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Kinetics of Enzyme-Catalyzed Alcoholysis of Soybean Oil in *n*-Hexane

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Abstract

This work investigated the production of fatty acid ethyl esters (FAEEs) from soybean oil using *n*-hexane as solvent and two commercial lipases as catalysts, Novozym 435 and Lipozyme IM. A Taguchi experimental design was adopted considering the variables temperature (35–65°C), addition of water (0–10 wt/wt%), enzyme (5–20 wt/wt%) concentration, and oil-to-ethanol molar ratio (1:3–1:10). It is shown that complete conversion in FAEE is achieved for some experimental conditions. The effects of process variables on reaction conversion and kinetics of the enzymatic reactions are presented for all experimental conditions investigated in the factorial design.

Index Entries: Alcoholysis; soybean oil; immobilized lipases; reaction kinetics; biodiesel.

Introduction

The potential of using vegetable oil fuels as either a diesel fuel additive or replacement is well documented in the literature *(1–4)*. The merit of biodiesel as an alternative to mineral diesel is that is a nontoxic, biodegradable, domestically produced renewable source. In addition, biodiesel possesses a higher cetane number compared to diesel from petroleum and a favorable combustion emissions profile, such as reduced levels of particulate matter and carbon monoxide and, under some conditions, nitrogen oxides *(5,6)*. Because of these environmental benefits,

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which mean a reduction in environmental investments, and also because of reduced import needs, biodiesel fuel can be expected to be a good alternative to petroleum-based fuel.

Among other processes used for biodiesel production such as pyro lysis and microemulsification, transesterification is the most common way to produce biodiesel *(1,2)*. 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 gly cerol as a byproduct. Conventionally, transesterification can be performed using alkaline, acid, or enzyme catalysts *(1,2,5)*. Alkali-catalyzed systems are very sensitive to both water and free fatty acid contents, so the gly cerides and alcohol must be substantially anhydrous because water makes the reaction partially change to saponification, which produces soaps. This leads to consumption of the catalyst and reduction in the catalytic efficiency, as well as causes an increase in viscosity, formation of gels, and difficulty in separations *(1,2,5)*. It has been found that when basic catalysts are used, the water content in the reaction medium should be kept below 0.06 wt/wt% and the vegetable oil should have an acidic number <1 *(7,8)*. Although transesterification using acid catalysts is much slower than that obtained from alkali catalysis, typically 4000 times slower, if high contents of water and free fatty acids are present in the vegetable oil, acid-catalyzed transesterification can be used *(1,2)*.

Although 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, recovery of glycerol may be difficult, the acid or alkaline catalyst must be removed from the product, alkaline wastewater requires treatment, and free fatty acids and water interfere with the reaction *(1)*.

The use of enzyme-catalyzed transesterification methods can overcome these problems, because oils with a high acid content can also be used without a pretreatment and no enzymatic activity loss is observed. Furthermore, as stated by Fukuda et al. *(1)* in a review on biodiesel fuel production, the byproduct, glycerol, can be easily recovered without any complex process, and also the free fatty acids contained in oil and fat wastes can be completely converted into fatty acid esters.

Enzymes are generally effective biocatalysts owing to a high substrate specificity property, functional group specificity, and stereospecificity in aqueous medium. In addition, chemical reactions can be conducted directly using lipases in organic medium. As a consequence of such favorable characteristics, enzymatic production of biodiesel has recently attracted great interest because of its waste-free process *(9)*. Although at present the high cost of enzyme production is the major obstacle to commercialization of enzyme-catalyzed processes, recent advances in enzyme technology, such as the use of solvent-tolerant lipases and immobilized lipases, making

possible the reutilization of catalyst of, have been made to develop cost-effective systems *(1,10)*. In this sense, several researches have reported an alternative method to produce esters through enzymatic reactions using lipases as catalysts *(11–18)*. Because biocatalysts have high specific activity and a low impact on the environment, they have become increasingly important for industry. For example, immobilized lipases are used as catalysts for reactions involving biomodification of triglycerides *(19)*.

The establishment of the Brazilian National Program on Biodiesel and the expectation of commercial availability of the product within 2 yr throughout Brazil 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 *(1,2)*. However, in the Brazilian context, ethanol has been the natural choice because Brazil is the world's largest ethanol producer, with a well-established technology of production and large industrial plant capacity installed throughout the country, and because 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 afforded higher yields when compared with the use of methanol *(20,21)*.

Based on these aspects, the main objective of this work was to investigate the production of fatty acid ethyl esters (FAEEs) from soybean oil, a raw material available almost worldwide, using *n*-hexane as solvent and two commercial lipases as catalysts, Novozym 435 and Lipozyme IM. For this purpose, a Taguchi experimental design was adopted considering the variables temperature *(35–65°C)*, addition of water (0–10 wt/wt%, by weight of oil), enzyme concentration (5–20 wt/wt%, by weight of oil), and oil-to-ethanol molar ratio (1:3–1:10). An empirical model was built to assess the main and cross-variable effects on the reaction conversion, as well as to maximize the biodiesel production for each enzyme. Besides conversion values, the kinetics of the enzymatic reactions are presented for all experimental conditions established in the experimental design.

Materials and Methods

Soybean Oil and Equipment

Commercial refined soybean oil (Soya; São Paulo, SP, Brazil) was used as purchased without any pretreatment. The fatty acid composition was determined using a gas chromatograph (Agilent 6850 Series GC System). The following instrumentation and conditions were used: a capillary column (DB-23 Agilent, 50% cyanopropyl-methylpolysiloxane, 60 × 0.25 mm id × 0.25-μm film thickness), a split ratio of 1:50, and an injection volume of 1.0 μL. The column temperature was programmed from 175–215°C at 5°C/min. Helium was the carrier gas, and the injection and detector temperatures were 250 and 280°C, respectively. The chemical composition of

Table 1
Chemical Composition of Soybean Oil Used

Fatty acid	Composition (wt%)
Palmitic	11.30 ± 0.01
Stearic	3.48 ± 0.03
Oleic	23.63 ± 0.11
Linoleic	54.71 ± 0.07
Linolenic	6.88 ± 0.01

the soybean oil is presented in Table 1; the fatty acids content is very similar to the typical values reported in the literature *(2,3)*. Ethyl alcohol (95 v/v%) (Merck) and *n*-hexane PA (Merck) were used as the substrate and solvent, respectively.

Enzymes

Two commercial immobilized lipases were kindly supplied by Novozymes Brazil (Araucária, PR, Brazil): *Mucor miehei* (Lipozyme IM) immobilized on a macroporous anion-exchange resin (0.15 U/g and 4 wt% water) and *Candida antarctica* (Novozym 435) immobilized on a macro porous anionic resin (0.12 U/g and 1.4 wt% water). The optimum activities were achieved at 40ºC for Lipozyme IM and 70ºC for Novozym 435 *(22)*.

Analytical Method

FAEE samples were analyzed through a GC/MSD (Shimadzu QP5050A), using a capillary column DB-5 (30 × 0.25 mm id × 0.25-μm film thickness), a split mode (split ratio of 1:20), and an injection volume of 0.5 μL. The column temperature gradient programming was 200–300°C at 5°C/min. Helium was the carrier gas, and the injection and detector temperatures were, respectively, 290 and 300°C. The compounds were identified and quantified through the injection of authentic standards (ethyl palmitate, stearate, oleate, linoleate, and linolenate) (Sigma) and injection of squalene (Sigma) as the internal standard, followed by comparison of the mass spectra and gas chromatography retention times. Detection was done in SCAN mode (at 70 eV). All analyses were replicated at least three times.

Experimental Procedure and Statistical Analysis

The experiments were performed in stoppered 300-mL Erlenmeyer flasks. Lipase was added to the mixture of oil-ethanol-solvent, the flasks were agitated at 200 rpm for 8 h in a shaker with temperature control, and samples were taken at each hour in order to follow the course of the reaction. Reaction mixtures were then filtered and submitted to solvent evaporation at mild temperature under moderate vacuum up to constant weight.

Table 2
Experimental Design and Conversions Obtained in Enzymatic Alcoholysis of Soybean Oil

	Experimental conditions				Conversion (%)[a]	
Run	*T* (°C)	[*E*] (wt/wt%)	[*W*] (wt/wt%)	*R*	Lipozyme IM	Novozym 435
1	35	5	0	1:3	23.0	1.0
2	35	5	10	1:10	0.3	0.3
3	35	20	0	1:10	37.7	1.8
4	35	20	10	1:3	95.6	0.8
5	65	5	0	1:10	13.4	11.8
6	65	5	10	1:3	25.3	6.2
7	65	20	0	1:3	30.2	9.8
8	65	20	10	1:10	1.9	5.8
9	50	12.5	5	1:6.5	25.4	1.0

[a]Average conversion values refer to 8 h of reaction.

The remaining solution was diluted in 5 mL of *n*-propanol (analytical grade) to promote phase separation, with the upper FAEE-rich phase being analyzed in a GC/MSD system. Based on previous works *(22–24)*, *n*-hexane was used as the solvent medium in a fixed amount of 40 mL with the purpose of reducing mass transfer limitations, thus promoting an efficient contact between substrates (oil and ethanol). In this work a Taguchi experimental design was adopted with two levels and four variables: temperature (*T*) (35–65ºC), addition of water (*W*) (0–10 wt/wt%, by weight of oil), enzyme concentration (*E*) (5–20 wt/wt%, by weight of oil), and oil-to-ethanol molar ratio (*R*) (1:3–1:10). The variable ranges adopted, as can be seen in Table 2, were based on previous results for a similar system and were chosen to cover the intervals commonly used *(22–24)*. The experimental runs were executed randomly, and duplicate runs were carried out for all experimental conditions, leading to an overall average standard deviation in FAEE conversion of about 5%. The process conversion was then modeled by a statistical model. From the kinetic data, it was possible to verify that for almost all experimental conditions the maximum conversions were obtained in 8 h. Thus, this time was used for evaluating the influence of the studied process variables and conversion optimization.

Results and Discussion

The experimental results for Lipozyme IM, which exhibits specifi city in the 1,3 triglyceride positions, and Novozym 435, a nonspecific lipase, are presented in Table 2, for 8 h of reaction. It can be observed that much higher yields were achieved in the experiments with Lipozyme IM when compared with Novozym 435 (95.6 and 11.8%, respectively).

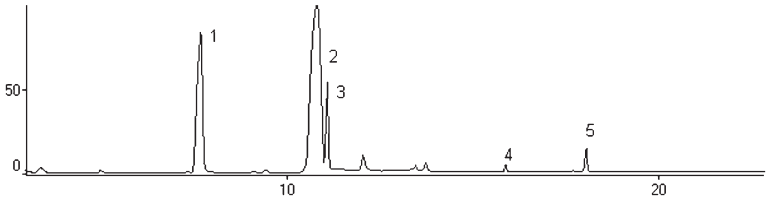

Fig. 1. Typical chromatogram obtained for lipase-catalyzed transesterification of soybean oil. Peak identification: 1, ethyl palmitate; 2, ethyl linoleate/oleate; 3, ethyl linolenate; 4, ethyl stearate; 5, squalene.

The enzymes exhibited different behavior probably because lipases gene rally have optimum working ranges and are affected mainly by the system temperature and water added to the reaction medium. In addition, the oil-to-ethanol molar ratio affected directly the process conversion, and the enzyme concentration had a positive effect on the ester production in the system catalyzed by Lipozyme IM. Furthermore, when Novozym 435 was used as catalyst, very low conversions were obtained. The results presented in Table 2 are similar to those presented by Oliveira and Alves *(22,23)* and Oliveira et al. *(24),* who performed lipase-catalyzed ethanolysis of palm and palm kernel oil and castor oil, respectively.

For the sake of brevity, Fig. 1 presents a typical chromatogram found for the biodiesel produced from soybean oil using Lipozyme IM (experimental condition 4). Figures 2 and 3 present kinetic curves obtained for the experimental conditions established on the factorial planning for each system studied up to 8 h of reaction. Undoubtedly, the knowledge of time evolution of the reaction plays an important role if one takes into account a possible scale-up of a continuous process.

Effect of Process Variables

The influence of the main variables—temperature, addition of water, enzyme concentration, and oil-to-ethanol molar ratio—as well as the cross interactions were investigated. To allow a direct comparison of each variable effect, the independent variables were normalized in the range of –1 to +1, according to

$$x_i = \frac{2(X_i - X_{\min})}{(X_{\max} - X_{\min})} - 1 \qquad (1)$$

in which x_i is the normalized value of the variable X at condition i; X_i is the actual value; and $X_{\min}$ and $X_{\max}$ are the lower and upper limit, respectively.

The "–1" level represents the lower limit, and the "+1" level represents the upper limit of each variable. A statistical modeling technique was used to obtain an empirical model able to reproduce the experimental data. Empirical models were built, the parameters related to each variable

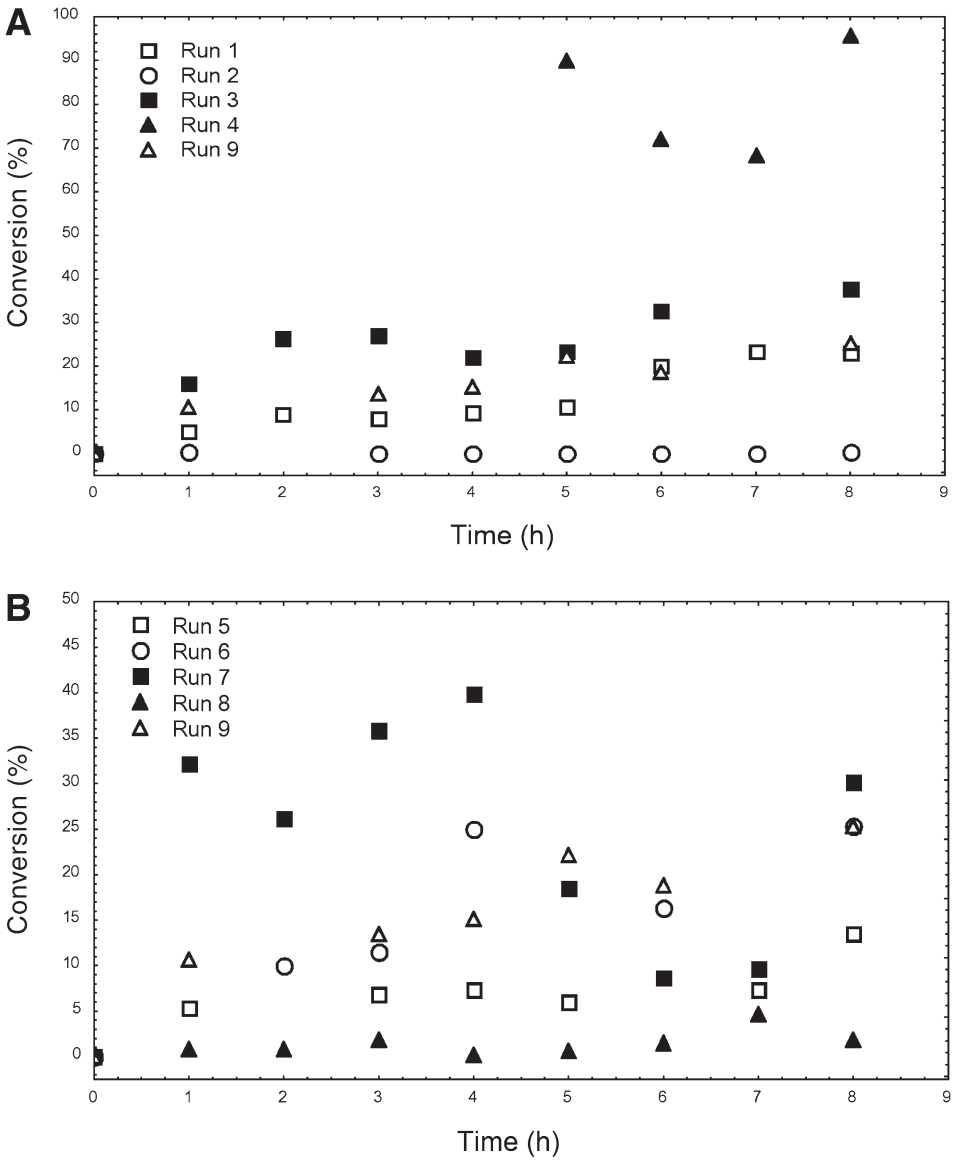

Fig. 2. Kinetics of alcoholysis of soybean oil catalyzed by Lipozyme IM in *n*-hexane: **(A)** runs 1–4 and 9; **(B)** runs 5–9.

interaction and main variable effects were estimated, and the meaningless parameters were discarded considering a confidence level of 95%, by using a student's '*t*'-test. The parameters were estimated using Statistica® 5.0 software (statsoft).

Reactions Catalyzed by Lipozyme IM

Regarding the system catalyzed by Lipozyme IM, one can note from Table 3 that the temperature and the oil-to-ethanol molar ratio, as well as

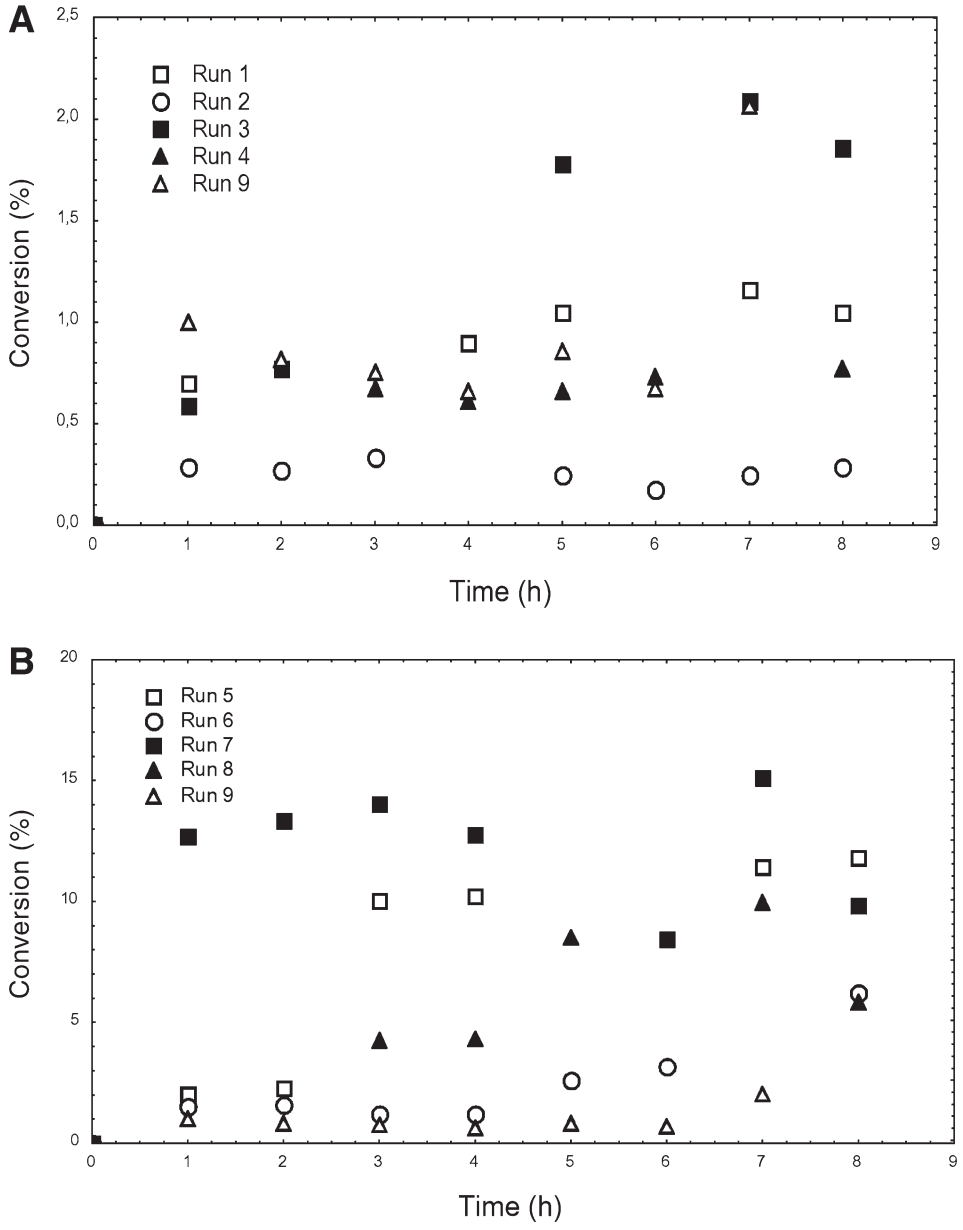

Fig. 3. Kinetics of alcoholysis of soybean oil catalyzed by Novozym 435 in *n*-hexane: **(A)** runs 1–4 and 9; **(B)** runs 5–9.

the interactions temperature-enzyme concentration and temperature-water concentration had a negative effect on the conversion. From these results, it can be concluded that an excess of ethanol may inhibit the enzymatic reaction, and the negative effect obtained for temperature corroborates the fact that for this enzyme the optimum temperature is about 40°C. Note also that in the range studied the enzyme concentration had a positive influence on

Table 3
Regression Results for Alcoholysis of Soybean Oil Using Lipozyme IM and Novozym 435 as Catalysts

Lipozyme IM: Conversion = $a_0 + a_1T + a_2E + a_3R + a_4TE + a_5TW + a_6TR$
Correlation coefficient = 0. 996

Effect	Parameter value	Parameter uncertainty
Independent	$a_0 = 28.09$	1.70
T	$a_1 = -10.73$	1.81
E	$a_2 = 12.93$	1.81
R	$a_3 = -15.10$	1.81
TE	$a_4 = -14.58$	1.81
TW	$a_5 = -6.45$	1.81
TR	$a_6 = 5.05$	1.81

Novozym 435: Conversion = $a_0 + a_1T + a_2W + a_3T^2 + a_4TE + a_5ER$
Correlation coefficient = 0.997

Effect	Parameter value	Parameter uncertainty
Independent	$a_0 = 1.00$	0.52
T	$a_1 = 3.71$	0.18
W	$a_2 = -1.41$	0.18
WW	$a_3 = 3.69$	0.55
TE	$a_4 = -0.46$	0.18
ER	$a_5 = -0.99$	0.18

biodiesel production. Once the effects of process variables were evaluated, optimization was carried out. For this system the optimum condition found from the experimental design was the same as observed for run 4 (see Table 2), with a conversion value predicted by the empirical model of 98.0%, which agrees satisfactorily well with the experimental one (95.6%).

Reactions Catalyzed by Novozym 435

From the results presented in Table 2, one can see that the experimental conversions obtained for the system catalyzed by Novozym 435 led to poor reaction yields. This fact can be associated with nonspecificity of the enzyme. In addition, it can be hypothesized that low conversions were owing to inactivation of lipase caused by the contact between the lipase and ethanol present in drops in the oil. Indeed, when the methanolysis of soybean oil was conducted with immobilized *Candida antarctica* lipase, the enzyme was inactivated irreversibly in the presence of more than 0.5 molar equivalents of methanol for the stoichiometric amount *(9)*. Table 3 reveals that the addition of water led to inhibition of the reaction. Concerning temperature, the result obtained confirms the fact that the optimum temperature for this enzyme is about 70°C. Note also that for this system no alcohol

inhibition was verified. Optimization of this system led to the following process variable values (not previously tested in the experimental design): T = 65°C, [E] = 5 wt/wt%, [W] = 0 wt/wt%, and R = 1:3, with a predicted maximum conversion of 12.1% in 8 h. The execution of the experiment at these conditions resulted in an experimental value of 9.1%, which is in reasonable agreement with the value predicted from the empirical model.

Conclusion

The use of an experimental design for the production of esters from the enzymatic reactions of vegetable oils proved to be a rational means to investigate the influence of process variables on the conversion. Empirical models were built to represent experimental data and to allow determination of the process variables that maximize the reaction conversion. The use of Novozym 435 as catalyst led to much lower conversions when compared with the systems using Lipozyme IM. These results are in agreement with those obtained by Oliveira and Alves *(22,23)* and Oliveira et al. *(24)*, who performed lipase-catalyzed ethanolysis of palm and palm kernel oil and castor oil, respectively. As already stated, this fact is probably associated with enzyme inactivation in this system. From the results obtained in our work and in the literature *(22–24)*, it can be concluded that the oil composition, the saturation level, as well as the physicochemical properties may interfere with enzyme activity and, accordingly, with the process conversion, affecting differently each system under investigation.

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References

1. Fukuda, H., Kondo, A., and Noda, H. (2001), *J. Biosci. Bioeng.* **92**, 405–416.
2. Ma, F. and Hanna, M. A. (1999), *Bioresour. Technol.* **70**, 1–15.
3. Srivastava, A. and Prasad, R. (2000), *Renewable Sustainable Energy Rev.* **4**, 111–133.
4. Altin, R., Çetinkaya, S., and Yucesu, H. S. (2001), *Energy Conversion Manage.* **42,** 529–538.
5. Zhang, Y., Dubé, M. A., McLean, D. D., and Kates, M. (2003), *Bioresour. Technol.* **89,** 1–16.
6. McCormick, R. L., Graboski, M. S., Alleman, T. L., and Herring, A. M. (2001), *Environ. Sci. Technol.* **35,** 1742–1747.
7. Wright, H. J., Segur, J. B., Clarck, H. V., Coburn, S. K., Langdon, E. E., and DuPuis, R. N. (1944), *Oil Soap* **21,** 145–148.
8. Ma, F., Clements, L. D., and Hanna, M. A. (1998), *Trans. ASAE* **41,** 1261–1264.
9. Watanabe, Y., Shimada, Y., Sugihara, A., and Tominaga, Y. (2002), *J. Mol. Catal. B* **17**, 151–155.
10. Iso, M., Chen, B., Eguchi, M., Kudo, T., and Shrestha, S. (2001), *J. Mol. Catal. B* **16,** 53–58.
11. Carta, G., Gainer, J. L., and Zaidi, A. (1995), *Biotechnol. Bioeng.* **48,** 601–605.
12. Abramowicz, D. A. and Keese, C. R. (1989), *Biotechnol. Bioeng*. **33,** 149–156.
13. Barzana, E., Karel, M., and Klibanov, A. M. (1989), *Biotechnol. Bioeng*. **34,** 1178–1185.
14. Brunt, J. V. (1986), *Biotechnology* **4,** 611–615.

15. Dordick, J. S. (1989), *Enzyme Microb. Technol.* **11,** 194–211.
16. Stevenson, D. E. and Storer, A. C. (1991), *Biotechnol. Bioeng*. **37,** 519–527.
17. Yamane, T. (1988), *Biocatalysis* **2,** 1–9.
18. Yamane, T., Ichiryu, T., Nagata, M., Ueno, A., and Simizu, S. (1990), *Biotechnol. Bioeng*. **36,** 1063–1069.
19. Basri, M., Ampon, W. M. Z., Razak, C. N. A., and Salleh, A. B. (1995), *J. Am. Oil Chem. Soc*. **72,** 407–411.
20. Mittelbach, M. (1990), *J. Am. Oil Chem. Soc*. **67,** 168–170.
21. Abigor, R., Uadia, P., Foglia, T., Haas, M., Jones, K., Okpefa, E., Obibuzor, J., and Bafor, M. (2000), *Biochem. Soc. Trans.* **28,** 979–981.
22. Oliveira, D. and Alves, T. L. M. (1999), *Appl. Biochem. Biotechnol.* **77–79,** 835–844.
23. Oliveira, D. and Alves, T. L. M. (2000), *Appl. Biochem. Biotechnol.* **84–86,** 59–68.
24. Oliveira, J. V., Di Luccio, M., Faccio, C., Dalla Rosa, C., Bender, J. P., Lipke, N. L., Amroginski, C., Menoncin, S., and Oliveira, D. (2004), *Appl. Biochem. Biotechnol.* **115,** 771–780.