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Use of novel acyl acceptors in lipase-catalyzed biodiesel synthesis

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Introduction

Biodiesel (fatty acid methyl esters) is an alternative fuel for diesel engines that is environmentally acceptable. From an environmental point of view biodiesel 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 polution.[1,2]Also, biodiesel is becoming increasingly important due to 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] 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 resolved 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. *C. Antarctica* B lipase, immobilized on the acrylic resin, commercially known as Novozym 435, was by far the most commonly used enzyme for the production of biodiesel. [7-9]

The enzymatic transesterification has been performed in a solvent and solvent-free media by various immobilized lipases.[7,10] However, enzymatic synthesis 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.[11]

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 could lead to serious inactivation of the enzyme. In order to overcome these drawbacks a stepwise addition of methanol to the reaction medium has been proposed.[12-14] However, operational stability of lipases in repeated cycles, in reactions with methanol, isn't very high.

The aim of the present work was to investigate novel acyl acceptors for 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.[1,12] However,

excess alcohol leads to inactivation of enzyme, and glycerol, a major by product, could block the immobilized enzyme resulting in low enzymatic activity.[13] This problem was solved by using methyl acetate as acyl acceptor.[14,15] Triacetylglicerol (triacetin) is produced instead of glycerol, and it has no negative effect on the activity of the lipase and much higher value than glycerol. It is a by-product that has widespread application in food, feed, printing, tanning, cigarette, cosmetic, pesticide and pharmaceutical industries. Triacetin can also 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.

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) and methanol, 2-propanol, n-butanol from Sigma (purity>99.8%, St. Louis, USA) were used as reactants for 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 reaction with three different alcohols: methanol, 2-propanol and n-butanol. The reaction mixture consisted of 5g of oil, 3% of enzyme based on oil weight and 6 molar equivalent of alcohol. The first portion of alcohol and whole amount of oil were added at the start of reaction; the second portion of methanol was added after 10 h, while the third portion was added after 25 h, according to previously obtained results. The reaction was carried out for 50h. The mixture was agitated on a shaker at 150rpm at 45°C.

Enzymatic interesterification

The reaction mixture with methyl acetate consisted of 5g of oil, 3% of 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 period. The reaction mixtures were agitated on a shaker at 150 rpm at 45°C.

Analysis of the samples

The methyl ester contents in the reaction mixture were quantified using a GS Varian 3400, connected to 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 2min. 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. However, as noted before, the enzyme was apparently inactivated when high molar equivalent of methanol content were added (Figure 1.). Therefore, methanol was added stepwisely 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 was added after 10 h, while the third portion was added after 25 h. The reaction was carried out for 50h. 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 reaction with 2-propanol and although a higher yield was achieved (65%), it is still not economically acceptable.

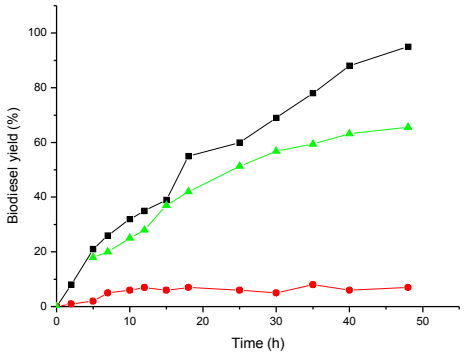

Figure 1. Effects of alcohols on biodiesel production. Reaction parameters: 45°C, 3% enzyme on oil weight, 50h, 6:1 alcohol to oil ratio;(■) three step addition of methanol,(●) one step addition of methanol, (▲) one step addition of 2-propanol

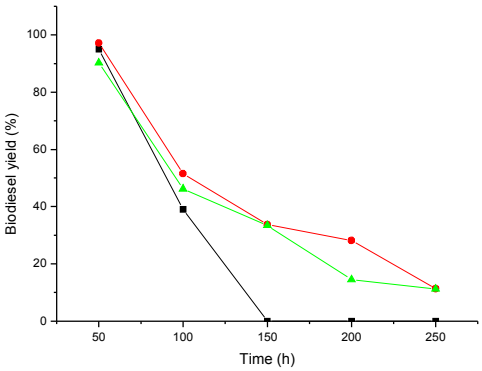

Figure 2. Operational stability of lipase. Reaction parameters 45°C, 3% enzyme on oil weight, 50h, 6:1 alcohol to oil ratio, (■)methanol, (●)2-propanol, (▲)n-butanol

Our study was focused on finding new acyl acceptors for the transesterification reaction. First, interest was focused on branched and longer chain alcohols such as 2-propanol and n-butanol. Research has shown that increase of carbon atoms increases 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 additives in fuel 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 (Figure 2). With all three acyl acceptors high initial yield was achieved. However, lipase exhibited poor activity during the repeated experiments. In reaction with methanol, production is non detectable even after the third cycle. Operational stability of lipase in transesterification reaction with 2-propanol and n-butanol is decreasing with time and after the third cycle, production of biodiesel is below 10%. This might be due to the inactivation effect caused by alcohol and the negative effect caused by by-product glycerol absorbed on the surface of the immobilized lipase. By-product glycerol is hydrophobic and insoluble in oil, so it is

easily adsorbed onto the surface of the immobilized lipase also leading to negative effect on lipase activity and operational stability.
In the second stage of our study, a novel acyl acceptor for biodiesel production, methyl acetate, has been developed. The usage of methyl acetate eliminates the risk of deactivation of enzyme by glycerol, since no glycerol is produced in the reaction. Triacetin, a by product in interesterification reaction has no negative effect on lipase activity and has greater value then glycerol, which makes this kind of production very promising. Since methyl acetate has no negative effect on enzyme stability a stepwise addition of methyl acetate wasn't needed.

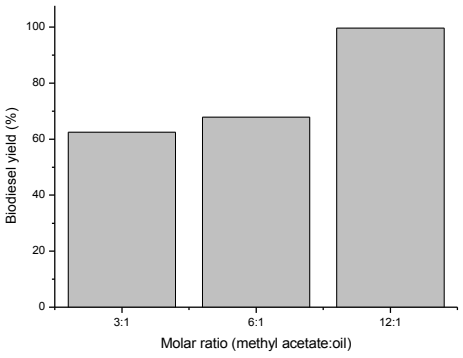

Figure 3. Effect of methyl acetate to oil molar ratio on biodiesel yield in interesterification Reaction parameters: 45°C, 3% enzyme on oil weight

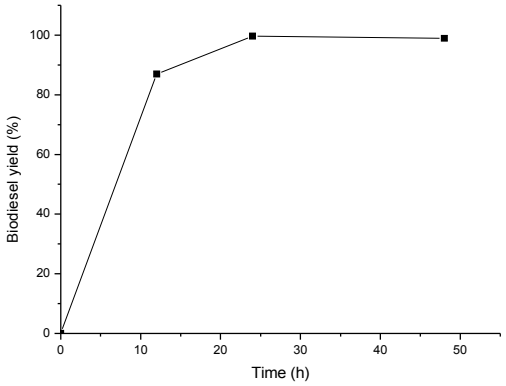

Figure 4. Time course of the formation of biodiesel during interesterification reaction. Reaction parameters: 45°C, 3%enzyme based on oil weight, 12:1 methyl acetate to oil molar ratio

First the effect of substrate ratio on biodiesel production was determined (Figure 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 *Candida antarctica)* based on oil weight. A large excess of methyl acetate was required in order to shift the interesterification in forward reaction. With these optimized reaction parameters, the methyl acetate were prepared by varying the reaction period. The yields of methyl esters were practically constant after 24h, indicating an optimum reaction period of 24h. With this reaction parameters yield of 99,6% biodiesel was obtained. Similar results were obtained by Due et al. They have obtained yield of 92% of methyl esters for 12h, however, the amount of enzyme in their study was 30% based on oil weight.[17]
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 (200h) without loosing 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 *Candida antarctica* deceased rapidly when methanol was adopted for biodiesel production and after the third cycle it is below 5% (Figure 5). Lipases exhibited poor activity during the repeated experiment, probably due, as noted before, to the inactivation effect caused by methanol and the negative effect caused by by-product glycerol absorbed on the surface of the immobilized lipase.

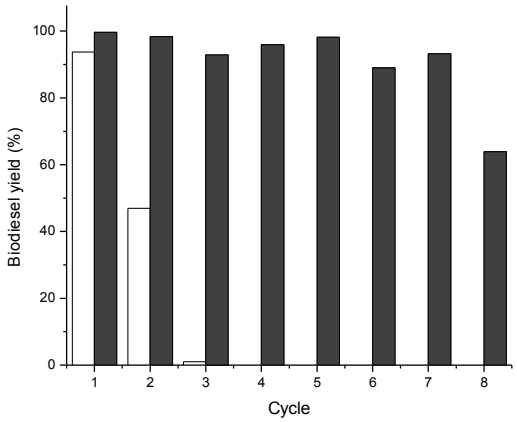

Figure 5. Operational stability of lipase in batch system. Reaction parameters: 45°C, 3% of enzyme based on oil weight, (■) methyl acetate (12:1 molar ratio) , (□) methanol (6:1 molar ratio)

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 form *Candida antarctica* 3% based on oil weight, 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 batch system..

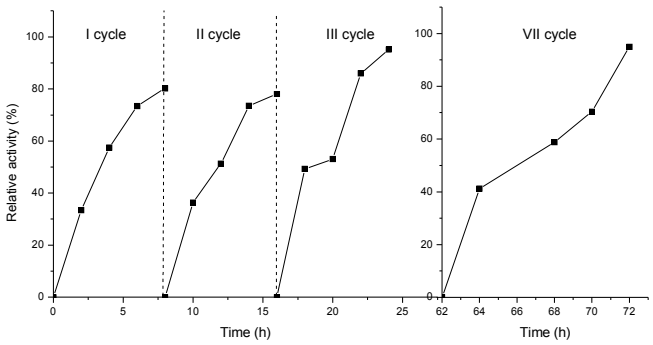

Figure 6. Operational stability in packed-bed reactor. Reaction parameters: 45°C, 3% of enzyme based on oil weight, 12:1 methyl acetate to oil molar ratio

Operational stability of lipase was found to be constant over seven repeated cycles, for 74h (Figure 6). It can be observed that almost same yield of biodiesel is obtained in first, as in seventh cycle. Results show that enzyme didn't loose 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 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 enzymatic activity. The yield of 99,6% biodiesel was achieved at 45°C, 3% of lipase from *Candida antarcticaI,*12:1 methyl acetate to oil molar ratio in a batch system. There was no loss detected in enzymatic activity after sever repeated cycles. From the results it was concluded that methyl acetate could be a suitable acyl acceptor. A by product, triacetin also has no negative effect on activity of the 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 shows that this process seems very promising for lipase-catalyzed large-scale production of biodiesel.

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