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Paper

Water-dependent kinetics guide complex lipase-mediated synthesis of biolubricants in a water activity control reactor†

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A water-dependent kinetic model for the lipase-mediated reaction with multiple substrates and products in a water activity controlled reactor was developed. Solvent-free esterification of trimethylolpropane (TMP), the products of which can be used as biolubricants, was investigated using a lipase from *Candida sp.* 99-125 as catalyst under variable water activities in a 5 L batch stirred tank reactor. The water activity control was accomplished at the set point by the introduction of dry air through the reaction medium at a digital-feedback-controlled flow rate. For the cases of esterification of TMP, the long-term (>72 h) control of water activity resulted in a considerable improvement of yield. By introducing a progressive water removal, and by combining the principles with an intrinsic kinetic model of Ping-Pong Bi-Bi mechanism, the integrated model can predict both the forward and reverse rates for TMP reactions. Kinetic parameters depending on the water activity were estimated by nonlinear regression fitting of experimental data. The proposed approach not only enables the optimization of the reaction under defined conditions, but also provides a solution for industrially upscaling similar environment-friendly biocatalytic processes.

Keywords: Kinetics; Water activity control; Lipase-mediated esterification; Biolubricant; Trimethylolpropare esters

Introduction

World demand for lubricants is expected to rise by 2.4 % annually, reaching a total demand of 43.6 million metric tons in 2017[1], primarily driven by the numerous applications of lubricants such as in motor vehicles, manufacturing, etc. Lubricants can be either mineral- or bio-based. The environmental impact of mineral-based lubricants has become an increasingly critical issue[2]. Approximately 50% of all lubricants sold worldwide end up in the environment as spills. Moreover, applications with total loss of the lubricants cause a severe pollution problem[3] and raising health hazards to humans by indirect routes through our environment[4]. The need for eco-friendly lubricants based on vegetable oils or synthetic esters, both considered rapidly biodegradable[4, 5], is considerably growing. The advantages of bio-based lubricants, including biodegradability, excellent lubricity, renewability of the source etc. are the primary driving force for their use. Conclusions from comparative life-cycle assessments showed that the use of bio-based lubricants could significantly reduce the environmental impact compared with mineral oils[6-8]. The current obstacle for bio-based lubricants is that they are two to three times more expensive than conventional mineral lubricants. Nevertheless, the market share for bio-based lubricants is increasing[5] since the bio-based lubricants have been already imposed in kinds of lubricant's usage (e.g. machinery in forestry, etc.) by various government bodies worldwide.

Despite the fact that vegetable oils possess most of the desired lubricity properties, they have significant drawbacks, particularly their poor low temperature performance and inadequate oxidative stability[3]. Synthetic esters (SEs) offer an appealing alternative in environmentally friendly lubricants; they are already widely used in many applications including automotive and marine engine oils, compressor oils, hydraulic fluids, gear oil and grease formulations[5]. In addition to the above-mentioned advantages, the SEs can be designed to have tailor-made properties, in order to use them in applications under various conditions. For example, polyol oleates are designed for lower viscosity, while polyol esters of saturated fatty acids have high oxidative stability, satisfactory hydrolytic stability and excellent low temperature performance[5]. Polyol esters, the most important kind of SEs, are composed of fatty acids attached to an organic polyol, such as trimethylolpropane (TMP), neopentyl glycol and

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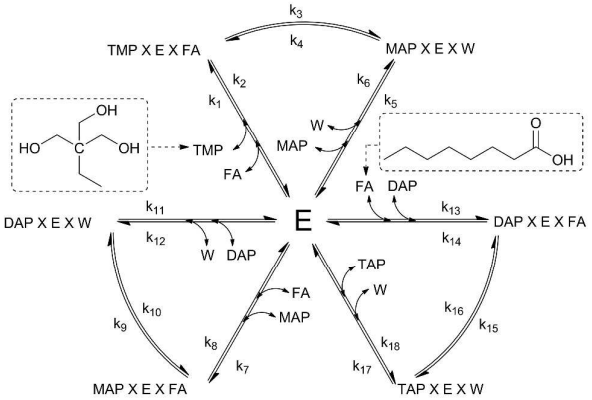
pentaerythritol. Among these polyhydric alcohols, TMP is attractive and well-studied, used as substrate in the synthesis of SEs. TMP esters have been demonstrated with better lubricity performance in formulating lubricants such as metal-working fluids[9], engine lubricants[10] and hydraulic fluids than conventional mineral oils.

The synthesis of TMP esters either by chemical[11-13] or by enzymatic[14-20] approaches has been described in literature. Lipases (EC 3.1.1.3), a sub-class of the α/β-hydrolases super family, have been used as biocatalysts for a wide range of applications such as waste treatment, fine chemistry, biofuel production and in pharmaceutical industry[21-24]. Lipase-mediated synthesis of TMP esters via acyl transfer[14] or direct esterification[15, 18-20] in solvent free systems was reported and these processes are considered as environmentally friendly productions. However, although some efforts, like modification of immobilized lipase and appropriate substrate feeding strategies[20] have been under investigation, the reported largest scale of direct esterification was limited to 100 g of substrate in a 500 mL reactor[15], which was still far away from the expectations of industrial productions. The two main hindrances causing this limitation were the lack of suitable bioreactor, especially for the efficient removal of the water produced, and the lack of an accurate kinetic model within the water balance, dealing with complex lipase catalytic reactions, which involves multiple steps with multi-substrates and multi-products.

The water content in the reaction medium is a key parameter in non-aqueous enzymology both for the maintenance of the three-dimensional structural integrity of the enzyme and also for the optimal catalytic activity of the enzyme. Water molecules can act simultaneously as a competitive inhibitor, a competitive substrate and a molecular lubricant. The structure of TMP (Scheme 1) is quite similar with glycerol, bearing an ethyl group substitution in the carbon atom in position 2, but it is a non-natural substrate for lipase. Due to the three-dimensional symmetric structure of TMP, the esterification of TMP with caprylic acid would result in tri-substituted TMP esters (due to the lack of steric hindrances) and 3 water molecules per TMP molecule would be produced, which means 10.5 g water would be produced for each 100 g tri-substituted TMP ester formatted, which would result to a water content of about 10 % (w/w), a condition which would strongly drive the equilibrium to the hydrolysis rather than the esterification. The *in situ* removal of water is difficult because it must be performed at relatively low temperatures to preserve the enzymatic activity.

Lipases are sensitive to water content and require different water contents for optimal activity[25, 26]. The efficient control of the water content becomes critical issue for scaling-up of lipase-catalyzed direct esterification. The first obstacle is the precise online measurement of the water content. Halling[27] proposed the measurement of thermodynamic water activity instead of water content since the water activity has good correlations with the amount of water bound to the enzyme in a wide range of conditions. A straightforward method to quantify the water activity is to use a sensor to continuously measure the relative humidity and temperature in the reactor[28]. Such sensors are commercially available[29], but only a few can be used in an environment with volatile organic solvents. Subsequently, the problem about how to control the water activity remains a critical subject. The commonly used method is pre-equilibration of the reaction components with saturated salt solutions. However, the final water activity may be disturbed when mixing the components to initiate the reaction. The water activity also could change during the reactions due to the produced or consumed water. Flowing a saturated salt solution through microporous and hollow fiber polypropylene membranes in the reactor[30] has been suggested to continuously control the water activity in the reactor. Another suggestion was equilibration between reactants and saturated salt solutions during reaction[31], but the slow mass transfer of water among solutions, air and reactants limits the feasibility of this method using in large-scale reactors. Novel membrane separation is an alternative candidate since the high-selective hydrophilic membrane can be available[32]. A computer-aided water activity control system with the introduction of air or nitrogen has been demonstrated as an efficient system maintaining constant water activity and adjusting the water content in reactants[28, 33], but till now no such system has been developed for controlling the water activity in a water abundant reaction like synthesis of TMP esters.



Scheme 1 Outline of the mechanism for lipase-mediated esterification of TMP and caprylic acid. The abbreviations can be found in the Notes and Reference part.

Kinetic modeling is an efficient tool to provide those parameters relevantly impacting on the productivity of enzymatic processes through guiding the processes' design and upscaling[34-36]. The most extended models are based on the application of *Michaelis-Menten* assumptions, which seem to be valid for simple enzymatic reactions. When substrates have more than one functional group, like TMP, their kinetic models become more complicated because the intermediate products (i.e. MAP, DAP) also act as substrates for subsequent reaction; all these reactions happen simultaneously. The increase of the number of reactants leads to the exponential growth of the number of the kinetic equation terms: for glycerolysis of olive oil, the kinetic model contains 18 terms[37] while a simple reaction model based on *Michaelis-Menten* assumptions has only four. The kinetic models for lipase-catalyzed esterifications with multiple reactants are sparse. Bornadel et al.[19] proposed a simplified model for lipase catalyzed esterification of TMP with oleic acid. In their work,

the effects of hydrolysis (reverse reaction) and the kinetic term for water were ignored, since water was continuously removed by using a vacuum pump. However, considering the important roles of water in lipase-mediated esterification and the extreme *in situ* difficult control of the water content in large scale, the effects of water should be included into the kinetic modeling. Moreover, it is necessary to propose a model that could predict both forward and reverse rate constants for esterification of TMP esters. To our knowledge, no such kinetic models considering water effects were reported for complex lipase-catalytic esterification.

The present research, for the first time, presents a kinetic model within a water balance that could predict both forward and reverse rate constants for complex lipase-mediated reactions of multiple substrates and products. The model was developed in a 5L bioreactor, equipped with a computer-aided water activity control system. By introducing a water removal rate and combination with an intrinsic kinetic model that follows the Ping-Pong Bi-Bi mechanism, an integrated kinetic model for complex lipase-catalyzed esterification was developed. Kinetic parameters depending on water activity were determined by fitting the experimental data to the model, using a Matlab script based on nonlinear regression. The effect of water activity on the performance of lipase was investigated through controlling the water activity at different levels.

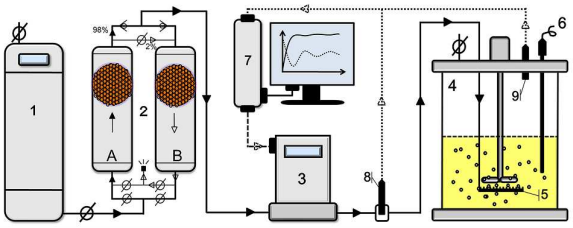


Fig. 1 Construction of water activity control system. 1-Air compressor; 2-Air dryer with automatically regeneration system of molecular sieves; 3-Air mass flow controller; 4-Stirred reactor; 5-Gas distributor; 6-Temperature sensor; 7-Water activity control program and computer; 8, 9-Relative humidity sensor measuring the inlet and outlet air, respectively. The black arrows show the direction of the air flow and the dashed arrows show the analogous signal direction. The molecular sieve auto-regenerated system is composed of two columns: when one of them is producing dry air, the other one is regenerated using dry air.

Experimental

General aspects

Trimethylolpropane with a melting range of 56-59 °C and caprylic acid with a purity of 98% were obtained from Fuchen Chemical Co. Ltd. (Tianjin, China). Ethyl acetate of chromatographic grade was purchased from Sigma-Aldrich. The other solvents and salts of analytical grade were obtained from Beijing Chemical Factory. The characterization and catalytic properties of lipase from *Candida sp.* 99-125 were described in the previous works[38, 39]. Lipase formulation (LS-20) from *Candida sp.* 99-125 was produced by Beijing CTA New Century Biotechnology Co., Ltd. The esterification activity of lipase formulation was estimated through a model esterification reaction of dodecanoic acid and dodecanol. A standard esterification procedure was described in previous works[40]. The specific esterification activity of lipase formulation was 420 ± 12 U/g.

Construction of water activity control system

A computer-aided system was developed to control the thermodynamic water activity in a 5 L BSTR for enzyme catalyzed synthesis; a schematic schedule of this system is shown in Fig. 1. The system mainly comprised 6 aspects as follows: air compressor and dryer, air mass flow controller, stirred reactor, relative humidity sensors and water activity control program.

Since water is produced continuously and should be removed efficiently during the esterification, instead of using humid air described in literature[28, 33], almost dry air (a_w: 0.04-0.10) was used and produced by a molecular sieve auto-regenerated air dryer, which was composed of two columns: when one of them is producing dry air, molecular sieve in the other one is under the ongoing desorption of water molecules by the pressure change of dry air. This system can operate for at least one year avoiding the frequent-change and high-cost reuse of molecular sieve. Water activity and temperature of the inlet and outlet air were measured in the gaseous phase by Hygroclip 2 sensors (Rotronic, Switzerland), and then the analogue signal of these values were received and monitoring by computer. The software *WACS-BUCT* based on Proportional-Integral-Differential (PID) control algorithm were written to maintain the water activity of the outlet air at a given point by varying the inlet air flow through an air mass flow controller (CS200A, Beijing Seven-Star Electronics Co., LTD.). The feedback time of water activity system was 15s.

Compared to the system with the constant flow using humid air, the present system with the air flow gradually decreasing in accordance with the decline of water production rate especially at the end of reaction can significantly save the cost of dry air supply, which is necessary to the reduction of industrial production cost.

Lipase mediated synthesis of TMP esters

Synthesis of TMP esters was carried out in a stirred reactor equipped with the water activity control system. 1.45 Kg of caprylic acid and 0.43 Kg of TMP with a molar ratio of 3.2:1 were mixed. About 25 min after addition of TMP to caprylic acid at 50 °C, a clear and homogeneous solution was observed. Heating to 60 °C can accelerate the dissolution of TMP. Subsequently, 200 g lipase powder was added and the water control system was started. The reaction was performed at 50 °C and 250 rpm for 80h. Paralleled experiments were performed and the errors were less than 3%: for clarity, error bars were omitted in most figures.

Analytical procedure

Samples were obtained at scheduled time (every 3 hours) and immediately heated to ensure lipase deactivation. The compounds of the reaction mixture, including TMP, caprylic acid, mono-, di-, and tri- substituted TMP esters were dissolved in ethyl acetate and quantified using a gas chromatography (GC-2010, Shimadzu Japan) equipped with a capillary column (DB-1ht, 30m×0.25mm×0.1um; J&W Scientific, USA) and a flame ionizing detector (FID). The column temperature was held at 110°C, heated to 125°C at 5°C/min and finally to 300°C at 30°C/min then maintained for 2 min. The temperatures of the injector and detector were both set at 300°C. The retention time of TMP, caprylic acid, mono-, di-, and tri-substituted TMP esters was 1.61 min, 1.76 min, 6.04 min, 8.22 min, and 9.71 min, respectively. Standard curves for each compound were used.

A Karl-Fischer moisture titrator equipped with a double platinum electrode from Beijing Xianqu Weifeng Technology Development Co. Ltd was used to quantify the water content of the samples. About 100 mg of sample was analyzed at room temperature with a one-component system using monopropellant Karl-Fischer reagent as titrating solution and methanol-dry as the solvent.

Theory

An integrated kinetic model was developed for lipase-mediated solvent-free esterification of TMP and caprylic acid carried out in 1.88 Kg scale of 5 L batch stirred tank reactor (BSTR) with water activity control system using formulation of a lipase from *Candida sp.* 99-125, on the basis of intrinsic kinetics and water balance equations.

Intrinsic kinetic modeling

General mechanisms for enzymatic reactions include three possibilities: (1) a random-order mechanism, (2) a Ping-Pong mechanism and (3) an ordered Bi-Bi mechanism. The lipase activity relies on its catalytic triad, formed by Ser, His and Asp/Glu residues. The reaction occurs via the formation of a tetrahedral acyl-enzyme intermediate, which undergoes a nucleophilic attack from the second substrate, leading to the product formation and release of the free enzyme[41]. Moreover, TMP and mono-, di-esters of TMP (MAP and DAP respectively) can simultaneously act as the subsequent substrates of acyl-enzyme intermediate while the tri-ester TMP (TAP) can be used as hydrolysis substrate from the lipase, in case the reverse reaction is favored. Combining these two points, we propose a Ping-Pong Bi-Bi mechanism for lipase-mediated esterification of TMP with fatty acid, as shown in Scheme 1.

Kinetic models for transesterification or glycerolysis/ hydrolysis of glycerol esters based on pseudo-equilibrium relationships have been well studied[37, 42, 43]. Here, our intrinsic kinetic model for esterification of TMP with caprylic acid was also assumed to follow the pseudo-equilibrium relationships since the concentration of the enzyme used was much lower than the concentration of substrates, so that the reaction rates for different enzymatic intermediate complexes were approximately zero. Another two assumptions were necessary for the simplicity of the kinetic model. Although the reaction rates might be controlled by the mass transfer for lipase formulation when aggregation happens due to high water content[25], these limitations can be neglected when investigating soluble enzyme system[44] under low water content; substrates or products inhibition were ignored in our kinetic model and non-enzymatic reactions were neglected due to the low reaction temperature.

All reaction rates of intrinsic kinetics for each compound can be expressed in terms of kinetic constants and concentrations of enzyme and compounds, as described in the Supplementary Information. The apparent reaction rates V_i and equilibrium constants K_i (i = 1 to 6) in Table 1 are functions of the constants k_i (i = 1 to 18).

Table 1 Kinetic parameters for rate equations of lipase-mediated esterification between TMP and caprylic acid based on intrinsic kinetic model.

Constants	Expressions	Constants	Expressions
K_1	$\frac{k_1(k_3+k_4+k_5)}{k_2k_4+(k_2+k_3)k_5}$	V_1	$\frac{k_1k_3k_5}{k_2k_4+(k_2+k_3)k_5}$
K_2	$\frac{k_6(k_2+k_3+k_4)}{k_2k_4+(k_2+k_3)k_5}$	V_2	$\frac{k_2k_4k_6}{k_2k_4+(k_2+k_3)k_5}$
K_3	$\frac{k_7(k_9+k_{10}+k_{11})}{k_8k_{10}+(k_8+k_9)k_{11}}$	V_3	$\frac{k_7k_9k_{11}}{k_8k_{10}+(k_8+k_9)k_{11}}$
K_4	$\frac{k_{12}(k_8+k_9+k_{10})}{k_8k_{10}+(k_8+k_9)k_{11}}$	V_4	$\frac{k_8k_{10}k_{12}}{k_8k_{10}+(k_8+k_9)k_{11}}$
K_5	$\frac{k_{13}(k_{15}+k_{16}+k_{17})}{k_{14}k_{16}+(k_{14}+k_{15})k_{17}}$	V_5	$\frac{k_{13}k_{15}k_{17}}{k_{14}k_{16}+(k_{14}+k_{15})k_{17}}$
K_6	$\frac{k_{18}(k_{14}+k_{15}+k_{16})}{k_{14}k_{16}+(k_{14}+k_{15})k_{17}}$	V_6	$\frac{k_{14}k_{16}k_{18}}{k_{14}k_{16}+(k_{14}+k_{15})k_{17}}$

Water balance

In our system, water was continuously produced by the esterification reaction, consumed by hydrolysis and removed from the system by the dry air flow. The rate of water produced or consumed could be calculated from intrinsic kinetics while the water removal rate could be calculated by the deviation of moisture content between inlet and outlet air. Parameters of the inlet and outlet air, including water activity a_w and temperature, were measured by water activity sensors. The air flow rate was obtained from an air mass flow controller. In order to derive the water balance equation, we assumed that humid air contains dry air (a_w=0) and moisture, something that satisfies state equation of ideal gas and Dalton's law of partial pressure.

Firstly, for dry air, $P_aV = m_aR_aT$; for moisture, $P_wV = m_wR_wT$, where $R_a = 0.287\ KJ/(Kg\cdot K)$ and $R_w = 0.461\ KJ/(Kg\cdot K)$.

The moisture content (d) in dry air was defined as the mass weight of moisture in 1 Kg dry air:

$$d = \frac{m_w}{m_a} = \frac{R_aT}{R_wT}\times\frac{P_wV}{P_aV} = 0.622\frac{P_w}{P_a} = 0.622\frac{a_w\times P_{w,s}}{P_a} \quad (1)$$

and then this equation can be rewritten as

$$m_w = 0.622 a_w \times \frac{P_{w,s}}{R_a T} \times V \quad (2)$$

The value of water vapor saturation pressure $P_{w,s}$ was estimated according to an equation reported in literature[45].

Hence, the water removal rate by the introduction of dry air can be calculated through the water mass weight deviation between inlet and outlet air as following:

$$K_{w,v} = \frac{dm_{w,v}}{dt} = \frac{d[\int_0^t (m_{w,in} - m_{w,out})dt]}{dt}$$
$$= 0.622 \times \left(a_{w,in} \times \frac{P_{w,s,in}}{R_a T_{in}} \times V_{in} - a_{w,out} \times \frac{P_{w,s,out}}{R_a T_{out}} \times V_{out}\right) \quad (3)$$

The kinetic term for water (equation *Seq26*) should be revised as following:

$$\frac{d[W]}{dt} = \frac{[ET]\begin{pmatrix} V_1[TMP][FA] - V_2[MAP][W] + V_3[MAP][FA] \\ -V_4[DAP][W] + V_5[DAP][FA] - V_6[TAP][W] \end{pmatrix}}{\begin{pmatrix} 1 + K_1[TMP][FA] + K_2[MAP][W] + K_3[MAP][FA] \\ +K_4[DAP][W] + K_5[DAP][FA] + K_6[TAP][W] \end{pmatrix}}$$
$$+K_{w,v} \quad (4)$$

Secondly, in order to verify the value of $K_{w,v}$, the water content in system was expressed in term of related parameters and compared with experimental values. Water production rate also can be calculated by the caprylic acid consumption rate because they are equimolar. Thus,

$$K_{w,g} = \frac{dm_{w,g}}{dt} = -d\left(\frac{C_{FA} \times m_{r,t} \times 18.02}{144.21 \times 100}\right)/dt$$
$$= -0.00125\left[K_{ca} \times m_{r,t} - K_{w,v} \times C_{FA}\right] \quad (5)$$

Finally, at a given time, the water content in reactants can be calculated as the ratio of total mass weights of initial, produced, consumed and removed water to total reactant weight:

$$C_{w,t} = \frac{m_{w,0} + m_{w,g,t} + m_{w,v,t}}{m_{r,t}}$$
$$= \frac{(m_{r,0} + m_E) \times C_{w,0} + \int_0^t K_{w,g}\, dt + \int_0^t K_{w,v} dt}{m_{r,0} + m_E + \int_0^t K_{wa,v} dt} \quad (6)$$

Model fitting

Nonlinear regression is a form of regression analysis in which experimental data are modeled by functions which are nonlinear relationships of the model parameters and depends on one or more independent variables; thus in this work, a Matlab script based on nonlinear regression of least square method was written, in order to compute both forward and reverse constants in Table 1 and fit the overall model (Supplementary Information equations *Seq21-25* and equation *4* or *Seq26*). Noticing that enzyme concentration *[ET]* and $K_{w,v}$ are not in terms of any kinetic parameters but functions of time, thus these two variables were firstly done by polynomial fit and then put into kinetic model. For the parameter fitting of kinetic model, 20 experimental data set comprising 120 experimental data points for *TMP*, *MAP*, *DAP*, *TAP*, *FA* and *W* concentrations were used.

Results and discussion

Control of water activity during TMP esters synthesis

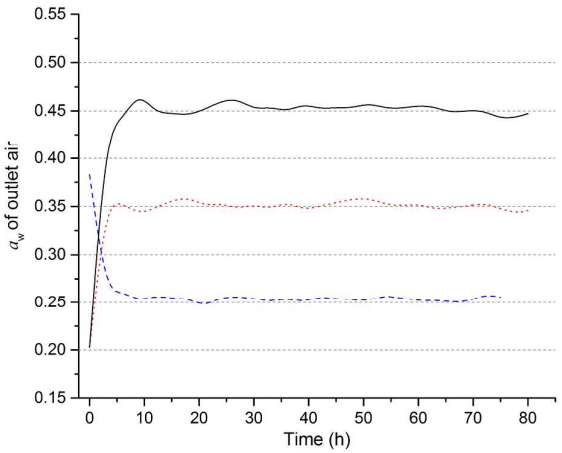


Fig. 2 Profiles of water activity in outlet air during syntheses of TMP esters controlled at different water activities 0.25 (dashed line), 0.35 (dotted line) and 0.45 (solid line).

In order to evaluate the water activity control system during the production of water from esterification process, lipase-mediated syntheses of TMP esters were performed at different controlled water activities in a 5 L BSTR. Ester synthesis by formulation of lipase from *Candida sp.* 99-125 was monitored at controlled a_w values of 0.25, 0.35 and 0.45; an a_w value of less than 0.25 could not be attained for the synthesis of TMP esters in our system due to the use of almost dry air (a_w: 0.04-0.10). The system was evaluated in a scale of 3.1 M TMP and 10 M caprylic acid, which after a full conversion would yield 9.3 M water. There are works were researchers successfully controlled the a_w , however in significantly lower scale (up to 1 M of substrates)[28, 33].

The profiles of a_w in the outlet air during synthesis of TMP esters can be seen in Fig. 2. The use of dry air makes it possible to induce a large a_w gradient and thereby a high water removal rate; as a result, the controlled water activity can quickly reach the set point. Our system, a 5 L BSTR with a reactant volume of 2.5 L, needed a period of 150-200 min response time to reach the desired value using only dry air, when controlling a lipase-catalyzed TMP esterification, starting from an a_w value which differs about 0.2 from the desired value. This response time is comparable with the literature[33], when taking the bigger volume of our system into consideration. Ujang et al.[46] reported that the change of water activity from 0.33 to 0.75 took 5 hours in a gas-phase hollow fiber reactor.

The system responded gently to the change of water activity, reaching to the desired value without sudden fluctuations, as observed in other systems[28]. Despite the fact that water was continuously produced by esterification, the

water activities were stable during the whole reaction (> 72h) and fluctuated only slightly around the set points giving a standard deviation of 0.004 for a_w 0.25 and 0.35, and 0.008 for a_w 0.45. In addition to the control algorithm itself, these acceptable fluctuations in practice can be caused both by the fluctuations of water activity and by temperature of inlet air and the temperature of reactor headspace.

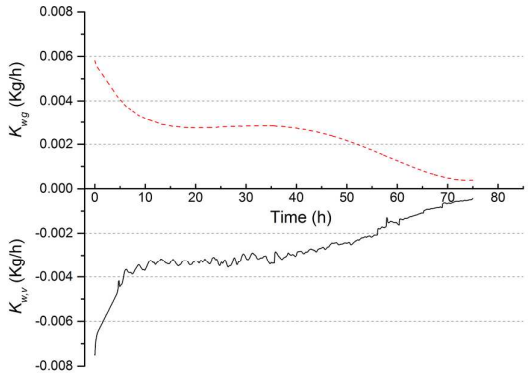


Fig. 3 Comparison of the removal rate ($K_{w,v}$, calculated by equation *3*, solid line) and produced rate ($K_{w,g}$, calculated by equation *5*, dashed line) of water during the whole reaction when a_w was controlled at 0.25.

Aimed to provide a deep insight in the mechanism of the water activity control system, comparison of the removed rate ($K_{w,v}$, calculated by equation 3) and produced rate ($K_{w,g}$, calculated by equation 5) of water during the whole reaction when a_w was controlled at 0.25 is depicted in Fig. 3. It can be easily seen that the two rates are changing simultaneously, leading to the stabilization of the a_w around the desired value. It needs to be mentioned that there was a small numerical difference of these two rates: the average $K_{w,v}$ value was −2.37 g/h while average $K_{w,g}$ was 2.18 g/h during the whole esterification. This difference could be due to the fact that the change of the reactant weight ($W_{r,t}$) due to the removal of produced water was not considered as a factor at the PID control algorithm.

Estimation of kinetic parameters

The validity of the $K_{w,v}$ value was identified through the comparison between observed and predicted (calculated from equation *6*) values of the water content in the medium. As shown in Fig. 4, the profiles of predicted water content are describing fairly the trends of experimental data. When $K_{w,v}$ value increases or decreases even only 10%, predicted curve of water content (data not shown) significantly deviates from experimental data. These results indicate that the $K_{w,v}$ value was valid in our study and can be used for kinetic model fitting. However, $K_{w,v}$ was not a function of kinetic terms but of time; thus, $K_{w,v}$ should be firstly done by 5-order polynomial fit and then put into kinetic equation 2. The same situation was for enzyme concentration so that [*ET*] values were also firstly 2nd-order polynomial-fitted, as shown by solid lines in Fig. 5.

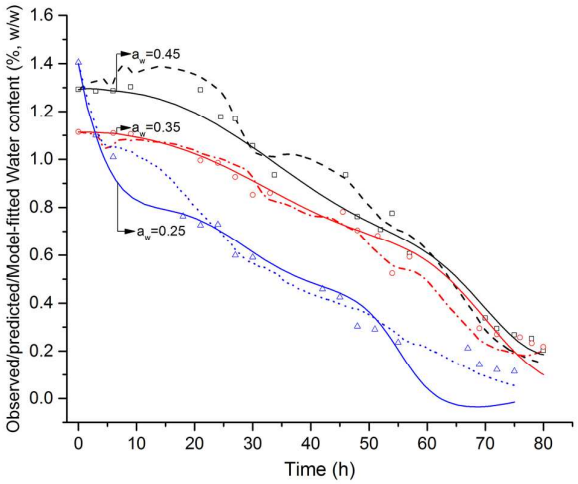


Fig. 4 Comparison of observed (symbols), predicted (dashed or dotted line, calculated from equation 6) and model-fitted (solid line, within water balance) water content in medium when the water activity was controlled at 0.25 (▲, blue), 0.35 (○, red) and 0.45 (□, black).

Subsequently, the overall reaction kinetic model, including water balance equation (equation *Seq21-25* plus equation *2*), was fitted to experimental data when water activity was controlled at different values. The fitting results are shown in Fig. 4 and Fig. 6 A-C; the correlation efficient for each compound between observed and calculated data is shown in Table S1. Generally, the values of correlation efficient over 0.963 show good agreement between experimental and model-fitted concentrations of *TAP*, *DAP*, *MAP*, *TMP*, *FA* and *W*. To highlight the advantage of the proposed model, we also fitted the experiment data using intrinsic kinetic model (equation *Seq21-25* plus equation *S26,* Fig. S1, S2) and the values of correlation efficient significantly decreased, to values as low as 0.655 (Table S2). All these results illustrate the extreme importance of comprehensive investigation of water, introducing both water removal and production rates into the kinetic model: for the kinetic modeling in large scale lipase-mediated complex direct esterification, water effects cannot be ignored.

All estimated values for kinetic parameters are listed in Table 2. Comparison of values of apparent rate constants V_i show that the forward rates were considerably larger than the reverse rates, especially V_1 was hundred times V_2. Due to the increase of molecular size from TMP to DAP, in other words, the increase of substrates' steric hindrance for *Candida* sp. lipase, the values of the forward rates V_1, V_3 and V_5 decreased gradually. This trend was also observed in the kinetic modeling of chemical transesterification of palm oil-based methyl esters with TMP[12, 13]. In a different view, *Michaelis* constants K_M for TMP, MAP and DAP were calculated by Bornadel et al.[19] when using Novozym 435 lipase and the values showed an gradual increasing tendency, which was in accordance with our results. The minimal reverse rate V_2 indicates the lowest hydrolysis

rate of mono-substituted TMP esters while V_4 and V_6 shared comparable values.

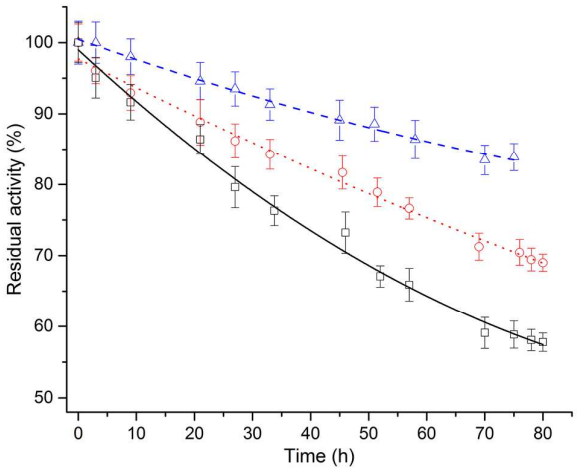


Fig. 5 Time-course profiles of residual activity of lipase when water activities were controlled at 0.25 (▲, dashed line), 0.35 (○, dotted line) and 0.45 (□, solid line). The solid lines were from 2nd.order polynomial fit of experimental data.

Effects of water activity

Water is always present in enzymatic processes, even in so-called non-aqueous systems and it has profound effects on the performance of lipases[27]. A molecular dynamics study of *Candida antarctica* lipase B in pure water and in organic solvents at five different water activities showed that similar water activities yielded similar enzyme hydration in the different solvents[47]. Thus, as in our system the accurate determination of the water activity is possible, we investigated the effect of water activity on the synthesis of TMP esters and lipase activity, by performing the reaction at different a_w levels.

Table 2 Values of kinetic parameters estimated by model within water balance when water activity was controlled at different values.

Items	Apparent forward rates			Items	Apparent reverse rates		
	0.25	0.35	0.45		0.25	0.35	0.45
V_1	3.54	3.14	2.86	V_2	0.0002	0.0002	0.017
V_3	2.70	2.01	1.95	V_4	0.47	0.29	0.43
V_5	1.08	0.74	0.77	V_6	0.11	0.11	0.22

In order to verify the a_w equilibrium, several factors should be considered. First, the a_w in the reaction medium did not significantly differ from that in the headspace[33]. This indicates that the dry air flow is sufficient to remove the produced water in a due time, keeping the a_w stable. Second, the flow rate of inlet air varied, following the water production rate. Lee et al.[33] concluded that equilibrium was established between the reaction medium and the outlet air despite the changes of air flow rate during the whole reaction. Third, the composition of reactant may be another factor that affects the equilibrium of water, because the concentrations of substrates and products are constantly changing during the reaction. The results revealed that the amount of water bound to enzymes is not directly related to the kind of solvents.

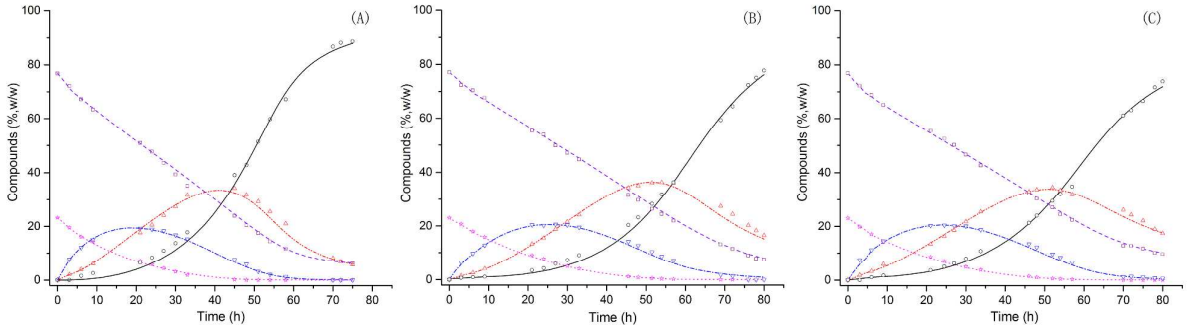


Fig. 6 Fitting of the kinetic model within water balance to the experimental data when the water activity was controlled at (A) 0.25, (B) 0.35 and (C) 0.45. ○: TAP; ▲: DAP; ▽: MAP; ☆: TMP; □: FA.

Time-course profiles of lipase-mediated esterification of TMP under controlled water activity are shown in Fig. 6. The reaction proceeded stepwise: the TMP concentration decreased to zero; the MAP and DAP concentration showed a "bell" shape, with MAP preceding DAP, as expected; TAP was produced later than DAP, indicated that it was not formed directly from TMP or MAP. The concentration of TAP at 72 h reached 88.2%, 63.2% and 61.1% and the final concentration of TAP was 88.8% (75h), 77.7% (80 h) and 73.8% (80 h) when water activity was regulated at 0.25, 0.35 and 0.45, respectively. These yield differences can be further confirmed by the apparent reaction rates V_i determined by kinetic model within water balance. From comparison of V_i (Table 2), it is obvious that the esterification rates decreased but the hydrolysis rates increased when the amount of water bound to lipase increased; something to be expected, as water is a substrate in the reverse reaction.

As shown in Fig. 5, the residual esterification activity of lipase continuously declined to 83.5%, 71.3% and 59.1% at 70 h under different controlled water activities 0.25, 0.35 and

0.45, respectively. More water molecules bound to enzyme induce higher enzyme internal flexibility since water can act as lubricant of enzyme. Inactivation of lipase through thermal aggregation mechanisms can be accelerated by the improved intrinsic flexibility.

The catalytic behavior of the lipase from *Candida sp.* 99-125 is beneficial under lower water activity, in terms of productivity and stability. A dependence between a_w and water content was observed when investigating lipase-catalyzed glycerolysis of fats and oils in ionic liquids[48]. In our case, despite that water activity was controlled at different levels, the values of water content in the medium decreased through time with no significant deviations, as shown Fig. 5. With the production of TMP esters, we have two different effects; from one side more water is produced, on the other side, the esters are more hydrophobic, resulting to less water capacity and thus without the water removal a two phase system may be formed. Clearly, it is not the water content but the water activity that plays a decisive role in the performance of lipase. This conclusion is also supported from Halling's research[27]: the optimal water contents in different solvents are different, while the optimal water activity is largely the same. Hence, it is essential and important to control the water activity instead of water content at the optimal value for lipase-mediated direct esterification.

Conclusions

This work provides a methodology that can develop an integrated kinetic model for water-dependent enzyme-catalyzed reactions that follow Ping-Pong bi-bi mechanisms in large scale. The developed model for synthesis of TMP esters comprehensively predicts both the forward and reverse rates and can thus be applied to guiding process design and upscaling aiming to improve the productivity and economy of these processes. A computer-aided water activity control system for upscaling solvent-free esterification was developed. Water activity control was achieved by the introduction of dry air through the reaction medium at a varied flow rate, which was digitally manipulated by an air mass flow controller. For cases of TMP esterification, the water activity was successfully controlled at the desired point in long-time operation (>72 h), resulting in a considerable improvement of the yield and the stability of lipase. The water activity dependence of kinetic constants, such as k_{cat} and K_{li}, can be determined at different concentrations of substrates and enzyme. In summary, the proposed methodology presents a solution for the kinetic model of non-aqueous enzymatic reactions and control system making it possible to optimize the reactions under well-controlled conditions.

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Notes and references

Nomenclature

TMP	Trimethylolpropane
FA	Fatty acid
MAP	Mono-substituted trimethylolpropane ester
DAP	Di-substituted trimethylolpropane ester
TAP	Tri-substituted trimethylolpropane ester
W	Water
[E]	free enzyme concentration (mmol/$g_{substrate}$)
[ET]	total enzyme concentration (mmol/$g_{substrate}$)
[A×E×B]	Ternary substrate A-enzyme-substrate B complex (mmol/$g_{substrate}$)
[A]	the concentration of compound A (mmol/$g_{substrate}$)
k_i	Kinetic constants
K_i	Equilibrium constant ($g_{substrate}^2$/$mmol^2$)
V_i	Apparent rate constants ($g_{substrate}^2$/(g_{enzyme}•mmol•min))
V_i^{max}	Maximum initial reaction rate (mmol/$g_{substrate}$•min)
a_w	Water activity
d	Moisture content
$K_{w,v}$	Water removal rate
$K_{w,g}$	Water generation rate
C_w	Water content in medium (w/w,%)
C_{FA}	Caprylic acid concentration in reactant (%)

Subscripts

w	Water
a	Air
in	Inlet air
out	Outlet air
t	time
r	reactant

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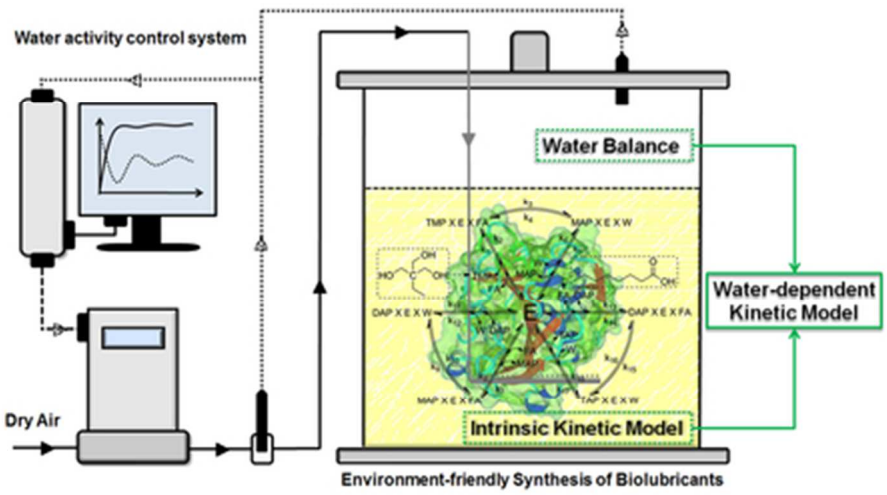

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