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ORIGINAL PAPER

Enzymatic Synthesis of Biodiesel from Transesterification Reactions of Vegetable Oils and Short Chain Alcohols

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Abstract Biodiesel synthesis by alcoholysis of three vegetable oils (soybean, sunflower and rice bran) catalyzed by three commercial lipases (Novozym 435, Lipozyme TL-IM and Lipozyme RM-IM), and the optimization of the enzymes stability over repeated batches is described. The effects of the molar ratio of alcohol to oil and the reaction temperature with methanol, ethanol, propanol and butanol were also studied. All three enzymes displayed similar reaction kinetics with all three oils and no significant differences were observed. However, each lipase displayed the highest alcoholysis activity with a different alcohol. Novozym 435 presented higher activity in methanolysis, at a 5:1 methanol:oil molar ratio; Lipozyme TL-IM presented higher activity in ethanolysis, at a 7:1 ethanol:oil molar ratio; and Lipozyme RM-IM presented higher activity in butanolysis, at a 9:1 butanol:oil molar ratio. The optimal temperature was in the range of 30–35 °C for all lipases. The assessment of enzyme stability over repeated batches was carried out by washing the immobilized enzymes with different solvents (*n*-hexane, water, ethanol, or propanol) after each batch. When washing with *n*-hexane, approximately 90% of the enzyme activity remained after seven synthesis cycles.

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Introduction

Biodiesel is composed of a mixture of fatty acid alkyl esters. It is a natural substitute for petroleum-derived diesel fuel and has similar or better specifications concerning density, viscosity, cetane number, flash point, among others. Because biodiesel is formed from renewable resources such as plant oils, it is considered CO_2-neutral, biodegradable and will help conserve fossil fuels. Compared to traditional diesel fuels, its combustion leads to a substantial reduction in polluting emissions [1, 2].

Industrially, biodiesel can be produced by the transesterification of vegetable oils and short chain alcohols, usually methanol, with alkaline or acid catalysts. The reaction products are a mixture of the desired esters, mono and diglycerides, glycerol, water and the catalysts. Compared to the process mediated by enzymes, this process is more energy-consuming. Due to the presence of soap by-products, separation and purification of the chemically produced biodiesel requires somewhat more complex steps than enzymatically produced biodiesel. Therefore, the use of biocatalysts could be an interesting alternative because it is more environmentally attractive because biodiesel synthesized enzymatically can be used directly without purification [3–5]. Lipase-catalyzed transesterification of vegetable oils has been investigated by many researchers in the last few years [4–12].

For cost reasons, methanol is the alcohol most frequently used for triglyceride transesterification. Nevertheless, other alcohols are also used. In Brazil, one the biggest world plant oil producers, biodiesel is obtained by ethanolysis of

triglycerides, since ethanol is a cheap and abundant commodity produced from the fermentation of sucrose from sugarcane. Alternatively, either propanol or butanol can also be used in this process, especially because these two alcohols promote a better miscibility between the alcohol and the oil phases [7].

The use of a triglyceride feedstock for biodiesel production depends on regional availability and economics and many vegetable oils can be used, such as soybean [3, 13–15], sunflower [16, 17], and rapeseed [3]. The main differences among these oils are their fatty acid compositions, which strongly affects some important properties of the biodiesel (cetane number, heat of combustion, melting point and viscosity) [1]. Oxidation of biodiesel is a common problem, depending on the source of vegetable oil. For instance, rice bran, sunflower, and soybean oils contain high contents of linoleic acid with low resistance to oxidation as a result of the presence of two double bonds [18].

In enzymatic catalysis, enzymes will lose their activity due to a series of factors that must be addressed. These factors include leakage of enzyme from supports to which they are attached when immobilized; inhibition by the substrate; thermal inactivation; and the loss of their spatial conformation leading to changes in the active site. Therefore, in order to reduce the cost of biocatalysts and make lipase-catalyzed biodiesel manufacture economically competitive for industrial scale, it is important to extend enzyme activity as long as possible. This can be done by developing methods for catalyst reuse in as many reaction cycles as possible.

The purpose of this work was to study the alcohol-enzyme specificity, the enzyme stability, and the optimal alcohol:substrate molar ratio on the enzymatic synthesis of biodiesel. We evaluated the reactions of three vegetable oils (soybean, sunflower and rice bran) and different alcohols (methanol, ethanol, propanol and butanol) in alcoholysis catalyzed by three immobilized lipases. We also investigated the effects of the alcohol concentration and temperature on the lipase activity. The stability of the immobilized lipases during repeated batches was investigated by washing the immobilized lipases with the solvents water, ethanol, propanol, and *n*-hexane.

Materials and Methods

Chemicals

Refined soybean, sunflower, and rice bran oils were purchased in a local market and used without any previous treatment. The acrylic resin immobilized lipase from *Thermomyces lanuginosus* (Lipozyme^{TM} TL-IM), the anion-exchange resin immobilized lipase from *Rhizomucor miehei* (Lipozyme^{TM} RM-IM), and the macroporous resin immobilized lipase from *Candida antarctica* (Novozym^{TM} 435) were kindly donated by Novozymes^{TM} Latin America (Araucária, Paraná, Brazil) and used in all experiments. Methanol, ethanol, 1-propanol and 1-butanol and other chemicals were of analytical grade. All experiments were performed in duplicate.

Synthesis Reaction

The reaction conditions were determined in a previous study [15]. To evaluate the different lipases, oils and alcohols, 2.5-g samples of each oil were mixed with each alcohol (7.5:1 alcohol:oil molar ratio), 15% (based on oil weight) of each immobilized lipase and 4% (based on oil weight) of water. The reactions were carried out in 50-mL Erlenmeyer flasks in an orbital shaker (200 rpm) at 30 °C for 6 h. After this time, 5 mL of distilled water was added to the tube reactions and centrifuged at 2,500 g for15 min at 4 °C. The lower phase containing glycerol was analyzed by HPLC. In the experiments to check the effects of alcohol concentration, the alcohol:oil molar ratio was varied from a stoichiometric ratio (3:1 molar ratio) to a large excess of alcohol (12:1 molar ratio). Temperatures were varied in the range of 20–50 °C, with 5 °C increments. In order to verify whether the glycerol could be related to the liberation of esters, free fatty acids in the vegetable oils and in the product reactions were periodically monitored by titration with NaOH [19]. This was necessary in order to quantify the degree of unwanted hydrolysis.

Enzyme Stability

After the transesterification reaction, the immobilized enzymes were separated from the reaction medium by filtration and submitted to different treatments before being reused. The treatments were performed by washing with different solvents. The solvents were *n*-hexane, propanol, ethanol, and water. The enzymes were washed with these solvents and after that dried for 24 h at 40 °C. As a control, a parallel experiment was carried out without solvent washing.

HPLC Analysis

Glycerol concentration was determined by HPLC with a refractive index (RI) detector (Perkin Elmer Series 200, USA) and a Phenomenex RHM monosaccharide column (300 × 7.8 mm), at 80 °C, using ultrapure water as the eluting solvent, with a flow of 0.6 mL min^{-1} and sample volume of 20 µL. The percentage yield conversion was calculated as follows:

$$\text{Conversion} = \left[\frac{\text{mmol glycerol}}{\text{mmol initial oil}}\right] \times 100\% \quad (1)$$

Results and Discussion

Control of Hydrolysis

In order to determine whether undesired hydrolysis, instead of transesterification, was liberating glycerol during the formation of biodiesel, free fatty acid formation in the medium reaction was measured by titration in control hydrolysis (no alcohol added) and transesterification. This was tested for all three lipases and in Fig. 1 results are shown for the transesterification and control hydrolysis of soybean oil catalyzed by Novozym 435. This behavior was observed for all system reactions tested in this research. As can be seen, free fatty acids were formed during hydrolysis (control reaction) but never during transesterification, indicating that it is possible to measure transesterification by the quantification of glycerol.

Screening of the Alcohols and Oils

Methanol is the most widely used alcohol in chemically catalyzed biodiesel production. Methanol is easily available and recoverable as an absolute alcohol. The high temperatures used in the chemical process improve the miscibility between methanol and oil. In contrast, when a biocatalyst process is used, the relatively low temperature (25–35 °C) of the process does not allow for a good mixing system. Therefore, we tested four short chain alcohols (methanol, ethanol, propanol and butanol) in the transesterification reaction catalyzed by three different immobilized lipases in order to compare their performances. We also compared the alcoholysis of various vegetable oils at a fixed temperature and substrate:enzyme ratio, and the results are summarized in Fig. 2.

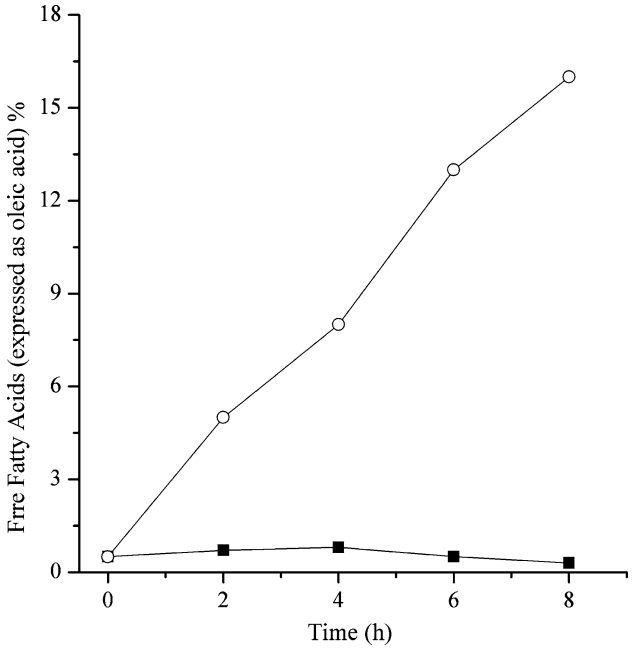

Fig. 1 Free fatty acid formation during (*filled squares*) transesterification and (*open circles*) hydrolysis of soybean oil catalyzed by Novozym 435. All reactions were carried out at $T = 30$ °C. Transesterification conditions: alcohol:oil molar ratio = 7.5:1; enzyme = 15%, water = 4%. Hydrolysis conditions: enzyme = 15%, water = 4%

Although the vegetable oils tested in this study have different fatty acid compositions [20], no significant differences were observed in the kinetics of their alcoholysis. The highest conversion yield was obtained in the transesterification with rice bran oil as compared with the soybean and sunflower oils. For enzymatic biodiesel production, almost all sources of triglycerides can be considered as enzyme substrates. The differences showed in the results may be due to the low viscosity of the rice bran oil, which facilitated the miscibility of the substrates in our solvent-free system. Each enzyme showed a different kinetic pattern depending on the alcohol used. Novozym 435 displays high activity in methanolysis, and the conversion yield was lower for other alcohols proportional to the increase in carbon chain length of the alcohol. Novozym 435 has been shown to be more active in the presence of low molecular weight alcohols, thereby facilitating its ability to catalyze methanolysis or ethanolysis reactions [21]. For Lipozyme TL-IM and Lipozyme RM-IM, the highest conversion yields were obtained with the higher molecular weight alcohols. Lipozyme TL-IM displayed no significant differences in reactions with ethanol, propanol, or butanol, and the conversion yield obtained with these alcohols were almost twice that obtained in methanolysis for all vegetable oils. Lipozyme RM-IM presented the highest conversion yield in butanolysis, possibly indicating that this enzyme is easily deactivated by substrates with low molecular weight alcohols (methanol and ethanol). Soybean oil was chosen for subsequent experiments. There were no significant differences between the vegetable oils in the transesterification reaction, and soybean oil is cheap and widely produced. One alcohol was selected for experiments with each enzyme: methanol for the experiments with Novozym 435, ethanol for Lipozyme TL-IM, and butanol for Lipozyme RM-IM.

Effects of the Concentrations of Alcohols

In order to verify the effects of the concentration of alcohol in the transesterification reaction, we tested the alcohol:oil molar ratio from a stoichiometric ratio (3:1 molar ratio) to a large excess of alcohol (12:1 molar ratio) (Fig. 3). Above

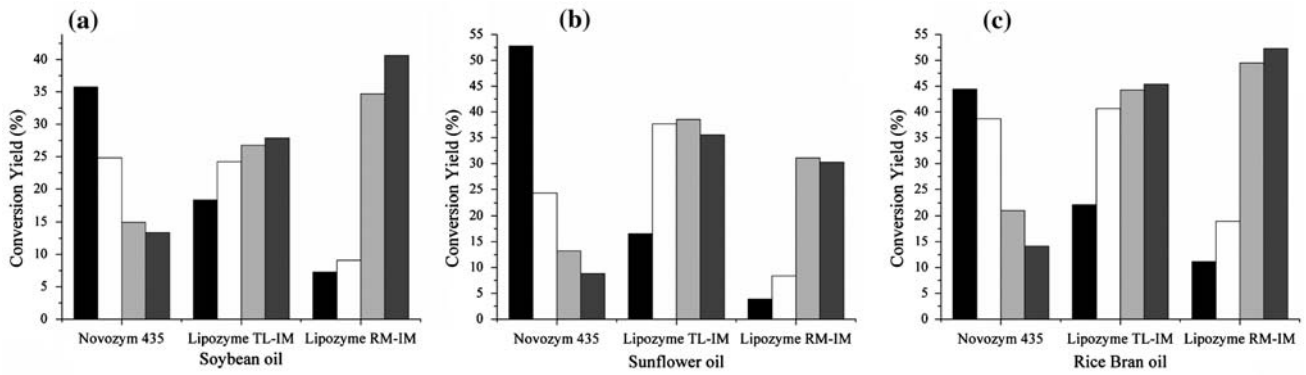

Fig. 2 Alcoholysis of vegetable oils catalyzed by the different lipases. **a** Soybean oil; **b** sunflower oil; **c** rice bran oil. *Black* methanol, *white* ethanol, *light gray* propanol, *dark gray* butanol. All reactions were carried out at 30 °C, alcohol:oil molar ratio = 7.5:1, enzyme = 15%, water = 4% for 6 h

certain alcohol concentrations, the transesterification reactions were inhibited. In the reaction catalyzed by Novozym 435, the highest yield conversion was obtained for a substrate molar ratio of 5:1. In the ethanolysis catalyzed by Lipozyme TL-IM, the highest yield conversions were obtained in the range of 7:1–8:1 in agreement with that previous work [15] on the optimization of ethanolysis of soybean oil catalyzed by a free lipase of *T. lanuginosus*. In the reaction catalyzed by Lipozyme RM-IM, large amounts of butanol (9:1) were necessary to obtain higher yields of conversions.

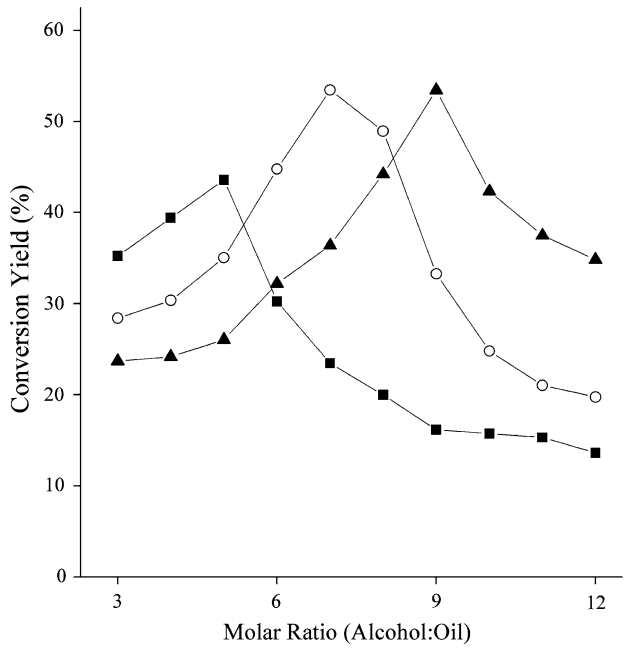

Fig. 3 Effect of alcohol concentration in the transesterification of soybean oil. *Filled squares* Novozym 435, methanol; *open circles* Lipozyme TL-IM, ethanol; *filled triangles* Lipozyme RM-IM, butanol. All reactions were carried out at 30 °C, enzyme = 15%, water = 4% for 6 h

The use of an excess of alcohol is necessary to ensure high reaction rates, to minimize diffusion limitations, and to keep the glycerol formed during the reaction in solution. This will reduce the glycerol-mediated deactivation of the immobilized lipase which can take place when glycerol liberated in biodiesel synthesis blocks the entrance of catalyst pores [8, 21]. However, high ratios of alcohol to oil increase the polarity of the medium, and this is often associated with the inactivation of the biocatalysts [9]. From these results, the alcohol:substrate molar ratios selected were: methanol, 5:1; ethanol, 7:1; and butanol 9:1, for subsequent experiments with Novozym 435, Lipozyme TL-IM, and Lipozyme RM-IM, respectively.

Effects of the Temperature of Reaction

The effects of temperature on the catalytic activity of the three immobilized lipases in the transesterification of soybean oil were investigated. Temperatures were varied in the range from 20 to 50 °C, with 5 °C increments at alcohol concentrations selected in the previous experiment. The highest conversion yields conversions were obtained in the range of 25–35 °C (Fig. 4). In all cases, catalytic activity decreased at temperatures above 40 °C, indicating a possible thermal deactivation of the biocatalysts. Because the inactivation of lipases is significantly greater at higher temperatures, we selected 30 °C for subsequent experiments with all the studied lipases.

Enzyme Stability

Immobilized enzymes have the advantage that they can be reused several times, but their activity eventually decreases due to many factors, such as desorption, substrate deactivation, and product inhibition. Therefore, we tried to improve the stability of the immobilized lipases after each use. After each transesterification reaction, the three lipases

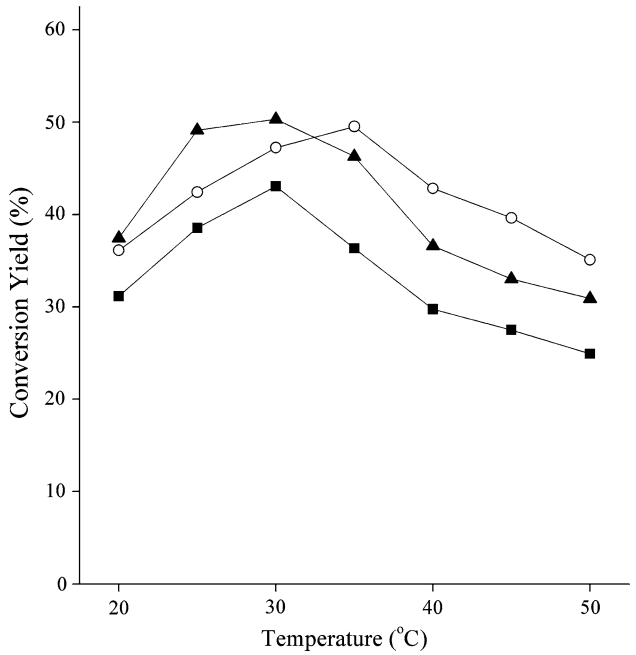

Fig. 4 Effect of the temperature in the transesterification of soybean oil. *Filled squares* Novozym 435, 5:1 methanol:oil; *open circles* Lipozyme TL-IM, 7:1 ethanol:oil; *filled triangles* Lipozyme RM-IM, 9:1 butanol:oil. All reactions were carried out with enzyme = 15%, water = 4% for 6 h

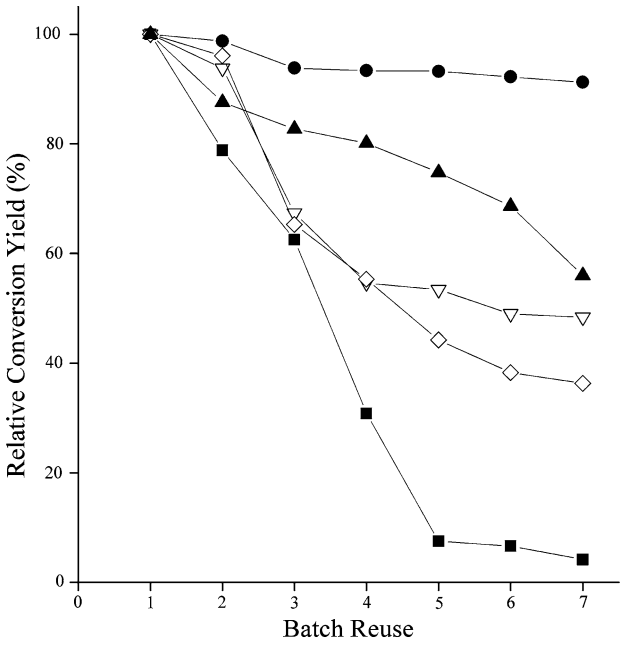

Fig. 5 Stability of Novozym 435 over repeated batches submitted to different treatments. *Filled squares* control, *filled circles* hexane, *filled triangles* ethanol, *inverted open triangles* propanol, *open diamonds* water. All reactions were carried out at 30 °C, 5:1 methanol:oil, enzyme = 15%, water = 4% for 6 h

were recovered by filtration and washed with different solvents before being reused under the optimal conditions previously obtained. Figures 5, 6, and 7 show the results of the relative yield conversions of the treatments with ethanol, propanol, *n*-hexane, water, and the control, expressed as percentage of the yield of the first batch reaction of each lipase preparation. In all cases, washing with *n*-hexane, the only non-polar solvent tested, caused greater retention of lipase activity than that obtained when washing with the polar solvents and the unwashed control. About 90% of the activity of Novozym 435 was maintained over seven batch reactions, while in the control the enzyme was almost completely deactivated (Fig. 5).

Lipozyme TL-IM and Lipozyme RM-IM retained 80% and 75% of their initial activity after seven batches with treatment by *n*-hexane, respectively (Figs. 6, 7). After washing with ethanol and propanol, 60 and 50%, respectively, of lipase activity was retained in reactions catalyzed by Novozym 435 and Lipozyme RM-IM. In the ethanolysis catalyzed by Lipozyme TL-IM, for all polar washing solvents (ethanol, propanol and water) used, the enzyme activity remaining was approximately 25% after seven batches.

During the repeated uses of lipases the formation of a separate layer was observed. On analysis, the layer was shown to be a heterogeneous mixture of oil and biodiesel. This substrate/product layer formed on the surface of the

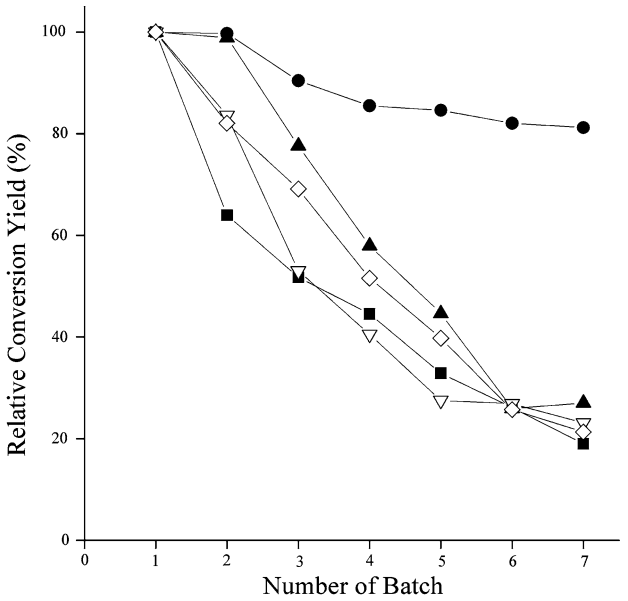

Fig. 6 Stability of Lipozyme TL-IM over repeated batches submitted to different treatments. *Filled squares* control, *filled circles* hexane, *filled triangles* ethanol, *inverted open triangles* propanol, *open diamonds* water. All reactions were carried out at 30 °C, 7:1 ethanol:oil, enzyme = 15%, water = 4% for 6 h

enzymatic support could cause loss of activity by limiting substrate and product diffusion [11]. As the main components of the mixture are non-polar (oil/biodiesel), the use of

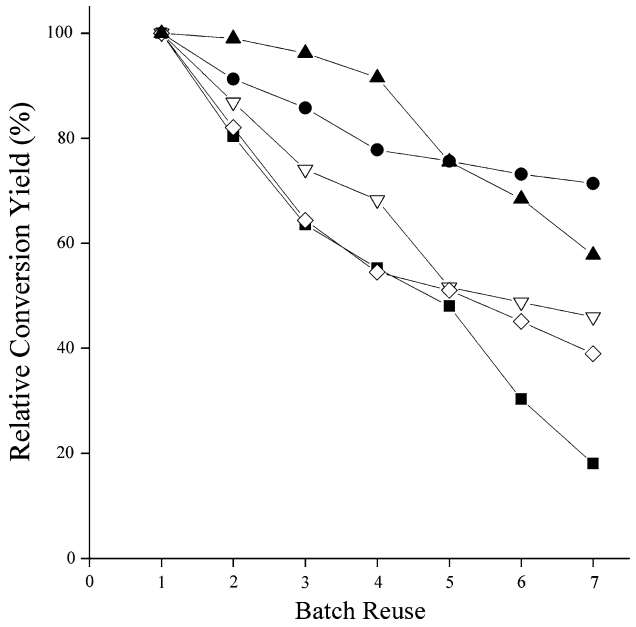

Fig. 7 Stability of Lipozyme RM-IM over repeated batches submitted to different treatments. *Filled squares* control; *filled circles* hexane; *filled triangles* ethanol; *inverted open triangles* propanol; *open diamonds* water. All reactions were carried out at 30 °C, 9:1 butanol:oil, enzyme = 15%, water = 4% for 6 h

a non-polar solvent to wash the immobilized lipase helped to remove the substrate/product layer formed on the enzyme surface and to preserve the enzyme activity.

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