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Enzyme Catalyzed Production of Biodiesel From Olive Oil

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Abstract

Biodiesel (fatty acid methyl esters) was produced by transesterification of triglycerides (triolein) present in olive oil with methanol and Novozym®435.

The effect of the molar ratio of methanol to triolein, semibatch (stepwise addition of methanol) vs batch operation, enzyme activity, and reaction temperature on overall conversion was determined. Stepwise methanolysis with a 3:1 methanol to triolein molar ratio and an overall ratio of 8:1 gave the best results. The final conversion and yield of biodiesel were unaffected by initial enzyme concentrations greater than 500 U/mL olive oil. The optimum reaction temperature was 60°C.

Comparison of conversion data between a test-tube scale reactor and a 2-L batch reactor revealed that the difference in conversion was within 10%. Experiments were also carried out with used cooking oil; the conversion with used cooking oil was slightly lower but no major differences were observed.

The efficacy of Novozym435 was determined by reusing the enzyme; although the enzyme's relative activity decreased with reuse, it still retained 95% of its activity after five batches and more than 70% after as many as eight batches.

Index Entries: Biodiesel; Novozym435; lipase.

Introduction

Biodiesel is a diesel fuel substitute produced from renewable triglyceride sources such as vegetable oils, animal fats, and recycled cooking oils. Chemically, it is defined as the mono alkyl esters of long-chain fatty acids derived from lipid/triglyceride sources. Biodiesel has become more attractive because of its environmental benefits. The chemical processes for

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production of biodiesel are well known. It is most commonly produced by reacting lipids (triglycerides) with a primary alcohol (e.g., methanol) and a base (sodium hydroxide). The reaction, known as transesterification, results in the production of biodiesel and glycerine. However, new biochemical routes to biodiesel production, based on the use of enzymes, have become very interesting *(1–6)*. The latter enzymatic route to produce biodiesel is the primary focus of this article. Most of the articles published have used a variety of substrates such as rice bran oil, canola, sunflower oil, soybean oil, and castor oil. In this article, the results of biodiesel production by transesterification of olive oil using lipase as a catalyst are reported.

The focus of this article is to determine the optimal conditions for transesterification so as to ensure the enzyme functions under operating conditions that minimize enzyme denaturation. The long-term performance of Novozym®435 during transesterification is evaluated. The reaction performance with used cooking oil is also reported. Finally, preliminary results of scale-up are discussed.

Previous experiments conducted with other types of alcohols, oils, and lipases are presented in Table 1 to allow for possible comparisons. Olive oil constitutes an interesting raw material because it is available in plenty in Mediterranean countries such as Spain and Italy and is in growing use in the United States.

Materials and Methods

Materials

Lipase B from *Candida antarctica* adsorbed onto a macroporous resin (Novozym435) was purchased from Sigma-Aldrich (St. Louis, MO). Pure, all natural, classic olive oil (Hannaford brand) was used in all the experiments. Hexane, isopropanol, and methanol were the elution solvents utilized during the analysis with high-performance liquid chromatography (HPLC). All three of them were HPLC grade and met American Chemical Society (ACS) standards. Hexane was obtained from Burdick and Jackson (Muskegon, MI), whereas methanol and isopropanol were purchased from Fisher Scientific (Fair Lawn, NJ). Methanol and hexane were also used as reactant and solvent, respectively, in the transesterification reactions.

Analytical Method

The standards, triolein, oleic acid, and oleic acid methyl ester were obtained from Sigma-Aldrich. The concentrations of both triolein and oleic acid methyl oleate were measured with a Hewlett Packard HP 1050 HPLC coupled to a ultraviolet (UV) detector. The HPLC unit contained a reverse phase, C_{18}, HPLC column. The analysis technique itself was developed experimentally. A wavelength of 230 nm was used in all the runs. Table 2 shows the gradient elution method that was used in the analysis. This method gave the best separation possible. In the analytical procedure, 100 µL of the

Table 1
Enzymatic Transesterification Using Different Alcohols and Lipases

Oil	Alcohol	Lipase	Conv. (%)	Solvent	Ref.
Rapeseed	2-Ethyl-1-hexanol	C. rugosa	97	None	*3*
Mowrah, mango, kernel, sal	C_4–C_{18} alcohols	M. miehei (Lipozyme IM20)	86.8–99.2	None	*3*
Sunflower	Ethanol	M. miehei (Lipozyme)	83	None	*3*
Fish	Ethanol	C. antarctica	100	None	*3*
Recycled restaurant grease	Ethanol	P. cepacia (Lipase PS-30) + C. Antarctica (Lipase SP435)	85.4	None	*3*
Tallow, soybean, rapeseed	Primary alcohols[a]	M. miehei (Lipozyme IM60)	94.8–98.5	Hexane	*3*
	Secondary alcohols[b]	C. Antarctica (Lipase SP435)	61.2–83.8	Hexane	
	Methanol	M. miehei (Lipozyme IM60)	19.4	None	
	Ethanol	M. miehei (Lipozyme IM60)	65.5	None	
Sunflower	Methanol	P. fluorescens	3	None	*3*
	Methanol		79	Pet ether	
	Ethanol		82	None	
Palm kernel	Methanol	P. cepacia (Lipase PS-30)	15	None	*3*
	Ethanol		72	None	
Canola	Methanol	Novozym 435	97.9	Water	*1*
Soybean	Methanol	P. cepacia Lipase	67	Water	*5*
	Ethanol		65		
Castor	Ethanol	Novozym 435	81.4	Hexane	*2*
		Lipozyme 1M	<98		

[a]Methanol, ethanol, propanol, butanol, and isobutanol.
[b]Isopropanol and 2-butanol.

Table 2
Gradient High-Performance Liquid Chromatography Method

Time (min)	Methanol (%)	Isopropanol (%)	Hexane (%)	Flow rate (m^3/s)
0	100	0	0	2.5e–9
19	100	0	0	2.5e–9
20	100	0	0	8.33e–9
35	64	20	16	8.33e–9
46	64	20	16	8.33e–9

sample was withdrawn and solvated with 1.2 mL of isopropanol. This was filtered through a 0.2- μL filter and then injected into the HPLC. Each run took about 50 min to complete. Standards were used to determine the retention times of the reactants and products and to establish a calibration curve. Conversion was calculated from the decrease in the triolein peak.

Experimental Setup

Conversion studies were performed in a batch reactor. The reactor consisted of a glass vial with a poly butyl terephthalate (PBT) open-top cap and a teflon-faced silicone septum. The vial measured 0.025 m in diameter by 0.057 m in height. The vial was placed vertically in the middle of a 0.0006 m^3 Pyrex beaker and held in a water bath by a Styrofoam ring. The beaker was set on top of a VWR Scientific Series 400HPS hot plate/stirrer. A crosshead magnetic stirring bar was used to stir the reactants.

Reaction Procedure

Triolein conversion was analyzed every 2.5 h for the first 10–12.5 h of the reaction. A final sample was drawn after 20–30 h to check for final conversion.

A parametric study was undertaken to determine the effect of different variables on conversion. The variables investigated were molar ratio of methanol to triolein, semibatch (stepwise addition of methanol) vs batch operation, mass of catalyst, and reaction temperature.

The following were the reaction conditions during the investigation of the influence of methanol to triolein molar ratio on conversion: 40°C, 100 rpm, and 500 U enzyme/mL oil. The amount of methanol was adjusted to achieve molar ratios of 2:1, 3:1, 4:1, 6:1, and 8:1, respectively. Unless stated otherwise, the volumes of hexane (4 mL) and oil (1 mL) remained constant.

While studying the effect of stepwise addition of methanol, the following reaction conditions were used: 40°C, 100 rpm, and 500 U enzyme/mL oil. During the two-step addition, 72 μL of methanol each were added to the reactor at times equal 0 and 5 h. In the four-step addition, 36 μL of methanol each were added at times 0, 2.5, 5, and 7.5 h.

To ascertain the influence of the amount of catalyst utilized on conversion, the following reaction conditions were maintained constant: 100 rpm, 60°C, and 144 µL of methanol addition. The enzyme activities in the runs were: 0, 500, 1000, 2000, and 3000 U enzyme/mL oil.

To determine temperature effects, experiments were conducted at 30, 40, 50, 60, and 70°C. Other reaction conditions such as mixing speed, catalyst amount, and methanol addition were maintained at 100 rpm, 500 U enzyme/mL oil, and 72 µL, respectively.

Next, experiments were conducted to determine the extent of enzyme deactivation. In this set of experiments, the same enzyme loading was used. Other reaction conditions were: 60°C, 1000 U enzyme/mL oil, 144 µL of methanol, and 100 rpm. A new batch of reactants was introduced every 24 h.

Conversion of used cooking oil was determined under the following conditions: 60°C, 1000 U enzyme/mL oil, 144 µL of methanol, and 100 rpm.

Scale-up studies were performed in a 2-L Virtis jacketed glass reactor. The reactants mixture consisted of 800 mL of hexane, 200 mL of oil, and 28.8 mL of methanol. These reactants were kept at 60°C, 100 rpm, and with 500 U enzyme/mL oil. Reaction conditions and reactants concentrations were the same as in the small-scale reactor.

Results and Discussion

Effect of Molar Ratio of Methanol to Triolein

Methanol plays a critical role in this reaction and its concentration has a considerable effect on the rate at which transesterification proceeds. In general, the reaction proceeds according to the following stoichiometry:

$$1\ \text{Triglyceride} + 3\ \text{Alcohol} \rightarrow 3\ \text{Biodiesel} + 1\ \text{Glycerol}$$

Even though methanol is a reactant, it also inhibits the enzyme *(1,3)*. Previous research indicates that in mixtures containing more than three molar equivalents of methanol with respect to triolein, Novozym435 starts to show deactivation *(3)*.

Figure 1 shows triolein conversion for different methanol to triolein molar ratios. The initial rates (slopes of the curves during the first 2.5 h) at ratios 4:1, 6:1, and 8:1 are almost identical, but lower than the rate corresponding to a ratio of 3:1. This indicates that the extent of inhibition of Novozym435 by methanol is limited and remains constant for methanol to triolein ratios greater than 4:1 (for the reaction conditions employed). Another interesting observation is that methanol in excess of the stoichiometric amounts leads to higher final conversions of triolein. This again is not a surprising result and may be attributed to the presence of other components in olive oil (such as mono- and di-glycerides) that also compete for methanol. Presence of other products (not identified) was revealed during analysis. Thus when methanol is present in excess, there is enough reactant available for almost complete conversion of triolein.

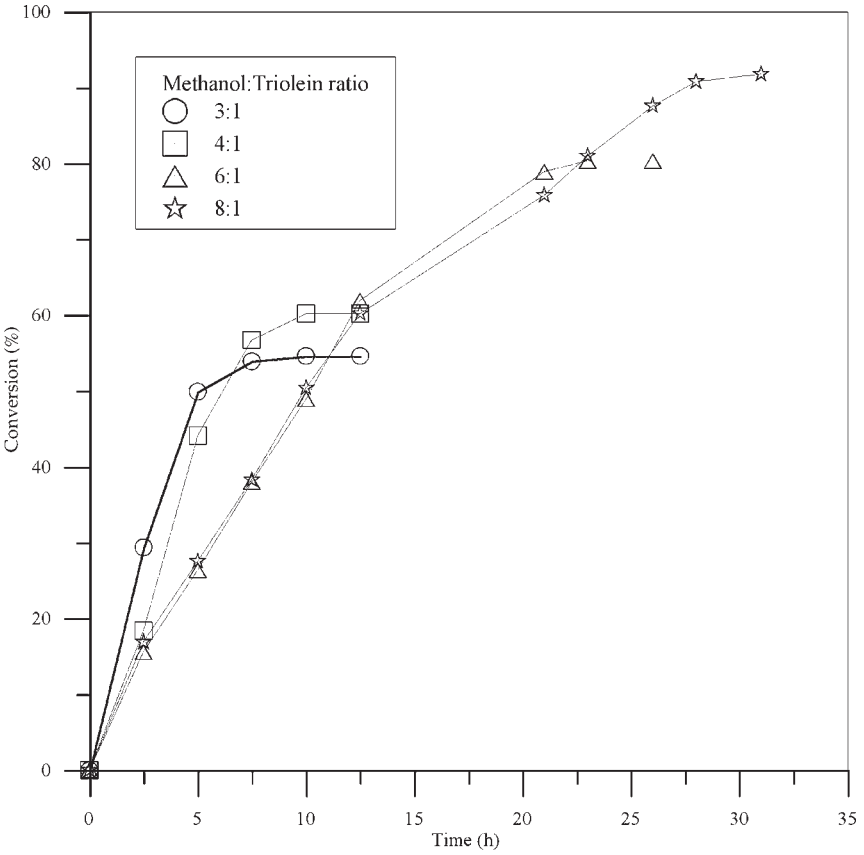

Fig. 1. Effect of molar ratio of methanol to triolein on conversion. Conditions: 40°C, 100 rpm, 500 U enzyme.

The results corresponding to a 4:1 ratio lead us to believe that enzymatic denaturation by methanol is a reversible process (suggesting inhibition rather than denaturation). This is in agreement with other findings *(1)*. After 2.5 h, when the methanol to triolein ratio has decreased to about 3:1 as a result of methanol consumption, it can be seen that the rate increases and approaches the rate corresponding to 3:1 (*see* Fig. 1). This decrease in the ratio is as a result of the presence of other components in olive oil besides triolein. In the case of the other ratios (6:1 and 8:1), the ratio never approaches this value (3:1) because of the excess amount of methanol present to start with.

Figure 2 shows a plot of initial rate vs methanol to triolein ratio. It is evident from the figure that the highest initial rate is obtained at a methanol to triolein ratio of 3:1. The reaction rate at methanol:triolein ratio of 2:1 is also lower. At higher ratios, the rate decreases because of either inactivation or inhibition of the enzyme by methanol.

Table 3 shows the influence of methanol to triolein molar ratio on initial rate, final conversion and yield. It is clear from Table 3 that both the

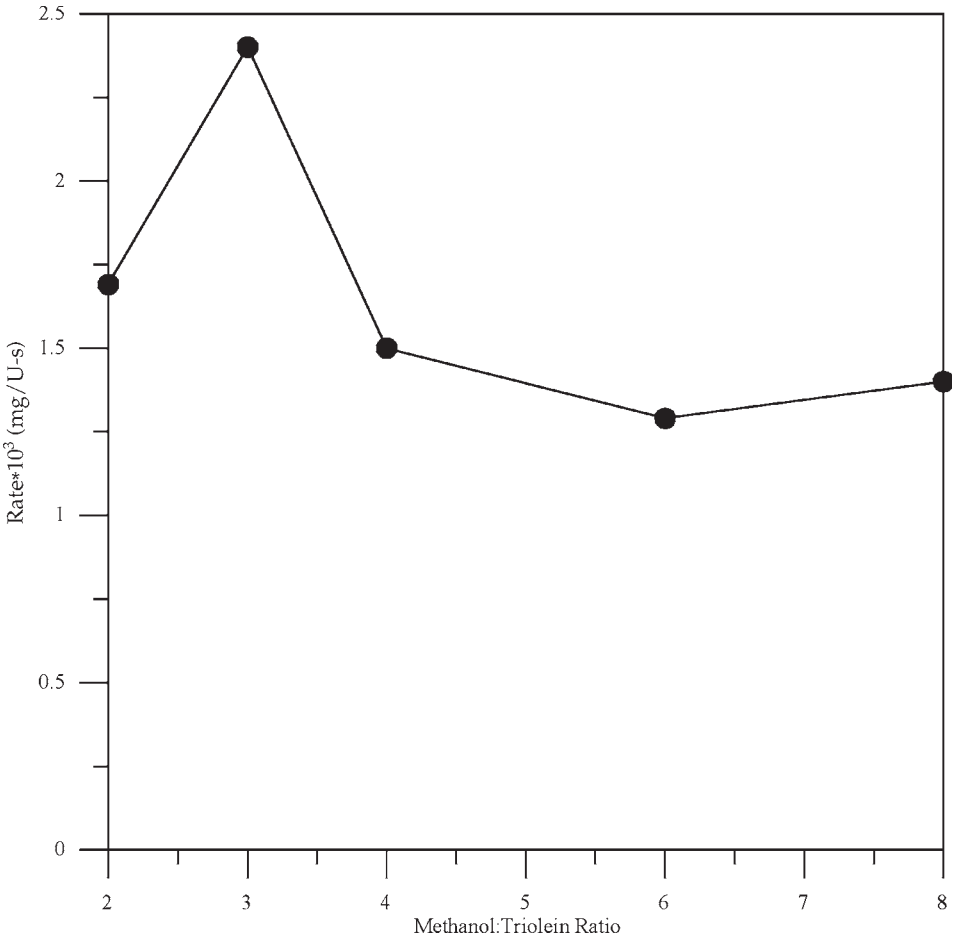

Fig. 2. Effect of molar ratio of methanol to triolein on initial rate. Conditions: 40°C, 100 rpm, 500 U enzyme.

Table 3
Effect of Methanol to Triolein Molar Ratio

Ratio	Initial rate $\times 10^3$ (mg/U·s)	Final conversion (%)	Yield (g biodiesel/g oil)
8:1	1.4	91.8	0.41
6:1	1.3	80.3	0.36
4:1	1.5	60.2	0.27
3:1	2.4	54.6	0.25

yield (defined as g biodiesel obtained per gram of oil at the end of the reaction), and final conversion, increase with increase in methanol to triolein ratio. However, the initial rate of reaction is highest when the ratio of methanol to triolein is 3:1.

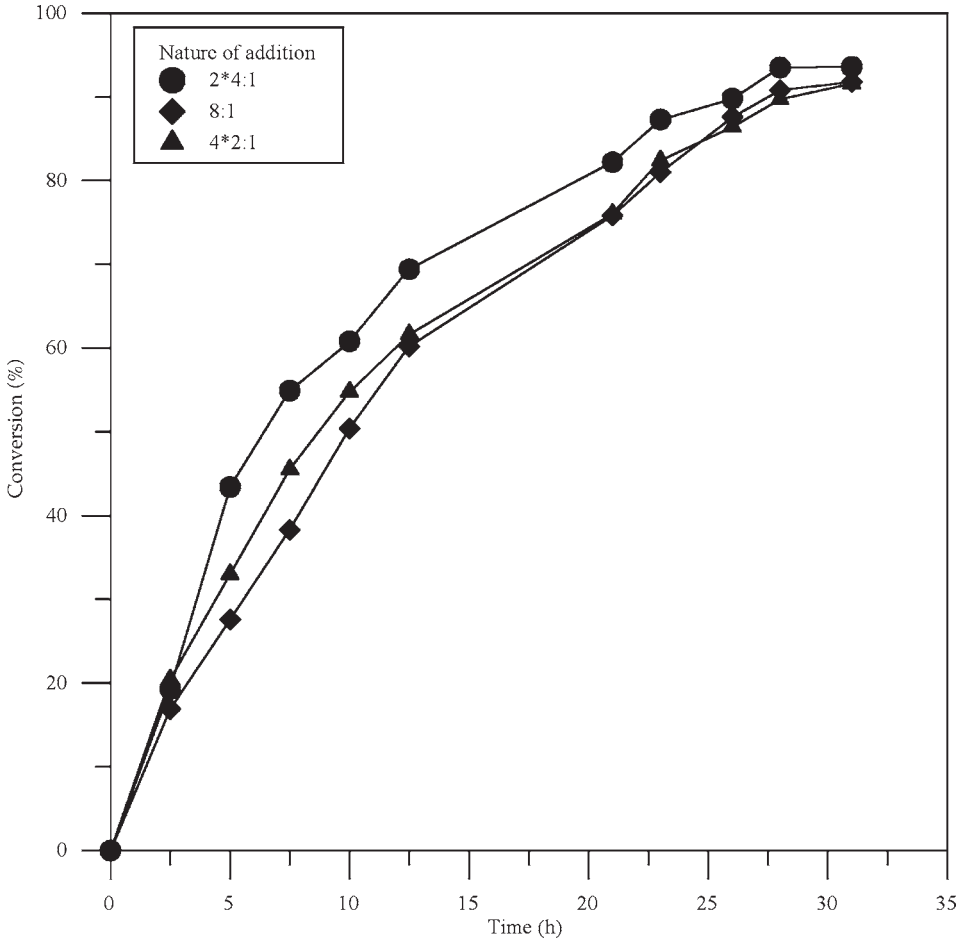

Fig. 3. Effect of stepwise addition of methanol on conversion. Conditions: 40°C, 100 rpm, 500 U enzyme.

Semibatch operation, or stepwise addition of methanol, was investigated because such an arrangement should maintain a low enough methanol concentration to avoid enzymatic denaturation or inhibition while providing enough alcohol (overall) to obtain high final conversions. Experiments were conducted to test this hypothesis.

Figure 3 shows the rate of conversion attained with an overall 8:1 molar ratio:

1. When 100% of the methanol is added in one step at zero time (ratio 8:1).
2. When the alcohol is added in two steps at times 0 and 5 h (2*4:1).
3. After a four-step addition at times 0, 2.5, 5, and 7.5 h (4*2:1).

Stepwise methanolysis indicates some improvement with respect to batch operation, especially when alcohol is added in a two-step process.

Thus:

1. A lack of methanol can be just as undesirable as enzymatic deactivation or inhibition; a four-step addition of methanol (4*2:1) keeps the ratio below the stoichiometric ratio, and the corresponding initial rate is not significantly higher than that for batch operation.
2. The final conversion in every case is nearly the same even though the path to reach this final level is different. When methanol is added batchwise, clearly there is more inhibition of the enzyme active sites. As the methanol is consumed in the reaction, especially as a result of the presence of other components in the oil, the final conversion level even in batch addition is only slightly less than the value for stepwise addition.
3. According to Fig. 1, the reaction rate for the 4:1 ratio remains fairly constant during the first 7.5 h, indicating that the reaction rate is close to the maximum during this period. In Fig. 3, because more methanol is added after the first 5 h for the 2*4:1 experiment, a sudden increase or jump in conversion is not observed (consistent with Fig. 1). This is also true for the 4*2:1 experiment in which methanol is added every 2.5 h; there is not a sufficient change in the rate to notice a jump.

Effect of Enzyme Concentration

Figure 4 shows the results obtained after solvating 1 mL of olive oil in 4 mL of hexane under the following reaction conditions: 60°C, 100 rpm, and 8:1 molar ratio of methanol to triolein. It is evident from the figure that the final conversion is independent of the enzyme concentration, in the range studied. However, as the enzyme concentration is increased, the final conversion of about 94% is reached much sooner. The yield obtained in each case was 0.43 g biodiesel/g oil.

Effect of Temperature

After solvating 1 mL of olive oil in 4 mL of hexane, experiments were conducted under the following reaction conditions: 500 U enzyme, 100 rpm, 4:1 molar ratio, and at temperatures of 30, 40, 50, 60, and 70°C.

Table 4 shows the influence of temperature on initial rate, final conversion, and yield. It can be seen that the initial reaction rate reaches a maximum at approx 60°C. The yield and final conversion are lower because of the lower methanol to triolein ratio. Final conversion remained constant up to a temperature of 60°C, which also confirms 60°C as the optimum reaction temperature. The results were verified for a different molar ratio of methanol to triolein (not shown). This result is different from what has been reported for transesterification of canola oil, in which the optimum temperature was found to be 38°C *(1)*. The higher optimum may be attributed to the fact Novozym435 is known to be thermally very stable and robust.

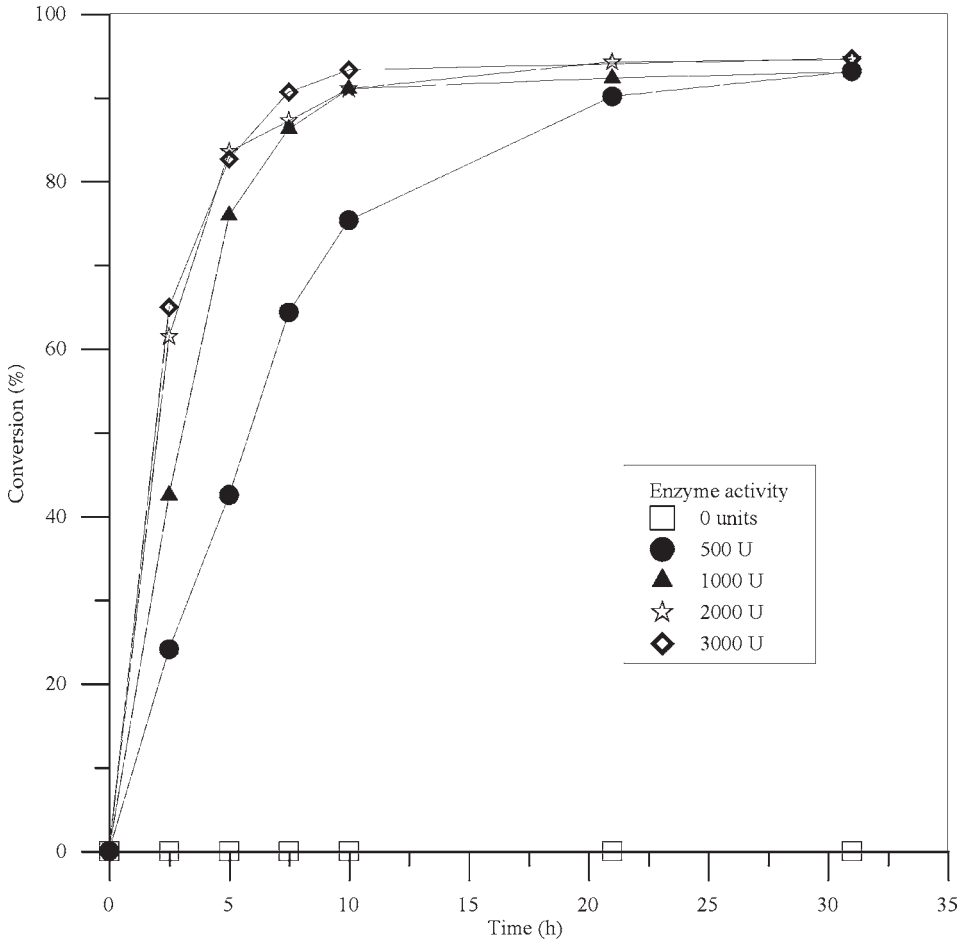

Fig. 4. Effect of catalyst activity on conversion. Conditions: 60°C, 100 rpm, 8:1 ratio.

Table 4
Effect of Temperature on Initial Rate, Final Conversion, and Yield

Temperature (°C)	Initial rate $\times 10^3$ (mg/U · s)	Final conversion (%)	Yield (g biodiesel/g oil)
30	0.23	31.7[a]	Not calculated
40	1.53	60.2	0.27
50	3.44	58.4	0.26
60	3.81	59.3	0.27
70	3.33	53.4	0.24

[a]Final conversion not reached—conversion was still increasing at this temperature.

Table 5
Effect of Catalyst Re-Utilization on Activity, Yield, and Productivity

Batch	Relative activity (%)	Yield (g biodiesel/g oil)	Productivity·10^5 (g biodiesel/U enzyme·h)
1	100	0.42	3.96
2	100	0.41	3.96
3	96	0.40	3.69
5	95	0.42	3.69
6	83	0.41	3.62
8	71	0.41	3.42
11	43	0.34	2.40

Re-Utilization of the Enzyme

Previous studies have indicated that during enzymatic transesterification of oil with short-chain alcohols, glycerol, as one of the major byproducts, has serious negative effects on lipase activity *(7)*. Research carried out while using Lipozyme TL IM show loss of lipase activity even after one batch *(8)*. Novozym435 is expected to be resistant to denaturation because its immobilization makes it a particularly robust and stable enzyme.

In this research, successive runs were performed to study the endurance of Novozym435 with respect to the number of batches used. A brand new reactor load, consisting of 1 mL of oil and 144 µL of methanol solvated in 4 mL of hexane, was introduced every 24 h. The same enzymatic load, 1000 U, was maintained throughout these experiments under the following conditions: 60°C, 100 rpm, and 8:1 ratio of methanol to triolein. Table 5 shows the results obtained.

It can be observed that the enzyme's activity decreases as it is re-utilized. However, it still retains 95% of its activity after five batches and more than 70% after as many as eight batches. It is also evident that the productivity and yield are more or less constant up to eight batches even though the activity of the enzyme sees some decline.

This demonstrates that Novozym435 is robust and stable; previous studies *(8)* show that other forms of lipase lose more than 50% of their activity after four batches under similar conditions.

Scale-Up

Kinetic studies were performed in a 2-L, jacketed batch reactor. The reactants consisted of 200 mL of oil and 28.8 mL of methanol solvated in 800 mL of hexane. Operating conditions were 60°C, 100 rpm, 8:1 ratio, and 500 U enzyme/mL oil. The reactions proceeded in a very similar manner during the first 2.5-h period (conversion was 24% in the small reactor vs 22% in the 2-L reactor). During the next 2.5 h, the conversions in the small and large reactors were 42.5 and 38.5%, respectively. However, after the first 5 h of reaction, the conversion became significantly different. This was

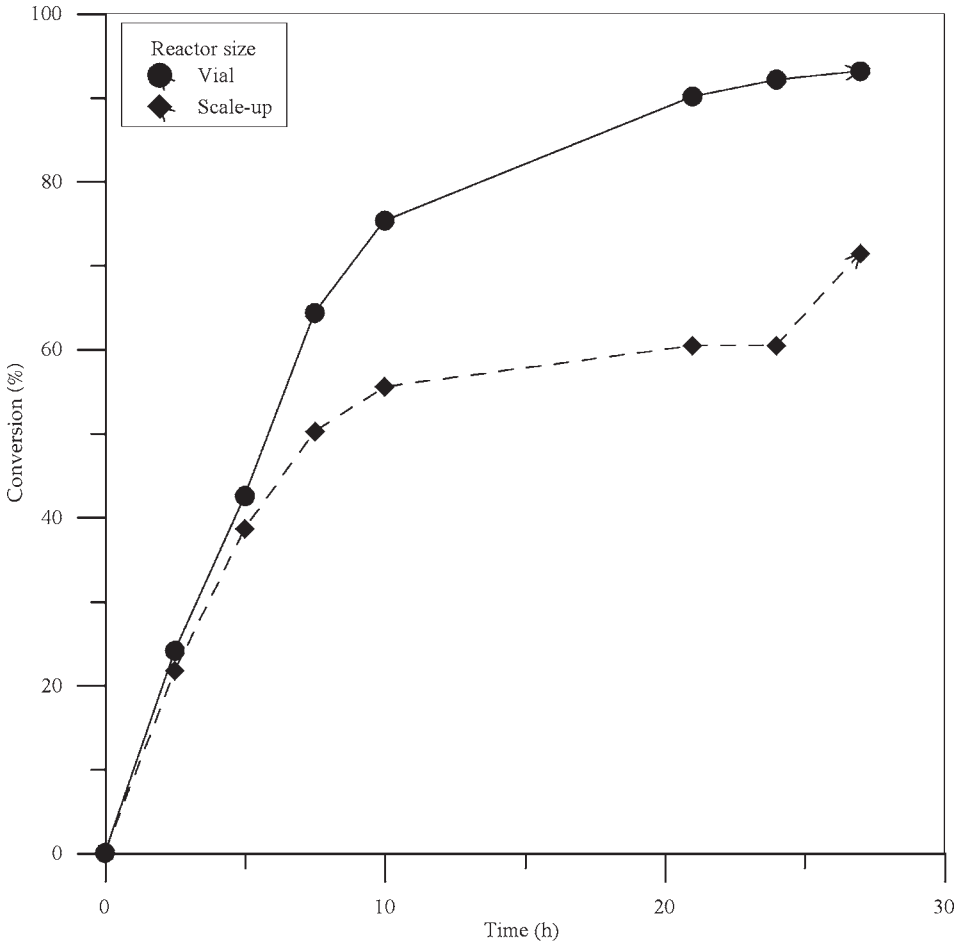

Fig. 5. Scale-up. Conditions: 60°C, 100 rpm, 500 U enzyme, 8:1 ratio.

attributed to a loss of methanol/hexane in the larger 2-L reactor because the reactor had many ports that could account for some losses. Also, the conversion appeared to be leveling off. To test this hypothesis, methanol was added to the reactor and the conversion was found to increase after the addition (*see* Fig. 5). Thus, as long as the reaction conditions are the same, the data obtained in the small-scale reactor can be extrapolated to the larger unit within ±10%.

Use of Used Cooking Oil

Previous studies have reported the enzymic methanolysis of restaurant grease using immobilized lipase from *Candida antarctica (9)*. *C. antarctica* supported on granulated silica and on a macroporous acrylic resin (SP435) were both tested. The corresponding yields after 48 h were 30 and 60%, respectively *(9)*.

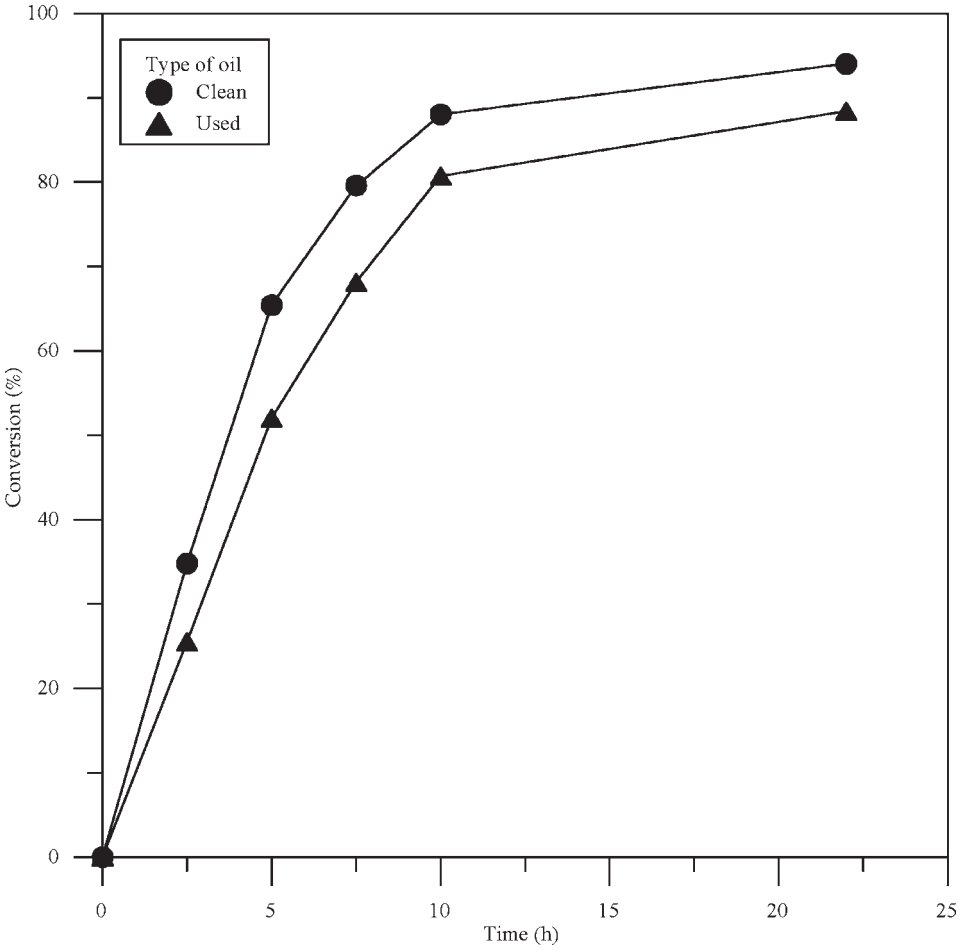

Fig. 6. Comparison of conversion when using fresh and used cooking oil. Conditions: 60°C, 100 rpm, 1000 U enzyme, 8:1 ratio.

Experiments were conducted with Novozym435 to determine the difference between used and clean oil as the raw material during biodiesel production. 1 mL of used oil was solvated into 4 mL of hexane under the following reaction conditions: 60°C, 100 rpm, 8:1 reactant molar ratio, and 1000 U of catalyst.

Figure 6 shows that both reactions proceeded in a similar manner. Even though the conversion of used cooking oil was somewhat lower, no major differences were observed, and the difference in the conversion was about 10% throughout.

Conclusions

It has been demonstrated that the molar ratio of methanol to triolein, semibatch (stepwise addition of methanol) vs batch operation, mass of catalyst, and reaction temperature have varied effects on lipase-catalyzed transesterification of olive oil.

Inhibition of Novozym435 by methanol is limited and the rate remains constant for methanol to triolein ratios greater than 4:1. Enzymatic inhibition caused by methanol is a reversible process. Methanol in excess of the stoichiometric amounts leads to higher conversions of triolein because of other components present in olive oil that react with it. Lack of methanol can be just as undesirable as enzymatic deactivation. Stepwise methanolysis indicates improvement to some degree with respect to batch operation.

The initial reaction rate reaches a maximum at approx 60°C. The final conversion and yield remain constant up to a temperature of 60°C.

The enzyme's relative activity decreases as it is re-utilized. However, it still retains 95% of its activity after five batches and more than 70% after as many as eight batches.

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