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Effect of monohydric alcohols on enzymatic transesterification for biodiesel production

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ABSTRACT

Biodiesel synthesis catalyzed by immobilized lipases in solvent-free and *tert*-butanol media was investigated in this study. The effect of two crucial parameters, type of biocatalysts (Novozym 435, Lipozyme TLIM and Lipozyme RMIM), and different alcohols (methanol, ethanol, propanol, isopropanol, isobutanol, isoamyl alcohol and fusel oil-like alcohol mixture), on conversion rate was comprehensively addressed. The results showed that each lipase presented a different kinetic pattern depending on the monohydric alcohols in solvent-free and *tert*-butanol systems. It was indicated that a possible use of fusel oil-like mixture as a raw material for biodiesel production was a promising procedure. In addition, a reaction kinetics model was developed for the methanolysis of waste baked duck oil using combined lipases of Novozym 435 and Lipozyme TLIM in solvent-free system. The kinetic parameters were estimated by fitting experimental data and deduced to be a pseudo-third-order reaction and the activation energy was 31.65 kJ/mol.

1. Introduction

Biodiesel derived from renewable vegetable oils and animal fats is an alternative biofuel in recent years. It consists of the monoalkyl esters formed by a catalyzed transesterification reaction of the triglycerides in the oils or fats with monohydric alcohols. Comparison to petroleum-based diesel, biodiesel has many favorable advantages. It is environmentally friendly, renewable and non-toxic [1]. So far, the most common way to produce biodiesel is by transesterification, which refers to alkali or acid catalyzed, enzymatically catalyzed and supercritical methanol methods. Basha et al. [2] reviewed biodiesel production, combustion, performance and emissions based on the literatures from 1980 to 2008.

The commercial industrial production of biodiesel is performed by transesterification of vegetable oils using alkaline catalysts. Chemical processes give high conversion rate of triglycerides to their corresponding esters but have drawbacks like energy intensive, difficult removal of glycerol, and the need for removal of alkaline catalyst from the product and treatment of alkaline wastewater. Unlike the conventional chemical routes, biocatalyst procedure permits the transesterification of a wide variety of oil feed stocks with high content of free fatty acids (FFAs). Separation and purification of biodiesel is easy because of the absence of soap byproduct [3].

It has been demonstrated that lipases exhibit high activity and operational stability in the hydrophobic solvent like *n*-hexane, but the hydrophilic substrate alcohol and byproduct glycerol are immiscible in hydrophobic reaction medium [4]. The problem of solubility results in the absorption of polar molecules onto the hydrophilic support leading to a low transesterification rate [5]. To solve this problem, *tert*-butanol was used as an ideal co-solvent. With a certain amount of *tert*-butanol as the reaction medium, both methanol and byproduct glycerol are soluble, so the negative effect caused by them on lipase catalytic activity can be totally eliminated [6].

However, so far, few studies have been comprehensively conducted to investigate the effect of monohydric alcohol on the kinetic pattern of lipases in solvent-free and *tert*-butanol reaction media. Therefore, in this study, the effect of the crucial variables, including type of commercial immobilized lipases (Novozym 435, Lipozyme TLIM and Lipozyme RMIM), and different monohydric alcohols (methanol, ethanol, propanol, isopropanol, isobutanol, isoamyl alcohol and fusel oil-like alcohol mixture), on conversion rate was systematically addressed with or without *tert*-butanol as co-solvent. Moreover, the kinetics model of methanolysis by combined lipases of Novozym 435 and Lipozyme TLIM in solvent-free medium was also highlighted on the conditions of excessive methanol. Previously, rapeseed oil, soybean oil and beef tallow were reported to synthesize biodiesel in literatures [2]. Therefore,

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Table 1
The physicochemical properties and fatty acid profile of WBDO ($n = 3$, average ± stdv).

Indexes	Unit	Results
Acid value (AV)	mg-KOH/g-oil	1.31 ± 0.01
Saponification value (SV)	mg-KOH/g-oil	201.16 ± 0.22
Specific gravity (25 °C)	g/cm^3	0.90 ± 0.01
Moisture content	%	0.17 ± 0.00
Free fatty acid (FFA)	%	0.65 ± 0.00
Fatty acid profile		
Palmitic acid ($C_{16:0}$)	%	23.4 ± 0.18
Stearic acid ($C_{18:0}$)	%	4.01 ± 0.11
Oleic acid ($C_{18:1}$)	%	50.4 ± 1.27
Linoleic acid ($C_{18:2}$)	%	5.8 ± 0.02
Linolenic acid ($C_{18:3}$)	%	16.4 ± 0.47

in this work we chose inexpensive feed stock, waste baked duck oil (WBDO) as the raw material so that the made biodiesel could be competitive in price compared to those produced from food grade vegetable oils and animal fats.

2. Materials and methods

2.1. Materials

WBDO was obtained locally, and its physicochemical properties and fatty acid profile were determined according to AOAC [7] and illustrated in Table 1. Novozym 435, Lipozyme TLIM and Lipozyme RMIM were purchased from Nov Norvisk (Bagsvaerd, Denmark). Standard methyl esters of chromatographic purity 99.9%, such as methyl palmitate, methyl stearate, methyl heptadecanoate, methyl oleate, methyl linoleate, and methyl linolenate were bought from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals obtained in China were of analytic grade.

2.2. Enzymatic transesterification for biodiesel production in solvent-free and tert-butanol media

The enzymatic transesterification reactions were carried out in a 50 mL shaking flask at 45 °C in a thermostat shaking bed with the speed rate of 250 rpm. The reaction mixture respectively consisted of WBDO, biocatalyst (Novozym 435, Lipozyme TLIM and Lipozyme RMIM), monohydric alcohol (methanol, ethanol, propanol, isopropanol, isobutanol, isoamyl alcohol and fusel oil-like alcohol mixture), with a molar ratio of alcohol:oil of 4:1 was allowed to react for a period of time with or without *tert*-butanol as co-solvent. The optimum operational parameters were achieved in our preliminary work: reaction temperature 45 °C, reaction time 20 h, oil to alcohol molar ratio 1:4, lipase dosage 5 wt%, and speed rate 250 rpm. In this work, more efforts were focused on the effect of monohydric alcohols on the yield of biodiesel by three different lipases in solvent-free and *tert*-butanol media. After reaction, 50 μL samples were taken from the reaction mixture and centrifuged. Then 5 μL withdraw from the supernatant and 295 μL of 1.0 mM methyl heptadecanoate (as internal standard) were precisely measured and mixed thoroughly for gas chromatography (GC) analysis for FFAs content. The GC conditions for the content determination of FFAs were detailed in our preceding work [8].

The conversion rate, described as the amount of FFAs formed in the transesterification reaction, was quantified in the presence of methyl heptadecanoate as internal standard. The conversion rate (%) was calculated with Eqs. (1) and (2):

$$\text{Conversion rate (\%)} = \frac{A_{\text{sample}} \times f_0}{A_{\text{internal}} \times W_{\text{internal}}} \qquad (1)$$

$$f_0 = \frac{W_{\text{sample}} \times A_{\text{internal}}}{W_{\text{internal}} \times A_{\text{sample}}} \qquad (2)$$

where: A_{sample} is the peak area of FFAs in sample; f_0 is the response factor; A_{internal} is the peak area of the internal standard; W_{internal} is the weight of the internal standard, g; W_{sample} is the weight of the sample, g.

2.3. Kinetics model for methanolysis of biodiesel production by combined lipases

Many researchers had demonstrated that kinetics model for transesterification reaction of biodiesel production catalyzed by lipases was Ping Pong Bi Bi model [9,10], in which the expression of reaction rate was employed with alcohol inhibition. In this work, the proposed kinetics model was based on the decrease of substrate concentration (including the oil substrate (C_A) and the methanol (C_B)), and the reaction rate was derived in the following Eq. (3).

$$r_A = k\ C_A^{\alpha} C_B^{\beta} \qquad (3)$$

where: r_A is the reaction rate of WBDO; k is reaction rate constant; C_A is the concentration of WBDO feedstock, mol/L; α is the reaction order of WBDO; C_B is the concentration of methanol, mol/L; β is the reaction order of methanol.

For the proposed kinetics model, it was presumed that methanol concentration was much excessive in comparison with the stoichiometry molar ratio of alcohol to oil 3:1. It was confirmed methanol inhibition and the transesterification reaction rate was more sensitive to changes in substrate concentration [11,12]. Therefore, kC_B^{β} was constant due to the excessive methanol concentration. Then, Eq. (3) was simplified as Eq. (4):

$$r_A = k_1\ C_A^{\alpha} \qquad (4)$$

$$\text{where,} \quad k_1 = k\, C_B^{\beta} \qquad (5)$$

It is assumed that x is the conversion rate from WBDO and the initial concentration of WBDO is C_{A0}, the concentration (C_A) of WBDO at any specific time is derived in Eq. (6):

$$C_A = C_{A0}(1 - x) \qquad (6)$$

Therefore,

$$r_A = -\frac{dC_A}{dt} = \frac{d(C_{A0}(1 - x))}{dt} = C_{A0}\frac{dx}{dt} = k_1 C_A^{\alpha} \qquad (7)$$

Combined Eq. (6) with (7), Eq. (8) can be obtained:

$$\frac{dx}{dt} = \frac{k_1}{C_{A0}} C_A^{\alpha} = \frac{k_1}{C_{A0}}[C_{A0}(1 - x)]^{\alpha} = k_2[C_{A0}(1 - x)]^{\alpha} \qquad (8)$$

where

$$k_2 = \frac{k_1}{C_{A0}} \qquad (9)$$

Using integral method, Eq. (10) was derived from Eq. (8):

$$\ln\frac{dx}{dt} = \alpha \ln[C_{A0}(1 - x)] + \ln k_2 \qquad (10)$$

In Eqs. (10) and (8), the kinetic parameters k_1, k_2 and α were calculated by the linear regression using the software OriginPro 7.5 (Origin Lab, USA).

2.4. Statistical analysis

All trials were carried out in triplicate and the data were analyzed by Origin 7.5 (Origin Lab, USA).

Table 2
Effect of monohydric alcohols on conversion rate in solvent-free medium ($n = 3$, average ± stdv).

Alcohols	Novozym 435	Lipozyme TLIM	Lipozyme RMIM
Methanol (%)	63.2 ± 1.3	19.7 ± 0.9	20.1 ± 1.0
Ethanol (%)	58.4 ± 1.0	62.7 ± 1.1	57.9 ± 2.4
Propanol (%)	43.5 ± 1.7	75.4 ± 2.0	65.8 ± 1.9
Isopropanol (%)	64.1 ± 0.8	37.6 ± 0.7	28.4 ± 1.0
Isobutanol (%)	79.6 ± 1.5	70.1 ± 0.9	72.3 ± 2.1
Isoamyl alcohol (%)	59.3 ± 2.6	82.4 ± 1.4	89.2 ± 3.0

Table 3
Effect of monohydric alcohols on conversion rate in *tert*-butanol medium ($n = 3$, average ± stdv).

Alcohols	Novozym 435	Lipozyme TLIM	Lipozyme RMIM
Methanol (%)	85.4 ± 2.7	78.5 ± 1.6	62.4 ± 3.0
Ethanol (%)	61.8 ± 1.9	43.4 ± 0.7	50.8 ± 2.5
Propanol (%)	61.5 ± 1.4	63.5 ± 1.2	49.9 ± 2.1
Isopropanol (%)	62.3 ± 1.7	26.7 ± 0.6	22.4 ± 2.3
Isobutanol (%)	78.6 ± 1.8	56.5 ± 0.9	54.7 ± 1.9
Isoamyl alcohol (%)	71.8 ± 1.1	72.4 ± 1.2	55.6 ± 2.0

3. Results and discussion

3.1. Effect of different monohydric alcohols on conversion rate in solvent-free medium

In order to demonstrate the effect of monohydric alcohols on biodiesel conversion rate from WBDO in solvent-free medium, six monohydric alcohols (methanol, ethanol, propanol, isopropanol, isobutanol and isoamyl alcohol) and three immobilized lipases (Novozym 435, Lipozyme TLIM and Lipozyme RMIM) were employed to compare their performances at the fixed conditions of temperature (45 °C), reaction time (20 h), lipase dosage (5 wt%, based on oil weight) and WBDO to alcohol molar ratio (1:4). The experimental results are shown in Table 2.

It can be seen in Table 2, each lipase shows a different kinetic pattern depending on the monohydric alcohols in solvent-free medium. For the straight chain monohydric alcohols, Novozym 435 displays the highest activity for methanolysis (63.2 ± 1.3%), and the lowest for propanolysis (43.5 ± 1.7%), and the conversion rate decreases with the increase of carbon chain length of the monohydric alcohols. For the branch chain monohydric alcohols, Novozym 435 generally shows higher activity than for the straight chain alcohols, and the highest conversion rate is observed with isobutanol (79.6 ± 1.5%). However, for Lipozyme TLIM and RMIM, the conversion rate increases with the carbon chain length regardless of straight or branch monohydric alcohols. Lipozyme TLIM displays high activity capacity in reactions with ethanol, propanol, isobutanol, or isoamyl alcohol, and the conversion rates obtained with these alcohols are almost three times than that obtained with methanol (19.7 ± 0.9%) and twice than that with isopropanol (37.6 ± 0.7%). Lipozyme RMIM presents the highest conversion rate in isoamyl alcoholysis (89.2 ± 3.0%). For the branch chain monohydric alcohols (isopropanol, isobutanol, and isoamyl alcohol), the conversion rate is higher than that for the straight chain alcohols with the same carbon number. The reason is probably attributed to the effluence of the steric hindrance for the transesterification of WBDO by lipases. It is indicated that isobutanolysis and isoamyl alcoholysis will be suitable for biodiesel production from WBDO by enzymatic transesterification.

Based on the above analysis, it is concluded that the conversion rate depends not only upon the kind of alcohols but also on lipase type. Specifically, for straight chain monohydric alcohols, the conversion rate of lipase-catalyzed transesterification reaction increases with the carbon chain length of alcohols for Lipozyme TLIM and RMIM. In contrast, the conversion rate of lipase-catalyzed transesterification reaction decreases with the carbon chain length of alcohols for Novozym 435. These observations are accordance with those reported by Rodrigues et al. [13], who demonstrated that Novozym 435, Lipozyme TLIM and Lipozyme RMIM displayed different alcoholysis activity with straight chain C_1–C_4 alcohols. Novozym 435 presented the highest activity in methanolysis, Lipozyme TLIM presented the highest activity in ethanolysis, and Lipozyme RMIM presented the highest activity in butanolysis. However, the observations in this work seem contradictory with those reported by Hanh et al. [14], who demonstrated that although the rate of the fatty acid ester formation depended upon the kind of alcohols, as the carbon chain length of alcohols increased, the conversion rate decreased under the conditions of molar ratio 6:1 (alcohol:triolein) and 25 °C in the presence of base catalysts (NaOH and KOH).

3.2. Effect of different monohydric alcohols on conversion rate in tert-butanol medium

Miscibility is an important factor in enzymatic transesterification for biodiesel production. The conventional enzymatic transesterification leads to a two-phase reaction which is mass-transfer limitation resulting in denaturation of lipase by methanol and glycerol byproduct. Specifically, the vegetable oils and methanol are not miscible. Thus, immobilized lipases are easily inactivated by contacting with insoluble methanol and glycerol byproduct. *Tert*-butanol, a moderately hydrophilic solvent, can solubilize oil, methanol and glycerol. Therefore, *tert*-butanol as a co-solvent improves the enzyme catalytic properties leading to high conversion rate by eliminating the negative effect of methanol and glycerol byproduct [15,16]. To test the above-mentioned phenomenon, the effect of different low carbon alcohols (methanol, ethanol, propanol, isopropanol, isobutanol and isoamyl alcohol) on conversion rate was carried out in *tert*-butanol medium, and the experimental results are shown in Table 3.

It can be seen from Table 3 that, in *tert*-butanol medium, each lipase also shows a different kinetic pattern depending on the monohydric alcohols as in solvent-free medium. For methanolysis, regardless of the lipase type, the conversion rate is higher in *tert*-butanol than that in solvent-free medium; the reason is ascribed to eliminating the negative effects of methanol and glycerol byproduct on lipases by the application of *tert*-butanol as a co-solvent, which enhances the enzyme activity [15,16]. In our preliminary experiments, it was found that about 83.3% glycerol byproduct was dissolved in *tert*-butanol, suggesting that *tert*-butanol could dissolve glycerol byproduct. In *tert*-butanol medium, for straight chain monohydric alcohols, Novozym 435, Lipozyme TLIM and Lipozyme RMIM display the highest conversion rate in methanolysis (62.4 ± 3.0% to 85.4 ± 2.7%), then ethanolysis (43.4 ± 0.7% to 61.8 ± 1.9%) and propanolysis (49.9 ± 2.1% to 61.5 ± 1.4%). For branch chain monohydric alcohols, Novozym 435 shows the highest conversion rate in isobutanolysis (78.6 ± 1.8%). However, in respect of Lipozyme TLIM and Lipozyme RMIM, the highest conversion rate is respectively observed with methanolysis (78.5 ± 1.6%) and isoamyl alcoholysis (72.4 ± 1.2%) for straight and branch chain monohydric alcohols in *tert*-butanol medium.

Combined Table 2 with Table 3, either in solvent-free or *tert*-butanol medium, for monohydric alcohols with same carbon number, lower conversion rate is observed with isopropanol (22.4 ± 2.3% to 37.6 ± 0.7%) than with propanol (49.9 ± 2.1% to 75.4 ± 2.0%) for Lipozyme TLIM and RMIM, respectively. On the contrary, Novozym 435 shows higher conversion rate with isopropanol (62.3 ± 1.7% to 64.1 ± 0.8%) than propanol (43.5 ± 1.7% to 61.5 ± 1.4%). The reason is due to the enzyme substrate stereospecificity. This observation is in good agreement with findings reported by Salis et al. [17] who investigated the effect of linear and branched short chain alcohols on the transesterification for biodiesel production by *Pseudomonas cepacia* lipase from triolein and short chain alcohols, and demonstrated the secondary alcohols usually were less reactive than primary alcohols. Nelson et al. [18] studied the alcoholysis of tallow and different vegetable oils with different alcohols using hexane as co-solvent, who found that immobilized *Rhizomucor miehei* lipase showed high conversion rate for linear alcohols, while *Candida antarctica* lipase B showed high conversion rate for the branched alcohols.

3.3. Effect of the fusel oil-like alcohol mixture on conversion rate catalyzed by combined lipases

As demonstrated above, Novozym 435 and Lipozyme TLIM showed high conversion rate with isobutanol and isoamyl alcohol either in solvent-free or in *tert*-butanol medium, which indicated that combined lipases of Novozym 435 and Lipozyme TLIM could be used as combined biocatalyst for fatty acid alkyl esters production from a fusel oil-like alcohol mixture. In addition, the advantage to choose combined lipases catalyst has been proven that Novozym 435 and Lipozyme TLIM show positive synergistic effect resulting in high conversion rate of biodiesel. As it is known, the enzymatic transesterification process for biodiesel production involves three steps, i.e., from triglyceride (TG) to diglyceride (DG), from diglyceride to monoglyceride (MG) and from monoglyceride to glycerol (GL). In our preliminary experiments (data not shown), for single Novozym 435 lipase, it was demonstrated that the reaction step from MG to GL was faster than the steps from TG to DG and DG to MG, and the slowest step from DG to MG was the rate-limiting step. While for single Lipozyme TLIM lipase, the rate-limiting step was the step from TG to DG. These indicated that combined lipases of Novozym 435 and Lipozyme TLIM could eliminate the rate-limiting step in biodiesel production. Moreover, it is worth noting that combined lipases of Novozym 435 and Lipozyme TLIM will reduce the cost of biodiesel production because a big dosage of Novozym 435 (the price is \$8500 per kg) is replaced by inexpensive Lipozyme TLIM (the price is \$800 per kg).

In addition, branched alcohols are important for improving the low temperature properties of biodiesel product, which is critical for diesel engine functioning in the winter seasons. Fusel oil has been recently used for the chemical esterification of oleic acid to produce lubricating oil [19] or biodiesel [17]. In this work, fatty acid alkyl esters production catalyzed by combined lipases was investigated from a fusel oil-like alcohol mixture and WBDO. The fusel oil-like alcohol mixture was reproduced in our laboratory by mixing suitable amounts of 15% isobutanol, 80% isoamyl alcohol and 5% methanol. The fusel oil-like alcohol mixture and WBDO was transesterified by combined lipases of Novozym 435 and Lipozyme TLIM in solvent-free and *tert*-butanol medium under the same experimental conditions. The results are shown in Fig. 1.

The global conversion rate of the process was evaluated as the sum of conversion rate for each alcohol, according to data reported in Table 2 and Table 3 for methanolysis, isobutanolysis and isoamyl alcoholysis. It can be seen from Fig. 1, the conversion rate increased with the proportion of Lipozyme TLIM for combined lipases, and the maximal conversion rate of WBDO to the corresponding alkyl esters reached over 90% in solvent-free medium and over 70% in *tert*-butanol medium catalyzed by combined lipases of Novozym 435 and Lipozyme TLIM with weight ratio of 2:4. Beyond the weight ratio of 2:4, the conversion rate decreased with the proportion of Lipozyme TLIM. The use of combined lipases for the synthesis of biodiesel from woody oils and fuel oil-like mixture as acyl acceptor is ongoing research in our laboratory. In view of cost saving of biodiesel production, it provides a promising alternative to use combined lipases as biocatalyst and fusel oil-like mixture as raw material for biodiesel synthesis.

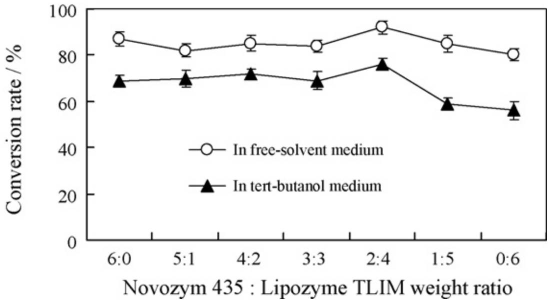


Fig. 1. Fatty acid alkyl esters production catalyzed by combined lipases using fusel oil-like alcohol mixture as acyl acceptors (conditions: oil weight 1 g, amount of combined lipases 5 wt% (based on oil weight), reaction temperature 45 °C, alcohol to oil molar ratio 4:1, speed rate 250 rpm).

3.4. Kinetics model of methanolysis of WBDO by combined lipases in solvent-free medium

As mentioned above, combined lipases of Novozym 435 and Lipozyme TLIM showed high catalyzed activity for biodiesel production in solvent-free medium. However, in comparison to other monohydric alcohols, methanol is inexpensive and easily available. For this reason, methanol is usually chosen as acyl acceptor for biodiesel production. Therefore, in this work, the reaction kinetics model of the transesterification of WBDO with methanol by combined lipases of Novozym 435 and Lipozyme TLIM in solvent-free medium was investigated, and the biodiesel conversion rate was described to the decrease of substrate concentration under the conditions of 1 g oil, 5 wt% amount of combined lipases of Novozym 435 and Lipozyme TLIM (based on oil weight), 9:1 methanol to oil molar ratio. In this reaction system, methanol was excess and the initial concentration of WBDO was 0.302 mmol/mL. The biodiesel conversion rate was depicted as the function of reaction time at 25 °C, 35 °C and 45 °C (Fig. 2).

Fig. 2 shows that the biodiesel conversion rate increases rapidly within first 5 h, and slightly increases during the reaction time ranges from 5 h to 15 h. Beyond 15 h, the biodiesel conversion rate keeps relatively stable and shows no significant increase, the reason is that the reaction equilibrium is attained at 15 h. In addition, higher reaction temperature results in higher biodiesel conversion rate, at 45 °C the conversion rate attained over 91% after 20 h, while

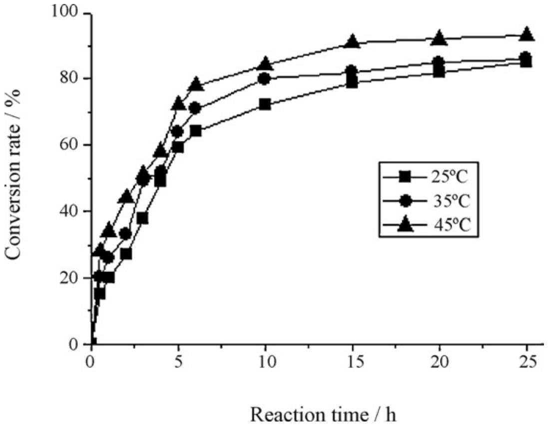
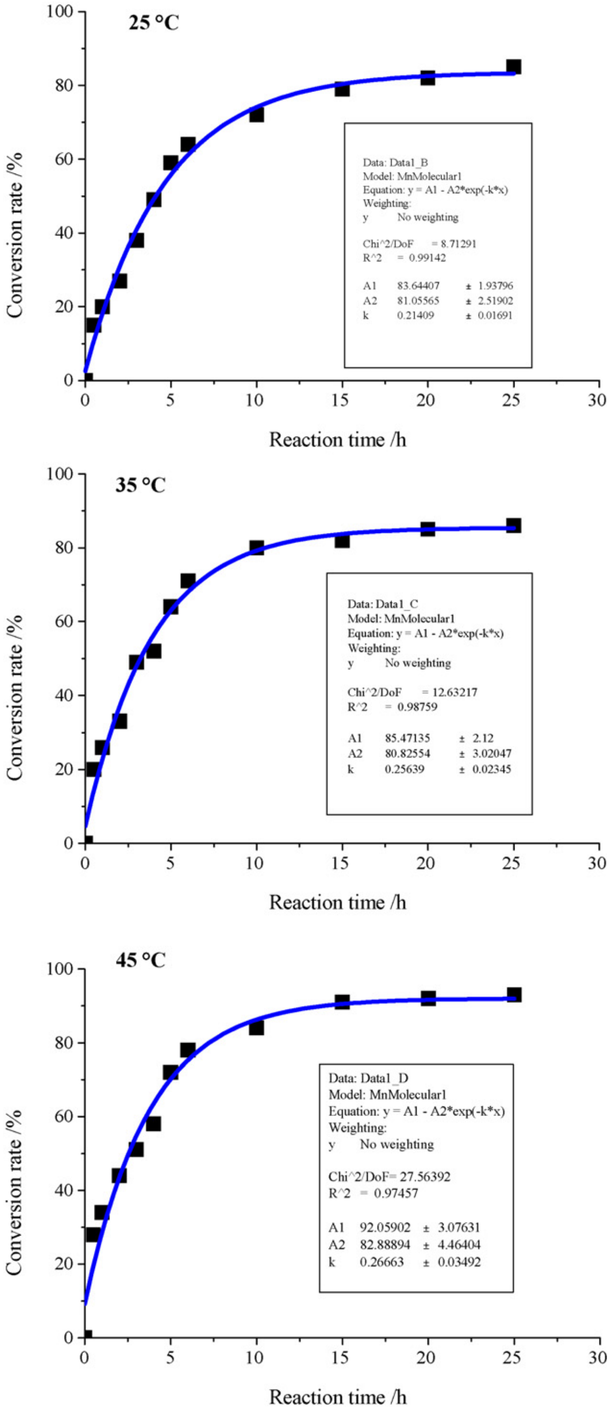


Fig. 2. The conversion rate as the function of reaction time at 25 °C, 35 °C and 45 °C (conditions: oil weight 1 g, amount of combined lipases of Novozym 435 and Lipozyme TLIM 5 wt% (based on oil weight), methanol to oil molar ratio of 9:1, and speed rate 250 rpm).



```
Data: Data1_B
Model: MnMolecular1
Equation: y = A1 - A2*exp(-k*x)
Weighting:
y         No weighting

Chi^2/DoF     = 8.71291
R^2    =  0.99142

A1      83.64407        ±  1.93796
A2      81.05565        ±  2.51902
k       0.21409         ±  0.01691
```

```
Data: Data1_C
Model: MnMolecular1
Equation: y = A1 - A2*exp(-k*x)
Weighting:
y         No weighting

Chi^2/DoF     = 12.63217
R^2    =  0.98759

A1      85.47135        ±  2.12
A2      80.82554        ±  3.02047
k       0.25639         ±  0.02345
```

```
Data: Data1_D
Model: MnMolecular1
Equation: y = A1 - A2*exp(-k*x)
Weighting:
y      No weighting

Chi^2/DoF= 27.56392
R^2  =  0.97457

A1    92.05902   ±  3.07631
A2    82.88894   ±  4.46404
k     0.26663    ±  0.03492
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Fig. 3. The non-linear fitting curve using Origin 7.5 at 25 °C, 35 °C and 45 °C.

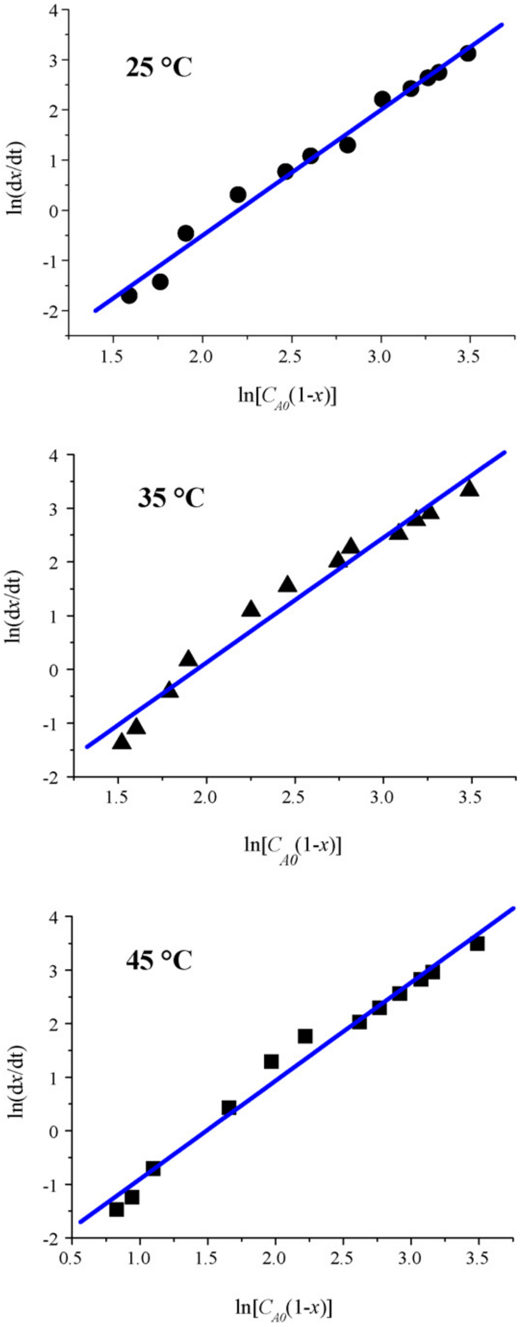

Fig. 4. The linear fitting curve between ln dx/dt and ln$[C_{A0}(1-x)]$ using Origin 7.5 at 25 °C, 35 °C and 45 °C.

the conversion rates were 83% at 35 °C and 80% at 25 °C, respectively. Three non-linear equation curves (Fig. 3) were obtained at 25 °C, 35 °C and 45 °C from the experimental data (Table 4) using Origin 7.5.

The derivative Eqs. (11), (12) and (13) are obtained at 25 °C, 35 °C and 45 °C from the non-linear equation as follows:

$$\text{At } 25^\circ\text{C} \quad \frac{dx}{dt} = 17.3532\, e^{-0.2141t} \tag{11}$$

$$\text{At } 35^\circ\text{C} \quad \frac{dx}{dt} = 20.7248\, e^{-0.2564t} \tag{12}$$

$$\text{At } 45^\circ\text{C} \quad \frac{dx}{dt} = 22.0985\, e^{-0.2666t} \tag{13}$$

From experimental data, the value of dx/dt and $[C_{A0}(1-x)]$ can be calculated at the function of reaction time at 25 °C, 35 °C and 45 °C. It can be deduced that the relationship between ln dx/dt and

Table 4
Non-linear equations between conversion rate and reaction time at different temperature.

Temperature/°C	Non-linear equations	Relative coefficient (R^2)
25	$x = 83.64 - 81.06e^{-0.2141t}$	0.97578
35	$x = 85.47 - 80.83e^{-0.2564t}$	0.98759
45	$x = 92.06 - 82.89e^{-0.2666t}$	0.97457

x: conversion rate, %; t: reaction time, h.

Table 5
The reaction rate order (α) and rate constant (k_1, k_2) at 25 °C, 35 °C and 45 °C.

Temperature/°C	Reaction rate order/α	k_1/min^{-1}	k_2/[L/(mol min)]
25	2.561	10.0722	30.8026
35	2.333	12.9368	39.5632
45	2.836	22.5736	69.0341

$\ln[C_{A0}(1-x)]$ is linear. The results are shown in Fig. 4.

Through Origin 7.5, a linear Eq. (14) is obtained at 25 °C.

$$\ln\frac{dx}{dt} = 2.561\ln[C_{A0}(1-x)] + 3.4276, \quad R^2 = 0.9758 \tag{14}$$

Hence, combined Eq. (10) with Eq. (14) the reaction kinetics constant at 25 °C is obtained. The reaction rate order (α) is equal to 2.561, which indicates that the reaction rate order is a pseudo-third-order, and the reaction rate constant k_1 is 10.072 min^{-1} and k_2 is 30.803 L/(mol min).

Similarly, the reaction rate order (α), the reaction kinetics constant k_1 and k_2 at 35 °C and 45 °C are respectively obtained, and are shown in Table 5.

According to Arrhenius Eq. (15), its derivative Eq. (16) is obtained through logarithm.

$$k_1 = A\ \exp\left(-\frac{E_a}{RT}\right) \tag{15}$$

$$\ln k_1 = -\frac{E_a}{R}\frac{1}{T} + \ln A \tag{16}$$

where: k_1 is the reaction kinetics constant, min^{-1}; A is the pre-exponential constant; E_a is the activation energy; R is the gas law constant, J/(mol K); T is temperature, K.

From experimental data of k_1 at 25 °C, 35 °C, and 45 °C, the value of $\ln k_1$ and $1/T$ can be calculated and fitted through Origin 7.5 (Fig. 5) to get the linear Eq. (17).

$$\ln k_1 = -3807.2\left(\frac{1}{T}\right) + 15.033, \quad R^2 = 0.9458 \tag{17}$$

The activation energy E_a and pre-exponential constant A are estimated from Eq. (16) and Eq. (17), which are 31.653 kJ/mol and 3.38×10^6, respectively.

Therefore, the reaction kinetics model is obtained as Eq. (18) for biodiesel production from WBDO catalyzed by combined lipases of Novozym 435 and Lipozyme TLIM in solvent-free medium.

$$r_A = 3.38 \times 10^6 e^{-31.653/RT} C_A \tag{18}$$

where: r_A is the reaction rate of WBDO, mol/(L min); R is the gas law constant, J/(mol K); T is temperature, K; C_A is the concentration of WBDO at specific reaction time, mol/L.

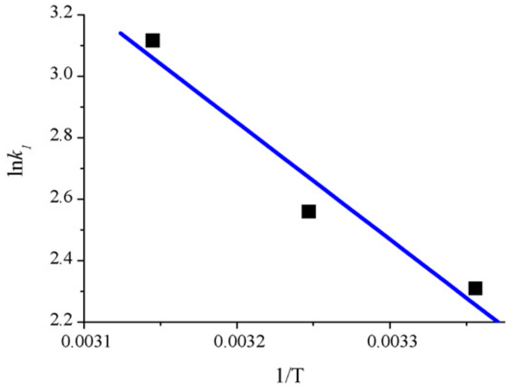

Fig. 5. The linear fitting curve between ln k_1 and $1/T$ at 25 °C, 35 °C and 45 °C.

4. Conclusions

Biodiesel synthesis from WBDO catalyzed by combined lipases has been considered as a promising procedure. WBDO is available with relatively cheap price for biodiesel production in comparison with fresh vegetable oil costs. In this study, more efforts are focused on the effect of monohydric alcohols on conversion rate by three different commercial immobilized lipases in solvent-free and *tert*-butanol system. It is demonstrated that selection of lipases and alcohols is of the utmost importance for biodiesel production by biocatalysts from renewable raw materials. It is interesting to discover that biodiesel production using fusel oil-like alcohol mixture as material is a promising alternative. The kinetics model of biodiesel production by combined lipases of Novozym 435 and Lipozyme TLIM in solvent-free medium has been addressed by decrease of substrate concentration and the parameters in the kinetic model are determined from experiments.

Annotations

A_{sample}	the peak area of fatty acid esters in sample
f_0	the response factor
A_{internal}	the peak area of the internal standard
W_{internal}	the weight of the internal standard (g)
W_{sample}	the weight of the sample (g)
r_A	the reaction rate of WBDO
k_1, k_2	constant coefficient
C_{A0}	the initial concentration of WBDO (mol/L)
C_A	WBDO concentration at a specific reaction time (mol/L)
α	reaction order of WBDO
C_B	methanol concentration (mol/L)
β	reaction order of methanol
x	conversion rate from WBDO (%)
t	reaction time (min)
A	the pre-exponential constant
E_a	activation energy
R	the gas law constant (J/(mol K))
T	temperature (K)

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