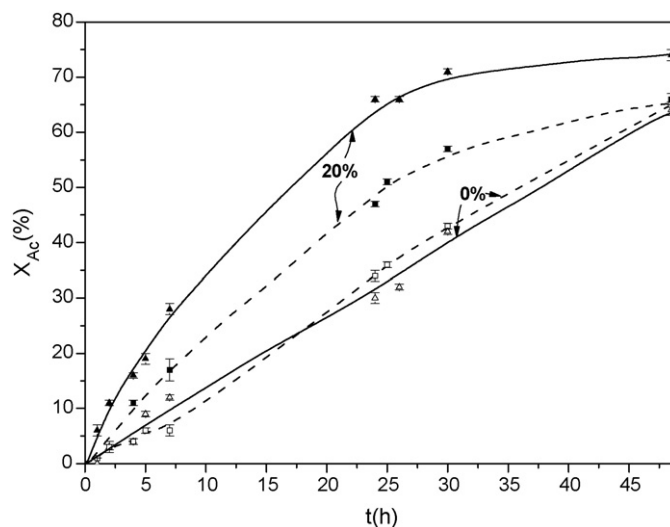
CA/CHIT/GLU 0.25. These biocatalysts were assayed in systems with $W = 0\%$ and $W = 20\%$. Once again, the conversions achieved in systems with $W = 20\%$ are superior to the ones measured in systems with no water addition, for all the chitosan supports used. Reactions catalyzed by CA/CHIT/GLU 0.025 showed the same behavior (data not shown). In reference to the relative activity of CA-chitosan derivatives, and similar to PF-chitosan derivatives, at $W = 20\%$ the biocatalyst immobilized on non-treated chitosan shows higher activity that the one that involved chemical bonds between lipase aminoacids and glutaraldehyde-modified chitosan.

In summary, Figs. 1 and 2 (see the first 8 h of reaction) and 3 evidence a significant increase in the esterification rate during the first hours of reaction for systems with increasing added water percentages. The behavior was observed in several supported and non-supported biocatalysts, indicating that the water effect is not related to the catalyst nature. Table 1 summarizes the effect of the added water percentage on the mean ester yield achieved in the reactions catalyzed by all the biocatalysts assayed (3 native lipases and 12 immobilized lipases). For all of them, the addition of high quantities of water to the reaction mixture not only does not favor ethyl oleate hydrolysis, but also promotes ester production. The generation of a two-liquid phase system upon addition of high quantities of water might explain the apparently anomalous phenomenon (see the Discussion section). In experiments with $W = 5\%$, $W = 10\%$ and $W = 20\%$ at the beginning of the reaction two liquid phases were distinguished on first sight. On the other hand, systems with no added water looked initially homogenous. These observations were confirmed by phase equilibrium calculations performed with UNIFAC [19,20]. For the studied reaction medium this thermodynamic model predicted the phase instability (i.e. two phases formation) of the systems with added water percentages higher than 1.5%.

Table 1

Conversions achieved with all the biocatalysts assayed for different amounts of water initially added to the reaction mixture. Operating conditions: 45 °C, stoichiometric ratio of substrates, 10 mg of native lipases or 50 mg of immobilized lipases

Biocatalyst	Time of measurement (h)	X_{Ac} (%)		
		$W=0\%$	$W=10\%$	$W=20\%$
CR	2	4	6	9
PF	2	5	5	7
CA	2	5	38	55
	7	14	73	78
CR/PP	2	3	5	7
PF/PP	2	8	8	12
CA/PP	2	8	36	37
	7	11	65	70
CR/CHIT	2	–	–	7
PF/CHIT	2	2	–	10
CA/CHIT	2	3	10	12
	7	12	20	28
CR/CHIT/GLU 0.025	2	2	–	9
PF/CHIT/GLU 0.025	2	2	3	7
CA/CHIT/GLU 0.025	2	4	–	9
	7	14	–	23
CR/CHIT/GLU 0.25	2	4	–	5
PF/CHIT/GLU 0.25	2	2	–	6
CA/CHIT/GLU 0.25	2	3	–	6
	7	6	–	17

4.2. Influence of water on reaction equilibrium

It is well-known that the addition of a product reduces the equilibrium conversion for reversible homogeneous reactions. Table 2 shows the experimental equilibrium conversions determined for ethyl oleate synthesis performed in systems with different W, at 45 °C, and with an initial stoichiometric ratio of substrates. To ensure that constant conversion measured was indeed a consequence of having reached equilibrium (and not due to the complete deactivation of the added catalyst), every time a plateau was registered in the conversion-time profile, an additional amount of biocatalyst was added. The procedure was repeated until the addition of biocatalyst did not lead to any conversion increment. To further guarantee the absence of significant deactivation/denaturation/inhibition effects that may partially o completely deactivate the biocatalyst and stop the reaction far from equilibrium, the biocatalyst chosen for the determinations was the extremely active and stable Novozym 435.

Table 2

Chemical equilibrium conversions for the synthesis of ethyl oleate. Operating conditions: Novozyme 435, 45 °C, stoichiometric ratio of substrates. W accounts for the water percentage added to the initial mixture of substrates

W (%)	Equilibrium conversion (%)
0	87
5	82
10	82
20	80

Results presented in Table 2 demonstrate that, *at equilibrium,* the fatty acid conversions follow the expected trend, i.e. as the added water percentage increases the equilibrium conversions get lower. The effect of the added water at the equilibrium can be also observed in Fig. 2. Even if rates measured for initially two-liquid phase systems are notably higher than initial reaction rates observed in homogenous systems ($W=0\%$), after 6–7 h of reaction, specific reaction rates follow the opposite tendency. Moreover, after the mentioned period, two-liquid phase systems show no significant conversion increments. On the other hand, the system with no added water exhibits a sudden increment of the reaction rate with significant ester generation during the following hours.

4.3. Influence of water on phase equilibrium

For the assayed conditions initial substrate mixtures with no water added are stable as a single liquid phase. However, even for initially homogeneous mixtures with $W=0\%$, thermodynamic calculations predict the generation of a second aqueous phase as the reaction proceeds. In the experimental conditions selected in this contribution, phase equilibrium calculations predict the phase split of the mixtures with no initial water for fatty acid conversions higher than 15.6%. Therefore, the rigorous kinetic modeling of ethyl oleate synthesis requires the consideration of the biphasic (two liquid phases) nature of the system. Monophasic approaches that take into account the existence of a single phase in which reactants and products coexist, have proven to be unable to fit experimental data that showed higher conversions for systems with increasing water contents [17,18].

In reference to the equilibrium of two-liquid phase systems (even for systems with no water addition two liquid phases are predicted to exist at equilibrium conditions), previous works have postulated expressions of a biphasic apparent equilibrium constant that varied with the water present in the system and with the initial molar ratio of substrates [23–25]. Following a different approach, in a previous work of our group simultaneous consideration of liquid-liquid and reaction equilibria led to the determination of a single classical temperature-dependent thermodynamic equilibrium constant that could be used in the kinetic modeling of the system [15]. This approach gave far better results in modeling biphasic solvent-free enzymatic esterifications than those provided by traditional global monophasic equilibrium constants [26–28].

The approach used for the modeling of the equilibrium of solvent-free ethyl oleate synthesis was also applied to the modeling of the complete kinetics of the reaction [17,18]. The simultaneous consideration of reaction and mass transfer between liquid phases permitted the obtention of a kinetic model that reproduced the experimentally observed trend of ester yield enhancement for systems with increasing added water percentages (in the first hours of reaction). The described model was used to fit the kinetic experimental data from CA/CHIT. Fig. 4 shows the evolution of oleic acid conversion in the reaction catalyzed by 50 mg of CA/CHIT, for $W=0\%$, $W=10\%$ and $W=20\%$, at 45 °C. It can be observed that the biphasic model is
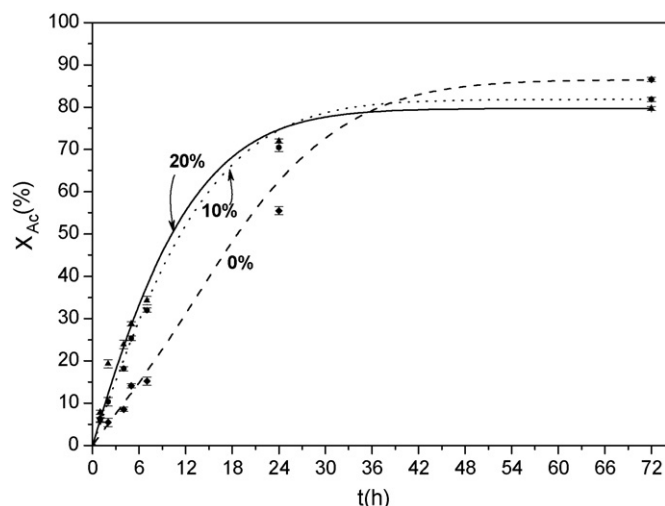
Fig. 4. Experimental and calculated conversions using the biphasic model for reactions at 45 °C, with different added water percentages and catalyzed by 50 mg of CA/CHIT. For experimental data: $W = 20\%$ (full triangles), $W = 10\%$ (full circles), and $W = 0\%$ (full rhombuses). Curves are simulated data.

able to adequately represent experimental data. The beneficial effect of water on initial reaction rates is also illustrated in Fig. 4.

The good capability of the model is based on *the amount of water effectively available in the organic phase* (reaction phase). Due to the hydrophobic-hydrophilic characteristics of oleic acid and water, phase equilibrium calculations reveal that only a very small fraction of the added/generated water remains in the organic phase, being this fraction insufficient to shift the equilibrium to reactants. Therefore, even when high global water contents are used, the ethyl oleate hydrolysis reaction cannot be favored as it would be predicted by a monophasic model. On the contrary, the availability of a second aqueous phase has a beneficial effect for ethyl oleate production, since it favors the migration of water from the reactive phase into the aqueous non-reactive phase.

5. General discussion

Several authors agree on the need of very small amounts of water to successfully employ lipases in esterification reactions in organic/solvent-free media [6,8–9,29,30]. In the synthesis of propyl oleate in non-aqueous conditions catalyzed by immobilized *Pseudomonas fluorescens* at 50 °C, Iso et al. (2001) found an optimum operating point at a water content of 0.2–0.3% [9]. Yadav and Piyush studied the effect of water on the reaction rate of the esterification of *n*-butanol and isobutyric acid using heptane as solvent and catalyzed by Novozym SP 435. The reaction rate was found to increase with an increment in the water content of up to 1.67%. However, when the water percentage was increased to 2.33%, the conversion was found to decrease, a behavior that was attributed to the equilibrium shift towards the reactants [8]. Linko et al. emphasized the importance of the control of water content in lipase-catalyzed esterifications. For the synthesis of butyl oleate in solvent-free media catalyzed by lipase from *Candida rugosa* and *Pseudomonas fluorescens* the authors recommended a water content of 3.2% [29]. In the

solvent-free synthesis of *n*-octyl oleate catalyzed by an immobilized form of *Rhizomucor miehei*, Rocha et al. reported that for non-dried enzymatic preparations the highest activity was obtained when 10 mm³ of water (equivalent to 0.15% v/v) were added to the reaction mixture. However, 100 mm³ of water (1.5% v/v) were required to maximize the activity of the vacuum-dried particles [6]. Kim and Lee studied the esterification of octanoic acid with methanol in a medium of cyclohexane and catalyzed by powdered *Candida rugosa* lipase. The authors determined an optimum water content of 0.67% (v/v) [30].

The previous examples describe the relatively small amounts of water reported as optimum by many authors dealing with enzymatic esterifications in organic/solvent-free systems. However, in the solvent-free synthesis of ethyl oleate in reaction mixtures with added water percentages from 0% up to 20%, the assay of 15 biocatalysts (native and immobilized lipases) showed a repetitive observation: esterification performed in media with added water percentages of 10% and 20% led to much higher esterification rates than systems with no/very little water added (Table 1).

In reference to equilibrium position, independently of the number of liquid phases present in the reaction medium, increments of the added water percentage reduce the final equilibrium conversion. Then, for reactions extremely fast, the water content has to be minimized in order to improve conversion. In reactions catalyzed by the extremely active biocatalyst Novozym 435, for example, the main effect of water on ester production is adverse due to the enhancement of ester hydrolysis [16].

On the other hand, if *the reaction rates* at conditions relatively far from equilibrium are analyzed, water has proven to have a positive effect in ethyl oleate synthesis. In this aspect, the lower activity of the native and immobilized catalysts assayed (compared to Novozyme 435), allowed to detect the beneficial effect of water developed in the first hours of the synthesis. Far from equilibrium, this contribution proposes that the beneficial effect of water is a consequence of two different reasons. Firstly, water is needed for the catalytic activity of lipases [2,3]. Insufficient biocatalyst hydration accounts for the low activity shown during the first hours of reaction by all biocatalysts assayed at $W = 0\%$. In the case of the native forms of CR and PF lipases, no activity was detected in media with no water addition. However, according to reported literature, the amount of water needed for enzyme hydration is much lower than those values that led to the highest oleic acid esterification rates in the experiments performed in this contribution. Moreover, reaction rates measured in systems with added water percentages as high as $W = 10\%$ (surely enough for biocatalyst hydration), showed to be lower than those measured for $W = 20\%$. Finally, the non-catalyzed reaction also showed higher esterification rates when performed in a two-liquid phases medium resulting from high water addition ($W = 20\%$) [17]. Definitely, the beneficial effect of water cannot only be assigned to biocatalyst hydration.

It is hereby proposed then, the existence of an additional beneficial effect of water which is a consequence of the characteristics of the medium in which the reaction takes place, and not only due to biocatalyst hydration. This effect, which is only distinguishable far from equilibrium, is related to the formation
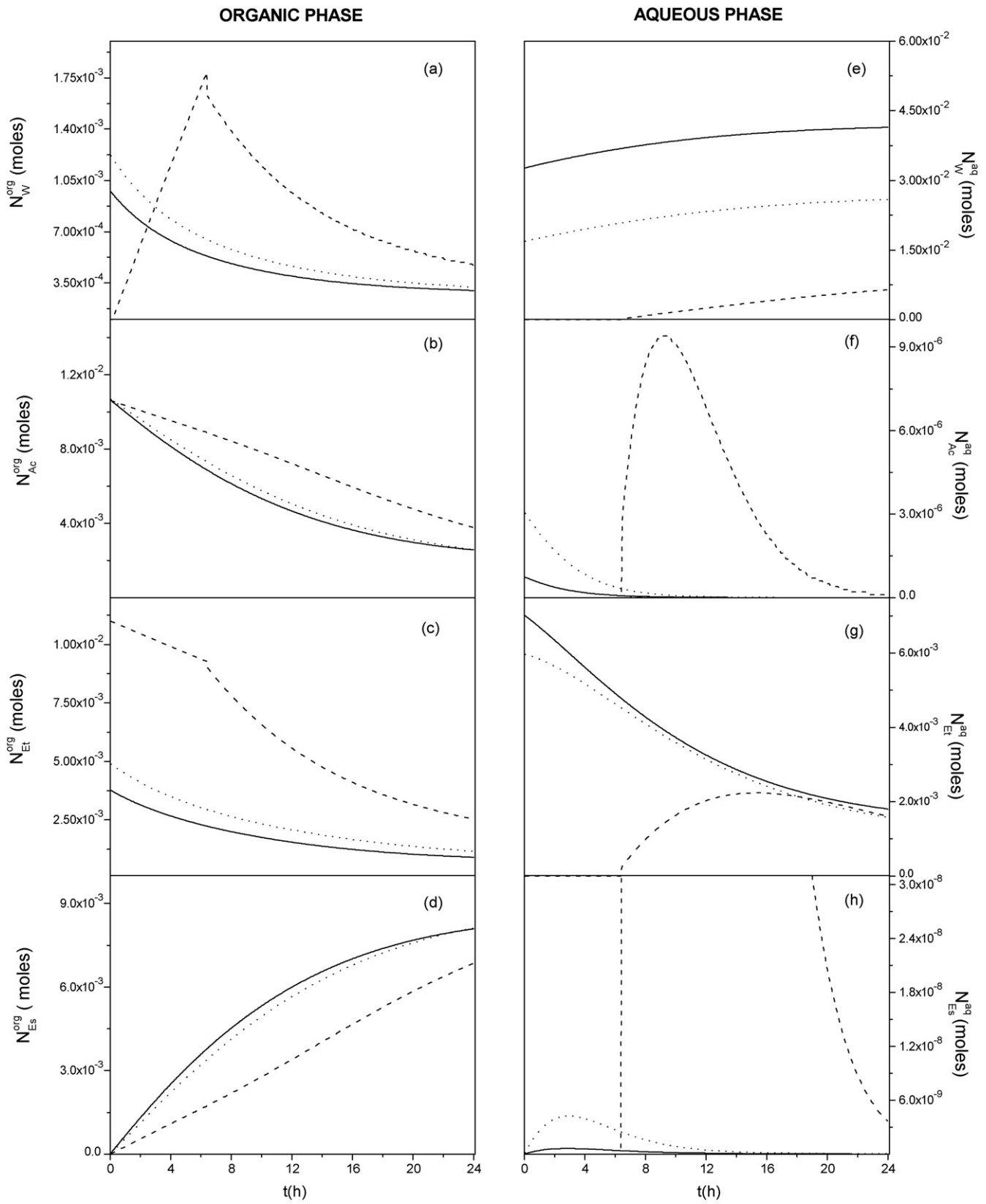
ORGANIC PHASE

AQUEOUS PHASE

Fig. 5. Evolution of the moles of water, oleic acid, ethanol and ethyl oleate in the organic and aqueous phases, calculated by the reactor simulation at 45 °C. Simulated reactions were catalyzed by 50 mg of CA/CHIT. $W = 20\%$ (—), $W = 10\%$ (– – –) and $W = 0\%$ (- - -).

of a two-liquid phase system. If all water added for $W = 20\%$ remained in a single liquid phase, the initial water concentration would equal 7.35 mol/l. However, thermodynamic calculations predict a water concentration in the organic reactive phase of just 0.27 mol/l, when the initial mixture splits.

Experimental data (Figs. 1–3) and also simulation data (Fig. 4), demonstrated that *the amount* of water added to the system is a key operating variable. As more water is added to substrate mixtures, more ethanol is solubilized in the aqueous phase and the capacity of the organic phase to solubilize water is reduced. In order to illustrate the point introduced, Fig. 5 shows the evolution of the moles of water, oleic acid, ethanol and ethyl oleate in the organic and aqueous phases, using 50 mg of CA/CHIT-calculated by the reactor simulation at 45 °C. In Fig. 5a the evolution of the moles of water in the organic phase for systems with $W = 0\%$, 10% and 20% is shown. In the case of the reaction performed in the system with $W = 0\%$, the low conversions measured in the first hours of reaction are a consequence of both insufficient biocatalyst hydration, and a continuously increasing water content within the reactive phase. Prior to the phase split of the reaction mixture into two liquid phases (after 6.4 h of reaction according to the reactor simulation), all water produced by the reaction remains in a unique phase favoring ester hydrolysis. On the other hand, the number of moles of water in the organic phase of initially two-liquid phase systems, continuously decreases as the reaction proceeds. Water concentration profiles are qualitatively identical to moles–time profiles. Although the esterification reaction generates water, for systems with $W = 10\%$ and $W = 20\%$ as the reaction extension increases, ester is produced and ethanol is consumed, making the organic phase more hydrophobic, and then favoring water extraction and, consequently, ester synthesis. The beneficial effect of the extraction of water to a second phase has also been illustrated by Svensson et al. [10]. In the referenced manuscript, even if there is no second liquid phase, the water activity in the reaction medium is kept low by transportation through a silicone tubing to a saturated salt solution phase [10].

Fig. 5b–d show the temporal evolution of the moles of oleic acid, ethanol and ethyl oleate in the organic phase; while Fig. 5e–h refer to the evolution of water, oleic acid, ethanol and ethyl oleate in the aqueous phase. The scales of Fig. 5b and f (oleic acid) are very different, indicating that most of the oleic acid remains in the organic phase. The ester presents the same behavior (Fig. 5d and h). On the other hand, as expected, in the aqueous phase more moles of water are present (Fig. 5a vs. e). The only compound that is present in similar quantities in both phases is ethanol (Fig. 5c and g).

In reference to the organic phase, the moles of the fatty acid continuously decrease basically by reaction (Fig. 5b). The initial amount of moles of ethanol in the organic phase (Fig. 5c) decreases over time due to reaction and mass transfer towards the aqueous phase. The sudden changes of ethanol and water moles in the organic phase for $W = 0\%$ are a consequence of both the formation of the second phase (at approximately 6.4 h of reaction), and the mass transfer of these compounds from the organic phase towards the aqueous one. As more water is added to the system, the number of ethanol moles in the organic

phase reduces. In fact, the alcohol becomes the limiting agent for the reaction in the organic phase of two-liquid phase systems. Finally, the evolution of the moles of ester in the organic phase (Fig. 5d) can be purely attributed to the reaction. Due to its high hydrophobicity, the ester practically confines its presence to the organic phase.

In initially two-liquid phase systems, the reduction of the concentrations of ethanol and water in the organic phase as W is increased, turns the organic phase more hydrophobic favoring the presence of the hydrophobic components. As a consequence, the *increase* of the global water content *reduces* the amounts of fatty acid and ester in the aqueous phase (Fig. 5f and h). Another distinct feature of Fig. 5f and h is the maximum predicted for the mixtures with no water initially added immediately after the phase split occurs. At the moment of the formation of the second aqueous phase, the moles of water in that phase are minimal with respect to the water present in the initially two-liquid phase systems (see Fig. 5e). Then, due to the still low amount of water present, the generated aqueous phase is able to solubilize a greater number of moles of the hydrophobic components than initially two-liquid phase systems are able to do. In reference to the ethanol in the non-reactive phase (Fig. 5g), two-liquid phase systems show that the moles of the alcohol in this phase diminish as a function of time. Since the model does not consider reaction in the aqueous phase, the reduction of ethanol is attributed to its transfer to the organic phase. When mass transfer between the phases is modeled after each reaction time step, the composition of both phases is redefined. The ethanol, that is initially transferred from the oily to the aqueous phase, is also transferred in the opposite direction as the reaction proceeds. For mixtures with $W = 0\%$ the transfer of ethanol to the new phase formed after 6.4 h of reaction (phase split) can be observed in Fig. 5g. However, after 16 h of reaction, the consumption of ethanol by the reaction in the organic phase causes the transfer of the alcohol from the aqueous phase to the organic one.

The final point to address is the reason why such low water contents are usually recommended for lipase-catalyzed esterifications in organic media. As previously mentioned, based on initial reaction rates or enzymatic activities measurements, several authors have found optimal enzymatic activity in systems with initial water contents within the range of approximately 0–3% [6,8,9,29,30]. However, these optima should have been found in homogenous single-liquid phase media, since the water contents tested were not high enough to promote the formation of a two-liquid phase system (the authors do not give any reference to the formation of a second aqueous phase). Although the specific water content necessary for phase split obviously depends on the conditions in which the synthesis is performed (hydrophobicity of substrates, temperature, concentrations, presence of solvent, etc.), it is probable that those recommended water contents correspond to local maxima found in lipase-catalyzed esterifications carried out in systems constituted by a single reactive liquid phase. However, the results presented in this contribution suggest that if the water added to the initial mixture was high enough to promote the formation of a two-liquid phase system, the initial rates (or the conversion measured in the first hours of reaction) would be higher than those

found for systems with minimal water contents. Although conversions measured with very active biocatalysts (like Novozym 435) are mainly controlled by equilibrium, the rate of esterifications performed with less active –and probably cheaper- biocatalysts can be greatly increased by the use of high water contents that promote water migration from the organic phase to the aqueous one, favoring ester synthesis in their evolution towards equilibrium.

Finally, an additional benefit of the generation of the two-liquid phase systems is that the presence of high amounts of water leads to an aqueous phase almost free of ester. In this way, the addition of water percentages usually considered too high for enzymatic esterifications, would also facilitate the recovery of higher quantities of the desired product in the downstream processing operations.

Acknowledgements

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