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Ectoine improves yield of biodiesel catalyzed by immobilized lipase

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ABSTRACT

To improve the production of biodiesel by enzymatic conversion of triglycerides in cottonseed oil, compatible solutes were added to the solvent-free methanolysis system to prevent competitive methanol inhibition on the immobilized lipase (Novozym® 435). The results indicated that the addition of ectoine increased biodiesel synthesis using a three-step methanol addition process. The concentration of methyl ester (ME) reached a maximum of 95.0% in the presence of 1.1 mmol/l ectoine, an increase of 20.9% compared to that in the absence of ectoine. On the other hand, excess ectoine decreased the ME concentration. Ectoine was also shown to enhance reuse of the immobilized lipase, significantly improving ME concentrations in each recycling test. Total concentrations of ME with added ectoine were about 1.5 times that without ectoine during five recycling tests (molar ratio of cottonseed oil to methanol, 1:4). Enzymatic reaction kinetics showed, in the concentration ranges of 0.8–1.14 mol/l and 0.03–8 mol/l for triglyceride and methanol, respectively, that ectoine had no effect on the initial reaction rates when methanol concentrations were below 0.5 mol/l. When methanol concentration exceeded 0.5 mol/l, the addition of 0.8 mmol/l ectoine increased the initial reaction rates, and the lipase exhibited a lower affinity for methanol and higher affinity for triglyceride (kinetic parameters of K_{mA} increase, K_{mTG} decrease). However, the initial reaction rates decreased significantly when 8 mmol/l ectoine was added, with the lipase having higher affinity for methanol and lower affinity for triglyceride (K_{mA} decrease, K_{mTG} increase). The supplementation of ectoine provided a new method for the purpose of improving yield of biodiesel catalyzed by enzyme.

1. Introduction

The development of biodiesel has proceeded rapidly in the last 20 years due to its promise as a renewable source of fuel. Biodiesel is composed of fatty acid methyl esters (FAME) or ethyl esters (FAEE) produced by alcoholysis of triglyceride such as animal fats or plant oils. Since biodiesel are generally similar to diesel on the characteristics, the former is a strong candidate to replace diesel [1].

The most common method for producing biodiesel involves triglyceride alcoholysis in the presence of chemical or enzymatic catalysts. Chemical processes give high conversions of triglyceride to their corresponding methyl esters in short reaction times but have drawbacks such as being energy intensive, difficulty in recovering glycerol, the need for removal of alkaline catalyst from the product and treatment of alkaline wastewater, and the interference of the reaction by free fatty acids and water [2]. Enzymatic methods can overcome the problems of conventional chemical processes mentioned above, using triglyceride lipases (E.C. 3.1.1.3) as biocatalysts catalyzing alcoholysis of triglyceride. In particular, glycerol can be easily recovered without any complex processing, free fatty acids contained in the oils can be completely converted to methyl esters and subsequent wastewater treatment is not required [3]. So, enzymatic conversion of triglyceride has drawn considerable attention due to its environmental friendliness.

However, enzymatic methods have not been industrialized due to the high cost of the enzymes and the negative effects of the substrate methanol and the co-product glycerol on enzyme activity and stability. These problems are the main bottleneck in biological enzymatic industrial production of biodiesel [4,5]. Most studies performed on the enzymatic production of biodiesel from vegetable oils focused on the effect of methanol on enzymatic stability. The effect of the molar ratio of oil to methanol on the initial reaction rates has also been investigated [6,7]. When methanol exceeded its solubility limits, the lipase was irreversibly inactivated by the methanol, which existed as drops in the oil [4]. Samukawa et al. [6] and Shimada et al. [8] have both reported the conversion of soybean oil to biodiesel using immobilized lipase (Novozym® 435). FAME production increased when methanol concentration increased up to oil to methanol ratio of 3:1 equivalents and then decreased when methanol concentration was further increased in the absence of organic solvents. This was also found by Noureddini et al. [9], who reported the oil to methanol ratio was 7.5:1

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equivalents using lipase from *Pseudomonas cepacia*. The reaction kinetics of enzymatic methanolysis for biodiesel has been studied as well. The kinetic mechanism for esterification and transesterification reactions is usually considered to be Ping Pong Bi Bi with competitive alcohol inhibition [10,11]. Samukawa et al. [6] and Al-Zuhair et al. [7] studied the kinetic mechanism of the production of biodiesel from plant oil using lipase, and proposed that the transesterification of plant oil with methanol catalyzed by lipase has been described by a Ping Pong Bi Bi model with competitive inhibition by methanol. Several methods were reported to decrease inhibition by methanol as follows: (1) Addition of non-polar organic media. Many solvents such as n-hexane [7], petroleum ether [12] and isooctane [13] were used to increase the dissolubility of methanol. (2) Stepwise addition of methanol. Shimada et al. [4] proposed adoption the stepwise methanolysis decreases the inhibition of lipase by methanol first time, that is, methanol was added to solvent-free systems with soybean oil by three successive additions of oil to methanol ratio of 1:3 equivalents during the reaction for 48 h. The three-step reaction converted more than 90% fatty acids in the oil to their corresponding methyl esters (ME). (3) Preincubation of immobilized lipase with organic solvent. Samukawa et al. [6] preincubated Novozym® 435 with methyl oleate and soybean oil penetrating into support matrix of immobilized enzyme, the enzyme activity and stability were all increased. (4) Use of immobilized lipase. Watanabe et al. [14] reported that immobilized lipase Novozym® 435 efficiently catalyzed the methanolysis of soybean oil without an organic solvent, which was necessary for the industrial production of biodiesel from an economical point of view. In addition, compatible solutes or stabilizers such as polyols have been applied to increase the stability and activity of the enzyme in organic solvents [15]. Hoq et al. [16] reported *Candida cylindracea* lipase was stabilized significantly by glycerol added to the mixture of olive oil and buffer solution in a microporous hydrophobic membrane bioreactor. Lee and Choo [17] reported, in the presence of polypropylene glycol, the rate of denaturation of *C. cylindracea* lipase decreased by 93%. Selmi and Thomas [18] and Nassreddine et al. [19] reported that silica gels could adsorb glycerol produced by transesterification of oil and alcohol, which improved the recycling activity of immobilized lipase in the solvent-free system. While the addition of compatible solutes improves the yield of biodiesel by increasing enzymatic activity, the effect on lipase stability has not been reported.

The cyclic amino acid ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid, chemical structure shown in Fig. 1 [20]), one of the representative compatible solutes with the characteristic of zwitterions, accumulates in halophilic and halotolerant bacteria in hypersaline environments, the role of which is to establish a balance between the osmotic pressures inside and outside the cells [21]. Previous studies indicated that ectoine functions on proteins, nucleic acids and cells as a stabilizer against some adverse conditions [20,22–24].

In this study, biodiesel was produced by methanolysis of cottonseed oil catalyzed by immobilized *Candida antarctica* lipase (Novozym® 435) in solvent-free systems. We examined whether the yield of biodiesel was enhanced by adding compatible solutes, particularly ectoine. In order to explore the mechanism of ectoine enhancement on the yield of biodiesel, the effect of ectoine on enzymatic reaction kinetics was investigated.

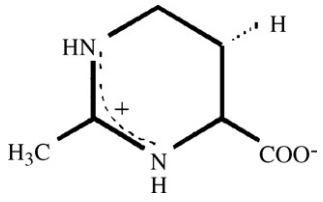

Fig. 1. Chemical structure of the ectoine.

2. Materials and methods

2.1. Materials

The cottonseed oil (palmitic acid 21.5%, stearic acid 2.2%, oleic acid 15.0%, linoleic acid, 61.3%) was purchased from a local grocery store and used as triglycerides. The molecular weight of the oil can be calculated from its saponification value. Ectoine is product of Biomol (Hamburg, Germany), its purity is over 95%. Novozym® 435 was provided by Novo Nordisk (Bagsvaerd, Denmark). Palmitic acid methyl ester, stearic acid methyl ester, oleic acid methyl ester, linoleic acid methyl ester and heptadecanoic acid methyl ester were chromatographically pure and purchased from Sigma (St. Louis, Mo, USA). Methanol, glycerol, and trehalose were of analytical grade and purchased from native chemical companies.

2.2. Methods

2.2.1. Methanolysis

A standard reaction mixture consisted of 5 g cottonseed oil and methanol (oil to methanol ratios were set according to the experiments), and 3.5% (w/w) immobilized lipase Novozym® 435 (based on the weight of reaction mixture). The reaction was conducted in a 50 ml screw-capped polypropylene test tube at 40 °C with shaking at 150 oscillations/min. Methanol was added by two ways. One was one-step methanolysis of oil, which was conducted by adding all methanol into the reaction system at the beginning of reaction at the ratio of cottonseed oil/methanol (1:4, mol/mol). The other was three-step reaction, which was conducted by adding the first part of methanol in a mixture of 1/3 molar ratio of methanol (the molar ratio of oil to methanol, 1:1.3); after 12 h, reaction was conducted by adding the second-part of methanol; after 24 h, methanolysis was done by adding the third-part of methanol (total reaction time, 36 h; total molar ratio of oil to methanol, 1:4). To investigate the yield of ME during reuse of the enzyme preparation, the immobilized enzyme was recovered from the reaction medium by filtration, and then washed with 5 ml hexane under agitation for 0.5 h and dried by evaporation of the hexane.

Methyl ester (ME) is the production of transesterification from cottonseed oil and methanol catalyzed by Novozym® 435. ME concentration (w/w, %) was defined as the percentage of ME content (g) per gram reaction liquid. The formula to calculate it from methanolysis experiment for biodiesel was as follows:

$$\text{ME concentration} = \frac{\text{ME content}}{\text{Reaction liquid mass}} \times 100\% \quad (1)$$

The initial reaction rate for methanolysis was determined within the limits of ME concentrations less than 5% in 40 min and it was represented as the millimolar concentration of biodiesel produced per minute, per liter (mmol/l min).

2.2.2. Analysis of methyl esters

Samples (0.018 g) of reaction liquid produced by methanolysis of cottonseed oil in a 50 ml screw-capped polypropylene test tube were withdrawn at different time intervals and mixed with 600 ml n-hexane and 600 ml of 1 mg/ml heptadecanoic methyl ester (served as internal standard) for gas chromatographic analysis. The contents of methyl ester were quantified on a HP6890 gas chromatograph equipped with a HP-5 capillary column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies, Palo Alto Calif., USA). An injection volume of 1.0 μl was employed. The column temperature was raised from 100 to 150 °C at 15 °C/min and

then raised to 200 °C at 8 °C/min, to 240 °C at 2 °C/min and maintained for 4 min, at last, the column temperature raised to 300 °C at 15 °C/min. The injector and detector temperatures were both set at 280 °C.

2.2.3. Kinetic model

Many authors have demonstrated that the kinetic mechanism for esterification and transesterification reactions was a Ping Pong Bi Bi with competitive alcohol inhibition [10,11]. The equation of this mechanism is given as follows [11,25]:

$$V = \frac{V_{max}[TG][A]}{K_{mTG}[A](1 + ([A]/K_i)) + K_{mA}[TG] + [TG][A]} \quad (2)$$

where V (mmol/l min) is the initial reaction rate; V_{max} (mmol/l min) the initial maximum velocity of the reaction; [TG] (mol/l) and [A] (mol/l) the initial molar concentrations of triglycerides and methanol, respectively; K_{mTG} (mmol/l) and K_{mA} (mmol/l) the apparent Michaelis constant for triglycerides and methanol, respectively; K_i (mmol/l) the apparent inhibition constant of methanol.

3. Results and discussion

3.1. Effect of the molar ratio of oil to methanol on the ME concentration

The effect of the molar ratio of oil to methanol on the methyl ester (ME) concentrations for biodiesel in a solvent-free system was examined. As shown in Fig. 2, the ME concentrations increased with increasing molar concentration of methanol when molar ratio of cottonseed oil to methanol was in the concentration range of 38:1–2:1 mol/l. The ME concentration reached the maximum value at a 2:1 molar ratio of oil to methanol. When the molar ratios were in the concentration ranges of 2:1–1:10 mol/l, the ME concentrations decreased with increasing molar concentration of methanol. In particular, the ME concentration was only 46% of the maximum value at a 1:4 molar ratio of oil to methanol which is necessary for the industrial production of biodiesel.

In the solvent-free methanolysis system for production of biodiesel, methanol is one of the substrates of lipase in the reaction mixture, and exhibits negative effects on the lipase at two levels. First, methanol displayed competitive inhibition on kinetics when the concentration of methanol was low (molar ratios of oil to methanol, 4:1–1:3), *i.e.*, an alcohol molecule reacting with the enzyme produced a dead-end enzyme-alcohol complex (E.A.) [7]. Second, when the concentration of methanol was high, it caused the destruction of the native conformation of the protein as denaturing agent, and resulted in the irreversible inactivation of the lipase [6,26]. In this study, the activity of lipase was inhibited by methanol when the molar ratios exceeded 2:1. However, to complete the bioconversion of cottonseed oil to the corresponding ME, at least 3–4 molar equivalents of methanol are necessary for the enzymatic production of biodiesel industrially (molar ratios of oil to methanol, 1:3–4) [6,27]. Hence, the study of protection for the lipase activity under this condition is significant.

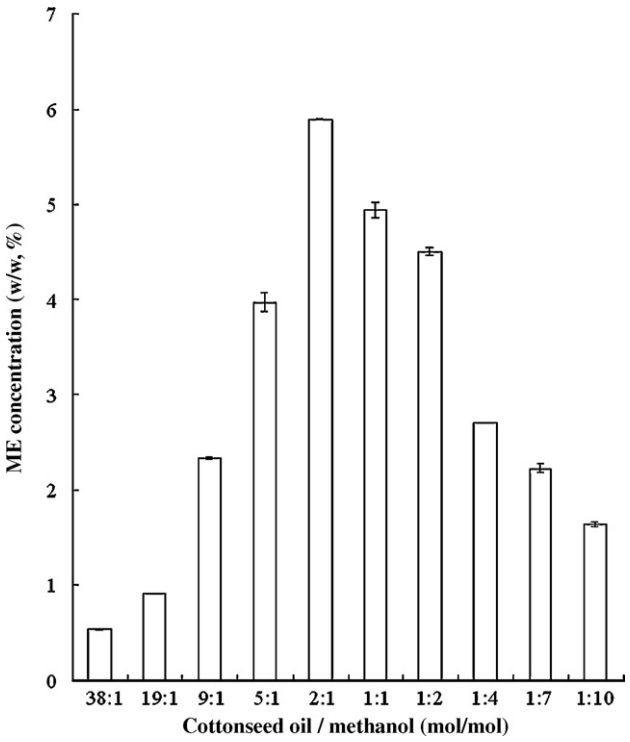

Fig. 2. Effect of the molar ratio of oil to methanol on the ME concentration. Reaction conditions: 5 g mixture of cottonseed oil (with concentrations of triglycerides between 0.8 and 1.14 mol/l) and methanol (with concentrations between 0.03 and 8 mol/l), the range of molar ratio of oil to methanol was 38:1–1:10, 3.5% Novozym® 435, 150 oscillations/min, 40 °C for 40 min. The ME concentration was the averages ± SD from three separate experiments.

3.2. Effect of the compatible solutes on the ME concentration

3.2.1. Comparison of the effects of compatible solutes on the ME concentration

The supplementation effects of representative compatible solutes such as glycerol, trehalose and ectoine at different concentrations were examined. As shown in Table 1, certain additional concentrations of three compatible solutes could improve ME concentrations, in which, the optimal concentrations were found to be 0.5, 0.5 and 0.8 mmol/l, respectively, and corresponding ME concentration increasing by 25, 6 and 24% compared to the control without above three compatible solutes, respectively. However, excess compatible solutes led to a decrease in the ME concentrations. For example, when the glycerol concentration was 1.1 mmol/l, the ME concentration decreased by 8.3%. Since glycerol is produced during the methanolysis for biodiesel, the concentration of glycerol increases gradually as the reaction progresses. Excess glycerol adsorbs on the surface of the lipase causes the formation of a hydrophilic layer around the enzyme. This results in limiting the diffusion of the hydrophobic substrate from the organic phase to the enzyme active center and enriching the concentration of methanol, and a serious negative effect on the enzymatic activ-

Table 1
Relative concentration (%) of ME in the presence of compatible solutes[a].

Compatible solute	Relative concentration of ME (%)				
Glycerol	114 ± 0.85 (0.2)	125 ± 2.26 (0.5)	108 ± 0.99 (0.8)	91.7 ± 0.42 (1.1)	
Trehalose	106 ± 1.04 (0.5)	105 ± 0.63 (0.8)	99.0 ± 0.70 (1.1)		
Ectoine	114 ± 0.78 (0.5)	124 ± 2.73 (0.8)	119 ± 2.46 (1.1)	102 ± 2.39 (1.4)	92.5 ± 4.45 (1.6)

[a] Different concentrations of glycerol, trehalose and ectoine were added to reaction mixture. Reaction conditions: 4.76 g cottonseed oil and 0.24 g methanol, 3.5% Novozym® 435, 150 oscillations/min, 40 °C for 6 h. The ME concentration was designated as 100% without compatible solute. Relative concentrations of ME were the averages ± SD from three separate experiments. The values in parentheses were compatible solutes concentrations (mmol/l).

ity [5]. Therefore, glycerol is not used as an additive. The addition of trehalose brought about lower protection efficiencies than those of ectoine and glycerol. Hence, trehalose is not used as an additive. The ME concentrations increased with the increase of ectoine concentration within the range of 0.5–1.4 mmol/l. It is interesting to note that the addition of ectoine significantly improved the ME concentration. Optimal amount of ectoine was 0.8 mmol/l, resulting in the ME concentration increasing by 24% compared to the control without ectoine. However, the ME concentration decreased when ectoine concentrations exceeded 1.6 mmol/l. We can deduce that excess ectoine has the same effect as excess glycerol, *i.e.*, excess ectoine causes the formation of a hydrophilic layer around the enzyme. This results in a serious negative effect on the enzymatic activity.

To examine ectoine causing efficiencies whether on substrates or on lipase, 1.1 mmol/l ectoine was added to the mixture containing 4.76 g cottonseed oil and 0.24 g methanol without immobilized lipase with shaking at 150 oscillations/min for 6 h. The results from gas chromatographic analysis show that no ME was synthesized, which indicated ectoine promoted the concentrations of ME for lipase-catalyzed methanolysis by protecting enzymatic activity.

3.2.2. Effect of ectoine on the ME concentration

Since a 1:3–4 molar ratio of oil to methanol is necessary for the industrial production of biodiesel, and methanol addition might inhibit lipase, we examined the effect of ectoine on the methanolysis of cottonseed oil using a three-step process for the addition of methanol. Methanol was added in three steps to maintain the molar ratio of oil to methanol at 1:1.3. As shown in Fig. 3, the addition of ectoine led to an increase in the synthesis of biodiesel, *i.e.*, the ME concentration reached 95.0% in the presence of 1.1 mmol/l ectoine, an increase of 20.9% compared to the control without ectoine, this result showed that supplemented ectoine efficiently improved activities of immobilized lipase with an appropriate amount of methanol.

The addition of ectoine could efficiently improve the yield of biodiesel using a three-step process for the addition of methanol. Based on the kinetic mechanism and the structural stability of enzyme by methanol [6,7,26], we can deduce that this yield increase could be due to the effect of ectoine on the lipase in methanol surroundings. On the one hand ectoine degrades competitive inhibition of lipase on kinetics by methanol, on the other hand it increases the stability of the native conformation of the protein in methanol surroundings, and decreases the irreversible inactivation of the lipase.

3.3. Effect of ectoine on additional amount of enzyme

The high cost of lipase is one of the most serious problems for the application of enzymatic processes to the industrial production of biodiesel fuel from vegetable oil [14]. Generally, the amounts of enzyme required were in the range of 4–30% to produce a high ME content [14,27]. Once the supplementation effect of 1.1 mmol/l ectoine on 3.5% (w/w) immobilized lipase was determined, the methanolysis catalyzed by 4 and 5% lipase (based on the weight of reaction mixture) was compared for 36 h. As shown in Fig. 4, the ME concentrations increased with the increase of enzyme quantity. When 1.1 mmol/l ectoine was added to the reaction mixture containing 3.5% enzyme, the ME concentration increased by 16.9 and 10.8%, compared to that catalyzed by 4 and 5% amounts of enzyme, respectively. These results indicated that the addition of an appropriate amount of ectoine allowed a lower amount of enzyme to be used for the same ME content.

3.4. Effect of ectoine on lipase reuse

To determine the effect of ectoine on the recycling activity of the immobilized lipase at different molar ratios of cottonseed oil to methanol (1:1, 1:4 and 1:7), five recycles of methanolysis were

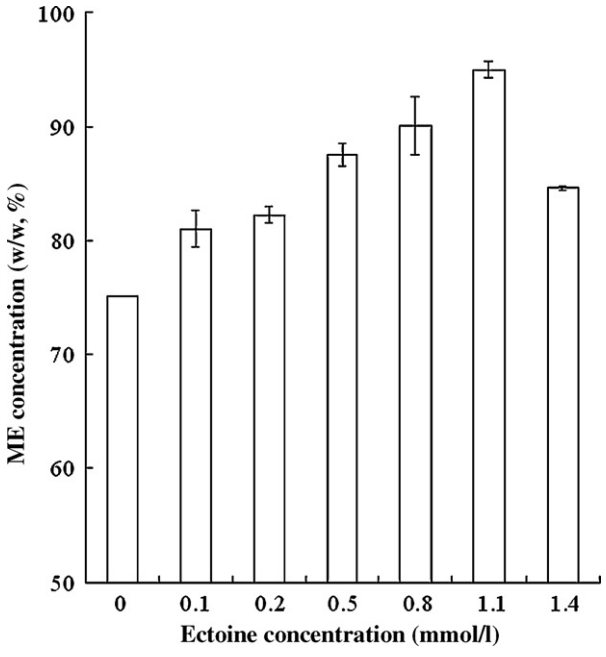

Fig. 3. Effect of ectoine on methanolysis by a three-step addition of methanol. Reaction conditions: 4.76 g cottonseed oil and 0.24 g methanol, 3.5% Novozym® 435, 150 oscillations/min, 40 °C. Different concentrations of ectoine were dissolved into the methanol. The reaction was conducted by adding the first part of methanol in a mixture of 1/3 molar ratio of methanol (the molar ratio of oil to methanol, 1:1.3); after 12 h, reaction was conducted by adding the second-part of methanol; after 24 h, methanolysis was done by adding the third-part of methanol (total reaction time, 36 h; total molar ratio of oil to methanol, 1:4). The ME concentration was the averages ± SD from three separate experiments.

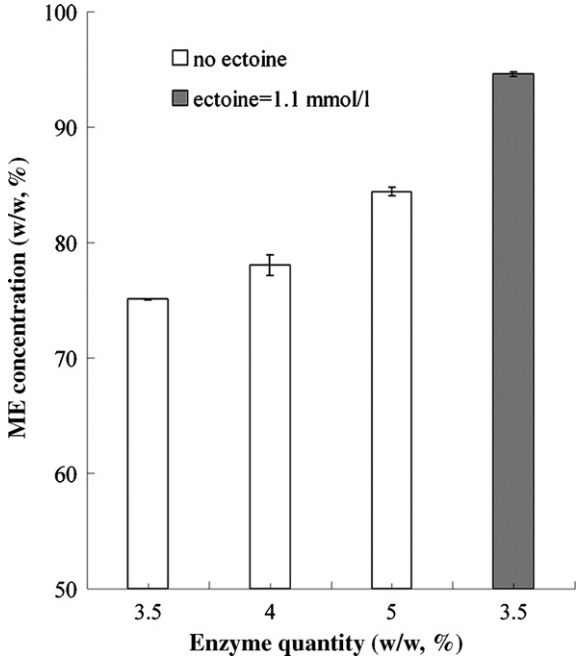

Fig. 4. Effect of ectoine on enzyme content by a three-step addition of methanol. Reaction conditions: 4.76 g cottonseed oil and 0.24 g methanol, 3.5% Novozym® 435, 150 oscillations/min, 40 °C. Reaction was conducted for 36 h by a three-step addition of methanol. No ectoine (open column), 1.1 mmol/l ectoine (shade column). The ME concentration was the averages ± SD from three separate experiments.

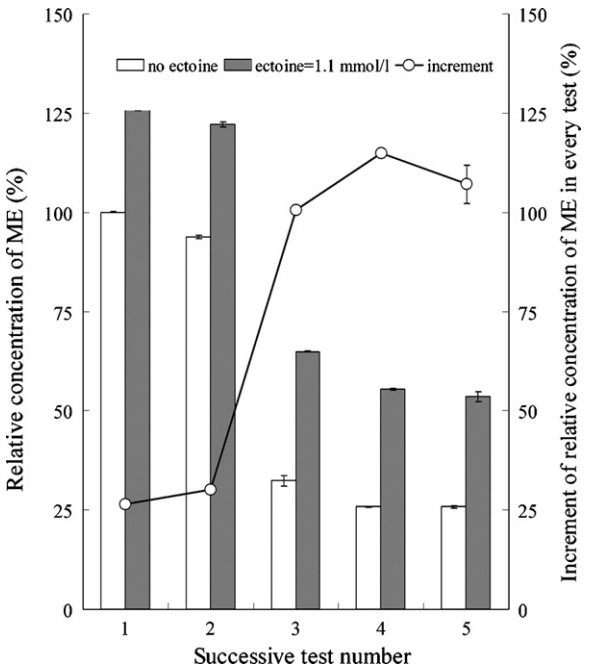

Fig. 5. Effect of ectoine on enzyme reuse. After each test the immobilized enzyme was recovered from the reaction medium by filtration, and then washed with 5 ml hexane under agitation for 0.5 h and dried by evaporation of the hexane. The reaction medium was operated under the same condition as in Fig. 3 during five tests. The ME concentration was designated as 100% without ectoine in test 1. The values on left *Y*-axis were relative concentration of ME (%). They were the averages ± SD from three separate experiments. No ectoine (open column), 1.1 mmol/l ectoine (shade column); the values on right *Y*-axis were increment of relative concentration of ME (%) in every test, which were expressed with a curve.

compared using the relative concentrations of ME synthesis under standard conditions, without additive, and with added 1.1 mmol/l ectoine, in which the ME concentration in test 1 without ectoine was designated as 100%. As shown in Fig. 5, when the molar ratio of cottonseed oil to methanol was 1:4, in the absence of ectoine, the relative concentrations of ME decreased with the increase of recycling test numbers, which were 100, 93.7, 32.4, 25.8 and 25.8% in tests 1, 2, 3, 4 and 5, respectively. The addition of ectoine resulted in increased ME synthesis during five recycling tests. Relative concentrations of ME reached 126.5, 122.1, 64.9, 55.4 and 53.5% in each successive test, which increase by 26.5, 30.2, 100.6, 114.9 and 107.1% compared to the control without ectoine. Total concentrations of ME with added ectoine were about 1.5 times that without ectoine during five recycling tests. Meanwhile, the result of immobilized lipase reuses at the molar ratio of 1:1 and 1:7 shows that the mode of lipase activity decrease and effect of ectoine on lipase reuse were similar with that at 1:4 molar ratio of oil to methanol (data not shown).

In the experiment of immobilized lipase reuse, ME concentrations decreased remarkably from the third test whether ectoine was added or not (Fig. 5), which could be explained by a loss in activity of the immobilized lipase due to the accumulation of the co-product glycerol [5]. To reduce the unfavorable effect of glycerol on immobilized lipase, Selmi and Thomas [18] reported the use of silica gels could improve significantly the yield of ethyl esters at equilibrium for the different runs in the transesterification of sunflower seed oil catalyzed by immobilized lipase, and proposed that the silica gel has a strong affinity for the glycerol, behaves as a glycerol "collector," and plays a protective role against the blockage of the immobilized enzyme by avoiding adsorption of the produced glycerol on the Lipozyme support. Nassreddine et al. [19] reported, during the five-test in the transesterification of sunflower seed oil catalyzed by a silica fibre reinforced aerogel encapsulated lipase, that the recycling activity increased by 40% after the first test, then it slowly decreased in further tests to reach an activity still 20% higher than initially during the fifth test. In our study, the addition of ectoine significantly improved ME concentrations in the recycling tests, showing that ectoine dramatically reduced the unfavorable effect of glycerol on the immobilized lipase in solvent-free systems.

3.5. Effect of ectoine on enzymatic kinetics

According to the results in Table 1, the ME concentration of biodiesel increased in the presence of 0.8 mmol/l ectoine, however, it decreased when ectoine concentrations were over 8 mmol/l (data not shown). Since only substrates not any extra organic solvent existed in the reaction system, the concentrations of the cottonseed oil and methanol were strictly interdependent, *i.e.*, the concentration of one substrate increased, the other would decrease. To investigate the effect of high and optimal concentrations of ectoine on the kinetics of the enzymatic methanolysis of triglycerides, two substrates were mixed at different molar ratios (38:1–1:10), while the total weight of substrates was kept constant (5 g). Since the concentrations of triglycerides and methanol were interdependent, [TG] could be expressed as a function of [*A*], and Eq. (2) was converted to Eq. (3) [25]:

$$V = \frac{V_{\max}[A](\rho_{\mathrm{mix}} - M_{\mathrm{A}}[A])/M_{\mathrm{TG}}}{K_{\mathrm{mTG}}[A](1 + ([A]/K_i)) + (K_{\mathrm{mA}} + [A])(\rho_{\mathrm{mix}} - M_{\mathrm{A}}[A]/M_{\mathrm{TG}})} \quad (3)$$

The weight of reaction mixture was 5 g, where $\rho_{\mathrm{mix}} = 900$ g/l was the density of reaction mixture; $M_{\mathrm{A}} = 32$ and $M_{\mathrm{TG}} = 872$ were the molecular weights of methanol and triglycerides, respectively. The kinetic parameters in Eq. (3) could be determined by nonlinear regression method, and the software Origin 7.0 was used to perform the nonlinear regression by Levenberg–Marquardt method.

The effect of ectoine on enzymatic kinetics was shown in Fig. 6. The addition of ectoine had no effect on the initial reaction rates when the molar concentrations of methanol were below 0.5 mol/l. However, when an excess amount of methanol was added, different concentrations of ectoine caused different effects on the initial reaction rates. The addition of 0.8 mmol/l ectoine led to an increase in the initial reaction rate. However, the initial reaction rate decreased

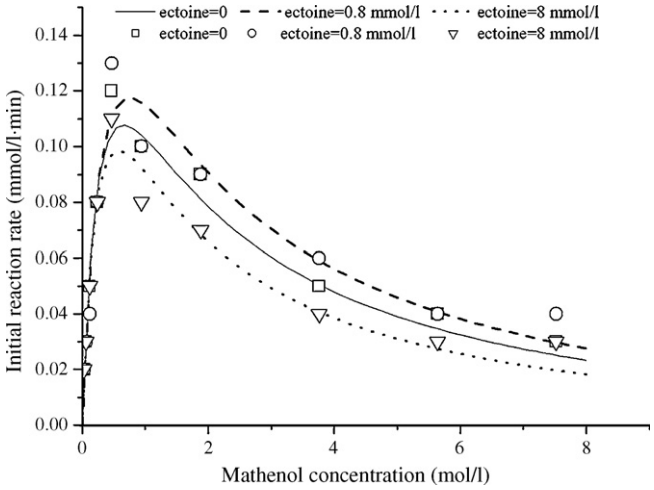

Fig. 6. Comparison of the calculated values and experimental data of initial reaction rates for the methanolysis of cottonseed oil. Reaction conditions: 5 g mixture of cottonseed oil and methanol (the range of molar ratios of oil to methanol were 38:1–1:10), 3.5% Novozym® 435, 150 oscillations/min, 40 °C for 40 min. Three calculated curves were fitted with Eq. (3). Lines: 0.8 mmol/l ectoine (- - - -), no ectoine (—), 8 mmol/l ectoine (....); Symbols: 0.8 mmol/l ectoine (○), no ectoine (□), 8 mmol/l ectoine (▽).

Table 2
Kinetic parameters in the present of different concentrations of ectoine[a].

Ectoine (mmol/l)	V_{max} (mmol/l min)	K_{mTG} (mmol/l)	K_i (mmol/l)	K_{mA} (mmol/l)	R^2	SD≤
0	0.498	1080	660	844	0.968	0.008
0.8	0.504	1050	720	873	0.917	0.014
8	0.426	1100	576	657	0.941	0.009

[a] Experimental data of initial reaction rates in Fig. 6 was put into the system of Eq. (3), and the kinetic parameters were determined using the nonlinear curve fitting software origin 7.0. SD was standard deviation of the calculated values and experimental data of initial reaction rates.

significantly when 8 mmol/l ectoine was added. To consider the effect of ectoine on the enzymatic kinetic mechanism, the experimental data in Fig. 6 was put into Eq. (3), and the kinetic parameters were determined using the nonlinear curve fitting software Origin.

The fitting results were shown in Table 2. When 0.8 mmol/l ectoine was added, the kinetic parameters V_{max}, K_{mA} and K_i all increased and K_{mTG} decreased compared to that in the case without ectoine. However, when 8 mmol/l ectoine was added, the kinetic parameters V_{max}, K_{mA} and K_i all decreased and K_{mTG} increased. These results indicated that supplementation with an appropriate amount of ectoine led to a decrease in the affinity of lipase for methanol and an increase for triglyceride, thus, the inhibition of lipase by methanol was decreased. However, there was an adverse effect when 8 mmol/l ectoine was added.

A simplified model was proposed to describe the effect of ectoine on the enzymatic kinetics of methanolysis of triglycerides. The results indicated that supplementation with an appropriate amount of ectoine led to a lower affinity of the lipase for methanol (K_{mA} increase) and a higher affinity for triglyceride (K_{mTG} decrease). Thus, based on the structure of ectoine shown in Fig. 1, we can infer that ectoine combines with the enzyme by hydrogen bonding, *i.e.*, ectoine adsorbs to the surface of the enzyme stabilizing its conformation and partially displaces methanol, which decreases the formation of the dead-end enzyme-alcohol complex (E.A.) and increases the reaction rates. However, excess ectoine inhibited the enzymatic activity. Based on the kinetic parameters of lipase-catalyzed methanolysis, the affinity for the lipase to methanol was increased (K_{mA} decrease), but that to triglycerol was decreased (K_{mTG} increase).

4. Conclusion

In the solvent-free methanolysis system for production of biodiesel by enzymatic conversion of triglycerides in cottonseed oil, methanol displays competitive inhibition of the lipase, even causes the destruction of the native conformation of the protein and leads to the irreversible inactivation of the lipase. We have shown that the addition of ectoine could efficiently improve the yield of biodiesel synthesis when immobilized lipase was used whether in once or in recycling tests. The results of the effect of ectoine on the enzymatic kinetics of methanolysis of triglycerides indicated that supplementation with an appropriate amount of ectoine led to a lower affinity of the lipase for methanol (K_{mA} increase) and a higher affinity for triglyceride (K_{mTG} decrease). The supplementation of ectoine provided a new method for the purpose of improving yield of biodiesel catalyzed by enzyme.

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