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Kinetics of Lipase-mediated Synthesis of Butyl Butyrate in *n*-hexane

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This paper reports experimental and modeling work concerning alcoholysis reactions between butanol and ethyl butanoate, catalyzed by Lipozyme™ in n-hexane, using a batch stirred system at 60°C. Description of the reaction kinetics was based on a postulated multisubstrate Ping Pong Bi Bi mechanism, and appropriate rate expressions were derived for all components in the reaction medium. Simplified models were fitted by nonlinear multiresponse regression analysis to data (experimental or calculated from mass balances, as appropriate) encompassing the concentrations of free butanol, ethyl butanoate, ethanol and butyl butanoate. Finally, incremental F-tests were performed to assess the simplest model form that was able to provide a statistically good fit throughout the entire reaction time frame.

Keywords: Alcoholysis; Enzyme; Ping Pong Bi Bi; Modeling

INTRODUCTION

Lipases (or acylglycerol acylester hydrolases, EC 3.1.1.3) are enzymes that have been tailored by nature to cleave ester bonds of acylglycerols, with concomitant consumption of water molecules (hydrolysis); however, under microaqueous conditions, such enzymes are also able to catalyze the reverse reaction (esterification). If such two basic processes are combined in a sequential fashion, a general set of reactions commonly designated as interesterification is obtained and, depending on the particular starting point in terms of substrates, one may be in the presence of acidolysis (where an acyl moiety is displaced between an acylglycerol and a carboxylic acid), alcoholysis (where an acyl moiety is displaced between an acylglycerol and an alcohol), or transesterification (where two acyl moieties are exchanged between two acylglycerols) (Balcão *et al.*, 1996).

Like other catalysts, lipases increase the rate of reaction with no net transformation over the time scale of the reaction, and without affecting the position of chemical equilibrium. Many studies have been carried out aimed at understanding the mechanism of interfacial activation of lipases (e.g. Brady *et al.*, 1990; Winkler *et al.*, 1990; Brzozowski *et al.*, 1991; van Tilbeurgh *et al.*, 1992; Jaeger *et al.*, 1994); it is currently accepted that not all lipases are activated by interfaces, as is the case of lipases from *Pseudomonas glumae* and *Pseudomonas aeruginosa* (Jaeger *et al.*, 1994), and that both the quality and quantity of interface play a role in the catalytic activity of these enzymes (Rogalska *et al.*, 1994). A comprehensive review pertaining to activation kinetics can be found elsewhere (Paiva *et al.*, 2000).

Development of rate expressions to model lipase-catalyzed reactions is intrinsically dependent on the complexity of the reaction mechanism postulated. Since lipases catalyze multisubstrate, multiproduct reactions, kinetic models based on the classical unisubstrate, Michaelis–Menten mechanism will hardly provide accurate descriptions of the prevailing chemical phenomena, and more involved kinetic mechanisms have accordingly been proposed. It has frequently been suggested that, as hydrolases, the catalytic action of lipases follows a two-step reaction mechanism, usually referred to as Ping Pong Bi Bi. Ping Pong mechanisms are usually assumed to be typical of group transfer, or "substituted enzyme"

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reactions (Segel, 1993); this is the case of lipase-catalyzed alcoholysis. A few alcoholysis reactions using immobilized lipases have been reported in the literature (e.g. Millqvist *et al.*, 1994; Fureby *et al.*, 1997; Shimada *et al.*, 1997; 1998; Gunnlaugsdottir *et al.*, 1998); nevertheless, the level of phenomenological modeling (e.g. Langrand *et al.*, 1988; Mukesh *et al.*, 1993; Jaeger *et al.*, 1994; Kierkels *et al.*, 1994) has lagged far behind that associated with its hydrolytic counterpart. Examples of the use of Ping Pong Bi Bi mechanisms in the literature comprise the modeling of esterification reactions (e.g. Chulalaksananukul *et al.*, 1990; Marty *et al.*, 1992; Stamatis *et al.*, 1993; Goto *et al.*, 1994; Ramamurthi and McCurdi, 1994; Yong and Al-Duri, 1996; Kamiya and Goto, 1997; Mukesh *et al.*, 1997; Basheer *et al.*, 1998; Huang *et al.*, 1999), transesterification reactions (e.g. Chulalaksananukul *et al.*, 1992; 1993; Rizzi *et al.*, 1992) and hydrolysis reactions (e.g. Malcata *et al.*, 1991; 1992a,b; 1993; Garcia *et al.*, 1992; van Tol *et al.*, 1995; Rice *et al.*, 1999).

This communication reports experimental work encompassing lipase-catalyzed alcoholysis between butanol and ethyl butanoate using a commercial immobilized lipase; reactions were carried out in an organic, hydrophobic solvent using a batch stirred processing set up. The data were eventually fitted to by non-linear, multiresponse regression of several increasingly simpler mechanistic models, based on the assumptions underlying the Ping Pong Bi Bi mechanism.

MATERIALS AND METHODS

Enzyme

A lipase from *Mucor miehei*, immobilized on a macroporous anion exchange resin (Lipozyme™ at 5 BAUN g^{-1}; BAUN: Batch Acidolysis Units Novo) was kindly provided by NOVO Nordisk (Bagsvaerd, Denmark) and was used without further purification. The water content of the enzyme, calculated by comparison of its weight before and after heating at 110°C for 24 h, was *ca.* 4.5% (w/w).

Chemicals

Ethyl butanoate (EtBu) and *n*-hexane were purchased from Merck (Darmstadt, Germany), 1-butanol (BuOH) from Romil Chemicals (Cambridge, UK), 1-butyl butanoate (BuBu) from Fluka (Buchs, Switzerland), and ethanol (EtOH) from AGA (Sacavém, Portugal). All chemicals were pro-analysis grade.

Reaction Conditions

Unless stated otherwise, lipase-catalyzed alcoholysis was carried out as follows. The immobilized enzyme was placed in 100 ml Erlenmeyer flasks containing the substrates dissolved in *n*-hexane, and then shaken at 150 rpm and 60°C. The flasks were closed by glass stoppers in order to prevent losses by evaporation, and the reaction was allowed to proceed for several hours. Samples (1.1 ml) were periodically withdrawn, and ultra-filtered to remove the enzyme and hence stop the reaction. Filtrates were analyzed by gas chromatography as described below.

Analytical Methods

The concentrations of butyl butyrate were determined by Gas Chromatography, GC (Perkin–Elmer Autosystem XL, USA) using FID. A capillary DB-1 column (J&W, USA), 60 m × 0.32 mm ID, with a stationary layer thickness of 0.25 μm of dimethylpolysiloxane (with a polarity of 5), was used as resolution medium.

The analytical procedure followed depended on the presence or not of *n*-hexane in the samples to be analyzed; hence, after injection of samples (1.0 μl), the temperature of the column was held constant at 130°C (when the samples did not contain *n*-hexane) or at 175°C (otherwise). The temperatures of the injector and detector were 250°C in both situations. In terms of flow rate of the carrier gas (He), when the samples did not contain *n*-hexane, the split was opened for the first minute at 32 ml min^{-1} and 85 kPa, whereas the split was opened for 15 sec at 225 kPa otherwise; the associated flow rates were then equal to *ca.* 1 ml min^{-1} and 6.2 ml min^{-1}, respectively. Quantitative data were collected and processed with the TurboChrom™ software.

When *n*-hexane was present, and because it has a retention time in the DB-1 column very similar to that of BuOH, the concentrations of the other components (i.e. EtOH, BuOH and EtBu) were calculated from the BuBu concentrations using mass balances based on the stoichiometry of the reaction. In this case, the calibration was with BuBu standards diluted in *n*-hexane, whereas in the other situation the calibration curves were prepared for all components to be analyzed.

RESULTS AND DISCUSSION

Equilibrium Studies

To determine the equilibrium constant (K_{eq}) for the model alcoholysis reaction, mixtures containing known concentrations of the two substrates were prepared, and the progress of ester formation was

monitored until equilibrium was eventually reached. The value obtained for K_{eq} of the reaction in the absence of n-hexane was thus 0.55 ± 0.18.

Effect of Amount of Enzyme

In order to evaluate the effect of the amount of enzyme on the kinetics of the alcoholysis reactions, experiments were performed in which different initial amounts of immobilized enzyme were added to equimolar mixtures of substrate (BuOH + EtBu), thus resulting in different initial concentrations of Lipozyme™ (i.e. 0, 4.6, 7.0, 10.0, 14.9 and 18.7 $g_{Lipozyme}\,l^{-1}$). An initial sample was withdrawn immediately after addition of Lipozyme™. The flasks (closed with a glass stopper to prevent evaporation) were then placed in a shaking bath at 150 rpm, and incubated at 60°C. Further samples were withdrawn at different times, and the samples were analyzed by GC in order to assay for their concentrations in BuOH, EtBu, EtOH and BuBu, as described previously. The experimental results were corrected for the amounts of sample withdrawn. As expected, and since no production of BuBu was detected, it was possible to conclude that no reaction took place in the absence of enzyme.

The evolution of the specific production of BuBu for each of the enzyme concentrations in the reaction medium is plotted in Fig. 1. Inspection of this figure indicates that for all initial concentrations of lipase tested, the reaction rate decreases as time elapses; this fact can derive from a combination of decrease in reactant concentration, increase in product concentration (thus inhibiting the forward reaction) and decrease in catalyst activity. Such a decrease of the reaction rate becomes more apparent when the reaction time exceeds 2 h. On the other hand, it can also be pinpointed in Fig. 1 that, under the reactional conditions tested, the optimum initial concentration of Lipozyme™ appears to lie in the range 7–10 $g_{Lipozyme}\,l^{-1}$; a decrease in the specific molar production of BuBu is indeed observed when the enzyme concentration is risen above 10 $g_{Lipozyme}\,l^{-1}$.

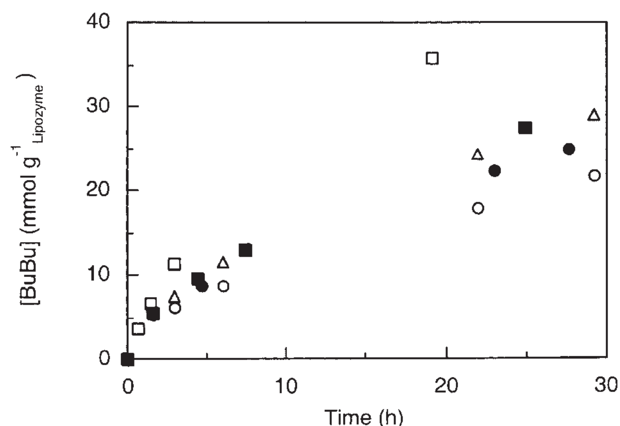

FIGURE 1 Effect of the amount of enzyme added on the specific concentration of BuBu, departing from the same initial equimolar substrate solution. The concentrations of Lipozyme used were 4.6 $g\,l^{-1}$ (○), 7.0 $g\,l^{-1}$ (●), 10 $g\,l^{-1}$ (□), 14.9 $g\,l^{-1}$ (■) and 18.7 $g\,l^{-1}$ (△).

Rate Expression of Reaction

In order to assess the effect of the concentration of either of the two substrates on the initial rate of reaction, preliminary experiments were performed in n-hexane solutions. Such solvent was chosen because, apart from keeping the water concentration low, it does not interfere with the spectrophotometric detection of BuBu after resolution by chromatography. The evolution of the concentration of BuBu produced in each situation (results not shown) indicated that BuOH acts as a competitive inhibitor of our lipase.

The Ping Pong Bi Bi mechanism (a well known and widely accepted mechanism for lipase-catalyzed reactions) coupled with competitive inhibition by one of the substrates was assumed as a basis for the modeling effort that follows; the description of this mechanism is schematically depicted in Fig. 2, and the associated rate equation is given by (Segel, 1993)

$$r = \frac{v_f v_r \left\{ [EtBu][BuOH] - \frac{[BuBu][EtOH]}{K_{eq}} \right\}}{\left\{ v_r K_{m,BuOH}[EtBu] + v_r K_{m,EtBu}[BuOH]\left(1 + \frac{[BuOH]}{K_{I,BuOH}}\right) + \frac{v_f K_{m,EtOH}}{K_{eq}}[BuBu] + \frac{v_f K_{m,BuBu}}{K_{eq}}[EtOH] + v_r[EtBu][BuOH] + \frac{v_f K_{m,EtOH}}{K_{eq}K_{i,EtBu}}[EtBu][BuBu] + \frac{v_f}{K_{eq}}[BuBu][EtOH] + \frac{v_r K_{m,EtBu}}{K_{i,EtOH}}[BuOH] \times [EtOH] \right\}} \quad (1)$$

Here v_f and v_r are the maximal velocities for the forward and reverse reactions, respectively, K_{eq} is the equilibrium constant, $K_{m,BuOH}$, $K_{m,EtBM}$, $K_{m,EtOH}$ and $K_{m,BuBu}$ are the Michaelis–Menten constants for BuOH, EtBu, EtOH and BuBu, respectively, $K_{I,BuOH,}$ is the inhibition constant for BuOH, and $K_{i,EtBu}$ and $K_{i,EtOH}$ are the dissociation constants for EtBu and EtOH from the specific enzyme inhibitor complex, respectively. Straight stoichiometry considerations associated with the reaction scheme, viz.

$$EtBu + BuOH \rightleftarrows EtOH + BuBu \quad (2)$$

allow a material balance to be written for the substrates (EtBu and BuOH) and products (EtOH and BuBu).

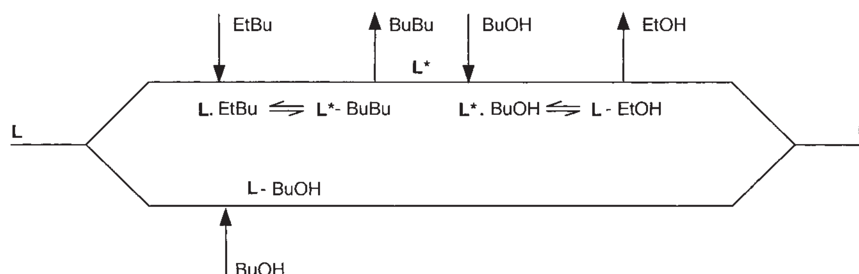

FIGURE 2 Cleland's representation of the mechanism of alcoholysis catalyzed by lipase. BuBu, butyl butyrate; BuOH, butanol; EtBu, butyl ethanoate; EtOH, ethanol; L, lipase; L*, modified lipase; L-BuOH, dead-end lipase–butanol complex.

Experimental molar concentrations of BuBu obtained in a new set of experiments (and concentrations of EtBu, BuOH and EtOH calculated therefrom *via* mass balances) were used to fit the postulated Ping Pong Bi Bi model *via* numerical integration of Eq. (1) (and associated mass balances). Reactions were carried out in *n*-hexane, where the initial EtBu concentrations were varied for four constant initial BuOH concentrations and for one initial Lipozyme™ concentration of *ca.* $10\,g_{Lipozyme}\,l^{-1}$.

The 10 parameters of the model were estimated by multiresponse, non-linear regression to the concentrations of free EtBu, BuOH, BuBu and EtOH, using the General REGression package (GREG) conceived by Caracotsios *et al.* (1985). At level 20, this package performs non-linear regression analysis of multiresponse data, using finite differences as approximants of the derivatives of the objective function with respect to each parameter, and using as objective function minimization of $|Z^{\top}Z|$, where Z is the matrix of the residuals between the model and known concentrations (either determined experimentally or calculated from experimental data).

Each form of the 10-parameter model depicted in Eq. (1) was duly fitted to 20 sets of experimental results, each consisting of several concentration data points which varied in number from 36 to 48. Such 20 sets of experiments were performed for constant initial BuOH concentrations of 0.5, 0.25, 0.125 and 0.05 M, and varying initial EtBu concentrations (ranging from 0.05 to 5 M, depending on the experiment). For the sake of book-keeping, and since our main goal was to obtain a full nested model which adequately describes all reactions at the same time, the estimates of the best model parameters obtained in the separate fitting of the kinetic models (with a total residual sum of squares of 7.161×10^{-2}) are not presented. Although interpretation of the parameter estimates obtained was not straightforward (since most of them conveyed confidence ranges much wider than the order of magnitude of the parameter estimates themselves), there were a few trends which could be perceived, namely that an increase in BuOH concentration promotes an increase both in v_f and $K_{m,BuOH}$, as well as a decrease in $K_{m,EtBu}$. Given that $K_{m,BuOH}$ and $K_{m,EtBu}$ are the Michaelis–Menten constants for BuOH and EtBu, respectively, and since they are related to the affinity of the enzyme for the substrate, higher values of said parameters do therefore unfold an inhibitory effect of BuOH. A further step taken was to fit a single (nested) model to the whole set of data obtained in the 20 experiments performed. The best estimates of the parameters obtained (as well as their confidence intervals, when available) are tabulated in Table I (with a total residual sum of squares of 8.975×10^{-2}). The appropriateness of the nested model to fit the whole set of 840 known data points was confirmed by the magnitude of residual sum of squares. The data produced are plotted in Figs. 3–6, laid down on the model predictions using the nested model with the parameter estimates in Table I.

In order to investigate whether such nested model (with only 10 parameters) could statistically substitute the full individual model (with $4 \times 5 \times 10 = 200$ parameters), likelihood ratio tests were performed on the incremental sum of squares between models, as described by Bates and Watts (1988). This procedure is summarized in Table II. The analysis was carried out by comparing the ratio s_e^2/s_f^2 with $F(\nu_e, \nu_f; \alpha)$: the nested model will be accepted if the calculated mean square ratio is lower than the Fisher distribution value tabulated; otherwise, the extra terms must be retained and the full model has to be

TABLE I Estimates of the nested model parameters

Model parameter	Value	95% confidence interval
v_f ($mol\,l^{-1}\,h^{-1}$)	1.522×10^{0}	4.183×10^{-1}
v_r ($mol\,l^{-1}\,h^{-1}$)	2.111×10^{0}	1.019×10^{0}
K_{eq} (–)	4.927×10^{-1}	5.850×10^{-2}
$K_{m,BuOH}$ ($mol\,l^{-1}$)	7.030×10^{-1}	4.040×10^{-1}
$K_{m,EtBu}$ ($mol\,l^{-1}$)	2.682×10^{-2}	–
$K_{I,BuOH}$ ($mol\,l^{-1}$)	6.809×10^{-3}	–
$K_{m,EtOH}$ ($mol\,l^{-1}$)	8.039×10^{-3}	–
$K_{m,BuBu}$ ($mol\,l^{-1}$)	0.000×10^{0}	–
$K_{i,EtBu}$ ($mol\,l^{-1}$)	7.591×10^{-3}	2.891×10^{-4}
$K_{i,EtOH}$ ($mol\,l^{-1}$)	1.620×10^{-4}	–
RSS* ($mol\,l^{-1}$)^2	8.975×10^{-2}	–

*Residual sum of squares.

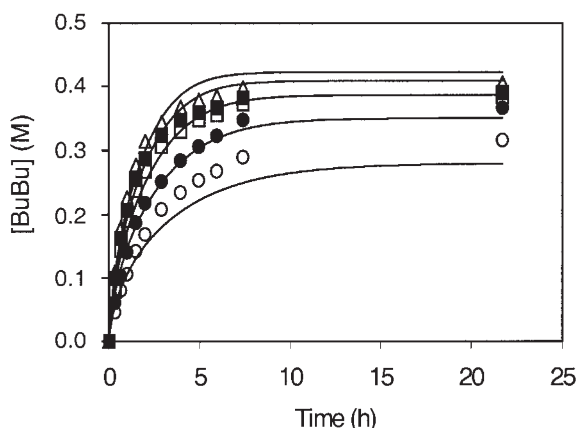

FIGURE 3 Evolution of the concentration of BuBu as a function of time for a constant initial concentration of BuOH (0.5 M) and several initial concentrations of EtBu: 1 M (○), 2 M(●), 3 M (□), 4 M (■) and 5 M (△).The fit by the nested model is also indicated (—).

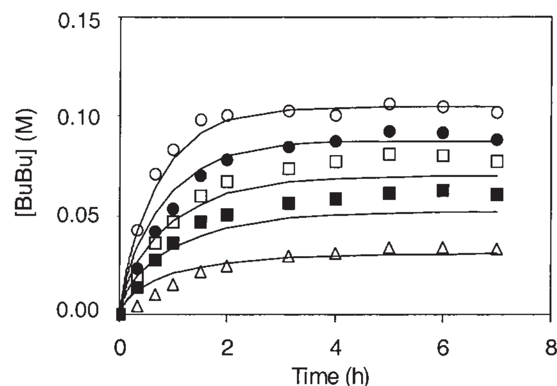

FIGURE 5 Evolution of the concentration of BuBu as a function of time for a constant initial concentration of BuOH (0.125 M) and several initial concentrations of EtBu: 0.05 M (○), 0.125 M (●), 0.25 M (□), 0.5 M (■) and 1 M (△). The fit by the nested model is also indicated (—).

used instead (Bates and Watts, 1988). Our results indicate that the null hypothesis holds; this hypothesis states that the nested model provides at least as good a statistical fit as the full model. Such realization therefore backs up use of such nested model to theoretically simulate the evolution of the molar concentrations of the various compounds in question. For the sake of clarity, only the evolution of the concentration of BuBu is plotted; it is apparent that the nested model provides in general a good fit.

From the inspection of Table I, one can see that the equilibrium constant lies between 0.434 and 0.551, as predicted by the nested model. This is in good agreement with the equilibrium constant found experimentally (0.55 ± 0.18) for the alcoholysis reaction performed in the absence of *n*-hexane. Values reported in the literature concerning $K_{m,BuOH}$ and $K_{m,EtBu}$ for this type of reaction are scarce; Chulalaksananukul *et al.* (1990), Rizzi *et al.* (1992) and Combes (1996) reported values for $K_{m,EtOH}$ of 190, <0.1 and 600 mmol l^{-1}, respectively, when using *Mucor miehei* lipase; these values are of the same order of magnitude of those found in our modeling effort. Carvalho *et al.* (1997) reported a value of 292 mmol l^{-1} for $K_{m,BuOH}$ concerning alcoholysis of hexanol and butyl acetate in a reverse micellar membrane bioreactor, as brought about by *Fusarium solani-pisi* lipase.

Values for v_f and v_r found in the literature for lipases range in 13–1056 mmol $l^{-1}h^{-1}$ (e.g. Miller *et al.*, 1991; Rizzi *et al.*, 1992; Carvalho *et al.*, 1997) and 41–1290 mmol $l^{-1}h^{-1}$ (e.g. Miller *et al.*, 1991; Rizzi *et al.*, 1992; Garcia *et al.*, 1992; Carvalho *et al.*, 1997), respectively, depending on the type of reaction, initial concentration of substrate, and source and amount of lipase used. In the present work, and since the initial Lipozyme™ concentration was *ca.* 10 $g_{Lipozyme}$ l^{-1}, specific v_f values found for the nested model (Table I) range from 110 to

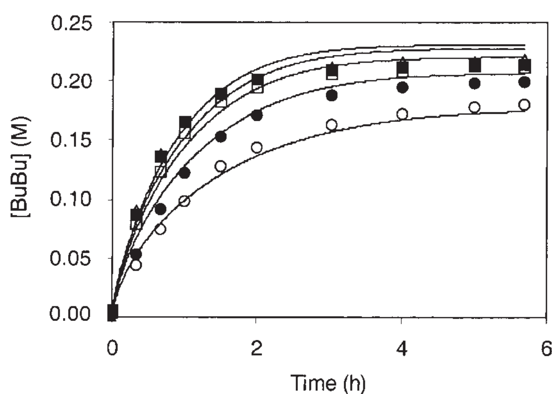

FIGURE 4 Evolution of the concentration of BuBu as a function of time for a constant initial concentration of BuOH (0.25 M) and several initial concentrations of EtBu: 1 M (○), 2 M (●), 3 M (□), 4 M (■) and 5 M (△). The fit by the nested model is also indicated (—).

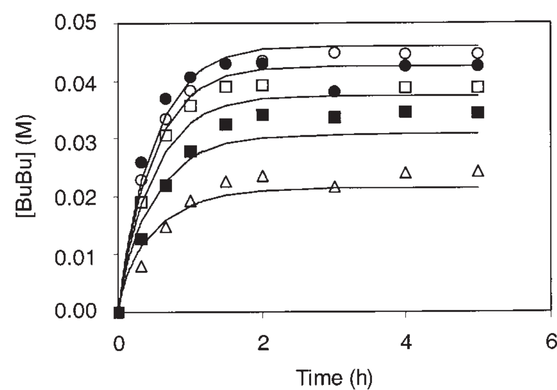

FIGURE 6 Evolution of the concentration of BuBu as a function of time for a constant initial concentration of BuOH (0.005 M) and several initial concentrations of EtBu: 0.05 M (○), 0.125 M (●), 0.25 M (□), 0.5 M (■) and 1 M (△). The fit by the nested model is also indicated (—).

TABLE II F-test results for model nesting

Source	Residual sum of squares (RSS)	Degrees of freedom (ν)	Mean square (s^2)	F-ratio	p-Value
Extra parameters (e)	$RSS_e = RSS_n - RSS_f = 1.814 \times 10^{-2}$	$\nu_e = P_f - P_n = 190$	$s_e^2 = RSS_e/\nu_e = 9.551 \times 10^{-5}$	$s_e^2/s_f^2 = 8.537 \times 10^{-1}$	9.05×10^{-1}
Full model (f)	$RSS_f = 7.161 \times 10^{-2}$	$\nu_f = N - P_f = 640$	$s_f^2 = RSS_f/\nu_f = 1.119 \times 10^{-4}$		
Nested model (n)	$RSS_n = 8.975 \times 10^{-2}$	$\nu_n = N - P_n = 830$			

TABLE III F-test results for simplified model nesting

Source	Residual sum of squares (RSS)	Degrees of freedom (ν)	Mean square (s^2)	F-ratio	p-Value
Extra parameters (e)	$RSS_e = RSS_n - RSS_m = 1.237 \times 10^{-2}$	$\nu_e = P_n - P_s = 4$	$s_e^2 = RSS_e/\nu_e = 3.093 \times 10^{-3}$	$s_e^2/s_m^2 = 28.60$	2.715×10^{-22}
Nested model (n)	$RSS_n = 8.975 \times 10^{-2}$	$\nu_m = N - P_n = 830$	$s_m^2 = RSS_m/\nu_m = 1.081 \times 10^{-4}$		
Simplified model ($K_{m,EtBu} \geqslant K_{m,j \neq EtBu}$) (s)	$RSS_s = 1.021 \times 10^{-1}$	$\nu_n = N - P_s = 834$			
Extra parameters (e)	$RSS_e = RSS_n - RSS_m = 1.163 \times 10^{-1}$	$\nu_e = P_n - P_s = 6$	$s_e^2 = RSS_e/\nu_e = 1.939 \times 10^{-2}$	$s_e^2/s_m^2 = 179.3$	4.401×10^{-146}
Nested model (n)	$RSS_n = 8.975 \times 10^{-2}$	$\nu_m = N - P_n = 830$	$s_m^2 = RSS_m/\nu_m = 1.081 \times 10^{-4}$		
Simplified model ($K_{m,BuOH} \geqslant K_{m,j \neq BuOH}$) (s)	$RSS_s = 2.061 \times 10^{-1}$	$\nu_n = N - P_s = 836$			
Extra parameters (e)	$RSS_e = RSS_n - RSS_m = 8.738 \times 10^{-4}$	$\nu_e = P_n - P_s = 6$	$s_e^2 = RSS_e/\nu_e = 1.456 \times 10^{-4}$	$s_e^2/s_m^2 = 1.347$	2.337×10^{-1}
Nested model (n)	$RSS_n = 8.975 \times 10^{-2}$	$\nu_m = N - P_n = 830$	$s_m^2 = RSS_m/\nu_m = 1.081 \times 10^{-4}$		
Simplified model ($K_{m,BuBu} \geqslant K_{m,j \neq BuBu}$) (s)	$RSS_s = 9.062 \times 10^{-2}$	$\nu_n = N - P_s = 836$			
Extra parameters (e)	$RSS_e = RSS_n - RSS_m = 1.045 \times 10^{-1}$	$\nu_e = P_n - P_s = 5$	$s_e^2 = RSS_e/\nu_e = 2.090 \times 10^{-2}$	$s_e^2/s_m^2 = 193.2$	1.832×10^{-136}
Nested model (n)	$RSS_n = 8.975 \times 10^{-2}$	$\nu_m = N - P_n = 830$	$s_m^2 = RSS_m/\nu_m = 1.081 \times 10^{-4}$		
Simplified model ($K_{m,EtOH} \geqslant K_{m,j \neq EtOH}$) (s)	$RSS_s = 1.942 \times 10^{-1}$	$\nu_n = N - P_s = 835$			

194 mmol $g_{Lipozyme}^{-1}$ h^{-1}, whereas v_r values range from 109 to 313 mmol $g_{Lipozyme}^{-1}$ h^{-1}. Specific v_{max} values found in the literature for Lipozyme™ are scarce, and encompass mainly esterification and transesterification reactions carried out in *n*-hexane, e.g. values of 2.125 and 342 mmol $g_{Lipozyme}^{-1}$ h^{-1} were reported by Chulalaksananukul *et al.* (1990; 1992).

Due to the still high mathematical complexity of the nested model considered (which involves 10 adjustable parameters), one has further studied the effect of dropping out some parameter(s) in order to produce a simpler model. In practical terms, $K_{m,i}$ (where i denotes either EtBu, BuOH, BuOH or BuBu) represents the Michaelis–Menten dissociation constant for each of the compounds from the enzyme-complex; this means that when such parameter assumes high values, there is no evidence of saturation in the concentration range studied or, in other words, there is no affinity of the enzyme to the compound in question. In order to study the adequacy of such hypothesis, the model equation was reformulated by assuming that the dissociation constants $K_{m,i}$ assume, one at a time, values much higher than those assumed by the remaining $K_{m,j\neq i}$ (mathematically, that corresponds to considering that $K_{m,i}$ assumes infinite values when compared to $K_{m,j\neq i}$), thus yielding four simpler models which were separately fitted to the known data (again using the GREG software).

Given the parameters obtained in each situation, F-tests were performed on the associated residual sum of squares in order to investigate the statistical likelihood of such simplifications when compared with the nested model; results obtained therefrom are represented in Table III. It can be concluded that, at the 5% level of statistical significance, only the model where $K_{m,BuBu}$ assumes values much higher than those associated with the other compounds can appropriately replace the nested model; in practical terms, this means that the enzyme exhibits negligible affinity for BuBu. The resulting rate expression reads

$$r = \frac{v_f v_r \left\{[EtBu][BuOH] - \frac{[BuBu][EtOH]}{K_{eq}}\right\}}{v_f \frac{K_{m,BuBu}}{K_{eq}}[EtOH] + v_r[EtBu][BuOH] + \frac{v_f}{K_{eq}}[BuBu][EtOH]} \quad (3)$$

From the same table, one also realizes that it is not possible to draw definite conclusions on the affinity of the enzyme towards the other components. Parameters obtained for such simplest model (as well as their confidence intervals) are presented in Table IV.

Comparing the values listed in Tables I and IV, one can see that, as expected, the values for K_{eq} are of the same order of magnitude. However, the values of both v_f and v_r obtained from the fitting of the simpler model are lower than those predicted by the nested model, which lie beyond the inference interval associated therewith.

TABLE IV Estimates of the simplest model parameters (when $K_{m,BuBu} \gg K_{m,l\neq BuBu}$)

Model parameter	Value	95% confidence interval
v_f (mol l^{-1} h^{-1})	2.909×10^{-1}	8.317×10^{-2}
v_r (mol l^{-1} h^{-1})	3.601×10^{-2}	1.193×10^{-2}
K_{eq} (–)	4.712×10^{-1}	8.910×10^{-2}
$K_{m,BuBu}$ (mol l^{-1})	2.514×10^{-1}	4.937×10^{-1}
RSS* (mol l^{-1})^2	9.062×10^{-2}	

* Residual sum of squares.

As a final remark, if one compares the value of $K_{m,BuBu}$ obtained in this simplification with the values for the various dissociation constants in Table I, one can see that the order of magnitude of the estimate of $K_{m,BuBu}$ is 10- to 100-fold higher (even though no confidence intervals could be calculated for $K_{m,EtBu}$, $K_{m,BuOH}$ and $K_{m,EtOH}$), thus supporting our previous rationale.

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NOMENCLATURE

BuBu	butyl butyrate
BuOH	butanol
C	molar concentration (mol l^{-1})
EtBu	ethyl butyrate
EtOH	ethanol
K_{eq}	equilibrium constant (dimensionless)
$K_{i,a}$	constant for the dissociation of compound a from the specific enzyme form–inhibitor complex (mol l^{-1})
$K_{I,i}$	inhibition constant for compound i (mol l^{-1})
$K_{m,i}$	Michaelis–Menten constant for compound i (mol l^{-1})
L	lipase
L*	modified lipase
M	number of measurements (dimensionless)
n	nested model
N	number of sampling times (dimensionless)
P	generic product
RSS	Residual sum of squares (mol^2 l^{-2})

s simpler model
S generic substrate
r rate of reaction ($mol\,l^{-1}\,h^{-1}$)
t reaction time (h)
v_0 specific initial reaction rate ($mmol\,g^{-1}\,h^{-1}$)
v_f maximal velocity for the forward reaction ($mol\,l^{-1}\,h^{-1}$)
v_r maximal velocity for the reverse reaction ($mol\,l^{-1}\,h^{-1}$)

References

Balcão, V.M., Paiva, A.L. and Malcata, F.X. (1996) "Bioreactors with immobilized lipases: state of the art", Enz. Microb. Technol. 18, 392–416.

Basheer, S., Cogan, U. and Nakajima, M. (1998) "Esterification kinetics of long-chain fatty acids and fatty alcohols with a surfactant-coated lipase in n-hexane", J. Am. Oil Chem. Soc. 75, 1785–1790.

Bates, D.M. and Watts, D.G. (1988) Nonlinear Regression Analysis and its Applications (Wiley, New York).

Brady, L., Brzozowski, A.M., Derewenda, Z.S., Dodson, E., Dodson, G., Tolley, S., Turkenburg, J.P., Christiansen, L., Huge-Jensen, B., Norskov, L., Thim, L. and Menge, U. (1990) "A serine protease triad forms the catalytic centre of a triacylglycerol lipase", Nature 343, 767–770.

Brzozowski, A.M., Derewenda, U., Derewenda, Z.S., Dodson, G., Lawson, D.M., Turkenburg, J.P., Bjorkling, F., Huge-Jensen, B., Patkar, S.A. and Thim, L. (1991) "A model for interfacial activation in lipases from the structure of a fungal lipase inhibitor complex", Nature 351, 491–494.

Caracatosios, M., Stewart, W.E. and Sørensen, J.P. (1985) GREG User's Manual (Department of Chemical Engineering, University of Wisconsin, Madison, WI).

Carvalho, C.M.L., Coonnah, P., Aires-Barros, M.R. and Cabral, J.M.S. (1997) "A performance study of reverse micellar membrane bioreactor for alcoholysis reactions catalyzed by cutinase", Proceedings of the International Meeting on Lipases and Lipids: Structure, Specificity and Applications in Biocatalysis, Como, Italy, 17–20 September, p. 50.

Chulalaksananukul, W., Condoret, J.S., Delorme, P. and Willemot, R.M. (1990) "Kinetic study of esterification by immobilized lipase in n-hexane", FEBS Lett. 276, 181–184.

Chulalaksananukul, W., Condoret, J.S. and Combes, D. (1992) "Kinetics of geranyl acetate synthesis by lipase-catalyzed transesterification in n-hexane", Enzyme Microb. Technol. 14, 293–298.

Chulalaksananukul, W., Condoret, J.S. and Combes, D. (1993) "Geranyl acetate synthesis by lipase-catalyzed transesterification in supercritical carbon dioxide", Enzyme Microb. Technol. 15, 691–698.

Combes, D. (1996) "Kinetics of lipase catalyzed reactions in non-conventional media", In: Malcata, F.X., ed, Engineering of/with Lipases (Kluwer, Dordrecht, The Netherlands), pp. 289–295.

Fureby, A.M., Tian, L., Adlercreutz, P. and Mattiasson, B. (1997) "Preparation of diglycerides by lipase-catalyzed alcoholysis of triglycerides", Enzyme Microb. Technol. 20, 198–206.

Garcia, H.S., Malcata, F.X., Hill, C.G. and Amundson, C.H. (1992) "Use of Candida rugosa lipase immobilized in a spiral wound membrane reactor for the hydrolysis of milkfat", Enzyme Microb. Technol. 14, 535–545.

Goto, M., Kamiya, N., Miyata, M. and Nakashio, F. (1994) "Enzymatic esterification by surfactant-coated lipase in organic media", Biotechnol. Prog. 10, 263–268.

Gunnlaugsdottir, H., Wannerberger, K. and Sivik, B. (1998) "Alcoholysis and glyceride synthesis with immobilized lipase on controlled-pore glass of varying hydrophobicity in supercritical carbon dioxide", Enzyme Microb. Technol. 22, 360–367.

Huang, S.Y. and Chang, H.L. (1999) "Kinetic study on the esterification of geraniol and acetic acid in organic solvents using a surfactant-coated lipase", J. Chem. Technol. Biotechnol. 74, 183–187.

Jaeger, K.E., Ransac, S., Dijkstra, B.W., Colson, C., van Heuvel, M. and Misset, O. (1994) "Bacterial lipases", FEMS Microbiol. Rev. 15, 29–63.

Kamiya, N. and Goto, M. (1997) "How is enzymatic selectivity of menthol esterification catalyzed by surfactant-coated lipase determined in organic media?", Biotechnol. Prog. 13, 488–492.

Kierkels, J.G.T., Vleugels, L.F.W., Gelade, E.T.F., Vermeulen, D.P., Kamphuis, J. and Wandrey, C. (1994) "Pseudomonas fluorescens lipase adsorption and the kinetics of hydrolysis in a dynamic emulsion system", Enzyme Microb. Technol. 16, 513–521.

Langrand, G., Baratti, J., Buono, G. and Trantaphylides, C. (1988) "Enzymatic separation and resolution of nucleophiles: a predictive kinetic model", Biocatalysis 1, 231–248.

Malcata, F.X., Hill, C.G. and Amundson, C.H. (1991) "Hydrolysis of butteroil by immobilized lipase using a hollow fiber reactor: part II. Uniresponse kinetic studies", Biotechnol. Bioeng. 39, 984–1001.

Malcata, F.X., Hill, C.G. and Amundson, C.H. (1992a) "Hydrolysis of butteroil by immobilized lipase using a hollow fiber reactor: part III. Multiresponse kinetic studies", Biotechnol. Bioeng. 39, 1002–1012.

Malcata, F.X., Hill, C.G. and Amundson, C.H. (1992b) "Hydrolysis of butteroil by immobilized lipase using a hollow fiber reactor: part IV. Effects of temperature", Biotechnol. Bioeng. 39, 1097–1111.

Malcata, F.X., Hill, C.G. and Amundson, C.H. (1993) "Hydrolysis of butteroil by immobilized lipase using a hollow fiber reactor: part VI. Multiresponse analysis of temperature and pH effects", Biocatalysis 8, 201–228.

Marty, A., Chulalaksananukul, W., Willemot, R.M. and Condoret, J.S. (1992) "Kinetics of lipase-catalyzed esterification in supercritical CO_2", Biotechnol. Bioeng. 39, 273–280.

Miller, D.A., Prausnitz, J.M. and Blanch, H.W. (1991) "Kinetics of lipase-catalyzed interesterification of triglycerides in cyclohexane", Enzyme Microb. Technol. 13, 98–103.

Millqvist, A., Adlercreutz, P. and Mattiasson, B. (1994) "Lipase-catalyzed alcoholysis of triglycerides for the preparation of 2-monoglycerides", Enzyme Microb. Technol. 16, 1042–1047.

Mukesh, D., Banerji, A.A., Newadkar, R. and Bevinkatti, H.S. (1993) "Mathematical modelling of enzymatic butanolysis of vegetable oils", Biocatalysis 8, 191–199.

Mukesh, D., Jadhav, S., Banerji, A.A., Thakkar, K. and Bevinakatti, H.S. (1997) "Lipase-catalyzed esterification reactions — experimental and modelling studies", J. Chem. Technol. Biotechnol. 69, 179–186.

Paiva, A.V., Balcão, V.M. and Malcata, F.X. (2000) "Kinetics and mechanisms of reactions catalyzed by immobilized lipases", Enzyme Microb. Technol. 27, 187–204.

Ramamurthi, S. and McCurdy, A.R. (1994) "Lipase catalyzed esterification of oleic acid and methanol in hexane—a kinetic study", J. Am. Oil Chem. Soc. 71, 927–930.

Rice, K.E., Watkins, J. and Hill, C.G. (1999) "Hydrolysis of menhaden oil by a Candida cylindracea lipase immobilized in a hollow fiber reactor", Biotechnol. Bioeng. 63, 33–45.

Rizzi, M., Stylos, P., Riek, A. and Reuss, M. (1992) "A kinetic study of immobilized lipase catalysing the synthesis of isoamyl acetate by transesterification in n-hexane", Enzyme Microb. Technol. 14, 709–714.

Rogalska, E., Ransac, S., Douchet, I. and Verger, R. (1994) "Lipase stereoselectivity depends on the 'interfacial quality'", Proceedings of the Closing Meeting of the EC BRIDGE Lipase T-Project: Characterization of Lipases for Industrial Application — 3D Structure and Catalytic Mechanism, Bendor Island, Bandol, France, 14–17 September, p. 44.

Segel, I.H. (1993) Enzyme Kinetics—Behavior and Analysis of Rapid Equilibrium and Steady-state Enzyme Systems (Wiley, New York).

Shimada, Y., Sugihara, A., Yodono, S., Nagao, T., Maruyama, K., Nakano, H., Komemushi, S. and Tominaga, Y. (1997) "Enrichment of ethyl docosahexaenoate by selective alcoho-

lysis with immobilized *Rhizopus delemar* lipase", *J. Ferment. Bioeng.* 84, 138–143.
Shimada, Y., Maruyama, K., Sugihara, A., Baba, T., Komemushi, S., Moriyama, S. and Tominaga, Y. (1998) "Purification of ethyl docosahexanoate by selective alcoholysis of fatty acid ethyl esters with immobilized *Rhizomucor miehei* lipase", *J. Am. Oil Chem. Soc.* 75, 1565–1571.
Stamatis, H., Xenakis, A., Menge, U. and Kolisis, F.N. (1993) "Kinetic study of lipase catalyzed esterification reactions in water-in-oil microemulsions", *Biotechnol. Bioeng.* 42, 931–937.
van Tilbeurgh, H., Sarda, L., Verger, L. and Cambillau, C. (1992) "Structure of the pancreatic lipase–procolipase complex", *Nature* 359, 159–162.
van Tol, J.B.A., Jongejan, J.A., Duine, J.A., Kierkels, H.G.T., Geladé, E.F.T., Mosterd, F., van der Tweel, W.J.J. and Kamphuis, J. (1995) "Thermodynamic and kinetic parameters of lipase-catalyzed ester hydrolysis in biphasic systems with varying organic solvents", *Biotechnol. Bioeng.* 48, 179–189.
Winkler, F.K., d'Arcy, A. and Hunziker, W. (1990) "Structure of human pancreatic lipase", *Nature* 343, 771–774.
Yong, Y.P. and Al-Duri, B. (1996) "Kinetic studies on immobilized lipase esterification of oleic acid and octanol", *J. Chem. Technol. Biotechnol.* 65, 239–248.