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Enzymatic production of biodiesel from cotton seed oil using *t*-butanol as a solvent

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Abstract

The enzymatic production of biodiesel by methanolysis of cottonseed oil was studied using immobilized *Candida antarctica* lipase as catalyst in *t*-butanol solvent. Methyl ester production and triacylglycerol disappearance were followed by HPLC chromatography. It was found, using a batch system, that enzyme inhibition caused by undissolved methanol was eliminated by adding *t*-butanol to the reaction medium, which also gave a noticeable increase of reaction rate and ester yield. The effect of *t*-butanol, methanol concentration and temperature on this system was determined. A methanolysis yield of 97% was observed after 24 h at 50 °C with a reaction mixture containing 32.5% *t*-butanol, 13.5% methanol, 54% oil and 0.017 g enzyme (g oil)$^{-1}$. With the same mixture, a 95% ester yield was obtained using a one step fixed bed continuous reactor with a flow rate of 9.6 ml h^{-1} (g enzyme)$^{-1}$. Experiments with the continuous reactor over 500 h did not show any appreciable decrease in ester yields.

Keywords: Biodiesel; Lipase; *t*-Butanol; Novozym 435; Methanol; Cotton seed oil

1. Introduction

Reserves shortage and price increase are causing a growing substitution of fossil fuels with fuels derived from vegetable origin such as ethanol or biodiesel. This substitution requires increased efforts in the research and development of alternative methods of producing of these fuels from renewable resources. This is the case for production of alkyl esters (biodiesel) from vegetable oils, which can be carried out using different catalytic processes (Fukuda et al., 2001). In the last few years the study of the enzymatic synthesis of biodiesel, encouraged by pollution and by product separation problems of the chemical catalyzed process, have shown significant progress (Shimada et al., 2002; Zhang et al., 2003). The main problem of the enzyme catalyzed process is the high cost of the lipases used as catalyst. However, high operational stability of the enzyme was reported in several studies (Chen and Wu, 2003; Shimada et al., 2002; Watanabe et al., 2002), making its recycle possible in a batch system, or its long use in a continuous one, which reduces the incidence of catalyst cost.

Among the possible raw materials for the production of biodiesel in Argentina, cotton seed oil must be considered due to local marketing problems. The selected alcohol for the synthesis would be methanol because of its low cost. The enzymatic methanolysis of cottonseed oil was studied recently (Kose et al., 2002). However, in that study, a large quantity of enzyme was necessary to obtain reasonable biodiesel yields.

Short chain alcohols, specially methanol, have low solubility in oils, therefore a new liquid phase appears in the system at moderate concentrations leading to an inactivation of the enzyme and decreased yields of ester (Shimada et al., 1999). This problem was overcome by the stepwise addition of methanol, since the solubility of methanol in the alkyl esters is greater than in the oil, and consequently

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limits enzyme deactivation (Shimada et al., 2002). The low solubility of glycerol in biodiesel is also a problem; a deposit of glycerol coating the immobilized catalyst is formed during the process, which reduces the enzymes activity (Dossat et al., 1999). The enzymatic alcoholysis of triglyceride also was studied in petroleum ether, hexane and gasoline solutions (Foglia et al., 1998; Haas, 1997; Oliveira and Oliveira, 2001); however, the solubility of methanol and glycerol in these solvents is low and the above problems probably persisted. Iso et al. (2001) found an appreciable increase in the yield of methanolysis of triolein when using 1,4-dioxane as co-solvent, which dissolves methanol; however, a high proportion of this solvent (90%) was necessary to obtain a reasonable conversion. The use of solvents in the enzymatic production of biodiesel is mentioned as being inconvenient, but solvent recovery is a common practice in the chemical catalyzed production of biodiesel. Accordingly, on an industrial scale, solvents can be recovered together with methanol after the enzymatic reaction.

We found that when using *t*-butanol as a solvent the enzymatic process is improved. *t*-Butanol dissolves both methanol and the glycerol and is not a substrate for the lipases because it does not act on tertiary alcohols. Moreover, *t*-butanol is a non-toxic solvent of relative low cost. In this paper, results of methanolysis of cottonseed oil using *t*-butanol as a solvent and immobilized *Candida antarctica* lipase (Novozym 435) as catalysts are reported.

2. Methods

2.1. Materials

A neutral cottonseed oil from a local firm (Vicentin SAIC, Argentina) was used as substrate. Methyl ester standards (methyl linoleate, methyl palmitate and methyl oleate) and *p*-nitrophenylstearate (PNPS) were from SIGMA (USA); other chemicals and solvents were of the highest purity. The enzyme Novozyme 435, an immobilized *C. antarctica* lipase, was a gift from Novozymes Latin America Limited (Brazil). The enzymatic test-kit for measuring triglycerides came from Wiener Lab, Argentina. The test included the following enzymes: lipoprotein lipase for hydrolyzing the glycerides; glycerol kinase to transform glycerol into glycerol-1-phosphate; glycerol phosphate oxidase which produces H_2O_2, and peroxidase to produce a color reaction.

2.2. Hydrolytic activity enzyme

Hydrolytic activity of the lipase was measured using the chromogenic substrate *p*-nitrophenylstearate (PNPS) according to a modification of the method described by Vorderwulbecke et al. (1992). A substrate emulsion was prepared by adding dropwise with vigorous stirring of 10 ml of 1 mg ml^{-1} PNPS dissolved in isopropanol to 10 ml of a solution containing 0.5 g gum arabic and 0.5 ml Triton X-100 in 40 ml of 50 mM Na–citrate/citric acid buffer, pH 5.0. The activity assay was conduced by adding 10 mg of immobilized lipase to 3 ml of substrate emulsion and incubating under stirring at 50 °C. After 30 min, 200 µl of the suspension were added to 3 ml of 0.5 M NaOH and the absorbance at 405 nm measured. One unit of activity was expressed as the µmol of *p*-nitrophenol released min^{-1} (mg of lipase)$^{-1}$ under the reaction conditions. The effect of *t*-butanol on lipase activity was tested by replacing isopropanol with this solvent in the described assay.

2.3. Methanolysis of cottonseed oil

2.3.1. Discontinuous system

In this system the reaction mixture, impelled by a peristaltic pump from a mixing reservoir, was passed through a thermostated fixed bed column reactor (6 cm long × 1.5 cm wide) containing the immobilized enzyme. The mixture was then recycled at 15 ml min^{-1} to the reservoir. Samples were taken at the outlet of the column. Using this system the following studies were carried out:

1. Effect of solvent on methanolysis at 50 °C using 18 g oil, 300 mg enzyme and 5 ml methanol. The amount of *t*-butanol was varied from 0% to 32.5% (vol%).
2. Influence of methanol at 50 °C using 18 g oil, 300 mg enzyme and 12 ml *t*-butanol. The amount of methanol was changed between 1.2 and 5 ml.
3. Effect of temperature between 25 and 50 °C, taking 5 ml methanol, 12 ml *t*-butanol, 18 g of oil and 300 mg enzyme.

2.3.2. Continuous reactor

A thermostatized column (18 × 0.6 cm) containing a mixture of immobilized enzyme plus milled glass in a ratio of 1:1.5 (w:w) was used as the fixed bed reactor. The reaction mixture was impelled once through the column using a peristaltic pump at selected flow rates. Using this reactor the following experiments were conduced:

1. Effect of flow rate in the conversion of a mixture of 54% oil, 13.5% methanol and 32.5% *t*-butanol (vol%), using 1 g enzyme at 50 °C.
2. Test of the operational enzyme stability by circulating the above mixture at 9.6 ml h^{-1} over 500 h at 50 °C. Enzyme stability was tested by conversion measurements carried out at 120 h intervals.

2.4. Analytical procedures

Methyl esters (ME), triacylglycerols (TAG) and intermediate methanolysis products (mono- and di-glycerides) were registered by HPLC with a C18 Phenomenex column (25 cm × 4.6 mm, 5 µm particle size) using UV/Visible detection (Gilson 218) at 210 nm. Two different operational conditions were used: (1) Linear methanol/hexane gradients for elution; during the first 10 min the methanol concentration was changed from 100% to 90%, in the next 5 min it was reduced from 90% to 70%, maintaining this value over

15 min at a flow rate of 1 ml min^{-1} during the first 5 min and then at 1.5 ml min^{-1}. (2) Isocratic elution using methanol/hexane (85:15) as solvent at a flow rate of 1.2 ml min^{-1}. Elution order of components was: FFA, MG, ME, DG, and TAG.

Acid number of oil and biodiesel were determinated by titration according the ASTM D 664 technique.

The residual glyceride content of the methanolysis product was estimated using the enzymatic-test for triglycerides (TAG), which also quantifies glycerol liberated by hydrolysis of diacylglycerols (DAG) and monoacylglycerols (MAG). For sample analysis, the solvent and methanol were separated from the biodiesel by vacuum distillation and glycerol was removed by decanting the biodiesel from the lower glycerol layer.

Taking in account results discussed below, the oil conversion (TAG consumption) was calculated as the (percent) diminution of the sum of the areas of all TAG HPLC peaks, and the ME production yield as the fraction (percent) of the sum of the areas of all ME HPLC peaks relative to that of samples with maximum conversion, considering its residual glyceride content.

All the analytical determinations were duplicated; and results are reported as the average ± the standard deviation.

3. Results and discussion

To permit an easy separation of the biocatalyst from the reaction medium, which favors its recycling, the use of an immobilized lipase is essential in the enzymatic production of biodiesel. Novozym 435, a *C. antarctica* lipase supported on a macroporous resin, which shows a high activity and stability, in transesterification reactions, is frequently used in numerous applications and was selected for this study. The catalyst was used in a fixed bed reactor because this type of reactor must be employed in an eventual industrial application to avoid destruction of the supported lipase by harsh treatment (Watanabe et al., 2000).

3.1. Effect of t-butanol and methanol on cottonseed oil methanolysis

To detect any possible effect of *t*-butanol on the *C. antarctica* lipase, its hydrolytic activity on PNPS was measured using isopropanol and *t*-butanol as solvents under the same conditions. At 50 °C and pH 5 no appreciable difference was observed, the values being 19.7 ± 0.7 IU and 19.8 ± 0.5 IU respectively.

The HPLC chromatogram of cottonseed oil showed the presence of a single peak followed by two separate groups of unresolved TAG peaks. Methanolysis experiments indicated that the areas of all oil peaks decreased nearly at the same rate during the reaction, and consequently the relative reduction of the sum of all TAG peak areas was taken as a measure of oil consumption.

Due to its low solubility in triglycerides, undissolved methanol inhibits enzymatic methanolysis at methanol to oil molar ratios >1. Therefore, solvent-free reaction systems were operated with the stepwise addition of this alcohol (Shimada et al., 1999). As shown in Fig. 1, cotton seed oil methanolysis, was completely inhibited when the methanol to oil molar ratio was 6:1. By adding *t*-butanol to the medium, the conversion of oil to ester noticeably increased, with 28% *t*-butanol giving the best yield. Under the optimum conditions in Fig. 1, an oil conversion of 90% was observed after about 10 h. In blank experiments without methanol we confirmed that *t*-butanol was not a substrate of the *C. antarctica* lipase, since an alcoholisis of the oil was not observed.

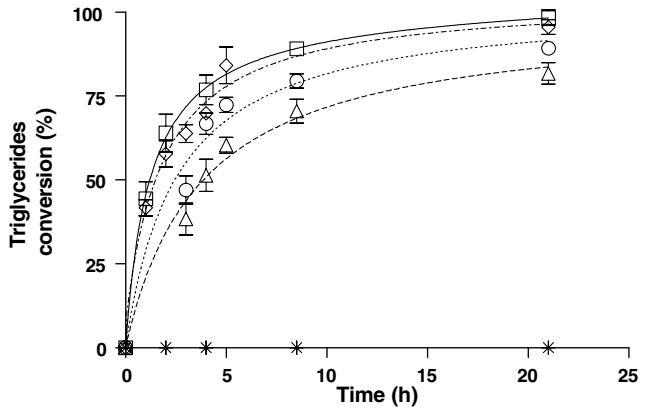

Fig. 1. Effect of *t*-butanol on oil conversion during the enzymatic methanolysis of cotton seed oil, using a discontinuous system with a 6:1 methanol to oil molar ratio. Reactions were performed at 50 °C with 18 g of oil and 300 mg enzyme; vol% of *t*-butanol in the mixture: 32.5% (□), 26.5% (◇), 19% (○), 11% (△), 0% (✳).

Immersion of lipases in *t*-butanol and other alcohols with carbon number ⩾3 was claimed as a pretreatment method to increase lipase activity in the synthesis of methyl esters (Chen and Wu, 2003; Wu and Chen, 2002). Therefore, it was not anticipated that *t*-butanol might gave the results shown in Fig. 1. In addition, this positive effect might be due to its ability to dissolve both methanol and glycerol (Dossat et al., 1999; Watanabe et al., 2000), thus avoiding the inhibitory effects produced on the lipase by these alcohols. The inhibitory effect of methanol is large at the beginning of the reaction, but with increasing oil conversion it decreases because its concentration decreases and its solubility is higher in the product methyl ester than in the triglyceride (Shimada et al., 1999). On the other hand, the inhibition due to the covering of catalyst by glycerol is absent at the beginning of the reaction and becomes larger at higher oil conversions.

In Fig. 2 it can be seen that in the presence of enough *t*-butanol, an increase in the oil initial consumption was produced when the methanol concentration was increased, and the best yields were obtained at a 3.6:1 molar ratio of methanol:oil.

3.2. Time course of oil methanolysis

The time course of the consumption of TAG under optimum conditions is shown in Fig. 3. It is seen that the oil

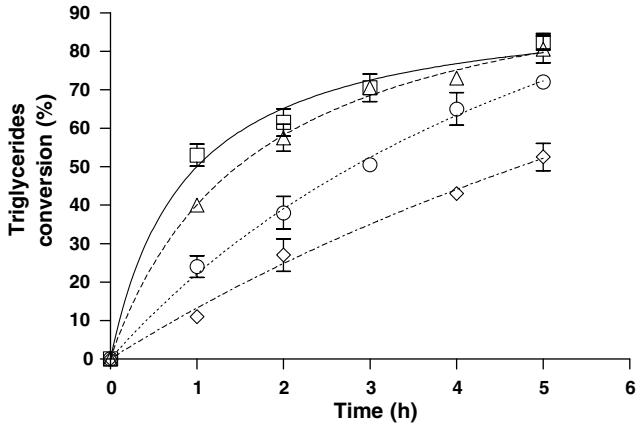

Fig. 2. Influence of the methanol to oil molar ratio on the initial rate of cotton seed oil conversion, using a discontinuous system, in the presence of *t*-butanol (0.67 ml (g oil)$^{-1}$). Reactions were performed at 50 °C with 18 g of oil and 300 mg enzyme. Methanol to oil molar ratio: 6 (□), 3.6 (△), 2.6 (○), 1.5 (◇).

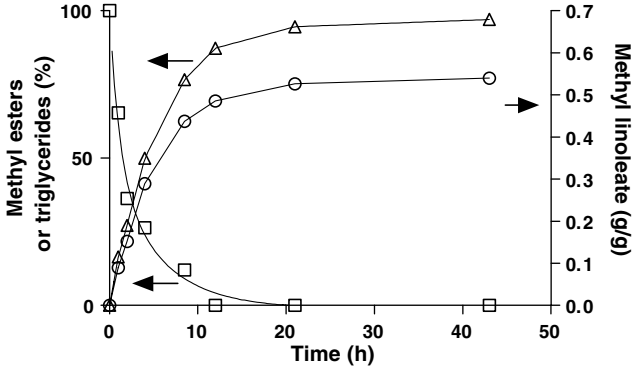

Fig. 3. Time dependence of triglycerides, methyl esters and methyl linoleate during methanolysis of cotton seed oil in the discontinuous system. Reactions were performed at 50 °C with a 6:1 methanol to oil molar ratio, 12 ml of *t*-butanol, 18 g of oil and 300 mg enzyme; (□) relative concentration of total TAG, (△) relative concentration of total ME (expressed as percent of the maximum value and considering the residual bounded glycerol), (○) methyl linoleate concentration g (g biodiesel)$^{-1}$.

consumption was almost complete after about 10 h. The time course of production of the methyl esters was also estimated by HPLC, the use of methyl ester standards indicated, as observed previously by Holapek et al. (2001), that methyl linoleate was detected as a single peak, whereas methyl oleate and methyl palmitate eluted together from this column. The relative concentration of the sum of all methyl esters also are plotted as a function of time, their maximum conversion was reached after about 24 h. Methyl linoleate concentration was also plotted in Fig. 3, its maximum concentration was 0.54 ± 0.03 g (g biodiesel)$^{-1}$, in accordance with the percent of linoleic acid (the most abundant fatty acid in cotton seed oil), which is about 50%. The relative rate of methyl linoleate production was comparable with that of the sum of the other methyl esters. The presence of intermediate products (MAG and DAG) was observed by HPLC, they eluted in the order observed by Holapek et al. (2001). During methanolysis they reached a maximum and then decreased to zero. The peaks of the second group were consumed in about 20 h, whereas total consumption of first intermediate peaks required a longer time, ≈30 h.

To determine if oil methanolysis was complete, the residual glycerides were determined enzymatically after removal of the free glycerol and solvent from the biodiesel. The method used was previously tested using oil samples. The obtained biodiesel had 1.4% bound glycerol (primarily as monoglycerides, as suggested by HPLC), and from this value it was estimated that the maximum yield of methyl esters was 97%.

Acidity increased slightly during the reaction. The neutralization number of cotton seed oil was 0.3 mg KOH g^{-1} and that of the final biodiesel was 1.1 mg KOH g^{-1}.

Using *C. antarctica* lipase in the enzymatic methanolysis of vegetable oils, Kose et al. (2002) and Watanabe et al. (2002) reported conversions >90%. The first authors achieved this yield after 7 h using cotton seed oil as substrate; however, they used a large amount of catalyst (30%). Watanabe et al. (2002) used soybean oil as substrate following a three-step methanol addition process to avoid inhibition of the lipase by undissolved methanol. They found that transesterification required about 48 h using 4% (w/w) immobilized lipase at 30 °C. With our *t*-butanol aided process ≈100% conversion was reached in about 24 h using 1.7% catalyst (oil weight %), at a temperature of 50 °C.

3.3. Influence of temperature on initial rate

The experimental results shown in Fig. 4 were used to determine the effect of temperature on the reaction rate, where v is the initial reaction rate expressed as mol of

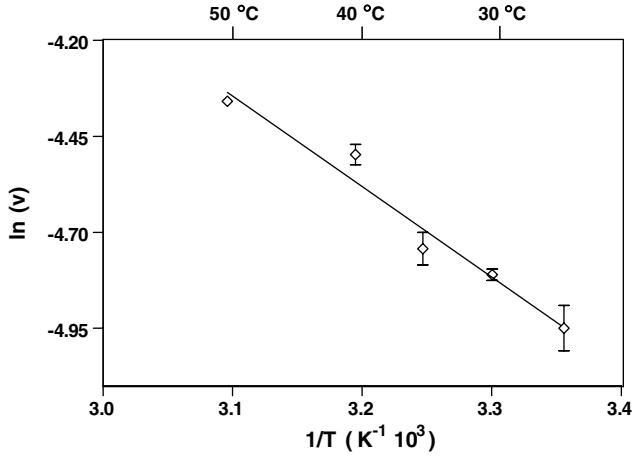

Fig. 4. Effect of the temperature on initial rate (mol L^{-1} (g enzyme)$^{-1}$ min^{-1}) of methyl linoleate production. Reactions were performed with a 6:1 methanol to oil molar ratio, 12 ml of *t*-butanol, 18 g of oil and 300 mg enzyme.

Table 1
Effect of flow rate on methyl esters production in the continuous reactor

Flow rate (ml h^{-1} (g enzyme)$^{-1}$)	Conversion ME[a] (%)
9.6	95[b]
12	74
14	60
18	53

t-butanol: 0.67 ml (g oil)$^{-1}$; methanol/oil molar ratio 6:1. Temperature 50 °C.
[a] ME: methyl linoleate + methyl oleate + methyl palmitate.
[b] Standard deviation of ME was lower than 2%.

methyl linoleate L^{-1} (g enzyme)$^{-1}$ min^{-1}. As the $\log v_i$ versus T^{-1} plot was linear, an "activation energy" of 19 ± 2 kJ/mol was calculated from these date, which corresponds to the direct reaction. Activation energy calculated from these data, was 19 ± 2 kJ/mol and corresponds to the reaction:

$$\text{TAG} + \text{MeOH} \rightarrow \text{ME (methyl linoleate)} + \text{DAG}$$

However, this was a lumped activation energy because TAG is a mixture of substrates, and it cannot be assigned to any rate constant because the reaction mechanism was not yet determined.

3.4. Continuous biodiesel production

The effect of flow rate on the oil conversion in the continuous reactor can be seen in Table 1.

The operational stability of the catalyst in the continuous process was tested at a methanol to oil molar ratio of 6:1, a solvent concentration of 32.5% and a flow rate of 9.6 ml h^{-1} (g enzyme)$^{-1}$, producing an oil conversion of 95%. This corresponds to a productivity of methyl ester of 4 g h^{-1} (g enzyme)$^{-1}$. The system was operated over 500 h without an appreciable loss in substrate conversion, which maintained a value of 95% during all the experiment. This is an essential result for the practical application of the process, since cost of the enzyme is high and its reuse appreciably reduces the final cost of the biodiesel.

Shimada et al. (2002) studied the continuous flow methanolysis of vegetable oil obtaining a 93% conversion at a flow rate of 6.0 ml h^{-1}. Considering the total amount of catalyst used in their system (three columns), the productivity referred to the mass of enzyme was significantly lower than that obtained with the *t*-butanol aided continuous reactor. Chen and Wu (2003) and Wu and Chen (2002) reported the results obtained with a continuous system with periodical regeneration of *C. antarctica* lipase by *t*-butanol washing. The productivity of this system was lower than that described here, their conversion was only 70%.

From our results it can be concluded that the use of *t*-butanol as a solvent in the enzymatic biodiesel production from cotton seed oil has the following advantages: (a) In the presence of this solvent, high reaction rates and yield are obtained. The quantity of enzyme needed to catalyze the reaction within a reasonable time periods is lower than that of other systems. (b) A very simple, one step continuous reactor can be used for the biodiesel production. (c) No catalyst regeneration steps are needed for lipase reuse. (d) The operational stability of the catalyst is high even at 50 °C.

The necessity of solvent recovery can be a draw back to the process. However, the following aspects should be considerer: *t*-butanol concentration for optimum conversion is not high and consequently the energy expense required for its recovery can be acceptable; solvent recovery is a common practice in the chemical catalyzed production of biodiesel and it is necessary in all cases to remove the excess methanol; the low boiling point of *t*-butanol makes for an easy separation of the solvent together with the methanol.

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