APTEFF, 39, 1-212 (2008)
DOI: 10.2298/APT0839161O
UDC: 665.3:665.75:665.058.6
BIBLID: 1450-7188 (2008) 39, 161-169
Original scientific paper

LIPASE-CATALYZED BIODIESEL SYNTHESIS WITH DIFFERENT ACYL ACCEPTORS

Nevena D. Ognjanović, Svetlana V. Šaponjić, Dejan I. Bezbradica and Zorica D. Knežević

Biodiesel is an alternative fuel for diesel engine that is environmentally acceptable. Conventionally, biodiesel is produced by transesterification of triglycerides and short alcohols in the presence of an acid or an alkaline catalyst. There are several problems associated with this kind of production that can be resolved by using lipase as the biocatalyst. The aim of the present work was to investigate novel acyl acceptors for biodiesel production. 2-Propanol and n-butanol have a less negative effect on lipase stability, and they also improve low temperature properties of the fuel. However, excess alcohol leads to inactivation of the enzyme, and glycerol, a major byproduct, can block the immobilized enzyme, resulting in low enzymatic activity. This problem was solved by using methyl acetate as acyl acceptor. Triacetylglycerol is produced instead of glycerol, and it has no negative effect on the activity of the lipase.

KEYWORDS: Biodiesel, lipase, methanol, 2-propanol, *n*-butanol, methyl acetate

INTRODUCTION

Biodiesel (fatty acid methyl esters) is an alternative fuel for diesel engines that is environmentally acceptable. From an environmental point of view it shows clear advantages over conventional fuel: it comes from renewable sources, and hence does not contribute to new carbon dioxide emission, it is biodegradable, its combustion products have reduced levels of particulates, sulphur oxides, carbon oxides, nitrogen oxides, and therefore, significantly reduces pollution (1, 2). Also, biodiesel is becoming increasingly important because of diminishing petroleum reserves.

Conventionally, biodiesel is produced by transesterification of triglycerides and short chain alcohols, such as methanol, in the presence of an acid or an alkaline catalyst (3).

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There are several problems associated with this kind of production. Removal of catalyst, excessive energy requirements, recovery of glycerol, undesirable side reactions are the major drawbacks for such chemical process. These problems can be overcome by using lipase as biocatalyst. The usage of lipases allows mild reaction conditions and easy recovery of glycerol without purification or chemical waste production (4-6). Lipases extracted from different sources have been successfully used in the production of biodiesel. *Candida antarctica* B lipase, immobilized on the acrylic resin, commercially known as Novozym 435, has been by far the most commonly used enzyme for the production of biodiesel (7, 8).

The enzymatic transesterification has been performed in a solvent and solvent-free media by various immobilized lipases (7, 9). However, enzymatic syntheses of biodiesel in organic solvents are not suitable from the application viewpoint because of toxicity, flammability of solvent, damaging effects on the environment and consequential requirement for its removal. Thus, to make the enzymatic process competitive, enzymatic solvent-free systems are being developed (10).

One of the main problems of implementing enzymes in biodiesel synthesis is low stability of the enzyme in the presence of the excess alcohol. The main obstacle in using methanol as the substrate, as several researches have reported, is that high methanol concentration can lead to serious inactivation of the enzyme (11-13). In order to overcome these drawbacks a stepwise addition of methanol to the reaction medium has been proposed. However, operational stability of lipases in repeated cycles, in reactions with methanol, is not very high.

The aim of the present work was to investigate novel acyl acceptors in biodiesel production. Longer chain alcohols, 2-propanol and n-butanol have less negative effect on lipase stability, and they also improve low temperature properties of the fuel (1, 11). However, excess alcohol leads to inactivation of the enzyme, and glycerol, a major by product, can block the immobilized enzyme, resulting in low enzymatic activity (12). This problem was solved by using methyl acetate as acyl acceptor (13, 14).

MATERIALS AND METHODS

Materials

The commercial lipase from *Candida antarctica* B lipase, immobilized on the acrylic resin (Novozym 435), refined sunflower oil "Sunce" (Sunce a.d. Sombor, Serbia) and methanol, 2-propanol, *n*-butanol from Sigma (purity>99.8%, St. Louis, USA) were used as reactants in the enzymatic reaction. Methyl myristate was purchased from Fluka (Buchs, Switzerland) and used as an internal standard. All other chemicals were reagent-grade.

Enzymatic transesterification

Synthesis was carried out in a 100 ml stoppered flasks, as a three-step process in the reaction with three different alcohols: methanol, 2-propanol and *n*-butanol. The reaction mixture consisted of 5 g of oil, 3% of the enzyme based on oil weight and 6 molar equivalents of alcohol. The first portion of alcohol and whole amount of oil were added at the

start of reaction; the second portion of methanol after 10 h, while the third portion was added after 25 h, according to previously obtained results. The reaction was carried out for 50 h. The mixture was agitated on a shaker at 150 rpm at 45°C for 50 hours.

Enzymatic interesterification

The reaction mixture with methyl acetate consisted of 5 g of oil, 3% of the enzyme based on oil weight and methyl acetate. The reaction conditions were optimized by carrying out different sets of experiments with varying methyl acetate to oil molar ratio and reaction time. The reaction mixtures were agitated on a shaker at 150 rpm at 45°C.

Analysis of the samples

At the end of the reaction, the enzyme was separated out by filtration and the filtrate was washed with distilled water and hexane after transferring it to a separating funnel. The ester phase, diluted with hexane, was mixed with methyl myristate, which served as the internal standard. The methyl ester content in the reaction mixture was quantified by gas-chromatography using a GS Varian 3400, equipped with a fused silica capillary column (30m x 0.32mm x 0.1μm). The column temperature was held at 150°C for 2 minutes, then heated to 190°C at 4°C/min, and held at that temperature for 3 minutes, heated again to 250°C at 5°C/min, held at that temperature for 5 minutes, and then raised to 300°C at 4°C/min and maintained at this temperature for 2 min. The temperatures of the injector and detector were set at 320°C and 330°C, respectively. Methyl myristate served as the internal standard.

RESULTS AND DISCUSSION

Methanol is the most commonly used alcohol in the biodiesel production. Since any excess of methanol, existing as drops in the oil, could cause enzyme inactivation, a multi-step addition of methanol has been developed. Shimada et al, achieved conversion of over 90% in a three-step methanolysis system with immobilized *Candida antarctica* lipase (4). Similar method has been developed by several other researches, and high yields have been achieved. Our previous research has shown that the best results with methanol were achieved with 6:1 methanol to oil molar ratio. However, as noted before, the enzyme was apparently inactivated when high molar equivalent of methanol content was added (Fig. 1). Therefore, methanol was added stepwise to maintain the methanol content at the desired level. In our study the first portion of methanol and whole amount of oil were added at the start of reaction; the second portion of methanol after 10 h, while the third portion was added after 25 h. The reaction was carried out for 50 h. When the three-step addition of methanol was employed the yield of biodiesel was 97%. When the whole amount of methanol was included in the system the yield was below 10%. It is clear that excess methanol leads to enzyme inactivation. One-step addition of alcohol was carried out also in the reaction with 2-propanol and, although a higher yield was achieved (65%), it is still not economically acceptable.

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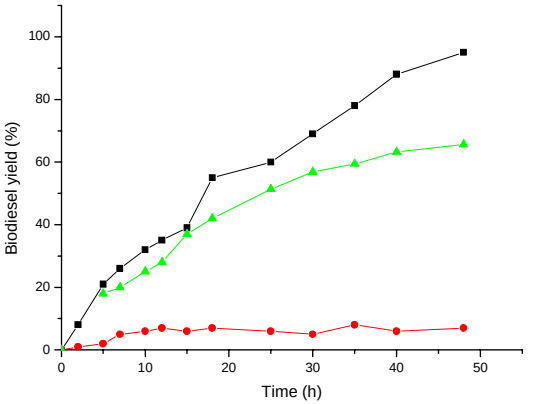

Figure 1. Effects of alcohols on biodiesel production. Reaction parameters: 45°C, 3% enzyme on oil weight, 50h, 6:1 alcohol to oil ratio;(■) three-step addition of methanol,(●) one-step addition of methanol, (▲) one-step addition of 2-propanol

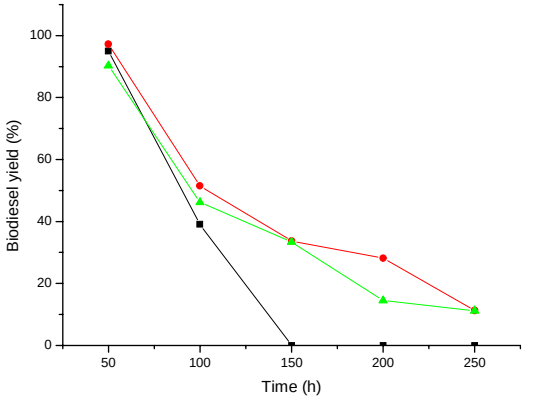

Figure 2. Operational stability of lipase. Reaction parameters 45°C, 3% enzyme on oil weight, 50h, 6:1 alcohol to oil ratio, (■)methanol, (●)2-propanol, (▲)*n*-butanol

Our study was focused on finding new acyl acceptors for the transesterification reaction. First, the attention was focused on branched and longer chain alcohols such as 2-propanol and *n*-butanol. Experiments showed that increase of the number of carbon atoms increased the cetane number as well as heat content of the fuel. Also, fatty acid esters of secondary or branched-chain alcohols can be used as fuel additives since they decrease the solidification point and, consequently, the high cloud point and pour point (1, 12). Operational stability of lipase was investigated in a three-step addition of alcohol, in a solvent-free system. The reaction time was 50 h, after which the enzyme is recycled and reused (Fig. 2). With all three acyl acceptors, a high initial yield was achieved. However, lipase exhibited poor activity during the repeated experiments. In the reaction

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with methanol, production was not detectable even after the third cycle. Operational stability of lipase in the transesterification reaction with 2-propanol and *n*-butanol was decreasing with time and after the third cycle, production of biodiesel was below 10%. This might be due to the inactivation effect caused by alcohol and the negative effect caused by byproduct glycerol adsorbed on the surface of the immobilized lipase. Byproduct glycerol is hydrophobic and insoluble in oil, so it is easily adsorbed onto the surface of the immobilized lipase, also exhibiting a negative effect on lipase activity and operational stability (15).

In the second stage of our study, a novel acyl acceptor for biodiesel production, methyl acetate, has been used. The usage of methyl acetate eliminates the risk of deactivation of enzyme by glycerol, since no glycerol is produced in the reaction. Triacetin, a byproduct in the interesterification reaction has no negative effect on lipase activity and has a greater value than glycerol, which makes this kind of production very promising. Triacetin can be used as a fuel additive, as an antiknock agent which can reduce engine knocking in gasoline, and to improve cold and viscosity properties of biodiesel (16, 17). Since methyl acetate has no negative effect on enzyme stability a stepwise addition of methyl acetate was not needed.

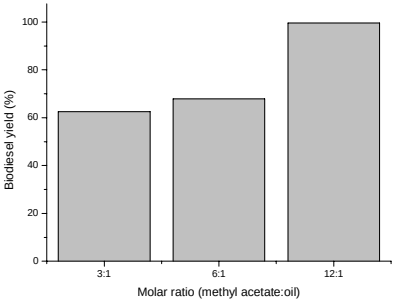

Figure 3. Effect of methyl acetate/oil molar ratio on biodiesel yield in interesterification.
Reaction parameters: 45°C, 3% enzyme based on oil weight

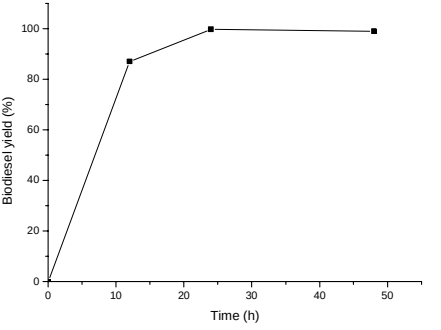

Figure 4. Time course of the formation of biodiesel during interesterification reaction.
Reaction parameters: 45°C, 3% enzyme based on oil weight, 12:1 methyl acetate to oil molar ratio

As first, the effect of substrate ratio on biodiesel production was determined (Fig. 3). The highest methyl ester yield was obtained at 12:1 molar ratio of methyl acetate to oil, at 45°C and 3% of enzyme (lipase from *C. antarctica)* based on oil weight. A large excess of methyl acetate was required in order to shift the interesterification in the forward direction. With these optimized reaction parameters, the optimized amount of methyl acetate was prepared by varying the reaction period. The yields of methyl esters were practically constant after 24 h, indicating an optimum reaction period of 24 h (Fig. 4).

With these reaction parameters yield of 99.6% biodiesel was obtained. Similar results were obtained by Du et al. (17). They have obtained a yield of 92% of methyl esters for 12 h, however, the amount of enzyme in their study was 30% based on oil weight.

The reusability of immobilized lipase over repeated cycles was analyzed with methyl acetate. The operational stability of lipase was found to be constant over seven repeated cycles (200 h) without losing its activity (yield of 95. 65 ± 2. 01%), whereas in the eight cycle the yield was lower, 63. 96%. However, the activity of immobilized enzyme from *C. antarctica* decreased rapidly when methanol was used for biodiesel production, and after the third cycle it was below 5% (Fig. 5). Lipases exhibited poor activity during the repeated experiment, probably, as noted before, due to the inactivation effect caused by methanol and the negative effect caused by byproduct glycerol adsorbed on the surface of the immobilized lipase.

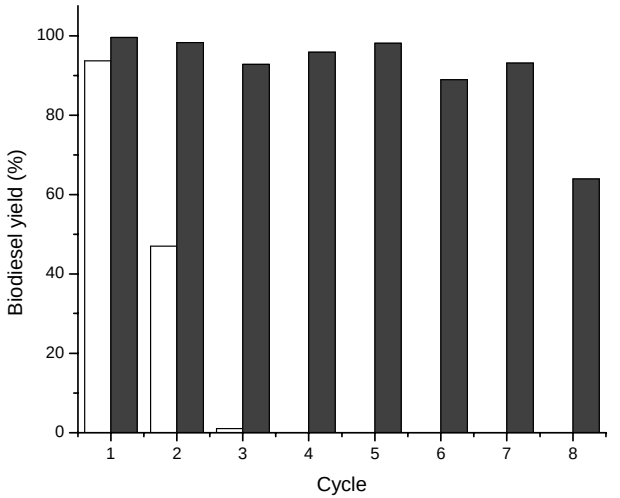

Figure 5. Operational stability of lipase in batch system. Reaction parameters: 45°C, 3% of the enzyme based on oil weight, (■) methyl acetate (12:1 molar ratio) , (□) methanol (6:1 molar ratio)

Based on these promising results, we have developed a packed-bed reactor for transesterification with methyl acetate as acyl acceptor. The packed reactors are the most frequent and the best production system which can minimize labor and costs for industrial application. The reactor was filled with immobilized lipase, under already mentioned conditions; working temperature was 45°C (the temperature was kept by recycling water from thermostat) and the molar ratio methyl acetate to oil was 12:1, according to previously obtained results in the batch system.

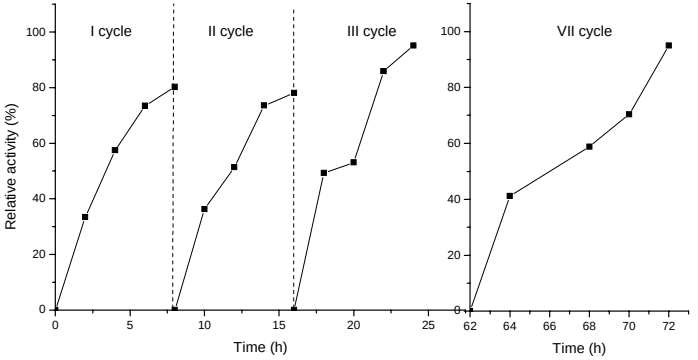

Figure 6. Operational stability in packed-bed reactor. Reaction parameters: 45°C, 3% of the enzyme based on oil weight, 12:1 methyl acetate to oil molar ratio

Operational stability of lipase was found to be constant over seven repeated cycles, for 74 h (Fig. 6). It can be observed that almost the same yield of biodiesel was obtained in the first, as in the seventh cycle. Results show that the enzyme did not lose its activity even in an excess of methyl acetate.

CONCLUSION

In this study, the use of different acyl acceptors in lipase-catalyzed biodiesel synthesis was studied. Short- chain alcohols such as 2-propanol and *n*-butanol have a less negative effect on lipase stability in comparison to traditionally used methanol, and they also improve low-temperature properties of the fuel. High yields can be achieved, but only with a stepwise addition of alcohol. Also, lipase exhibited poor activity during the repeated cycles. However, methyl acetate as novel acyl acceptor showed no negative effect on the enzymatic activity. The yield of 99.6% biodiesel was achieved at 45°C, 3% of lipase from *Candida antarctica,* 12:1 methyl acetate to oil molar ratio in a batch system. There was no loss detected in the enzymatic activity after seven repeated cycles. From the results it was concluded that methyl acetate could be a suitable acyl acceptor. The byproduct, triacetin, also has no negative effect on the activity of lipase and has a greater value than glycerol. Based on these results, a packed-bed reactor for interesterification with methyl acetate as acyl acceptor has been developed. Operational stability over repeated cycles showed that this process may be very promising for lipase-catalyzed large-scale production of biodiesel.

ACKNOWLEDGEMENT

This work was supported by the Ministry of Science and Technological Development of the Republic of Serbia (Project No TR 20064)

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ПРИМЕНА НОВИХ АЦИЛ АКЦЕПТОРА У ПРОЦЕСУ ЕНЗИМСКИ КАТАЛИЗОВАНЕ СИНТЕЗЕ БИОДИЗЕЛА

Невена Д. Огњановић, Светлана В. Шапоњић, Дејан И. Безбрадица и Зорица Д. Кнежевић

У овом раду испитана је могућност примене различитих ацил акцептора као полазних реактаната уместо метанола у синтези биодизела у циљу смањења инхибиције ензима, повећања његове стабилности и могућности значајног поједностављивања извођења процеса. Прво је испитана могућност коришћења различитих алкохола: 2-пропанол и n-бутанол који имају мањи утицај на денатурацију ензима. Осим тога, применом ових алкохола добија се гориво бољег квалитета, пошто се са повећањем броја угљеникових атома повећава октански број, температура паљења, као и садржај топлоте. Други део истраживања био је оријентисан ка испитивању могућности коришћења метилацетата као погодног нуклеофила. За разлику од алкохола, применом овог ацил акцептора омогућен је једностепени поступак синтезе биодизела, што значајно смањује време трајања реакције, као и комплексност исте. Такође, применом метилацетата повећава се стабилност биокатализатора, што омогућава вишекратну употребу. Наиме, у реакцији са метилацетатом као нуспродукт се не ствара глицерол већ триацетилглицерол, који нема негативан ефекат на стабилност липазе.

Received 14 July 2008
Accepted 27 October 2008