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Exploration on the effect of phospholipids on free lipase-mediated biodiesel production

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ABSTRACT

Free lipase-mediated biodiesel production has been drawing great attention for its lower cost and faster reaction rate compared to immobilized lipase. Since biodiesel derived from vegetable oils may cause competition with food supply, the exploration of crude vegetable oils as well as microbial oils as the feedstock has aroused great interest worldwide. It is reported that those crude oils usually contain some amount of phospholipids, typically with content varying from 2% to 10%. Our previous study showed that phospholipids content within 2% in the oil feedstock was even beneficial to free lipase NS81006-catalyzed biodiesel production. However, there was no research about the influence of much higher phospholipids content on free lipase-mediated methanolysis for biodiesel production. In this paper, the effect of varied content of phospholipids on free lipase NS81006-mediated biodiesel production as well as the related mechanism was investigated for the first time. It was found that both the catalytic performance and reuse stability of free lipase NS81006 were inhibited with more than 5% phospholipids contained in the oil feedstock. Exploration on the related influence mechanism revealed that either phospholipids or methanol (with appropriate addition strategy) alone did not have negative effect on the catalytic performance of the lipase, while the coexistence of phospholipids and methanol in the system led to significant inhibitory effect on the lipase. The further exploration indicated that with the increase of phospholipids content, the amount of solubilized methanol gradually increased in the micelle, which may subsequently result in negative effect on the enzyme's catalytic performance.

1. Introduction

Nowadays, the need to search for alternative sources of energy has been greatly raised due to concerns over the fast declining supply of petroleum reserves, increasing threat to the environment and continuous global warming. Biodiesel, namely methyl or ethyl esters of fatty acids, can be used as an alternative fuel to reduce the use of petroleum diesel in existing engines [1]. Biodiesel is a sustainable clean bioenergy as it can be derived from natural and renewable feedstock such as vegetable oils, animal fats and microbial oil [2,3]. Basically, biodiesel can be produced through either chemical or enzymatic pathways. In recent years, the employment of lipase as biocatalyst has drawn worldwide attentions due to its distinctive merits such as lower oil quality requirement, mild reaction condition and waste-free process [4–6]. Compared with commonly used immobilized lipase, the utilization of free lipase with lower cost is recently undergoing a rapid development [7–9]. It was reported that free lipase NS81006 can efficiently catalyze the alcoholysis of refined soybean oils for biodiesel production with faster reaction rate and a final biodiesel yield of more than 90% can be obtained [10,11].

At present, the main bottleneck for the commercialization of biodiesel is the high cost of raw materials, which account for over 85% of the total operating cost [12,13]. In recent years, the utilization of crude vegetable oils, which refers to non-degummed oils, provides a potential approach to reduce biodiesel production cost by saving the expense on degumming [14]. However, due to lack of degumming, impurities such as phospholipids still exist in the crude oil. For example, the phospholipids content in the crude soybean oil may range from 1.1% to 3.5% [15]. Another alternative oil source for the production of biodiesel is through oleaginous microorganisms, which are capable of accumulating more than 20–25% lipids [16]. Except triglycerides, other lipids represented by phospholipids also exist in these oils. Compared with crude vegetable oils, the phospholipids contents of microbial oils are much higher. For example, lipids extracted from some fungi or algae are reported to compose more than 10% phospholipids [17,18].

The presence of phospholipids in oil feedstock has been found to affect the lipase activity. For instance, more than 0.5%

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phospholipids contained in the oil would result in a reduced reaction rate and biodiesel yield during immobilized lipase-mediated biodiesel production, and the presence of 1% phospholipids could even cause the irreversible deactivation of immobilized lipase in some reactions [19–21]. However, as to free lipase, our previous study showed that low amount of phospholipids (within 2%) contained in the oil feedstock even has positive effect on the catalytic performance of free lipase NS81006 in methanolysis process [22]. To further promote the application of free lipase in biodiesel production, the effect of varied content of phospholipids on NS81006-mediated methanolysis for biodiesel production was investigated systematically for the first time in this paper. Besides, the possible influence on the mechanism was also explored.

2. Materials and methods

2.1. Materials

Free lipase NS81006 from the genetically modified *Aspergillus niger* (lipase activity 3300 LU/ml), and free phospholipase A1 Lecitase Ultra from the genetically modified *Aspergillus oryzae* (phospholipase activity 12,000 LU/ml) were both kindly donated by Novozymes (Denmark). One unit of lipase/phospholipase activity (LU) is defined as the amount of lipase/phospholipase 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 provided by 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

Phospholipids-containing oils were prepared by adding certain amount of soybean phospholipids into the refined soybean oil. The enzymatic process was performed in a 500 ml three-neck round-bottom flask equipped with mechanical stirrer in a water bath at 55 °C. The reaction mixture contained 100 g soybean oil, soybean phospholipids (2–10 wt%, based on oil weight), 10 g water, 20 ml methanol (the mole ratio of methanol/oil is 4.4:1) and 1.5 ml free lipase NS81006. The reaction was conducted under stirring speed of 1200 rpm. Methanol was added stepwise and the addition strategy was as follows: 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. Preparation of phospholipids degradation product

The degradation of phospholipids was performed at 45 °C in the above mentioned three-neck round-bottom flask with stirring at 1000 rpm.The reaction mixture contained 100 g soybean oil, soybean phospholipids (2–10 wt%, based on oil weight), 10 g water, and 0.5 ml free phospholipase A1 Lecitase Ultra. After 5 h reaction, the resultant mixture was prepared for HPLC analysis of phospholipids.

2.2.3. Free lipase-catalyzed hydrolysis of phospholipids-containing oil

The hydrolysis of phospholipids-containing oil catalyzed by free lipase NS81006 was conducted following the procedures for methanolysis without the addition of methanol. Samples were taken from the reaction mixture 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). Heptadecanoic acid methyl ester was served as the internal standard. 50 mg of the upper layer and 0.6 ml of 0.7 mg/ml heptadecanoic acid methyl ester (ethanol as the solvent) were mixed thoroughly. The resultant mixture of 1 μl 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 °C/min and maintained for 6 min. Injector and detector temperatures were set at 245 °C and 250 °C, respectively.

2.3.2. HPLC analysis of phospholipids

The phospholipids components of PE, PC and LPC in the reaction mixture were analyzed by a Shimadzu 20A HPLC system (Shimadzu Corp., Kyoto, Japan) fitted with an ELSD-LTII low temperature-evaporative light scattering detector. Agilent Prep Sil Scalar column (4.6× 150 mm, 10 μm) (Agilent Technologies, USA) was used for the separation and controlled at 37 °C. The mobile phase consisted of solvent A (*n*-hexane/isopropanol, 3:4, v/v) and solvent B (*n*-hexane/isopropanol/water, 3:4:0.75, v/v/v), and it was pumped with a gradient elution program at the rate of 1.2 ml/min. The drift pipe temperature was controlled at 40 °C and the nitrogen pressure was controlled at 340 kPa, respectively.

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. Before HPLC analysis, the oil phase and water phase were diluted to a proper concentration with solvent A and solvent B, respectively. After mixing thoroughly, the resultant mixture of 20 μl was injected for related analysis.

2.3.3. Determination of acid value in oil

The acid value in oil was determined according to Chinese national standard GB5530-2005. All assays were carried out in triplicate for the calculation of the mean value.

2.4. Micelle solution preparation and micelle size determination

Phospholipids micellar solution was prepared by mixing phospholipids with soybean oil. The methanol solubilization process of phospholipids micelles was conducted according to the method proposed by Edris and Abd El-Galeel [23]. Methanol was slowly titrated dropwise in the micellar solution. The whole solution was mixed in the shaker at 55 °C, 200r/min for 5 min. The titration was continued as long as the whole solution still remained clear and transparent after rest. The weight of the titrated methanol was determined continuously after each addition. The end point of titration was determined by the first drop of methanol that turns the solution turbid. Then the maximum amount of methanol solubilized in the system is determined and the titration process stopped. Finally, the phospholipids micellar solution with maximum solubilized methanol was subjected to size analysis.

The mean micelle size (hydrodynamic diameter) was measured by HORIBA SZ-100 (Japan) Dynamic Light Scattering Particle Size