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Enzymatic synthesis of ibuprofen monoglycerides catalyzed by free *Candida antarctica* lipase B in a toluene–glycerol biphasic medium

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The enzymatic esterification of glycerol and ibuprofen in different organic media to obtain a monoester of ibuprofen was studied in an open and close system, selecting an adequate kinetic model in both cases. The solubility of ibuprofen was tested in various solvents, leading to the selection of toluene as the most appropriate organic medium. Preliminary runs led to fixing the concentration of lipase CALB-L at 2 g L^{-1}, stirring speed at 720 rpm, a water content of 6% v/v and a glycerol to toluene volume ratio of 20/5. Kinetic runs were performed at several ibuprofen initial concentrations (20 to 100 g L^{-1}) and temperatures (50 to 80 °C). Two systems were defined, one of which contemplated continuous removal of water with toluene. Considering kinetic and thermodynamic data, several kinetic models were proposed and fitted to all available data making use of physical and statistical criteria to select the best ones. In the first system, the chosen model was an irreversible hyperbolic model, whereas in the other system, the selected model was a Michaelis–Menten-based reversible model of pseudo-first order with respect to the concentration of ibuprofen and the monoester.

Introduction

At the present time, the production of glycerol as the main by-product of the biodiesel industry is considerably greater than the demand of such products.[1] Due to this overproduction, its price has undergone a very steep decrease in the market. For this reason, glycerol has been widely reported as a building block in a broad variety of processes using inorganic, organic and enzymatic catalysts of homogeneous and heterogeneous nature.[2] Glycerol-(1,2,3-propanetriol) is a highly hygroscopic polyalcohol with very high boiling point and viscosity that offers considerable reactivity at moderate temperature and pressure conditions and, in addition, can be considered of renewable nature due to its biological origin.[3]

Thus far, studies have been published focusing on the synthesis of ibuprofen ester by esterification of glycerol with ibuprofen catalyzed by free *Candida antarctica* lipase B,[4] the production of monobenzoate of glycerol (α-MBG) by esterification of benzoic acid and glycerol using free *Candida antarctica* lipase B[5] and the transesterification of 2-methyl heptanoate catalyzed by immobilized *Candida antarctica* lipase B in glycerol.[2]

In recent years, prodrugs (bioreversible derivates) have been developed to block the carboxylic acid group of non-steroidal anti-inflammatory drugs (NSAIDs) as a way of reducing or avoiding the gastrointestinal (GI) side effects.[6] Esterification of the NSAIDs can be a synthetic strategy to prepare the prodrugs because esters exhibit reasonable chemical stability *in vivo*.[7] Besides, some hydrophilic esters are more soluble and bioavailable than the parental drug.[8] Ibuprofen (2-(4-isobutyilphenyl) propionic acid) belongs to the family of profens and is a widely used NSAID.[9] Ibuprofen esters are obtained by Fischer esterification and lipase-catalyzed esterification.

Lipases are very profusely used biocatalysts as they offer the possibility to operate under mild operating conditions in addition to offering a very broad range of substrate specificities and high stability and selectivity, even in organic media. Lipases from various origins have been studied with promising results in the esterification of NSAID, such as lipase from *Candida rugosa*,[7] *Rhizomucor miehei*,[10] and *Candida antarctica*.[11] The lipase-catalyzed esterification of NSAID has usually been performed in inorganic media, because the thermal stability of the enzymes and the different polarity of organic solvents can improve the enantioselectivity and stability of lipases; besides, the solubility of hydrophobic substrates and the thermodynamic equilibrium can be enhanced by shifting the reaction to the products.[12–15] According to literature, thus far, the enzymatic esterification of NSAID has widely been studied using immobilized lipase in an organic medium. Nevertheless, Ong *et al.*[16] have studied the performance of free *Candida antarctica* lipase B in the esterification of R-ketoprofen in *n*-hexane. In our study, the esterification of glycerol and ibuprofen catalyzed by free *Candida antarctica* lipase B in organic medium is proposed, as depicted in Scheme 1.

In this context, several authors have researched the effect of organic solvents on the performance of lipases; usually, the

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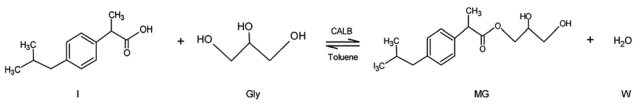


Scheme 1 Esterification reaction between ibuprofen (I) and glycerol (Gly) to yield the corresponding ester (MG) and water (W).

hydrophobicity of solvents and the water content are the most important factors affecting the enhancement of lipases.[17] The hydrophobicity of solvents is expressed by the value log *P* (octanol–water partition coefficient), from whose values it has been determined that the enzymatic activity in organic solvents is low at log $P < 2$, medium between $2 < \log P < 4$ and high at log > 4.[18] In general, the catalytic activity takes place at log $P > 2$. Arroyo *et al.*[19] have obtained high esterification rates of 2-aryl-propionic acid with nonpolar solvents (log $P > 2$). Also, in the synthesis of (*S*)-naproxen hydroxyalkyl ester, the *Candida rugosa* lipase had higher enantioselectivity and activity in cyclohexane ($\log P = 3.2$) and hexane ($\log P = 3.5$).[20] Also, the enzymatic esterification of ibuprofen has been performed employing solvents like cyclohexane,[21] isooctane ($\log P = 4.5$)[11,21] and toluene ($\log P = 2.5$)[21,22], among others.

As mentioned above, water content is one of the most important factors affecting the catalytic activity and stability of the lipase, being some water content necessary to keep the enzyme flexible and hydrated within an organic solvent.[23] However, in esterification reactions, water is a by-product; an excess of water in the reaction medium can eventually increase the available interfacial area of lipase, making it more flexible and facilitating the hydrolysis of the ester. Therefore, the optimal presence of a certain water content required will depend on the type of lipase, the organic solvent employed and the immobilized support.[24] Various methods have been used extensively in solventless systems to remove water, such as adding molecular sieves, salt hydrates or pervaporation.[5] Gubicza *et al.* have used azeotropic distillation for water removal in the synthesis of ethyl acetate from ethanol and acetic acid catalyzed by lipase Novozym 435 in hexane: a strategy to intensify the process and increase productivity by equilibrium shift.[25]

According to literature, ping–pong bi–bi or double displacement mechanism is widely accepted for describing esterification reactions catalyzed by lipases. Duan *et al.*[26] have proposed this mechanism featuring the dead-end inhibition of alcohol in the esterification of ketoprofen with propanol catalyzed by Novozym 435 inorganic medium. In a previous work,[4] we have considered ping–pong bi bi mechanism in the esterification of glycerol with ibuprofen using free *Candida antarctica* lipase B (CALB) in solventless medium, yet simplifying this complex mechanism to a reversible uni–uni kinetic model due to the presence a large excess of glycerol and water. Therefore, our study aims at performing the esterification of ibuprofen with glycerol catalyzed by the same CALB industrial preparation as described elsewhere,[4] though using an organic solvent as reaction medium. The reaction was carried out in a batch reactor to elucidate the effect of operational conditions like temperature, reagents, enzyme load, stirring speed and water concentration on the chemical equilibrium and, at the selected conditions, a solvent selection study is made. Several kinetic models have been proposed to discriminate the most adequate one on the basis of physical and statistical criteria. Finally, these models were fitted to all the experimental data obtained from the two systems considered, hence evaluating the goodness of fit.

Results and discussion

Preliminary experiments

Solubility of ibuprofen in different solvents. The solubility of ibuprofen was determined in different solvents for a fixed amount of ibuprofen at room temperature and atmospheric pressure. As can be seen in Fig. 1, among all the solvents tested, the solubility of ibuprofen was higher in 2-butanone, toluene and xylene compared to other solvents, reaching 0.25, 0.37 and 0.28 g ibuprofen per g solvent, respectively. 2-Butanone was dismissed because its boiling point (79.4 °C) is very close to the maximum temperature fixed in the esterification of glycerol and ibuprofen (80 °C). Toluene and xylene can be used as organic media due to their partition coefficient log *P* being 2.50 (toluene) and 3.15 (xylene). In order to choose between them, the esterification of glycerol and ibuprofen catalyzed by free CALB-L with toluene or xylene was carried out; the conversion to the monoester of ibuprofen with both solvents proved almost identical at low times, though beyond 300 min, the conversion of monoester is slightly larger in toluene than in xylene (data no shown). For this reason, toluene was chosen as the organic medium for further experiments. In addition, toluene had previously been employed as the solvent in esterification of profens using free and immobilized CALB; for instance, esterification of naproxen[28] and flurbiprofen[29] with propanol in this solvent catalyzed by immobilized enzyme Novozym 435 was achieved reaching conversion values of 34% for naproxen and 20% for flurbiprofen. Also, the synthesis of different esters, polyesters and polycaprolactones has been reported making use of toluene as an ideal medium for this purpose.[30]

After selection of the organic medium, the solubility of ibuprofen in toluene was determined at temperatures ranging from 50 to 80 °C. Fig. 2b shows that the solubility of ibuprofen in

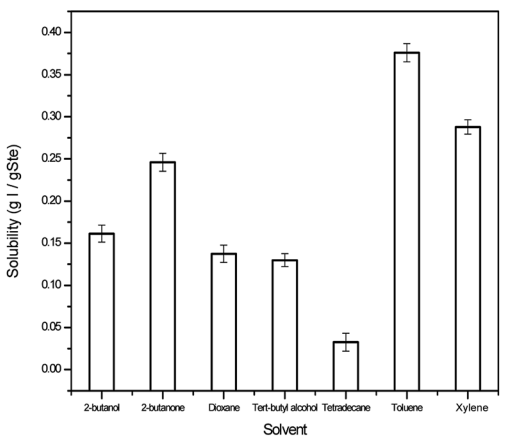


Fig. 1 Solubility of ibuprofen in various solvents at room temperature and pressure ($n = 3$).

toluene increases linearly with temperature, reaching the value of 2.56 g I per g Tol at 80 °C. Besides, the partition ratio of ibuprofen (K_p) between two immiscible solvents (toluene and glycerol) was calculated using eqn (2). Fig. 2c shows a decreasing value of K_p as temperature escalates from 50 to 80 °C, which indicates that the ibuprofen achieves a higher concentration in the toluene-rich phase than in the glycerin-rich phase at this temperature. In a previous paper,[4] we have determined the solubility of ibuprofen in glycerol, which proves to increase exponentially with temperature, as can be seen in Fig. 2a.

On the other hand, it was observed that the yield of ibuprofen monoglyceride increased alongside with the ratio Gly/Tol (data not shown). In this way, when there is a lower amount of toluene (5 mL) and 20 mL of glycerol, CALB does not suffer from low activity, because the aqueous phase formed by glycerol and the components present in the enzyme preparation can form a layer around the enzyme particles. Therefore, the direct contact between the enzyme and the organic phase can be reduced, an effect which has previously been described in the literature.[31] In addition, *C. antarctica* lipase B is widely known as a very stable enzyme in organic solvents.[32] Thus, experiments were set at volume ratio of 20/5.

Effect of enzyme concentration. The effect of enzyme concentration on the initial reaction rate of esterification of glycerol and ibuprofen was tested between 0.7 and 2.7 g L^{-1}. This rate was calculated by fitting interpolation functions to data at several reaction times and deriving such functions at zero time. As observed in Fig. 3, the initial reaction rate increased with the amount of enzyme following a hyperbolic trend, as observed in a previous paper.[4] Given the fact that the higher the enzyme concentration the more active sites for substrate binding are available, it is inferred that the reaction rate increases at such concentrations. However, little improvement is observed at values exceeding 2 g L^{-1}, so this value was selected as the enzyme concentration for further runs.

Effect of stirring speed. The effect of the stirring speed in the esterification of glycerol with ibuprofen in organic media was studied by varying the stirring speed between 120 and 960 rpm. In this way, the possibility of mass transfer limitation in the biphasic reaction system can be observed and determined. As shown in Fig. 4a, the initial rate of esterification increases with stirring speed. At low speeds, however, the contact between the phases is very poor due to the immiscibility of toluene with the glycerol and water present in the reaction medium. This contact increases to a certain extent when incrementing the stirring speed up to values of 700–800 rpm, yet no significant changes are observed in the initial rate of esterification. On the other hand, as can be seen in Fig. 4b, which illustrates the intensification of stirring speed from 120 to 920 rpm, the highest values of the final yield to MG was achieved from 720 rpm. Additional augmentation of stirring speed beyond 720 rpm did not show any change of the final yield to monoester, which in turn confirms that an optimum stirring speed is reached at which mass transfer limitations can be neglected. Therefore, the value of 720 rpm was chosen for subsequent experimental runs.

Effect of temperature and initial concentration of ibuprofen. In this study, several experiments were carried out in an open system varying temperature from 50 to 80 °C and the initial concentration of ibuprofen between 20 and 100 g L^{-1}. Enzyme concentration (2 g L^{-1}), volume ratio (Gly/Tol = 20/5) and water content (C_{WO} = 6.4% v/v) were kept constant in all cases. Fig. 5 shows the initial rate of esterification *versus* the initial ibuprofen concentration; as can be seen in this graph, at any temperature considered, the initial rate increased with the initial ibuprofen concentration, being this effect more notorious at higher substrate concentrations. This behavior was also observed in the esterification of glycerol and ibuprofen

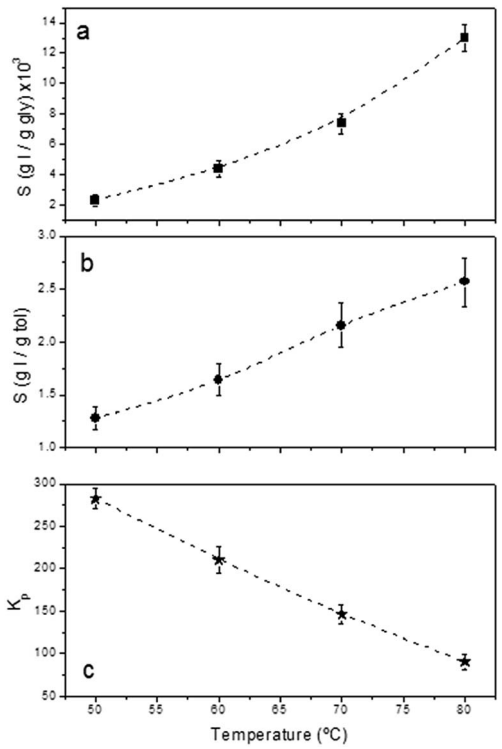


Fig. 2 Solubility of ibuprofen in (a) glycerol, (b) toluene and (c) partition ratio I–Tol–Gly (n = 3).

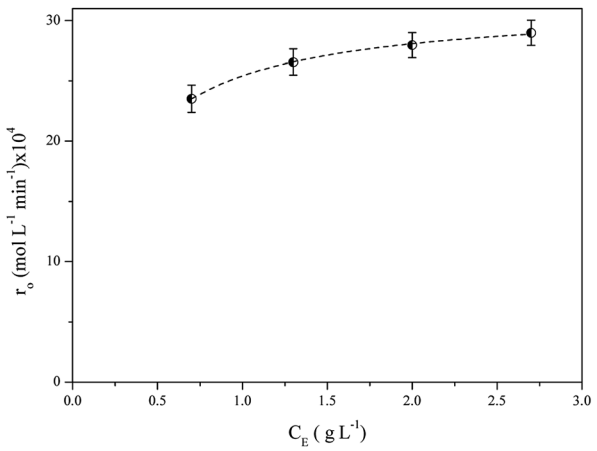


Fig. 3 Effect of enzyme concentration on the enzymatic esterification of glycerol and ibuprofen. Reaction conditions: T = 80 °C, C_{I0} = 100 g L^{-1}, C_{WO} = 6% v/v, volume ratio Gly/Tol = 20/5, N = 720 rpm (n = 3).

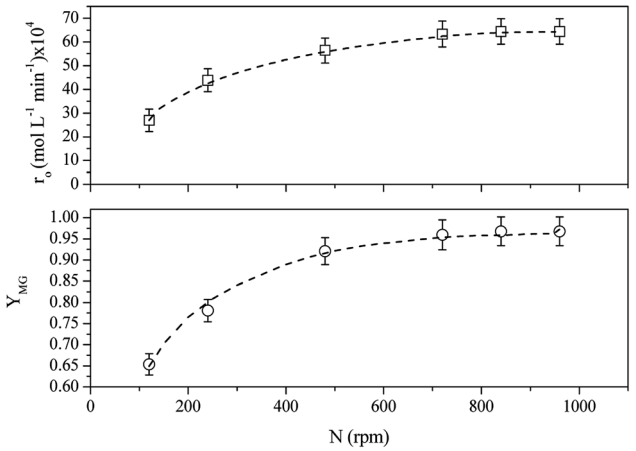


Fig. 4 Effect of stirring speed on the enzymatic esterification of glycerol and ibuprofen. Reaction conditions: $T = 80$ °C, $C_{I0} = 100$ g L^{-1}, $C_E = 2$ g L^{-1}, $C_{WO} = 6\%$ v/v, volume ratio Gly/Tol = 20/5 ($n = 3$).

catalyzed by CALB-L in a solventless medium.[4] In addition, no fast deactivation was observed in any case, not even at high temperature and acid concentrations. However, Poojari *et al.* have studied the thermal stability of the enzyme free CALB-L in toluene between 40 up to 100 °C, finding that the maximum activity for esterification of 1-octanol and lauric acid took place at 40 °C, above which a drop in the relative activity was observed with an increase of temperature up to 90 °C, at which the free CALB showed no catalytic activity in toluene.[30] In our case, as previously remarked, the presence of glycerol in the reaction medium is able to maintain the hydration of enzyme at high temperature and thus, improve the performance of esterification. In this way, Kobayashi *et al.* have studied the thermal stability of immobilized lipase CALB (Chirazyme L-2) in glycerol with added water at 80–100 °C, so the immobilized of CALB at 80 °C is stable using glycerol in the reaction medium.[33]

Effect of water content. The presence of water is very important for keeping the enzyme active because in this way it is more hydrated and flexible. The effect of the addition of water in the esterification of glycerol and ibuprofen was studied within the range from 6 to 13% v/v. Runs were performed in the presence of certain amount of water in all cases considering that the enzymatic preparation solvents include glycerol, sorbitol and water, as shown in the Materials section, being important the volume water added in each run. As can be observed in Fig. 6, the addition of large amounts of water at zero time decreased significantly the initial reaction rate. The presence of hydrophobic solvent in the reaction medium could allow the accumulation of water near the active site of enzyme, around the polar amino acids; therefore, addition of an amount of water could increase the size of interfacial area of reaction and facilitate the hydrolysis of the monoester, whereby its production would be reduced.[34,35] Following these results, the value of water content was selected at $C_{WO} = 6\%$ v/v for further experiments.

Equilibrium conversion

In order to achieve equilibrium conditions in the esterification reaction, kinetic runs performed at temperatures from 50 to 80 °C and all initial concentration of ibuprofen were left to proceed for 72 hour, in open and closed reacting systems, withdrawing several samples by that time and analyzing them by HPLC. Fig. 7b indicates that, including experimental error, total conversion was not reached in the esterification of glycerol and ibuprofen for the closed system, with the conversion values reaching final average values at equilibrium between 0.6 and 0.78. With the open system (Fig. 7a), much higher conversions at equilibrium were obtained; it is remarkable that total conversion is achievable at all temperatures tested if error intervals are taken into account. This equilibrium shift is due to water withdrawal by co-evaporation with toluene.

Proposal of kinetic models

The esterification of glycerol with ibuprofen catalyzed by CALB involves two reactants (substrates) and two products. In this

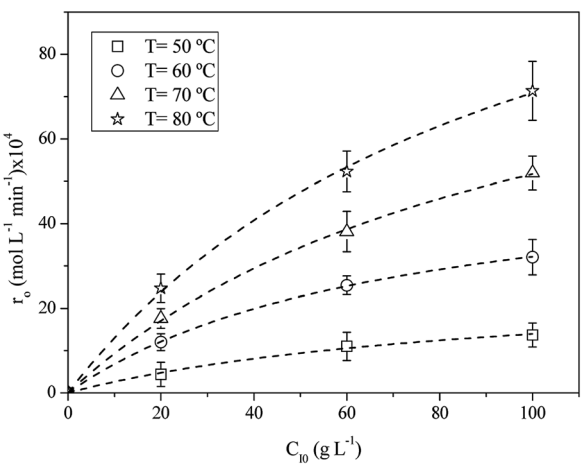


Fig. 5 Effect of temperature and initial concentration of ibuprofen on the initial reaction rate. $C_{WO} = 6\%$ v/v, volume ratio Gly/Tol = 20/5, $N = 720$ rpm ($n = 3$).

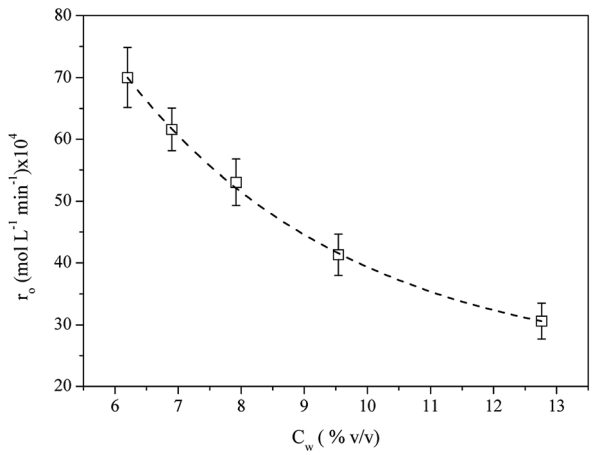


Fig. 6 Effect of stirring speed on the enzymatic esterification of glycerol and ibuprofen. Reaction conditions: $T = 80$ °C, $C_{I0} = 100$ g L^{-1}, $C_E = 2$ g L^{-1}, $C_{WO} = 6\%$ v/v, volume ratio Gly/Tol = 20/5 ($n = 3$).

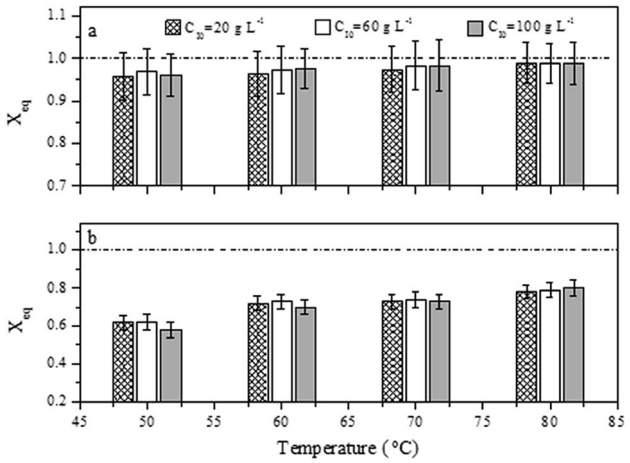


Fig. 7 Equilibrium conversion at 72 h, for the enzymatic esterification of glycerol and ibuprofen at all temperatures and ibuprofen initial concentrations tested (a) open system (system A) and (b) closed system (system B). C_E = 2 g L^{-1}, volume ratio Gly/Tol = 20/5 and N = 720 rpm (n = 3).

way, ping–pong a bi–bi mechanism has been proposed by lipase-catalyzed esterification as indicated in the introduction. The general schematic diagram of ping–pong bi–bi mechanisms goes as follows:[36]

$$E + I \leftrightarrow EI,\ k_1,\ k_2$$

$$EI \leftrightarrow E^*W,\ k_3,\ k_4$$

$$E^*W \leftrightarrow E^* + W,\ k_5,\ k_6$$

$$E^* + Gly \leftrightarrow E^*Gly,\ k_7,\ k_8$$

$$E^*Gly \leftrightarrow EMG,\ k_9,\ k_{10}$$

$$EMG \leftrightarrow E + MG,\ k_{11},\ k_{12}$$

where: E, E*, I, Gly, MG and W, k_i stand for enzyme, intermediate enzyme, ibuprofen, glycerol, ibuprofen monoglyceride, water and the kinetic constants, respectively. By taking into account the steps involved in the above reaction scheme, a general kinetic equation for the esterification reaction is:[36]

$$r = \frac{k_a C_I C_{Gly} - k_b C_{MG} C_W}{aC_I + bC_{Gly} + cC_{MG} + dC_W + eC_I C_{Gly} + fC_{MG} C_W + gC_I C_{MG} + hC_{Gly} C_W} \quad (1)$$

where

$$k_a = k_1 \times k_3 \times k_5 \times k_7 \times k_9 \times k_{11} \times C_E$$

$$k_b = k_2 \times k_4 \times k_6 \times k_8 \times k_{10} \times k_{12} \times C_E$$

$$a = k_1 \times k_3 \times k_5 \times k_8 \times k_{11}$$

$$b = k_2 \times k_5 \times k_7 \times k_9 \times k_{11}$$

$$c = k_2 \times k_4 \times k_6 \times k_8 \times k_{11}$$

$$d = k_2 \times k_5 \times k_8 \times k_{10} \times k_{12}$$

$$e = k_1 \times k_5 \times k_7 \times k_{11} \times (k_3 + k_9)$$

$$f = k_2 \times k_5 \times k_8 \times k_{12} \times (k_4 + k_{10})$$

$$g = k_1 \times k_6 \times k_8 \times k_{11} \times (k_3 + k_4)$$

$$h = k_2 \times k_5 \times k_7 \times k_{12} \times (k_9 + k_{10})$$

According to the experimental observation, two systems are taken into account: one of them is open (system A), while the other is closed (system B). In order to confirm the observations in each, several kinetic models were fitted to experimental data. In this way, the proposed kinetic models in system A consider the following facts:

• The concentration of glycerol is constant, due to this compound being in large excess compared to ibuprofen ($C_{Gly} \gg C_I$).

• The concentration of water in the reaction medium is lower, owing to the presence of toluene allows remove the water formed in the esterification.

• The inhibition of ibuprofen monoglyceride was considered insignificant. In a previous work,[4] we have demonstrated that the presence of monoglyceride can, even, improve the performance of esterification reaction.

• The reaction can be considered irreversible, including the experimental error taking into account that the equilibrium conversion is approximately 1 (Fig. 7a).

Accordingly, the esterification reaction shown in eqn (1) can be simplified as indicated in eqn (2), which, after rearrangement, results in eqn (3):

$$r = \frac{k_a C_I C_{Gly}}{aC_I + bC_{Gly} + eC_I C_{Gly}} \quad (2)$$

$$r = \frac{k'_1 C_I}{1 + K_I C_I} \quad (3)$$

where: $k'_1 = \frac{k_a}{b}$; $K_I = \frac{a}{bC_{Gly}} + e$

By considering that $K_I C_I \ll 1$, in this way, eqn (3) leads to the following equation:

$$r = k'_1 C_I \quad (4)$$

Therefore, model 1 was proposed as a potential model of pseudo-first-order with respect to the concentration of ibuprofen—eqn (11). A first-order model has been utilized in

the esterification of ibuprofen with 1-butanol catalyzed by immobilized lipase *Rhizomucor miehei*.[10] Moreover, a second model proposed, model 2, assumes an irreversible Michaelis–Menten kinetic model, considering zero order for glycerol and water, given by eqn (3).

Concerning the proposed kinetic models for system B, they take into account the following assumptions:

• Owing to the fact that it is a closed system, water is not removed from the reaction medium due to evaporation. For this reason, its concentration is in large excess compared to that of the monoglyceride ($C_W \gg C_{MG}$), so its variation with time can be considered negligible.

• The concentration of glycerol can be considered constant for a similar reason, *i.e.*, it is present in an enormous excess compared to ibuprofen, the other reagent ($C_{Gly} \gg C_I$).

• According to our results, the esterification reaction is reversible, as ibuprofen conversion at very high time values is clearly lower than 1 (Fig. 7b).

In view of the aforementioned items, the kinetic rate equation of esterification defined by eqn (1) can be rearranged as follows:

$$r = \frac{k_1^* C_I - k_2^* C_{MG}}{1 + K_I^* C_I + K_{MG}^* C_{MG}} \quad (5)$$

$$k_1^* = \frac{k_a}{b \times y} \quad ; \quad k_2^* = \frac{k_b \times C_W}{b \times y \times C_{Gly}}$$

$$y = 1 + \frac{d \times C_W}{b \times C_{Gly}} + \frac{h \times C_W}{b} \quad ; \quad K_I^* = \frac{a}{b \times y \times C_{Gly}} + \frac{e}{b \times y}$$

$$K_{MG}^* = \frac{c}{b \times y \times C_{Gly}} + \frac{f}{b \times y \times C_{Gly}} + \frac{g}{b \times y \times C_{Gly}}$$

On these grounds, the first proposed kinetic model for the closed batch reactors (system B) is a model (model 3) that considers no inhibition by the presence of ibuprofen monoglyceride and is represented by eqn (6). A second and more complex model was put forth to fit to experimental data, model 4, which is based on Michaelis–Menten kinetics and involves terms in the denominator for the substrates, ibuprofen and its monoglyceride. The latter is given by eqn (5).

$$r = \frac{k'_1 \, C_I - k'_2 C_{MG}}{1 + K_I C_I} \quad (6)$$

Discrimination of kinetic models and parameter estimation

Kinetic parameters related to the aforementioned kinetic models were calculated by nonlinear fitting coupled to numerical integration to all experimental data. First, each model was fitted to experimental data at individual temperatures to estimate the activation energies of the kinetic constants. Afterwards, for each model, further fitting to experimental data at all temperatures was carried out to obtain optimal parameters and a model valid for the entire experimental interval of all the variables studied both in the open and closed system.[27]

To select the best proposed model for systems A and B, physicochemical criteria like activation energy values and statistical criteria related to goodness-of-fit and parameter confidence intervals were used. All mentioned statistical parameters and final kinetic constants, together with their standard errors, are compiled in Tables 1 and 2. The first physical criterion is the value of the activation energy of each kinetic constant, shown in Tables 1 and 2. For system A, all kinetic constants have activation energies around 58 kJ mol^{-1}, while for system B the activation energies range from 14 to 45 kJ mol^{-1}. Their values for both systems lie within the 2–200 kJ mol^{-1} interval, typical of most chemical reactions. Another criterion to be accounted for is the positive sign in all equilibrium constants, as can be seen in Tables 1 and 2: all kinetic constants have positive sign for all proposed models. Therefore, as physical criteria are met by all models, model discrimination has to be performed on the sole basis of statistical criteria.

The first statistical criterion is that the absolute standard error of a given kinetic constant must be lower than the constant itself. Constants and parameters for all models showed very low standard errors compared to constant values, in other words, very low *t*-values and narrow intervals for possible constant values at 95% confidence. Therefore, from a statistical point of view, only goodness-of-fit and information criteria, such as RMSE, *F*-value, VE (%), BIC and AICc, would represent sufficient reasons to choose the adequate kinetic model for each system. That being acknowledged, for system A,

Table 1 Kinetic constants (with their standard errors) and statistical parameters for goodness-of-fit obtained for the models proposed when fitting them to data from the enzymatic esterification of glycerol and ibuprofen in open reactors (open system)[a]

	Model	Kinetic parameters	*F*-Value	SQR	AICc	BIC	RMSE	VE (%)
System A	1	$\ln k'_{10} = 15.16 \pm 0.43$ $E_a k'_1/R = 6874 \pm 146$	15 308	0.350	−1120	−6.19	0.044	98.14
	2	$\ln k'_{10} = 15.09 \pm 0.39$ $E_a k'_1/R = 6812 \pm 133$ $K_I = 0.68 \pm 0.14$	11 852	0.302	−1145	−6.31	0.041	98.39

[a] Note: Arrhenius expression according to: $k_i = \exp\left[\ln k_o - \frac{E_a/R(k_i)}{T + 273.16}\right]$.

Table 2 Kinetic constants (with their standard errors) and statistical parameters for goodness-of-fit obtained for the models proposed when fitting them to data from the enzymatic esterification of glycerol and ibuprofen in close reactors (close system)[a]

	Model	Kinetic parameters	*F*-Value	SQR	AICc	BIC	RMSE	VE (%)
System B	3	$\ln k_{10}^{*} = 11.71 \pm 0.45$ $E_a k_1^{*}/R = 5394 \pm 152$ $\ln k_{20}^{*} = 2.71 \pm 0.79$ $E_a k_2^{*}/R = 2637 \pm 267$ $K_I = 17.70 \pm 1.26$	22 615	0.122	−1699	−7.41	0.023	98.99
	4	$\ln k_{10}^{*} = 11.42 \pm 0.44$ $E_a k_1^{*}/R = 5274 \pm 149$ $\ln k_{20}^{*} = 2.30 \pm 0.75$ $E_a k_2^{*}/R = 2468 \pm 255$ $K_I = 0.86 \pm 0.29$ $K_{MG} = 16.92 \pm 1.26$	19 633	0.117	−1715	−7.43	0.023	99.04

[a] Note: Arrhenius expression according to: $k_i = \exp\left[\ln k_o - \frac{E_a/R(k_i)}{T + 273.16}\right]$.

the values of RMSE, AICc and BIC reach the lowest values for model 2, while the *F*-value is the highest for model 1 and VE (%) is the highest for model 2. In the case of system B, the values of RMSE, AICc and BIC are lower for model 4, while the *F*-value is the highest for the model 1 and VE (%) is the highest for the model 4. Therefore, both models have a goodness-of-fit in

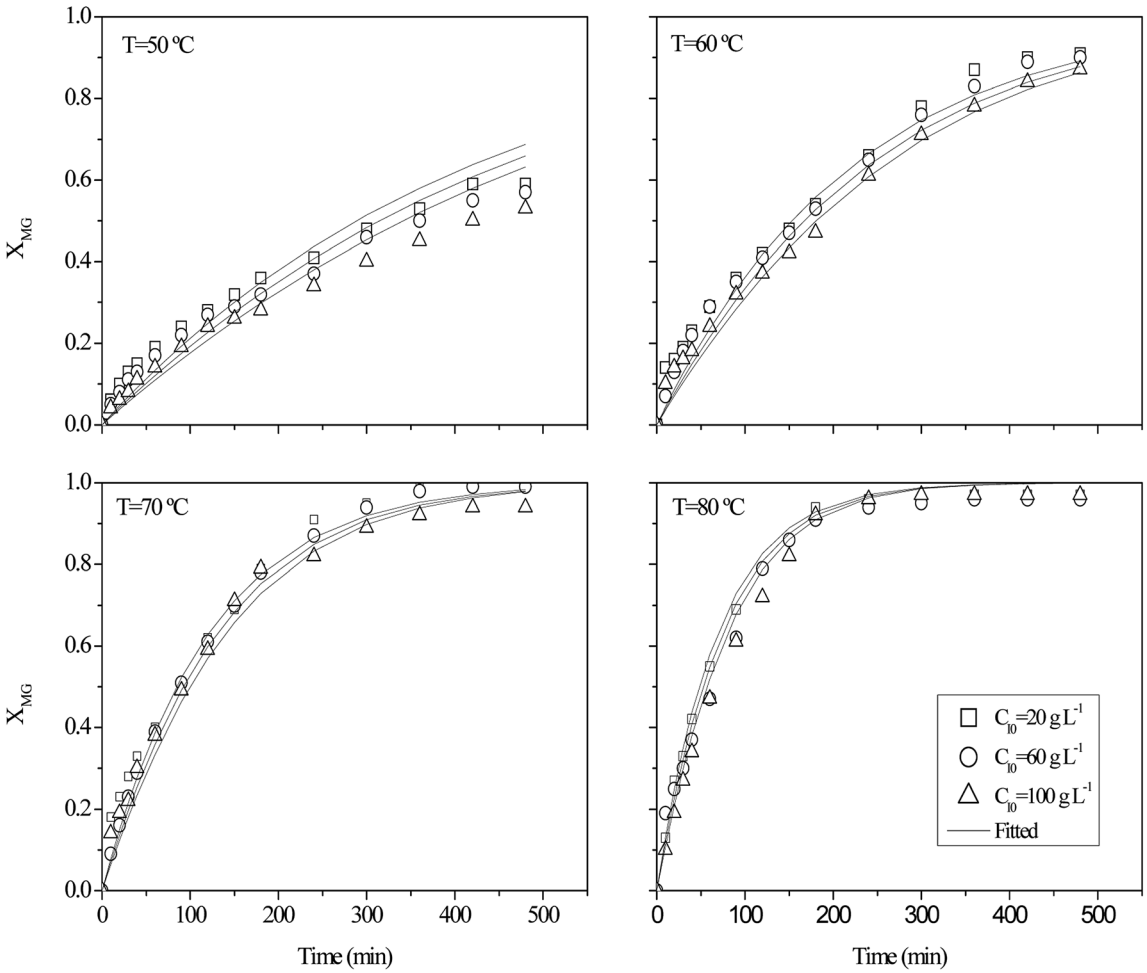


Fig. 8 Multivariable fitting of the selected kinetic model 2 to experimental at 50 to 80 °C for open reactors (open system). $C_E = 2$ g L^{-1}, volume ratio Gly/Tol = 20/5 and $N = 720$ rpm.

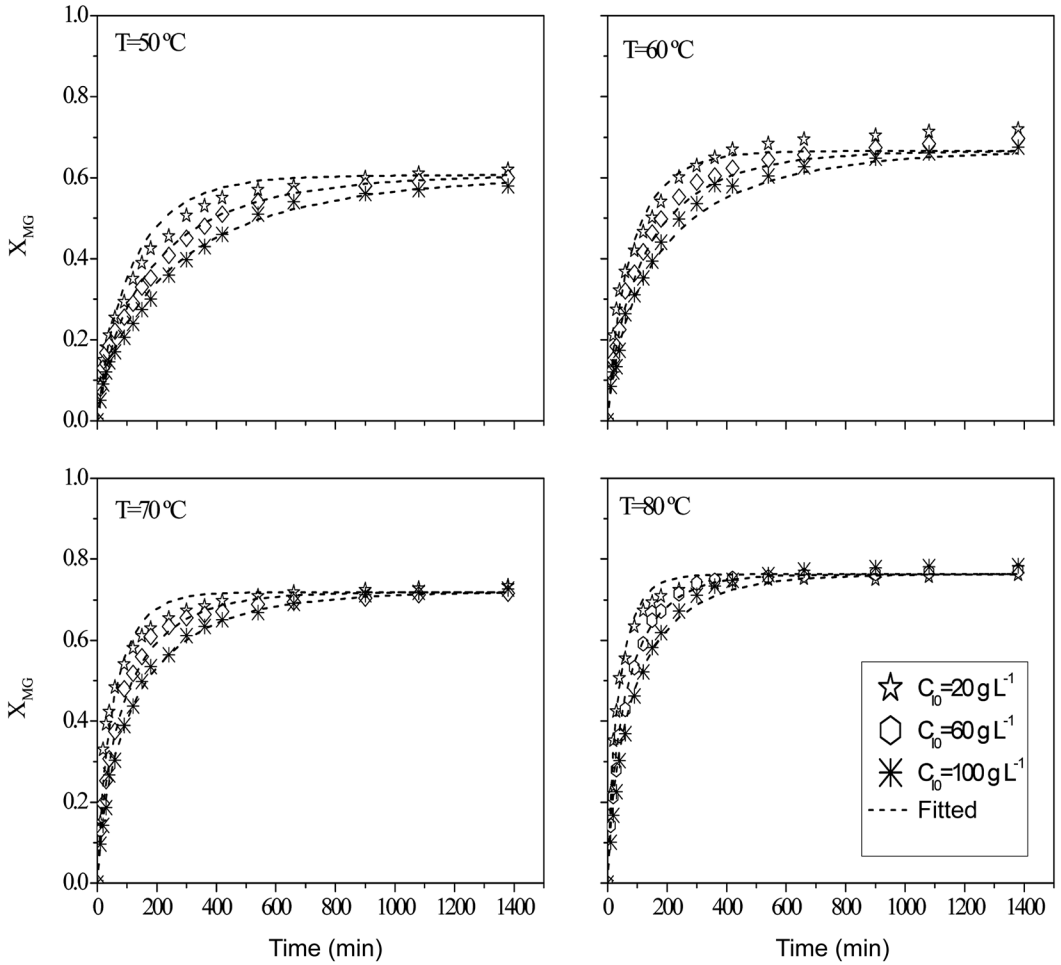


Fig. 9 Experimental results of the kinetic runs performed in closed reactors (close system) using model 4. Conditions: temperature varied from 50 to 80 °C using C_{WO} = 6% v/v, a volume ratio Gly/Tol = 20/5, while agitation was kept constant at 720 rpm.

a narrow interval for the kinetic constants, as well as other statistical parameters. These results suggest an irreversible hyperbolic model for system A (model 2) and a reversible simple Michaelis–Menten model for system B (model 4) in the esterification of glycerol and ibuprofen to yield the monoester in an open and closed system, respectively. Fig. 8 and 9 show the fitting of models 2 and 4 to all data, evidencing its adequacy in the experimental conditions interval studied. The following equations compile the kinetic information for said models:

System A

$$r = \frac{k'_1 C_I}{1 + K_I C_I} \quad (7)$$

where

$$k'_1 = \left[(15.09 \pm 0.39) - \frac{6812 \pm 133}{T}\right]$$

$$K_I = 0.68 \pm 0.14$$

System B

$$r = \frac{k_1^* C_I - k_2^* C_{MG}}{1 + K_I^* C_I + K_{MG}^* C_{MG}} \quad (8)$$

where

$$k_1^* = \left[(11.42 \pm 0.44) - \frac{5274.14 \pm 148.63}{T}\right]$$

$$k_2^* = \left[(2.30 \pm 0.75) - \frac{2468.39 \pm 255.28}{T}\right]$$

$$K_I^* = 0.86 \pm 0.29$$

$$K_{MG}^* = 16.92 \pm 1.26$$

When comparing both models, it is interesting to note that the kinetic constant for the esterification of ibuprofen and glycerol is much more affected when water and a small part of

toluene are removed from the reacting medium by distillation (system A). In fact, this action seems to reduce the rate of this reaction 3 to 4 times, though it is compensated by the absence of the reverse reaction. Moreover, the influence of ibuprofen concentration in the denominator, and, therefore, in the forward reaction rate value is relatively low when compared to unity, with values varying from 0.07 to 0.34. Thus, a first-order kinetic model (model 1) is not very far for being adequate to be fitted to experimental data, and the ibuprofen effect on reaction rate *via* the denominator term is almost negligible. In this regard, if a high concentration of ibuprofen is employed at 70 and 80 °C (the highest temperature values tested), monoglyceride productivity in system A is 2 to 3 times higher than when a reacting system without toluene is employed, with or without an optimal initial added amount of water.[4] Most probably, this is due to a better contact between the lipase and their substrates promoted by toluene. This aspect is also reflected in Fig. 3, where the initial rate values are three times higher for the reacting system with toluene compared to a system without added toluene for identical concentrations of lipase (2 mg L^{-1}).

Curiously, if water (and part of toluene) is not withdrawn from the reacting liquid, apart from the onset of the reverse reaction and an equilibrium conversion lower than unity, kinetic constant values are less affected by temperature and are 3–4 times higher. This is further indication that an adequate amount of toluene is needed for a rapid forward reaction. Nevertheless, the presence of water is deleterious, not only for the presence of the reverse reaction, but also for the profound inhibition caused by the monoglyceride.

If models 2 and 4 (systems A and B) are compared, the effect of ibuprofen in the denominator term is similar, but, in the case of system B (closed bath reactor), the reduction of the reaction rate value due to the monoglyceride term in the denominator is evident. The value of the latter term fluctuates between 1.7 and 8.5, compared to the unity term and the 0.07–0.34 value of the ibuprofen term. Further prove of this assertion is given by Fig. 6, which depicts an exponential reduction of initial reaction rate if water is initially added in addition to that present in the enzyme preparation.

Experimental section

Materials

Lipozyme® CALBL (free lipase B from *Candida antarctica* expressed in an *Aspergillus niger* host) was kindly gifted by Novozymes A/S (Denmark) and contains mainly glycerol (25%), sorbitol (25%), water (40%) and proteins (4%), with sodium benzoate (0.2%) and potassium sorbate (0.1%) as additives. Ibuprofen sodium salt (α-methyl-4-(isobutyl) phenylacetic acid) was purchased from Sigma-Aldrich. Ibuprofen (I) was obtained by precipitating this sodium salt with a hydrochloric acid solution (0.1 M) and subsequent filtration. Other reagents and their suppliers were: extra pure glycerol (Gly) 99.98% reagent grade and methanol HPLC grade (Fisher Scientific UK Ltd.), hydrochloric acid (35% w/w, Panreac Quimica, Spain), and dimethylsulfoxide-d6 deuteration degree 99.8% (Scharlau), toluene (Tol) (99% Panreac), 1,4-dioxane (99% Alfa Aesar GmbH, Germany), tetradecane (99% Aldrich), 2-butanone (99,7% Sigma-Aldrich), *o*-xylene (98% Sigma-Aldrich), *tert*-butyl alcohol (99% Alfa Aesar GmbH, Germany), 2-butanol (99% Alfa Aesar GmbH, Germany) and hydrochloric acid (35% w/w, Panreac Quimica, Spain).

Study of ibuprofen solubility

In the enzymatic esterification of ibuprofen with glycerol, several factors were considered to establish the operating conditions: selection of solvent, ibuprofen solubility in glycerol and solvents, stirring speed, initial water content and enzyme concentration. Firstly, ibuprofen solubility in various solvents was determined to find the most appropriate. Approximately 50 mg of ibuprofen were placed in a vial and the solvents were added dropwise with a micropipette into the vial with stirring at 720 rpm and room temperature, until the ibuprofen was dissolved.

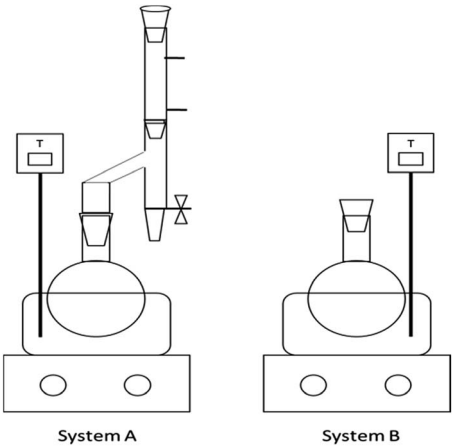
Secondly, the solubility of ibuprofen in toluene was determined adding a certain amount of ibuprofen in 5 mL of toluene until saturation in glass flasks placed inside a glycerin bath (IKA Yellow Line, model MSC basic C) with temperature and magnetic agitation control. The mixtures were stirred at 720 rpm during 24 h, after which 1 mL of the suspensions was withdrawn and passed through a 0.22 μm filter. 10 μL of the filtrate were diluted in DMSO-d6 for ^{1}H-NMR analysis. The signals of toluene and ibuprofen were identified at shifts of 2.32 ppm and 0.85 ppm, respectively.

Finally, the partition ratio between the mixture ibuprofen–glycerol–toluene was determined as follows: 2.5 g of ibuprofen (C_{I0} = 100 g L^{-1}) were dissolved in a flask containing 5 mL toluene at boiling conditions; then, 20 mL of extra pure glycerol were added. The flask was placed inside a glycerin bath at least 24 h, where temperature (50, 60, 70 and 80 °C) and magnetic agitation (720 rpm) were controlled. Afterwards, this mixture was placed in a separation funnel where the glycerol-rich (bottom) and the toluene-rich (top) phases were separated owing to their difference of density. 1 mL of each phase was filtered through a 0.22 μm filter; thereafter, 10 μL of each filtrated phase were diluted with DMSO-d6 and the concentration of each phase was determined by ^{1}H-NMR at 300 MHz. The partition ratio was calculated using the following equation:

$$K_p = \frac{\left(\frac{\text{mol I}}{\text{V Tol}}\right)_1}{\left(\frac{\text{mol I}}{\text{V Gly}}\right)_2} \quad (9)$$

Enzymatic esterification of ibuprofen ester

In the esterification reaction, two systems were considered, as shown in Scheme 2. In system A, the water formed during the lipase-catalyzed reaction is removed by toluene, which is returned to liquid reacting media by using a Dean–Stark apparatus; whereas in system B, the water formed remains in the reaction medium. In each system, the amount of ibuprofen (20; 60 to 100 g L^{-1}) was dissolved in 5 mL of toluene and then 20



Scheme 2 Schematic representation of the device employed for the kinetic experiments.

mL of pure glycerol were added (either anhydrous or with added water). Likewise, the reaction took place in a reactor placed inside a glycerin bath (IKA Yellow Line, model MSC basic C), where temperature and magnetic agitation were controlled, with the reaction being initiated by addition of the biocatalyst. Samples of 250 μL were withdrawn at several time values and frozen in order to stop the enzyme action. Prior to analysis, these samples were mixed with 750 μL of pure methanol and centrifuged. Further dilution was performed by taking 100 μL of the diluted sample and mixing it with additional 900 μL of pure methanol.

Analytical methods

Diluted samples were analyzed by reverse phase chromatography in a JASCO HPLC modular system. The components were separated using a Mediterranean Sea-18 column (Teknokroma) at 35 °C and a mixture of methanol : H_2SO_4 5 mM (pH 2.2) in a volume ratio of 83 : 17 flowing at 0.8 mL min^{-1} as the eluent phase. Detection of ibuprofen and its esters was performed with a JASCO diode array detector (model MD 2010) at 220 nm. The compounds were quantified using the area normalization method. Taking into account that their UV-vis spectra are identical, the conversion of ibuprofen was calculated using the following equation:

$$X = \frac{A_{MG}}{A_I + A_{MG}} \tag{10}$$

where MG stands for ibuprofen monoglyceride and I for ibuprofen.

Statistical methods

For previous experiments, calculation of initial reaction rate was performed by curve fitting to hyperboles (X data *vs.* time) followed by numerical differentiation (finite interval method) using Origin 8.5 software. Fitting of the data was made by numerical integration of the differential equations of the models using a 4^{th} order Runge–Kutta algorithm coupled to nonlinear regression fitting by a Marquardt–Levenberg algorithm. Subroutines for both algorithms are included in Aspen Custom Modeler v8.0. First, the proposed models were fitted step-by-step to data at a given temperature; then, simultaneous correlation of all the data sets at all temperatures was made.

Comparison of models and final selection of the most appropriate one were made following statistical and physical criteria. Among the latter, adequate values for activation energies are of very high relevance for model selection. Statistical considerations include narrow error intervals of the model constants computed and reasonable values of the information and goodness-of-fit criteria. Regarding statistical criteria, several parameters related to the amount of experimental data, the number of kinetic parameters and the total sum of residuals are considered for the selection of the most adequate model, which give precise account of the goodness of fit and over-parameterization. As indicated by Ravelo *et al.*,[4] Tamayo *et al.*[5] and Esteban *et al.*,[27] these parameters are: the square root of the mean square error (RMSE) (eqn (11)), Fischer's F-value (eqn (12)), Akaike information criteria corrected for a low data number to parameter number ($N/K < 40$) or AICc (eqn (13)), Bayesian information criterion (BIC) (eqn (14)) and the percentage of variation explained (% VE) (eqn (15)).

$$\mathrm{RMSE} = \sqrt{\sum_{i=1}^{N} \frac{(y_e - y_c)^2}{N}} \tag{11}$$

$$F - \mathrm{Value} = \frac{\sum_{n-1}^{N} \frac{(y_c)^2}{K_t}}{\sum_{n-1}^{N} \frac{(y_e - y_c)^2}{N - K_t}} \tag{12}$$

$$\mathrm{AICc} = N \times \ln\left(\frac{\mathrm{SQR}}{N}\right) + 2 \times K_t + \frac{2 \times K_t \times (K_t + 1)}{N - K_t - 1} \tag{13}$$

$$\mathrm{BIC} = \ln\left(\frac{\mathrm{SQR}}{N}\right) + \frac{K_t}{N} \times \ln(N) \tag{14}$$

$$\mathrm{VE}(\%) = 100 \times \left(1 - \frac{\sum_{j=1}^{j} \mathrm{SSQ}_j}{\sum_{j=1}^{j} \mathrm{SSQ}_{\mathrm{mean}\,j}}\right) \tag{15}$$

Conclusions

A thorough study of the esterification of glycerol with ibuprofen is herein presented as a means to valorize the former material and produce a prodrug of the latter one.

The solubility of ibuprofen in toluene increased with temperature, while, in the mixture ibuprofen–toluene–glycerol, the ibuprofen is better distributed in the toluene-rich phase. Toluene was used for water removal during esterification leading to an improvement of the yield to monoester and intensification in its production. Moreover, the solubility of

ibuprofen was enhanced with the presence of toluene in the reaction medium.

In the enzymatic esterification of glycerol and ibuprofen using free CALB as catalyst in organic medium, the effects of various variables on the activity of the enzyme were observed. Activity increases hyperbolically with enzyme concentration, stirring speed, and initial concentration of ibuprofen, and decreased exponentially with water content. Therefore, the most favourable operational conditions were established at an enzyme concentration of 2 g L^{-1}, stirring speed of 720 rpm, water content of 6% v/v and volumetric ratio Gly/Tol = 20/5, thus avoiding mass transfer limitations and fast deactivation of the enzyme. Furthermore, thermodynamic and kinetic studies were performed with temperature ranging from 50 to 80 °C and initial concentration of ibuprofen varying from 20 to 100 g L^{-1}.

The reaction is endothermic owing to the increment of the equilibrium conversion with temperature for both systems. As for the kinetic studies, adequate reaction rate equations based on modifications of well-known models were proposed and fitted to experimental data. Goodness-of-fit parameters and standard errors of constants and conversion at equilibrium (90% to 98%) suggest an irreversible hyperbolic model with pseudo-zero order for glycerol for the open system. On the other hand, for the closed system, a reversible simple Michaelis–Menten model accounting for the esterification and hydrolysis reaction with pseudo-first order for ibuprofen and monoester proved valid. Both models were fitted to all experimental data in the wide experimental interval studied in the corresponding case with excellent results.

Nomenclature

Components

I	Ibuprofen
E	Enzyme
Gly	Glycerol
MG	Ibuprofen ester (monoglyceride)
Tol	Toluene
W	Water

Nomenclature

A	Peak area of chromatogram
AICc	Modified Akaike's information criterion
BIC	Bayesian information criterion
C	Concentration (mol L^{-1} or g L^{-1} or % v/v)
$E_a(k_i)$	Activation energy (kJ mol^{-1})
F-Value	Statistical Fischer's *F* estimated considering eqn (3)
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
HPLC	High-performance liquid chromatography
k_i	Kinetic constant (mol L^{-1} min^{-1})
K	Equilibrium constant (mol L^{-1})
K_p	Partition ratio (mol mol^{-1})
K_t	Number of parameters in kinetic model eqn (3)–(6)
k_{i0}	Preexponential factor of the kinetic constant
log *P*	Partition coefficient
N	Total number of components
N	Total number of data to which a model is fitted, eqn (3)–(6)
p-Value	Value of the probability of getting a better model
R	Reaction rate (mol L^{-1} min^{-1})
R	Ideal gas constant (J mol^{-1} K^{-1})
RMSE	Square root of the mean of standard errors
SQR	Sum of quadratic residues
S	Solubility (g substrate per g solvent)
Ste	Solvent
T	Temperature (°C)
X	Conversion, as defined by eqn (2)
X_{eq}	Equilibrium conversion
V	Volume (mL)

Subscripts

0	Relative to the start of the reaction, time equals zero
1	Kinetic direct constant or kinetic constant
2	Kinetic reverse constant

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References

1 M. Pagliaro and M. Rossi, The future of glycerol, *Focus on Catalysts*, 2008, 8.
2 A. Wolfson, C. Dlugy and Y. Shotland, Glycerol as a green solvent for high product yields and selectivities, *Environ. Chem. Lett.*, 2007, **5**, 67–71.
3 A. Wolfson, A. Atyya, C. Dlugy and D. Tavor, Glycerol triacetate as solvent and acyl donor in the production of isoamyl acetate with *Candida antarctica* lipase B, *Bioprocess Biosyst. Eng.*, 2010, **33**, 363–366.
4 M. Ravelo, E. Fuente, Á. Blanco, M. Ladero and F. García-Ochoa, Esterification of glycerol and ibuprofen in solventless media catalyzed by free CALB: Kinetic modelling, *Biochem. Eng. J.*, 2015, **101**, 228–236.
5 J. J. Tamayo, M. Ladero, V. E. Santos and F. Garcia-Ochoa, Esterification of benzoic acid and glycerol to alpha-monobenzoate glycerol in solventless media using an industrial free *Candida antarctica* lipase B, *Process Biochem.*, 2012, **47**, 243–250.
6 P. Halen, M. Prashant, R. Giridhar and M. R. Yadav, Prodrug designing of NSAIDs, *Mini-Rev. Med. Chem.*, 2009, **9**, 124–139.

7 C. Jean-Ching and T. Shau-Wei, Enantioselective synthesis of (s)-ibuprofen ester prodrug in cyclohexane by *candida rugosa* lipase immobilized on accurel mp1000, *Biotechnol. Prog.*, 2000, **16**, 986–992.
8 A. M. Qandil, Prodrugs of nonsteroidal anti-inflammatory drugs (NSAIDs), more than meets the eye: a critical review, *Int. J. Mol. Sci.*, 2012, **13**, 17244–17274.
9 Y. Liu, F. Wang and T. Tan, Effects of alcohol and solvent on the performance of lipase from *Candida* sp. in enantioselective esterification of racemic ibuprofen, *J. Mol. Catal. B: Enzym.*, 2009, **56**, 126–130.
10 A. Sánchez, F. Valero, J. Lafuente and C. Solà, Highly enantioselective esterification of racemic ibuprofen in a packed bed reactor using immobilised *Rhizomucor miehei* lipase, *Enzyme Microb. Technol.*, 2000, **27**, 157–166.
11 M. L. Foresti, M. Galle, M. L. Ferreira and L. E. Briand, Enantioselective esterification of ibuprofen with ethanol as reactant and solvent catalyzed by immobilized lipase: experimental and molecular modeling aspects, *J. Chem. Technol. Biotechnol.*, 2009, **84**, 1461–1473.
12 Y.-C. Xie, H.-Z. Liu and J.-Y. Chen, *Candida rugosa* lipase catalyzed esterification of racemic ibuprofen with butanol: racemization of r-ibuprofen and chemical hydrolysis of s-ester formed, *Biotechnol. Lett.*, 1998, **20**, 455–458.
13 M. Arroyo and J. Sinisterra, High enantioselective esterification of 2-Arylproponic acids catalyzed by immobilized lipase from *Candida antarctica*: A mechanistic approach, *J. Org. Chem.*, 1994, **59**, 4410–4417.
14 C.-S. Chang, C.-C. Su, J.-R. Zhuang and S.-W. Tsai, Enhancement of enantioselectivity on the synthesis of (S)-naproxen morpholinoalkyl ester prodrugs in organic solvents using isopropanol-dried immobilized lipase, *J. Mol. Catal. B: Enzym.*, 2004, **30**, 151–157.
15 P. Trodler and J. Pleiss, Modeling structure and flexibility of *Candida antarctica* lipase B in organic solvents, *BMC Struct. Biol.*, 2008, **8**, 1–10.
16 A. L. Ong, A. H. Kamaruddin, S. Bhatia, W. S. Long, S. T. Lim and R. Kumari, Performance of free *Candida antarctica* lipase B in the enantioselective esterification of (R)-ketoprofen, *Enzyme Microb. Technol.*, 2006, **39**, 924–929.
17 P. d. O. Carvalho, F. J. Contesini and M. Ikegaki, Enzymatic resolution of (R, S)-ibuprofen and (R, S)-ketoprofen by microbial lipases from native and commercial sources, *Braz. J. Microbiol.*, 2006, **37**, 329–337.
18 K. Faber, *Biotransformations in organic chemistry: a textbook*, Springer Science & Business Media, 2011.
19 M. Arroyo and J. V. Sinisterra, High enantioselective esterification of 2-arylpropionic acids catalyzed by immobilized lipase from *candida antarctica*: a mechanistic approach, *J. Org. Chem.*, 1994, **59**, 4410–4417.
20 C.-S. Chang and C.-S. Hsu, Lipase-catalyzed enantioselective esterification of (S)-naproxen hydroxyalkyl ester in organic media, *Biotechnol. Lett.*, 2003, **25**, 413–416.
21 D.-t. Zhao, E.-n. Xun, J.-x. Wang, R. Wang, X.-f. Wei, L. Wang and Z. Wang, Enantioselective esterification of ibuprofen by a novel thermophilic biocatalyst: APE1547, *Biotechnol. Bioprocess Eng.*, 2011, **16**, 638–644.
22 P. Carvalho, F. Contesini, R. Bizaco, S. Calafatti and G. Macedo, Optimization of enantioselective resolution of racemic ibuprofen by native lipase from *Aspergillus niger*, *J. Ind. Microbiol. Biotechnol.*, 2006, **33**, 713–718.
23 A. M. Klibanov, Improving enzymes by using them in organic solvents, *Nature*, 2001, **409**, 241–246.
24 B. C. Páez, A. R. Medina, F. C. Rubio, P. G. Moreno and E. M. Grima, Modeling the effect of free water on enzyme activity in immobilized lipase-catalyzed reactions in organic solvents, *Enzyme Microb. Technol.*, 2003, **33**, 845–853.
25 L. Gubicza, A. Kabiri-Badr, E. Keoves and K. Belafi-Bako, Large-scale enzymatic production of natural flavour esters in organic solvent with continuous water removal, *J. Biotechnol.*, 2000, **84**, 193–196.
26 G. Duan, C. B. Ching, E. Lim and C. H. Ang, Kinetic study of enantioselective esterification of ketoprofen with n-propanol catalysed by an lipase in an organic medium, *Biotechnol. Lett.*, 1997, **19**, 1051–1055.
27 J. Esteban, E. Fuente, M. Gonzalez-Miquel, A. Blanco, M. Ladero and F. Garcia-Ochoa, Sustainable joint solventless coproduction of glycerol carbonate and ethylene glycol via thermal transesterification of glycerol, *RSC Adv.*, 2014, **4**, 53206–53215.
28 R. Morrone, N. D'Antona, D. Lambusta and G. Nicolosi, Biocatalyzed irreversible esterification in the preparation of S-naproxen, *J. Mol. Catal. B: Enzym.*, 2010, **65**, 49–51.
29 R. Morrone, G. Nicolosi, A. Patti and M. Piattelli, Resolution of racemic flurbiprofen by lipase-mediated esterification in organic solvent, *Tetrahedron: Asymmetry*, 1995, **6**, 1773–1778.
30 Y. Poojari and S. J. Clarson, Thermal stability of *Candida antarctica* lipase B immobilized on macroporous acrylic resin particles in organic media, *Biocatal. Agric. Biotechnol.*, 2013, **2**, 7–11.
31 P. B. L. Fregolente, L. V. Fregolente, G. M. F. Pinto, B. C. Batistella, M. R. Wolf-Maciel, and R. Maciel Filho, Monoglycerides and diglycerides synthesis in a solvent-free system by lipase-catalyzed glycerolysis, *Biotechnology for Fuels and Chemicals*, Springer, 2008, pp. 285–292.
32 F. Secundo, G. Carrea, C. Soregaroli, D. Varinelli and R. Morrone, Activity of different *Candida antarctica* lipase B formulations in organic solvents, *Biotechnol. Bioeng.*, 2001, **73**, 157–163.
33 T. Kobayashi, T. Matsuo, Y. Kimura and S. Adachi, Thermal stability of immobilized lipase from *Candida antarctica* in glycerols with various water contents at elevated temperatures, *J. Am. Oil Chem. Soc.*, 2008, **85**, 1041–1044.
34 A. Gog, M. Roman, M. Toșa, C. Paizs and F. D. Irimie, Biodiesel production using enzymatic transesterification–current state and perspectives, *Renewable Energy*, 2012, **39**, 10–16.
35 T. Tan, J. Lu, K. Nie, L. Deng and F. Wang, Biodiesel production with immobilized lipase: A review, *Biotechnol. Adv.*, 2010, **28**, 628–634.
36 S. Cha, A simple method for derivation of rate equations for enzyme-catalyzed reactions under the rapid equilibrium assumption or combined assumptions of equilibrium and steady state, *J. Biol. Chem.*, 1968, **243**, 820–825.