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Enzyme deactivation during biodiesel production

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ABSTRACT

Enzymatic conversion of vegetable oils into biodiesel offers an environmentally more attractive option to the conventional processes. However, lipase can be deactivated by lower linear alcohols, such as methanol and ethanol, conventionally used in biodiesel process. In this work, transesterification reactions of waste frying oil were carried out in presence of two different acyl acceptors in order to analyze its influence on the free and immobilized lipase activity.

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1. Introduction

Currently, the lack of conventional fossil fuels and the increase of the polluting emissions generated by combustion have increased the necessity for alternative fuels, such as biodiesel. Biodiesel is biodegradable, non-flammable and non-toxic. It generates less carbon and sulphur dioxides than conventional diesel [1]. In general, biodiesel is an alternative fuel obtained from plants (a renewable resource). According to the ASTM norm D 6751, biodiesel is a diesel engine fuel comprised of monoalkyl esters of long-chain fatty acids derived from vegetable oils or animal fats. It is produced by a transesterification reaction.

The transesterification reaction consists of the triglycerides transformation into fatty acid alkyl esters in presence of a short-chain alcohol, such as methanol, ethanol or butanol, and a catalyst, obtaining additional glycerin as a by-product [2]. The stoichiometry of the reaction is 3:1 alcohol to lipids. However, in practice this ratio is usually increased to 6:1 to raise the product yield [3]. Catalysts commonly used in transesterification are:

1. Alkali, such as sodium hydroxide, potassium hydroxide or alkali methoxide, being potassium hydroxide considered the best for the transesterification of fried oils [4]. This last catalyst is currently the most widely used for the production of biodiesel. The major disadvantage of the alkaline transesterification is the separation of biodiesel and glycerine after the reaction due to the formation of soaps produced in the reaction [5]. Also, the free fatty acids (FAA) content in the oil used to obtain biodiesel must be less than 0.5% in order to obtain yields higher than 99% [6].
2. Acids, such as sulphuric or sulphonic acids. Iron sulphate has also been used recently. Acid-catalyzed transesterification is more suitable for waste or unrefined oil [7]. This process is not very appealing because the transesterification reaction is slow compared to the alkali-catalyzed one and increases the biodiesel cost.
3. Enzymes, whose role in the biodiesel production is now being determined as studies have started recently. Lipases, which are derived from microorganisms such as fungi and bacteria [8], are usually employed in this process. Lipases hydrolyze triglycerides to fatty acid and glycerine. The enzymatic synthesis of biodiesel is usually carried out in a temperature range of 20–60 °C. Once the transesterification process is complete, the lower phase (glycerine) is simply separated from the upper phase (biodiesel). Neither deodorization nor neutralization of the product is necessary [9]. These advantages are the reason why enzymatic catalysts are proposed more and more for the production of biodiesel.

Enzymatic synthesis of biodiesel can be carried out either in organic solvents or in solvent-free. Lipases catalyze not only hydrolysis, but also esterification and transesterification of triacylglycerols with lipase thus it is considered one of the more effective reactions for production of biodiesel fuel from waste edible oil [10]. Studies commonly include enzymatic transesterification optimization variables such as type of solvent, temperature, pH, and type of microorganism that produces the enzyme to be used in the process. However, the reaction yields as well as the reaction times are still unfavourable compared to the base-catalyzed reaction systems [11] because of deactivation of the enzyme [12,13].

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Table 1
Waste frying oil characterization.

Properties	Unit	WFO
Acid value	mg KOH/g oil	1.35
Saponification value	mg KOH/g oil	180.79
Iodine value	mg I_2/100 g oil	93.20
Water content	%	0.044
Density	g/ml	0.96
Molecular weight	g/mol	940.01

In Europe, the main raw material for the biodiesel production is rapeseed oil. Its transesterification with methanol and ethanol (in systems with organic solvents) has been catalyzed by lipases from *Mucor miehi* [12] and *Mucor circinelloides.* In solvent-free systems transesterification of rapeseed oil with methanol have been catalyzed by lipases from *Thermomyces lanuginosa* (lipozyme TL IM), *M. circinelloides* and others [9].

The major disadvantage of biodiesel is the cost of raw material and also the fact that these materials are intended primarily for food production. Therefore, an alternative is to obtain biodiesel from used cooking oil. Regarding the raw materials to obtain biodiesel, waste cooking oils are available at relatively low cost, compared to fresh vegetable oil, and can be used for biodiesel production [2]. Moreover, the use of waste cooking oil as a biodiesel source can lead to a potential reduction of the CO_2, particulate matter and other greenhouse gases as the carbon contained in biomass-derived fuels is largely biogenic and renewable [14].

Bearing in mind these considerations, the aim of the present study is to investigate the deactivation of free and immobilized lipases in solvent-free media when they are used for the alcoholysis of waste frying oil in different reaction conditions. The deactivation studies will be based on the reaction yield.

2. Methods

2.1. Materials

The waste frying oil (WFO) was procured from local restaurants. Samples were mixed to obtain a homogenous oil mixture. The samples of waste frying oil were filtered to remove the suspended matter. The results of the oil characterization are illustrated in Table 1. The fatty acid composition of the samples (Table 2) was obtained by gas chromatography according to Section 2.3.

Commercial enzymes used were: free *Candida antarctica* lipase B (Calb L), with an activity of 5000 LU/g (laurate units/g), and *C. antarctica* lipase B immobilized on acrylic resin (Novozym 435), with an activity of 10000 PLU/g (propyl laurate units/g), both provided by Novozymes A/S (Denmark). Methanol and 2-propanol were used as acyl-acceptor and were supplied by Panreac. Standard fatty acid methyl esters were taken as reference and purchased from Supelco. All other chemicals were obtained commercially and were of analytical grade.

Table 2
Fatty acid composition (wt%) of methyl esters prepared from the WFO.

Component	Composition (wt%)
C6:0	0.067
C8:0	0.094
C12:0	0.001
C14:0	0.474
C16:0	9.203
C16:1	0.892
C17:0	0.846
C18:0	4.636
C18:1	53.940
C18:2	26.697
C18:3	0.357
C20:0	0.269
C20:1	0.316
C20:2	0.428
C22:2	0.809

2.2. General procedure for transesterification process

The enzymatic transesterification reactions were carried out in a test tube that contained 2 g of waste frying oil, 0.2 g of enzyme (Novozyme 435 or Lipozyme Calb L) (10%) and different alcohol-to-oil molar ratios. The molar amount of the oil was calculated from the saponification value. In order to reduce the enzyme deactivation, alcohol-to-oil molar ratios under the stoichiometric ratio were used. The reaction was carried out in an incubator at 50 °C for 8 h with constant stirring at 150 rpm. Additional experiments were carried out to analyze the influence of different parameters, such as alcohol to oil molar ratio and reaction time, on enzyme activity.

At the end of the reaction period, 500 μl were taken from the reaction mixture and centrifuged in order to obtain the upper layer that was analyzed by gas chromatography.

2.3. Analytical procedure

The methyl and propyl ester contents were quantified using a gas chromatograph Agilent 6890N connected to a forte BP-20 capillary column (0.25 mm × 30 m) from SGE. The temperature program was as follows: 155 °C for 1 min and programmed from 155 to 180 °C at a rate of 2 °C/min, kept for 2 min, and finally raised to 220 °C at 4 °C/min and maintained for 6 min. The injector was set up for 250 °C and the FID detector at 260 °C. Nitrogen was used as carrier gas, at constant flow of 1.6 ml/min. Methyl and propyl heptadecanoate were used as an internal standard [15].

The fatty acid composition of the WFO was determined by gas chromatography measurement of fatty acid methyl esters prepared by transmethylation with trimethylsulfonium hydroxide: 10 mg of oil were dissolved in 500 μl of methyl *t*-butyl ether and then adding 500 μl of a methanol solution of 0.2 mol/l trimethylsulfonium hydroxide. Fatty acid methyl esters obtained were quantified according the chromatographic method previously described.

3. Results and discussion

As said in Section 1, the aim of this study is to analyze the influence of the type of alcohol and other reaction parameters on the free and immobilized enzyme deactivation taking into account the obtained conversion of methyl esters.

For a better comparison between the obtained methyl esters yield, the experience with the highest methyl esters percentage (Novozyme 435 in presence of 1:2 methanol to oil molar ratio) has been defined by as a reference for the relative calculations. Then, relative activity represents the percentage of the methyl ester yield obtained in each experience with respect to the reference experience.

First of all, some experiences were carried out in order to check the effect of reaction time on the relative activity of both enzymes during the transesterification process using methanol like acyl acceptor. The reaction was carried with a shortage of methanol (1:40 methanol to oil molar ratio) and 0.2 g of enzyme (Fig. 1). Alcohol under the stoichiometric ratio (1:3) was used in order to reduce the enzyme deactivation, since excess alcohol levels may inhibit the enzyme activity and thereby decrease its catalytic activity toward the transesterification reaction.

The results show that the relative activity is higher when the enzyme is immobilized. These results agree with the study of Ranganathan et al. [16] who explains that the immobilization of

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