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Synthesis of Ibuprofen Monoglyceride Using Novozym®435: Biocatalyst Activation and Stabilization in Multiphasic Systems

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Abstract: This work was focused on the enzymatic esterification of glycerol and ibuprofen at high concentrations in two triphasic systems composed of toluene+ibuprofene (apolar) and glycerol or glycerol–water (polar) liquid phases, and a solid phase with the industrial immobilized lipase B from *Candida antarctica* named Novozym®435 (N435) acting as the biocatalyst. Based on a preliminary study, the concentration of the enzyme was set at 30 g·L^{-1} and the stirring speed at 720 r.p.m to reduce external mass transfer limitations. To obtain more information on the reaction system, it was conducted at a wide range of temperatures (50 to 80 °C) and initial concentrations of ibuprofen (20–100 g·L^{-1}, that is, 97 to 483 mM). Under these experimental conditions, the external mass transfer, according to the Mears criterion (Me = 1.47–3.33·10^{-4} << 0.15), was fast, presenting no limitation to the system productivity, regardless of the presence of water and from 50 to 80 °C. Considering that the enzyme is immobilized in a porous ion-exchange resin, limitations due to internal mass transfer can exist, depending on the values of the effectiveness factor (η). It varied from 0.14 to 0.23 at 50 to 80 °C and 0.32–1 mm particle diameter range in the absence of water, and in the same ranges, from 0.40 to 0.66 in the presence of 7.4% *w*/*w* water in the glycerol phase. Thus, it is evident that some limitation occurs due to mass transfer inside the pores, while the presence of water in the polar phase increases the productivity 3–4 fold. During the kinetic study, several kinetic models were proposed for both triphasic reacting systems, with and without first-order biocatalyst deactivation, and their fit to all relevant experimental data led to the observation that the best kinetic model was a reversible hyperbolic model with first-order deactivation in the anhydrous reaction system and a similar model, but without deactivation, for the system with added water at zero time. This fact is in sharp contrast to the use of N435 in a water-glycerol monophasic system, where progressive dissolution of ibuprofen in the reacting media, together with a notable enzyme deactivation, is observed.

Keywords: ibuprofen; monoglyceride; toluene; biphasic system; Novozym®435; ester prodrug

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1. Introduction

After the Sharm el-Sheikh Climate Change Conference (27th COP) of November 2022, it is more evident that global temperatures will increase to 1.5 °C in the period 2030 to 2052 [1]. This reality is boosting the use of renewable resources, causing a drop in fossil fuel consumption and stimulating novel sustainable energy systems as well as energy transition, climate change and clean energy initiatives. The growth of renewables poses new design and operational challenges for the transition to a 100% renewable energy goal [2]. Renewable energy includes the production of biofuels, mainly bioethanol (from first and second generation biorefineries) and biodiesel (by means of homogeneous, heterogeneous and enzymatic catalytic processes) [3]. Glycerol is a by-product of biodiesel production, accounting for approximately 10% of the mass of the triglyceride source. Thus, novel applications of this C3 platform chemical are being sought [4,5].

The lipase (EC.3.1.1.3) from *Candida antarctica* (commonly known as CALB) is a major workhorse in biocatalytic organic synthesis [6], due to its notable activity, stereospecificity and stability [7,8]. Novozym®435 (N435) is an industrial preparation of this enzyme on Lewatit VP OC 1600 manufactured by Novozymes A/S. This biocatalyst is most active in the esterification of profens [9] and, in particular, ibuprofen [10–12]. For several decades, immobilization techniques have been applied to lipases, profiting from their ability to act as interfacial biocatalysts, although there is still space for further enhancement [12,13]. The wise choice of the immobilization approach dramatically affects the activity, selectivity and stability of the biocatalyst [14,15]. Enzyme immobilization permits its stabilization and an easy reuse after liquid-solid separation or its use in flow reactors. Selectivity and, in particular, stereoselectivity can also be affected [16]. However, the presence in the bioreactor of a solid phase enhances the effects of mass and heat transfer phenomena as they become slower [12,17]. Mass transfer, outside the particle and in the pores, should be tackled to reduce productivity limitations. External mass transfer can be sped up by combining low viscosities and high kinetic energy; internal mass transfer can be useful to establish a stable regime under limited productivity (global transformation rate) conditions or it can be circumvented by reducing particle size, increasing pore size or using only external surfaces or zones in nanoparticles and porous particles.

One of the most relevant effects on enzymes due to immobilization is stabilization. Stabilization can also be achieved or enhanced by a particular choice of enzyme origin such as extremophiles or expressing their proteins in mesophilic hosts [18]. It can also be achieved by modifying the liquid environment of the enzyme, choosing the correct solvent, cosolvent, and additives mixture in a monophasic system or creating, in a similar way, the adequate interfacial chemical composition in a multiphasic system [19,20].

Profens are effective medications for the reduction of pain, inflammation and fever. They are inhibitors of cyclooxygenases (COX), which inhibit the production of prostaglandins. Prostaglandins are synthesized by most of the body cells de novo following a mechanical trauma or other stimuli and act as autocrine mediators of acute inflammation [21]. Two isoforms of COX encoded by two different genes (*Ptgs-1* and *Ptgs-2*) have been identified. Both isoforms have different functions [22,23]. COX-1 is a housekeeping enzyme that is needed for basic requirements and is produced constitutively in the body [24]. However, COX-2 is an inducible enzyme, which is generally synthesized in response to the secretion of other chemical messengers such as toxins, cytokines, or mitogens [25].

Apart from the therapeutic potential of ibuprofen against pathological situations associated with enhanced inflammatory changes and pain, it has also been evaluated for its efficacy in cancer, neurodegeneration and even as a complement to make stem cell therapy more effective [26–29]. There is experimental evidence that establishes the anti-inflammatory, apoptosis-inducing, and tumor-suppressing properties of ibuprofen. Additionally, a large number of publications have shown that this drug may exert a potent neuroprotective effect on different cellular or animal models of several neuronal diseases [30]. In these models, it has been demonstrated that ibuprofen suppresses the neuronal loss linked with neurodegenerative disorders. For example, in Alzheimer's disease, the accumulation of amyloid-beta aggregates leads to activation of microglia and astrocytes, which may further cause the initiation of inflammatory signals. Ibuprofen helps in both suppression of these signals as well as in the clearance of amyloid plaques [30]. Several studies have also indicated the positive responses to treatment with ibuprofen in neuronal cells, mice models, and human patients with Parkinson's disease [31].

Ibuprofen may also produce a high number of physiological changes in the body, which lead to abnormalities in the heart, intestine, and kidney [30]. The main explanation behind these toxic effects is the non-selective inhibition of cyclooxygenases. The best-known negative effect when ibuprofen and its derivatives are administered for a prolonged time are the gastrointestinal problems, which are produced due to the suppression of the synthesis of COX-1. Symptomatic peptic ulcer formation and perforations followed by bleeding in the gastrointestinal tract may lead to the death of patients taking these drugs for a long time [32].

The administration of lower doses of the active stereoisomers (for example, S-ibuprofen) instead of the racemic mixture and the use of profen prodrugs, including their esters, allow for a slow release of the drug into the body, reducing unwanted side effects [10–12,33]. In this way, bioprocesses based on the esterification of glycerol or other polyols and profens increase the pharmacological value of the polyol and the easy production of prodrugs with an increased bioavailability (by increasing the profen solubility in aqueous media); they also limit the deleterious effect on health due to local effects on the gastrointestinal system and controlled pharmacokinetics [10,12,34]. The use of multiphasic reacting systems with glycerol as both solvent, substrate and stabilizer leads to high conversion to the prodrug at moderate to high temperatures [34].

Kinetic modelling is based on several multivariable statistics regressions coupled to numerical methods of integration for the differential equations (generally ODEs) expressing the evolution of a chemical system with time. This mathematical expression of reality permits the simulation, optimization, scale-up and control of chemical processes, including those driven by enzymatic biocatalysts. Our recent work with Lipozyme®CALB L (free enzyme) and Novozym®435 allows us to couple chemical reactions driven by the lipase, substrate solubilization, mass transfer phenomena and enzyme deactivation [12,34,35]. All kinetic models were based on Michaelis–Menten assumptions, the ping-pong bi-bi mechanism and its simplification due to the large excess of one of the reagents, glycerol.

The objective of this work is to shed light on the esterification of glycerol and ibuprofen catalyzed by Novozym®435 in triphasic systems with toluene only or with water as cosolvents, as shown in Figure 1. To achieve this aim, preliminary experiments were conducted to understand the roles of water content in glycerol, reagent concentration, temperature, and enzyme concentration on enzyme activity and, for temperature, on enzyme stability. This information was critical to proposing and fitting several kinetic models, and applying the models to select the most adequate in view of physicochemical and statistical criteria.

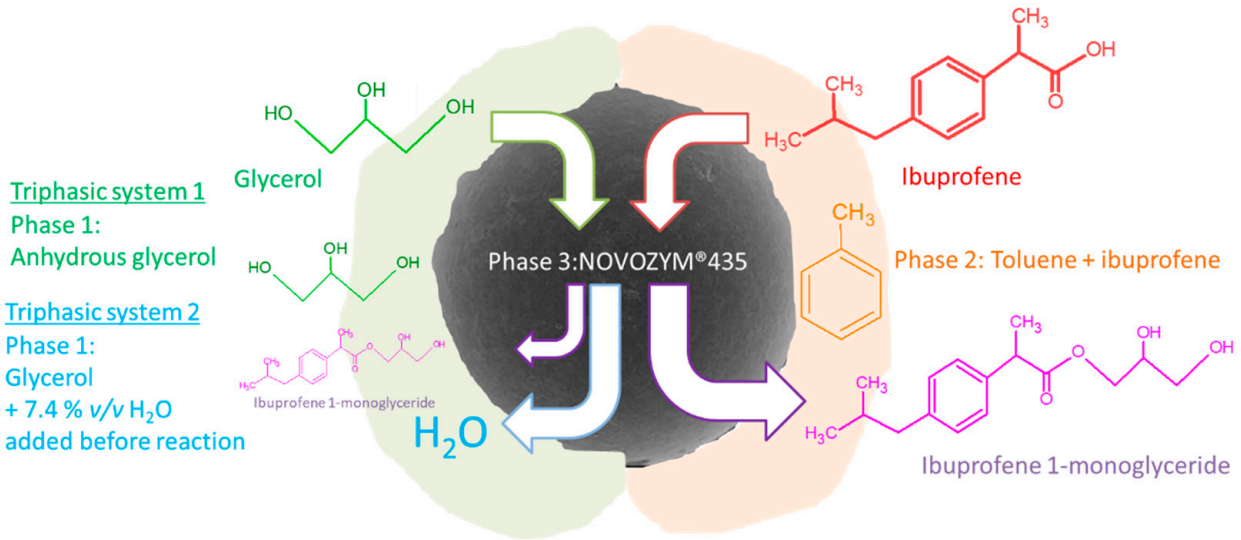

Figure 1. Reactions in triphasic L-L-S systems for the esterification of glycerol and ibuprofen with Novozym®435.

2. Results and Discussion

2.1. Effect of Enzyme Concentration

In the enzymatic esterification of glycerol with ibuprofen catalyzed by the preparation N435 in the presence of a cosolvent (toluene), the effect of enzyme concentration between 10 and 40 $g \cdot L^{-1}$ was studied. Figure 2a shows the initial rate of esterification of glycerol with ibuprofen versus the concentration of enzyme used.

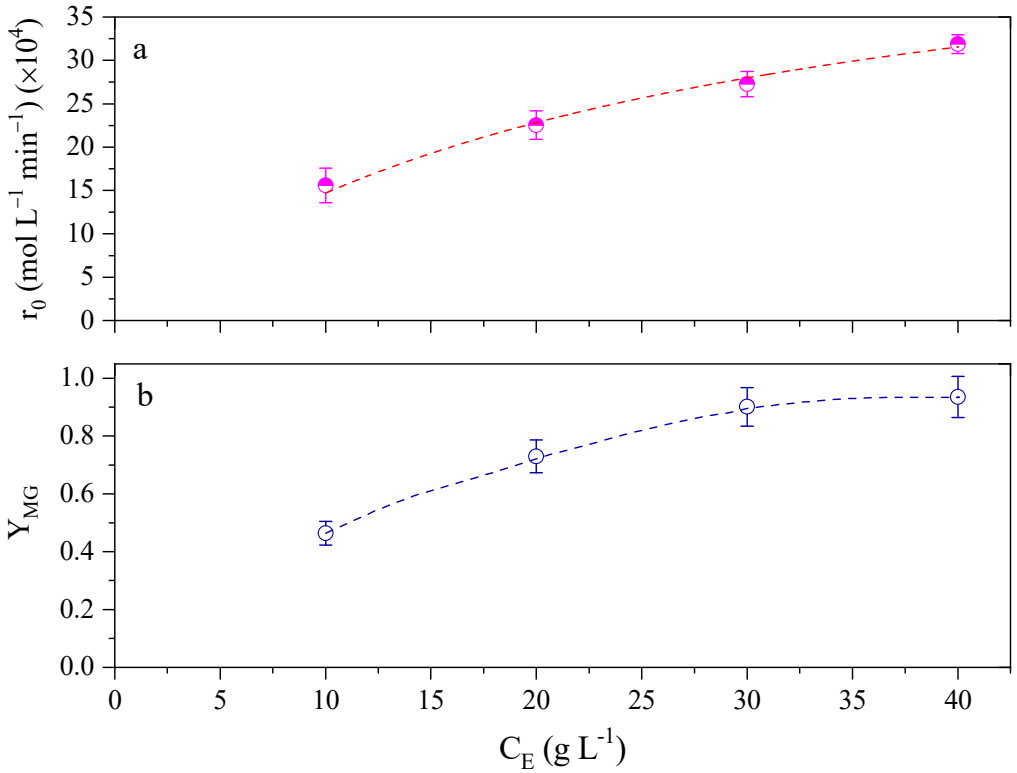

Figure 2. Effect of enzyme concentration on initial rate of esterification of ibuprofen with glycerol using toluene as solvent in the apolar phase at T = 70 °C, C_{I0} = 60 g·L^{-1}, N = 720 rpm over initial reaction rate (**a**) and monoglyceride yield at 24 h (**b**).

It can be seen that the initial esterification rate increases steadily with enzyme concentration from 16·10^{-4} to 33·10^{-4} mol L^{-1} min^{-1}, but there is a slightly hyperbolic trend, which indicates the effects of substrate distribution on the inner surface where the enzymes are immobilized. This trend was also observed for the free enzyme, which accommodates onto the interfacial interface between the apolar ibuprofen and the polar glycerol [34]. However, as presented in Figure 2b, with increasing enzyme concentration the yield to monoester increases but remains almost unchanged from an enzyme concentration of 30 g·L^{-1} (circa 2.4 g·L^{-1} pure *Candida antarctica* lipase B), showing that a thermodynamical limit is reached at a yield value near 0.93 (an equilibrium position), even in conditions in which ibuprofen concentration is 0.29 mol·L^{-1} and glycerol is in a high excess (glycerol concentration: 13.7 mol·L^{-1}).

2.2. Effect of the Volume Ratio Glycerol: Toluene

The influence of the glycerol to toluene ratio (Gli/Tol) on the esterification of glycerol with ibuprofen catalyzed by the preparation Novozym®435 was analyzed using the same Gli/Tol ratios considered in the system with the free enzyme CALB-L [35]; this Gli/Tol ratio was changed, taking the following values: 1/10; 10/10 and 20/5. As can be seen in Figure 3, for a ratio of Gli/Tol = 1/20 the initial esterification rate is low, but increases as the ratio of glycerol to toluene increases. This behavior is very similar to that observed when the free CALB-L is present in the medium; consequently, the volume ratio Gli/Tol = 20/5 was chosen for further experimentation. Furthermore, the values of the initial esterification rate are higher when the N435 preparation is present in the medium than with the free CALB-L enzyme, which may be due to the fact that the concentrations of compounds around the enzyme are more suitable for its activity or, alternatively, that the effective concentration of enzyme is higher on a solid surface (the Lanxess VPOC 1600 resin used in the N435 preparation) that distributes it adequately.

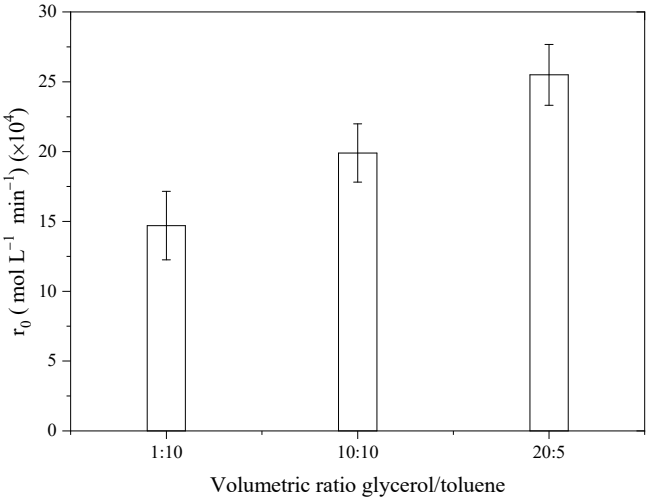

Figure 3. Effect of the volume ratio of the solvents on the esterification of ibuprofen with glycerol using toluene as solvent in the apolar phase at T = 70 °C, C_{I0} = 60 g·L^{-1}, N = 720 rpm over initial reaction rate.

2.3. Influence of the Addition of Water as a Polar Cosolvent

Water is one of the products of the reaction, while glycerol is highly hygroscopic. Therefore, it can have an inhibitory effect on the esterification as it promotes the hydrolysis reverse reaction, while its capacity as solvent and plasticizer affects the viscosity and, thus, the molecular diffusion of ibuprofen and glycerol into the pores. Therefore, we studied the effect of added water in a concentration range that varied from 0 to 7.4% v/v under the following experimental conditions: a stirring speed of 720 rpm, a fixed temperature of 80 °C and an initial ibuprofen concentration of 100 g·L^{-1}, and an enzyme concentration of 30 g·L^{-1}. The results are compiled in Figure 4. In Figure 4a we observed that the initial esterification rate increases up to a water concentration value of 2% v/v, and no significant changes are observed above this value. Considering the final yield to ibuprofen monoester, in Figure 4b a very similar behavior to that of the initial esterification rate can be seen.

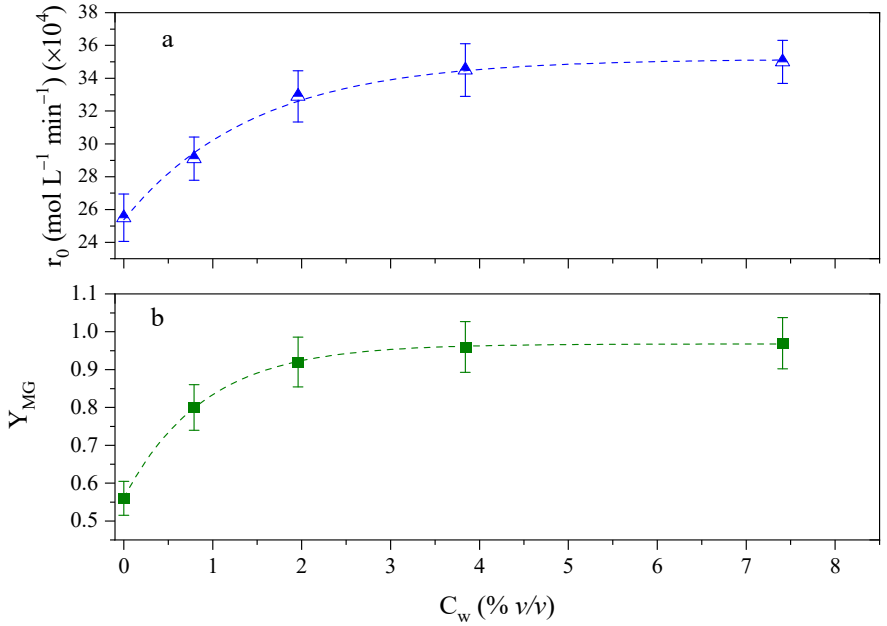

Figure 4. Effects of the addition of water on the triphasic reaction system and the esterification of ibuprofen with glycerol at T = 80 °C, C_{I0} = 100 g·L^{-1}, N = 720 rpm and Gly/Tol 20/5 over initial reaction rate (**a**) and monoglyceride yield (**b**).

2.4. Influence of Temperature and Initial Concentration of Ibuprofen on Activity and Stability

The possible inhibitory effect of the reagent (ibuprofen) on the enzymatic esterification of ibuprofen with glycerol was studied by varying the initial concentration of ibuprofen between 20 to 100 g·L^{-1}, and the possible deactivation of the enzyme was analyzed by varying the temperature between 50 and 80 °C with a constant concentration of the enzyme preparation Novozym®435 (30 g·L^{-1}) and a fixed volumetric glycerol/toluene ratio of 20/5. Figure 5a shows the initial esterification rate for each of the ibuprofen concentrations and different operating temperatures in a triphasic reaction system without added water. As it can be seen, for any temperature considered, the initial rate of esterification increases with the initial concentration of ibuprofen in a hyperbolic manner, following the typical trend according to a Michaelis–Menten model, as occurs in systems employing the enzyme in its free form (CALB-L) in the absence and presence of a cosolvent [34,35]. Likewise, as the temperature rises, the curves of the enzyme activity with the initial concentration of ibuprofen tend to be less hyperbolic and more linear. This behavior suggests that the value of K_M trends to higher values, showing an apparent lower affinity of the enzyme for the ibuprofen at higher temperatures.

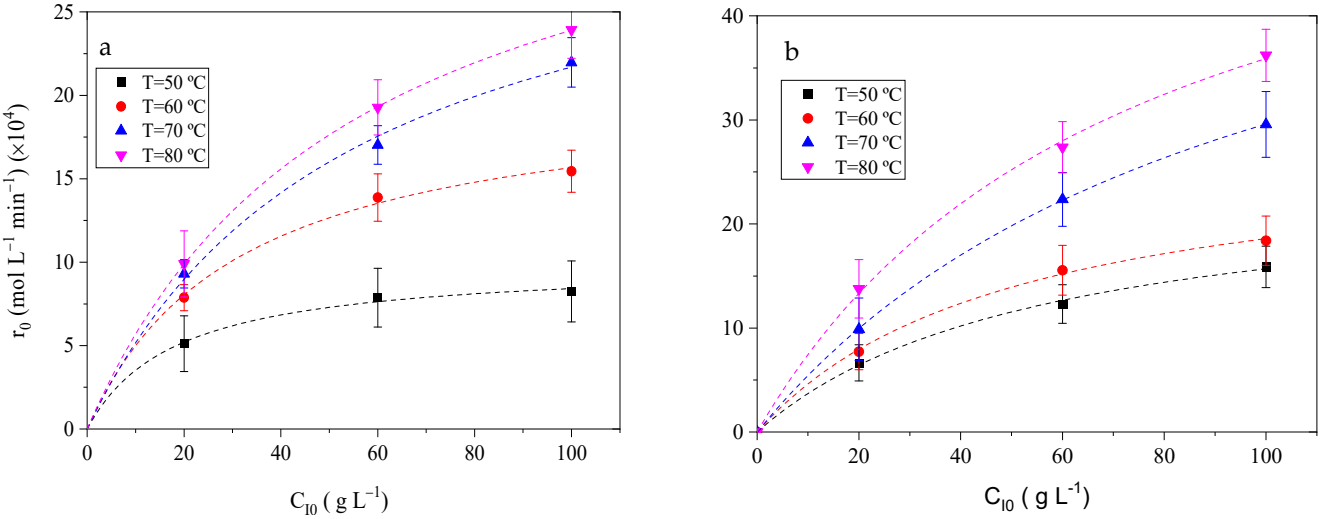

Figure 5. Effect of temperature on the initial rate of esterification for various initial concentrations of ibuprofen in a triphasic glycerol-toluene-N435 system with no added water (**a**) and with 7.4% *v*/*v* water at zero time (**b**).

In a similar triphasic system, we added water to a final concentration volumetric ratio of 7.4% *v*/*v* with respect to glycerol. In Figure 5b, we can observe that, at all the temperatures tested, the initial rate of esterification increases with the initial concentration of ibuprofen in a hyperbolic manner, following the typical trend explained by a Michaelis–Menten model. This behavior is very similar to that found in the anhydrous system. However, the presence of a concentration of water in the reaction medium results in a higher value of the monoester yield at an ibuprofen concentration of 100 g·L^{-1} compared with the value at 60 g·L^{-1}, at all temperatures. This is very likely due to a reduction in viscosity with a concomitant increase in mass transfer rate and, thus, in the observed enzyme activity. Moreover, more linear curve trends can be seen in this figure, suggesting slightly lower affinities of the enzyme for ibuprofen in the presence of water at zero time.

2.5. Influence of Mass Transfer

2.5.1. External Mass Transfer

Substrate mass transfer is known to limit the global transformation rate when this dynamic phenomenon is slow (or takes a longer time to occur than the chemical reactions), so enhancing it usually leads to process intensification in multiphasic systems [36]. We

studied substrate mass transfer in the film surrounding the immobilized enzyme particle (known as the mass transfer limit layer), performing several experiments at a range of stirring speeds from 120 to 840 rpm, keeping the temperature constant at 50 or 80 °C and with an initial concentration of ibuprofen of 60 g·L^{-1}.

We can see in Figure 6a,b, for an initially anhydrous triphasic system at the temperatures considered, that the initial esterification rate increases with increasing stirring speed. At low stirring speed, the contact between the phases is poor, due to a low kinetic energy, but the mixing improves with increasing stirring speed. It can also be appreciated that the initial esterification rate presents lower values at a temperature of 50 °C than at 80 °C. This behavior is consequence of the Arrhenius exponential increment of the global rate and the viscosity of each liquid phase: at low temperature (T = 50 °C), the viscosity of the glycerol–toluene mixture is high but its value decreases considerably when the temperature is increased to 80 °C. Furthermore, it can be seen that at 80 °C, for an agitation value of 600–840 rpm, there are no significant changes in the initial rate of glycerin esterification with ibuprofen; at the lower temperature, it can be considered that there are no changes in the initial rate above 720 rpm. Figure 6c,d display the results obtained for identical experiments run in a triphasic system with 7.4% v/v water added at zero time. At a temperature of 50 °C, the initial esterification rate increases with the increase in the stirring speed; the same behavior occurs at 80 °C, and it is logical that, at this higher temperature, the values of the reaction rate are higher than at 50 °C. It can be observed again that, at low stirring speeds, the contact between the phases is deficient due to the low kinetic energy of the system, even with some water present in the reaction medium from the beginning, but this mixing increases to a point where there is no significant change in the initial rate of esterification of glycerin with ibuprofen, for stirring values of 600–840 rpm. When we compare both systems, the trends are evidently similar, but, as expected, the presence of water from the beginning increases the observed activity of N435.

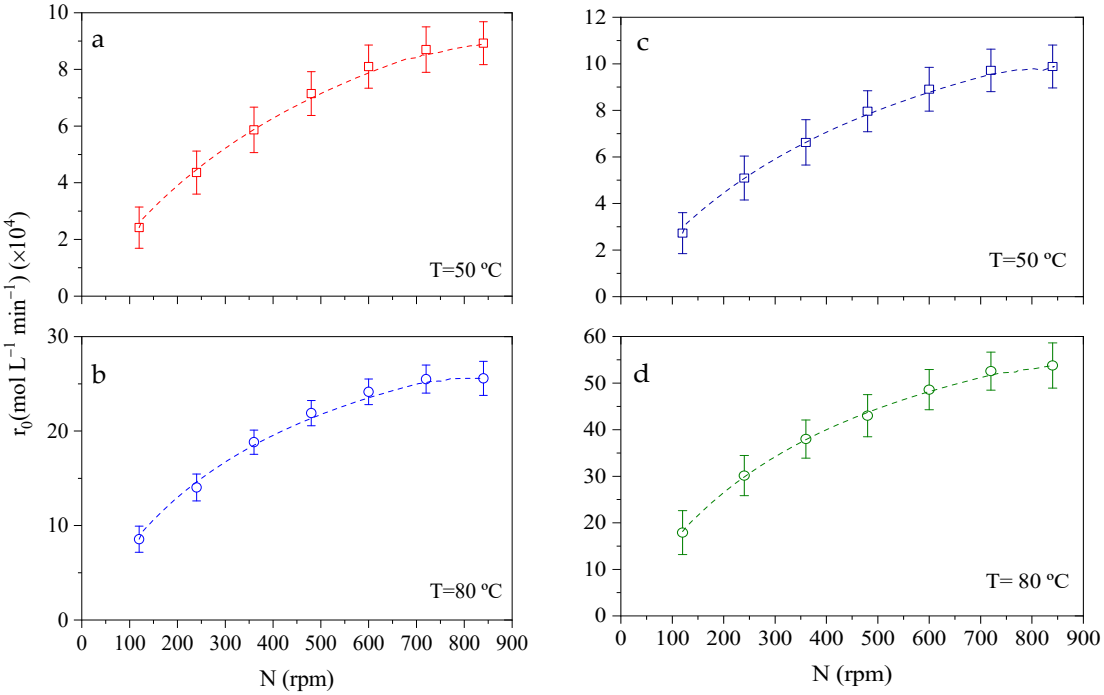

Figure 6. Influence of the stirring speed (mixing) on the initial rate of esterification in a triphasic glycerol-toluene-N435 (L-L-S) system with no added water at zero time: (**a**) 50, (**b**) 80 °C, and with 7.4% v/v of water added initially (**c**,**d**). In both systems for 50 (□) and 80 °C (○) and fixed operational conditions: C_{I0} = 100 g·L^{-1}, C_E = 30 g·L^{-1}.

In Figure 7a, we find that, for an initially low-water system at 50 °C, an increase in stirring speed up to 600 rpm produces an increase in the final yield of ibuprofen monoester (MG); however, with a further increase in stirring speed from 720 to 840 rpm, the final yield of monoester (MG) shows no significant changes, at least above the estimated experimental error. At 80 °C, above 480 rpm, an increase in stirring speed does not lead to noticeable changes in the final monoester yield. Therefore, it can be stated that the possible limitations of external matter transport are not significant above 720 rpm in the small round flask reactor system with a magnetic agitator and the amount of ibuprofen monoester (MG) formed is independent of the stirring speed above this value. Under these conditions, the biochemical esterification reaction that yields the monoglyceride and water is the slowest phenomenon and, therefore, limits or controls the global transformation rate. When a small amount of water is added before running the reaction, we can observe (Figure 7b) an increase in stirring speed up to 600 rpm, leading to an increment to the final yield to monoester of ibuprofen (MG); however, with a further increase in stirring speed from 720 to 800 rpm, the yield to monoester does not show significant changes, at least above the estimated experimental error. Furthermore, the addition of a 7.4% v/v concentration of water leads to an increase in the final MG yield compared with the anhydrous system. Therefore, it can be assumed that possible external matter transport limitations are not significant if stirrings from 720 rpm are used as the amount of ibuprofen monoester (MG) formed is independent of the stirring speed under the described conditions.

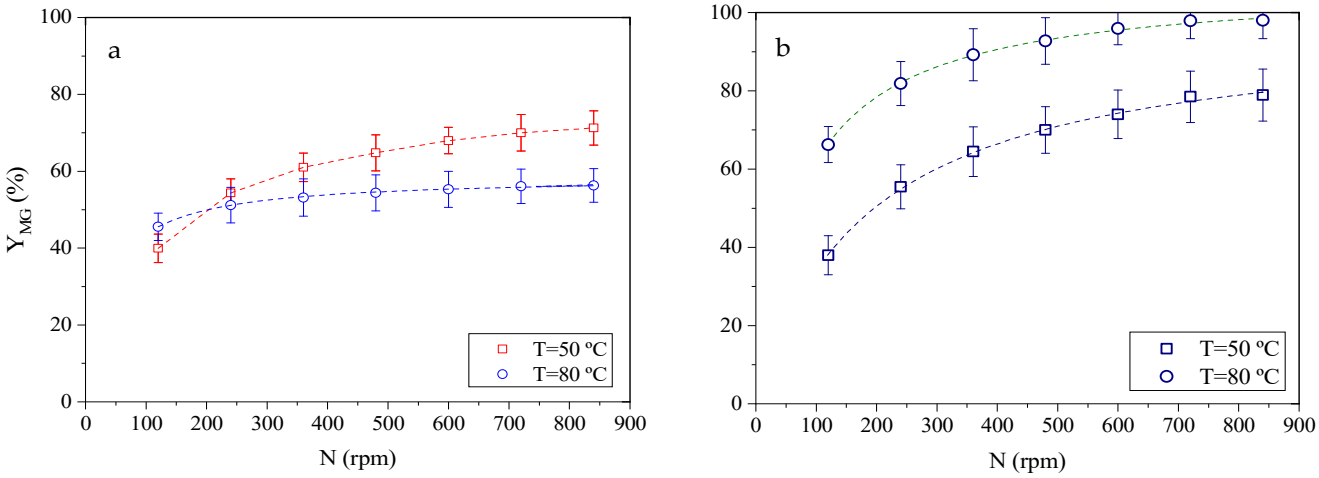

Figure 7. Influence of the stirring speed (mixing) on the yield to ibuprofen monoglyceride in a triphasic glycerol-toluene-N435 (L-L-S) system with no added water at zero time (**a**) and with 7.4% v/v added water **(b)**. Run operational conditions: T = 50 and 80 °C, C_{I0} = 100 g·L^{-1}, C_E = 30 g·L^{-1}.

To corroborate these results, the Mears modulus defined by Equation (1) was applied. The molecular diffusivity (D_{ij}) was calculated using Equation (2), the Wilke–Chang equation (diverse expressions needed for the calculus of parameters contained in these equations can be found elsewhere, as well as the definition of each parameter and the corresponding nomenclature [12]). For both systems, the viscosity of the glycerin–toluene mixture with and without added water was experimentally determined using a concentric cylinder viscometer. Table 1 shows the values of the viscosity of the glycerin–toluene mixture (μ_m) and the molecular diffusivity (D_{ij}). It can be seen that the viscosity of the mixture decreases significantly with increasing temperature, and consequently, the molecular diffusivity values increase with increasing temperature.

Table 1. Molecular diffusivity (D_{ij}) and viscosity (μ_m) values of the glycerin–toluene mixture (without and with 7.4% *v*/*v* added water) estimated over the operating temperature range.

Triphasic L-L-S System with No Added Water		
T(°C)	**$D_{ij} \times 10^{11}$ ($m^2\ s^{-1}$)**	**μ_m ($kg\ m^{-1}\ s^{-1}$)**
50	4.50	0.053
60	8.24	0.030
70	14.8	0.017
80	21.5	0.012
Triphasic L-L-S System with 7.4% *v*/*v* Added Water		
50	6.27	0.038
60	18.3	0.014
70	28.9	0.009
80	36.4	0.007

The Mears criterion states that if Me < 0.15, external matter transfer limitations can be neglected. We can see that for an initial ibuprofen concentration of 100 $g \cdot L^{-1}$, the values of maximal matter transfer flux ($k_L \cdot a \cdot C_{i,0}$) are much higher than the initial esterification rate, and the Mears value is significantly lower than 0.15 (Table 2); therefore, the external transfer limitations are not significant under the aforementioned conditions. Thus, to ensure that the global transformation rate is controlled by the chemical reaction, 720 rpm was chosen as the stirring speed for the subsequent experimentation.

$$Me = \frac{-r_{obs} \cdot d_p \cdot n}{2 \cdot k_L \cdot C_{i,0}} \quad (1)$$

$$D_{ij} = \frac{7.4 \cdot 10^{-8} \cdot (\phi \cdot M_j)^{0.5} \cdot T}{\mu_m \cdot V_i^{0.6}} \quad (2)$$

Table 2. Measured initial reaction rate (r_{obs}), mass transfer flow as a product of the volumetric mass transfer coefficient ($k_L \cdot a$) and the ibuprofen concentration in bulk (C_{I0}) and Mears parameter (Me) of the glycerin–toluene mixture (without and with 7.4% *v*/*v* added water) estimated over the operating temperature range.

Triphasic Systems		**With No Added Water**			**With 7.4% *v*/*v* Added Water**		
T (°C)	**N (rpm)**	**$r_{obs} \times 10^4$ (mol $L^{-1}min^{-1}$)**	**$k_L \cdot a \cdot C_{I0}$ (mol $L^{-1}min^{-1}$)**	**Me ($\times 10^4$)**	**$r_{obs} \times 10^4$ (mol $L^{-1}min^{-1}$)**	**$k_L \cdot a \cdot C_{I0}$ (mol $L^{-1}min^{-1}$)**	**Me ($\times 10^4$)**
50	600	7.79	2.01	1.94	17.7	2.65	3.33
	720	8.76	2.20	1.99	19.5	2.90	3.36
	840	9.08	2.38	1.91	19.8	3.14	3.18
80	600	23.9	7.36	1.62	48.6	11.5	2.12
	720	25.5	8.06	1.58	52.6	12.5	2.10
	840	25.6	8.70	1.47	53.8	13.6	1.98

2.5.2. Internal Mass Transfer

To study the effect of matter transfer in the pores, experiments were carried out by changing the particle size of the biocatalyst used. The rate of the process was experimentally measured at two temperatures (50 and 70 °C) using the following particle size fractions:

0.32–0.5; 0.5–0.7 and 0.7–1 mm. Figure 8 shows the initial esterification rate versus different values of the mean particle diameter (d_p) for the system under study. It can be seen that as the particle size increases, the initial esterification rate decreases significantly; therefore, there must be diffusion problems within the biocatalyst particle. This behavior is almost identical to that observed in the system without toluene as solvent [12]; however, in this system the initial esterification rate is an order of magnitude higher with respect to the initial rate values achieved in the system without the presence of solvent (toluene). This improvement in the initial esterification rate is due to the fact that the presence of solvent in the reaction medium improves the solubility of ibuprofen, and therefore, the transfer of the substrate to the active center of the enzyme is improved.

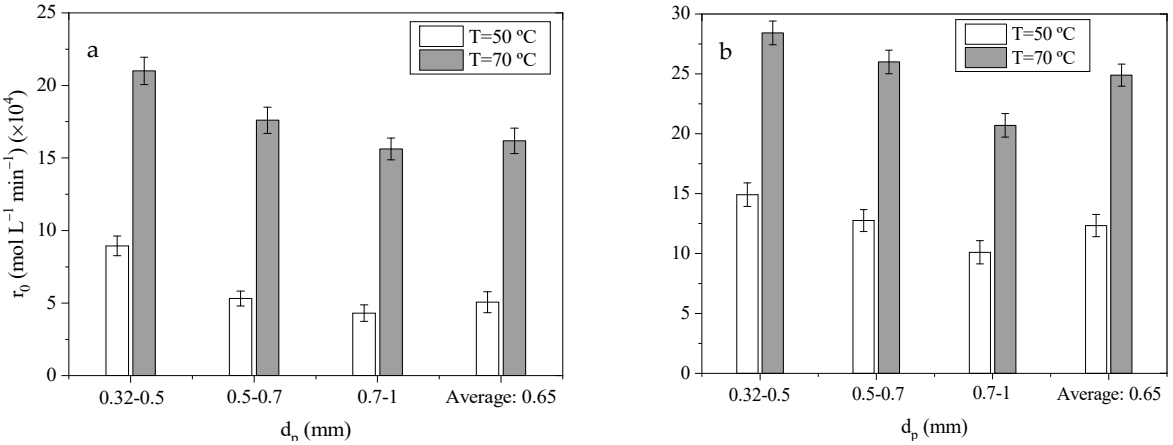

Figure 8. Internal mass transfer in the esterification of ibuprofen in reactive L-L-S triphasic systems without (**a**) and with added water (**b**) at zero time: initial observed reaction rate vs. particle diameter for 50 °C (white) and 70 °C (grey). Other operational conditions: C_{I0} = 60 g·L^{-1}, C_E = 30 g·L^{-1}, N = 720 rpm.

The effectiveness factor (η) is the ratio between the observed global process rate and a rate of reference that is usually the maximum reaction rate at the surface of the particle, that is, the reaction rate for the concentration of ibuprofen in that location. To avoid the calculation of the reference rate, we proposed a system of equations for each fraction considered in Equation (3), and we added to the equation system the mean fraction results through Equation (4) to establish an identical number of equations and unknowns. Table 3 displays the values of effectiveness factor calculated by this strategy for the two triphasic systems studied here: one with no added water at the beginning of the process and the other with a small amount of water added before running the reaction. The values varied between 0.14 and 0.23 for the first system (no added water), indicating that the global rate was 14–23% of the maximum achievable rate, which is a clear sign of internal mass transfer limitation. However, it should be noted that for the triphasic glycerol-ibuprofene-N435 system (thus, with no toluene in the apolar liquid phase), we were using only 8–12% of the maximum rate [12]. Thus, the addition of toluene boosts the internal mass transfer rate. Considering the second half of Table 3 (results for a triphasic system with added toluene and water), the effectiveness factor changes from 40 to 66%, so the addition of water produces a dramatic improvement in internal mass transfer flux, reducing the mass transfer limitations. In addition, the average effectiveness factor values, at 50 and 70 °C, are very close to those for the particle size 0.5–0.7, as expected.

$$\frac{\eta_i}{\eta_j} = \frac{r_{obs,i}}{r_{obs,j}} \qquad i = 1,2;\ j = 2,3,1 \tag{3}$$

$$\overline{\eta} = \sum_{i=1}^{n} x_i \cdot \eta_i \tag{4}$$

where i, j corresponds to each particle size fractions, η is the effectiveness factor of the mean fraction, and x_i is the mass percentage of a given particle size fraction.

Table 3. Values of effective diffusion coefficient (D_{ij}), effectiveness factor (D_e), and Weisz–Prater criterion at 50 and 70 °C.

T (°C)	$D_{ij} \times 10^{11}$ ($m^2\ s^{-1}$)	$D_e \times 10^{13}$ ($m^2\ s^{-1}$)	Size Fraction % *w*/*w*	Size Particle (mm)	$r_{obs} \times 10^4$ Novozym®435 (mol L^{-1} min^{-1})	η	We-Pt
Triphasic glycerol-toluene-N435 (L-L-S) system with no added water at zero time							
50	4.50	6.75	13	0.32–0.5	11.6	0.23	4.0
			52	0.5–0.7	6.92	0.15	5.4
			35	0.7–1	5.65	0.12	8.0
			Average	0.3–1	6.60	0.14	4.5
70	14.80	22.20	13	0.32–0.5	21.0	0.18	2.3
			52	0.5–0.7	17.6	0.15	4.2
			35	0.7–1	15.6	0.13	7.5
			Average	0.3–1	16.2	0.14	4.4
Triphasic glycerol-toluene-N435 (L-L-S) system with 7.4% *v*/*v* added water at zero time							
50	6.27	3.13	13	0.32–0.5	14.9	0.66	1.15
			52	0.5–0.7	12.8	0.57	2.14
			35	0.7–1	10.1	0.45	3.42
			Average	0.3–1	12.3	0.55	2.38
70	28.9	14.5	13	0.32–0.5	28.4	0.45	0.47
			52	0.5–0.7	26.0	0.41	0.94
			35	0.7–1	20.7	0.32	1.52
			Average	0.3–1	24.9	0.40	1.04

As in the case of the external mass transfer, we applied a well-known criterion to appreciate the importance of limitations due to mass transfer inside the pores: the Weisz-Prater criterion [12], through Equation (5), while the effective diffusivity coefficient, D_e, can be given as Equation (6). For the systems here studied, the particle porosity ε was considered to be 0.5, the tortuosity to be 13 and the constriction factor to be 0.4. It is known that the N435 preparation tends to swell when present in organic media. Poojari et al. (2013) have determined the degree of swelling ("swelling") of Novozym®435 in the presence of the following solvents: toluene, diphenyl ether and isooctane; in this study it was observed that the acrylic resin of the support shows considerable swelling with the three solvents used, which it does not present in its contact with glycerin. Furthermore, they conclude that the degree of swelling depends on the type of solvent, temperature and contact period [37].

$$We - Pt = \frac{r_{obs} \cdot d_p^2}{D_e \cdot C_{i,o}} \quad (5)$$

$$D_e = \frac{D_{ij} \cdot \varepsilon_p \cdot \sigma}{\tau} \quad (6)$$

In Equations (5) and (6) above, r_{obs} is the observed or measured rate reaction ($mol \cdot L^{-1} \cdot s^{-1}$), d_P is the diameter of biocatalyst particle (m), $C_{i,0}$ is the bulk concentration of the substrate ($mol \cdot L^{-1}$), D_{ij} is the molecular diffusivity estimated from the Wilke–Chang equation (Equation (2)), ε_p is the porosity of the particle, σ is the constriction factor, and τ is the tortuosity of the particle.

We can see that all values for the Weisz–Prater parameter (We-Pt) are very near or higher than three for the glycerol-toluene+ibuprofene-N435 system with no added water. The calculated values of the We-Pr number are all higher and they slightly increase with

temperature as expected with increasing mass transfer limitations, even if the diffusivity increases with the reduction of viscosity due to a higher temperature. Mass transfer enhancement comes in hand with reaction rate enhancement, so the effectiveness factor only slightly decreases with temperature. This can also be observed for the triphasic system with added water, but the Weisz–Prater parameter is clearly lower than three. As a result, the values of We-Pt > 3 and effectiveness factor η < 1 suggest that mass transfer limitations are significant in the system without water at zero time, while the higher values for η and lower values for the Weisz–Prater criterion indicate a certain overcoming of mass transfer limitations due to water, in particular when using the smaller size particle fraction of Novozym®435. As expected, the mass transfer limitations are higher as the temperature rises. This is due to the effect of temperature on the physical phenomenon, the mass transfer, and the chemical reaction. Mass transfer is usually explained by the mass transfer coefficient, which slightly increases with temperature (apparent activation energies of 2–8 kJ mol^{-1}). The kinetic constant of (bio)chemical reactions depends much more strongly on temperature (20–100 kg mol^{-1}). Thus, at higher temperatures, the biochemical reaction rate increases strongly, whereas the mass transfer flux only accelerates slightly, being in our case, a slow, limiting kinetic phenomenon. As a consequence, the limitation due to mass transfer is more evident at 70 °C than at 50 °C (Table 3).

2.6. Kinetic Modelling

2.6.1. Triphasic L-L-S System Glycerol-toluene(+ibuprofene)-N435

In this system, a total of 12 runs were performed. The operating conditions chosen for the kinetic study were as follows: temperature range of 50 to 80 °C, initial ibuprofen concentration of 20 to 100 $g{\cdot}L^{-1}$, enzyme concentration of 30 $g{\cdot}L^{-1}$, stirring speed of 720 rpm and no addition of water (water concentration of 0% v/v at zero time). The relevant experimental data is given in the Supplementary Material (Tables S1–S5).

Four kinetic models have been proposed to describe the experimental results of the enzymatic esterification of glycerol and ibuprofen, all based in the ping-pong mechanism explained in detail elsewhere [12]. In order to select the most appropriate one, these proposed models were fitted to the obtained experimental data, comparing and discriminating between them according to relevant physical and statistical criteria: positive values of the kinetic constants, reasonable orders of magnitude for their activation energies, good values for the goodness-of-fit parameters (as indicated in Section 3.2.3. in Materials and Methods) and narrow error intervals for the kinetic constant values. The enzymatic esterification reaction of glycerol with ibuprofen catalyzed by the preparation N435 in the presence of a cosolvent (toluene) was carried out with an excess of glycerol with respect to the initial concentration of ibuprofen ($C_{glycerol} >> C_{ibuprofen}$). In this system, in principle, the direct reaction, esterification, and the reverse, hydrolysis, must be considered. Furthermore, it is assumed that the concentration of the products of the esterification reaction are equal ($C_{water} = C_{ester}$), leading to an expression very close to the typical Michaelis–Menten expression for a substrate and a product, as represented by Equation (7). We have observed that, for this system, the effectiveness factor (η) considered in the kinetic models is approximately 0.14, corresponding to the average value calculated for the commercial preparation formed by different particle size fractions. This parameter is contained in the values for the kinetic constants in the numerator of the models:

$$r = \frac{k'_1 \cdot C_{acid} - k'_2 \cdot C_{ester}^2}{1 + K_{acid} \cdot C_{acid} + K_{ester} \cdot C_{ester}} \quad (7)$$

where k'_1 is the kinetic constant of the direct reaction (min^{-1}), k'_2 is the kinetic constant of the reverse reaction ($L{\cdot}mol^{-1}{\cdot}min^{-1}$), K_{acid} is the adsorption constant for the acid species ($mol{\cdot}L^{-1}$) and K_{ester} is the adsorption constant for the monoglyceride species ($mol{\cdot}L^{-1}$). In this case, using the immobilized form of CALB (N435) as described in the previous experiments section, the enzyme seems to be deactivated by the effect of acid concentration (ibuprofen) and temperature. Therefore, for some models, a mechanism of total irreversible

deactivation is proposed, as has been suggested in a previous paper [12], leading to first-order residual activity loss, usual in enzyme deactivation kinetics. The kinetic equations of the proposed models are in Table 4, and the models can be described as follows:

Table 4. Kinetic equations corresponding to the proposed kinetic models to fit to the triphasic glycerol-toluene (+ibuprofen)-N435 data (fitting each model to all retrieved data).

Model	Kinetic Equation(s)
1	$r = \frac{k_1 \cdot C_{E0} \cdot C_I - k_2 \cdot C_{E0} \cdot C_{MG}^2}{1 + K_I \cdot C_I + K_{MG} \cdot C_{MG}}$
2	$r = \frac{k_1 \cdot C_{E0} \cdot a_R \cdot C_I - k_2 \cdot C_{E0} \cdot a_R \cdot C_{MG}^2}{1 + K_I \cdot C_I + K_{MG} \cdot C_{MG}}$ $-\frac{da_R}{dt} = k_d \cdot a_R \cdot C_I$
3	$r = \frac{k_1 \cdot C_{E0} \cdot a_R \cdot C_I}{1 + K_I \cdot C_I + K_{MG} \cdot C_{MG}}$ $-\frac{da_R}{dt} = k_d \cdot a_R \cdot C_I$
4	$r = \frac{k_1 \cdot C_{E0} \cdot a_R \cdot C_I}{1 + K_I \cdot C_I + K_{MG} \cdot C_{MG}}$ $-\frac{da_R}{dt} = k_{dT} \cdot a_R + k_d \cdot a_R \cdot C_I$

Model 1: This is a reversible hyperbolic pseudo-first-order model with respect to the total enzyme activity (C_{E0}) and the ibuprofen concentration (C_I), and of order two with respect to the monoglyceride concentration (C_{MG}).

Model 2: This model is similar to the previous one, with the difference that it considers a pseudo-first-order reaction with respect to the ibuprofen concentration and, in addition, it assumes an irreversible first-order deactivation mechanism of the lipase, expressed as residual activity (a_R); it also implies that the influence of the ibuprofen concentration and the remaining activity are linear, i.e., of order 1.

Model 3: This model, similar to the previous one, takes into account the reversibility of the esterification reaction and also a total irreversible deactivation.

Model 4: This model is similar to model 3, but adds a deactivation of the lipase enzyme due to the action of two effects: ibuprofen concentration and temperature, the rate of deactivation by temperature being of order 1.

Table 5 shows the parameters for each of the proposed kinetic models, in this case for the anhydrous system with toluene as cosolvent and N435, the immobilized form of CALB. In the selection and discrimination of the proposed models, the first physical criterion considered is the range of values of the activation energies (E_a) of the kinetic constants. Considering models 1, 2 and 3, in the hydrolysis reaction (reverse), the kinetic constants have an activation energy of 177.32; 10.77 and 189.03 kJ·mol^{-1}, respectively. As for the values of the kinetic constants of the direct reaction, models 1, 2, 3 and 4 have activation energies between 31 and 34 kJ·mol^{-1}. Thus, all the proposed models have activation energy values of the hydrolysis reaction that are within the range of 2–200 kJ·mol^{-1}, normally accepted as valid in kinetic models for chemical reactions. Another criterion applied was the positive sign in all the adsorption or equilibrium constants, which was achieved for all the proposed models, as can be seen in Table 5. Therefore, the discrimination of the models has to be carried out according to the statistical or fitting criteria, since the physical criteria are fulfilled in all the models. However, the first statistical criterion considered is the absolute value of the standard error (displayed in Table 6), which remains low for all the kinetic and thermodynamic constants of the proposed models, so that at 95 % confidence, the zero value is not included in the confidence interval of any constant, since these intervals are narrow. In addition, the p parameter for all the proposed models is low ($p < 0.0001$; this criterion has also been used in the case of the preparation Novozym®435 [12].

Table 5. Parameter values, with standard error, for each of the proposed kinetic models of glycerol esterification with ibuprofen for the anhydrous L-L-S system with toluene as apolar cosolvent. The selected kinetic model is highlighted in bold font.

Model	Kinetic Parameter	Value	Standard Error
1	$\ln k'_{10}$	16.67	4.25
	$Ea_{k'1}/R$	3884	162
	$\ln k'_{20}$	96.88	21.51
	$Ea_{k'2}/R$	21,329	2175
	K_I	5.06	0.51
	K_{MG}	54.87	6.20
2	$\mathbf{\ln k'_{10}}$	**4.57**	**0.28**
	$\mathbf{Ea_{k'1}/R}$	**3888**	**93**
	$\mathbf{\ln k'_{20}}$	**−3.94**	**1.91**
	$\mathbf{Ea_{k'2}/R}$	**1296**	**637**
	$\mathbf{\ln k_{d0}}$	**72.86**	**7.90**
	$\mathbf{Ea_{kd}/R}$	**27,207**	**2795**
	$\mathbf{K_I}$	**9.90**	**0.89**
	$\mathbf{K_{MG}}$	**44.36**	**3.90**
3	$\ln k'_{10}$	8.91	1.34
	$Ea_{k'1}/R$	4124	110
	$\ln k_{d0}$	71.40	8.64
	Ea_{kd}/R	22,738	1655
	K_I	5.45	0.32
	K_{MG}	60.20	4.67
4	$\ln k'_{10}$	4.00	0.31
	$Ea_{k'1}/R$	3754	103
	$\ln k_{d0}$	104.89	21.86
	Ea_{kd}/R	38,591	7730
	$\ln k_{dT0}$	0.29	1.42
	Eak_{dT}/R	2406	475
	K_I	8.46	0.60
	K_{MG}	29.48	2.85

Table 6. Goodness-of-fit statistical data at 95% confidence for the proposed kinetic models to fit to the triphasic glycerol-toluene(+ibuprofen)-N435 data (fitting each model to all retrieved data). In bold font, data related to the selected model: model 2.

Model	F-Fisher	N_{dexp}	K	SQR	N/K	AICc	BIC	RMSE	VE(%)
1	12,578	300	6	0.481	50	−1918	−1930	0.040	98.26
2	**38,313**	**300**	**8**	**0.118**	**37.5**	**−2335**	**−2351**	**0.020**	**99.57**
3	29,399	300	6	0.211	50	−2165	−2177	0.027	99.24
4	36,180	300	8	0.127	37.5	−2314	−2331	0.205	99.54

As for other statistical discrimination criteria (see Table 6), the lowest values of the corrected Akaike information criterion (AICC) and Bayesian information criterion (BIC) are those of Model 2. The highest F value is also from Model 2, and the percentage of variation explained (%VE) of this kinetic model is also the highest, although similar to that of Model 4. To decide whether Model 2 or 4 is the most convenient, it is interesting to remember that total conversion of ibuprofen into its monoglyceride was not achieved in a triphasic system without toluene as a cosolvent [12]. This fact, in addition to the statistical evidence, suggests the higher adequacy of Model 2, expressed as:

$$r = \frac{\exp\left((4.57 \pm 0.28) - \frac{3888 \pm 93}{T}\right) \cdot C_{E0} \cdot a_R \cdot C_I - \exp\left((-3.94 \pm 1.91) - \frac{1296 \pm 637}{T}\right) \cdot C_{E0} \cdot a_R \cdot C_{MG}^2}{1 + (9.90 \pm 0.89) \cdot C_I + (44.36 \pm 3.90) \cdot C_{MG}} \quad (8)$$

$$-\frac{da_R}{dt} = \exp\left((72.86 \pm 7.90) - \frac{27207 \pm 2795}{T}\right) \cdot C_I \quad (9)$$

We can see in Figure 9, the reproduction provided by model 2 of the experimental data for all the initial ibuprofen concentrations and all the temperatures studied for the anhydrous system or without added water at the beginning of the reactive process. As can be seen, the fits are very good for all the ibuprofen concentrations and temperatures established, although small deviations are observed for an ibuprofen concentration of 60 g·L^{-1}, at times below 400 min, both at T = 60 °C and at T = 80 °C, and at longer times above 800 min.

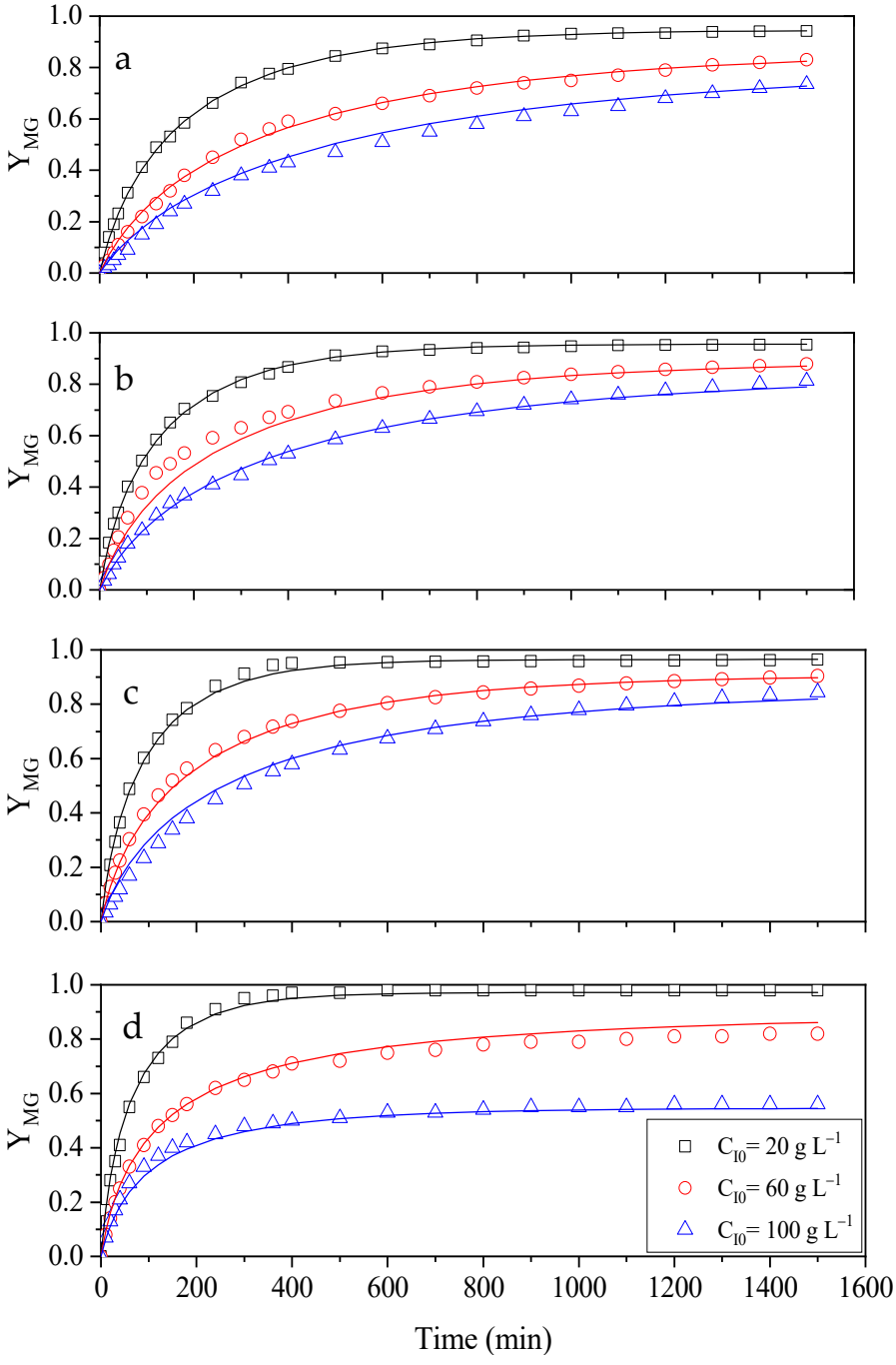

Figure 9. Esterification of ibuprofen and glycerol in reactive L-L-S triphasic systems without water at zero time: Model 2 multivariable fit to all relevant kinetic data: (**a**) 50 °C, (**b**) 60 °C, (**c**) 70 °C and (**d**) 80 °C.

2.6.2. Triphasic L-L-S System Glycerol(+water)-toluene(+ibuprofene)-N435

In this system, 7.4% v/v of water was added to the system before performing the reaction (that is, before adding the biocatalyst N435). A total of 12 runs were carried out, choosing the following conditions for the kinetic study runs: temperature range of 50 to 80 °C, initial ibuprofen concentration of 20 to 100 g·L^{-1}, enzyme concentration of 30 g·L^{-1}, and a stirring speed of 720 rpm to avoid any interference from external mass transfer. The relevant experimental data is given in the Supplementary Material (Tables S6–S10).

Regarding the proposed kinetic models, in this system we can assume that the glycerol and the water concentrations are much higher (in excess) than the concentrations of ibuprofen and monoglyceride ($C_{ibuprofeno} >> C_{ester}$ y $C_{agua} >> C_{ester}$). Therefore, the proposed models should contain a kinetic equation for the esterification reaction and its reverse hydrolysis reaction according to:

$$r = \frac{k_1 \cdot C_{acid} - k_2 \cdot C_{ester}}{1 + K_{acid} \cdot C_{acid} + K_{ester} \cdot C_{ester}} \quad (10)$$

Thus, we can assume that the key species in the active site of the enzyme will be the acid and the ester. As in this case, the yields to ibuprofen monoglyceride reach very high values (almost 100% at 80 °C), deactivation can be present or not. In particular, data at the highest temperature do not lead to the proposal of any deactivation due to the effect of ibuprofen as a deactivation agent. Therefore, the kinetic models proposed (as shown in Table 7) were as follows:

Table 7. Kinetic equations corresponding to the proposed kinetic models to fit all data from the triphasic L-L-S system composed of glycerol(+water)-toluene(+ibuprofen)-N435.

Model	Kinetic Equation(s)
5	$r = \frac{k_1 \cdot C_{E0} \cdot C_I - k_2 \cdot C_{E0} \cdot C_{MG}}{1 + K_I \cdot C_I + K_{MG} \cdot C_{MG}}$
6	$r = \frac{k_1 \cdot C_{E0} \cdot a_R \cdot C_I - k_2 \cdot C_{E0} \cdot a_R \cdot C_{MG}}{1 + K_I \cdot C_I + K_{MG} \cdot C_{MG}}$ $-\frac{da_R}{dt} = k_d \cdot a_R \cdot C_I$
7	$r = \frac{k_1 \cdot C_{E0} \cdot a_R \cdot C_I}{1 + K_I \cdot C_I + K_{MG} \cdot C_{MG}}$
8	$r = \frac{k_1 \cdot C_{E0} \cdot a_R \cdot C_I}{1 + K_I \cdot C_I + K_{MG} \cdot C_{MG}}$ $-\frac{da_R}{dt} = k_{dT} \cdot a_R + k_d \cdot a_R \cdot C_I$

Model 5: Pseudo-first-order reversible hyperbolic model with respect to ibuprofen concentration and monoester concentration without lipase deactivation due to the effect of ibuprofen concentration and temperature.

Model 6: Pseudo-first-order hyperbolic model with respect to ibuprofen concentration and monoester concentration; it also assumes irreversible deactivation of the enzyme, being of first order with respect to ibuprofen concentration and remaining activity.

Model 7: Model similar to model 3 in the previous L-L-S system (no added water), but does not consider any enzyme deactivation.

Model 8: Model identical to model 4 in the previous L-L-S system (no added water).

As in the previous subsection, the NL2SOLV algorithm for non-linear regression coupled to a variable interval Gauss algorithm for the numerical integration of the differential equations in each model were applied to fit the kinetic models to the experimental data. Table 8 shows the kinetic constants and their errors calculated for each of the kinetic models proposed for this system with water addition. Following the same methodology as in the model selection and discrimination used previously, the first physical criterion considered is the range of values of the activation energies (E_a) of the kinetic constants, shown in the mentioned table. In this system with water addition, the kinetic constants of the direct reaction have an activation energy between 39.6 and 43.2 kJ·mol^{-1}; however, for the hydrolysis reaction, the values of the kinetic constants have an activation energy of 14.0 kJ·mol^{-1}. As

in the case of the anhydrous system, the kinetic constants of the proposed models have activation energy values that are within the range of 2–200 kJ·mol^{-1} usually considered acceptable in kinetic models for chemical reactions. Another criterion applied was the positive sign in all the adsorption or equilibrium constants, which was not reached for all the proposed models; as can be observed in Table 8, the values of the constants are positive for models 5 and 6, but in models 7 and 8, the value of K_I takes a negative value; therefore, these last models are not considered in the subsequent discrimination. From this point on, the discrimination of models 5 and 6 has to be performed only on the basis of statistical criteria, since both of them fulfil the physical criteria.

Table 8. Parameter values, with standard error, of the proposed kinetic models of glycerol esterification with ibuprofen for the triphasic L-L-S system with toluene as apolar cosolvent and water as polar cosolvent. The selected model parameters are indicated in bold font.

Model	Kinetic Parameter	Value	Standard Error
5	**$\ln k'_{10}$**	**6.69**	**0.38**
	$Ea_{k'_1}/R$	**4766**	**129**
	$\ln k'_{20}$	**−5.00**	**1.73**
	$Ea_{k'_2}/R$	**1689**	**583**
	K_I	**1.55**	**0.31**
	K_{MG}	**14.70**	**0.98**
6	$\ln k'_{10}$	7.04	0.43
	$Ea_{k'_1}/R$	4889	146
	$\ln k'_{20}$	−5.10	1.75
	$Ea_{k'_2}/R$	1695	589
	$\ln k_{d0}$	225	9097
	Ea_{kd}/R	81,550	3,212,980
	K_I	1.56	0.31
	K_{MG}	14.00	1.10
7	$\ln k'_{10}$	7.35	0.45
	$Ea_{k'_1}/R$	5032	150
	K_I	−0.39	0.32
	K_{MG}	7.35	0.45
8	$\ln k'_{10}$	7.82	0.54
	$Ea_{k'_1}/R$	5194	184
	$\ln k_{d0}$	104	274
	Ea_{kd}/R	38,668	96,710
	K_I	−0.32	0.31
	K_{MG}	18.27	1.66

The first statistical criterion considered is the absolute value of the standard error (Table 9), which remains constant for all the parameters of the proposed models. However, it is observed in model 6 that the standard error for the deactivation constant ($\ln k_{d0}$) and the deactivation activation energy (Ea_{kd}) is very large. In fact it comprises the zero value in the confidence interval; therefore, model 6 cannot be adopted. For a similar reason, the Ea_{kd} parameter in model 8 is also unacceptable. Regarding other discrimination statistical criteria (Table 9), model 5 has the highest value of Fisher's F and model 8 has the lowest values of the Akaike information criterion (AICC) and Bayesian information criterion (BIC). Both models present very similar values of the percentage of variance explained (%VE), and as for RMSE, the value is lower in model 5; however, the value of RSS is lower in model 8.

Table 9. Goodness-of-fit statistical data at 95% confidence for the proposed kinetic models fitted to the L-L-S triphasic glycerol (+water)-toluene (+ibuprofen)-N435 data (fitting each model to all retrieved data). In bold font we observed the statistical parameters for the selected kinetic model.

Model	F-Fisher	N_{dexp}	K	SQR	N/K	AICc	BIC	RMSE	VE(%)
5	**28,318**	**300**	**6**	**0.268**	**−2093**	**−2106**	**0.040**	**99.03**	**28318**
8	21,436	300	8	0.030	−2749	−2765	0.205	99.05	21436

According to the above, the best model of those proposed for the system with water is model 5, which suggests a pseudo-first-order reversible hyperbolic model with respect

to ibuprofen concentration and monoester concentration, without deactivation, which is represented by the equation:

$$r = \frac{\exp\left((6.69 \pm 0.38) - \frac{4766 \pm 129}{T}\right) \times C_{E0} \times C_I - \exp\left((6.69 \pm 0.38) - \frac{4766 \pm 129}{T}\right) \times C_{E0} \times C_{MG}}{1 + (1.55 \pm 0.31) \times C_I + (14.70 \pm 0.98) \times C_{MG}} \quad (11)$$

Figure 10 shows the reproduction of the experimental data by model 5, for the system with added water, at all the concentrations and temperatures studied. As can be seen, with the proposed model the adjustments are quite good, although certain deviations are observed in the interval from 100 to 600 min for an ibuprofen concentration of 20 g·L^{-1} at a temperature of 60 and 70 °C; similarly, for an ibuprofen concentration of 100 g·L^{-1} and temperatures of 50 and 60 °C, the goodness of fit is very good at short times; however, at times longer than 200 min, certain deviations from the experimental data can be observed, although they are always small and lower than the estimated experimental error for both systems (10–15 %).

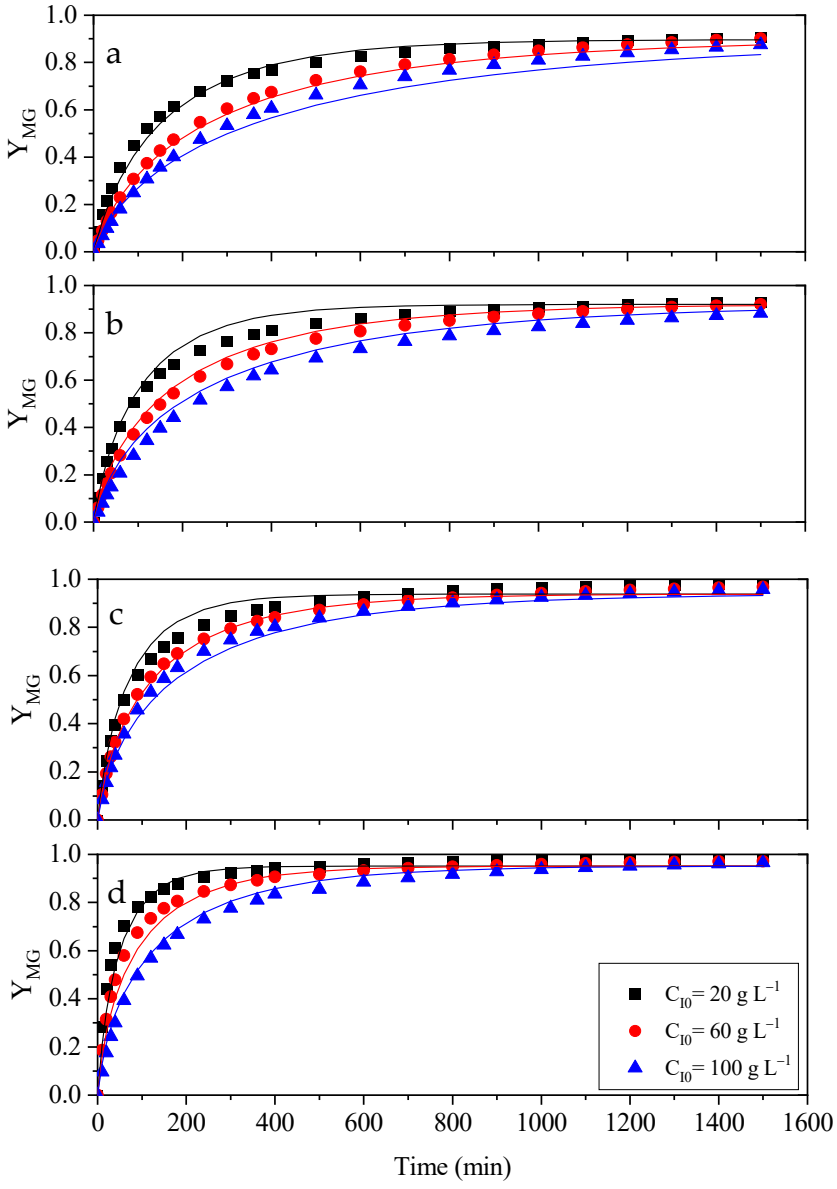

Figure 10. Esterification of ibuprofen in reactive L-L-S triphasic systems with toluene and water at zero time as cosolvents: Model 5 multivariable fit to all relevant kinetic data: (**a**) 50 °C, (**b**) 60 °C, (**c**) 70 °C and (**d**) 80 °C.

Comparing the values of the kinetic parameters obtained in the proposed model in the esterification of glycerol with ibuprofen catalyzed by N435 in the presence of cosolvents for the anhydrous system and with water, it can be observed that, for the system with water (model 5), the activation energies (E_a) of the direct and inverse reaction present an increase with respect to the anhydrous system. Therefore, the addition of water improves the transfer of ibuprofen, which is reflected in the higher value of the effectiveness factor in the presence of water (from 0.1–0.2 to 0.5–0.6). Therefore, we move from a purely matter transfer controlled regime to a mixed control regime. In addition, the values of the activation energies for the kinetic constant of the direct reaction and for the reverse reaction are higher when water is added to the reaction medium. Another effect of the addition of water is the reduced tendency of ibuprofen and monoglyceride to adsorb on the enzyme, apparently implying that their dissolution in the liquid medium (in one liquid phase or the other) is favored by the presence of water. The most important effect, however, is the apparent absence of thermal or chemical deactivation when water is added at the temperature and ibuprofen concentration conditions under which we operate. This can be the reason behind the absence of a deactivation equation in the kinetic model, suggesting that the water stabilizes the enzyme and very high values of conversion to monoglyceride can be achieved even at moderately high temperatures (80 °C).

Now, if we compare the results of the esterification of glycerol with ibuprofen in the presence of a cosolvent (toluene) using the preparation Novozym®435 with the results obtained when using the free enzyme Lipozyme CALB-L [35] (Table 10), we can appreciate that the activation energy values of the kinetic constants with the CALB-L enzyme are higher than with N435, suggesting a total absence of limitation by mass transfer in the case of the free enzyme and a mixed regime, at best, for the immobilized enzyme. Moreover, the addition of water for the immobilized enzyme system results in an increase of the activation energy, approaching the values obtained with the free enzyme; in other words, water addition helps to move from a diffusion-controlled reaction regime to a chemical reaction-controlled regime. This shift towards a chemically-controlled regime reflects also on the activity of N435 in triphasic systems with ibuprofen: when no cosolvent is used [12], the initial reaction rate r_0 is in the range 2–9·10^{-4} mol MG·L^{-1}·min^{-1}. Adding toluene facilitates an apolar liquid phase at all temperatures from 50 to 80 °C and modifies ibuprofen transport into N435 increases r_0 twofold (4–20 mol MG·L^{-1}·min^{-1}). Further addition of 7.4% water to the glycerol phase results in another r_0 increase (12–35 mol MG·L^{-1}·min^{-1}). Thus, this solvent engineering approach increases the practical activity of N435 in this system by four to six times depending on the operation temperature.

Table 10. Comparison of kinetic models and parameters for triphasic systems using toluene as cosolvent in the liquid apolar phase ([35], this work).

Enzyme	Water a t = 0	ln k'_{10}	$Ea_{k'_1}/R$	ln k'_{20}	$Ea_{k'_2}/R$	ln k_{do}	Ea_{kd}/R	K_I	K_{MG}
N435	0% *v*/*v*	4.57	3888	−3.94	1296	72.86	27.21	9.90	44.36
	7.4% *v*/*v*	6.69	4766	−5.00	1689	-	-	1.55	14.70
Lipozyme CALB-L	7.4.% *v*/*v*	11.42	5274	2.30	2468	-	-	0.86	16.92

Moreover, as observed for the systems in the absence of cosolvent, the immobilized enzyme presents a lower specific activity than the free enzyme. Working at enzyme concentrations of 2 g·L^{-1} in the case of the free enzyme [34,35] and 30 g·L^{-1} for the experiments with the immobilized preparation needs to be considered, with specific productivities of 0.486 g MG·g E^{-1}·min^{-1} for Lipozyme®CALB L and 0.0234 g MG·g E^{-1}·min^{-1} for the industrial immobilized Novozym®435. These specific activities were calculated considering that Lipozyme®CALB L has a 0.24% *w*/*w* protein content (2.4 mg of protein per gram of solution) [38], whereas N435, by elemental analysis, contains up to 82 mg of protein per gram of solid [39]. The highest effectiveness factor obtained was 0.66 in the triphasic system with glycerol + 7.4% *w*/*w* water as the liquid polar phase, ibuprofen+toluene as the liquid

apolar phase and N435 as solid biocatalyst, suggesting a 66% effectiveness in comparison with an ideal reference (runs driven at 50 °C). The reaction with immobilized enzyme occurred in the presence of some internal mass transfer limitation so the specific activity ratio N435/Lipozyme®CALB-L was 0.048, or 4.8%. By a simple calculation considering this number and the effectiveness factor value previously mentioned, this comparison suggests that the enzyme retains, upon immobilization and in the absence of mass transfer limitations, only 7.2% of its activity in this particular esterification reaction. Considering that the concentration of protein in the solid is high and most of the protein is at or near the particle surface [40], it is reasonable to think that most active centers are not accessible to the substrate as the protein is immobilized in multilayers. However, using Lipozyme®CALB-L in a previous work, we observed a very stable emulsion (for months) created during the esterification process; in this case, no emulsion was observed, with most of the ibuprofen monoglyceride extracted to the toluene–ibuprofen phase (>90%, according to RP-HPLC analysis of the apolar liquid phase) so a novel apolar phase was generated throughout the process, containing the product, toluene and the remaining ibuprofen.

This fact, together with the very notable stability of the immobilized lipase in the water-containing L-L-S system, suggests that this system can be a suitable approach for a flow or continuous production of the prodrug with N435 contained in a fixed bed and both liquid phases entering well-mixed into such a reactor (for example, by using a static mixer at the reactor inlet). This could consist of a reactor that can be easily coupled to a settling/centrifugation unit to recover the polar and dense glycerol-rich phase and a subsequent distillation unit to treat the light apolar phase, recover toluene in the column head and, depending on the reactor temperature, an almost pure monoglyceride in the tail section of the column. Continuous production of drugs in small-sized factories maintains the flexibility of batch processing while optimizing process time use and safe drug handling and manufacture [41].

However, further studies working under continuous or flowing conditions with the improved multiphasic system addressed here are needed to ensure the absence of enzyme leaching or reductions caused by increased biocatalyst immobilization (for example, by temporal evolution of the enzyme once immobilized). Enzyme leaching is a particular effect that affects Novozym®435 due to the hydrophobic nature of the bonds of the enzyme CALBL and the ion exchanger that serves as support, Lewatit VP OC 1600 [8]. This industrial preparation suffers from enzyme leaching at high temperatures in non-polar solvents, especially when using triglycerides as substrates, even if the fatty acids are short (acetic, butyric), because tri- and diglycerides usually act as biosurfactans, disrupting the hydrophobic bonds that link the enzymes among them and to the support surface [8]. Although in this work, the main phase is very polar (glycerol with a notable percentage of water), long-term in-flow experiments in fixed-bed or basket stirred tank reactors under a diversity of operational conditions should be the subject of further research in view of the process insights reported here.

3. Materials and Methods

3.1. Materials

Novozym®435 (immobilized lipase B from *Candida antarctica* on macroporous polyacrylate Lewatit VPOC 1600) was a kind gift of Novozymes A/S (Bagsværd, Denmark). The ibuprofen sodium salt was purchased from Sigma-Aldrich. Pure ibuprofen was obtained by acid precipitation using HCl 36% *w*/*w* (from Sigma-Aldrich, St. Louis, MO, USA), filtration and drying at 50 °C for 24–48 h till constant weight, as described elsewhere [12,34,35]. We use also extra pure glycerol 99.98% UPS grade and methanol HPLC grade purchased from Fisher Scientific UK Ltd. (Loughborough, UK).

3.2. Methods

3.2.1. Enzymatic Esterification of Ibuprofen Ester

In every run, we mixed a certain mass of ibuprofen (0.5 to 2.5 g) with an adequate volume of toluene, a solvent in which the ibuprofen has a very high solubility. Usually, 5 mL of toluene was used unless otherwise stated. This solution was dispersed in 20 mL of glycerol, creating a liquid–liquid phase that contains both reagents. The mixture, contained in a 50 mL round flask, was agitated with a circular double-cross magnet in the bottom to avoid attrition of the biocatalyst and set on an aluminum jacket for heating with an IKA Yellow Line, model MSC basic C device, thus controlling both controlled temperature and agitation. The run started when the specified amount of biocatalyst was added. During the run, several 250 μL samples were withdrawn and immediately refrigerated to stop the reaction. After thawing, the samples were diluted with 750 μL of methanol 99% and centrifuged to remove any N435 particles. A second dilution step was performed by mixing 100 μL of the diluted sample and with 900 μL of the same methanol. These double-diluted samples (dilution factor = 40) were analyzed by reverse-phase HPLC.

3.2.2. Analytical Methods

We used a RP-HPLC technique developed in a JASCO HPLC to determine the concentrations of ibuprofen and its monoglyceride, separating the cosolvent toluene, as can be observed in the chromatogram shown in the Supplementary Material. It can be seen that the first peak is ibuprofen monoester (t_R = 6.9 min), the second peak corresponds to the solvent (t_R = 7.6 min) and the last peak is ibuprofen (t_R = 8.6 min). The conditions of the method used are presented below:

- Column: Teknokroma "Mediterranea Sea" C-18 column 25 × 0.46 cm d_p 5 μm.
- Mobile phase: Mixture: 83% methanol, 17% acidified water (the eluents used are milli-Q water acidified with sulfuric acid to pH 2.2 and high purity methanol).
- Column temperature: 30 °C.
- Flow rate: 0.8 mL/min.

Diode array detector (DAD) at 220 nm was used to measure the ibuprofen, toluene and the monoglyceride. We added the areas of ibuprofen and its monoglyceride to create an internal standard, as the UV spectra of both compounds are identical; this value was used to correct the percentage areas of the analytes. With the corrected values of the %Area of ibuprofen, its conversion can be easily calculated by:

$$X = \frac{\%Area_0 - \%Area_t}{\%Area_0} \quad (12)$$

3.2.3. Statistical Non-Linear Regression Methods

In the determination of the kinetic parameters of the enzymatic esterification reaction of glycerol with ibuprofen, the fit of the models to the experimental data obtained was carried out by non-linear regression, using the NL2SOLV algorithm coupled to a numerical integration of the kinetic model differential equation (or equations) with a variable step Gauss algorithm. In fact, integral data and monoglyceride yield changes with time were handled using Aspen Custom Modeler v11, which includes subroutines for each algorithm [12,34,35]. The process of discrimination between kinetic models to select the model that best fits the experimental results was out in several stages. First, in the adjustment of the kinetic models, the evaluation of experiments run at a constant temperature was considered. Subsequently, the parameters obtained for each temperature were used to obtain initial values of the neperian logarithms of the pre-exponential factors and activation energies using the linearized form of the Arrhenius equation -see Equation (13)-. The last step consisted of multivariate fitting of the kinetic models including all the experimental data at the same time, with temperature as an independent variable included in the differential equations through Arrhenius-type equations for each kinetic constant. At each

stage of discrimination, several physical and statistical criteria were considered to select the model that best fits the experimental data. The Arrhenius equation is as follows:

$$\ln k_i = \ln k_{i,o} - \frac{E_{a,k}}{R \times T} \tag{13}$$

where k_i stands for the kinetic constant considered, $k_{i,0}$ is the preexponential factor of the Arrhenius equation, R is the constant of ideal gases (8.31 $J \cdot mol^{-1} \cdot K^{-1}$) and T is the absolute temperature (K). For the discrimination of the kinetic models, physical and statistical criteria were considered to select the most representative model. The main physical criterion was to obtain an adequate value for the activation energy, which must always be positive and be greater than 20 $kJ \cdot mol^{-1}$and not greater than 300 $kJ \cdot mol^{-1}$. As for the statistical criteria, the residual root mean squared error (RMSE), Fisher's F value (F), the corrected Akaike information criterion (AICc) and the Bayesian information criterion (BIC) were considered. To check the goodness of fit of the models, F must be high, while RMSE, AICc and BIC have to be very low values. These parameters are defined in the following paragraphs and equations.

The root mean squared error (RMSE) is a measurement of the squared difference between the experimental values of the dependent variable (the monoglyceride yield in this case) and the values calculated with the kinetic model being used for fitting. To reduce overparameterization, this value is the Squared Sum of Residual or Variances (SSR) and is divided by the difference between the experimental data number (N) and the parameter number of the model (K). The ideal value of this goodness-of-fit parameter is zero (exact coincidence between experimental and calculated values). Equation (14) indicates how to calculate RMSE:

$$RMSE = \sqrt{\sum_{i=1}^{N} \frac{(y_e - y_c)_i^2}{(N - K)}} \tag{14}$$

The Fisher's F-value is calculated using Equation (15). This value should be over a critical value of the N number of data and the K number of model parameter at a given confidence value, usually 95%. In our case, this critical value is between 20 and 32. Any F-value over this critical threshold indicates that the model is valid and passes the null hypothesis. Moreover, when comparing different models, the model with a higher F-value is the one with the best fit to the experimental data. Similar to RMSE, F-value is also a goodness-of-fit parameter that takes overparametrization into account.

$$F - Fisher = \frac{\sum_{n=1}^{N} \frac{(y_c)^2}{K}}{\sum_{n=1}^{N} \frac{(y_e - y_c)^2}{N - K}} \tag{15}$$

The AICc and BIC information criteria are goodness-of-fit parameters that also solve the problem of overparameterization by introducing a penalty term in the number of parameters in the model. The correction (AICc), Equation (16), is suitable for a ratio of number of data (N) to number of model parameters (K) less than 40 [12]. The BIC is defined in Equation (17).

$$AICc = N \times Ln\left(\frac{SSR}{N}\right) + 2 \times K + \frac{2 \times K \times (K + 1)}{N - K - 1} \tag{16}$$

$$BIC = Ln\left(\frac{SSR}{N}\right) + \frac{K}{N} Ln(N) \tag{17}$$

The percentage of explained variation, %VE, is given by Equations (18)–(21). The Aspen Custom Modeler program, by default, considers the heteroscedasticity parameter (l) equal to unity [12].

$$\%VE = 100 \times \left(1 + \frac{\sum_{l=1}^{L} SSQ_l}{\sum_{l=1}^{L} SSQpromedio_l}\right) \quad (18)$$

$$SSQ_l = \sum_{i=1}^{N} \frac{\left(y_{i,e} - y_{i,c}\right)^2}{y_{i,c}^{\gamma_l}} \quad (19)$$

$$SSQpromedio_l = \sum_{i=1}^{N} \frac{\left(y_{i,e} - \overline{y}_{i,c}\right)^2}{y_{i,c}^{\gamma_l}} \quad (20)$$

$$y_{i,e} = \frac{\sum_{i=1}^{N} \frac{y_{i,e}}{y_{i,c}^{\gamma_l/2}}}{\sum_{i=1}^{N} \frac{1}{y_{i,c}^{\gamma_l/2}}} \quad (21)$$

4. Conclusions

The enzymatic esterification of ibuprofen and glycerol using immobilized Novozym®435 as a biocatalyst with the use of minor volumes of cosolvents (toluene and, in the second system, water) results in the activation (compared with the system without cosolvents) and stabilization of the biocatalyst. When studying the transfer of matter at the interface in both systems, anhydrous and with water, it was determined that at speeds above 600 rpm, the initial esterification rate does not show significant changes; this was also confirmed by the application of the Mears criterion. The Weisz–Prater criterion indicated the presence of internal mass transfer limitations, but lower than in a system without added cosolvents. The addition of toluene to the ibuprofen phase increased the activity two-fold and, therefore, the effectiveness factor. Further addition of water to the glycerol phase resulted in a two- to three-fold additional increment of these parameters, showing a mixed controlled regime in the best case.

Likewise, toluene addition led to a lower deactivation of N435 compared with the situation without cosolvents [12]. This deactivation, at least for the operating time employed here, is altogether avoided when adding, in addition, water to the glycerol phase. Both situations are clearly explained by the selected kinetic models for each triphasic reacting biosystems, models that are discriminated by applying a non-linear regression algorithm coupled to an ODEs numerical integration algorithm. Each selected model fits very accurately to all the retrieved data for the relevant reaction biosystem, according to several goodness-of-fit parameters.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/catal12121531/s1, Figure S1: example of RP-HPLC chromatogram; Table S1: Kinetic runs performed for the anhydrous triphasic system; Tables S2–S5: Experimental results of the enzymatic esterification of glycerin with ibuprofen immobilized enzyme N435 at T = 50 °C for the anhydrous system (50–80 °C); Table S6: Kinetic runs performed for the hydrated triphasic system; Tables S7–S10: Experimental results of the enzymatic esterification of glycerin with ibuprofen immobilized enzyme N435 at T = 50 °C (and 60, 70, 80 °C) for the system with added water.

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