Study on Reaction Parameters in Lipase-Catalyzed Methanolation of Plant Oil

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Biodiesel fuel, as fatty acid alkyl ester, which is produced by the transesterification of plant oil or animal fat sources with an alcohol, is an attractive alternative to petroleum-based fuel because it is produced from renewable resources. In this research, the enzymatic esterification of plant oil and methanol was studied. The reaction was catalyzed by commercial immobilized lipase from candida antarctica in solvent-free media. The influences of several reaction parameters, i.e. the amount of enzyme, molar ratio of methanol to oil and reaction temperature, on methanolation reaction were investigated. The inhibitory effect of undissolved methanol on lipase activity was eliminated by stepwise addition to the reaction mixture. The optimum conditions for the reaction were as follows: enzyme amount 4%, molar ratio of methanol to oil 3:1, and temperature 35 °C in three-step addition of methanol. The maximum methyl ester yield of 84.4% was obtained after 72 h of reaction at optimum conditions.

1. Introduction

Biodiesel fuel, as fatty acid alkyl ester, which is produced by the transesterification of plant oil/animal fat with an alcohol, i.e. alcoholysis, is an alternative to petroleum-based fuel because it is produced from renewable resources. Moreover, biodiesel is biodegradable, non-toxic, and has a low emission profile. Enzymatic production of biodiesel from plant oils has drawn a great attention because of some advantages over conventional chemical catalysis such as having mild operating conditions, high purity of the products, and eliminated environment pollution (Fukuda et al., 2001). In number of studies, enzymatic synthesis of fatty acid esters in presence of organic solvents has been proposed (Nelson et al., 1996). However, in order to avoid solvent separation, toxicity, flammability and costs of organic solvents, a solvent-free system would be the process of choice (Selmi and Thomas, 1998). Methanol is the most commonly used alcohol as

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the acyl acceptor in the transesterification reaction because of its low cost and high reactivity. Lipases from various sources, such as *Candida antarctica*, *Rhizopus oryzae*, *Rhizomucor miehei* and *Pseudomonas cepacia* have been used for esterification reaction (Kaieda et al., 1999, 2001; Noureddini et al., 2005; Soumanou and Bornscheuer, 2003). Several researchers have reported that the immobilized *C. antarctica* lipase can be effectively used for the enzymatic production of biodiesel (Hernandez-Martin and Otero, 2008).

The aim of the present study was to investigate the influence of several operating conditions such as the amount of enzyme, molar ratio of methanol to oil and reaction temperature on the methyl ester synthesis. Commercial immobilized lipase from *C. antarctica*, Novozym 435, was employed as the biocatalyst to perform the methanolysis reaction of canola oil in a solvent free system.

2. Materials And Methods

2.1 Materials

Immobilized lipase from *C. antarctica*, commercially known as Novozym 435, was kindly donated by Novo Nordisk (A.S., Denmark-Tehran office). Refined canola oil was purchased from Behshahr Industrial Co. (Tehran, Iran). According to the gas chromatography analysis, the fatty acid composition was: palmitic acid 4.8%, stearic acid 2.3%, oleic acid 60.9%, linoleic acid 22.6%, arachidic acid 0.6%, linolenic acid 7.3%, gadoleic acid 1.2% and behenic acid 0.3%. From this composition, an average molecular weight of 881.6 for the canola oil was determined. All other chemicals were of analytical grade.

2.2 Methanolysis reaction

Methanolysis reaction of canola oil was performed in a 30 ml screw-capped bottle with a working volume of 10 ml. The reaction mixture consisted of canola oil, methanol and immobilized lipase at the conditions of experiment. This mixture was incubated in a shaker at 130 rpm and at specified temperature for 72 h. After the end of reaction, the lipase enzyme was removed by decantation from the reaction mixture and glycerol as the lower phase, was separated from the mixture by centrifugation at 5000 rpm for 10 min. The methanolysis yield was measured based on the amount of methyl ester present in the upper phase of the reaction mixture.

2.3 Analysis of methyl esters

The yield of methyl esters contained in the reaction mixture was determined using $^1$H nuclear magnetic resonance (NMR) spectroscopy according to the method described by Gelbard et al. (1995). $^1$H NMR spectra were recorded at 500 MHz on a Bruker Avance DRX-500 spectrometer using CDCl$_3$ as the solvent.
3. Results And Discussion

3.1. Stepwise addition of methanol

The stoichiometric ratio of reactants for the transesterification reaction is normally 3 mole alcohol/1 mole triglyceride. The effect of methanol-addition manner to the reaction mixture on methyl ester yield was studied. As shown in Fig. 1, stepwise addition of methanol is more effective than one-step methanolysis reaction.

![Graph showing methyl esters yield (%) vs. number step addition of methanol](image)

*Fig. 1. Effect of number step addition of methanol on lipase-catalyzed methanolysis reaction. Reacting conditions: amount of enzyme 4%, temperature 35 °C, molar ratio of methanol to oil 3:1.*

If a stoichiometric amount of methanol was added at the beginning of the reaction, a low methyl ester yield (i.e. 43.7%) was obtained. However, the yield of reaction could be significantly increased to more than 84% when the same amount of methanol was added in a three-step to the reaction mixture. The short-chain alcohols, specially methanol may damage lipases on account of its intense polarity and the hyrophilic property. Because the immobilized lipase was irreversibly inactivated by contact with insoluble methanol which exists as drops in the oil, the enzyme activity could be significantly decreased (Shimada et al., 1999). We thus hypothesized that immobilized lipase was not inactivated at lower methanol concentrations. Therefore, subsequent experiments were thereafter carried out using a three-step addition of methanol to the reaction mixture, i.e. the first portion at the beginning of the reaction, the second and third portions after 24 and 48 h reaction time, respectively.

3.2. Enzyme quantity

To study the effect of the enzyme quantity on methyl ester formation, different quantities of immobilized lipase ranging from 1% to 6% were added to the starting reaction mixture. The obtained results are shown in Fig. 2.
Fig. 2. Effect of enzyme amount on lipase-catalyzed methanolysis reaction. Reaction conditions: temperature 35°C, molar ratio of methanol to oil 3:1, and three-step addition of methanol.

The yield of reaction increased with increasing the lipase amount and reached to 84.4% using 4% of immobilized lipase. However, further increase in lipase amount did not show any increase in methyl ester yield. It is concluded that at low enzyme loadings, enzyme quantity would be the rate-limiting factor of methanolysis reaction. This achievement is consistent with results reported by Modi et al. (2006; 2007) that maximum conversions for esterification of vegetable oils were obtained at enzyme dosage of more than 10%.

3.3 Molar ratio of substrates
Methanolysis reaction was performed using different substrate molar ratio of methanol to oil varying in the range 3–6. The results (Fig. 3) demonstrate that the yield of methanolysis reaction significantly decreases with increasing the methanol to oil molar ratio. The highest methyl ester yield of 84.4% was achieved at a methanol to oil molar ratio of 3:1, and decreased to 40.8% when the molar ratio of 6:1 was utilized. This is in agreement with the earlier observation that excessive methanol concentration lead to lipase enzyme inactivation. The addition of methanol more than stoichiometric amounts, exerts an inhibitory effect on enzyme performance. This could be due to the fact that the immiscible methanol was accumulated around the lipase structure including its active sites, reaching a concentration level sufficient to cause a denaturation of the protein. This phenomenon might lead to enzyme inactivation.
Fig. 3. Effect of methanol to oil molar ratio on lipase-catalyzed methanolysis reaction. Reactin conditions: temperature 35 °C, amount of enzyme 4%, and three-step addition of methanol.

3.4 Effect of temperature

The effect of temperature on the methanolysis reaction was determined in the range 25–55 °C. As shown in Fig. 4, the methyl esters yield initially increases with temperature from 25 to 35 °C and reaches its maximum at 35 °C. However, a further increase in temperature leads to decrease in the reaction yield. This decrease in methyl ester yield can be explained by enzyme thermal denaturation.

Fig. 4. Effect of temperature on lipase-catalyzed methanolysis reaction. Reaction conditions: amount of enzyme 4%, molar ratio of methanol to oil 3:1, and three-step addition of methanol.
4. Conclusions

The enzymatic transesterification of canola oil with methanol in a solvent free media is carried out using an immobilized lipase as catalyst. The influence of several operation conditions on the reaction yield was analyzed. The inactivation of the lipase enzyme was successfully avoided by addition of methanol in step by step manner. The optimum reaction conditions was achieved by setting the experiment with the amount of enzyme at 4%, molar ratio of methanol to canola oil 3:1, and temperature 35 ºC in three-step addition of methanol. The maximum methyl ester yield of 84.4% was obtained after 72 h reaction at optimum conditions.

5. References