Fatty acid ethyl esters production using a non-commercial lipase in pressurized propane medium

Produção de ésteres etílicos de ácidos graxos utilizando uma lipase não comercial em propano pressurizado

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Abstract

The objective of this work is to investigate the production of fatty acid ethyl esters from soybean oil in compressed propane using a non-commercial lipase from *Yarrowia lipolytica* and two commercial ones as catalysts, Amano PS and Amano AY30. The experiments were performed in the temperature range of 35-65 °C. at 50 bar, enzyme concentration of 5 wt%, oil to ethanol molar ratio of 1:6 and 1:9, and solvent to substrates mass ratio of 2:1 and 4:1. The results indicated that low reaction conversions were generally obtained with the use of commercial and non-commercial lipases in pressurized propane medium. On the other hand, the aspects of low solvent to substrates mass ratio and mild temperature and pressure operating conditions used to produce ethyl esters justify further investigations to improve reaction yields.

Keywords: *biodiesel; fatty acid ethyl esters; lipase; propane; soybean oil.*

Resumo

O principal objetivo deste trabalho foi investigar a produção de ésteres etílicos de ácidos graxos a partir de óleo de soja em propano pressurizado, utilizando uma lipase não comercial (obtida por fermentação submersa de *Yarrowia lipolytica*) e duas comerciais, Amano PS e Amano AY30. Os experimentos foram conduzidos no intervalo de temperatura de 35-65 °C, em pressão de 50 bar, concentração de enzima de 5 m/v%, razão molar óleo etanol de 1:6 e 1:9 e razão molar substratos solvente de 2:1 e 4:1. Os resultados obtidos indicaram que baixas conversões foram geralmente obtidas com o emprego das lipases testadas em propano pressurizado. Por outro lado, os aspectos de baixas razões molares entre o solvente e os substratos (óleo e etanol) e condições amenas de temperatura e pressão usadas na produção dos ésteres etílicos possam justificar investigações futuras no sentido de aumentar a conversão do processo.

Palavras-chave: *biodiesel; ésteres etílicos de ácidos graxos; lipase; óleo de soja; propano.*

1 Introduction

The potential of using vegetable oil fuels either as a diesel fuel additive or as a replacement is well documented in the literature (FUKUDA; KONDO; NODA, 2001; MA; HANNA, 1999; ALTIN; ÇETINKAIA; YUCESU, 2001; SRIVASTAVA; PRESAS, 2000). The merits of using biodiesel as an alternative to mineral diesel include the fact that it is a non-toxic, biodegradable, domestically produced, renewable source. In addition, biodiesel possess higher cetane number compared to diesel obtained from petroleum and favorable combustion emissions profile such as reduced levels of particulate matter and carbon monoxide and, under some conditions, nitrogen oxides (McCORMICK et al., 2001; ZHANG et al., 2003). Because of these environmental benefits, which mean reduction of environmental investments, and also due to the relief reliance on import needs, biodiesel fuel can be considered a good alternative to petroleum-based fuel.

Among other processes used for biodiesel production such as pyrolysis and micro-emulsification, transesterification is the most common way to produce biodiesel (FUKUDA; KONDO; NODA, 2001; MA; HANNA, 1999). Transesterification, also called alcoholysis, refers to a catalyzed reaction involving the displacement of alcohol from an ester by another alcohol to yield fatty acid alkyl esters (i.e., biodiesel) and glycerol as a by-product. Conventionally, transesterification can be performed using alkaline, acid, or enzyme catalysts. Since alkali-catalyzed systems are very sensitive to both water and free fatty acids contents, the glycerides and alcohol must be substantially anhydrous because water makes the reaction change partially to saponification, which produces soaps thus consuming the catalyst and reducing the catalytic efficiency, as well as causing an increase in viscosity, formation of gels, and difficulty in achieving separations (FUKUDA; KONDO; NODA, 2001;

Recebido para publicação em 10/12/2007
Aceito para publicação em 3/1/2009 (003084)

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MA; HANNA, 1999; ZHANG et al., 2003). It has been found that when basic catalysts are used, the water content in the reaction medium should be kept below 0.06 wt% and the vegetable oil should have an acid number less than 1 (MA; CLEMENTS; HANNA, 1998). Although transesterification using acid catalysts is much slower than that obtained from alkali catalysis, typically 4000 times, if high contents of water and free fatty acids are present in the vegetable oil, acid-catalyzed transesterification can be used (FUKUDA; KONDO; NODA, 2001; MA; HANNA, 1999).

Despite the fact that chemical transesterification through alkali-catalyzed processes provides high conversion levels of triglycerides to their corresponding fatty acid alkyl esters in short reaction times, it suffers from several drawbacks: it is energy intensive, the recovery of glycerol may be difficult, the acid or alkaline catalyst has to be removed from the product, alkaline wastewater requires treatment, and free fatty acids and water interfere with the reaction separations (FUKUDA; KONDO; NODA, 2001). The use of enzyme-catalyzed transesterification methods can overcome these problems since the by-product - glycerol - can be easily recovered without any complex process, and also the free fatty acids contained in oil and fats wastes can be completely converted to fatty acid esters (FUKUDA; KONDO; NODA, 2001). Enzymes are generally effective biocatalysts due to their high substrate specificity property, functional group specificity, and stereo specificity in aqueous medium. Thus, several studies have reported an alternative method to produce esters through enzymatic reactions using lipases as catalysts (WATANABE et al., 2002; CARTA; GAINER; ZAIDI, 1995; ABRAMOWICZ; KUSE, 1989; BARZANA; KAREL; KLIBANOV, 1989; STEVENSON; STORER, 1991; YAMANE, 1988; YAMANE et al., 1990). Because biocatalysts have high specific activity and a low impact on the environment, they have become increasingly important for industries. For example, immobilized lipases have been used as catalysts for reactions involving the biomodification of triglycerides (BASRI et al., 1995).

The establishment of the Brazilian National Program on Biodiesel and the expectation of commercial availability of the product throughout the country have prompted several studies on biodiesel production using different techniques and a variety of vegetable and animal sources. Methanol has been the most commonly used alcohol to perform transesterification in alkali, acid, and enzyme-catalyzed reactions (FUKUDA; KONDO; NODA, 2001; MA; HANNA, 1999). However, in the Brazilian context, ethanol has been the natural choice since Brazil is the world's biggest ethanol producer with a well-established technology of production, large industrial plant capacity installed throughout the country, and due to the fact that ethanol also comes from a renewable resource. Moreover, it has been found that in the conversion of palm kernel oil and sunflower oil to alkyl esters using lipase-catalyzed reaction, ethanol produced higher yields when compared to the use of methanol (MITTELBACH, 1990; ABIGOR et al., 2000).

For a long time enzymes were believed to work efficiently only in aqueous solutions. Consequently, their utilization in organic synthesis was rather scarce since the low water solubility of many substrates represented a serious obstacle (GARCIA-JUNCEDA et al., 2004). This disadvantage, nevertheless, stimulated the search for systems based on the use of non-aqueous solvents in order to increase the solubility of hydrophobic substrates, and as a consequence, non-aqueous biocatalysis has rapidly become a standard approach in the development of strategies for organic synthesis (HABULIN; KNEZ, 2001; KAEWTHONG; KITTIKUN, 2004; OLIVEIRA et al., 2004; OLIVEIRA et al., 2005).

Since the first reports on enzyme-catalyzed reactions in supercritical fluids, much attention has been paid to the use of dense gases, mainly supercritical carbon dioxide as potential alternatives to conventional organic solvent (GARCIA-JUNCEDA et al., 2004; HABULIN; KNEZ, 2001; CHIO; NAKAMURA; YANO, 1988; OLIVEIRA; OLIVEIRA, 2000; OLIVEIRA; OLIVEIRA, 2001). Nevertheless, other compressed gases seem also to be adequate for biocatalysis (HAMMOND et al., 1985). Actually, the comparable dielectric constant of compressed propane to carbon dioxide, and the higher-pressure phase transition values generally found in systems formed by carbon dioxide with high molecular weight (e.g. triglycerides) compounds support a firm belief that propane may also be suitable as a reaction medium for enzyme-catalyzed bioconversions (HABULIN; KNEZ, 2001; CHEN; YANG, 2000). Besides, compared to higher homologue hydrocarbons and liquid solvents at ambient conditions, the use of propane offers the advantages of low separation costs and also solvent-free products (LANZA et al., 2005; NDIAYE et al., 2006a; NDIAYE et al., 2006b).

Although at present the high cost of enzyme production may be the major obstacle to the commercialization of enzyme-catalyzed processes, recent advances in enzyme technology, such as the use of solvent-tolerant lipases and immobilized lipases – making catalyst re-utilization possible, have been made to develop cost-effective systems (FUKUDA; KONDO; NODA, 2001; ISO et al., 2001). Furthermore, the possibility of using non-commercial lipases obtained from relatively low-cost and renewable raw materials renders additional importance towards conducting studies on enzyme-catalyzed production of biodiesel (OLIVEIRA et al., 2006a, b).

Based on these aspects, the main focus of this work is to investigate the production of fatty acid ethyl esters from soybean oil in compressed propane using a non-commercial immobilized lipase from *Yarrowia lipolytica*. All the experiments were performed in a batch mode using a laboratory-scale unit. A comparison with two commercial, free and immobilized, lipases from Amano (Amano PS and AY 30) in terms of biodiesel production is also provided.

2 Material and methods

2.1 Materials

Commercial refined soybean oil (Soya), ethanol (Merck 99.9%), lauric acid (Vetec, 98%), n-propanol Synth (99.5% of purity), sodium hydroxide (Quimex, 97.0%) and acetone (Quimex, analytical grade) were used without further treatment.

A non-commercial lipase from *Yarrowia lipolytica* was obtained as described below. Two commercial free and immobilized lipases (Amano Pharmaceutical Co. Ltd.), purchased from Sigma-Aldrich, were also used as process catalysts: lipase PS Amano (*Pseudomonas cepacia*), and lipase AY Amano 30 (*Candida rugosa*), both non-specific enzymes with optimum temperature in the range of 50-60 and 40-50 °C, respectively.

2.2 Production of lipases from Yarrowia lipolytica

A lipase from *Yarrowia lipolytica* was produced in a 2,000 L fermentor containing (wt/v) 1.0% of glucose, 3.0% of whey powder, 0.8% of ammonium sulfate, 1.0% of corn steep syrup and 0.5% of olive oil. After 30 hours of fermentation, the culture broth was centrifuged and the supernatant was dried by lyophilization (OLIVEIRA et al., 2006a, b).

2.3 Lipases immobilization

The lyophilized preparation was solubilized in phosphate buffer (0.05 M pH 7.0) and submitted to preferential immobilization by physical adsorption on a hydrophobic support (Accurel® MP 1000) (FREIRE et al., 1997). The carrier preparation was performed by adding 10 mL of ethanol to 1 g of the carrier. After 30 minutes, the supernatant was poured out and the carrier was washed repeatedly with distilled water until ethanol was completely removed. Enzyme immobilization was performed at a relation soluble enzyme (mL): support (mg) of 1:25 and the activity was in the range from 30 to 260 U_H/mL. Immobilization was performed with magnetic stirring in an ice cooler and aliquots were sampled periodically (0, 1, 5, 10, 15, 20, 30, 60, 90, 120 minutes) for protein content assay. The supernatant and carrier with enzyme were also assayed for enzyme activity. The lipase activity was determined as described previously (FREIRE et al., 1997). One lipase activity unit (U_H) was defined as the amount of enzyme that produces 1 µmol of fatty acids per minute, under the assay conditions.

2.4 Lipases activity

The enzyme activity was determined as the initial rates in esterification reactions between lauric acid and propanol at a molar ratio of 3:1, temperature of 60 °C and enzyme concentration of 5 wt% in relation to the substrates. At the beginning of the reaction, samples containing the mixture of lauric acid and propanol were collected, and the lauric acid content was determined by titration with 0.04 M NaOH. After the addition of the enzyme to the substrates, the mixture was kept at 60 °C for 40 minutes. Then, the lauric acid consumption was determined. One lipase activity unit was defined as the amount of enzyme necessary to consume 1 µmol of lauric acid per minute under the established experimental conditions presented previously. All enzymatic activity determinations were replicated at least three times.

2.5 Analytical methods

Samples collected from the reactor at the end of each experiment were first submitted to ethanol evaporation to constant weight in a vacuum oven (65 °C, 0.5 bar) and then diluted with 2 mL of ethanol and 8 mL of n-heptane. The reaction products were determined using a gas chromatograph (Varian, STAR 3400 CX). The following instrumentation and conditions were used: capillary column DB-5 (methyl siloxane with 5 % phenyl groups, with 30 m × 0.25 mm × 0.25 µm film thickness); split ratio: 1:50; injection volume: 2.0 µL. The column temperature was programmed from 150 °C, holding 1 minute, heating to 180 °C at 15 °Cmin^{-1}, to 210 °C at 4 °Cmin^{-1}, holding 1 minute, and to 250 °C at 15 °Cmin^{-1}, holding 3 minutes. Helium was the carrier gas and the injection and detector temperatures were 250 and 280 °C, respectively. The identification and quantification of the compounds were accomplished through the injection of authentic standards (ethyl palmitate, stearate, oleate, linoleate and linolenate) (Sigma-Aldrich) and methyl palmitate (Sigma-Aldrich) as the internal standard. All analyzes were replicated at least three times.

2.6 Apparatus and experimental procedure

The experimental setup used for ethyl esters production from soybean oil with immobilized and free lipases in pressurized propane, schematically presented in Figure 1, consists basically of a jacketed 50 mL reactor (Parr, model 4843) with mechanical agitation, a propane cylinder, and a syringe pump (Isco, model 260D).

The charge of a known amount of propane was performed with the help of the syringe pump (resulting accuracy of ± 0.005 g in propane loadings) until the pre-established pressure was achieved. Based on the uncertainty in propane loading, substrates weighing and predictions in solvent feed, the propane to substrates ratio varied approximately 5 % from the desired value. The experiments were performed in a laboratory-scale unit in the temperature range of 35-65 °C, at 50 bar, enzyme concentration of 5 wt%, oil to ethanol molar ratio of 1:6 and 1:9 and solvent to substrates mass ratio of 2:1 and 4:1. The agitation and reaction time were kept fixed for all experimental conditions at 600 rpm and 8 hours, respectively.

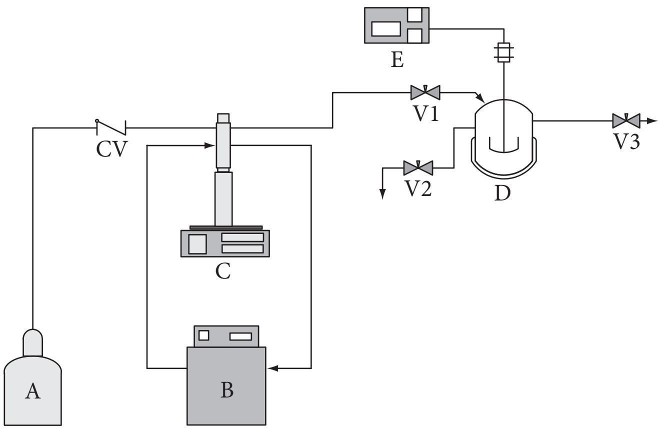

Figure 1. Schematic diagram of the experimental apparatus: a - solvent reservoir; b- thermostatic bath; C - syringe pump; D - reactor vessel; E - reactor control system; CV - check valve; V1, V2, and V3 - needle valves.

It may be important to mention that the samples were collected from the whole resulting medium at the end of each run after complete system depressurization.

3 Results and discussion

3.1 Effect of temperature

To evaluate the effect of temperature on the reaction conversion, the following reaction variables were kept fixed as follows: solvent/substrates mass ratio of 2:1, oil to ethanol molar ratio of 1:6, enzyme concentration of 5 wt%, and pressure of 50 bar. Figure 2 presents the results of these experiments.

One can see from this figure that low reaction conversions were obtained for the conditions studied. This trend is in agreement with that found by Knez et al. (1998) in the synthesis of oleyl oleate with Lipozyme IM in compressed gases from 40 to 60 °C under mild pressure conditions. The increase in temperature from 35 to 50 °C at constant pressure leads to a decrease in solvent density thus favoring diffusion process reducing mass transfer limitations. Possibly, above this temperature, the improvement in mass transfer was suppressed by over passing optimum enzyme activity condition.

3.2 Solvent to substrates mass ratio

The effect of solvent to substrates mass ratio was evaluated with 2:1 and 4:1 at 50 °C, oil to ethanol molar ratio of 1:6 and enzyme concentration of 5 wt% (by weight of substrates). As it can be seen in Figure 3, an increase in the solvent to substrates mass ratio from 2:1 to 4:1 led to a noticeable decrease in the reaction conversion indicating that the system is not mass-transfer limited at the condition investigated and that the interaction between enzyme particles and solvent is not of primary importance. This result may be of technological importance since a relatively small amount of solvent seems to be sufficient for conducting enzymatic production of biodiesel with clear advantage over organic liquid solvents (OLIVEIRA; OLIVEIRA, 2001).

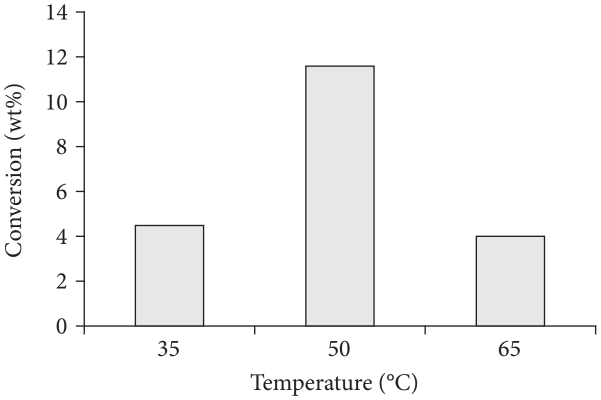

Figure 2. Effect of temperature on the ethyl esters production from soybean oil in propane medium with lipase from Yarrowia lipolytica.

3.3 Oil to ethanol molar ratio

The effect of oil to ethanol molar ratio on the reaction conversion was assessed keeping the temperature at 50 °C, solvent to substrates mass ratio of 2:1, and enzyme concentration of 5 wt%, varying the oil to ethanol molar ratio of 1:6 and 1:9. It can be seen in Figure 4 that the best conversion (~12%) was achieved at the molar ratio of 1:6, which means that higher values of alcohol to oil molar ratio seem to cause inhibition by ethanol.

3.4 Comparison with commercial lipases

Reactions were also accomplished using two commercial lipases (Amano PS and Amano AY 30) in the free and immobilized forms. The conditions were fixed at 50 °C, solvent to substrates mass ratio of 2:1, enzyme concentration of 5 wt%, oil to ethanol molar ratio of 1:6, agitation of 600 rpm, and 8 hours of reaction.

The results are presented in Table 1, where it can be observed that the use of free enzymes led to better conversion values compared to immobilized form. In addition, as in the case of lipase from *Yarrowia lipolytica*, low reaction conversions were

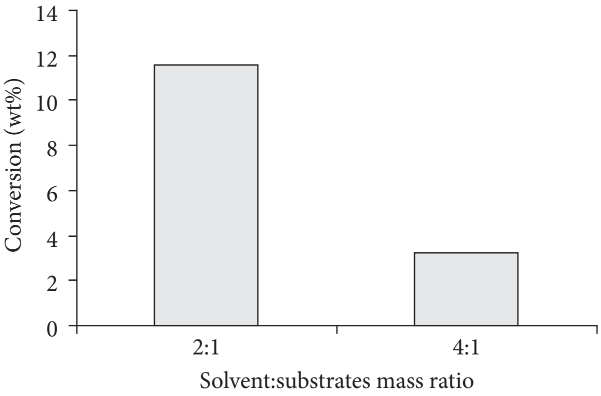

Figure 3. Effect of solvent/substrates mass ratio on the ethyl esters conversion from soybean oil with lipase from Yarrowia lipolytica.

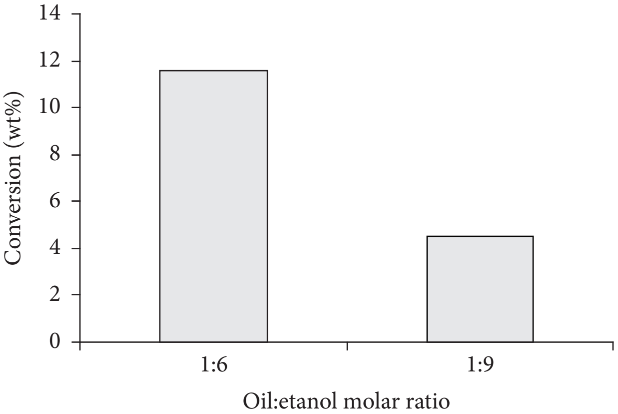

Figure 4. Effect of the oil to ethanol molar ratio on the ethyl esters conversion from soybean with lipase from Yarrowia lipolytica.

Table 1. Comparison of the performance of all lipases used in this work for biodiesel production.

Lipase	Conversion ± σ*
Immobilized lipase from *Yarrowia lipolytica*	11.61 ± 0.83
Free Amano PS	6.32 ± 1.32
Immobilized Amano PS	3.21 ± 0.67
Free Amano AY 30	8.17 ± 0.04
Immobilized Amano AY 30	5.13 ± 0.63

*Standard deviation.

obtained. In fact, measured values of esterification activity for all lipases tested in this work were one order of magnitude lower than those observed for Lipozyme IM and Novozym 435 in compressed propane (OLIVEIRA et al., 2006a; OLIVEIRA et al., 2006b). However, as enzyme stability and activity may depend on the enzyme species, characteristics of compressed fluid, water content of the enzyme/support/reaction mixture and process variables manipulated, it seems not possible a priori to provide safe predictions on the performance of a given enzyme submitted to pressurized fluid medium.

Furthermore, it was experimentally observed that almost complete enzyme inactivation occurred at the end of the reaction revealing a deleterious effect of the reaction medium, as a whole, on the enzyme activity. The fatty acid ethyl esters conversions obtained in this work can be considered negligible compared to those obtained by Oliveira and Oliveira (2000) and Oliveira and Oliveira (2001) when dealing with Lipozyme IM and Novozym 435 in the production of biodiesel from palm oil in supercritical carbon dioxide medium.

4 Conclusions

In this work, the effects of some process variables on the production of fatty acid ethyl esters from soybean oil in compressed propane were evaluated. Although low reaction conversions were obtained for all conditions and enzymes tested, further investigations on lipase-catalyzed transesterification of vegetable oils with different enzymes in pressurized propane should be performed. The reason is that according to a literature review while there is relatively abundant data regarding activity and stability of enzymes in carbon dioxide, there is a lack of corresponding experimental information for other fluids, like propane. This subject is in fact being studied by our working group using other commercial and non-commercial lipases as catalysts.

Acknowledgements

The authors are grateful for the financial support provided by CNPq, CAPES, and PETROBRAS.

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