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# Free lipase-catalyzed biodiesel production from phospholipids-containing oils

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ABSTRACT

Free lipase-catalyzed biodiesel has drawn more and more attentions in recent years because of its advantages of lower cost and faster reaction rate. Utilizing free lipase to convert low quality oils such as crude vegetable oils and microbial oils is beneficial to further reduce the cost of biodiesel production. However, these oils typically contain some amount of phospholipids. Phospholipids were found to affect the lipase-catalyzed process and further influence the enzyme's thermal stability in biodiesel production process. In this work, free lipase NS81006-mediated biodiesel production from oils containing phospholipids at varied temperature was investigated systematically. It was found that the presence of phospholipids at high temperature led to a decreased fatty acid methyl esters (FAME) yield and poor reuse stability of the lipase during NS81006-catalyzed biodiesel production process. The higher the temperature was, the greater negative effect was observed. This inhibitory effect was found to be mainly caused by the coexistence of phospholipids and methanol in the system. Based on this finding, a novel two-step enzyme-mediated process was further developed, with which the above-mentioned inhibitory effect was eliminated, and a FAME yield of 95.1% could be obtained with oils containing 10% phospholipids even at high temperature of 55 °C.



## 1. Introduction

As a renewable and clean bioenergy, biodiesel has been attracting great interest worldwide [1,2]. So far, one of the most critical challenges for the commercialization of biodiesel is the cost of oil feedstock, which accounts for more than 85% of the total manufacturing cost [3,4]. One possible solution is to use crude vegetable oils directly to reduce the cost of refining. However, considering the world-wide food shortage, the use of vegetable oils for biodiesel production is still questionable. Recently, the development of microbial oil is expected to offer new opportunities to solve the food versus fuel controversy [5]. Exploring the feasibility of converting these oils into biodiesel is of great significance. However, comparing with refined oil, crude vegetable oils and microbial oils have some impurities, one important of which is phospholipids. For example, the mass fraction of phospholipids in crude soybean oil may range from 1.1% to 3.5% [6]. In addition, much higher mass fraction of over 10% phospholipids was reported in some microbial oils [7,8].

Recently, much attention has been paid to adopt lipase as biocatalysts for biodiesel production due to its mild reaction condition and environmental friendly process [9–11]. When using lipase to catalyze the transesterification of crude vegetable oils or microbial oils, the influence of phospholipids

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should also be considered. The effect of phospholipids on immobilized lipase-catalyzed biodiesel production has already been discussed in many papers. It is reported that 0.2–2% phospholipids contained in the oil feedstock have negative effects on immobilized lipase's catalytic performance as well as its reuse stability [12–14]. This inhibitory effect may result from the binding of phospholipids with immobilized lipase/carrier, thereby affecting the interaction between substrates and the active site of the enzyme [12]. Compared with immobilized lipase, free lipase has the merits of lower preparation cost and faster reaction rate, offers a promising approach for biodiesel production and has drawn increasing attentions in recent years [15–17]. Different from immobilized lipases, in our previous study, the presence of phospholipids (within 2% mass fraction of the dry oil) was found to have positive effect on free lipase catalyzed biodiesel production due to the better emulsification with phospholipids [18]. On the other side, the presence of phospholipids may further affect the thermal stability of lipase. Patel et al. [19] reported that during the hydrolysis of milk fat with the presence of phospholipids, the optimum temperatures of *Rhizopus javanicus* lipase and *Candida rugosa* lipase both increased by about 20 °C. In this work, free lipase NS81006-mediated biodiesel production from oils containing phospholipids at varied temperature was investigated for the first time and the possible influencing mechanism was also explored.

## 2. Materials and methods

### 2.1. Materials

Free lipase NS81006 from the genetically modified *Aspergillus niger* (lipase activity 3300 LU $cm^{-3}$) was kindly provided by Novo Industries (Denmark). One unit of lipase activity (LU) is defined as the amount of lipase that produces 1 μmol free fatty acid per minute. Heptadecanoic acid methyl ester as GC standard and phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC) as HPLC standards were chromatographically pure and purchased from Sigma–Aldrich (St. Louis, MO). Soybean phospholipids (containing 19.84% PE, 20.96% PC, 4.03% LPC, 13.2% phosphatidylinositol, 7.5% phosphatidic acid) were purchased from a local company. All other chemicals and solvents were obtained commercially of analytical grade.

### 2.2. Enzyme catalysis procedure

#### 2.2.1. Free lipase-catalyzed methanolysis for biodiesel production

The free lipase-catalyzed methanolysis process was conducted in a 500 $cm^3$ three-neck round-bottom flask equipped with a stirrer and immersed in a thermostat water bath of 45–50 °C. The reaction mixture comprised soybean oil (100 g), soybean phospholipids (2–10 wt%, based on dry oil weight), water (10 g), methanol (20 $cm^3$, the mole ratio of methanol/oil is 4.4:1) and free lipase NS81006 (1.5 $cm^3$). The reaction was performed under the stirring speed of 20 Hz for 8 h. The addition of methanol followed a stepwise strategy: 35%, 20%, 15%, 15%, 10% and 5% of the total methanol was added into the reaction mixture at 1 h interval from 0 h to 5 h. Samples were taken from the reaction mixture at specified times and then centrifuged to get the upper layer for gas chromatography analysis. All determinations were carried out in duplicates.

#### 2.2.2. Free lipase-catalyzed hydrolysis of phospholipids-containing oil

The hydrolysis of phospholipids-containing oil catalyzed by free lipase NS81006 was conducted following the same procedures of methanolysis without the addition of methanol. Samples were taken at specified times and then centrifuged to get the upper layer for acid value analysis.

### 2.3. Sample analysis

#### 2.3.1. GC analysis of FAME

The fatty acid methyl esters (FAME) contained in the reaction mixture was detected by Agilent 7890A GC system (Agilent Technologies, Santa Clara, USA) equipped with a CP-FFAP CB capillary column (25 m × 0.32 mm × 0.30 μm, Agilent Technologies, USA). The carrier gas (nitrogen) was maintained at a constant pressure of 100 kPa. Heptadecanoic acid methyl ester was used as the internal standard. 50 mg of the oil layer and 600 $mm^3$ of 700 g $m^{-3}$ heptadecanoic acid methyl ester (ethanol as the solvent) were mixed thoroughly. The resultant mixture of 1 $mm^3$ was injected for analysis. The initial column temperature was set at 180 °C and held for 0.5 min, then heated to 250 °C at the rate of 10 K $min^{-1}$ and maintained for 6 min. Injector and detector temperatures were set at 245 °C and 250 °C, respectively. FAME yield was calculated as the percentage of the actual amount of methyl esters detected in the reaction process divided by the theoretical quantity of methyl esters from soybean oil.

#### 2.3.2. HPLC analysis of phospholipids

After the completion of reaction, all the reaction mixture was heated at 80 °C for 10 min to deactivate the enzyme, then transferred into a tap funnel and left to settle to separate the oil phase and water phase for phospholipids determination. The HPLC analysis of phospholipids was conducted according to the procedure described in our earlier paper [18]. The HPLC elution profile of phospholipids is presented in Fig. 1.

#### 2.3.3. Determination of acid value in oil

Acid value is used to determine the percent of free fatty acid (FFA) in the oil, and it is defined as the amount of potassium hydroxide in mg required to neutralize the free acid in 1 g of the oil sample. The acid value in oil was determined according to Chinese national standard GB5530-2005. In a typical procedure, 0.05–0.1 g of oil sample was weighted into a 250 $cm^3$ flask, followed by adding 25 $cm^3$ of anhydrous ethanol, which was pre-neutralized by KOH standard solution. After mixing, 2 drops of phenolphthalein indicator was added, and then the mixture was titrated with KOH standard solution until a pink color which persists for 30 s can be observed. The acid value can be calculated according to the following equation:

$$\text{Acid Value} = \frac{V \times N \times 56.1}{m}$$