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Microwave-assisted fatty acid methyl ester production from soybean oil by Novozym 435†

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The production of fatty acid methyl ester (FAME) from soybean oil and methanol through transesterification by Novozym 435 (*Candida antarctica* lipase B immobilized on polyacrylic resin) was conducted under two different conditions—microwave irradiation and conventional heating—to compare their overall effects. It was found that, compared to conventional heating, microwave irradiation significantly increased the reaction rate by enhancing the activity of Novozym 435. The effect of the reaction conditions, such as water activity (a_w), organic solvents, the ratio of solvent/oil, the ratio of methanol/oil, the pre-treatment method, methanol deactivation kinetics, enzyme dosage and temperature, on the activity of Novozym 435 were also investigated. Under the optimum conditions (a_w of 0.53, *tert*-amyl alcohol/oil volume ratio of 1 : 1, methanol/oil molar ratio of 6 : 1, 3% Novozym 435 and 40 °C), a 94% yield of FAME could be achieved in 12 h under microwave irradiation, compared to 24 h under conventional heating. Furthermore, the repeated use of Novozym 435 for five cycles under microwave irradiation resulted no obvious loss of enzyme activity, suggesting that this enzyme is stable under microwave irradiation conditions. These results show that microwave irradiation is a fast and efficient method for FAME production.

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Introduction

Biodiesel (monoalkyl esters of fatty acids) produced from vegetable oils, animal fats and microalgal oils by transesterification or esterification with short chain alcohols has been viewed as a promising renewable source of fuel because of its biodegradability, low toxicity, renewability and lower dependence on petroleum products.[1,2] The conventional method for biodiesel production involves using acid and base catalysts to form fatty acid alkyl esters.[3] The most often used method for the industrial production of biodiesel today is alkaline transesterification, because of its high activity under mild reaction conditions and relatively low cost.[4,5] However, these homogeneous alkaline catalysts can easily react with free fatty acids in the feed oil to form unwanted soaps and water, which would adversely affect the quality of the biodiesel and require an expensive separation step for biodiesel purification.[6] Soap formation can be avoided by using acid catalysts that can simultaneously catalyze both transesterification of the triglyceride and esterification of the free fatty acid to produce FAME. However, acid catalysts are rarely used compared to alkaline catalysts because they are corrosive and may cause damage to equipment. Rates of reactions catalyzed by acid catalysts are also observed to be low.[7]

Recently, the enzymatic production of biodiesel has attracted considerable interest, since it is more efficient and highly selective, involves less energy consumption, and produces fewer side products or waste.[8,9] Furthermore, in comparison with chemical methods, no complex operation is needed for the recovery of the glycerol, and the elimination of the catalyst and salt.[10] It has been reported in the literature that some lipases can effectively catalyze the methanolysis of vegetable oils and fats to biodiesel.[11] Most of the recent research has focused on determining the best enzyme source and optimizing the reaction conditions (substrate molar ratio, solvent or no solvent, temperature, water content, free fatty acid level, percent conversion, acyl migration and substrate flow rate in packed-bed bioreactors) to improve the yield of biodiesel for possible industrial scale-ups and use.[12,13]

However, from an economic point of view, a major problem with lipase catalytic biodiesel production is the low activity of the enzymes compared with chemical catalysts;[3] therefore it is necessary to find a suitable method to increase the reaction rate so as to promote the application of enzyme catalytic biodiesel production. Microwave irradiation, which has proved to be a clean, fast and convenient energy source,[14] has been widely used in organic chemistry.[15] Traditionally, organic synthesis is carried out by conductive heating with an external heat source. This method is comparatively slow and inefficient for transferring energy into a reaction system as it depends on the thermal conductivity of the media and the temperature of the reaction vessel is higher than that of the reaction mixture. In contrast, microwave irradiation produces efficient internal heating by direct coupling of the microwave energy with the solvent, reagent and catalyst molecules in the reaction mixture, and the reaction

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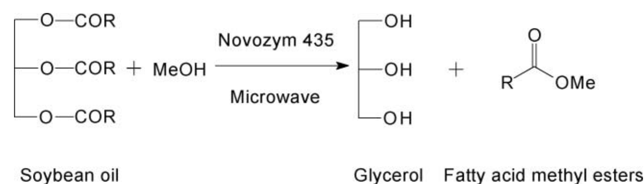
time is usually shortened with a higher yield.[16] The microwave-assisted, lipase-catalyzed reaction method is therefore a significant development. The enhancement of enzyme activity and the stability of Novozym 435 to microwave irradiation are well covered in the literature.[17,18] Microwave irradiation also offers a fast, easy route to biodiesel production by the use of acid or base catalysts; a satisfactory transesterification conversion can be achieved in a few seconds under microwave irradiation, compared to a few hours under conventional heating.[19] However, little research has been done so far on the application of microwave irradiation in lipase catalytic biodiesel production, and the demonstration of its utility remains a pressing concern. Our study for the first time describes temperature-controllable microwave-assisted FAME production from soybean oil and methanol through transesterification with Novozym 435 as the catalyst (Scheme 1). A comparison between conventional heating and microwave irradiation is also conducted. The effect of the reaction conditions on the enzyme activity, an enzyme kinetic analysis and an enzyme stability analysis have also been investigated.



Scheme 1

Experimental

Materials

Refined soybean oil was obtained locally and Novozym 435 (EC 3.1.1.3) from *Candida antarctica* was a gift from Novo-Nordisk (Bagsværd, Denmark). The reference substances (methyl oleate, methyl linoleate, methyl stearate and methyl palmitate) and methyl *p*-hydroxybenzoate (MP) were purchased from Sigma and chromatographically pure. Other chemicals were obtained commercially and were of analytical grade.

Microwave equipment

Reactions were carried out in a commercial multimode microwave reactor (MCR-3, Shanghai JieSi Microwave Chemistry Corporation). The machine consisted of a continuous focused microwave power delivery system with an operator-selectable power output from 0 to 800 W. The temperature of the reaction mixture was monitored and kept constant (± 1 °C) by using a contact Teflon platinum resistance temperature transducer inserted directly into the reaction mixture. The contents of the vessel were stirred by means of a rotating magnetic plate located below the floor of the microwave cavity and a Teflon-coated magnetic stirring bar in the vessel.

Experimental procedures

Enzymatic transesterification of soybean oil. The transesterification reactions were performed in a three-necked round-bottomed flask. The reaction mixture was placed into the microwave cavity, with an immersion well placed into one of the necks of the flask and a glass connector (linking the flask inside the cavity to a reflux condenser outside the cavity) into another. A temperature probe was placed into the immersion well. The reaction mixture was heated to the desired temperature. Conventional heating reactions were performed using a digital heating circulating water bath (Changzhou Electrical Instrument Factory, HH-42) equipped with a magnetic stirrer. A standard reaction mixture consisted of a water activity of 0.53, a *tert*-amyl alcohol/oil volume ratio of 1 : 1, a methanol/oil molar ratio of 6 : 1, 3% immobilized lipase based on the oil weight, 40 °C and 200 rpm. One unit (*U*) of enzyme specific activity was defined as the amount of enzyme necessary to produce 1 μmol of FAME per minute in the first 2 h.

All experiments were performed in triplicate, and the results are reported as the mean ± standard deviation. All reactants were dehydrated by 4 Å molecular sieves before use.

Water activity setting. All the reaction mixture components were pre-equilibrated to the water activity (a_w) of the experiment through the vapor phase with saturated salt pair solutions at 25 °C. The following salts were used: LiBr (a_w = 0.06), LiCl (a_w = 0.11), CH_3COOK (a_w = 0.24), $MgCl_2$ (a_w = 0.33), $Mg(NO_3)_2$ (a_w = 0.53), NaCl (a_w = 0.75) and K_2SO_4 (a_w = 0.97).[20] In all reactions with salt hydrates, 10 g L^{-1} of each form of the pair were used, and the equilibration was performed overnight.

Reusability of the enzyme. After each round of the reaction, the Novozym 435 was filtered and washed with *tert*-amyl alcohol three times and dried in air at room temperature. The recycled enzyme was then used in the next reaction.

Analytical procedures

The FAME content was analyzed using a gas chromatograph (GC 2014, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) and an Rtx-1 capillary column (0.25 mm × 30 m, 0.25 μm; Restek, USA). Samples were withdrawn from the reaction mixture at specified time intervals and centrifuged at 8000 rpm for 3 min to remove the enzyme. 100 μl methyl *p*-hydroxybenzoate stock solution (5 mg ml^{-1}) in pyridine was added as an internal standard to 100 μl of the reaction mixture, 1 μl of the mixture was then injected in the GC. The injector was used in split mode with a 40 : 1 split ratio. Nitrogen was used as the carrier gas at a set flow rate of 100 ml min^{-1} and a back pressure of 100 kPa. The column temperature was kept at 50 °C for 1 min, raised to 180 °C at 15 °C min^{-1}, after that at 7 °C min^{-1} to 230 °C and at 200 °C min^{-1} to 350 °C. The temperatures of the injector and detector were maintained at 280 and 350 °C, respectively. The yield of products was identified by comparing the peak areas of standard methyl *p*-hydroxybenzoate at particular retention times. Quantification of the final product (FAME) was done according to the calibration curves of pure reference substrate.

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Results and discussion

Microwave irradiation *vs.* conventional heating for FAME production

A comparison between the transesterification of soybean oil and methanol by Novozym 435 under conventional heating and microwave irradiation conditions was performed. As shown in Fig. 1, under microwave irradiation, the reaction rate improved up to 1.5-fold in the first 4 h compared to that under conventional heating. The reaction reached equilibrium after 12 h under microwave radiation and the yield of FAME was 94 ± 3 wt%, while the reaction didn't reach equilibrium until 24 h by using conventional heating with the same yield. This indicates that to achieve the same yield of FAME, a shorter time was needed under microwave radiation compared to conventional heating. The effect of the "microwave absorbing character of the feed" might contribute to the faster reaction rate.[18,21] It was presumed that microwave heating involved directed energy absorption by the functional groups that bear ionic conductivity or a dipole rotational effect. In the reaction mixture, methanol may be a good microwave radiation absorption material. Its dipole may quickly reorientate under microwave radiation, which would destroy the two-tier structure of the interface between the methanol and the oil, making the functional groups much more highly reactive.[22] Microwave irradiation might also increase the emulsification speed, which might result in an accelerated transportation of reactants or products due to greater contact between the enzyme and the substrate, and thus an improved reaction rate. It is also possible that the enzyme behaves slightly differently and becomes more active if a conformation change in the enzyme facilitates the substrate to approach the active site of the enzyme more easily under microwave irradiation than that under conventional heating. However, only irreversible effects can be measured by comparing the properties of the enzyme before and after microwave irradiation; real time detection of the effect of microwave radiation on proteins cannot easily be achieved because of the limited measurement technology available.[23] Moreover, the heating mode had no effect on the conversion rate of the reaction, as the calculated equilibrium constant (K_{eq} = 17.2) was the same under both conventional heating and microwave irradiation.

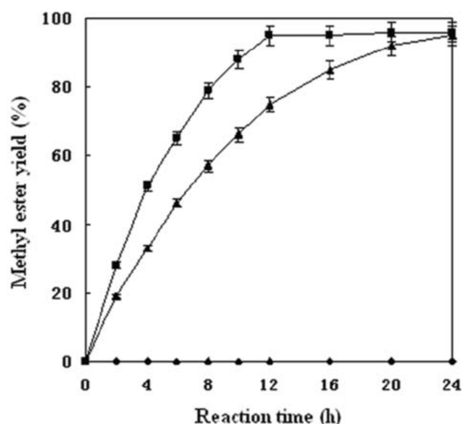

Fig. 1 Comparison of reaction process without enzyme under microwave irradiation (◆), with enzyme under microwave irradiation (■), and with enzyme under conventional heating (▲). *Conditions:* Reactions were carried out in *tert*-amyl alcohol (40 ml), soybean oil (40 mmol), methanol (240 mmol), Novozym 435 (3% based on the oil weight), water activity of 0.53 at 40 °C and 200 rpm under microwave irradiation and conventional heating, respectively.

Conditions that affect the Novozym 435-catalyzed production of FAME under microwave irradiation

The activity of Novozym 435 in FAME production under microwave irradiation was evaluated by changing the water activity, solvents, solvent ratio (solvent/oil), substrate ratio (methanol/oil), enzyme dosage and temperature of the reaction.

Effect of water activity on FAME production. Enzyme hydration is one of the critical parameters affecting enzyme activity in low-water environments. Water plays a crucial role in minimizing solvent-induced conformational rigidity, which is one of the causes of the reduced catalytic activities observed in non-aqueous media.[24] Water activity reflects the water level associated with the enzyme. In the present study, the thermodynamic water activity (ranging from 0.06 to 0.97) of the reactants was used to study its effect on enzyme-catalyzed FAME production.

As shown in Fig. 2, the enzyme activities exhibited a bell-shaped curve with changing water activity. No transesterification was detected with dried enzymes. At low water activity (0.06–0.11), low enzyme activity was observed. When the water activity was <0.53, the enzyme activity increased according to the increase of water activity. When the water activity was 0.53, the Novozym 435 exhibited its highest activity. With a further increase in water activity to 0.97, the enzyme activity decreased dramatically. In non-aqueous media, a certain amount of water was necessary for the enzyme to maintain its proper conformation, so as to keep its catalytic activity. At low water activity, the conformation of the Novozym 435 was probably excessively rigid, disturbing the "induced-fit" process of the enzyme and decreasing its activity.[25] At higher water activity (>0.53), the decrease in enzyme activity could be attributed to the observed enzyme particle aggregation, which might consequently lead to a more limited access of the substrate to the enzyme active site.[26] An alternative possibility is that the conformation of Novozym 435 is more flexible at higher

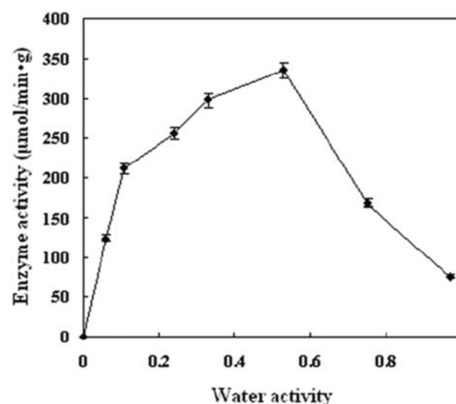

Fig. 2 Effect of water activity on FAME production. *Conditions:* Reactions were carried out in *tert*-amyl alcohol (40 ml), soybean oil (40 mmol), methanol (240 mmol), Novozym 435 (3% based on the oil weight) at 40 °C and 200 rpm under microwave irradiation in 2 h. The water activity varied from 0.06 to 0.97.

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water activity, and so the water in the reaction mixture acts as competing nucleophile that might suppress the expected acyl transfer and cause an unfavorable equilibrium position in the reversed hydrolysis. Overall, these results suggested that water activity strongly influences the hydration level of the enzyme, which consequently affects the transesterification activity for FAME production.

Effect of solvents on FAME production. It is well described in the literature that enzyme activity is strongly affected by organic solvents.[27] In the present study, the effects of several organic solvents with various log *P* (the partition coefficient of the solvent for the standard octanol/water two-phase system) values, dielectric constants, solubility parameters and other parameters were investigated for the transesterification of soybean oil.

As shown in Table 1, there was an increase in enzyme activity as the solvent hydrophobicity increased in the range of −1.1 to 0.42. Klibanov has pointed out that a minimum amount of water is required to preserve the conformation of the enzyme.[28] In hydrophilic solvents such as acetone and 1,4-dioxane, water has a higher affinity for the hydrophilic solvent, rather than for binding to the enzyme. As a consequence, the enzyme might alter its conformation due to the lack of bound water and thus lose its activity.[29] The explanation of the enhanced enzyme activity with the increasing hydrophobicity might be that stronger hydrophobicity protects the enzyme from deactivation by these alcohols.[30]

No apparent correlation between log *P* and enzyme activity was observed when log *P* >0.8. The enzyme activity was greatly influenced by the different functional groups and molecular structures of the solvents. Enzyme activities were improved by adding the solvents with hydroxyl groups because they solubilised both the methanol and glycerol. Higher enzyme activities could be achieved when alcohols with branched molecular structures were used as solvents, especially tertiary alcohols like *tert*-butanol and *tert*-amyl alcohol, compared to the linear ones, as shown in Table 1. This phenomenon might be attributed to differences in miscibility between alcohols and the triglyceride. As an indication of the solubility parameters, for alcohol molecules with the same number of carbons, branched examples had a better miscibility with the triglyceride (solubility parameter of 7.43) than their linear isomers. The enzyme activity might be even higher with long chain tertiary alcohols. Other small alcohol molecules, such as n-pentanol, 2-pentanol, n-butanol and 2-propanol, were inappropriate to use because they served not only as solvents, but also as acyl acceptors in the transesterification reaction with the triglyceride, resulting in the corresponding by-products (fatty acid amyl ester, fatty acid isoamyl ester, fatty acid butyl ester and fatty acid isopropyl ester). Particularly noticeable differences in enzyme activity were found when using solvents with very similar viscosity values (n-pentanol and *tert*-amyl alcohol), dielectric constants (n-pentanol and 2-pentanol) and solubility parameters (*tert*-amyl alcohol and 1,4-dioxane). Therefore, viscosity, dielectric constant, solubility parameter or even log *P*, by themselves, do not appear to be reliable parameters for assessing the effect of the solvent on enzyme activity. All solvent parameters should therefore be taken into consideration when choosing an appropriate solvent. As *tert*-butanol has a higher freezing point (25 °C) compared to *tert*-amyl alcohol, and need for it to be incubated beforehand, *tert*-amyl alcohol was chosen as the solvent for simplification of the operation, also considering the higher enzyme activity it permitted.

Effect of *tert*-amyl alcohol concentration on FAME production. FAME synthesis was greatly influenced by the addition of *tert*-amyl alcohol as a solvent to the reaction mixture. Different amounts of *tert*-amyl alcohol were added to the reaction mixture in order to observe the effect of *tert*-amyl alcohol concentration on the transesterification reaction. The enzyme activity was very low in a solvent-free system due to the toxicity of excessive methanol on the enzyme activity, as shown in Fig. 3. The enzyme activity increased greatly with increasing *tert*-amyl alcohol/oil volume ratio, and a maximum activity value was achieved at a ratio of 1 : 1. Further increasing the *tert*-amyl alcohol concentration resulted in no enhancement of the enzyme activity. As *tert*-amyl alcohol in the system could improve the solubility of methanol in the reaction mixture, the inhibitory effects of methanol in FAME production were eliminated, and lipase

Table 1 The effect of solvent on the enzyme activity in FAME production[a]

Solvent	Log *P*	Viscosity	Dielectric constant	Solubility parameter	Enzyme activity/μmol min^{-1} g	Yield of other esters(%)[b]
n-Hexane	3.50	0.30	1.89	7.22	56.94 ± 1.71	—
n-Pentanol	1.51	3.31	13.92	11.10	78.23 ± 2.35	6.05 ± 0.18
tert-Amyl alcohol	1.50	3.70	15.44	10.43	339.50 ± 10.18	—
2-Pentanol	1.42	3.86	14.71	10.91	287.88 ± 8.64	2.81 ± 0.08
Cyclohexane	1.20	0.89	2.02	8.20	56.41 ± 1.69	—
n-Butanol	0.80	2.95	17.70	11.46	95.04 ± 2.85	6.87 ± 0.21
tert-Butanol	0.80	4.31	12.47	10.65	332.85 ± 9.98	—
2-Propanol	0.42	2.37	17.94	11.63	134.66 ± 4.04	3.26 ± 0.10
Acetone	−0.23	0.31	20.71	9.67	97.57 ± 2.93	—
1,4-Dioxane	−1.10	1.30	2.21	10.11	23.70 ± 0.71	—

[a] Conditions: Reactions were carried out in 40 ml solvent, soybean oil (40 mmol), methanol (240 mmol), Novozym 435 (3% based on the oil weight), a water activity of 0.53 at 40 °C and 200 rpm under microwave irradiation. Data source for the solvent parameters: refs. 31 and 32. [b] GC conditions: Injector and detector temperatures were 280 and 350 °C, respectively (split 40 : 1). Nitrogen was used as the carrier gas (100 ml min^{-1}, 100 kPa). The column temperature was kept at 50 °C for 1 min and then programmed from 50 to 180 °C at 15 °C min^{-1}, 7 °C min^{-1} to 230 °C and 200 °C min^{-1} to 350 °C. The yield of esters was identified by comparing the peak areas of standard methyl *p*-hydroxybenzoate at particular retention times. Quantification of the ester was done according to the calibration curves of the pure reference substrate.

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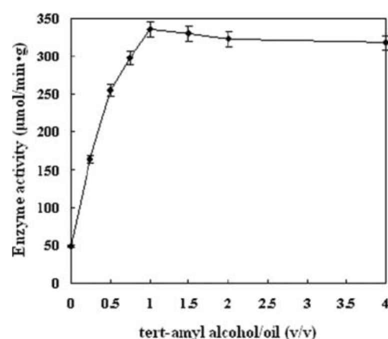

Fig. 3 Effect of *tert*-amyl alcohol concentration on FAME production. *Conditions:* Reactions were carried out in soybean oil (40 mmol), methanol (240 mmol), Novozym 435 (3% based on the oil weight) and water activity of 0.53 and 200 rpm at 40 °C under microwave irradiation in 2 h. The *tert*-amyl alcohol/oil volume ratio varied from 0 to 4.

maintained a high activity, even with large amounts of methanol present in the system.

Effect of substrate ratio on FAME production. The rate of the enzyme catalytic reaction depended on the concentrations of the enzyme and substrate. Variation of the substrate ratio might have a significant effect on the rate of reaction. In the present experiments, the substrate ratio (methanol/oil) was changed by keeping the amount of soybean oil constant at 40 mmol and changing the molar ratio of methanol/oil from 3 to 18. When keeping the amount of the enzyme constant, the enzymatic activities increased mildly with increasing substrate ratio from 3 : 1 to 6 : 1, and it reached a maximum of 325 μmol min^{-1} g^{-1} when the substrate ratio was 6 : 1 (Fig. 4). As the amount of methanol increased, the substrate concentration increased and thus the enzyme activity improved. After this point (6 : 1), with further increases to the substrate ratio, the enzyme activity decreased gradually. This is probably due to the inhibitory effect of methanol on the enzyme, which becomes more significant with higher concentrations of methanol. Hence, a molar ratio of 6 : 1 was considered as the optimum substrate ratio.

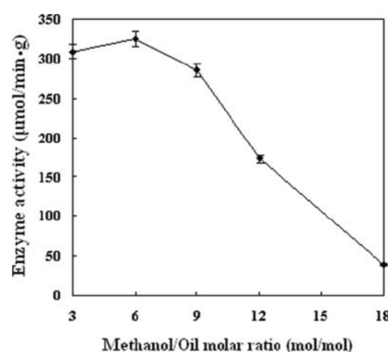

Fig. 4 Effect of substrate ratio on FAME production. *Conditions:* Reactions were carried out in *tert*-amyl alcohol (40 ml), soybean oil (40 mmol), Novozym 435 (3% based on the oil weight) and water activity of 0.53 at 40 °C and 200 rpm under microwave irradiation in 2 h. The molar ratio of methanol/oil varied from 3 to 18.

Effect of pre-treatment on FAME production. It has been reported that the activity of Novozym 435 is greatly inhibited by methanol, and that this inhibitory effect could be eliminated by adding a certain amount of *tert*-butanol.[33] The immersion of lipases in *tert*-butanol and other alcohols with a carbon number ≥ 3 is claimed as a pre-treatment method to increase lipase activity in the synthesis of methyl esters.[34] Therefore, it was very interesting to investigate if there was a positive effect of *tert*-amyl alcohol incubation on the enzyme and whether the methanol inhibitory effect was reversible. As shown in Table 2, there was no change of enzyme activity after incubation in *tert*-amyl alcohol. When incubated in methanol, the enzyme was almost completely deactivated and the enzyme activity could not be regenerated. This is because methanol caused a irreversible change in the flexible conformation of the immobilized enzyme, which results in a permanent loss of enzyme activity.[35] We also found that when the enzyme was pre-incubated in *tert*-amyl alcohol, it was less easily deactivated by methanol. After being sequentially incubated in *tert*-amyl alcohol and methanol for 2 h, a 75.8% enzyme activity was preserved. Although the *tert*-amyl alcohol could not regenerate the enzyme activity from methanol inhibition, it could protect the enzyme when *tert*-amyl alcohol and methanol were added together. It is possible that the methanol dissolved in *tert*-amyl alcohol preserves the conformation of the enzyme.

Enzymatic deactivation kinetics in methanol. Since there was an enzymatic deactivation effect caused by methanol and this effect was irreversible, the enzymatic deactivation kinetics were investigated in this study. As shown in Fig. 5, the deactivation of Novozym 435 in methanol obeyed first-order deactivation kinetics. In addition, we have calculated the k_d, the half-life time ($t_{\frac{1}{2}}$) and the free energy of activation (ΔG_d) for the deactivation process. The enzyme deactivation constant (k_d = 1.99 h^{-1}) was calculated from the slopes of the best-fit curves obtained by linear regression when log ($A/A°\times100$) was plotted against pre-incubation time, where A is the residual enzymatic activity obtained after pre-incubation and $A°$ is the enzymatic activity obtained without pre-incubation. The half-life ($t_{\frac{1}{2}}$ = 0.158 h^{-1}) was the time required for the enzyme to lose 50% of its activity, whereas ΔG_d = 96.3 kJ mol^{-1} was calculated from the following equation:

$$\Delta G_d = RT\ln[k_d h/k_B T]$$

Where k_d is the deactivation constant (h^{-1}), k_B is Boltzmann's constant (1.38×10^{-23} J K^{-1}), h is Planck's constant (1.84×10^{-37}), R is the gas constant (8.314 J mol^{-1} K^{-1}) and T is the absolute temperature.[17]

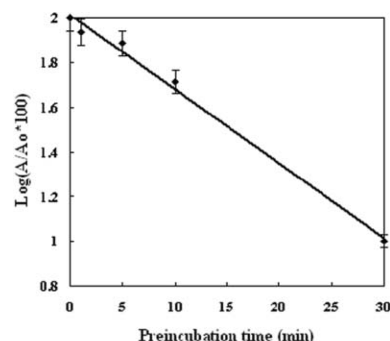

Fig. 5 Linear regression curves obtained from logarithmic representation of residual activity *versus* preincubation time.

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Table 2 The effect of pre-treatment on transesterification under different immersion conditions[a]

Type of enzyme pre-treatment method	Enzyme activity/μmol min^{-1} g^{-1}
Control (untreated, standard reaction)	335 ± 9
Immerse in *tert*-amyl alcohol for 2 h	332 ± 9
Immerse in *tert*-amyl alcohol for 2 h then in methanol for another 2 h	254 ± 7
Immerse in methanol for 2 h	14 ± 1
Immerse in methanol for 2 h then in *tert*-amyl alcohol for another 2 h	13 ± 1

[a] The enzyme was first pre-treated as described in Table 2, after being filtered and dried in air at room temperature; the pre-treated enzymes were used in standard reactions, respectively.

Effect of enzyme dosage on FAME production. The effect of Novozym 435 dosage on FAME production was studied under microwave irradiation. The molar ratios of the reactants were kept constant while the amount of enzyme was changed from 0 to 4% based on the oil weight. As the loading of Novozym 435 increased, the yield of FAME also increased (Fig. 6). Considering the cost of the enzyme, 3% of Novozym 435 proved to be the most efficient amount (before this point, the yield of methyl ester witnessed a steep increase, while after this point, the yield increase was much slower) and was adopted in the experiments.

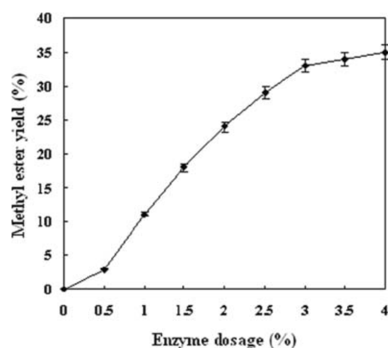

Fig. 6 Effect of enzyme dosage on FAME production. *Conditions:* Reactions were carried out in *tert*-amyl alcohol (40 ml), soybean oil (40 mmol), methanol (240 mmol), water activity of 0.53 at 40 °C and 200 rpm under microwave irradiation in 2 h. The amount of enzyme varied from 0 to 4% based on the oil weight.

Effect of temperature on FAME production. Theoretically, an elevated temperature could help the substrate molecules obtain adequate energy to pass over the energy barrier and enhance the reaction rate. In contrast, enzymes are very sensitive to temperature and easily deactivated at high temperature. The effect of temperature on the activity of Novozym 435 was examined in the range 30–70 °C. The results in Fig. 7 show that the enzyme activity increased as the temperature increased from 30 to 40 °C, followed by a decrease at higher temperature. As the reaction temperature elevates, the chance of a collision between the enzyme and substrate molecules increases, which might help form enzyme–substrate complexes and then led to an increase in enzyme activity. Moreover, the protein molecules could fluctuate more with increasing temperature to relieve steric repulsion; such a fluctuation could contribute to a rate acceleration at elevated temperatures. As for the decrease of enzyme activity with further increasing the temperature above 40 °C, it is mostly likely to be due to the denaturation (alteration) of the

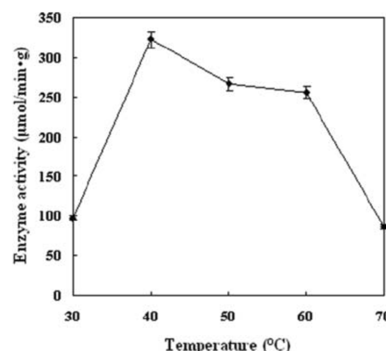

Fig. 7 Effect of temperature on FAME production. *Conditions:* Reactions were carried out in *tert*-amyl alcohol (40 ml), soybean oil (40 mmol), methanol (240 mmol), Novozym 435 (3% based on the oil weight) and water activity of 0.53 and 200 rpm under microwave irradiation in 2 h. Temperature varied from 30 to 70 °C.

protein structure resulting from a heat-induced destruction of non-covalent interactions (a breakdown of the weak ionic and hydrogen bonding that stabilizes the three dimensional structure of the enzyme).[36]

Reusability of Novozym 435. The reusability of the enzyme is an essential factor for the production of biodiesel in industry due to cost management. Catalyst reusability studies were carried out to investigate the stability of the enzyme under microwave irradiation and under conventional heating. As shown in Fig. 8, Novozym 435 was not deactivated or denatured by microwave irradiation; only a slightly decrease (4%) in enzyme activity was observed after five cycles of use. However, 10% enzyme activity was lost after five cycles of use under conventional

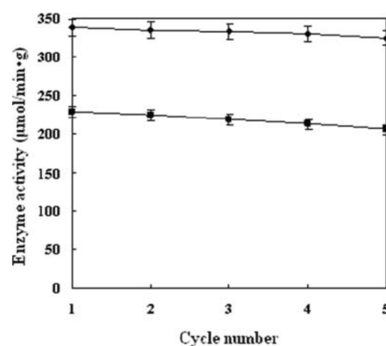

Fig. 8 Reusability of enzyme on FAME production. *Conditions:* Each cycle was carried out in *tert*-amyl alcohol (40 ml), soybean oil (40 mmol), methanol (240 mmol), Novozym 435 (3% based on the oil weight) and water activity of 0.53 at 40 °C and 200 rpm for 2 h under microwave irradiation (◆) and under conventional heating (■).

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heating. The higher stability of the enzyme under microwave irradiation compared to conventional heating might be because the interactions between the enzyme and its microenvironment are improved in a microwave field. These results suggested that Novozym 435 was suitable for catalytic use under microwave conditions.

Conclusions

In the present study, we have shown that microwave irradiation is an efficient method for enzymatic fatty acid methyl ester production compared to conventional heating. Under microwave irradiation, the enzyme activity of Novozym 435 was enhanced in a transesterification reaction of soybean oil and methanol. The microwave-assisted reaction achieved an equivalent yield of FAME in a relatively short time compared to that of conventional heating. Under the optimum conditions (a_w of 0.53, *tert*-amyl alcohol/oil volume ratio of 1 : 1, methanol/oil molar ratio of 6 : 1, 3% immobilized lipase based on the oil weight and 40 °C), a 94% yield of FAME could be achieved in 12 h under microwave irradiation. For the first time, we found that the methanol inhibitory effect was irreversible by analyzing the deactivation kinetics parameter. The enzyme used was also microwave-stable, as no obvious loss in lipase activity was observed after repeated use for five cycles under microwave irradiation.

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References

1 A. Demirbas, *Prog. Energy Combust. Sci.*, 2007, **33**, 1–18.
2 L. C. Meher, D. Vidya Sagar and S. N. Naik, *Renewable Sustainable Energy Rev.*, 2006, **10**, 248–268.
3 C. C. Akoh, S. W. Chang, G. C. Lee and J. F. Shaw, *J. Agric. Food Chem.*, 2007, **55**, 8995–9005.
4 B. Freedman, E. H. Pryde and T. L. Mounts, *J. Am. Oil Chem. Soc.*, 1984, **61**, 1638–1643.
5 C. C. Akoh and B. G. Swanson, *J. Food Process. Preserv.*, 1988, **12**, 139–149.
6 F. Ma and M. A. Hanna, *Bioresour. Technol.*, 1999, **70**, 1–15.
7 M. Canakci and J. H. Van Gerpen, *Trans. ASAE.*, 1999, **42**, 1203–1210.
8 L. Fjerbaek, K. V. Christensen and B. Norddahl, *Biotechnol. Bioeng.*, 2009, **102**, 1298–1315.
9 S. Shah, S. Sharma and M. N. Gupta, *Indian. J. Biochem. Biophys.*, 2003, **40**, 392–399.
10 J. M. Marchetti, V. U. Miguel and A. F. Errazu, *Renewable Sustainable Energy Rev.*, 2007, **11**, 1300–1311.
11 L. Nelson, T. Foglia and W. Marmer, *J. Am. Oil Chem. Soc.*, 1996, **73**, 1191–1195.
12 K. Nie, F. Xie, F. Wang and T. Tan, *J. Mol. Catal. B: Enzym.*, 2006, **43**, 142–147.
13 J. F. Shaw, S. W. Chang, S. C. Lin, T. T. Wu, H. Y. Ju, C. C. Akoh, R. H. Chang and C. J. Shieh, *Energy Fuels*, 2008, **22**, 840–844.
14 C. O. Kappe, *Angew. Chem., Int. Ed.*, 2004, **43**, 6250–6284.
15 M. Nüchter, B. Ondruschka, W. Bonrath and A. Gum, *Green Chem.*, 2004, **6**, 128–141.
16 B. A. Roberts and R. S. Christopher, *Acc. Chem. Res.*, 2005, **38**, 653–661.
17 B. Réjasse, S. Lamare, M. D. Legoy and T. Besson, *Org. Biomol. Chem.*, 2004, **2**, 1086–1089.
18 H. Yuan, B. L. Yang and G. L. Zhu, *Energy Fuels*, 2009, **23**, 548–552.
19 V. Lertsathapornsuk, R. Pairintra, K. Aryusuk and K. Krisnangkura, *Fuel Process. Technol.*, 2008, **89**, 1330–1336.
20 L. Ma, M. Persson and P. Adlercreutz, *Enzyme Microb. Technol.*, 2002, **31**, 1024–1029.
21 D. H. Yu, Z. Wang, P. Chen, L. Jin, Y. M. Cheng, J. G. Zhou and S. G. Cao, *J. Mol. Catal. B: Enzym.*, 2007, **48**, 51–57.
22 X. L. Zhang and D. O. Hayward, *Inorg. Chim. Acta*, 2006, **359**, 3421–3433.
23 A. B. Copty, Y. Neve-Oz, I. Barak, M. Golosovsky and D. Davidov, *Biophys. J.*, 2006, **91**, 1413–1423.
24 P. J. Halling, *Biotechnol. Tech.*, 1992, **6**, 271–276.
25 P. J. Halling, in *Enzyme Catalysis in Organic Synthesis: A Comprehensive Handbook: Volume I*, ed. K. Drauz and H. Waldmann, Wiley-VCH Verlag GmbH, Weinheim, Germany, 2nd edition, 2002, pp. 259–286.
26 S. Karboune, A. Archelas and J. Baratti, *Enzyme Microb. Technol.*, 2006, **39**, 318–324.
27 C. Laane, S. Boeren, K. Vos and C. Veeger, *Biotechnol. Bioeng.*, 1987, **30**, 81–87.
28 A. M. Klibanov, *Nature*, 2001, **409**, 241–246.
29 R. Perraud and F. Laboret, *Appl. Microbiol. Biotechnol.*, 1995, **44**, 321–326.
30 V. Dossat, D. Combes and A. Marty, *Enzyme Microb. Technol.*, 1999, **25**, 194–200.
31 *CRC Handbook of Chemistry and Physics*, ed. D. R. Lide, CRC Press/Taylor and Francis, Boca Raton, FL, 87th edn., 2006.
32 F. Secundo, S. Riva and G. Carrea, *Tetrahedron: Asymmetry*, 1992, **3**, 267–280.
33 W. Du, L. Wang and D. H. Liu, *Green Chem.*, 2007, **9**, 173–176.
34 J. W. Chen and W. T. Wu, *J. Biosci. Bioeng.*, 2003, **95**, 466–469.
35 A. M. Klibanov, *Nature*, 1995, **374**, 596.
36 G. D. Yadav and P. S. Lathi, *Enzyme Microb. Technol.*, 2006, **38**, 814–820.

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