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Enzymatic synthesis of biodiesel from fatty acids. Kinetics of the reaction measured by fluorescent response of Nile Red

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ABSTRACT

Production of biodiesel (B) from free fatty acids (F) was investigated. Different amounts of F dissolved in B were esterified by methanol or ethanol with help of lipase Novozym 435. The kinetic model included (i) steady-state scheme; (ii) reversible inhibition of the enzyme by alcohols; and (iii) aggregation of water $W + W \leftrightarrow WW$. The aggregated form WW imitated large water droplets with low chemical activity. Forward and backward reactions were recorded using the calibrated fluorescent signal from Nile Red. The relevant rate constants were calculated and used in computer simulations. The model demonstrated that content of F could be decreased below the specification level of 0.25% by means of the enzymatic conversion exclusively. It was found that the reaction could be accomplished in one step starting from content of $F \leq 1\%$, water = 100 ppm and MeOH = 6% or EtOH = 13%. The higher levels of F ($\geq 4\%$) would require three cycles where MeOH ($\geq 4\%$) or EtOH ($\geq 7\%$) are added at the beginning of each step, and water is dried to 100 ppm between the steps. The designed kinetic scheme is a part of the general biodiesel reaction model, which is currently under construction.

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1. Introduction

Biodiesel (B) is a sustainable fuel produced in the reaction of (trans)esterification, where fatty acids (F) of vegetable oil are conjugated to an alcohol giving the corresponding esters [1–6]. The oil substrates of the reaction are: F, triglycerides (T), diglycerides (D) and monoglycerides (M). The alcohol of typical choice is either methanol (MeOH, C_1) or ethanol (EtOH, C_2), though long-chain alcohols are also considered [1–6]. The reaction requires a catalyst, where inorganic alkali are used most often [1–6].

Recently, application of the immobilized enzymes (e.g. lipase CALB, trademark Novozym 435) attracted much attention due to affordable prices [1,3,4]. Enzymatic transesterification has a number of advantages including (a) minimal industrial waste; (b) reusability; (c) low sensitivity to the composition of feedstock;

and (d) high velocity of the reaction even at ambient temperatures and low alcohol concentrations [1,3,4]. Optimization of such process is, however, not an easy task. First of all, the reaction is reversible, and increasing concentrations of alcohol are required to shift the equilibrium toward the products (preferably compliant with the specifications for biodiesel [3]). Yet, excess of MeOH or EtOH causes both reversible inhibition and irreversible inactivation of lipases [1,3,4]. A good knowledge of the reaction kinetics and inhibition patterns is necessary to reach a high conversion degree preserving at the same time the enzymatic activity.

Kinetic analysis of the biodiesel reaction usually follows the two below strategies. The first one is an empiric approach, where the final yield is presented as a polynomial function of the reaction conditions [7,8]. Yet, reliability of such method is low because the polynomial function does not truly reflect any physical or chemical law and might significantly deviate from the true chart course. Another approach is based on the correct equations of enzyme kinetics [9,10]. This method is potentially much more perspective, but the conclusions are often compromised by a cumbersome experimental design. For example, description of the global scheme requires a very complex kinetic mechanism with at least 15–20 parameters. Such model is usually applied to a limited number of experimental points (30–50), which severely reduces reliability of the calculated rate constants.

Abbreviations: B, biodiesel; B_1 , methanol based B; B_2 , ethanol based B; CALB, Candida antarctica lipase B; C, alcohol; C_1 , MeOH, methanol; C_2 , EtOH, ethanol; E, enzyme; EX, enzyme with attached fatty acid; FAEE, fatty acid ethyl ester; FAME, fatty acid methyl ester; F, fatty acid (free); m/m, mass per mass; m/v, mass per volume; FR, fluorescent ratio; OA, oleic acid; T/D/M, tri-/di-/mono-glyceride; TLC-FID, thin layer chromatography assisted by flame ionization detector; v/v, volume per volume; W, water.

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Fig. 1. Kinetic schemes. (A) Full scheme of the ping-pong mechanism, where concentrations of P_1 and P_2 are close to zero. (B) Shortened scheme of the ping-pong reaction. (C) The analyzed reaction scheme presented as a King-Altman master pattern. Notation is as follows: B – biodiesel (either B_1 or B_2), C – alcohol ($C_1 = CH_3OH$, $C_2 = C_2H_5OH$), E – free enzyme, EX – the enzyme with attached fatty acid, F – fatty acid, W – finely dispersed water, WW – water layer. The rate coefficients labeled by stars are sensitive to enzyme inhibition, see the main text for further details.

The above reasoning emphasizes necessity to (i) simplify the kinetic scheme within the reasonable limits; (ii) reduce the number of reactants in the examined reactions; and (iii) collect as many points as possible. Several perspective approaches are sketched below and will be examined in the current publication.

Simplification of the kinetic scheme can be done as follows. Any elementary lipase reaction involves at least two substrates (S_1 , S_2 , e.g. F and C) and two products (P_1 , P_2 , e.g. B and W). Such reaction follows the ping-pong mechanism (Fig. 1A) with four steps present [9,10]. Yet, the scheme can be condensed to two steps if assuming low affinity of the ligands for the enzyme (Fig. 1B). This is seemingly the case according to the available data on kinetics of the enzymatic biodiesel production [9]. The reduced scheme will be, therefore, explored as a fair approximation of the classical ping-pong mechanism.

Analysis of kinetics becomes much easier if working with partial reactions, where the number of ligands is confined to the absolute minimum. The most convenient system is esterification of free F in the presence of an alcohol $F + C \rightleftharpoons B + H_2O$ (all dissolved in biodiesel). This reaction is isolated from conversions of glycerides $M \rightleftharpoons D \rightleftharpoons T$, and it can be described by relatively simple equations. Therefore, the reaction of esterification was chosen as a good starting point to construct the global model.

Enzymatic conversion of F to B presents a considerable interest for the industrial purposes. For example, many feedstocks have high initial content of F, which makes these oils inapplicable for the direct alkaline esterification. Fatty acids are also major contaminants of biodiesel produced from a vegetable oil treated by aqueous EtOH in the presence of an enzyme [11]. Decrease of F content below 0.25% (recalculated acid value of EN 14214 standard [3]) is difficult and requires detailed quantification of the reaction kinetics. This subject will be explored in the current publication.

Collection of numerous points is a prerequisite of any successful kinetic analysis. Yet, the well established methods of gas-chromatography, HPLC and TLC are relatively lengthy (discussed earlier [11]). In this publication we will examine application of the fluorescent probe Nile Red for monitoring of the reaction progress. Nile Red is soluble in organic liquids and changes its quantum yield in the presence of hydrophilic groups dissolved in the medium [12,13]. For example, organic alcohols (C) reversibly inter-

act with Nile Red via hydrogen bonding and cause a noticeable red shift of its emission spectrum. Appearance or disappearance of those reactants can be recorded using emission of the fluorophore. The reaction of esterification $F + C \rightarrow B + H_2O$ presents a very convenient example, where both substrates have hydrophilic groups and are active toward Nile Red. They disappear in course of reaction and produce two products – a hydrophobic molecule of B (“neutral” toward fluorophore) and water (excluded from the oil phase). A noticeable change of Nile Red emission can be expected. Correlation of the fluorescent signal with concentration of a reactant will facilitate monitoring of the reaction progress.

In the current publication we have analyzed kinetics of the enzymatic reaction $F + C \rightleftharpoons B + H_2O$ using the calibrated fluorescent response from Nile Red. The obtained data were used in design of a computer model, where reduction of F in “raw” biodiesel was simulated and optimized. This work describes the first stage in reconstruction of the full scheme of the enzymatic biodiesel production.

2. Materials and methods

2.1. Materials

All salts and solutions were purchased from Sigma–Aldrich. TLC-FID Chromarods S III were from SES GmbH – Analysensystem (Germany). TLC plates Polygram Sil G 20×20 cm (gel 0.2 mm) were from Macherey-Nagel (Germany). Preparation of F (oleic acid, 98% purity) was purchased from Danisco (Denmark). Biodiesel of 96–97% purity was prepared as described below using rapeseed oil from Danish supermarket. Immobilized lipase Novozym 435 was kindly provided by Novozymes (Denmark).

2.2. Methods

2.2.1. Glossary of the model and molecular characteristics of the reactants

A one letter code was adopted in the kinetic schemes and equations: B_1 (biodiesel FAME), B_2 (biodiesel FAEE), C_1 (CH_3OH), C_2 (C_2H_5OH), E (the immobilized enzyme CALB, trademark Novozym 435), EX (the enzyme with conjugated fatty acid), F (free fatty acid), W (water as a fine micella), WW (water as a large droplet or layer), $\sum W$ (total water, $W + 2 \cdot WW$). The molar concentrations (M) of the corresponding compounds were indicated as small letters, e.g. $b_1 = [B_1]$ etc. The notation of rate constants followed the above one-letter code, see Fig. 1C. The calculations were based on the following assumptions of the molecular mass and density (g/mol, g/mL, respectively): B_1 (296, 0.88), B_2 (310, 0.88), C_1 (32, 0.79), C_2 (46, 0.79), F (282, 0.90), W (18, 1.0). Pure solutions were assumed to have the below molarity (mol/L): B_1 (3.0), B_2 (2.84), C_1 (24.7), C_2 (17.2), F (3.2), W (55.5). The biodiesel solutions used in the experiments were of $\approx 96\%$ purity, i.e. 2.85 M B_1 and 2.72 M B_2 . Most reactions were performed in the presence of 4% (m/v) Novozym 435 at $30^\circ C$ and 200 rpm, where molar concentration of the enzyme CALB was estimated as $100 \mu M$ based on the information provided by manufacturers (≈ 85 mg of CALB with $M_w \approx 34,000$ bound per 1 g of the carrier). Other conditions are specified in the text.

2.2.2. Preparation of biodiesel solutions

Biodiesel preparations (both B_1 and B_2) were produced from rapeseed oil using a uniform method with Novozyme 435 (5%, m/v, $37^\circ C$, 200 rpm in shake-flasks). Alcohol (either MeOH or dry EtOH) was added at zero time (1/3 equivalent of full conversion, i.e. 1 mol of alcohol per 1 mol of T), after 24 h of incubation (1/3

equivalent) and after 48 h (1/2 equivalent). After the overall incubation time of 72 h, the mixture was taken out of the incubator, and glycerol was allowed to settle as a separate layer under the enzyme particles (3 h). Glycerol was removed by aspiration, 1/3 equivalent of alcohol was added, and the reaction was continued overnight. The liquid product was separated from the enzyme and centrifuged, whereupon excessive alcohol was evaporated by incubation in open shake-flasks (37 °C, 200 rpm, overnight). Some amount of glycerol was additionally adsorbed on glass walls of the shake flask. The typical composition of the final product was as follows (% m/m): B = 96%, T ≈ 0.6%, D ≈ 1.1%, M ≈ 1.5%, F ≈ 0.8% based on TLC or TLC-FID measurements [11]. The endogenous water was evaluated as 500–800 ppm according to Karl Fisher titration.

2.2.3. Reaction of B with water

Hydrolysis of B (either B₁ or B₂) to free fatty acid and alcohol was conducted under the below conditions. A mixture of biodiesel and water (14 mL in total) was placed into a standard 15 mL tube and pre-warmed to 30 °C (approximately 30 min). Before the reaction was started, the mixture was vigorously shaken, and a sample of 0.6 mL was collected. Then 560 mg of the enzyme beads (Novozym 435, 4%, m/v) were added, and the reaction was continued over 1 h (30 °C and 200 rpm). The reaction tubes were placed horizontally in the shaking incubator to improve the mixing. At time intervals, the tubes were vigorously shaken, the enzyme particles were allowed to settle for 10–15 s, whereupon the samples of 0.6 mL were collected from the top. They were kept in closed 0.6 mL vials to prevent evaporation of alcohol. The time of collection was compensated for the increasing enzyme concentration, see Section 2.2.5.

2.2.4. Reaction of F with alcohol

Conversion of F to B in the presence of alcohol (MeOH or EtOH) was conducted as described in previous paragraph. In short, the mixture of either F (oleic acid) + B + alcohol or pure F + alcohol was incubated with Novozyme 435 particles. The samples of oil phase (without the enzyme beads) were collected at time intervals and subjected to analysis as described below.

2.2.5. Time correction

The volume of the reaction mixture gradually decreased because of the sample collection. This caused the proportional increase of the enzyme concentration (*e*) in the mixture. In order to use the constant *e* throughout the whole experiment, the original time of collection was corrected assuming a direct proportionality of the accumulate product (*dp*) to both the enzyme (*e*) and the time interval (*dt*), e.g. $dp = k_s \cdot s \cdot e \cdot dt$. Each time interval *dt* was corrected as follows: $dt_{corr} = dt \cdot V_0 / V_X$, where *V*₀ and *V*_X represent the volumes of reaction mixture at zero time and after collection of the corresponding sample.

2.2.6. Analysis of the collected samples

The fluorescent response and composition of the collected samples were analyzed in the following way. The fluorescent probe Nile Red (2.5 μL of 140 μM solution in dimethylformamide) was added to 0.6 mL of the reaction sample (final concentration of 0.58 μM). The mixture was vigorously shaken and centrifuged to precipitate the water phase (2 min, 5000 rpm). The upper oil phase (0.5 mL) was collected and placed into a disposable plastic cuvette for fluorescent measurements. After measurement of fluorescence (Section 2.2.7), the composition of several selected samples was analyzed by either TLC on plates or/and TLC-FID as described earlier [11]. Depending on the reaction conditions, the change of either F or B was followed over the time and correlated with the fluorescent signal.

Table 1

The elementary steps and constants of the model (Fig. 1C).

Reaction	Forward <i>k</i> , M ⁻¹ min ⁻¹	Backward <i>k</i> , M ⁻¹ min ⁻¹
F + E ↔ W + EX	<i>k_f</i> = 1500	<i>k_w</i> = 2600
C ₁ + EX ↔ B ₁ + E	<i>k_{c1}</i> = 2000	<i>k_{b1}</i> = 180
C ₂ + EX ↔ B ₂ + E	<i>k_{c2}</i> = 1200	<i>k_{b2}</i> = 150
F + W + E ↔ WW + EX (C ₁)	<i>k_{fw}</i> = 10 or ≤50	<i>k_{ww1}</i> = 490
F + W + E ↔ WW + EX (C ₂)	<i>k_{fw}</i> = 10 or ≤50	<i>k_{ww2}</i> = 80
E + C ₁ ↔ EC ₁	<i>k_{+ic1}</i> = 100 ^a	<i>k_{-ic1}</i> = 140 ^a <i>K_{ic1}</i> = 1.4 M
E + C ₂ ↔ EC ₂	<i>k_{+ic2}</i> = 100 ^a	<i>k_{-ic2}</i> = 40 ^a <i>K_{ic2}</i> = 0.40 M
W + W ↔ WW (for C ₁)	<i>k_{+w1}</i> = 100 ^a	<i>k_{-w1}</i> = 17 ^a <i>K_{ww1}</i> = 0.17 M
W + W ↔ WW (for C ₂)	<i>k_{+w2}</i> = 100 ^a	<i>k_{-w2}</i> = 31 ^a <i>K_{ww2}</i> = 0.31 M

^a The rate constants were deduced from the corresponding equilibrium dissociation constants. The value of the binding rate constants *k₊* was assigned to guarantee a near-equilibrium state of this process at any moment of the reaction.

2.2.7. Measurement of the fluorescent signal

A stock solution of Nile Red was prepared and diluted with dimethylformamide to approximately 140–150 μM. The concentration was evaluated by absorbance of Nile Red in methanol on Varian Cary 50 spectrophotometer (coefficient of molar absorbance $\epsilon_{552} = 45,000 \text{ M}^{-1} \text{ cm}^{-1}$ [12]). Nile Red solution was added to the reaction sample (0.58 μM final concentration, Section 2.2.6) and the fluorescent signal was recorded on Varian Eclipse fluorometer using the below settings: excitation at 519 nm (slit 5 nm); emission spectrum 550–700 nm (slit 5 nm) or emission at 570 nm and 630 nm; photomultiplier 580 V, see Section 4 for further details.

2.2.8. Nonlinear regression analysis

The approximation of nonlinear curves was done by computer program KyPlot 5 (KyensLab Inc., Japan) using quasi-Newton method of least squares.

2.2.9. Computer simulations

The progress of a reaction over the time was simulated by computer program COPASI 4.5 [14] supplied with the reaction scheme, the initial concentrations of the reactants and the required rate constants, see Table 1.

3. Theory

A typical lipase reaction follows the ping-pong mechanism (Fig. 1A). If the enzyme operates at a very low saturation level ($s_1 \ll K_{m1}$, $s_2 \ll K_{m2}$), the “classical” velocity equation of the ping-pong scheme can be simplified, see Appendix (Eqs. (A1) and (A2)). Introduction of the correction coefficients extends the working range of the simplified equation to 0–40% of maximal velocity as discussed elsewhere [15]. The simplified scheme is shown in Fig. 1B, where each reaction step (braces in Fig. 1B) is interpreted as an efficient collision between the ligand and either E (free biocatalyst) or EX (a biocatalyst molecule with covalently attached fatty acid). Each rate constant of the simplified model (Fig. 1B) corresponds to the combination of constants in the full model (Fig. 1A), e.g. $k_{s1} \approx k_{p1} / (1.3 \cdot K_{s1})$.

The designed reaction scheme of esterification is presented in Fig. 1C as a King-Altman master pattern. One letter code was adopted, where e.g. F and f corresponded to the molecule of F and its concentration, respectively (see also Section 2.2.1). Apart from the main reaction cycle, two additional equilibrium patterns were added. For example, association of micellar water (W) into large droplets or a layer (WW) was imitated by the binding scheme $W + W \leftrightarrow WW$ (dissociation constant *K_{ww}*). Water remained partially active even as a layer due to reactions at its interface, and hydrolysis of biodiesel by WW was described by the scheme $B + WW \rightarrow C + F + W$ with the rate coefficient *k_{ww}*. An opposite reaction (“enzymatic synthesis” of WW with the rate constant *k_{fw}*) was also considered to avoid the thermodynamically awkward

Fig. 2. Spectral properties of Nile Red. (A) Absorbance spectrum of Nile Red (18 μ M) in biodiesel with or without 1 M MeOH (4%, v/v), dash-dotted and solid lines, respectively. The excitation spectrum of discolored oil or biodiesel corresponded to zero line. The excitation spectrum of endogenous pigments in raw oil or biodiesel (emission at 673 nm) is shown by dashed line. (B) The emission spectra of Nile Red (0.58 μ M) in biodiesel with 0.0, 0.1, 0.35, 0.6, 0.85, 1.1 M of MeOH added, solid lines. The emission spectrum of biodiesel from discolored oil corresponded to zero line. The emission spectrum of biodiesel with endogenous pigments (excitation at 519 nm) is shown by dashed line.

assumption of irreversibility. Velocity of such conversion is obviously very low. The total concentration of water ($\sum w = w + 2 \cdot ww$) was expressed as its mass related to the total volume of the reaction mixture.

Distribution of alcohol C between oil and water phases was detected. Yet, formation of CW, CWW etc. complexes was ignored to prevent an extensive branching of the model. The alcohol concentration was treated as its total contents in the reaction volume disregarding phase distribution. At the same time, we assumed the effect of alcohols on formation (K_{ww}) and chemical activity (k_{ww}) of the aggregated water WW, containing C as a “hidden” element. In such way, redistribution of alcohols between oil and water phases was “encoded” as properties of WW different in MeOH and EtOH related reactions. This subject will be additionally addressed in Sections 4.6 and 5.1.

The reversible inhibition of the enzyme by alcohol was considered as the unproductive binding $E + C \leftrightarrow EC$ (dissociation constant K_{ic}). All reactions were performed under conditions, where no irreversible inactivation of the enzyme was detected, i.e. $\text{MeOH} \leq 2$ M and $\text{EtOH} \leq 3$ M. A partial inactivation of Novozym 435 was found at MeOH and $\text{EtOH} \geq 4$ M (not shown).

The velocity equation of the full scheme is presented in Appendix B.

4. Results

4.1. Absorbance and fluorescence of Nile Red

Absorbance spectra of Nile Red in B_1 (FAME) with or without MeOH (1 M, 4%, v/v) are shown in Fig. 2A. The absorbance remained constant at 519 nm, which made this wavelength convenient for excitation of the probe. Biodiesel in all below experiments was produced from discolored oil, where the endogenous pigments were at a background level (not shown). Yet, raw oil was also tested. The performed test revealed presence of a fluorescing pigment, whose excitation spectrum (Fig. 2A, dashed line) partially overlapped the absorbance spectrum of Nile Red.

Emission spectra of Nile Red in B_1 with different amounts of MeOH are shown in Fig. 2B. As anticipated, they revealed high sensitivity of the probe to the presence of hydrophylic groups. The background fluorescence (or light scattering) of biodiesel from discolored oil was very low (not shown). The emission of pigments in raw oil (or biodiesel from raw oil) was, however, noticeable (Fig. 2B, dashed line). This makes the products from raw oil less suitable for fluorescent measurements, though, the pigment emission can be subtracted from the Nile Red emission spectrum. In the below

kinetic experiments only discolored oil (or biodiesel) was used to avoid any ambiguity.

The fluorescent ratio $FR = F_{630}/F_{570}$ (i.e. the ratio of emission measured at 630 nm and 570 nm) was chosen as a good indicator of Nile Red response to the changing composition of the medium. The ratio is independent of the amount of Nile Red, and all potential errors associated with dilution of the probe and varying turbidity of the samples can be ignored.

4.2. Calibration of the fluorescent signal

The fluorescent response was correlated with the concentration of chosen analyte (e.g. F) in the backward and forward reactions for both MeOH (C_1) and EtOH (C_2). For this purpose, the samples were collected during the reaction, whereupon they were analyzed in terms of both Nile Red fluoresce and the chemical composition [11]. The change of the fluorescent ratio was presented in absolute units $\Delta FR = |FR - FR_0|$ ($FR = F_{630}/F_{570}$), where FR_0 and FR were measured before and under the reaction, respectively. The value of ΔFR was plotted versus the concentration changes (see Fig. 3 with calibrations).

All panels in Fig. 3 were analyzed similarly following the below logic. The experimental points were fitted by a suitable nonlinear function with two parameters (e.g. $\Delta FR = a \cdot (-\Delta f)^n$ in Fig. 3C). At the preliminary stage, an unconstrained fit was performed, and parameters of the best approximation were calculated. It appeared that one of the parameters fluctuated around an average value, whereas the other one was dependent on the initial concentrations of the reactants. After establishing this pattern, the fit was repeated, where the parameter without a clear tendency was fixed at its average value (e.g. $n = 1.8$ in Fig. 3C) and the other one (a in Fig. 3C) was calculated again for each dataset. The obtained coefficients covered a broad range of the reaction conditions and connected the change of fluorescence (ΔFR) with the reactant (e.g. $-\Delta f = (\Delta FR/a)^{1/1.8}$, Fig. 3C). When the initial conditions differed from the described cases, the calibration coefficients were deduced by interpolation, see the functions for a and n coefficients in legend to Fig. 3 for further details. The discussed scheme of analysis was applied to all panels in Fig. 3, see below.

The calibration curves for the reactions $B_1 + \sum W \rightarrow \dots$ and $B_2 + \sum W \rightarrow \dots$ at different total water concentrations ($\sum w$) are shown in Fig. 3A and B (endogenous water was considered).

The calibration data for the reaction $F + C_1 \rightarrow \dots$ (Fig. 3C) were obtained in either biodiesel mixtures (B_1 acting as a solvent-product) or pure F ($f = 3.2$ M). Different initial combinations of F, B_1 and MeOH were used. One calibration curve approximated all

Fig. 3. Change of the fluorescent ratio $\Delta FR = |FR - FR_0|$ (absolute units) as a function of the concentration change. (A) Calibration curves for the reaction $B_1 + \sum W \rightarrow \dots$ at different initial water ($\sum w_0$, M), 0.03 M corresponds to the endogenous water. The fitting equation was $\Delta FR = a/(1 + 1.4/\Delta f)$ where coefficient a depended on initial water $\sum w_0$ as $a = 5.77 - 3.54/(1 + 0.776/\sum w_0)$. Change in f was calculated as $\Delta f = 1.4/(a/\Delta FR - 1)$. (B) Calibration curves for the reaction $B_2 + \sum W \rightarrow \dots$ at different initial water ($\sum w_0$, M), 0.05 M corresponds to endogenous water. The fitting equation and other calculations were as in panel A except for $a = 4.80 - 2.77/(1 + 0.99/\sum w_0)$. (C) Calibration curves for the reaction $F + C_1 \rightarrow \dots$ in the mixtures of F, MeOH and B_1 . Pure F corresponded to 3.2 M. The fitting equation was $\Delta FR = a(-\Delta f)^{1.8}$, where coefficient a depended on the initial F concentration f_0 as $\log_{10}(a) = 3.01 - 0.271 \cdot x - 2.07 \cdot e^{-5.44/f_0}$. Change in F was calculated as $-\Delta f = (\Delta FR/a)^{1/1.8}$. (D) Calibration curves for the reaction $F + C_2 \rightarrow \dots$ in the mixtures of F (0.52 M), EtOH and B_2 . The fitting equation was $\Delta FR = 10.3 \cdot (-\Delta f)^n$, where coefficient n depended on the initial EtOH concentration c_0 as $n = 1.95 + 0.711/(1 + (2.37/c_0)^{3.5})$. Change in F was calculated as $-\Delta f = (\Delta FR/10.3)^{1/n}$.

reactions with the same initial F disregarding MeOH (see legend to Fig. 3C), at least within the tested limits of 0.5–1 M MeOH and 0.125 M–3.2 M F.

The calibration curves for the reaction $F + C_2 \rightarrow \dots$ (Fig. 3D) covered a narrower span of conditions (0.52 M of initial F at different concentrations of EtOH). The fitting equations were used to calculate the change in F (legend to Fig. 3D). Fluorescent signal under the EtOH reaction with pure F (3.2 M) was ambiguous, because no reliable change of fluorescence was detected at the initial stage of the reaction (not shown).

In the mixtures $F + B$, purity of the added oleic acid (98%) and presence of endogenous F in biodiesel (0.025 M in B_1 , 0.03 M in B_2) were considered when calculating f .

Coefficients of determination R^2 for the produced calibration curves (Fig. 3) varied in the range of 0.86–0.99 (mean value of 0.97). Residual standard error of the fluorescent measurements was lower than that of TLC data by factor 1.7, when both variables were plotted versus time (not shown). Therefore, the detailed analysis of the time curves was based mainly on the fluorescent records except for the cases with $\Delta FR \approx 0$, where TLC data were used instead.

The fluorescence of Nile Red in the artificial mixtures of substrates and products was tested and compared to the corresponding reaction samples. A deviation between the two datasets was found (not shown). It was probably caused by adsorption of some reactants on the surface of the enzyme particles leading to different composition of the oil phase in the artificial mixtures and the true reaction samples. This means that the substrate–product mixtures, though prepared at a correct proportion, are inapplicable for calibration of the reaction progress.

4.3. Kinetic analysis of water-dependent hydrolysis of biodiesel (backward reaction)

Different concentrations of water were added to biodiesel, whereupon Novozym 435 catalyzed hydrolysis of B to F and alcohol. Accumulation of free F was recorded using the fluorescent signal ΔFR and the calibration curves in Fig. 3A and B. The biodiesel preparations contained endogenous water assessed as 0.03 M (B_1) and 0.05 M (B_2).

The changes of Δf over time (Fig. 4A and C) were fitted by exponential functions, whereupon the initial velocities (exponential slopes at zero time, $-v = +\Delta f/\Delta t = -\Delta b/\Delta t$) were calculated for each water concentration. Negative notation of $-v$ refers to the general expression of the reaction velocity $v = v_{\text{forward}} - v_{\text{backward}}$, see Appendix Eq. (B1). The velocities were used to produce dependencies of $-v/e_0$ versus total water $\sum w$ (endogenous water included). The reached equilibrium levels were also evaluated and used later under analysis of K_{eq}^{app} , see Section 4.7. The experiments with $\sum w = 0.03$ M and 0.05 M (Fig. 4A and C, respectively) corresponded to hydrolysis caused by endogenous water.

The relative velocities of backward reaction at zero time ($-v/e_0$, min^{-1}) were plotted as functions of the total water (Fig. 4B and D). It was assumed that only biodiesel and water were present in the appreciable amounts. This caused simplification of the general equation (Appendix B) to the below expression:

$$-\frac{v}{e_0} = \frac{k_b \cdot b \cdot (k_w \cdot w + k_{ww} \cdot ww)}{k_b \cdot b + (k_w \cdot w + k_{ww} \cdot ww)}; \quad w = F(\sum w, K_{ww});$$

$$ww = F(\sum w, K_{ww}) \quad (1)$$

Fig. 4. Kinetics of biodiesel hydrolysis. (A) Accumulation of F over time under hydrolysis of B₁ at different total water (0.03 M corresponds to the endogenous water). (B) Velocity of B₁ hydrolysis (reversible reaction with global velocity of $-v/e_0$) as a function of the total water concentration. Dashed and dotted lines indicate the boundaries of $k_{b1} \cdot b_1$ and $k_w \cdot w$ evaluated under the preliminary analysis. The arrow shows the true level of $k_{b1} \cdot b_1$. Solid line shows prediction of the final model (Table 1). (C) Accumulation of F over time under hydrolysis of B₂ at different total water (0.05 M corresponds to endogenous water). (D) Velocity of B₂ hydrolysis ($-v/e_0$) as a function of the total water concentration. Dashed and dotted lines indicate the boundaries of $k_{b2} \cdot b_2$ and $k_w \cdot w$ evaluated under the preliminary analysis. The arrow shows the true level of $k_{b2} \cdot b_2$. Solid line shows prediction of the final model (Table 1).

where b stands for the biodiesel concentration (either B₁ or B₂), w corresponds to the apparent concentration of micellar water, and ww represents the aggregated water. Both w and ww are directly connected to the total concentration $\sum w$ via the equilibrium constant K_{ww} and the binding equation (Appendix, Eqs. (C1) and (C2)). Initial concentration of biodiesel decreased with increasing water in accordance with the expressions: $b_1 = 2.85 - 0.0513 \cdot \sum w$, and $b_2 = 2.72 - 0.049 \cdot \sum w$ (all in M). The initial values of 2.85 M and 2.72 M corresponded to B₁ and B₂ of 96% purity.

The obtained velocity function (Eq. (1)) was too complex to make a reliable fit of the available data in Fig. 4C and D. Yet, it was possible to evaluate two rate constants, because Eq. (1) got simplified to $-v/e_0 \approx k_w \cdot w$ at $\sum w \rightarrow 0$ ($w \approx \sum w \gg ww$) and $-v/e_0 \approx k_b \cdot b$ at $\sum w \rightarrow \infty$. One can expect that the true value of $k_b \cdot b$ is situated above the experimental points, because dilution of biodiesel by water causes a downward deviation of the curve creating a plateau below its true level. The value of k_{b1} was estimated as $180 \text{ M}^{-1} \text{ min}^{-1}$ (boundaries $160\text{--}200 \text{ M}^{-1} \text{ min}^{-1}$), Fig. 4B. The value of k_{b2} was assessed as $150 \text{ M}^{-1} \text{ min}^{-1}$ (boundaries $130\text{--}160 \text{ M}^{-1} \text{ min}^{-1}$), Fig. 4D. The value of k_w had probable boundaries of $1000\text{--}3000 \text{ M}^{-1} \text{ min}^{-1}$ ($k_w \approx 2000 \text{ M}^{-1} \text{ min}^{-1}$).

The solid lines in Fig. 4B and D indicate predictions of the final model when all parameters of Eq. (1) were calculated (Table 1).

4.4. Conversion of F at different alcohol concentrations (forward reaction)

In these experiments, a constant amount of F (mainly OA) was reacted with different concentrations of alcohol (either MeOH or EtOH) in the presence of Novozym 435. The preparations of F (OA) were either diluted with biodiesel B₁ and B₂ (containing 0.025 M and 0.03 M of endogenous F, respectively) or used in the pure OA.

The reaction progress was monitored by the fluorescent response transformed to the concentration of the consumed F (produced B) with help of the calibration charts in Fig. 3C and D. Fluorescence data were equivocal when working with the mixtures of pure F and MeOH < 0.3 M, or EtOH in the whole region of concentrations. Therefore, the corresponding reaction was analyzed by TLC. Examples of F utilization ($-\Delta f = +\Delta b$ over the time) are shown in Fig. 5A (reaction of 1 M F with MeOH) and Fig. 5C (reaction of 0.52 M F with EtOH). The time curves were approximated by exponential functions, whereupon the slopes $-\Delta f/\Delta t$ were calculated at zero time (slope 0) as well as at $-\Delta f = 0.2 \text{ M}$ for EtOH (slope 0.2), where 0.2 M water was produced in course of reaction.

Dependencies of the normalized velocity (v/e_0) on alcohol concentration (c) are depicted in Fig. 5B (MeOH) and Fig. 5D (EtOH). It was assumed that only F, B and C were present in the medium at the moment of the initial velocity measurements (slopes at t_0). Consequently, the general equation of the model in Appendix (B1) and (B2) was reduced to the below form:

$$\frac{v}{e_0} = \frac{k_f^* \cdot f \cdot k_c \cdot c}{k_b^* \cdot b + k_f^* \cdot f + k_c \cdot c}; \quad k_x^* = \frac{k_x}{1 + c/K_{ic}} \quad (2)$$

where reversible inhibition by alcohol was considered as expressions k_f^* and k_b^* . The constant concentration of F ($f = 0.52 \text{ M}$, 1.02 M in Fig. 5C and D, respectively) was maintained on the expense of biodiesel. The latter became dependent on alcohol as $b_1 = 1.95 - 0.11 \cdot c_1$ and $b_2 = 2.40 - 0.166 \cdot c_2$ (all in M), mind different initial concentrations of F in these two cases. In the experiments with pure OA, f decreased proportionally to the added alcohol in accordance with the expressions $f = 3.2 - 0.04 \cdot c_1$ and $f = 3.2 - 0.058 \cdot c_2$ (M).

A semi-quantitative graphical examination of Eq. (2) was undertaken. It was obvious that the initial slope at small c

Fig. 5. Conversion of fatty acids in the presence of variable alcohol. (A) Time dependence of F utilization ($f_0 = 1.02$ M) at different MeOH concentrations (c_1) dissolved in B_1 . (B) Initial velocity of F conversion (v/e_0 at zero time) as a function of MeOH concentration. Dashed line (○) indicates the best fit (Eq. (2)) for F + MeOH in B_1 with $k_f = 1222$, $k_{c1} = 2296 \text{ M}^{-1} \text{ min}^{-1}$; and $K_{ic} = 1.77 \text{ M}$, ($k_{b1} = 180$ fixed). Results for pure F are shown as (▲). Solid lines describe behavior of the final model (Table 1). (C) Time dependence of F utilization ($f_0 = 0.52$ M) at different EtOH concentrations (c_2) dissolved in B_2 . (D) Velocity of F conversion as a function of EtOH concentration. The values of v/e_0 correspond to zero time (○) and the intermediate step of reaction at $-\Delta f = 0.2$ M (◇). Dashed line (○) indicates the best fit (Eq. (2)) for the reaction F + EtOH in B_2 with $k_f = 2125$, $k_{c2} = 799 \text{ M}^{-1} \text{ min}^{-1}$; and $K_{ic} = 0.26 \text{ M}$, ($k_{b2} = 150$ fixed). Velocities at the intermediate step (◇) had the best fit coefficients of $k_f = 993$, $k_{c2} = 1050$, $k_w^{app} = 674 \text{ M}^{-1} \text{ min}^{-1}$; and $K_{ic} = 0.55 \text{ M}$, ($k_{b2} = 150$ fixed). Results for pure F are indicated as (▲). Solid lines describe behavior of the system according to the final model (Table 1).

($v/e_0 \approx c \cdot k_c / (1 + (k_b \cdot b) / (k_f \cdot f))$) should be somewhat below k_c depending on the balance between f , b and their rate constants (k_b being relatively small). A rough evaluation pointed to the slope of $\approx 0.7 \cdot k_c$. More graphical information was obtained after transformation of Eq. (2) to Eq. (2A), where b was assumed to be approximately constant:

$$\frac{v}{e_0} = \frac{k_f \cdot f}{1 + (K_1/c) + (c/K_2)}; \quad K_1 = \frac{k_f \cdot f + k_b \cdot b}{k_c}; \quad K_2 = K_{ic} \quad (2A)$$

Dependence of v/e_0 on c has a maximum at $c = \sqrt{K_1 \cdot K_2}$. The values of K_1 and K_2 should be of comparable magnitude to get the observed shapes of the curves. In such case, the maximal velocity becomes equal to $v/e_0 \approx 1/3 \cdot k_f \cdot f$. Difference in K_1 and K_2 does not affect this value very much, e.g. $v/e_0 = 0.5 \cdot k_f \cdot f$ at $K_1/K_2 = 1/4$ and $v/e_0 = 0.2 \cdot k_f \cdot f$ at $K_1/K_2 = 4$.

Graphical examination revealed the magnitudes of the relevant constants, whereupon the nonlinear regression analysis revealed parameters with higher precision. One of the rate constants in Eq. (2) was determined earlier during hydrolysis of biodiesel by water (k_b). Therefore, only three parameters (k_f , k_c , and K_{ic}) in Eq. (2) were subjected to optimization. The best fits are shown by dashed lines in Fig. 5B and D, and the optimal coefficients are presented in the figure legend. Later on, the optimal curves were substituted by the global model approximations, where the data from different experiments were considered. For example, the value of k_f was expected to be identical in Fig. 5B (○), Fig. 5D (○) as well as in the following experiments, see Fig. 5D (◇), Fig. 6C and D. The value of $k_f \approx 1500 \text{ M}^{-1} \text{ min}^{-1}$ was satisfactory for all the mentioned datasets. Repeated fitting with fixed k_f gave more accurate values of k_c , and K_{ic} for each alcohol (Table 1). The simulations based on the final model are shown by solid lines (endogenous water and F were considered).

Another set of reaction velocities (Fig. 5D, ◇) was calculated from the slopes at $-\Delta f = 0.2$ M in Fig. 5C. Such setup required introduction of water ($0.2 + 0.03$ M) to the fitting equation:

$$\frac{v}{e_0} = \frac{k_f^* \cdot f \cdot k_c \cdot c - k_b^* \cdot b \cdot k_w^{app} \cdot w_x}{k_b^* \cdot b + k_f^* \cdot f + k_c \cdot c + k_w^{app} \cdot w_x}; \quad k_x^* = \frac{k_x}{1 + c/K_{ic}} \quad (3)$$

where all concentrations were adjusted on the value of consumed F, e.g. $f = f_0 - 0.2$; $b = b_0 + 0.2$ M etc. Water was interpreted as existing in the unknown form (w_x) characterized by the apparent coefficient k_w^{app} ($k_w > k_w^{app} > k_{ww}$). The rate constant k_{fw} was ignored as being much less than all other coefficients. It should be noted that intersection with x -axis takes place not at zero but at $k_f \cdot f \cdot k_c \cdot c = k_b \cdot b \cdot k_w^{app} \cdot w_x$. During the computer fitting (Fig. 5D, ◇) k_f was fixed at $1500 \text{ M}^{-1} \text{ min}^{-1}$ as discussed in previous paragraph. The optimal fit is shown by dashed line over symbols ◇ and the corresponding parameters are presented in the legend to Fig. 5C. The newly introduced water-related coefficient was equal to $k_w^{app} = 675 \text{ M}^{-1} \text{ min}^{-1}$, which agreed with the assumption of $k_w^{app} < k_w \approx 2000 \text{ M}^{-1} \text{ min}^{-1}$ (Fig. 4B and D). Another constant (k_{c2}) was set to $1200 \text{ M}^{-1} \text{ min}^{-1}$ based on all data in Fig. 5D. The simulations of the general model (Table 1) are shown by solid lines.

The reactions in pure F demonstrated high dispersion of points (▲ in Fig. 5B and D), which made difficult an accurate interpretation. The general model (Table 1) provided satisfactory approximations (solid lines over ▲), however.

4.5. Conversion at varying F and constant MeOH (forward reaction)

In this setup, the reaction scheme from previous section was examined in a different way. Variable amounts of F were diluted

Fig. 6. Conversion of fatty acids (variable) in the presence of constant alcohol. Time dependencies of F utilization dissolved in B_1 in the presence of (A) 1 M MeOH (c_1) and (B) 0.5 M MeOH (c_1). (C) Initial velocity of F conversion (v/e_0 at zero time) as a function of F concentration. Solid lines describe behavior of the final model (Table 1). Dash-dotted lines demonstrate contradiction of fits if inhibition by MeOH is absent ($K_{ic} \rightarrow \infty$). Dotted line shows the maximal slope at $f \rightarrow 0$. (D) Velocity of F conversion (v/e_0 at $-\Delta f = 0.2$ M) as a function of F concentration. Solid lines describe behavior of the final model (Table 1). Dotted line shows the maximal slope at $f \rightarrow 0$.

with biodiesel (B_1) and exposed to the constant MeOH in four separate setups with initial MeOH = 1, 0.75, 0.5, 0.25 M. Changes in the F concentrations were recorded over time (examples for 1 M and 0.5 M MeOH are presented in Fig. 6A and B, respectively). The slopes $-\Delta f/\Delta t$ were calculated at zero time in all four experiments and at the level of $-\Delta f = 0.2, 0.15$ and 0.15 M for MeOH = 1, 0.75 and 0.5 M, respectively. The obtained velocities v/e_0 were plotted as functions of F-concentration in Fig. 6C and D.

Analysis of v/e_0 versus f employed Eqs. (2) and (3) from previous section, except for f set now as a variable and c set as a constant. Concentration of biodiesel became, therefore, connected to fatty acids via expressions $b_1 = 2.74 - 0.89f$ ($c_1 = 1$ M), $b_1 = 2.79 - 0.89f$ ($c_1 = 0.5$ M) etc. At a small f , the initial slope of the velocity function (Eq. (2)) was expected to be somewhat below k_f . A rough evaluation pointed to $v/e_0 \approx 0.5 \cdot k_f \cdot f$. As discussed above, the value of k_f should be identical in all experiments (Figs. 5B, D and 6C, D), and the value of $k_f = 1500 \text{ M}^{-1} \text{ min}^{-1}$ was assigned as discussed in Section 4.4. The alcohol inhibition ($K_{ic} \approx 1.77$ M according to Fig. 6B) could not be determined in any of the separate fits in Fig. 6C, 6D because c was constant for each curve. Therefore, the dissociation constant K_{ic} was assigned in steps as ∞ (no inhibition), 5, 2, 1.5, 1 M. Then, the remaining rate constants k_{c1} (Fig. 6C and D) and k_w^{app} (Fig. 6D) were subjected to optimization. If K_{ic} was assigned correctly, all the curves will be described by similar values of k_{c1} and k_w^{app} . If not, the set of parameters optimal for one curve will be completely inconsistent with another one. The latter statement was evidently demonstrated for $K_{ic} \rightarrow \infty$ (Fig. 6C, dash-dotted lines), where a good fit for the dataset labeled as diamonds $\diamond$ provided a very poor fit for the dataset labeled as circles $\circ$. After several trials, the value of $K_{ic} = 1.4$ M was accepted as the best tradeoff between Figs. 5B and 6C, D.

Optimization of other parameters pointed to $k_{c1} = 1700\text{--}2100 \text{ M}^{-1} \text{ min}^{-1}$ (Fig. 6C and D, Eq. (2)) and $k_w^{app} = 700\text{--}1200 \text{ M}^{-1} \text{ min}^{-1}$ (Fig. 6D, Eq. (3)), these temporary fits are not shown. Afterward, the value of $2000 \text{ M}^{-1} \text{ min}^{-1}$

was assigned to k_{c1} as a fair compromise between all the experiments. The water-related constants were still implemented as the apparent coefficient k_w^{app} , the latter being dependent on the current water concentration. The true constants k_w , k_{ww} and K_{ww} were established in Section 4.7 where also k_{fw} was conjectured. Afterward, the solid curves of the final model (Table 1) were generated to demonstrate a satisfactory global fit (Fig. 6C and D).

4.6. Distribution of alcohols between oil and water phases

Measurements of Nile Red fluorescence in biodiesel with 1 M alcohol (with or without 2 M water added) indicated transition of alcohols to water phase. The amounts of MeOH and EtOH remaining in oil phase were different: 1/3 and 2/3, respectively. This means that the kinetic schemes for MeOH and EtOH should be treated somewhat differently. Assumption of a complex-formation between water and alcohol, like the molecular species of CW, CWW, CCWW etc., was abandoned as leading to an explosive branching of the model. Yet, the difference in MeOH and EtOH properties was interpreted via water-related constants, assuming that redistribution of MeOH and EtOH affects association and activity of the aggregated water, e.g. $W + W \leftrightarrow WW + C \leftrightarrow CWW$. For the sake of simplicity, C was not incorporated into WW in the scheme, but the characteristics of WW were assumed to be different for MeOH and EtOH.

4.7. Equilibrium analysis

The equilibrium concentrations of f , b , c , and $\sum w$ were calculated from the data in Figs. 4A, C, 5A, C and 6A, B and the two other experiments analogous to those in Fig. 6A and B, but performed at 0.75 M, 0.25 M MeOH (not shown). The concentration changes Δf determined the total material balance $-\Delta f = -\Delta c = +\Delta b = +\Delta \sum w$, which made possible calculation of all equilibrium concentrations based on $-\Delta f$ record, e.g. $f_{eq} = f_0 - \Delta f$. Finally, the apparent con-

Fig. 7. The apparent equilibrium constant of the reaction $F + C \leftrightarrow B + \sum W$ plotted as a function of equilibrium $\sum w$. The data for (A) MeOH and (B) EtOH reactions. Dashed lines indicate the best fits, and the solid lines show the approximations reached by the final model (Table 1).

stant $K_{eq}^{app} = (b_{eq} \cdot \sum w_{eq}) / (f_{eq} \cdot c_{eq})$ was calculated and plotted as a function of $\sum w_{eq}$ (Fig. 7A and B).

The apparent equilibrium ratio depends on equilibrium $\sum w$ according to the below function (see also Appendix (C1)–(D2)):

$$K_{eq}^{app} = \frac{b \cdot \sum w}{c \cdot f} = \frac{k_c \cdot (k_f + k_{fw} \cdot w)}{k_b \cdot (k_w(w/\sum w) + k_{ww}(ww/\sum w))};$$

$$w = F(\sum w, K_{ww}); \quad ww = F(\sum w, K_{ww}) \quad (4)$$

where w and ww were calculated from $\sum w$, see Appendix C. The above equation contains three known parameters (k_b , k_c , k_f) and four unknown water-related constants k_w , k_{ww} , k_{fw} and K_{ww} (see the scheme in Fig. 1C). One of the constants (k_{fw}) describes a process $E + F + W \rightarrow EX + WW$. Velocity of this conversion is expected to be very low, because probability of formation of EX complex under simultaneous interaction of the enzyme with fatty acid and water is not high. We assigned $k_{fw} = 10 \text{ M}^{-1} \text{ min}^{-1}$, though any $k_{fw} \leq 50 \text{ M}^{-1} \text{ min}^{-1}$ can be used with a very little effect on the model.

After assignment of k_b , k_c , k_f and k_{fw} , the unconstrained optimization of k_w , k_{ww} and K_{ww} was performed separately for MeOH (Fig. 7A) and EtOH (Fig. 7B), dashed lines. Different behavior of water in MeOH and EtOH reactions was assumed because of different solubility of alcohols in biodiesel when water was also present, see Section 4.6. The optimal values of the water-related constants (Fig. 7) were afterward subjected to adjustments. Thus, the value of k_w was assumed to be the same in both MeOH and EtOH reactions, because no alcohol redistribution occurs at low water content. Two other coefficients k_{ww} and K_{ww} were treated as being affected by MeOH and EtOH. The approximations based on the final model (Table 1) are shown in Fig. 7A and B as solid lines.

4.8. Temperature dependence

Comparison of the reaction $F + C \rightarrow B + W$ performed at 30 °C and 40 °C pointed to 1.55 acceleration of the velocity for both alcohols (not shown).

4.9. Modeling of the reaction

In the previous sections we have obtained the relevant parameters of the model, which are summarized in Table 1. These coefficients were used to explore behavior of the system under different conditions. Among the addressed questions were (i) possibility to reduce the contents of fatty acids in 96% biodiesel from 4% to 0.2% F by means of the enzymatic conversion; and (ii) selection of the optimal scenario for such conversion.

To answer the first question, the equilibrium equation (Eq. (4)) was solved in terms of f_{eq} to produce a dependency of free fatty acids

on the equilibrium values of c_{eq} and $\sum w_{eq}$. Concentration of b_{eq} was eliminated via its material balance $b_{eq} = 3 - f_{eq}$ or $b_{eq} = 2.84 - f_{eq}$ (M) for MeOH and EtOH reactions, respectively. The below expression was reached (example for MeOH):

$$f_{eq} = \frac{3 \cdot \sum w_{eq}}{K_{eq}^{app} \cdot c_{eq} + \sum w_{eq}}; \quad K_{eq}^{app} = F(\sum w_{eq}, K_{ww}) \quad (5)$$

where K_{eq}^{app} is a function of the total water (Eq. (4)). Then, Eq. (5) was used to simulate the theoretical curves, (Fig. 8A and C). Relations between the molar concentrations and concentrations in percent are as follows: 0.1 M H_2O corresponds to 2000 ppm (m/m); 0.1 M F corresponds to 3% (m/m); complete conversion of 0.1 M F gives 2000 ppm of water; 1 M alcohol is equivalent to 4% (v/v) of MeOH and 5.8% (v/v) of EtOH.

The results in Fig. 8A and C clearly demonstrated that a mixture of 96% B and 4% F (with 100–200 ppm of water) cannot be converted to 99.8% B + 0.2% F in course of a one-step enzymatic reaction, because the shift of equilibrium will require presence of 5 M MeOH (20%) or 8 M EtOH (46%). Thus is far above the safe levels for Novozym 435 estimated as $\text{MeOH} \leq 2 \text{ M}$ and $\text{EtOH} \leq 3 \text{ M}$. A single-step conversion of F from 1% to 0.2% can be, however, reached, because 6% MeOH and 13% EtOH are required.

F at concentrations above 1% should be processed in several steps, where both alcohol and water are evaporated in a separated from the enzyme unit. The industrial drying equipment provides ≈ 100 ppm of residual water in 30–60 min. Under these assumptions, the conversion of 96% B + 4% F might be achieved in three steps as shown in Fig. 8B (MeOH) and Fig. 8D (EtOH). The presented simulations corresponded to 5% (m/v) Novozym 435 at 35 °C. The stages of conversion in Fig. 8B should be interpreted as follows: (0 min) 4% MeOH is added to the mixture and the reaction proceeds; (30 min) the mixture is transferred to a drying unit and both water and MeOH are evaporated (100 ppm of residual water is reached, where kinetics is not specified); (60 min) the mixture is transferred to the reaction unit and 4% MeOH is added; (90 min) next drying is performed; (120 min) final reaction is started with 3% MeOH. The process with EtOH proceeds in a similar way except for higher concentrations of alcohol required at each step.

The data from Table 1 can be used for modeling of other scenarios, e.g. processing of pure F to B or reduction of F contents in oils to the level acceptable for alkaline conversion. In the later case, T (as the main component of oil) can be treated as a “neutral” substance, because reactivity of T with Novozym 435 is much slower than that of F according to our preliminary data. It also appears that conversion of F in T-solution is twice as slow as in B-solution, which is presently interpreted as formation of unproductive complexes between E and T.

Fig. 8. Computer simulations of the reaction $F + C \leftrightarrow B + \Sigma W$. (A) Dependence of the equilibrium fatty acid concentration (f_{eq}) on the equilibrium water concentration (ΣW_{eq}) in the presence of different MeOH concentrations. The targeted level of F (0.2%) is shown by dotted line. (B) Stepwise conversion of F dissolved in biodiesel in the presence of MeOH (C_1 , % v/v), Novozym 435 (5%, m/v) at 35 °C. Levels of F and H₂O are depicted by solid and dashed lines, respectively. The reaction steps are alternated with water drying (drying kinetics is not specified, final water of 100 ppm). (C) and (D) EtOH used instead of MeOH, descriptions as in panels A and B, respectively.

5. Discussion

5.1. The analyzed scheme

The current work investigated the enzymatic esterification of fatty acids by MeOH and EtOH in the reaction $F + C \leftrightarrow B + \Sigma W$ (see Fig. 1C for a detailed description). This reaction is an important part of the global scheme related to production of biodiesel from vegetable oils [1–6]. We have already discussed in the Introduction that the global kinetic scheme is too complex to be solved in the full-component mixture. Our strategy was based on the investigation of one individual reaction with a limited number of ligands, where biodiesel had a dual role as both the medium and the product. Such approach allows a gradual assembly of the whole model, where novel elements will be introduced at the next stage of our work.

Conversion of F to B presents a very convenient starting point of investigation, because this segment of the process does not produce the cross-reacting ligands (T, D, M), and complexity of the scheme can be kept at a reasonable level. The rate constants for alcohols and biodiesel determined under F-reaction could be used in all models of the future analysis. Examination of F-conversion was important also as a separate study, because decrease of F below the specification level of 0.25% (m/m) by means of the enzymatic catalysis appeared to be the most challenging task according to our previous experiments [11]. The global strategy of enzymatic B production might consider the initial conversion of oil to biodiesel of 90–96% purity [11] followed by “polishing” cycle(s), where the remaining contaminations are converted to B. In this publication we examine the “polishing” process with particular focus on F.

The scheme of F-conversion was based on a shortened presentation of the ping-pong mechanism, where the original four-step process (Fig. 1A) was condensed to a two-step scheme (Fig. 1B), see Appendix (A1) and (A2). The model also considered reversible inhibition of the enzyme by alcohol and aggregation of water forming a separate layer WW with low chemical activity.

5.2. Monitoring of the reaction

Monitoring of the concentration changes was based on a novel approach, where the fluorescent signal from Nile Red (Fig. 2) was correlated with the reactant of interest (e.g. F) via parallel records of fluorescence and concentrations (Fig. 3). The chosen method made possible prognosis of the fluorescence for various reaction conditions even at a few calibrations shown in Fig. 3. Additionally, dispersion of the fluorescent signal was lower than that of TLC, which increased reliability of the data.

Presence of multiple calibration curves for exactly the same reaction seems to be strange at a first look. Yet, this fact can be explained by effects of phase separation. Water (either added or generated under the reaction) causes redistribution of alcohols between oil and water phases. In such case, the amount of alcohol in the oil phase depends on both the chemical stoichiometry ($F + C \leftrightarrow B + W$) and the equilibrium with water (e.g. $C + W \leftrightarrow CW$). Alcohol in the hydrophobic phase affects the fluorescence of Nile Red, making the calibration dependent on the redistribution effects. Additionally, enzyme particles appear to adsorb some reactants on their surface. This means that (i) one cannot use the artificial mixtures to prepare a calibration, and (ii) different enzymes might require separate calibrations.

5.3. Stepwise solution of the kinetic scheme

Analysis of data had been done in steps. In the beginning, the concentrations were recorded over time (e.g. Δf vs t in Fig. 4A and C), and the charts were fitted by exponential functions. Good exponential approximations indicated that the enzyme is indeed unsaturated, and the suggested simplification of the ping-pong mechanism (Fig. 1B) is valid. The initial velocities were calculated, normalized to the enzyme concentration (v/e_0) and plotted as functions of the corresponding substrate concentration (e.g. Fig. 4B and C).

The meticulously planned combinations of substrates were used to disassemble the reaction scheme in Fig. 1C into several segments, where only a few constants were evaluated per time. For example, analysis of the backward reaction $B + \sum W \rightarrow \dots$ (Fig. 4) helped to establish the values of k_b for MeOH and EtOH, as well as make a rough evaluation of k_w . The other rate constants were ignored at this stage.

Then, experiments with the forward reaction were conducted using two different schemes of substrate supply: (i) variable alcohol concentration at a constant F; or (ii) variable F on the background of a constant alcohol. The velocity dependencies on either c or f are shown in Figs. 5B, D and 6C, D. All these charts had k_f as a common parameter, and a comparative examination revealed its value (Table 1). The curves in Fig. 5B and D assisted in finding of k_c and K_{ic} , which were additionally verified for MeOH in Fig. 6C and D.

When the above stage was accomplished, the water-related constants became the subject of investigation. Determination of the true water constants was done in an equilibrium assay (Fig. 7) where the ratio $(b \cdot \sum w)/(f \cdot c)$ was plotted as a function of $\sum w$, see Appendix (D1) and (D2) and Eq. (4). Despite the same water in all experiments, we treated the water constants as being different for MeOH and EtOH. This was done to compensate redistribution of alcohols between oil and water phases, which was not identical for MeOH and EtOH. Such compensation could have been done either by introducing water–alcohol complexes (CW, CWW, etc.) or assuming different association and activity properties of water droplets WW in the presence of MeOH or EtOH. The latter approach was by far less troublesome, and it was used with satisfactory results. Absence of any kinetic manifestation of alcohol redistribution in the alcohol-related rate constants might imply, that MeOH and EtOH occupy the surface of water droplets and remain exposed, whereas water itself becomes hidden in the bulk. The results of performed analysis are summarized in Fig. 7 and Table 1.

5.4. Modeling of F conversion

The detailed characterization of the F conversion was used to examine various scenarios of this reaction. It has previously been found that a single-step conversion is not sufficient to make a biodiesel product conforming to the specifications [1–3]. For example, introduction of water to the reaction mixture (necessary for high activity of the enzyme Lipozyme TL HC) caused accumulation of F in B [11]. Therefore, a “polishing” step seemed to be necessary, where biodiesel with a few percents of F should be subjected to additional treatment with Novozym 435. The final contents of F was chosen as 0.2% as being well below the specification of 0.25%. Numerous variants of the process were examined by computer modeling. The equilibrium curves are shown in Fig. 8A and B, and they indicate that conversion of F to 0.2% in a single “polishing” step is unrealistic if the initial contents of F exceeds 1%. The shifting of equilibrium would require a high alcohol concentration, resulting in an

scenarios are possible if using the data from Table 1 and a suitable computer program (e.g. COPASI [14]).

6. Conclusions

Enzymatic conversion of fatty acids to biodiesel was examined and quantified in terms of the relevant kinetic constants. The effect of MeOH and EtOH on the overall equilibrium and the enzymatic activity was examined. Separation of water into an individual phase was approximated by a simple association scheme. A functional computer model was designed, and a number of “virtual experiments” allowed a fast optimization of the process. The current model is a part of a full biodiesel scheme being in preparation.

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Appendix A. Simplification of the ping-pong mechanism

The “classical” velocity expression for the ping-pong mechanism (Fig. 1A) in the absence of any products can be presented as follows:

$$\frac{v}{e_0} = \frac{V_+}{1 + K_{m1}/s_1 + K_{m2}/s_2}; \quad V_+ = \frac{k_{p1} \cdot k_{p2}}{k_{p1} + k_{p2}}; \quad K_{m1} = \frac{K_{s1} \cdot k_{p2}}{k_{p1} + k_{p2}}; \\ K_{m2} = \frac{K_{s2} \cdot k_{p1}}{k_{p1} + k_{p2}} \quad (A1)$$

This equation becomes simplified if the concentrations of substrates are much lower than the corresponding Michaelis constants:

$$\frac{v}{e_0} \approx \frac{k_{s1} \cdot k_{s2} \cdot s_1 \cdot s_2}{k_{s1} \cdot s_1 + k_{s2} \cdot s_2}; \quad k_{s1} \approx \frac{k_{p1}}{1.3 \cdot K_{s1}}; \quad k_{s2} \approx \frac{k_{p2}}{1.3 \cdot K_{s2}} \quad (A2)$$

This expression corresponds to the scheme shown in Fig. 1B. It should be noted that introduction of the correction coefficients (e.g. 1.3) to k_{s1} and k_{s2} expressions extends applicability of the velocity Eq. (A2) from a very low saturation level to $v \leq 0.4 \cdot V_{max}$ as discussed elsewhere [15]. The elementary two-step mechanism in Fig. 1B was used to draw the full reaction scheme in Fig. 1C.

Appendix B. Global model

The King-Altman master pattern of the considered steady-state reaction is shown in Fig. 1C. It is supplemented by two equilibria: (i) an unproductive binding of alcohol to the free enzyme and (ii) aggregation of water. The below general equation was derived:

$$\frac{v}{e_0} = \frac{N_+ - N_-}{D} = \frac{k_f^* \cdot f \cdot k_c \cdot c + k_{fw}^* \cdot f \cdot w \cdot k_c \cdot c - k_b^* \cdot b \cdot k_w \cdot w - k_b^* \cdot b \cdot k_{ww} \cdot ww}{k_f^* \cdot f + k_c \cdot c + k_b^* \cdot b + k_w \cdot w + k_{fw}^* \cdot f \cdot w + k_{ww} \cdot ww} \quad (B1)$$

irreversible damage to the enzyme. The “safe” limits for Novozym 435 were estimated as 1.5 M MeOH (6%) and 2 M EtOH (12%).

The decrease of F from 4% to 0.2% could be done in three steps instead of one; by alternating the reaction with water drying (final level of water ≈ 100 ppm). The two convenient scenarios for MeOH and EtOH were simulated in Fig. 8C and D with the overall reaction times of 150 min and 240 min, respectively. The scheme with a simultaneous evaporation of water and alcohol is also feasible but requires a continuous supply of alcohol to maintain high velocity of forward reaction (not shown). Simulations of numerous other

where concentrations of the two water forms w and ww are functions of the total water concentration $\sum w$ (see below Appendix C). Simultaneous presence of substrates and products means that the total velocity ($v = db/dt$) is expressed as $v = v_{forward} - v_{backward}$, and its value might become negative. The “star” parameters (k_x^*) in Eq. (B1) are equal to the below expression (f_e standing for the fraction of E):

$$k_x^* = k_x \cdot f_e = \frac{k_x}{1 + c/K_{ic}} \quad (B2)$$

Appendix C. Water equilibrium

Association of water can be described by the below equilibrium equation and the material balance.

$$K_{ww} = \frac{w^2}{ww}; \quad \Sigma w = w + 2 \cdot ww = w + 2 \cdot \frac{w^2}{K_{ww}} \quad (C1)$$

Solution of the quadratic equation provides the expressions for both w and ww as functions of Σw :

$$w = \frac{-1 + \sqrt{1 + 8 \cdot \Sigma w / K_{ww}}}{4 / K_{ww}}; \quad ww = \frac{1}{2} \cdot (\Sigma w - w) \quad (C2)$$

Appendix D. The apparent reaction equilibrium

The full reaction scheme in Fig. 1C describes two isolated equilibriums for W and WW molecular species (both dependent on total water). The equilibrium requires $N_+ - N_- = 0$ in Eq. (A1). This leads to the below transformations:

$$N_+ - N_- = k_f^* \cdot f \cdot k_c \cdot c + k_{fw}^* \cdot f \cdot w \cdot k_c \cdot c - k_b^* \cdot b \cdot k_w \cdot w - k_b^* \cdot b \cdot k_{ww} \cdot ww = 0 \quad (D1)$$

$$k_c \cdot (k_f + k_{fw} \cdot w) \cdot f \cdot c = k_b \cdot \left(k_w \cdot \frac{w}{\Sigma w} + k_{ww} \cdot \frac{ww}{\Sigma w} \right) \cdot b \cdot \Sigma w \quad (D2)$$

The equilibrium concentrations of f , c , b , Σw should be regrouped on one side and all the rate constants (as well as water species w and ww) on another one leading to Eq. (4) presented in Section 4.7.

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