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BIODIESEL PRODUCTION FROM SUNFLOWER OIL WITH *CANDIDA ANTARCTICA* LIPASE B

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ABSTRACT. In this paper, we have evaluated the efficacy of an enzymatic method for the transesterification process of sunflower oil with methanol in the presence of *tert*-butanol. *tert*-Butanol was used as the reaction medium to eliminate both negative effect caused by excessive methanol and glycerol resulted as by product. Using the following reaction conditions: 1% Novozym 435 based on oil weight, methanol / oil molar ratio 6:1, *tert*-butanol/ oil volume ratio 6:1, the FAME reached its maximum level after 3h, with a value of 78.6% (m/m).

Keywords: *biodiesel, tert-Butanol, Novozym 435, enzymatic transesterification*

INTRODUCTION

Biodiesel, having a chemical structure of fatty acid alkyl esters (usually fatty acid methyl ester, FAME), has recently become an alternative to petroleum-based diesel fuel. It is renewable, biodegradable, non-inflammable, and non-toxic. It also has a favorable combustion emission profile, producing much less carbon monoxide, sulphur dioxide and unburned hydrocarbons than petroleum-based diesel [1]. Biodiesel is derived from abundant and renewable substances such as vegetable oils, animal fats, algae, industrial acid oil, waste cooking oil etc. In conventional chemical processing, synthesis of these esters is achieved by an alkaline esterification reaction encountered with lowered selectivity, leading to undesirable side reactions. Moreover, the process is not environmentally friendly. The disadvantages caused by chemical catalysts are largely prevented by the lipases which allow mild reaction conditions and easy recovery of glycerol without purification, avoiding in this way the formation of chemical waste [2, 3]. Because of the high energy cost of the conventional chemical process and additional purification step of glycerol, application of lipase in

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the oleo chemical industry has become more attractive. Although enzymatic approaches have become more and more attractive, their use at industrial scale has not been realized yet, due to the relatively high price of lipases and their short operational life caused by the negative effects of excessive methanol and by-product glycerol [4, 5].

It has been demonstrated that more than 1/2 molar equivalent methanol are insoluble in vegetable oils and the immobilized lipases are easily inactivated by contacting with insoluble methanol existing as drops in the oils [6]. For example, Shimada et al. [7] found that immobilized *Candida antarctica* lipase was inactivated in a mixture containing greater than 1.5 molar equivalents of methanol in oil when using a solvent-free system. Stepwise addition of methanol or using some hydrophobic solvents such as *n*-hexane or petroleum ether as reaction media have been proposed to reduce the negative effect of methanol on lipase activity [8, 9]. But in another study, although n-hexane was used as reaction medium for the methanolysis of salad oil with *Candida* sp., Nie et al. [10] found that the lipase was denatured when methanol/oil molar ratio exceeded 1:1. This was caused by methanol poor solubility in this hydrophobic solvent, so the negative effects of methanol on lipase activity and stability could not be eliminated. Several hydrophilic organic solvents have also been tested for lipase-catalyzed biodiesel production and proved much less useful, as strongly interact with the essential water layer coating enzyme molecules [4, 11]. Other studies reported that when using hydrophilic 1,4-dioxane and *tert*-butanol the enzymatic process was improved [4, 9, 11-13].

In our study, we conducted the enzyme-catalyzed methanolysis of sunflower oil using Novozym 435, a well-known lipase that facilitates reactions between a wide variety of substrates and a remarkably heat-tolerant enzyme. A moderate polar solvent, *tert*-butanol, was adopted as reaction medium for lipase-catalyzed methanolysis of sunflower oil in order to eliminate the negative effect caused by methanol and glycerol on lipase activity [11, 12].

RESULTS AND DISCUSSION

In this study, *tert*-butanol was used as reaction medium for lipase-catalyzed methanolysis of sunflower oil because it dissolves both methanol and glycerol and is not a substrate for lipases (lipases do not act on tertiary alcohols). Moreover, *tert*-butanol is a non-toxic solvent of relative low cost.

The influence of *tert*-butanol was examined for the enzymatic methanolysis of sunflower oil and the result was shown in Figure 1. Total methyl ester content was very low in solvent-free system due to the toxicity of excessive methanol on lipase activity. But when *tert*-butanol was added into the reaction mixture the ester content significantly increased. The highest content of methyl esters of 70% was obtained when the volume ratio of *tert*-butanol/oil reached 6:1 therefore this ratio was used for further studies.

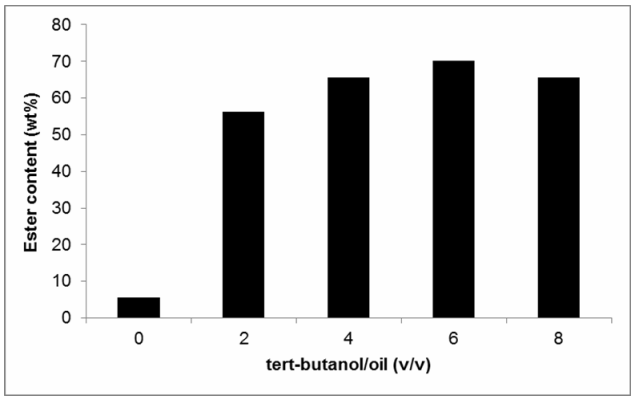

Figure 1. Effect of *tert*-butanol quantity on the methanolysis of sunflower oil. Reaction conditions: methanol/oil molar ratio 6:1, 10% Novozym 435 based on oil weight, 8 h reaction time.

Effect of molar ratio of methanol/oil was studied and the results are shown in the Figure 2. It can be seen that total methyl ester content increased with the increasing of methanol/oil molar ratio and that the presence of *tert*-butanol as solvent allowed the use of methanol in high excess without lipase deactivation.

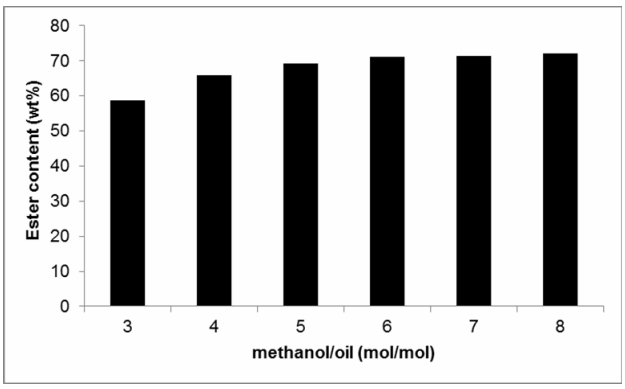

Figure 2. Effect of methanol to oil molar ratio on the methanolysis of sunflower oil. Reaction conditions: 10% Novozym 435 based on oil weight, *tert*-butanol/ oil volume ratio 6:1, 8 h reaction time.

The effect of Novozym 435 dosage on the methanolysis of sunflower oil was shown in Figure 3. The methyl ester content was increased by increasing lipase dosage and when lipase dosage reached 10%, a content of 72% could be given at 8 h. Further increase of lipase dosage did not have that much effect on ester content.

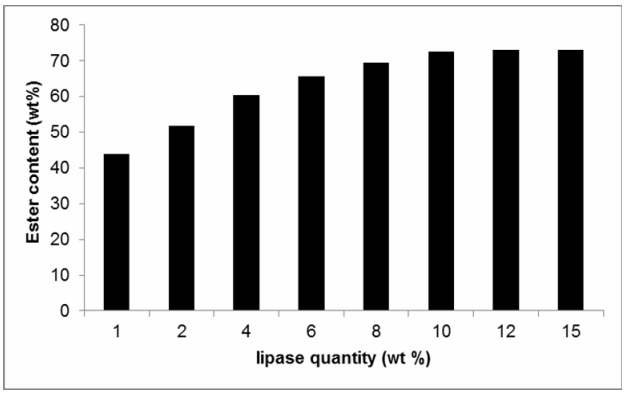

Figure 3. Effect of lipase dosage on the methanolysis of sunflower oil. Reaction conditions: methanol /oil molar ratio 6:1, *tert*-butanol/oil volume ratio 6:1, 8 h reaction time.

These optimized conditions (10% Novozym 435 based on oil weight, *tert*-butanol/oil volume ratio 6:1, methanol/oil molar ratio 6:1) were further used for the enzymatic methanolysis of sunflower oil for 12 h reaction time. The results obtained are summarized in Table 1 and the time course methanolysis of sunflower oil was shown in Figure 4.

Table 2. Fatty acid methyl ester content resulted for the enzymatic methanolysis of sunflower oil using *tert*-butanol as reaction medium

Time [min]	Total methyl ester content [wt %]
15	9.19
30	34.78
45	50.21
60	56.14
90	63.76
180	78.60
360	77.06
480	76.71
540	70.74
660	55.40

It was observed that the FAME content reached its maximum after 3h, with a value of 78.6% (m/m). After reaching this point the FAMEs content was maintained almost constant for another 2h and in the end a decrease of FAMEs content was observed.

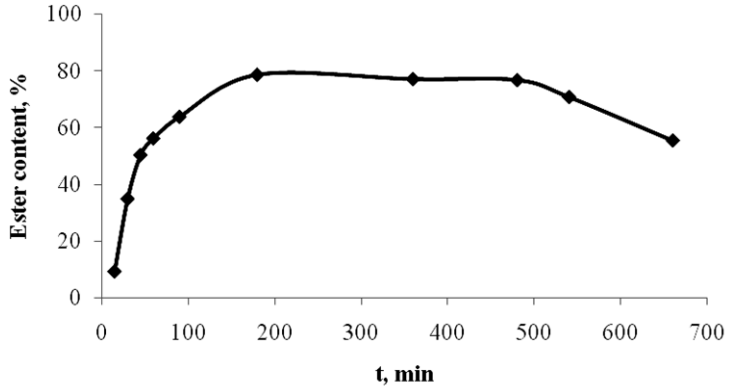

Figure 1. Time course methanolysis of sunflower oil. Reaction conditions: 10 % Novozym 435 based on oil weight; methanol /oil molar ratio 6:1; tert-butanol/ oil volume ratio 6:1

This decrease was caused by the mechanical disruption of the enzyme due to the physical agitation of the reaction mixture with the magnetic stirrer. Thus, the particles resulted from the enzyme and from the carrier determined a dilution effect for the samples withdrawn after 8h. The results obtained showed a high activity of the lipase even in the presence of methanol in high excess and this high activity was maintained because of the *tert*-butanol used as reaction medium. However, disruption of the enzyme carrier by the physical agitation force may not promise the use of immobilized lipase for a long period of time. Thus, in case of immobilized lipases the solution would be the use of mechanical shakers instead of magnetic stirrers, or other reactor configurations such as packed-bed reactor.

The widely accepted mechanism for triglycerides alcoholysis follows a Ping-Pong Bi Bi mechanism, as each product is released between addition of the substrates [14-16]. When fitting to experimental results, simplifications such as Michaelis–Menten kinetics can also be applied [17]. The effect of substrate concentration on the initial reaction rate (V) catalyzed by the Novozym 435 lipase was studied using sunflower oil as the substrate. Thus, using Michaelis–Menten model, Michaelis constant (K_m) and the maximum reaction rate (V_{max}) of Novozym 435 were calculated from the Lineweaver-Burk plot given in Figure 2. The experiment was run using substrate concentration in the range of 0.1 –0.6 M, at a constant methanol concentration of 3 M and in the presence of *tert*-butanol as reaction medium. The values obtained for initial reaction rate V_{max} and the Michaelis constant K_m were 0.0165 M min^{-1} and 0.7118 M respectively.

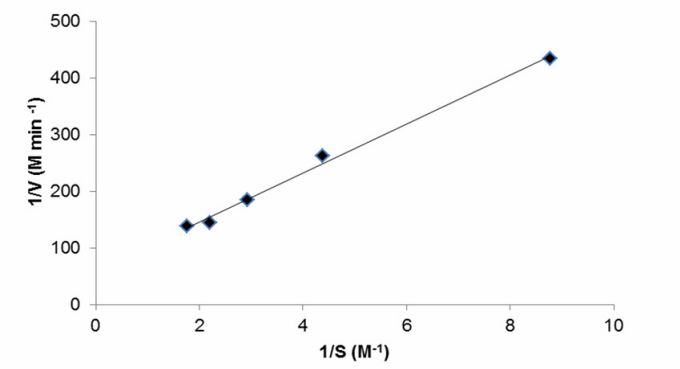

Figure 2. Lineweaver-Burk plot of sunflower oil methanolysis reaction for variation in triglycerides concentration in the range of 0.1 –0.6 M.
The value of R^2:0.9893

CONCLUSIONS

For the enzymatic methanolysis of sunflower oil a batch reactor was used and the reaction was performed under continuous agitation using a magnetic stirrer. The enzyme used was Novozym 435 and the reaction was performed in the presence of *tert*-butanol in order to avoid the inactivation of enzyme caused by methanol or by the glycerol formed during the reaction. The FAME content was 78.6% (m/m) after 3h, when the following optimized conditions have been used: 10% Novozym 435 based on oil weight, *tert*-butanol/oil volume ratio 6:1, methanol/oil molar ratio 6:1. The effect of substrate concentration on the initial reaction rate was studied using sunflower oil concentration in the range of 0.1 –0.6 M and the values obtained for initial reaction rate V_{max} and Michaelis constant K_m were 0.0165 M min^{-1} and 0.7118 M respectively.

The main disadvantage of the batch reactor used was the enzyme denaturation in time caused by mechanical degradation produced by the magnetic stirring but the presence of *tert*-butanol improved the solubility of methanol in the reaction mixture, and thus lipase retained a high level of activity with all methanol added for lipase-catalyzed methanolysis.

EXPERIMENTAL SECTION

Materials

The Novozym 435, lipase B from *Candida antarctica*, immobilized on macroporous acrylic resin, 1–2% water content, 10,000 propyl laurate units/g) was purchased from Novozymes (Vienna, Austria). The sunflower oil was obtained locally and its characteristics are summarized in Table 1. Methyl

ester standards (palmitic acid methyl ester, stearic acid methyl ester, oleic acid methyl ester, linoleic acid methyl ester, linolenic acid methyl ester, arachidic acid methyl ester, eicosane acid methyl ester, docosane acid methyl ester and heptadecanoic acid methyl ester) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were chromatographically pure; other chemicals and solvents were of the highest purity.

Table 1. Fatty acid composition for the sunflower oil used for biodiesel production

Systematic name	Common name	Abbr.	Content [%] (m/m)
Hexadecanoic acid	Palmitic acid	C16:0	6.2
Octadecanoic acid	Stearic acid	C18:0	3.9
cis-9-Octadecenoic acid	Oleic acid	C18:1n9	26.3
cis,cis-9,12-octadecadienoic acid	Linoleic acid	C18:2n6	62.7
6,9,12-octadecatrienoic acid	γ-Linolenic acid	C18:3n6	0.1
Icosanoic acid	Arachidic acid	C20:0	0.2
Docosanoic acid	Behenic acid	C22:0	0.6

GC-FID analysis

The fatty acid methyl ester contents in the reaction mixture were determined using an Agilent 7890A GC (Agilent Technologies) gas chromatograph equipped with a DB-WAX capillary column (30m × 0.32mm × 0.5μm) and a flame ionization detector. The instrumental configuration and the experimental conditions are given in Table 2.

Methanolysis of sunflower oil

Methanolysis reaction was carried out under continuous stirring in a 50 mL round-bottomed flask, maintained at room temperature. In order to avoid the direct contact of lipase with methanol drops, methanol was mixed with *tert*-butanol and oil first, followed by the addition of lipase into the mixture. The reaction conditions were the following: 10% Novozym 435 based on oil weight; methanol /oil molar ratio 6:1; tert-butanol/ oil volume ratio 6:1.

The methanolysis reaction using a batch reactor was monitored for 12 h. Samples from reaction mixture were withdrawn at specific periods of time and analyzed to determine the ester content. Before analysis, the samples were processed as the following: a volume of 100 μL sample was introduced in a mixture that contained 400 μL hexane, 400 μL methyl heptadecanoate (used as internal standard) and 100 μL water, and the solution obtained was centrifuged at 1000 rpm for 15 min. 1 μL from the organic phase was injected in the gas chromatograph to determine the FAME content (m/m).

Tabel 2. Instrumental configuration and experimental conditions used for GC-FID analysis of fatty acid methyl esters

Instrumental configuration	Agilent 7890N GC - FID
GC Parameters	
Injector	Split/Splitless
Column	DB-WAX 30 m x 0,25 mm(i.d.), 0,25µm
Detector	FID
Experimental conditions	
Inlet temperature	250°C
Injection mode	Split (Split ratio: 1/50)
Injection volume	1 µl
Carrier gas	He
Pressure	53 kPa
Oven temperature	50, 1 min 25°C/min to 200°C 3°C/min to 230°C, 18 min
Detector temperature	280°C
Detector gases	H2:40 ml/min; Air: 450 ml/min; He make-up: 30 ml/min

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