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Regular article

Kinetic studies on the transesterification of sunflower oil with 1-butanol catalyzed by *Rhizomucor miehei* lipase in a biphasic aqueous-organic system

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ABSTRACT

The kinetics of sunflower oil transesterification with 1-butanol using a homogeneous lipase (*Rhizomucor miehei*) in an aqueous-organic biphasic system were studied in a stirred batch reactor set-up. An initial screening study was performed to optimize relevant process conditions (enzyme concentration, stirring speed, 1-butanol to oil ratio). A kinetic model was developed based on concentration time profiles. Important reaction parameters were the oil concentration in *n*-hexane, the enzyme concentration, the 1-butanol to oil molar ratio; the organic to an aqueous solvent volume ratio, the temperature, the pH setting using a phosphate buffer and the agitation rate. The experimental data were modelled using the Ping Pong Bi Bi mechanism with non-competitive inhibition by 1-butanol and a term for irreversible enzyme deactivation during reaction. Agreement between model and experiments was good ($R^2 = 0.99$, average error 4.2%).

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1. Introduction

Biodiesel is commercially produced by a transesterification reaction of vegetable oil with an alcohol, in most cases methanol, in the presence of a catalyst. Alkaline catalysts, such as sodium hydroxide, are commonly used due to their low costs and high reaction rates. However, such catalysts also have several drawbacks. For instance, the alkaline catalyst has to be removed from the product, alkaline waste water requires treatment, and free fatty acids and water interfere with the reaction. An alternative for basic catalysts is the use of enzymes. Enzymes like triacylglycerol lipase (EC 3.1.1.3) effectively catalyze the transesterification of a triglyceride in either aqueous or non-aqueous systems. The advantages of enzyme catalysis include a lower reaction temperature, ease of glycerol work-up, and the possibility to process oils with a high free fatty acid and water content [1,2].

Enzymatic conversions of vegetable oils or acids derived thereof may be carried out in a single phase, using a co-solvent or a biphasic liquid-liquid system. Both free, soluble enzymes as well as immo-

bilized enzymes may be applied. Well known examples are lipases from *Candida rugosa* and *Rhizomucor miehei* [2]. In the research reported here, a biphasic liquid-liquid approach is used using a free enzyme. Advantages of the use of free enzymes instead of immobilized enzymes are the absence of possible intraparticle mass transfer limitations that may limit the overall reaction rate [3] and a lower price [4]. A disadvantage for practical applications of free enzymes in terms of reutilization, as compared to immobilized enzymes, is the more elaborate recovery of the enzyme. Another possible disadvantage of free enzymes is a lower thermostability, both at storage and operating conditions [5]. Generally a higher reaction performance can be achieved with immobilized enzymes in non-aqueous media [6]. Despite this, under anhydrous conditions the free lipase of *Rhizomucor miehei* is stable and has a high esterification activity as compared to other free lipases [7]. The biphasic aqueous-organic system for enzymatic transesterification of triglycerides offers several advantages when compared to a single phase system. The presence of water during the transesterification reaction is known to increase the enzyme activity and to prevent hydrophobic substrate and/or product inhibition [8–10]. However, the use of water may lead to incomplete conversions due to equilibrium constraints and the formation of fatty acids. By using a biphasic concept, equilibrium constraint may be partly overcome

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Nomenclature

BuOH	Butanol
C	Concentration (gr L^{-1})
DG	Diglyceride
FABE	Fatty acid butyl ester
FFA	Free fatty acid
Gly	Glycerol
GlyOH	Glycerol hydroxyl group
k	Kinetic reaction constant ($\text{mol L}_{\text{aq}} \text{L}_{\text{org}}^{-1} \text{g}^{-1} \text{s}^{-1}$)
k_i	Kinetic deactivation constant ($\text{mol L}_{\text{aq}} \text{L}_{\text{org}}^{-1} \text{g}^{-1} \text{s}^{-1}$)
K_i	Inhibition constant (mol L^{-1})
K_m	Michaelis Menten constant (mol L^{-1})
MG	Monoglyceride
n	Number of moles (mol)
P	Partition coefficient
R^2	Coefficient of determination
RML	<i>Rhizomucor miehei</i> lipase
r_p	Formation rate of fatty acid butyl ester (FABE) ($\text{mol L}_{\text{org}}^{-1} \text{min}^{-1}$)
t	Time (minute)
V	Volume (L)
V_{max}	Maximum enzyme velocity ($\text{mol L}_{\text{org}}^{-1} \text{s}^{-1}$)

Subscripts

A	1-butanol
Ac	Acyl
aq	Aqueous phase
DG	Diglyceride
eff(t)	Effective at t
enz	Enzyme
FABE	Fatty acid butyl ester
MG	Monoglyceride
org	Organic phase
S	Oil
TG	Triglyceride
0	Initial

and the rate of hydrolysis may be reduced. In addition, the use of a biphasic system also make glycerol and enzyme recovery from the reaction mixture easier and increases the possibility of reusing the enzyme [10,11].

Kinetics studies on the transesterification of vegetable oils with methanol using enzymes have been reported [12–14]. Most of the studies involve the use of an immobilized lipase. In some cases, an additional organic solvent is added and for these systems also a number of kinetic studies are available [15–17]. Al-Zuhair et al. studied vegetable oil methanolysis catalyzed by *R. miehei* lipase in *n*-hexane as the organic solvent. The results were modelled using a Ping Pong bisubstrate mechanism with competitive inhibition of methanol and oil [17]. Confidence factors of this model were not reported.

A limited number of (kinetic) studies on the transesterification of vegetable oils catalyzed by enzymes in oil-water biphasic systems have been reported (Table 1). The reaction is assumed to involve two-steps, viz hydrolysis of the triglyceride to free fatty acids (FFA) followed by a subsequent esterification of the FFAs with the alcohol. Competitive inhibition of oil and alcohol were observed [8,18,19]. However, kinetic modelling to obtain rate equations for the individual reactions was performed in one case only (Table 1). To the authors best knowledge, studies on the enzymatic transesterification of vegetable oils using 1-butanol in a biphasic aqueous-organic system have not been reported so far.

Fig. 1. Sunflower oil transesterification with 1-butanol.

Of relevance for this study are related enzyme catalyzed esterification reactions of fatty acids (oleic acid) and short chain acids with alcohols in biphasic systems. We have recently reported a kinetic model for the esterification of oleic acid 1-butanol using *R. miehei* lipase in a biphasic *n*-heptane-water system. The data were modelled using a Ping Pong mechanism with alcohol inhibition. The model included a constant partition coefficient of 1-butanol over the two phases. Parameter estimations were performed with high accuracy [20].

In this study we report a kinetic study on the sunflower oil transesterification with 1-butanol using a free lipase (*Rhizomucor miehei*) in aqueous-organic biphasic system given fatty acid butyl esters (FABE) and glycerol (Fig. 1). 1-Butanol is an interesting chemical for transesterification of vegetable oils because it can be obtained in a sustainable way via fermentation of biomass. This way it can also be made available in remote areas in developing countries. From a chemical point of view, the use of 1-butanol can be beneficial because of its greater solubility in the organic phase thereby facilitating the transesterification reaction that is assumed to take place in the organic phase.

In the first exploratory phase, the effect of substrate, product and enzyme concentration on the initial rate was determined. In the second stage, a kinetic study was performed for the reaction at variable oil intakes with all other parameters set at optimum values as determined during the exploratory studies. The experimental data were modelled using a Ping-Pong Bi-Bi mechanism.

2. Materials and methods

2.1. Materials

Commercial sunflower oil produced by Vandermoortele BV, Belgium was used as the substrate. Ethanol (absolute) and acetic acid (100%) were obtained from EMSURE®, 1-butanol (99%) and ethyl oleates (98%) were from Sigma-Aldrich. The *Rhizomucor miehei* lipase in solution ($\geq 20,000$ Unit/g) was obtained from Sigma-Aldrich. *n*-Hexane (analytical reagent) was obtained from Lab-Scan. *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (98.5%), pentadecane (99%), and buffer compounds ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 99% and $\text{NaH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$, 98%) were obtained from Sigma-Aldrich. For GC-calibration analytical standards (palmitic, linoleic, oleic and stearic acid, $\geq 99\%$) were obtained from Sigma-Aldrich, as well as ethyl palmitate ($\geq 99\%$), ethyl linoleate ($\geq 99\%$), ethyl oleate (98%), ethyl stearate ($\geq 99\%$ for capillary GC), 1-oleoyl-glycerol ($\geq 99\%$), 1,3-diolein ($\geq 99\%$ for GC) and triolein.

2.2. Methods

2.2.1. Experimental set up

Experiments were performed in a 300 mL batch reactor made of glass. The reactor was equipped with a heating jacket connected to a temperature controlled water bath. Stirring was provided by a twin agitator (Fig. 2). The reactor was pre-heated to 40 °C before adding the substrates. Oil, alcohol and organic solvent were added

Table 1
Overview of kinetic studies of biphasic aqueous oil lipase catalyzed (trans)esterification reactions.

System	Reaction	Substrates	Enzyme	Mechanism	Model	Ref.
Organic solvent	Trans-esterification	Palm oil, methanol	<i>R. miehei</i> lipase	Ping Pong	yes	[17]
Biphasic; aqueous-oil	Trans-esterification	Sunflower oil, methanol	<i>R. miehei</i> lipase	–	no	[8]
Biphasic; aqueous-oil	Trans-esterification	Soybean oil, methanol	NS81006	4 step equilibrium	yes	[18]
Biphasic; aqueous-oil	Trans-esterification	Soybean oil, ethanol	NS81006	–	no	[19]
Biphasic; aqueous-organic	Esterification	Oleic acid, butanol	<i>R. miehei</i> lipase	Ping Pong	yes	[20]
Biphasic; aqueous-organic	Esterification	Oleic acid, ethanol	<i>R. miehei</i> lipase	Ordered bisubstrate	yes	[21]
Biphasic; aqueous-organic	Esterification	Butyric acid, methanol	<i>R. miehei</i> lipase	Ping Pong	yes	[22]

Fig. 2. Schematic representation of the batch reactor set-up.

to the reactor first, followed by the aqueous enzyme solution. The stirring speed was set to 800 rpm based on a previous study [20].

2.2.2. Effect of the enzyme concentration on the initial rates

Initial experiments were performed to identify the enzyme concentration that is most suitable for the kinetic experiments. The conditions were as follows: *n*-hexane (45 mL), aqueous phosphate buffer (15 mL, 50 mM, pH 6 (6.80 g L⁻¹ Na₂HPO₄·2H₂O and 1.72 g L⁻¹ NaH₂PO₄·7H₂O)), sunflower oil (40 g L_{org}⁻¹), 1-butanol (alcohol to oil molar ratio of 6) and lipase concentrations of 10, 20, 50 and 200 g L_{aq}⁻¹.

2.2.3. Kinetic experiments

Experiments were performed in a biphasic system consisting of *n*-hexane (45 mL) and an aqueous phosphate buffer (15 mL, 50 mM, pH 6.0, with a lipase concentration of 20 g L_{aq}⁻¹). Eight experiments were performed with a variable 1-butanol to oil molar ratio (0–12 mol/mol) with a constant oil concentration of 40 g L_{org}⁻¹. In addition, eight experiments were performed at different oil concentrations (15–100 g L_{org}⁻¹) with a constant 1-butanol to oil molar ratio of 6. The experimental error in the initial rates for the kinetic experiments was determined for an experiment performed in triplicate at 40 g L_{org}⁻¹ oil concentration and 1-butanol to oil molar ratio of 6. This error was used as an estimation for the relative error of the other data points.

To study possible inhibition of the oil, hydrolysis experiments using various initial oil concentrations (10–100 g L_{org}⁻¹) were performed in the presence of lipase (20 g L_{aq}⁻¹). Glycerol inhibition was investigated by doing experiments with initial glycerol concentrations from 0 to 100% mol/mol relative to the amount of oil with a constant oil concentration of 40 g L_{org}⁻¹, a constant 1-butanol to oil molar ratio of 6, and a lipase concentration of 20 g L_{aq}⁻¹.

Ester inhibition was investigated by performing experiments with initial ethyl oleate concentration of 0, 50, and 100% mol/mol

compared to the amount of oil using 40 g L_{org}⁻¹ oil, 20 g L_{aq}⁻¹ enzyme, and a constant alcohol to oil molar ratio of 6. In this case, ethanol was used instead of 1-butanol. Experimental errors for oil, glycerol and ester inhibition experiments were based on experiments performed in duplicate.

2.2.4. Analytical methods

The composition of the organic phase was determined using GC-FID. Samples (1 mL) were taken in duplicate from the reaction mixture at several time intervals with a syringe. These were immediately quenched with absolute acetic acid (0.1 mL) to inactivate the enzyme. The samples were shaken and centrifuged for 5 min to separate the organic and aqueous layers. For GC sample preparation, a fixed amount of a pentadecane solution in *n*-hexane as internal standard (2000 ppm) was added and MSTFA was used for derivatization. A sample of the organic layer (20 µL) was transferred to an auto sampler vial and pentadecane solution (80 µL), MSTFA (20 µL) and *n*-hexane (1500 µL) were added. Subsequently the samples were heated to 65 °C for 1 h in an oven.

A Hewlett-Packard (HP) 5890 series II GC equipped with 15 m long J&W Select Biodiesel (UltiMetal) column from Agilent Technologies was used for the measurements. Helium was used as a gas carrier at a column pressure of 22.1 kPa. Before injection, the injection needle was washed with hexane for 3 times and a cold injection of 1 µL was done on-column at room temperature. The oven temperature was initially set on 40 °C for 1 min. Temperature was increased to 180 °C at a rate of 15 °C min⁻¹. Subsequently the rate was increased to 20 °C min⁻¹ until the temperature reached 380 °C, this was held for 5 min. The detector temperature was set on 400 °C and the injector temperature was set to 40 °C. The average relative error was 2.76% based on the standard deviations of 115 data points derived from multiple samples and analyses.

GC measurements with pure components were carried out for product identification. Sample solutions of glycerol, palmitic, oleic, linoleic and stearic acid as well as ethyl palmitate, ethyl linoleate, ethyl oleate, ethyl stearate, 1-oleoyl-glycerol, 1,3-diolein, triolein and sunflower oil in *n*-hexane were prepared (100 mg in 10 mL). 20 µL of these solutions were taken and 20 µL MSTFA, 80 µL pentadecane solution (2000 ppm) and 1500 µL *n*-hexane were added. Butyl esters were not measured independently but were identified by their corresponding shifts in retention time compared to the measured ethyl esters.

The amounts of butyl esters and free fatty acids were quantified using GC. For this purpose, sample solutions containing 100 mg oleic acid and ethyl oleate in 10 mL of *n*-hexane were made. A number of GC samples were prepared at different concentrations by taking a certain amount of the sample solutions (5–80 µL) and by subsequently adding 20 µL MSTFA, 80 µL pentadecane solution in *n*-hexane (2000 ppm) and 1500 µL *n*-hexane. Measurements of these solutions allowed calculation of the relative response factors (RRF) of oleic acid and ethyl oleate. It is assumed that RRF for the different free fatty acids and for the butyl and ethyl esters are essentially similar when applying a correction for the difference in the number of C-atoms [23].

Fig. 3. Effect of enzyme concentration on initial rates for fatty acid butyl ester (FABE). Experimental conditions: *n*-hexane=45 mL; phosphate buffer=15 mL, pH 6.0; sunflower oil=40 g L⁻¹; 1-butanol to oil molar ratio of 6; lipase=5–200 g L⁻¹; stirrer speed=800 rpm; 40 °C. Line is for illustrative purpose only.

2.2.5. Modelling tools

The first phase of the kinetic study involved evaluation of initial reaction rates for FABE. The initial reaction rate for each experiment was obtained from the experimental concentration-time profile by linear regression of the data over the first two minutes. For the subsequent development of the kinetic model, experimental concentration-time profiles were used as input.

The kinetic equations were fitted to the data points using MATLAB Mathworks 2014. The model was solved using the numerical integration toolbox *ode45*. Kinetic parameters were estimated by minimization of the sum of squared errors between the experimental data and simulated data from the model. Error minimization was done using a *lsqnonlin* toolbox. Confidence limits (CF) of the different parameters for the complete fit were calculated.

3. Results and discussion

3.1. Evaluation of enzyme concentrations on the initial reaction rates

The effect of enzyme concentration on the initial rate of FABE formation was determined by performing a number of experiments at variable enzyme concentration (5–200 g L⁻¹) while keeping all other variables at a constant value (*n*-hexane (45 mL); aqueous phosphate buffer (15 mL, pH 6.0); sunflower oil (40 g L⁻¹); 1-butanol to oil mol ratio of 6; stirrer speed of 800 rpm; 40 °C). The results (Fig. 3) showed that the initial rates increase linearly with the enzyme concentrations when the lipase concentration is below 50 g L⁻¹.

Higher concentrations of enzyme did not lead to an increase in the initial rate and the rate seems to be independent of the enzyme concentration at lipase concentrations above 50 g L⁻¹. The observed relationship between the initial rate and enzyme concentration indicates that the enzyme becomes saturated at a concentration above about 50 g L⁻¹. A possible explanation is the available surface area for reaction. The phase ratio (*V*_{org}/*V*_{aq}) used in the experiments is 3, and as such the system may be regarded as a water-in-organic micro-emulsion or reversed micellar system [24]. The enzyme is expected to be present at the interface of the two immiscible liquids [10]. It is well possible that at an enzyme concentration of 50 g L⁻¹, the surface is fully covered with enzyme. As such, higher enzyme concentrations will not lead to higher rates, as experimentally observed. For the kinetic experiments, an enzyme

Fig. 4. Effect of the 1-butanol to oil molar ratio on the initial rate of fatty acid butyl ester (FABE) (■) and free fatty acids (FFA) (●). Experimental conditions: *n*-hexane=45 mL; phosphate buffer=15 mL, pH 6.0; sunflower oil=40 g L⁻¹; lipase=20 g L⁻¹; stirrer speed=800 rpm; 40 °C. Lines are for illustrative purpose only. See experimental section for error estimation.

concentration of 20 g L⁻¹ was used. The concentration was low enough to avoid enzyme saturation and the initial reaction rate of FABE using this concentration is in the linear regime (Fig. 3).

3.2. Effect of initial substrate concentrations on initial reaction rate

The influence of 1-butanol to oil ratio on the initial rates of FABE and FFA formation is given in Fig. 4. According to the reaction stoichiometry, a minimum of 1-butanol to oil molar ratio of 3 is required for the complete conversion of the oil to FABE. The results show that the initial rate of FABE formation reaches a maximum at 1-butanol to oil ratios between 3 and 4.5, which coincides with the stoichiometric ratio needed for complete oil conversion. At higher amounts of 1-butanol, the initial rates are reduced. A possible explanation is inhibition of the enzyme by the alcohol (*vide infra*).

Besides the formation of FABE, free fatty acids (FFA) were also observed in the course of the reaction (Fig. 4). Thus, besides transesterification, the enzyme also catalyzes the hydrolysis reaction of the oil to FFA. The rate of FFA formation is highest at low 1-butanol to oil ratios. In the absence of 1-butanol, FABE was not formed; hence in this case the FFA initial rate is at its maximum. The lowering in the initial rate for FFA with increasing amounts of 1-butanol indicates that the formed FFA are converted further into FABE, and reaches a minimum value when the FABE initial formation rate reaches its maximum value. It appears that the FFA formation is low compared to FABE formation when the 1-butanol to oil ratio is between 3 and 6.

The influence of the oil concentration in the organic phase on the FABE initial formation rates is given in Fig. 5. The initial rates increase with increasing oil concentrations between 15 and 40 g L⁻¹, and then decrease at higher oil concentrations. For all experiments a constant 1-butanol to oil molar ratio of 6 was used. Therefore the decrease of the FABE initial formation rate at higher oil concentrations can be caused by either a high oil or high 1-butanol concentration (see also Fig. 4). In order to check for enzyme inhibition by the oil, another set of experiment using various oil concentration but now without any 1-butanol were conducted, see Fig. 6. The initial rate of free fatty acids (FFA) increased linearly with increasing oil concentrations. As such, these findings indicate that the oil does not inhibit the enzyme. Though not proven directly, we also assume that FABE, like FFA formation is not inhibited by the oil. Based on these results, it can be concluded that lipase inhibi-

Fig. 5. Effect of the oil concentration on the initial reaction rate of FAE. Experimental conditions: *n*-hexane = 45 mL; phosphate buffer = 15 mL, pH 6.0; 1-butanol to oil molar ratio of 6; lipase = 20 g L_{aq}⁻¹; stirrer speed = 800 rpm; 40 °C. Line is for illustrative purpose only, error estimations are given in the experimental section.

Fig. 6. Effect of the oil concentration on initial formation rate of free fatty acids (FFA). Experimental conditions: *n*-hexane = 45 mL; phosphate buffer = 15 mL, pH 6.0; sunflower oil = 0–100 g L_{org}⁻¹; g L_{org}⁻¹; 1-butanol to oil molar ratio of 6; lipase = 20 g L_{aq}⁻¹; stirrer speed = 800 rpm; 40 °C. Line is for illustrative purpose only, error estimations are given in the experimental section.

tion at higher oil concentrations is most likely caused by 1-butanol inhibition.

Previously it was suggested that the enzymatic esterification of fatty acids can be described by a Ping Pong Bi mechanism with competitive alcohol inhibition [10,25]. The product formation rate, r_p , according to the Ping Pong bi-substrate reaction mechanism of substrates S and A with competitive inhibition of both substrates is described by the following equation:

$$r_p = \frac{V_{max} \cdot C_S \cdot C_A}{C_S \cdot C_A + K_{m,S} \cdot C_A \cdot \left(1 + \frac{C_A}{K_{i,A}}\right) + K_{m,A} \cdot C_S \cdot \left(1 + \frac{C_S}{K_{i,S}}\right)} \quad (1)$$

Here C_S denotes the oil concentration, C_A the 1-butanol concentration, $K_{m,S}$ and $K_{m,A}$ denote the dissociation constants of S and A and $K_{i,S}$ and $K_{i,A}$ the inhibition constants of S and A, respectively. When the total concentration of substrates S and A is very high,

Fig. 7. Effect of the ethyl oleate initial concentrations on initial formation rate of fatty acid ethyl ester (FAEE). Experimental conditions: *n*-hexane = 45 mL; phosphate buffer = 15 mL, pH 6.0; sunflower oil = 40 g L_{org}⁻¹, g L_{org}⁻¹; ethanol to oil molar ratio of 6; lipase = 20 g L_{aq}⁻¹; stirrer speed = 800 rpm; 40 °C. Line is for illustrative purpose only, error estimations are given in the experimental section.

i.e. $C_S \geq K_{i,S}$ and $C_A \geq K_{i,A}$, then the initial product formation rate reduces to:

$$r_p = \frac{V_{max} \cdot C_S \cdot C_A}{C_S \cdot C_A + C_A^2 \cdot \left(\frac{K_{m,S}}{K_{i,S}}\right) + C_S^2 \cdot \left(\frac{K_{m,A}}{K_{i,A}}\right)} \quad (2)$$

In the experiments with variable oil concentrations, a constant initial substrate ratio was used, thus $C_S = x C_A$. Substitution of the concentration ratio $C_S/C_A = x$ reduces Eqs. (2)–(3):

$$r_p = \frac{V_{max}}{1 + \frac{1}{x} \cdot \left(\frac{K_{m,S}}{K_{i,S}}\right) + x \cdot \left(\frac{K_{m,A}}{K_{i,A}}\right)} = \text{constant} \quad (3)$$

From Eq. (3) it follows that the rate only depends on the kinetic parameters and the concentration ratio x . As these are all constant, the initial reaction rate should also be constant, provided that the substrates concentrations are high and their concentration ratio is constant. However, as illustrated by the experimental results (see Fig. 4 and Fig. 5), the initial rates decrease when using high substrates concentrations, indicating that the reaction does not follow the competitive inhibition mechanism. Instead, the results suggest that inhibition of 1-butanol is non-competitive. This type of inhibition occurs when the inhibitor binds to both the unbound enzyme and the enzyme-substrate complex. Then, although the substrate is still able to bind to the enzyme, the main reaction cannot take place [26].

3.3. Effect of the product concentrations on the initial reaction rates

The effects of products on the initial rate of reactions were determined by performing experiments with both products (glycerol and ester) present at the beginning of a standard transesterification experiment. The initial FAE formation rates were plotted against the initial concentrations of ester (Fig. 7) and glycerol (Fig. 8). The experiments with esters were not performed with a FAE mixture but with ethyl oleate as a model compound in ethanol instead of 1-butanol. Ethyl oleate is readily available, in contrast to *n*-butyl oleate. These choices are rationalised by assuming that the reactivity of sunflower oil with 1-butanol and 1-ethanol proceed via a similar reaction mechanism.

Fig. 8. Effect of the initial glycerol concentration on initial formation rate of fatty acid butyl ester (FABE). Experimental conditions: *n*-hexane = 45 mL; phosphate buffer = 15 mL, pH 6.0; sunflower oil = 40 g L⁻¹; g L⁻¹; 1-butanol to oil molar ratio of 6; lipase = 20 g L⁻¹; stirrer speed = 800 rpm; 40 °C. Line is for illustrative purpose only, error estimations are given in the experimental section.

The results show that the initial concentration of ester in the form of ethyl oleate has no major influence on the initial FABE formation rate, see Fig. 7. Similarly, the effects of the initial glycerol concentration on the initial FABE rates are also small, see Fig. 8, and are assumed to be negligible in the further discussions.

In biphasic systems such as the one used here, the active site of the enzyme is expected to be directed towards the organic phase [27]. With this assumption, the absence of glycerol inhibition can be explained, as glycerol dissolves preferentially in the water phase and is therefore not present in the same phase as the active sites of the enzyme. This conclusion is supported by several studies using monophasic organic systems for enzymatic oil transesterification. In these studies inhibition effects of glycerol were observed [28–30], rationalised by the fact that the glycerol formed is inevitably present in the same (organic phase) as the enzyme, or by physical blockage of the enzyme due to surface coverage by glycerol.

The product esters potentially can have an inhibiting effect on enzyme activity, likely by hydrogen bonding with the active site of the enzyme [31]. In our study inhibition by esters was not observed, probably because the amounts of esters produced here are relatively low and they are highly diluted in the organic solvent.

3.4. Kinetic modelling

A kinetic model was developed for the transesterification of sunflower oil with 1-butanol catalyzed by *Rhizomucor miehei* lipase. For this purpose, concentration time profiles for 13 experiments (101 data points) were modelled. A number of representative experimental profiles is given in Fig. 9.

The kinetic model was set-up using the following assumptions:

- 1) The active enzyme is present at the interface of the aqueous and organic phases, with the active site of the enzyme directed towards the organic phase [27]. Therefore it is assumed that the reaction occurs in the organic phase. Thus the concentrations of substrates used in the kinetic modelling are all in the organic phase.
- 2) The solubilities of water, glycerol and enzyme in the organic phase are assumed negligible. Also the solubilities of hexane,

Fig. 9. Representative FABE concentration- time profiles for different sunflower oil concentrations. Symbols: experimental results. Lines: model lines. Experimental conditions: *n*-hexane = 45 mL; phosphate buffer = 15 mL, pH 6.0; sunflower oil = 50, 30, 15 g L⁻¹ for lines 1–3, respectively; 1-butanol to oil molar ratio of 6; lipase = 20 g L⁻¹; stirrer speed = 800 rpm; 40 °C. Error estimations are given in the experimental section.

triglyceride, diglyceride, monoglyceride, FFA and FABE in the aqueous phase are assumed negligible. 1-butanol is distributed over the two phases according to its partition coefficient *P*, however since the *P* value was not determined experimentally, it will be integrated into a model parameter (see below).

- 3) Based on previous studies of the hydrodynamics of the reactor [20] it is assumed that the aqueous and organic phases are ideally mixed, mass transfer limitations are absent in either phase so that the concentrations at the interface are equal to the bulk concentrations, and the interfacial area is constant. Following these assumptions the enzyme activity is only affected by the substrates or products involved in the reaction. Since the enzymatic reaction is intrinsically slow the assumption that mass transfer is much faster than the reaction rate, and therefore mass transfer limitations are absent, is a mild one. Thus the observed rates are assumed to be solely dependent on reaction kinetics.
- 4) Fatty acid type does not influence the reaction rate. Sunflower oil consists of 4 major fatty acid types, with oleic and linoleic acid being the most abundant ones. It is assumed that the different fatty acids react in the same manner and with the same rate. This assumption is supported by the measured relative amounts of the butyl esters of the various fatty acids present in the system. Fig. 10 shows a typical example of the analysis results, the fractions of the different butyl esters are plotted versus the total FABE yield, which is defined as:

$$\text{FABE yield} = \frac{n_{\text{Ac},0}}{n_{\text{FABE}}} \quad (4)$$

where $n_{\text{Ac},0}$ is the initial amount of reactive acyl groups (mol) and n_{FABE} is the amount of butyl esters formed (mol). Although the total amount of a specific butyl ester increases with time, and consequently the total yield of FABE, see Fig. 9, the ratio between various fatty acids remains constant. Apparently the formation rates of the various FABE's is approximately equal. Note that with the analytical methods employed here it was difficult to distinguish between oleic and linoleic butyl esters, therefore the sum of these two is shown in Fig. 10.

- 5) Instead of considering triglyceride, diglyceride and monoglyceride as different components, the total amount of reactive acyl

Fig. 10. Relative amounts of butyl oleate/linoleate (1), butyl palmitate (2) and butyl stearate (3) formed for different total FABE yields during a single experiment. Lines are for illustrative purposes only.

groups, n_{Ac} , and alcoholic glycerol moieties, n_{Gly} , are considered in the kinetic modelling:

$$n_{Ac} = 3n_{TG} + 2n_{DG} + n_{MG} \quad (5)$$

$$n_{GlyOH} = 3n_{Gly} + 2n_{MG} + n_{DG} \quad (6)$$

where n_{Ac} (mol) is the amount of unreacted acyl groups, n_{GlyOH} (mol) the amount of reacted acyl groups and n_{Gly} , n_{MG} , n_{DG} and n_{TG} are the amount of glycerol, mono-, di- and triglycerides (mol) respectively. The transesterification reaction can be written as:

Rhizomucor Miehei lipase is an *sn*-1,3-specific enzyme and does not recognize the *sn*-2 position significantly, meaning that it only reacts with acyl groups on the outer positions of the glycerol moiety. However, acyl migration can modify the configuration by forming 1,3-DG from 1,2-DG and 1-MG from 2-MG, allowing full conversion of triglycerides [7]. Acyl migration is especially prominent in aprotic hydrophobic solvents such as *n*-hexane, which was used in this research [32]. Because of the favourable conditions for acyl migration and the relatively low rate of the RML catalyzed transesterification reaction, it is assumed that acyl migration does not influence the transesterification rate. The much faster acyl migration, as compared to the transesterification, is implicitly incorporated into the model here, see Eqs. (5)–(6), where we assume that all acyl groups of the glycerides are equally available for transesterification.

- 6) The kinetic modelling only includes measurements with alcohol to oil molar ratios above 4. The minimum ratio for full conversion should be at least 3 because of the reaction stoichiometry. Also, for low alcohol to oil ratios, considerable amounts of FFA are formed as a side product (see Fig. 4). For ratios 4 and above, formation of FFA can be neglected entirely and is therefore excluded from the model.
- 7) It is assumed that the enzyme inhibition by alcohol is non-competitive. Oil, ester, and glycerol did not inhibit the enzyme according to our results.

The Ping Pong mechanism with non-competitive inhibition of 1-butanol can be expressed by [26]:

$$r_p = \frac{k_{enz} \cdot C_{enz, aq}}{\left(1 + \frac{K_{m,S}}{C_{S,org}} + \frac{K_{m,A}}{C_{A,org}}\right) \cdot \left(1 + \frac{C_{A,org}}{K_{I,A}}\right)} \quad (8)$$

Here subscription S denotes the oil while A denotes 1-butanol. The term in the denominator with the dissociation constant of 1-butanol ($K_{m,A}$) can be neglected because the rate accelerating effect

Table 2

Parameter estimates for rate model Eqs. (11) and (14) and their 95% confidence limits.

Parameters	Value
k_{enz} ($\times 10^{-3}$ mol L _{aq} ⁻¹ g ⁻¹ s ⁻¹)	0.794 ± 0.003
$k_{i,enz}$ ($\times 10^{-3}$ mol L _{aq} ⁻¹ g ⁻¹ s ⁻¹)	0.007 ± 0.001
$K_{m,S}$ (mol L _{org} ⁻¹)	0.355 ± 0.003
$PK_{I,A}$ (mol L _{org} ⁻¹)	0.005 ± 0.0003
R^2	0.99

Fig. 11. Parity plot of FABE reaction rate based on Eq. (11).

of 1-butanol is much lower than its inhibiting effect within the range of the model (see Fig. 4), hence Eq. (8) simplifies to:

$$r_p = \frac{k_{enz} \cdot C_{enz, aq}}{\left(1 + \frac{K_{m,S}}{C_{S,org}}\right) \cdot \left(1 + \frac{C_{A,org}}{K_{I,A}}\right)} \quad (9)$$

From an overall mass balance for 1-butanol and its partition coefficient, $P_A = C_{A,org}/C_{A,aq}$, the concentration of 1-butanol in organic phase, $C_{A,org}$ (g L_{org}⁻¹), is obtained as:

$$C_{A,org} = \frac{n_A}{\left(\frac{V_{aq}}{P_A}\right) + V_{org}} \quad (10)$$

This result allows the rate Eq. (9) to be written as

$$r_p = \frac{k_{enz} \cdot C_{enz, aq}}{\left(1 + \frac{K_{m,S}}{C_{S,org}}\right) \cdot \left(1 + \frac{n_A}{PK_{I,A}}\right)} \quad (11)$$

where r_p is formation rate of fatty acid butyl ester (FABE) (mol L_{org}⁻¹ min⁻¹), k_{enz} is the kinetic reaction constant (mol L_{aq}⁻¹ g⁻¹ s⁻¹), $C_{enz, aq}$ is enzyme concentration in water (g L_{aq}⁻¹), $K_{m,S}$ is dissociation constants of substrate (mol L_{org}⁻¹), $C_{S,org}$ is substrate concentration in organic solvent (g L_{org}⁻¹), and n_A is total number of moles of 1-butanol in the system:

$$n_A = n_{A,0} - C_{P,org} \cdot V_{org} \quad (12)$$

$PK_{I,A}$ (mol L_{org}⁻¹) is a combined parameter containing the partition coefficient of 1-butanol (P_A), the aqueous (V_{aq}) and organic volumes (V_{org}), and the inhibition constant $K_{I,A}$:

$$PK_{I,A} = \left(\left(\frac{V_{aq}}{P_A}\right) + V_{org}\right) \cdot K_{I,A} \quad (13)$$

A parity plot of the data revealed a systematic deviation of the modelled reaction rates from the experimental data, see Fig. 11. This phenomenon is explained by assuming that a slow irreversible deactivation of the enzyme occurs throughout the course of an

Table 3

Comparison of kinetic parameters with related (trans-)esterification reactions.

Reaction	RML transesterification [This work]	RML transesterification [17]	RML esterification [20]	RML esterification [21]
Substrates	Sunflower oil butanolysis	Palm oil methanolysis	Oleic acid butanolysis	Oleic acid ethanolysis
System	Biphasic; aqueous-organic	Organic solvent	Biphasic; aqueous-organic	Biphasic; aqueous-organic
k_{enz} ($\times 10^{-3}$ mol/L _{aq} /L _{org} g s)	0.794 ± 0.003	0.041 (V_{max}) ^a	7.384 ± 0.001	0.057 (V_{max}) ^b
$k_{i,enz}$ ($\times 10^{-3}$ mol/L _{aq} /L _{org} g s)	0.007 ± 0.001	–	–	–
$K_{m,S}$ ($\times 10^{-1}$ mol/L _{org})	3.351 ± 0.027	4.30	0.678 ± 0.003	18.0
$K_{m,A}$ ($\times 10^{-1}$ mol/L _{org})	–	3.50	2.536 ± 0.011	17.9
$K_{i,A}$ ($\times 10^{-1}$ mol/L _{org})	0.050 ± 0.003 ($PK_{i,A}$)	445	1.277 ± 0.004	0.637^c
$K_{i,S}$ ($\times 10^{-1}$ mol/L _{org})	–	330	–	0.633^c
K_A ($\times 10^{-1}$ mol/L _{org})	–	–	–	3.96

^a V_{max} in mol/m³ min^{−1}.^b V_{max} in M h^{−1} mg^{−1}.^c $K_{S(ET)}$ and $K_{S(OI)}$ in mmol h^{−1} mg^{−1} M^{−1} as used in [21]. **Fig. 12.** Parity plot of FABE production rates obtained with Eqs. (11) and (14).

experiment, possibly caused by the shearing action of the stirrers [33].

Consequently, a new parameter, $k_{i,enz}$, was introduced into Eq. (11) to incorporate the enzyme deactivation into the model. The parameter correlates the effective enzyme concentration ($C_{enz,eff}(t)$) according to following equation:

$$C_{enz,eff}(t) = C_{enz,0} \cdot e^{(-k_{i,enz} \cdot t)} \quad (14)$$

where $k_{i,enz}$ is enzyme deactivation constant. The parity plot of the data fitting including irreversible enzyme deactivation (Eq. (14)) is given in Fig. 12, while the final estimated parameters and their confidence limits are presented in Table 2. The parity plot, Fig. 12, does not show any systematic deviation anymore. The results of several representative experiments were plotted together with the accompanying calculated profiles and presented in Fig. 9.

With the parameter estimates of Table 2, the coefficient of determination was obtained as $R^2 = 0.99$, with an average error of 4.2%, which suggests that the data are well represented by the model.

The kinetic parameters estimated in this study are difficult to compare with literature data because the enzymatic transesterification of vegetable oils using 1-butanol in biphasic water-organic systems have not been reported before. In Table 3 we present a comparison of our kinetic parameters and literature data for a number of related (trans-)esterification reactions. In general, the kinetic reaction constant k_{enz} is lower for the transesterification reaction than for esterification. From this it can be concluded that the enzyme performs the transesterification of oil more slowly than esterification of oleic acid. It should be taken into account that in this research a 3:1 organic to aqueous solvent volume ratio is used compared to a 1:1 ratio in the esterification research by Kraai et al. [21]. The value

for the oil dissociation constant ($K_{m,S}$) is within the range of most Michaelis-Menten constants for enzymes, which is 10^{-1} – 10^{-7} M [34]. The substrate dissociation constant obtained in this work with butanol, $K_{m,S} = 3.351 \times 10^{-1}$ mol/L_{org}, is comparable to the value obtained with methanol, $K_{m,S} = 4.30 \times 10^{-1}$ mol/L_{org} [17] although the latter value is obtained in with an organic solvent for the oil as opposed to the biphasic system used in this work.

4. Conclusions

The kinetics of the transesterification of sunflower oil with 1-butanol catalyzed by a soluble *R. miehei* lipase in an aqueous-organic biphasic system was studied and shown to be best described by a Ping-Pong Bi model with non-competitive inhibition of 1-butanol. The model is in good agreement with the experimental data as is evident from the R^2 of 0.99 and an average error of 4.2%. Inhibition by the oil, ester or glycerol at the conditions employed here was not observed. Consistent with the experimental data, a slow, irreversible deactivation of the enzyme was incorporated into the model.

The results from this study can be used as input for reactor modelling of transesterification reactions (a.o. biodiesel) catalyzed by free enzymes in biphasic systems. For example, it will allow reactor modelling of transesterification reactions in advanced continuous centrifugal contactor separators (CCCS) [21,35–37].

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