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Biodiesel Fuel Production by the Transesterification Reaction of Soybean Oil Using Immobilized Lipase

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Abstract

The enzymatic alcoholysis of soybean oil with methanol and ethanol was investigated using a commercial, immobilized lipase (Lipozyme RM IM). The effect of alcohol (methanol or ethanol), enzyme concentration, molar ratio of alcohol to soybean oil, solvent, and temperature on biodiesel production was determined. The best conditions were obtained in a solvent-free system with ethanol/oil molar ratio of 3.0, temperature of 50°C, and enzyme concentration of 7.0% (w/w). Three-step batch ethanolysis was most effective for the production of biodiesel. Ethyl esters yield was about 60% after 4 h of reaction.

Index Entries: Enzyme; ethanol; immobilized lipase; methanol; solvent; soybean oil.

Introduction

The use of an alternative fuel becomes necessary owing to the reduction of oil reserves and, consequently, of the diesel oil supplies and to the increasingly higher amount of gases produced in the combustion reaction of its derivatives. The indirect use of vegetable oils or mixtures of them is usually regarded as impracticable and insufficient for direct/indirect injection in diesel engines because of their high viscosity, the presence of free fatty acids, and the presence of gum from the oxidation and polymerization of diesel oil during their storage and combustion *(1)*. Recently, biodiesel, defined as a mixture of monoalkyl esters derived from fatty acids, has become a more interesting option because of its environmental benefits, as it comes from a renewable source, is biodegradable, and nontoxic.

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Biodiesel fuel can be obtained through different reaction pathways. The transesterification of an oil or fat in the presence of an acid or alkaline catalyst is commercially used. The lipase-catalyzed enzymatic production of biodiesel under milder conditions unfolds as a promising option. Under optimum conditions, the cost of production can be reduced and the conversion yield improved *(2)*. Moreover, that reaction uses fewer complex steps for product isolation as well as avoids the elimination of the catalyst and salt produced in the first process *(3)*. This work investigated the effect of reaction parameters such as: temperature, oil/alcohol molar ratio, alcohol, enzyme concentration, solvent, as well as lipase reuse on the transesterification reaction of soybean oil by short-chain alcohols, using an immobilized lipase (Lipozyme RM IM, Novozymes A/S, Denmark).

Materials and Methods

Materials

The commercial enzyme used, Lipozyme RM IM, was provided by Novozymes. Other reagents used were commercial soybean oil (Sadia), analytical grade ethanol, methanol, and hexane (Merck, Darmstadt, Germany). Methyl heptadecanoate (a chromatographic standard) was acquired from Sigma (St. Louis, MO).

Measurement of Lipase Activity

The esterification activity of Lipozyme RM-IM was measured by the consumption of oleic acid at 45°C in the esterification reaction with butanol (oleic acid/butanol molar ratio of 1) with the enzyme concentration of 3% (w/w). One esterification unit of Lipozyme was defined as 1 μmol of oleic acid consumed/min (U) under the experimental conditions described herein. The enzyme used in this work has esterification activity of 3000 U/g.

Reaction System

The transesterification reactions between soybean oil and alcohol were conducted in closed 15-mL batch reactors, with constant mechanical stirring, coupled to condensers in order to avoid alcohol loss by volatilization. The water circulating in the condenser was cooled by a thermostatic bath. The reaction temperature was kept constant by circulating ethylene glycol from a thermostatic bath (Haake DC 10-B3) into the reactor's jacket. Reaction progress was monitored by taking duplicate samples, which were diluted in hexane and analyzed by gas chromatography.

Chromatography Analysis

The samples were injected into a Varian gas chromatograph (CP–3380 model), equipped with a flame ionization detector and a CP WAX 52 CB capillary column 30 m × 0.25 mm × 0.25 μm, and split injection system

with a 1 : 20 ratio. Injector and detector temperatures were kept at 250°C. The heating rate was 20°C/min. The oven was initially maintained at 200°C for 4.5 min, then was heated up to 210°C, and was kept constant at this temperature for 0.5 min. After that, it was heated to 220°C for 0.5 min. The oven was heated again to 250°C at a 30°C/min rate and maintained at this temperature for 1.5 min. Hydrogen was used as the carrier gas at a 1.8 mL/min flow rate; column pressure was set at 12 psi. A computer loaded with the Star Workstation 6.2 software was connected to the GC by a Star 800 Module Interface to automatically integrate the peaks obtained. Methyl heptadecanoate was the internal standard used.

Transesterification Reaction

The reaction medium consisted of a mixture of the commercial soybean oil, alcohol, and enzyme. The biodiesel production was also evaluated in the presence of a solvent. In this work, the transesterification reactions took place in the presence of hexane (50% [v/v]). The alkyl esters (biodiesel) synthesis was evaluated as a function of temperature (40, 50, and 60°C), enzyme concentration (3, 5, 7, 9, 11, and 20% [w/w]), alcohol/soybean oil molar ration (3, 6, and 10), type of alcohol used (methanol or ethanol), and in respect to stepwise addition of alcohol (single addition or two or three consecutive alcohol additions, at different times). One molar equivalent of ethanol was 0.72 g for 12.70 g soybean oil.

Results and Discussion

Enzymatic Transesterification in the Presence of a Solvent

Effect of Reactants Molar Ratio

The effect of the ethanol/soybean oil molar ratio over the transesterification reaction was evaluated initially using a 7% (w/w) commercial, immobilized lipase (Lipozyme RM IM) in the presence of hexane (50% [v/v]). An increase in the concentration of any reactant results in a higher yield of ester, because the ethanolysis is a reversible reaction. At least three mols of ethanol are required in the ethanolysis reaction to accomplish a complete conversion of the soybean oil into its ethyl esters. An alcohol/oil molar ratio of six is commonly used in industrial processes to obtain higher yields of esters *(4–6)*. In such case, ethanol/soybean oil molar ratios of 6 and 10 were tested in reactions conducted at 40°C in a closed batch reactor. The results obtained are shown in Table 1 and indicate that the excess of alcohol reduced the ethyl esters production, even in the presence of a nonpolar solvent, hexane. It is well known that, usually, proteins are unstable in a reaction medium containing short-chain alcohols, such as ethanol and methanol. An excess of alcohol can promote the inhibition and/or deactivation of the lipases *(3,7–9)* as observed in Table 1 where the reduced ethyl esters yield is related to the increase in the alcohol molar ratio.

Table 1
Effects of Enzyme Concentration, Alcohol/Soybean Oil Molar Ratio, Type of Alcohol and Solvent Addition on Reaction Yield

Enzyme concentration (% [w/w])	Alcohol/ soybean oil molar ratio	Alcohol	Solvent addition (% [v/v])	Ester yield (%)
7	6	Ethanol	Hexane 50	25.0
7	10	Ethanol	Hexane 50	12.0
20	10	Ethanol	Hexane 50	24.7
7	6	Methanol	Hexane 50	5.1
7	6	Ethanol	No addition	3.5
7	10	Ethanol	No addition	3.3
7	3	Ethanol	No addition	16.9

The results were obtained after 8 h of reaction at 40°C.

Effect of Enzyme Concentration

The effect of the enzyme concentration in the ethyl esters production in the reaction was investigated using an ethanol/soybean oil molar ratio of 10 in the presence of hexane (50% [v/v]), at 40°C, in a closed batch reactor. According to the results shown in Table 1, there was an increase in the yield and in the rate of the reaction when 20% (w/w) Lipozyme was used. After 8 h of reaction, the yield obtained was approx 25%, whereas for an enzyme concentration of 7% (w/w), the yield was only 12%. However, taking into account the cost of process and the operational difficulty of working with such a high enzyme concentration (20% [w/w]), the concentration chosen to conduct the experiment was 7% (w/w).

Effect of Type of Alcohol

The transesterification of triglycerides with methanol is the preferred enzyme-catalyzed reaction for biodiesel production. Nevertheless, the enzymatic alcoholysis of triglycerides using other alcohols, including ethanol, *n*-propanol, isopropyl alcohol, butanol, and pentanol has also been investigated *(4)*. Usually, methanol is the alcohol of choice because of its lower cost in various countries. However, the importance of ethyl alcohol for the Brazilian energy market is well known. The effect of the kind of alcohol used (methanol or ethanol) over the reaction, at 40°C, was investigated using an alcohol/oil molar ratio of 6 and 7% (w/w) Lipozyme RM IM. The reactions were conducted in the presence of 50% (v/v) solution of hexane.

According to the results presented in Table1, the reaction yield when ethanol was used was five times higher than that when methanol was used. This can be explained by the greater enzyme deactivation by an alcohol with fewer carbon atoms. Methanol is a highly hydrophilic solvent, thus able to solubilize and remove the essential water layer that cover the enzymes, which can result in loss of lipase's catalytic activity *(10,11)*.

Effect of Solvent Addition

The use of organic solvents is not indicated for biodiesel production because of the high risk of explosion and the need for an additional step for solvent removal *(7)*. On the other hand, immobilized lipases show high conversion rates in nonpolar organic solvents, therefore there are several studies of triglyceride enzymatic alcoholysis in organic solvents *(9)*. The biocatalysis of synthetic reactions, such as the biodiesel production reaction, are usually considered possible in solvents immiscible in water and with logP (logP is one of the parameters used to determine the hydrophobicity of a solvent, and is defined as the partition coefficient of a solute in a standard biphasic system formed by water and 1-octanol) more than 4 *(10,11)*. Hexane is one of the most commonly used solvents in synthetic reactions using lipases (logP for hexane = 3.5).

The effect of the addition of 50% v/v hexane was evaluated on the transesterification reaction of soybean oil with ethanol, at 40°C, with 7% (w/w) Lipozyme RM IM. According to the results presented in Table 1, the use of hexane helped the biodiesel production. However, because of the cost and operational difficulties of the process conducted in the presence of a solvent, the effects of certain reaction parameters on the solvent-free transesterification reaction were also investigated.

Enzymatic Transesterification in a Solvent-Free Medium

Effect of the Reactants Molar Ratio

The effect of the ethanol/soybean oil molar ratio was evaluated in the transesterification reaction conducted at 40°C with 7% (w/w) Lipozyme. The results obtained (Table 1) show that the stoichiometric molar ratio of the reactants (ethanol/oil molar ratio = 3) allowed a higher yield of ethyl esters, which confirms the prejudicial effect of a high concentration of ethanol on the lipase activity. Similar results *(3,12)* were observed for the methanolysis of vegetable oils using Novozym 435 (commercial, immobilized *Candida antarctica* lipase). According to Köse et al. *(12)*, the alcohol can remove the essential water layer which stabilizes the immobilized enzyme, and can form inhibiting binary alcohol-lipase complexes.

Effect of the Stepwise Addition of Ethanol

At least the stoichiometric amount of ethanol has to be used in order to achieve total conversion of triglycerides into their ethyl esters. Even under these conditions, the yield was low (16.9% in 8 h), as seen in Table 1. The stepwise addition of ethanol (three consecutive steps) was studied in order to avoid the lipase deactivation by a high initial alcohol concentration. The reactions were conducted with 7% (w/w) Lipozyme at 40°C and with the reactants stoichiometric ratio. The results are shown in Fig. 1. When ethanol was added in a stepwise manner (i.e., 1/3 added at time 0, 1/3 after 4 h, and 1/3 after 6 h), the yield (60%) was much higher than that

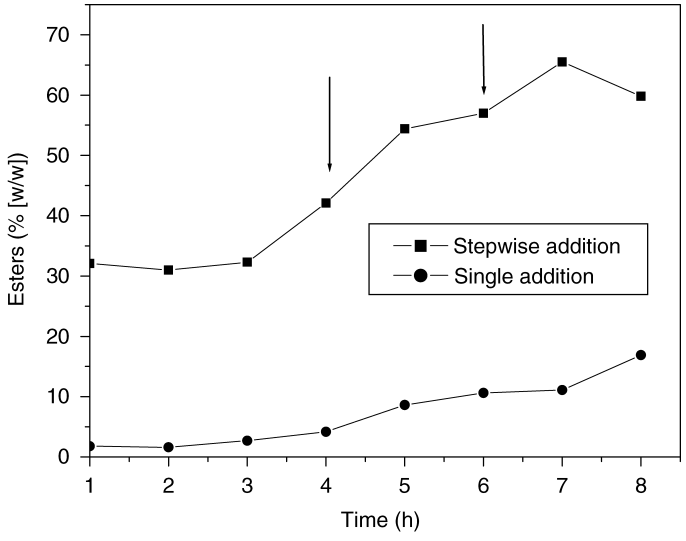

Fig. 1. Effect of stepwise ethanol addition on the transesterification of soybean oil using an ethanol/soybean oil molar ratio of 3, 7% (w/w) Lipozyme RM IM at 40°C.

observed when ethanol was added in a single step at the beginning of the reaction.

Shimada et al. *(7)* also verified a higher conversion in the methanolysis reaction of vegetable oil with immobilized *C. antartica* lipase, and three consecutive methanol additions (0, 10, and 24 h).

The stepwise addition also allowed a higher ethyl esters yield, even when an excess of alcohol was used (ethanol/soybean oil molar ratio = 6), as seen in Fig. 2. In this case, ethanol was added in six steps (time: 0, 30, 60, 90, 120, and 150 min). According to the results presented in Figs. 1 and 2, the best molar ratio is the stoichiometric ratio, even when ethanol is added in a stepwise manner.

Because the stepwise addition of ethanol, using the reactants stoichiometric ratio afforded the highest yield of ethyl esters, different ethanol addition times were also investigated. The results show that the ethanol addition at shorter times (0, 30, and 60 min) allowed the highest reaction yield (58.2%) to be reached more rapidly—after about 3 h.

Effect of Enzyme Concentration

The effect of the enzyme concentration on the reaction yield was investigated under the best reaction conditions established by the previously reported results, (i.e., molar ratio of reactants equal to 3, no solvent, stepwise ethanol addition after 0, 30, and 60 minutes of reaction). According to the results presented in Fig. 3, the highest ethyl esters yield was obtained with 7% (w/w) Lipozyme. The ethanolysis reaction mixture requires constant stirring because of the low solubility of the vegetable oil in ethanol. Initially, the reactants formed a two-phase system, which becomes a three-phase system

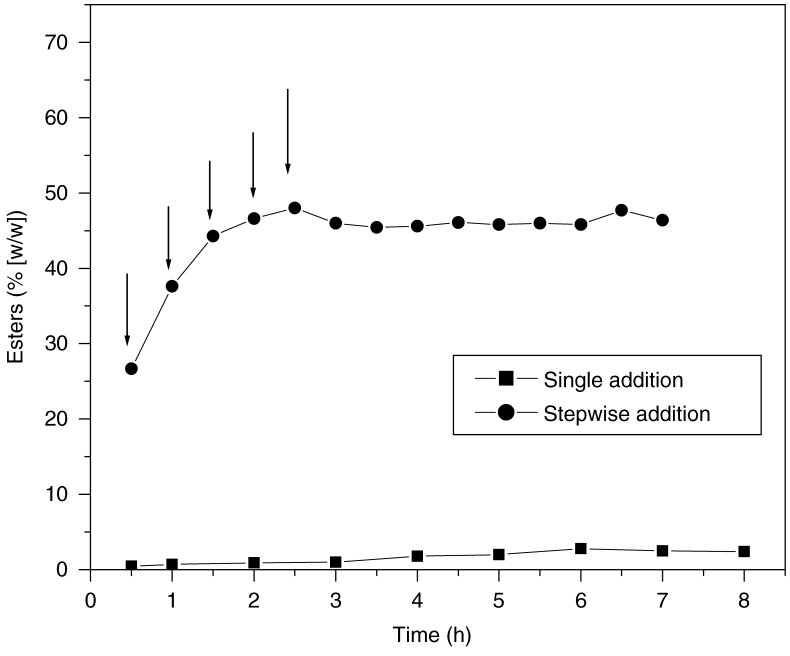

Fig. 2. Effect of stepwise ethanol addition on the transesterificaction reaction of soybean oil using an ethanol/soybean oil molar ratio of 6, 7% (w/w) Lipozyme RM IM at 40°C.

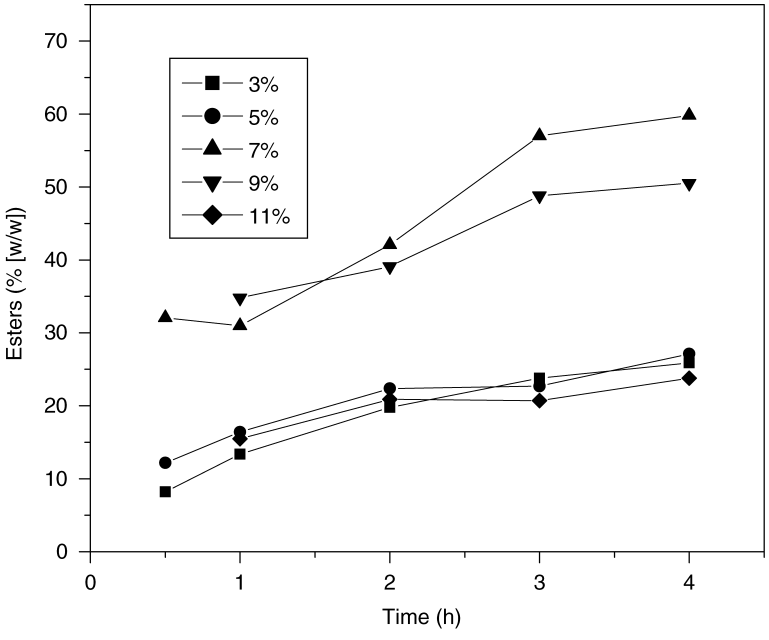

Fig. 3. Effect of Lipozyme RM IM concentration on the transesterification of soybean oil using an ethanol/soybean oil molar ratio of 3, with stepwise ethanol addition (1/3 at 0 h, 1/3 after 0.5 h, and 1/3 after 1 h) at 40°C.

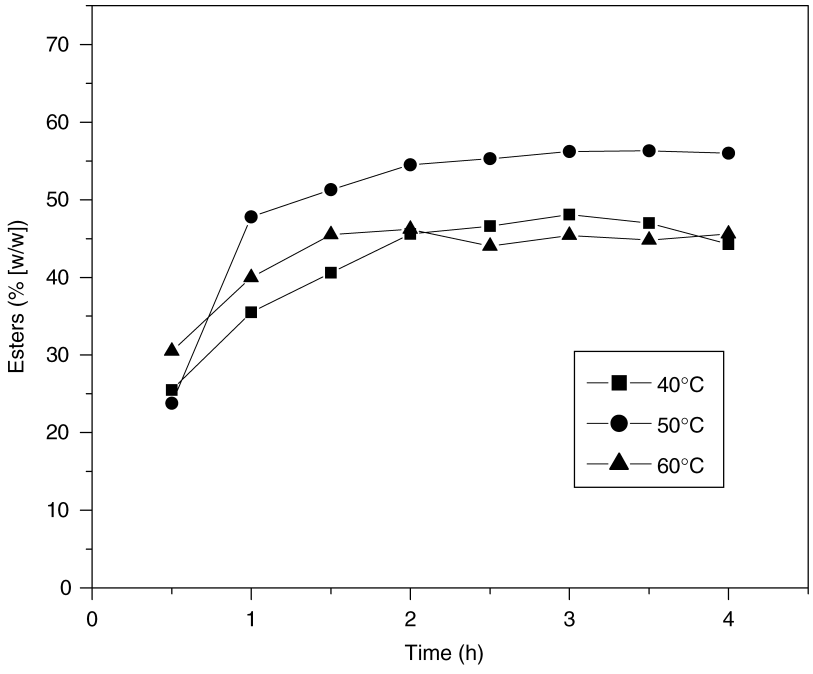

Fig. 4. Effect of temperature on the transesterification of soybean oil using an ethanol/soybean oil molar ratio of 3, with stepwise ethanol addition (1/3 at 0 h, 1/3 after 0.5 h, and 1/3 after 1 h), and 7% (w/w) Lipozyme RM IM.

once the immobilized enzyme is added. Higher Lipozyme concentrations (9 and 11% [w/w]) did not allow an appropriate system homogenization with the stirring system used, which can explain the lower ethyl esters yield shown in Fig. 3. After 8 h of reaction, the final yield was basically the same for the reactions conducted with Lipozyme at 7, 9, or 11% (w/w) (these results are not shown in the figures).

Effect of Temperature

Temperature is another factor that can possibly interfere in the immobilized lipase-catalyzed alcoholysis of vegetable oils. The biodiesel production was investigated at temperatures of 40, 50, and 60°C for reactions using a stoichiometric ratio of reactants and 7% (w/w) Lipozyme. The results obtained show maximum yield at 50°C (Fig. 4). An increase in temperature increases the equilibrium conversion, as verified in the reactions conducted at 40 and 50°C. However, temperatures higher than 50°C deactivate the enzyme as confirmed by the lower yield obtained at 60°C. According to Illanes *(10)*, the thermal stability of enzymes decreases as higher amounts of water are present in the organic solvents, i.e., hydrophilic solvents, such as ethanol, tend to deactivate enzymes by reducing their thermal stability. Köse et al. *(12)* also observed a higher methyl ester yield at 50°C for the immobilized *C. antartica* lipase-catalyzed methanolysis reaction of cottonseed oil.

Table 2
Lipozyme Reuse in the Transesterification of Soybean Oil With Ethanol Using a Reactant Stoichiometric Molar Ratio, Stepwise Ethanol Addition (1/3 at 0 h, 1/3 after 0.5 h, and 1/3 after 1 h), at 50°C

Batch number	Ester yield after 4 h of reaction (%)
First	56.0
Second (before the enzyme was reused, it was dried in oven at 50°C for 12 h)	4.1
Second (before the enzyme was reused, it was stored in a desiccator at room temperature for 12 h)	6.5

Effect of the Stepwise Addition of Enzyme

In order to avoid enzyme deactivation by the ethanol present in the reaction medium, the stepwise addition of Lipozyme (in two steps: 0 and 2 h) to the reaction conducted at 50°C, using the reactants stoichiometric ratio (with stepwise addition of ethanol) and 7% (w/w) Lipozyme was studied. The results show that the final yield after 4 h of reaction was similar for both cases tested. The initial rate of reaction was, evidently, much higher for the reaction in which the whole enzyme was added at time 0 h. In this case, the equilibrium seems to have been reached after about two hours of reaction.

Enzyme Reuse

One of the major advantages of using immobilized enzymes is the possible reuse of the enzyme preparation, a means of reducing the total costs of reaction. The ethanolysis reaction of soybean oil under the optimum conditions previously reported ($T = 50$°C, $R = 3$ with stepwise ethanol addition, no solvent, 7% [w/w] Lipozyme) was used to investigate the possibility of reusing the enzyme. The enzyme was recovered from the reaction medium, washed with hexane, and placed in a desiccator for 12 h or dried in an oven at 50°C (for 12 h) to remove all the water accumulated on the immobilization support. After this treatment, the enzyme was reused. The results presented in Table 2 indicate that:

- The enzyme drastically loses its activity after the reaction described previously.
- Yields lower than 10% are obtained after the second reuse and after the two types of treatment tested.

According to Lima et al. *(13)*, the loss of lipase's catalytic activity, after being successively reused in the transesterification reactions, is owing to the deposit of water on the enzyme support. The authors have confirmed this effect when the enzyme recovered its catalytic activity after being dried back to its initial water content. However, the results shown in Table 2 indicate that the enzyme deactivation was not only caused by the water

accumulated on the immobilization support during the reaction, because in one of the experiments, the recovered enzyme was stored in an oven at 50°C for 12 h, reaching constant weight. Glycerol, one of the reaction products, could also be adsorbed on the immobilization support, which would promote a change on the enzyme microenvironment, and consequently a decrease in its activity *(14,15)*. According to Soumanou and Bornscheuer *(9)*, glycerol can also inhibit the reaction by limiting the product and substrate diffusion because it is not soluble in oil.

Conclusions

It is possible to conduct a transesterification reaction of soybean oil with ethanol using a commercial, immobilized lipase in a solvent-free reaction medium as demonstrated by the work presented herein. Under mild reaction conditions (temperature of 50°C, atmospheric pressure), yields higher than 50% were achieved after less than 4 h for a reaction using 7% (w/w) of the biocatalyst with the stepwise addition of ethanol in three steps and using the reactants stoichiometric ratio.

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