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ORIGINAL PAPER

Study on Free Lipase-Catalyzed Ethanolysis for Biodiesel Preparation in an Oil/Water Biphasic System

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Abstract Using free lipase as the catalyst for biodiesel production has drawn increasing attention in recent years due to its advantages of lower cost and faster reaction rate compared to immobilized lipase. Our previous study showed that free lipase NS81006 could effectively catalyze the methanolysis of renewable oil. Ethanol, derived from renewable biomass, has a greater potential for biodiesel production. In this paper, ethanol was explored for the first time as the acyl acceptor for free lipase-mediated biodiesel preparation. The effect of stirring rate, water content, molar ratio of ethanol to oil and ethanol adding strategy was investigated systematically during the process of free lipase NS81006-catalyzed ethanolysis. An ethyl ester yield of 90% was obtained under the optimized conditions. Further study showed that the free lipase could be repeatedly used by simple separation of the water phase from the oil phase and there was no obvious loss in lipase activity after five repeated uses.

Keywords Biodiesel · Ethanolysis · Lipase catalysis · Renewable oil · Repeated use

Introduction

Biodiesel, as an alternative to mineral diesel, has huge potential application as a substitute for petroleum diesel because of the increasing worldwide concern for environmental protection and diminishing petroleum resources. The merits of biodiesel such as biodegradability and low emission profiles are well documented in the literature [1, 2]. Currently, industrial-scale synthesis of biodiesel is generally performed by the transesterification of renewable oils with methanol catalyzed by chemical catalysts especially alkaline-based catalysts.

Although chemical catalyst-mediated transesterification has realized the industrialization of biodiesel production in many countries, the reaction has several drawbacks: high energy consumption, difficulty in recovering by-product glycerol, the removal of the alkaline catalyst from reaction mixtures, and the treatment for alkaline waste water, etc. [3]. Moreover, free fatty acid and water will interfere with the reaction to a great extent [1–3]. Recently, enzymatic approaches for biodiesel production have received much attention due to its moderate reaction conditions, lower ratio of alcohol to oil and easier product recovery process [1–4].

Methanol has been the most commonly used alcohol for biodiesel production. Compared with methanol, ethanol can be derived from biomass sources, such as wheat, sugar beet, corn, straw, and wood. Using ethanol as the acyl acceptor for biodiesel production is drawing an increasing interest [5–9], among which there are some literatures regarding using immobilized lipase-mediated ethanolysis for biodiesel preparation. Rosa et al. [5] investigated the continuous production of fatty acid ethyl esters from soybean oil in compressed carbon dioxide, propane and *n*-butane, respectively, using immobilized Novozym 435 as the catalyst. They found that in compressed propane, high reaction conversions of more than 90% could be obtained. Moreira et al. [8] dealt with the transesterification of palm oil with ethanol in a solvent free system and the best performance was attained with the lipase from *Pseudomonas fluorescens*.

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Compared to immobilized lipase, free lipase is easier to prepare with a much lower preparation cost, offering an alternative approach to enzyme-catalyzed biodiesel production. In this paper, free lipase NS81006-mediated ethanolysis for biodiesel production was explored for the first time in a biphasic water–oil system. The effects of temperature, stirring rate, water content, molar ratio of ethanol to oil, ethanol adding strategy and repeated use of the free lipase were systematically investigated.

Materials and Methods

Materials

Free lipase NS81006 (lipase activity 3,270 LU/ml) from the genetically modified *Aspergillus niger* microorganism, was a generous gift from Novo Industries (Denmark). One unit of lipase activity (LU) is the amount of lipase that catalyzes the hydrolysis of triglyceride of butyric acid into 1 μmol butyric acid per minute. Heptadecanoic acid methyl ester was purchased from Sigma-Aldrich (St. Louis, MO) and was chromatographically pure.

General Procedure of Free Lipase-Mediated Ethanolysis for Biodiesel Preparation

The enzymatic process was conducted in a 250-ml three-neck round-bottom flask which was equipped with a stirrer and immersed in a thermostat water bath. The reaction mixtures comprised 100 g soybean oil, 1.5 ml free lipase NS81006 and some amount of water, and ethanol specified in the reaction conditions. Samples were taken from the reaction mixtures at specified times and centrifuged to obtain the upper layer for GC analysis. Ethanol was added stepwise at intervals of 0.5 h from 0 to 4 h unless otherwise specified. All experiments were carried out in duplicate and there was no statistical difference in the measurements.

Analytical Analysis

The ethyl esters contained in the reaction mixture were analyzed using a GC-14B gas chromatography (Shimadzu, Kyoto, Japan) equipped with a CP-FFAP CB capillary column (25 m × 0.32 mm × 0.30 μm) supplied by Agilent. The resultant upper layer (50 mg) and 1 ml of 10 mg/ml heptadecanoic acid methyl ester (served as the internal standard) were mixed thoroughly for gas chromatography analysis. The column temperature was kept at 180 °C for 0.5 min, heated to 250 °C at 10 °C/min., and then maintained for 6 min. The temperatures of the injector and detector were set at 245 °C and 250 °C, respectively. Biodiesel yield (ethyl ester%, EE%) was calculated as the percentage of the actual amount of ethyl esters detected in the reaction process divided by the theoretical quantity of ethyl esters.

Results and Discussion

Effect of Temperature on NS81006-Mediated Ethanolysis for Biodiesel Production

The effect of temperature on soluble lipase NS81006-catalyzed preparation of ethyl esters in the biphasic water–oil systems was investigated (Fig. 1). The ethyl ester yield increased as temperature increasing from 35 to 45 °C; while after the temperature exceeded 45 °C, a decrease in biodiesel yield was observed. The influence trend of temperature was generally in accordance with earlier researches with methanol as the acyl acceptor for biodiesel production [3, 4].

Effect of Stirring Rate on Reaction Rate and Biodiesel Yield

It has been found that free lipase NS81006 can perform alcoholysis in water/oil biphasic system and interaction interface is very critical for soluble lipase-mediated methanolysis [2, 10, 11]. Herein, effect of stirring rate on soluble NS81006-mediated ethanolysis for biodiesel production was explored and the related results were presented in Fig. 2.

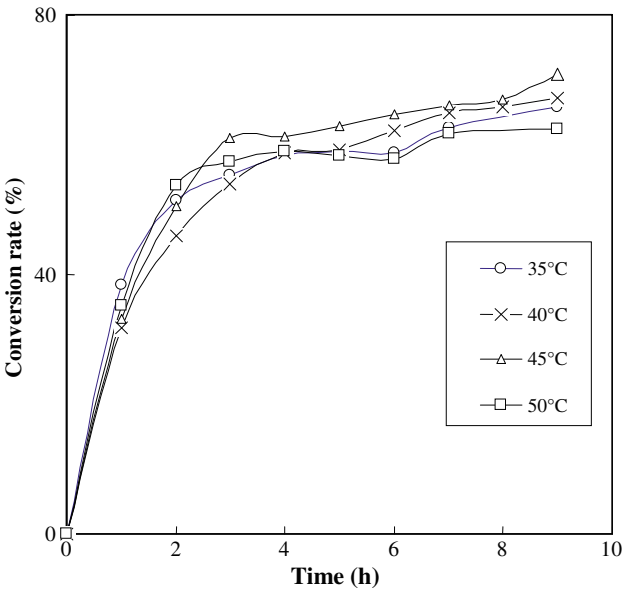

Fig. 1 Effect of temperature on soluble lipase-mediated ethanolysis for biodiesel. Reaction conditions: 1,000 rpm, the mole ratio of ethanol to oil of 4.5, stepwise ethanol adding performed with 11.5, 8.7, 5.8 and 3.6 ml at 1 h interval

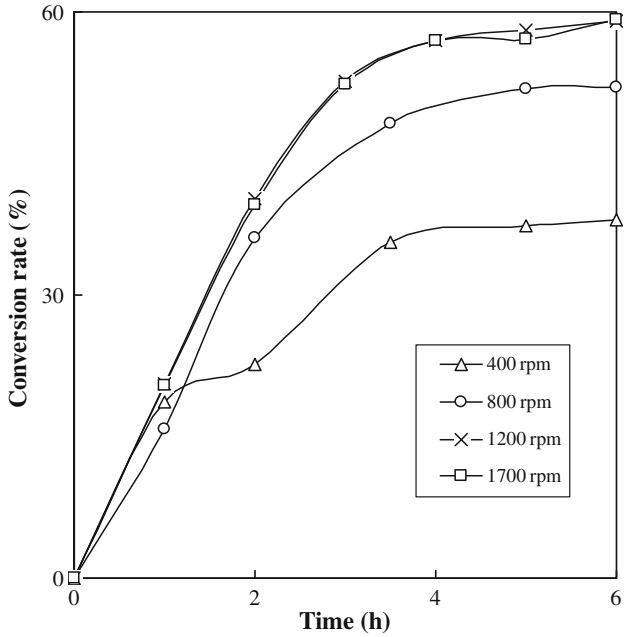

Fig. 2 Effect of stirring rate on soluble lipase-mediated ethanolysis for biodiesel. Reaction conditions: 45 °C, water content 10% by weight of soybean oil and mole ratio of ethanol to oil of 5.0

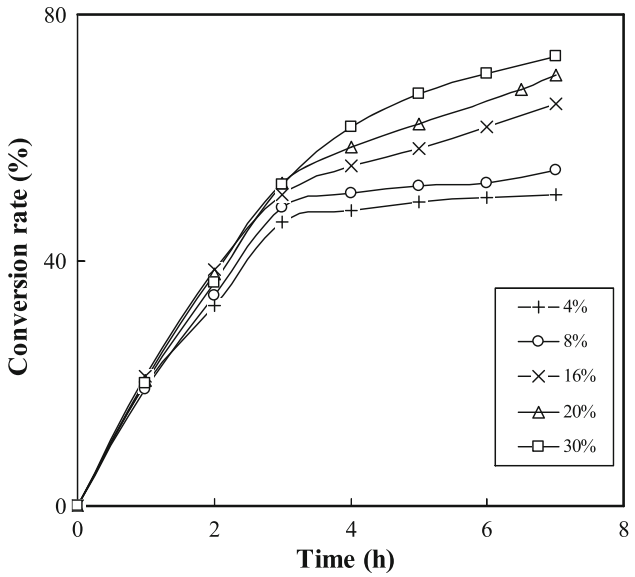

Fig. 3 Effect of water content on soluble lipase-mediated biodiesel production. Reaction conditions: 45 °C, 1,200 rpm, mole ratio of ethanol to oil of 5.0

It could be noted that the reaction rate was accelerated with the increasing of stirring rate until it reached 1,200 rpm. Further increments in the stirring rate had negligible impact on the reaction rate and biodiesel yield.

Effect of Water Content on NS81006-Mediated Ethanolysis

With methanol as the acyl acceptor for soluble lipase-catalyzed biodiesel production, it was demonstrated that a certain amount of water present in the system plays key role in the maintenance of lipase activity [2, 10].

The effect of water content on soluble lipase NS81006-mediated ethanolysis was shown in Fig. 3. Interestingly, it was found that there was not much difference in biodiesel yield in first 3 h reaction with water content ranging from 4 to 30 wt%. While after 3 h reaction, the biodiesel yield increased to a varied extent with different initial water content.

For the system with a water content ranging from 4 to 8 wt%, no obvious increase in biodiesel yield was observed after 3 h of reaction, which probably resulted from the deactivation of the lipase caused by the accumulated excessive ethanol in limited water-containing system, which was confirmed by further analysis of lipase activity (data not shown).

Nevertheless, with the water content ranging from 8 to 30 wt%, the biodiesel yield increased with the increment of water content, which was different from the results with methanol as the acyl acceptor for biodiesel production, where 10 wt% was optimal for achieving a high methyl ester yield [2, 10, 11].

Effect of Molar Ratio of Ethanol to Oil on NS81006-Mediated Biodiesel Production

Excessive methanol was found to easily deactivate the enzyme in either immobilized lipase or free lipase-mediated process for biodiesel production [5–11]. Here the effect of molar ratio of ethanol to oil on free lipase NS81006-mediated ethanolysis for biodiesel production was explored (Fig. 4).

It was found that with the enhancement of the molar ratio of ethanol to oil, the initial reaction rate increased to a varied extent. While after 3 h reaction, the ethyl esters with molar ratio of ethanol to oil over 5.0 were even much lower than those with lower ethanol to oil molar ratio.

Optimization of Ethanol Adding Strategy

The adding strategy of acyl acceptor might have pronounced influence on the reaction rate as well as the final biodiesel yield [5–10]. Considering too much ethanol present in the system would deactivate lipase seriously, different ethanol addition strategies were compared systematically which were based on the consumption rate of ethanol as well as its minimal effect on lipase's activity (Table 1). It could be seen from Table 1 that the molar ratio of ethanol to oil had obvious influence on the final biodiesel yield. With molar ratio of ethanol to oil being

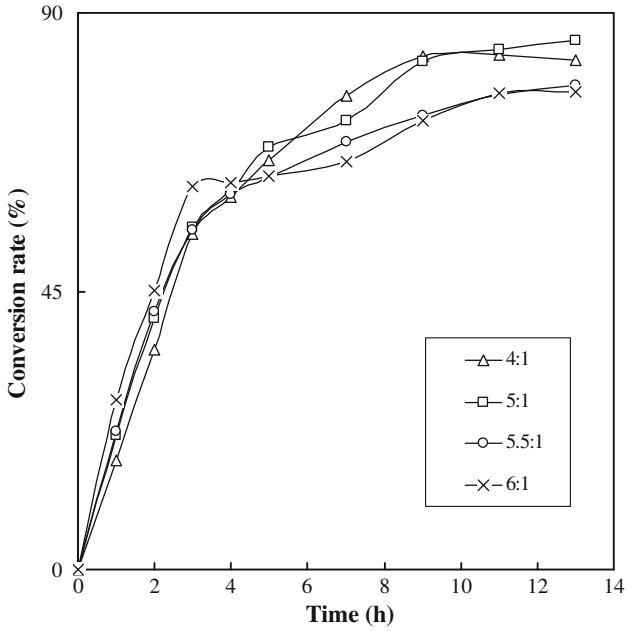

Fig. 4 Effect of molar ratio of ethanol to oil on soluble lipase-mediated biodiesel production. Reaction conditions: 45 °C, 1,200 rpm, water content of 20% by weight of oil

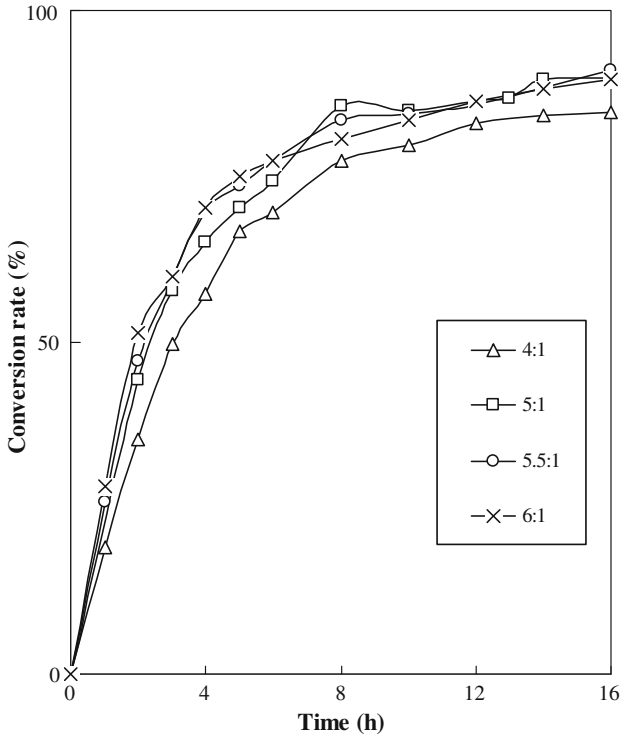

Fig. 5 The change of ethyl ester concentration with optimized ethanol addition strategy with varied molar ratio of ethanol to oil (water content 20%)

increased from 4.0 to 5.5, the final biodiesel yield increased to a varied extent. However, further increase of molar ratio of ethanol to oil would not further enhance the biodiesel yield.

An optimized ethanol addition approach was further proposed with molar ratio of ethanol to oil of 5.5 as follows: ethanol of 4.03 ml at intervals of 0.5 h from 0 to 2 h, ethanol of 4.03 ml at intervals of 1 h from 2 to 3 h and ethanol of 4.03 ml at intervals of 1.5 h from 3 to 7.5 h. The corresponding results with water content of 20 wt% were shown in Fig. 5.

Compared to the results presented in Fig. 3, it could be seen that with the optimized ethanol addition strategy, much better results could be obtained with a biodiesel yield of around 90% at 8 h.

Reuse of Free Lipase

It was found that most of lipase accumulated in the water phase after the reaction completed and the phase separation could be easily performed by the natural gravity force. The reuse of the free lipase was realized though the directly repeated use of the whole water phase. Just as indicated in Fig. 6, the results showed the biodiesel yield could remain

Table 1 Effect of different ethanol addition strategy

Molar ratio of ethanol to oil	4.0:1	5:0:1	5.5:1	6.0:1
Ethanol addition (ml)	2.93 (0 h)	3.66 (0 h)	4.03 (0 h)	4.39 (0 h)
	2.93 (0.5 h)	3.66 (0.5 h)	4.03 (0.5 h)	4.39 (0.5 h)
	2.93 (1 h)	3.66 (1 h)	4.03 (1 h)	4.39 (1 h)
	2.93 (1.5 h)	3.66 (1.5 h)	4.03 (1.5 h)	4.39 (1.5 h)
	2.93 (2 h)	3.66 (2 h)	4.03 (2 h)	4.39 (2 h)
	2.93 (2.5 h)	3.66 (2.5 h)	4.03 (3 h)	4.39 (3 h)
	2.93 (3 h)	3.66 (4 h)	4.03 (4.5 h)	4.39 (4.5 h)
	2.93 (4 h)	3.66 (5 h)	4.03 (6 h)	4.39 (6 h)
	2.93 (5 h)	3.6 (6 h)	4.03 (7.5 h)	4.39 (7.5 h)
Conversion rate (%, 16 h)	84.6	89.9	91.1	89.6

Reaction conditions: 1,000 rpm, 45 °C, water content 20% by weight of soybean oil

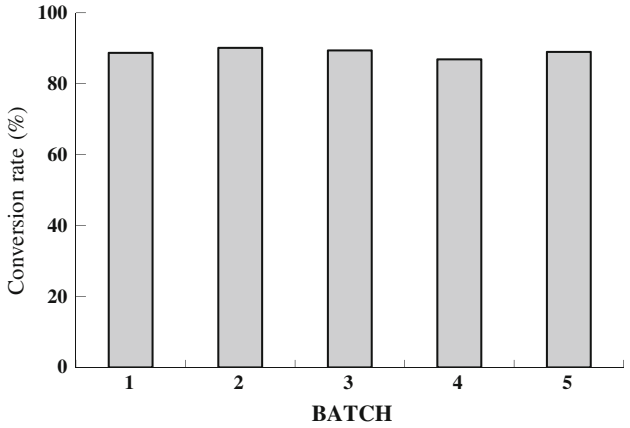

Fig. 6 Study on the reusability of soluble lipase for ethyl ester production

at around 90% after five times repeated use of the whole water phase, which indicated that the simple direct use of the whole water phase containing lipase might be very promising for the practical use of free lipase for biodiesel production.

Compared to immobilized lipase-mediated biodiesel production [1, 3–9], free lipase has the advantages of lower production cost and faster reaction rate, showing the greater prospect in the field of biodiesel production especially by taking the rather easy recovering the lipase for repeated use into consideration just as demonstrated in this study.

Conclusion

Free lipase NS81006-mediated biodiesel production was studied systematically with ethanol as the acyl acceptor in a biphasic aqueous-oil system. It was found that temperature, stirring rate, water content, molar ratio of ethanol to oil and ethanol adding strategy had varied influence on the reaction rate and final biodiesel yield. A biodiesel yield of over 90% could be obtained at the optimized reaction conditions with temperature of 45 °C, stirring rate of 1,200 rpm, water content of 20 wt% and ethanol to oil mole ratio of 5.5 with the proper adding strategy of 4.03 ml ethanol at intervals of 0.5 h from 0 to 2 h, 4.03 ml ethanol at intervals of 1 h from 2 to 3 h and 4.03 ml ethanol at intervals of 1.5 h from 3 to 7.5 h The free lipase could be repeatedly used by simple separation of the water phase from the oil phase and there was no obvious loss in lipase activity after five repeated uses, which indicated that free lipase might have a great prospect in biodiesel production especially by taking its easier preparation and lower production cost into consideration.

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