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Influence of alcohol: oil molar ratio on the production of ethyl esters by enzymatic transesterification of canola oil

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The influence of alcohol:oil molar ratio on the canola oil transesterification reaction in solvent-free medium using free lipase from *Thermomyces lanuginosus* and *Burkholderia cepacia* was studied. The experiments conducted in batch reactor for 72 h at 37°C in cosolvent-free reaction system with ethanol addition in three steps showed great potential for ester production. The stepwise addition of ethanol allowed increasing yield throughout the total period of the reaction, even if the course has limited reaction at times, minimizing possible deleterious effects of the alcohol on the enzyme structure. The highest yields were achieved with lipase from *T. lanuginosus*, despite presenting lower activity values than those of *Burkholderia cepacia* lipase, which proved to be less selective for ester production. In the reaction medium containing lipase from *T. lanuginosus*, 100 % yield was obtained using a molar ratio of 12:1. For *B. cepacia* lipase, the highest yield was 90.73% at a molar ratio of 6:1. In all cases studied, at least 92% of the triacylglycerols from canola oil were consumed.

Key words: Free lipase, *Thermomyces lanuginosus, Burkholderia cepacia,* molar ratio, solvent-free medium, transesterification reaction, triacylglycerols, diacylglycerols, monoacylglycerols, ester.

INTRODUCTION

A special kind of enzymes that can be used in the production of esters are the lipases, which are present in living organisms and are used for the hydrolysis and synthesis of triacylglycerols. The enzymatic route is preferred over the chemical route due to the environmental appeals of green chemistry, the possibility of esterifying oils with high fatty acids content, and also the feasibility of conducting the reaction under mild conditions of temperature and pH. In most ester production processes in the world, the alcohol used is methanol, as it is more reactive and reduces emulsification problems, that is, facilitates product purification. However, ethanol

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Abbreviations: HPLC, High performace liquid chromatography; **A,** ultra-pure water; **B,** acetonitrile; **C,** 5:4 (v/v) isopropanol:hexane; **TAG,** triacylglycerols; **DAG,** diacylglycerols; **MAG,** monoacylglycerols.

is more interesting from the viewpoint of technological sustainability, as long as it comes from renewable sources, while methanol is usually a petroleum derivative. In addition, ethanol is produced in large scale in Brazil.

In reactions that use inorganic catalysts, excess alcohol is used to ensure high conversion and minimize diffusional restrictions.

In enzymatic synthesis, however, excessive levels of alcohol can inhibit the enzyme and decrease its catalytic activity during the reaction. According to the study of Salis et al. (2005) higher alcohol:substrate ratio means higher polarity of the medium that may be associated with the inactivation of the biocatalyst or even the possibility of destabilizing the essential water layer of its catalytic site (Köse et al., 2002). In order to minimize the effects of enzyme deactivation by excess alcohol, Watanabe et al. (2001) addition into the reaction medium was performed stepwise. Given the above, this work was planned to investigate the influence of ethanol in the reaction medium on ethyl esters production by transesterification of canola oil in solvent-free medium, using free lipases from *Thermomyces lanuginosus* and *Burkholderia cepacia*.

MATERIALS AND METHODS

Reagents

Amano P.S. lipases from *B. cepacia* and *T. lanuginosus*, 2-phenethyl alcohol, vinyl acetate, 2-phenethyl acetate, and diisopropyl ether were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. Absolute ethanol 99.9% was from Cinética, Brazil. Commercial canola refined oil was purchased in a local supermarket. The reagents used in high performance liquid chromatography (HPLC) were n-hexane, n-propyl alcohol and acetonitrile (J.T. Baker). The standards tri-olein, di-olein, mono-olein and methyl-oleate were purchased from Accustandard. Other materials and reagents were of analytical grade.

Methods

Transesterification reaction

The reactions of ethyl esters synthesis were performed in a jacketed cylindrical batch reactor with a capacity of 50 mL of reaction medium, which contained the oil used in the experiment, ethanol at alcohol:oil molar ratios of 3:1, 6:1, 9:1, and 12:1, and free lipase (5 % of the oil mass). The reaction was performed for 72 h at 37°C at 250 rpm. Ethanol was added in three steps (equal amounts at the beginning, after 12 and 24 h).

The reaction sample were washed with hot water to inactivate enzyme, centrifuged, dried and storage at -20°C. Production of ethyl esters and consumption of triacylglycerols were monitored by HPLC.

Determination of transesterification activity by 2-phenethyl acetate production

Transesterification activity in the reaction of 2-phenethyl acetate formation using 2-phenethyl alcohol and vinyl acetate was measured by high performance liquid chromatography (HPLC, Varian 920-LC) with ODS-C18 column and UV-VIS detector at 254 nm, using acetonitrile and water (42:58) as mobile phase, room temperature, injection of 10 µL, and flow rate of 1.0 $mL.min^{-1}$. The reaction mixture was prepared by adding 0.6 mL of 2-phenethyl alcohol and 2.4 mL of vinyl acetate to 20 mg of the biocatalyst and kept under stirring for 20 min at 37°C at 200 rpm. The method of transesterification activity by 2-phenethyl acetate production was proposed by the supplier Amano P.S. as assay method of transesterification activity.

Analytical method

A combined linear gradient with aqueous/organic and non-aqueous phases, adapted from Holčapek et al. (1999) was used for HPLC. The total time of the gradient was 42 min, using ultra-pure water (A), acetonitrile (B), and 5:4 (v/v) iso-propanol:hexane (C). The method started with 70% A + 30% B, going to 100% A in 15 min and then to 50% A + 50% C in 11 min, followed by 10 min of isocratic elution with 50% A + 50% C.

Over the last 6 min the initial equilibrium condition was restored (70% A + 30% B). The column was operated at 40°C with UV-VIS detection at 205 nm, with injection of 20 µL and flow rate of 1 $mL.min^{-1}$. Quantification of triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), and ester was carried out by the sum of peak areas, with subsequent application in the calibration curves obtained with external standards. Triacylclycerol consumption and yield of product formation were determined by Equations 1 and 2, respectively,

$$C_T = \frac{\text{moles of TAG consumption}}{\text{moles of TAG initial}} \quad (1)$$

$$Y_i = \frac{\text{moles of product i formed}}{\text{moles of product i that can be formed}} \quad (2)$$

Where, C_T, is the Triacylclycerol consumption; Y_i, is the yield of product formation.

Ester production activity and triacylglycerol consumption activity were determined from the initial velocities of ester production and triacylglycerol consumption. A unit of activity was defined as the amount of enzyme required for producing 1 µmol of product per minute.

RESULTS AND DISCUSSION

Transesterification activity

It is difficult to characterize lipase enzymes activities since most enzymes carry out only one specific reaction (Babtie et al., 2010). Thus, the activity can be measured and compared by means of a single standard reaction. Ordinarily, lipases are promiscuous enzymes (Kapoor and Gupta, 2012), in other words, can catalyze both the hydrolysis of triacylglycerols and their formation. Thus it is difficult to characterize and compare the potential activity of lipases and prevents further discuss phenomena involved or compare lipases of different papers. Given

Table 1. Triacylglycerol consumption activity (A_{TC}), ethyl esters production activity (A_{EP}), consumption of TAG (C_T), and esters yield (Y_E).

Alcohol:oil molar ratio	*Thermomyces lanuginosus* lipase				*Burkholderia cepacia* lipase			
	A_{TC} (U/g)	A_{EP} (U/g)	C_T (%)	Y_E (%)	A_{TC} (U/g)	A_{EP} (U/g)	C_T (%)	Y_E (%)
3:1	9.44	22.15	100	86.81	36.50	106.70	95.07	60.78
6:1	8.52	22.42	100	99.94	22.50	20.04	100	53.28
9:1	11.79	59.62	100	97.38	30.54	110.46	95.64	67.26
12:1	3.49	20.14	100	97.66	18.88	19.01	95.95	54.54

this difficulty, Frédéric et al. (2000) reviewed a large number of methods to measure the hydrolytic activity of lipases, Sörensen et al. (2010) quantifies the activity by the method of 4-nitrophenyl acetate as well as Dhake et al. (2013) even though the methods used were different. Given the difficulty in characterizing, Chen et al. (2012) using two methods to quantify the activity of the immobilized lipase activity is a method of hydrolysis of olive oil and the other is the esterification of decanoic acid. Rodríguez-Contreras et al. (2012) also uses the method of hydrolytic activity equivalent to that used by Chen et al. (2012). In this work, lipase activity is measured by transesterification activity. Thus we can represent classes of reactions that are involved in this work.

The transesterification activity per unit mass of protein in the reaction of formation of 2-phenethyl acetate using 2-phenethyl alcohol and vinyl acetate were 73.93 and 284.16 U·g-1 for *T. lanuginosus* and *B. cepacia* lipases, respectively. Additionally, we chose to also discuss triacylglycerol consumption activity (A_{TC}) and ethyl esters production activity (A_{EP}), which is described in Table 1. Also, we construct an extensive discussion on the values of the enzyme in the reaction compared to the values of yield ester and triacylglycerol consumption.

Optimization of alcohol:oil molar ratio

The different behaviors shown by lipases from *T. lanuginosus* and *B. cepacia* can be observed in Figures 1 and 2, respectively, with variation of the amount of alcohol in the reaction medium. Figure 1A shows a rapid consumption of TAG up to 120 min, when there is a reduction in the consumption of these compounds. It is also possible to observe that the yield of esters and DAG grows markedly in the same period and then the reaction slows down, indicating that the stepwise addition of alcohol caused limitation of this reagent in the reaction medium, leading to a decrease in reaction rate. After 720 min of reaction, a new portion of ethanol was added and then the reaction resumed, but at lower speed. At the end of the tests, 86.81% ester yield was achieved, with complete consumption of TAG and only DAG (12.38%) and MAG (8.45%) remaining in the reaction medium. The same behavior is repeated in Figure 1B, but for the alcohol:oil molar ratio of 6:1 the decrease in reaction rate occurred after 240 min. It is important to note that even with low alcohol levels, between 240 and 720 min, discrete consumption of DAG forming MAG and esters was still observed. After the second addition of alcohol at 720 min, all reaction intermediates were rapidly consumed, reaching 99.94% ester yield, with little amounts of MAG and no DAG or TAG. Small amounts of TAG, DAG and MAG is important for application of esters as biodiesel, because these compounds must be removed in the purification step.

In Figure 1C, the reduction of both ester production and triacylglycerol consumption rates occurred at 360 min, but the rate of DAG production was exceeded by the rate of consumption after around 120 min. In this configuration, with alcohol:oil molar ratio of 9:1, no lack of alcohol was expected in the reaction medium, as the amount of alcohol added in the first step was enough for the reaction to be completed. That was not the case, though, as between 360 and 600 min the reaction showed a decrease in the rate of consumption of reagents. After the addition of more alcohol, triacylglycerol and diacylglycerol consumption was resumed and low amounts of intermediates were found after 1440 min of reaction. At the end of the experiment 97.38% of the esters had been formed. As for alcohol:oil molar ratio of 12:1 (Figure 1D), no decrease in reaction rate was observed and 360 min of reaction led to 99% of TAG consumption and 85.95% of ester yield. After 480 min, TAG were depleted and 97.97% of the esters had been formed. It is clear from Figures 1A, 1B, and 1C that within 12 h of reaction there was a lack of ethanol in the reaction medium, even with alcohol:oil molar ratio of 9:1 in which the minimum amount of alcohol was added early in the reaction. This indicates that the alcohol is not only a reactant of the reaction medium, but also plays the role of the solvent reaction medium. Indeed Avelar et al. (2013) which verified a yield reduction on the increasing of concentration of the oil in the reaction medium. This effect was attributed to aggregation of the oil droplets that eventually resulted in destabilization of the emulsion.

However, the lack of ethanol in the reaction medium did

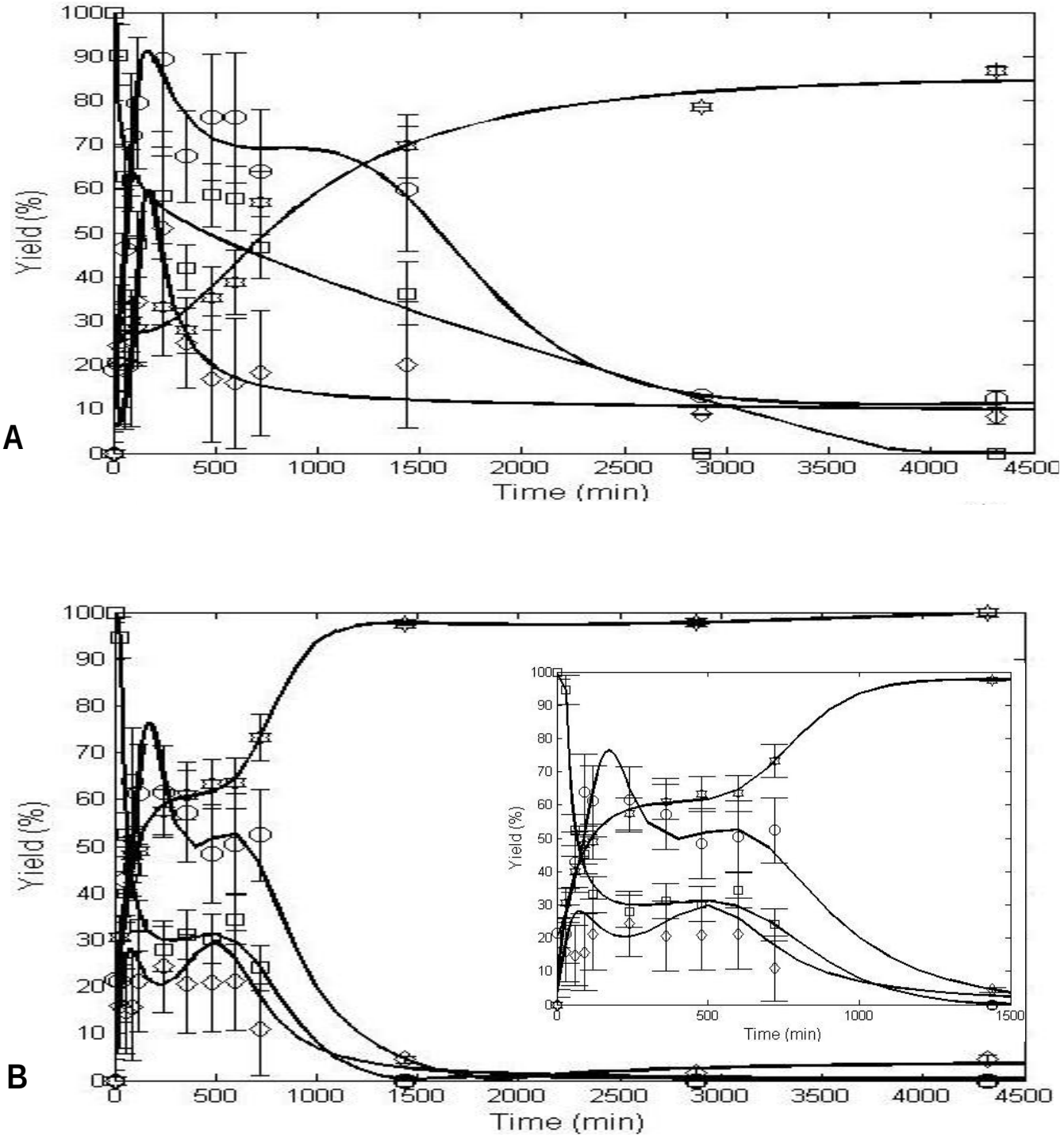
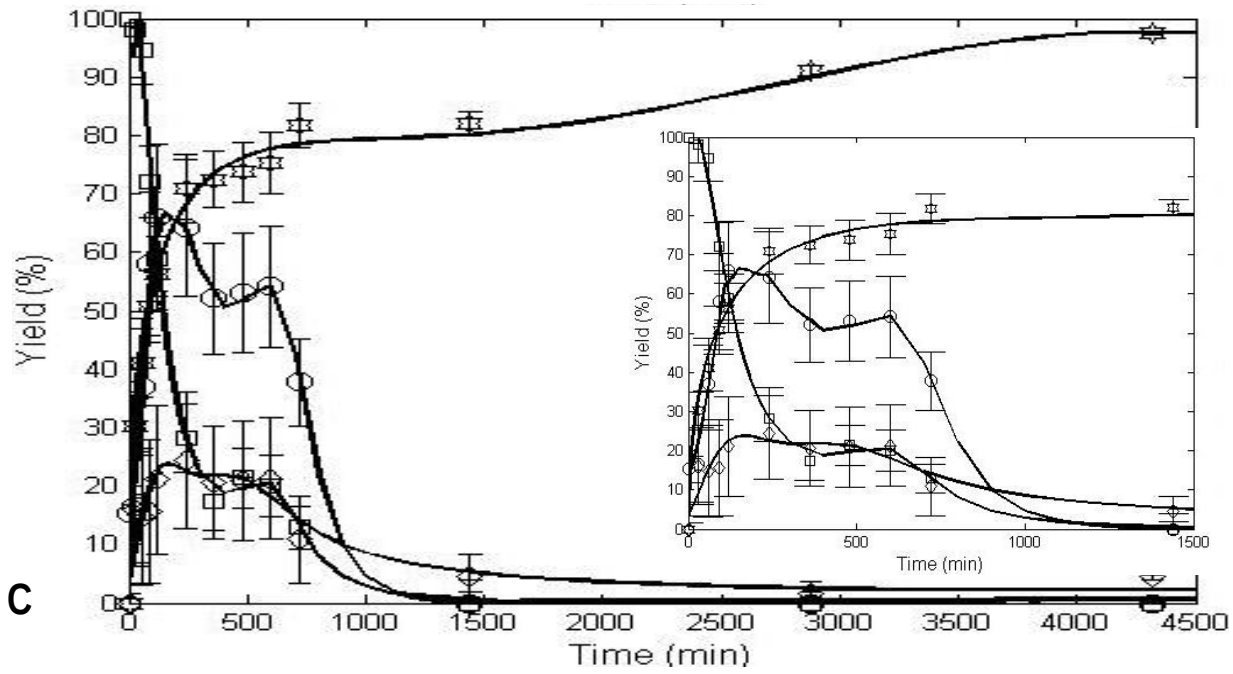
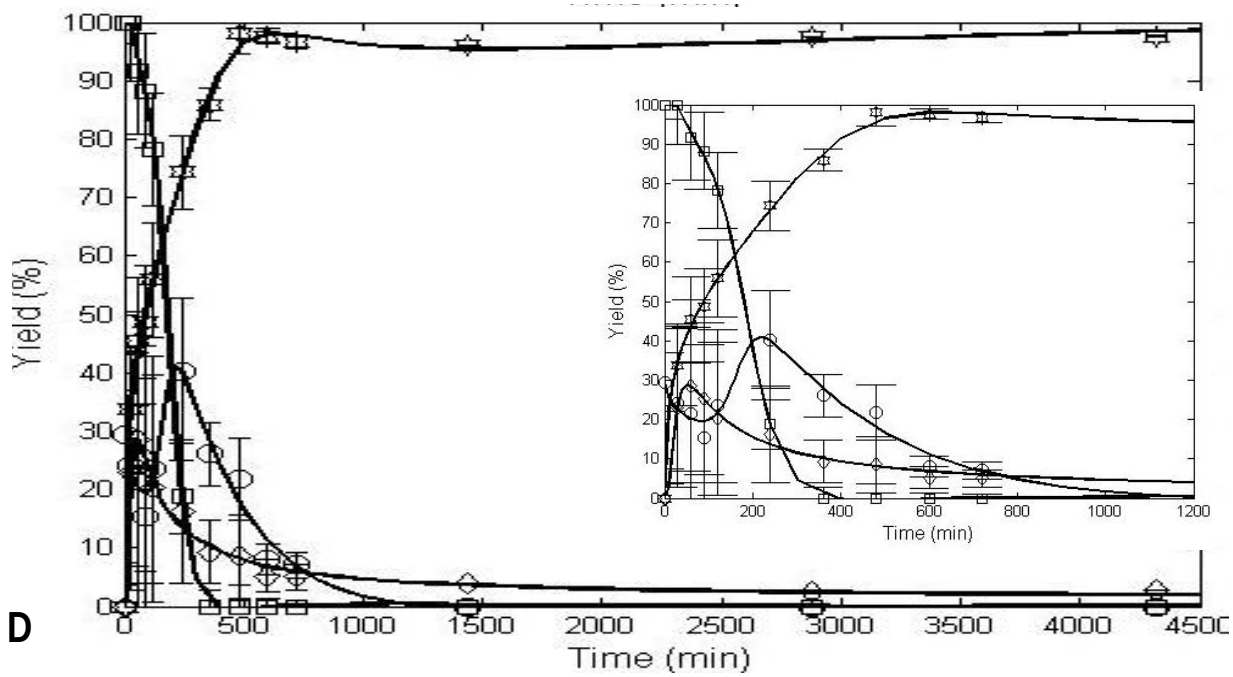


Figure 1. Yield profile of the transesterification reaction products using lipase from *Thermomyces lanuginosus*: Triacylglycerol (□), diacylglycerol (Δ), monoacylglycerol (○), and ethyl esters (◌). Alcohol:oil molar ratio: 3:1 (A), 6:1 (B), 9:1 (C), and 12:1 (D).

not affect the reaction yield because when new ethanol aliquots were added, all reactions proceeded. Figures 3 and 5 show that even after the second addition of alcohol the reactions with lower amounts of ethanol proceeded more slowly. It is also interesting to note in Figure 1 that the concentration of MAG was always smaller than that of DAG, indicating that the slow step of the reaction was the transesterification of DAG to esters and MAG. As a





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matter of fact, Shu et al. (2011) studying the kinetics of biodiesel production using a solid acid catalyst, found that transesterification is the slowest step in the reaction of ester production. The results for lipase from *T. lanuginosus* show that high alcohol:oil molar ratios provide better yields, and then high amounts of ethanol

rapidly promotes the reaction. However, ethanol should be used cautiously. Excess alcohol can cause deformation of the essential water layer that stabilizes the enzyme active site, or destabilization of the alcohol/oil interface where the catalytic action of the enzyme takes place (2, 4, 16). However, this was not observed in the results, since the production of esters increased throughout the test period for all analyzed conditions.

Verdugo et al. (2011) investigating the production of ethyl esters from sunflower oil with high fatty acid content using lipase from *T. lanuginosus* as catalyst obtained maximum ester yield of 70%. Studying biodiesel production from canola oil and methanol, Dizge et al. (2009) used *T. lanuginosus* lipase immobilized as catalyst obtained yield of 97% in 24 h batches at 50°C, with stepwise addition of alcohol, using molar ratio of 6:1. Dizge and Keskinler (2008) obtained a maximum yield of methyl ester of 90% at 40°C with alcohol:oil molar ratio of 6:1 and adding 0.1 g of water into the reaction medium. Comparing the results of the present study with those reported by the authors mentioned above, it is clear that the best ester yield (greater than 99%) was found with alcohol:oil molar ratio of 6:1. The results were consistent with those obtained by the aforementioned authors even with the use of free enzyme in the reaction medium, considering that the results were obtained at 37°C and after 72 h of reaction.

Ethyl ester production from *B. cepacia* is described in Figure 2. Fast consumption of TAG can be observed up to 720 min in Figure 2A. After that, reaction progress became very slow and by the end of the experiment, triacylglycerol consumption was 95%. Ester yield profile showed a very similar behavior, with fast production up to 31.84% yield at 360 min, followed by a slow production period which led to the maximum yield of 60.78% after 4320 min. Diacylglycerol production increased rapidly up to 240 min (60%) and decreased to 22.74% between 480 and 720 min. By the end of the experiment its value was 13.88%. MAG showed maximum yield of 10.08%. Figure 2B shows the rapid consumption of TAG between 30 and 720 min. After 2880 min of reaction they had been completely converted. Ester yield showed a sharp growth up to 480 min, followed by a slow evolution until reach 53.28% yield. The maximum yield of DAG was 48.37% after 600 min, and by the end of the experiment, its value was 10.53%. MAG showed a maximum yield of 23.67% and at the end of the reaction the yield was 19.61%. Figure 2D showed the highest yields of MAG, when compared with the other molar ratios, reaching a maximum yield of 33.48% after 1440 min and 20% at the end of the reaction. The maximum yield of DAG was of 43.96% at 480 min. TAG were rapidly consumed up to 1440 min, reaching 93%, and then remained constant until the end of the reaction. The highest yield of esters was found to be 67.26%. Analysis of Figure 2 shows that in all cases DAG yield profiles were higher than those of MAG, indicating that the slow step in the reaction was the conversion of DAG to MAG and ethyl esters, unlike what happens for the reaction using *T. lanuginosus*. All alcohol:oil molar ratios tested with lipase from *B. cepacia* showed equivalent ester yields, except the molar ratio 9:1, whose yield of 67% was slightly higher than the others. Alcohol:oil molar ratio also had little influence on the consumption of TAG; 100 and 96% were consumed for 6:1 and 12:1 molar ratios, respectively.

Studying a new strain of *B. cepacia*, Yang et al. (2007) conducted the transesterification of soybean oil at 40°C with methanol:oil molar ratio 3:1, 5% water, and 3% enzyme. The authors obtained up to 88% ester yield after 72 h of reaction. Da Ros et al. (2010) evaluated the catalytic properties of *B. cepacia* lipase immobilized for the synthesis of biodiesel at 50°C for 48 h with ethanol:oil molar ratio of 12:1 and using 20% of immobilized enzyme. The best ester yields obtained with babassu oil and tallow were 100 and 90%, respectively. Salum et al. (2010) produced biodiesel in fixed bed reactor with lipase from *B. cepacia* LTEB11 and obtained ester yield of 40% from soybean oil after 48 h of reaction at 37°C with alcohol:oil molar ratio of 6:1, and using n-heptane as cosolvent.

Ester yields obtained in the present work were lower than those reported by the above cited authors. However, the present results were obtained with no addition of cosolvent to the reaction medium. Lipase may be inhibited in the reaction of ethyl esters production by transesterification of TAG from oils when there is no addition of cosolvents (Royon et al., 2007; Véras et al., 2011; Hama et al., 2007; 2009; Fernandes et al., 2007). This inhibition occurs as short-chain alcohols and glycerol are capable of forming a hydrophilic coat on the surface of the enzyme that excludes the triacylglyceride from the active site (Véras et al., 2011; Hama et al., 2007; Adachi et al., 2011; Tan et al., 2010). The cosolvent is believed to reduce the interfacial tension that arises in solution, mainly due to increase of triacylglycerol and ethanol solubility (Véras et al., 2011; Tan et al., 2010). Faster reactions are then possible due to the higher stability of the active site.

Figures 3 and 4 show the rapid consumption of TAG up to 360 min for *T. lanuginosus* lipase and up to 720 min for *B. cepacia* lipase, which is quantified by the triacylglycerol consumption activity (A_{TC}) shown in Table 1. *B. cepacia* lipase was more efficient than that from *T. lanuginosus* in the conversion of TAG, as it presented higher values of A_{TC}. Table 1 also shows triacylglycerol consumption (C_T) for both enzymes. It is interesting to note that in spite of showing higher A_{TC} values, *B. cepacia* lipase presented lower C_T values than that from *T. lanuginosus*, except for the 6:1 molar ratio, in which the consumption was the same for both enzymes. Low activity values indicate hindering of the reaction progress by a deleterious effect on the structure of the enzyme.

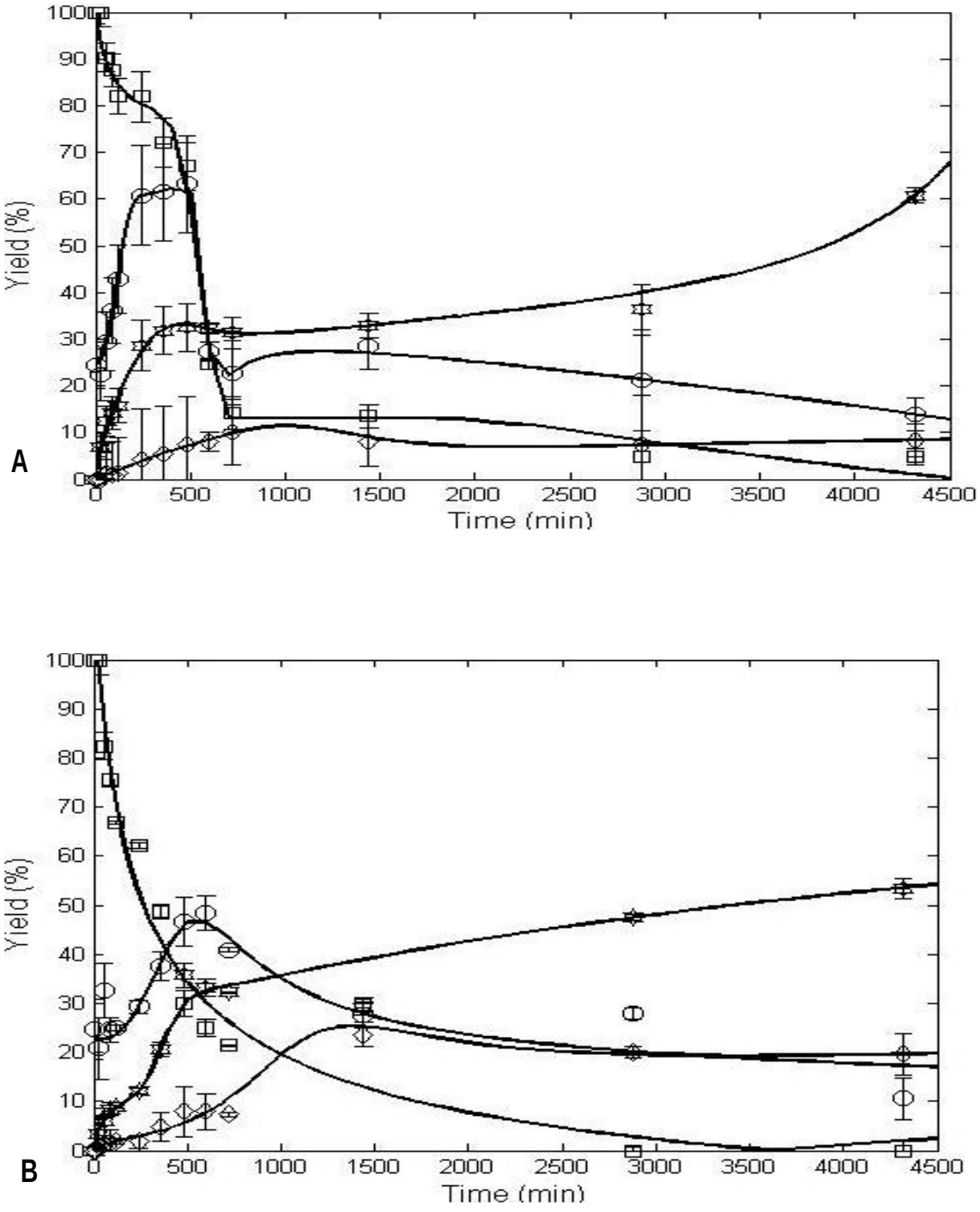
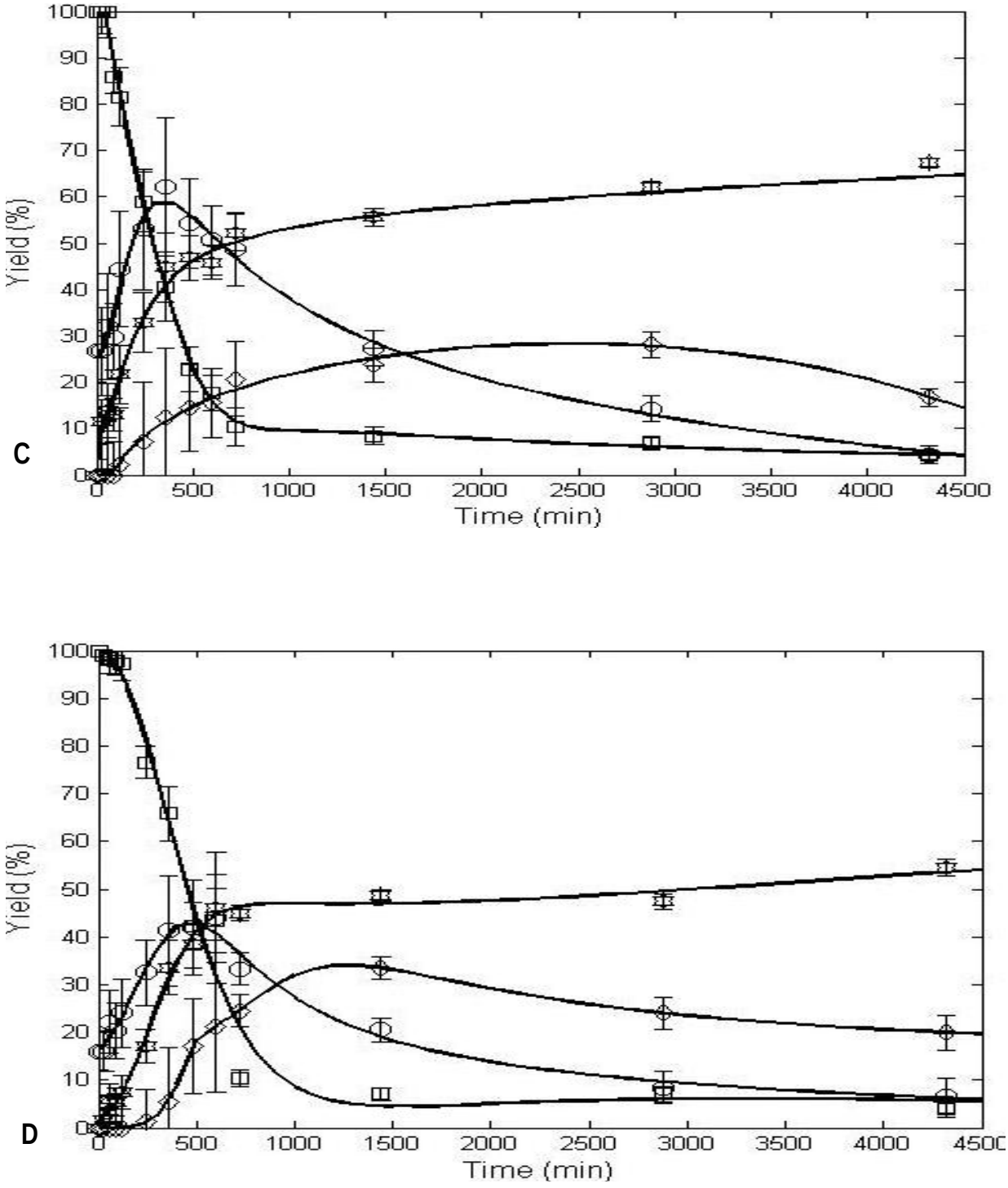


Figure 2. Yield profile of the transesterification reaction products using lipase from *Burkholderia cepacia*: Triacylglycerol (□), diacylglycerol (Δ), monoacylglycerol (○), and ethyl esters (۞).. Alcohol:oil molar ratio: 3:1 (A), 6:1 (B), 9:1 (C), and 12:1 (D).

This would result in a slower reaction, but the opposite behavior was observed; higher levels of triacylglycerol consumption were reached by the enzyme that showed lower activity. This suggests that the reaction medium inhibits the lipase from *B. cepacia* and not that from *T. lanuginosus*.

Figures 5 and 6 show ester yield progress during the reaction. There was stagnation in production for lipase



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from *T. lanuginosus*, probably due to lack of alcohol in the reaction medium because of the stepwise addition of this reagent. After addition of the second ethanol aliquot, the reaction proceeded normally, but with a decrease in reaction speed, except for the molar ratio of 12:1, in which the effect of rate reducing was not observed. When lipase from *B. cepacia* was used, no stagnation or reduction in reaction speed was observed. Interestingly,

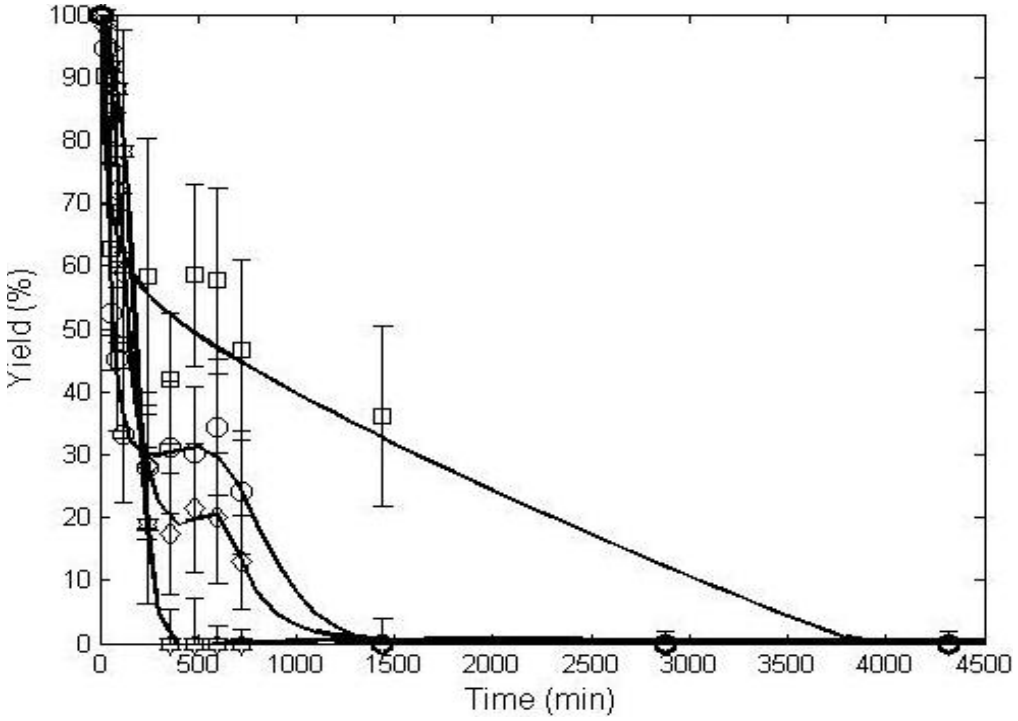

Figure 3. Consumption of triacylglycerols by *Thermomyces lanuginosus* lipase. Ethanol:oil molar ratio 3:1 (□), 6:1 (○), 9:1 (◊), and 12:1 (۞).

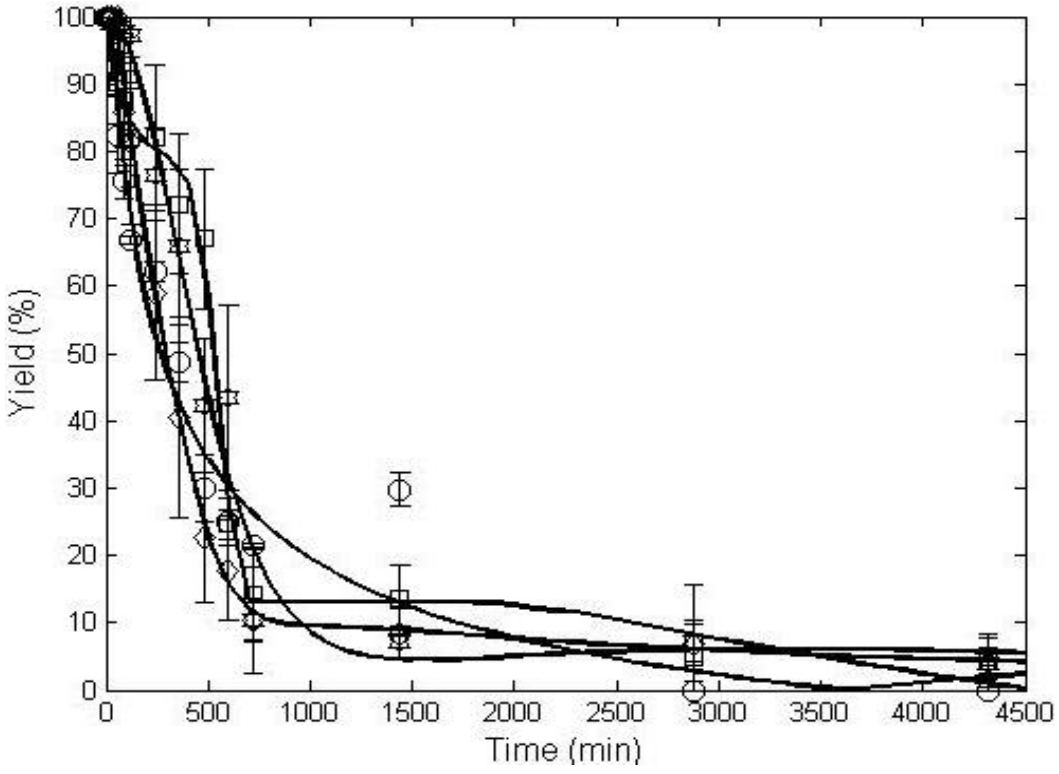

Figure 4. Consumption of triacylglycerols by *Burkholderia cepacia* lipase. Ethanol:oil molar ratio 3:1 (□), 6:1 (○), 9:1 (◊), and 12:1 (۞).

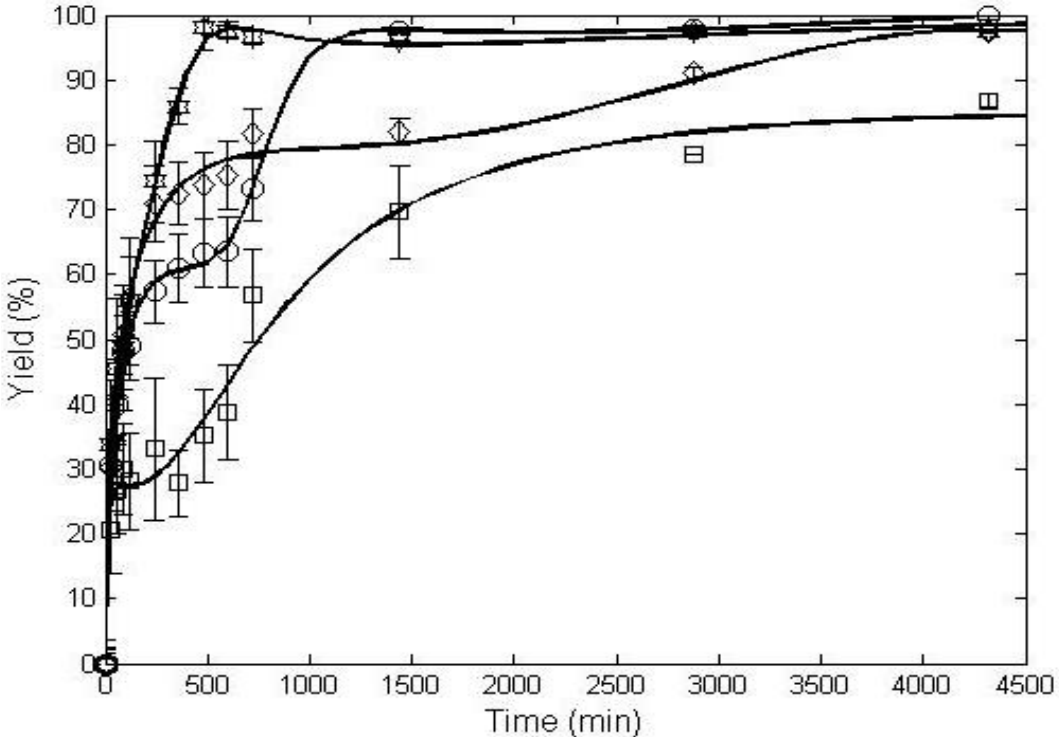

Figure 5. Ethyl esters production by *Thermomyces lanuginosus* lipase. Ethanol:oil molar ratio 3:1 (□), 6:1 (○), 9:1 (◊), and 12:1 (۞).

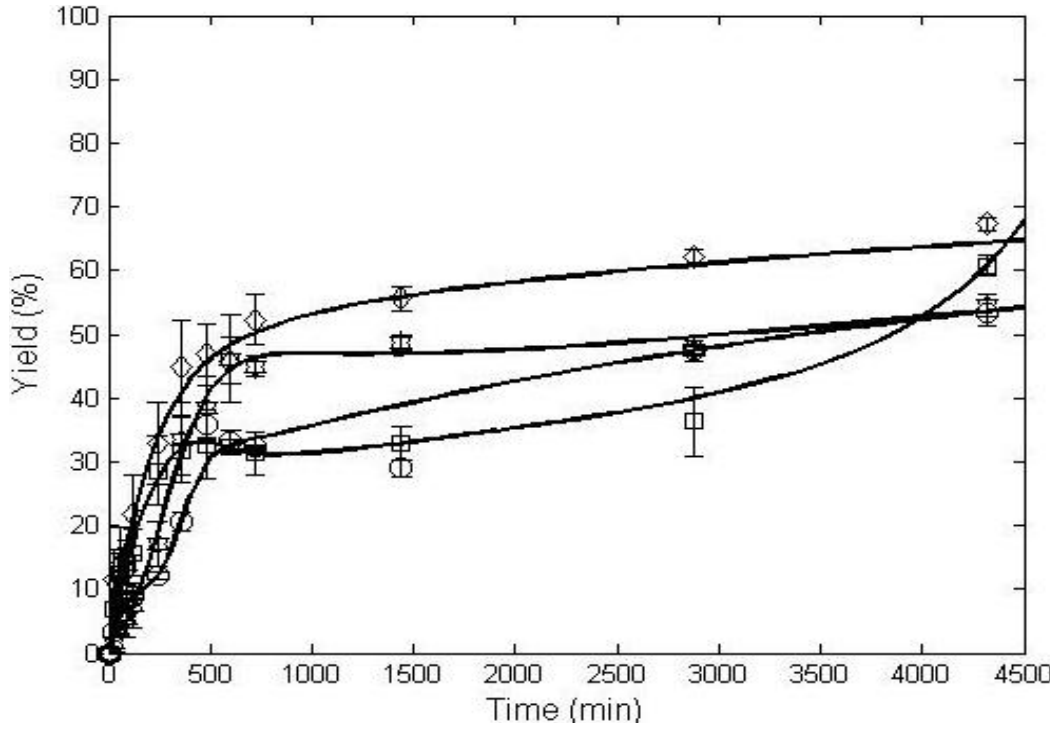

Figure 6. Ethyl esters production by *Burkholderia cepacia* lipase. Ethanol:oil molar ratio 3:1 (□), 6:1 (○), 9:1 (◊), and 12:1 (۞).

at alcohol:oil molar ratio of 9:1 with *B. cepacia* lipase, no lack of alcohol was observed in the reaction medium, as the minimum amount of alcohol required had been added in the first aliquot. On the other hand, this lack of alcohol was observed when using *T. lanuginosus* lipase, as shown in Figure 5, with a decrease in the rate of production of esters at around 500 min. This rate reduction suggests that the enzymes present different mechanisms of action, which is confirmed by the finding that the slow step in the reaction was the conversion of DAG to MAG and ethyl esters, unlike what happens for the reaction using *T. lanuginosus*. The effect of reaction rate reduction with *T. lanuginosus* lipase is also evident in Table 1, where higher ester production activity is observed for *B. cepacia* lipase than for *T. lanuginosus* lipase, except for alcohol:oil molar ratios of 12:1 and 6:1, which shows equivalent activities. Despite presenting lower activity values, *T. lanuginosus* lipase showed higher ester yields than *B. cepacia* lipase, as already observed for triacylglycerol consumption.

In a 2^2 factorial design experiment using *Burkholderia cepacia* lipase, Fernandes et al. (2007) observed little influence of alcohol:oil ratio in the reaction medium on the production of ethyl esters of corn oil at 37°C with the use of cosolvent. The yield changed very little with a twofold increase in the molar ratio. These results corroborate the indication that for *B. cepacia* lipase an increase in alcohol:oil molar ratio not necessarily leads to a significant increase in ester yield.

Conclusions

Lipase from *B. cepacia* was less selective for ethyl esters production than that from *T. lanuginosus*. The differences in efficiency of esters production may be directly related to the different reaction mechanisms of each enzyme. The slow step of the transesterification reaction was the conversion of monoacylglycerol to ester for *T. lanuginosus* lipase and the production of ethyl esters and monoacylglycerol from diacylglycerols for *B. cepacia* lipase despite the stepwise addition of ethanol to the reaction medium in order to minimize the effects of enzyme deactivation. The lower activity for ester production by *T. lanuginosus* lipase when compared with *B. cepacia* did not impair the progress of the reaction, with 99.94% ester yield for alcohol:oil ratio of 6:1. The highest yield achieved with lipase from *B. cepacia* was 67.26%, with molar ratio of 9:1. The conversion of triacylglycerols in the reaction medium with lipase from *T. lanuginosus* was complete in all conditions, while for the reaction medium using lipase from *B. cepacia* 100% conversion was achieved only with alcohol:oil molar ratio of 6:1. The results indicated the potential of lipase to produce esters in cosolvent-free media.

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