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SUCCESSIVE CYCLES OF UTILIZATION OF NOVOZYM 435 IN THREE DIFFERENT REACTION SYSTEMS

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Abstract - The main focus of this work was to investigate the residual esterification activity and the product conversion after 10 successive cycles of utilization of a commercial lipase in three systems: esterification of 2-ethyl hexanol and palmitic acid in a solvent-free system; esterification of ascorbic acid and palmitic acid in tert-butanol; and transesterification of glycerol and methyl benzoate in 2-propanol. These systems were chosen based on previous results by our research group in terms of product conversion. Before scale-up, there is a need for evaluating several cycles of utilization of the biocatalyst. The esterification of 2-ethyl hexanol showed that after 10 cycles the enzyme retained 90% of its activity. The system consisting of ascorbic acid, palmitic acid, Novozym 435 and tert-butanol showed that a reduction in enzyme activity was accompanied by a reduction in reaction conversion; the same behavior was not observed for the third system.
Keywords: Lipase; Transesterification; Reuse.

INTRODUCTION

Lipases, also known as triacylglycerol ester hydrolases, are enzymes that cleave the ester bonds of triacylglycerols, with the subsequent release of free fatty acids, diacylglycerols, monoacylglycerols and glycerol. Lipases are also able to catalyze the reverse reactions (esterification, interesterification and transesterification), provided that the aqueous medium is replaced by an organic or a biphasic aqueous/organic medium. Although ester synthesis can be chemically catalyzed by acids or bases, the use of enzyme technology offers environmental advantages and a reduction in energy consumption. Furthermore, lipases show high selectivity, including stereo-selectivity, and give products of high purity and improved quality (Kato et al., 1999; Richetti et al., 2010; Richetti et al., 2010; Barros et al., 2010; Pereira et al., 2003).

A range of fatty acid esters is now being produced commercially using immobilized lipase in non-aqueous solvents. For example, esters produced from long-chain fatty acids (12–20 carbon atoms), as well as short-chain fatty acids (2–8 carbon atoms), are increasingly used in the food, detergent, plasticizer, lubrificant, cosmetic and pharmaceutical industries (Vedejs and Jure, 2005; Oliveira et al., 2006; Gottor, 1999).

Loss of enzyme activity with time can be caused by deactivation of the enzyme as a consequence of thermal effects and product concentration. Knowledge of the rate law governing the deactivation process is often important in enzyme-catalyzed processes and of interest in process design.

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One of the problems that arises when one tries to evaluate the loss of enzyme activity in batch reactors is the fact that the time of the reaction and the time at which the set of experiments is started are equivalent. This fact does not permit one to separate the rate of the reaction from the rate at which the enzyme is losing activity because both processes occur simultaneously. One strategy to overcome this problem is to carry out several cycles of reaction with the same batch of lipase. Hence, it is possible to distinguish between the time characteristic of the reaction (reaction time) and that corresponding to lipase deactivation (the time elapsed since the start of the first experiment). Each cycle involves the same elapsed time for the reaction, but this elapsed time will differ from the time during which the lipase has been susceptible to deactivation (Theil, 1995; Saxena et al., 2003; Ceni et al., 2010).

In this context, the main focus of this work was to investigate the residual esterification activity and the product conversion after 10 successive cycles of utilization of a commercial immobilized lipase (Novozym 435) in three important reaction systems: esterification of 2-ethyl hexanol and palmitic acid in a solvent-free system for 2-ethylhexyl palmitate production;, esterification of ascorbic acid and palmitic acid in tert-butanol for ascorbyl palmitate production; and transesterification of glycerol and methyl benzoate in 2-propanol for production of 1-glyceryl benzoate. These systems were chosen after recent works published by our research group (Richetti et al., 2010; Ceni et al., 2010; Lerin et al., 2011), where promising results were obtained in terms of product conversion and the fact that, before the scale-up of the enzymatic process, there was a need to evaluate several cycles of utilization of the biocatalyst by measurement of residual activity and reaction conversion after each cycle of use of the enzyme.

L-Ascorbic acid, a natural hydrophilic antioxidant, has been used but its application is limited in hydrophobic foods and cosmetics. Due to the steadily growing demand for natural materials, the synthesis of esters of ascorbic acid by lipase-catalyzed reactions has become of current commercial interest to be used as an antioxidant in the food, cosmetic and pharmaceutical industries (Viklund et al., 2003).

Fatty acid esters of 2-ethyl hexanol, such as 2-ethylhexyl palmitate, are of great interest due to their applications in the cosmetics, pharmaceutics, food and chemical industries. They are used, for example, as low temperature plasticizers for polyvinyl chloride, vinyl chloride, copolymers, polystyrene, ethyl cellulose and synthetic rubber, and also in the manufacture of water-resistant lubricants or as solvents (He et al., 2002; Tan et al., 2006).

1-Glyceryl benzoate is a medically important compound since it is a natural choice to replace epichlorohydrin as a green intermediate for beta-blocker production, typified by carvedilol and propranolol (Brockerhoff and Jensen, 1974). The antagonists of beta-adrenergic receptors (beta-blockers), such as propranolol (Howe and Rao, 1968) and carvedilol, are important active pharmaceutical ingredients (APIs) used in the treatment of diseases like hypertension, cardiopathies, some kinds of hyh and glaucoma.

MATERIALS AND METHODS

Substrates and Solvents

For Enzymatic Esterification of 2-Ethyl Hexanol and Palmitic Acid in Solvent-Free System

Commercial palmitic acid (Vetec, 98% purity) and 2-ethyl hexanol (Merck, 99% purity).

For Enzymatic Esterification of Ascorbic Acid and Palmitic Acid in Tert-Butanol System

Commercial palmitic acid (Vetec, 98% purity) and L-(+)-ascorbic acid (Vetec, 99% purity) and tert-butanol (HPLC grade, Vetec) as solvent.

For Enzymatic Transesterification of Glycerol and Methyl Benzoate in 2-Propanol System

Glycerol and methyl-benzoate (Vetec, both 99.5% purity) and n-propanol (HPLC grade, Vetec) as solvent.

Enzyme

Commercial lipase from *Candida antarctica* (Novozym 435), immobilized on a macroporous anionic resin (0.12U/g, 1.4% water, diameter in the range of 0.3–0.9mm and optimum temperature of 70°C), was purchased from Novozymes (Araucária, PR, Brazil) and used as catalyst in all tested systems.

Experimental Conditions for Enzyme Reuse in Each Experimental System

The optimized experimental conditions for each reaction system were previously determined in

recent works published in the literature (Richetti et al., 2010; Ceni et al., 2010; Lerin et al., 2011). In summary, the following conditions were used here for successive cycles of utilization of Novozym 435.

For Enzymatic Esterification of 2-Ethyl Hexanol and Palmitic Acid in Solvent-Free System

The optimum conditions for the production of 2-ethylhexyl palmitate were found to be: acid to alcohol molar ratio of 1:5.5, stirring rate of 150rpm, 70°C, enzyme concentration of 10.5wt% and 6 hours of reaction, resulting in a 2-ethylhexyl palmitate conversion of about 93% (Richetti et al., 2010).

For Enzymatic Esterification of Ascorbic Acid and Palmitic Acid in Tert-Butanol System

The optimum production of ascorbyl palmitate were achieved at an ascorbic acid to palmitic acid molar ratio of 1:9, stirring rate of 150rpm, 70°C, enzyme concentration of 5wt% at 17 hours of reaction, using tert-butanol as solvent, resulting in an ascorbyl palmitate conversion of about 67% (Lerin et al., 2011).

For Enzymatic Transesterification of Glycerol and Methyl Benzoate in 2-Propanol System

The optimum conditions were found to be a methyl benzoate to glycerol molar ratio of 1:1, stirring rate of 150rpm, 50°C, enzyme concentration of 10wt% at 24 hours of reaction, using 2-propanol as solvent, with a resulting conversion to 1-glyceryl benzoate of about 29% (Ceni et al., 2010).

Experimental Procedure for Enzyme Reuse

Using the experimental conditions presented previously, successive cycles of utilization of Novozym 435 were carried out. A total of ten cycles was evaluated for each reaction system. Before starting the first reaction cycle, the enzyme was activated at 40°C for 60 minutes. After the pre-defined reaction time, established above for each different system, the enzyme was recuperated and kept in a desiccator for at least 24 hours. Then the enzyme was used in a new batch. The enzymatic activity was measured before and after each cycle and the residual activity was determined following the methodology described below. The product conversion in each cycle was also evaluated.

Analytical

Quantification of Reaction Products

For Enzymatic Esterification of 2-Ethyl Hexanol and Palmitic Acid in a Solvent-Free System

Quantitative analyses of the products were conducted using an HPLC system from Agilent, equipped with a refractive index detector. The following instrumentation and conditions were used: Zorbax C18 column (4.6mm x 250 mm, 5μm), flow rate of 1.0mL/min, column temperature of 35°C and the mobile phase, acetonitrile:methanol:H_20 (75:25:5, v/v/v). The mobile phase was used as the sample dissolving solvent and the injection volume was 20μL. Quantification was carried out using authentic standards of 2-ethylhexyl palmitate (Sigma-Aldrich). Calibration curves were constructed with the following concentrations 1410; 2820; 5640; 11280; 16920; 22560; 28200 and 33840 ppm. Reaction conversion was calculated based on the content of 2-ethylhexyl palmitate in the sample analyzed and on the reaction stoichiometry (Richetti et al., 2010).

For Enzymatic Esterification of Ascorbic Acid and Palmitic Acid in Tert-Butanol

Quantitative analyses of the products were conducted using an HPLC system from Agilent, equipped with a refractive index detector. The following instrumentation and conditions were used: Zorbax C_{18} column (4.6m x 250mm, 5μm), flow rate of 1.0mL/min, column temperature of 35°C; the mobile phase, acetone:methanol:H_20 with 0.5% acid acetic (75:25:5, v/v/v). The mobile phase was used as the sample dissolving solvent and the injection volume was 20μL. Quantification was carried out using authentic standards of ascorbyl palmitate (6-O-palmitoyl-L-ascorbic acid) (Sigma-Aldrich). Calibration curves were built with the product concentrations of 240; 480; 960; 1920; 2880; 3840 and 4800 ppm. Reaction conversion was calculated based on the content of ascorbyl palmitate in the sample analyzed and the reaction stoichiometry (Lerin et al., 2011).

For Enzymatic Transesterification of Glycerol and Methyl Benzoate in 2-Propanol

The reactional medium, without enzyme, was rotary-evaporated and diluted in methanol for the chemical analysis, performed with a gas

chromatograph (Shimadzu Model GC 17-A) equipped with a capillary column of Wcot Fused Silica (30m x 0.32mm) containing GP-Sil 5CB. The column was kept at 40ºC for 1 minute, heated to 125ºC at a rate of 10ºC/min (2min) and, then, heated again to 250ºC at the same rate (10ºC/min). The detector and injector temperatures were kept at 300ºC. The column pressure and the hydrogen flow rate were 8KPa and 3.5mL/min, respectively. The split ratio was 1:60. The standard sample of 1-glyceryl benzoate was synthesized in our laboratory and the structure of the compound was confirmed by NMR: ^{1}H-NMR registered on a Varian NMR (200MHz) in $CDCl_3$ solution with $CHCl_3$ as internal reference and ^{13}C-NMR on a Varian NMR (50MHz) in $CDCl_3$ solution with $CHCl_3$ as internal reference (Ceni et al., 2010).

Lipase Esterification Activity

The enzyme activity was determined as the initial rate of the esterification reaction between lauric acid and n-propanol at a molar ratio of 3:1, temperature of 60°C and enzyme concentration of 5wt% in relation to the substrates. At the beginning of the reaction, samples containing the mixture of lauric acid and n-propanol were collected and the lauric acid content was determined by titration with 0.04N NaOH. After the addition of the enzyme to the substrates, the mixture was kept at 60°C for 15min. Then, the lauric acid consumption was determined. One unit of activity (U) was defined as the amount of enzyme necessary to consume 1µmol of lauric acid per minute (Oliveira et al., 2006). All enzymatic activity determinations were performed in at least three replicates.

Recuperation of Enzymes After Each Cycle of Utilization

After each cycle of utilization, the biocatalyst was separated from the reaction medium by filtration. Then, the catalyst was washed twice using 10mL of n-hexane (99%, Vetec) and the sample was vacuum filtered. This procedure is a modification of the method proposed by Castro & Anderson (1995) that used n-heptane as the washing agent. The enzyme was then dried in oven at 40°C for 1 hour. The recovered enzyme was kept in a desiccator for 24 hours and, after this period of time, an aliquot of 0.2g of the enzyme was used for determination of esterification activity. The remaining amount of the enzyme used in the previous batch was reutilized in a subsequent cycle for each reaction system evaluated in this work. The residual esterification activity was defined as the ratio of (Final activity/Initial activity) x 100 and determined after each cycle of utilization of the enzyme.

RESULTS AND DISCUSSION

The use of immobilized enzymes in synthetic reactions presents, as a main advantage, the possibility of reuse in successive cycles. Here 10 cycles of utilization of a commercial immobilized lipase were carried out for three different reaction systems, aiming at evaluating the residual enzyme activity and reaction conversion. Figures 1, 2 and 3 present the results obtained for each system.

Figure 1 indicates that the reaction system using palmitic acid and 2-ethyl hexanol as substrates and Novozym 435 in a solvent-free system presented a residual enzyme activity of almost 100% after 5 cycles of utilization. After 10 cycles of utilization, a residual activity of about 90% was obtained. Conversion remained practically constant after these 10 cycles. Because a solvent-free system was tested, a low reduction in enzyme activity was observed.

Figure 2 shows that, for the reactional system of ascorbic acid and palmitic acid, with Novozym 435 and tert-butanol as catalyst and solvent, respectively, the residual enzyme activity was nearly 100% after 4 cycles of utilization (each cycle of 17 hours). As of the fifth cycle, there was a reduction in enzyme activity, accompanied by a reduction in reaction conversion. Here it seems that the enzyme activity is directly related to the product conversion.

In the experiments on enzymatic production of 1-glyceryl benzoate, Figure 3 shows that the enzyme kept its initial activity for 4 cycles of utilization (about 100% of residual activity after each cycle of 24 hours). From the fifth cycle on, a loss of enzyme activity was observed (from 30 to 40%, until 10 cycles of utilization). The conversion to 1-glyceryl benzoate remained constant after 10 cycles of enzyme utilization (about 13.5%). In the range evaluated, the reduction in enzyme activity did not affect the product conversion. A possible explanation for this fact in this reaction system would be an excess of enzyme under the initial experimental conditions, so that a reduction on enzyme activity was not enough to reduce the process conversion.

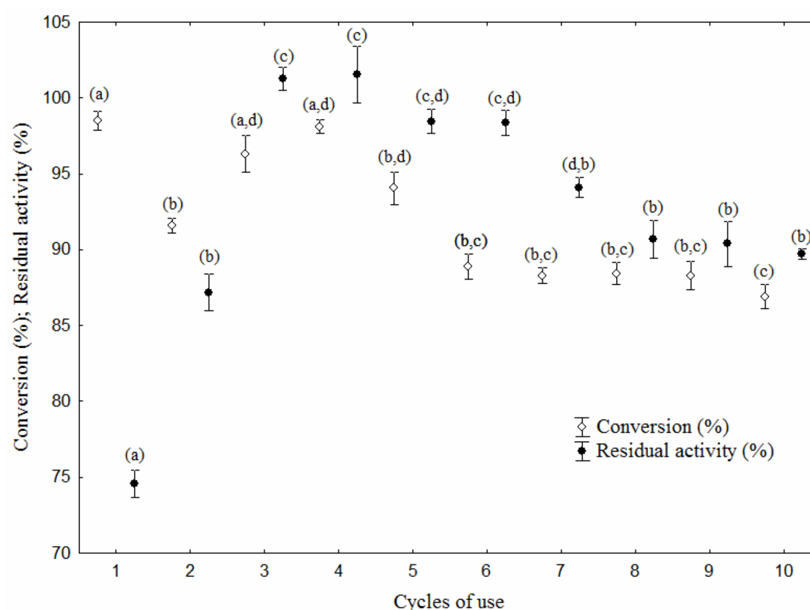

Figure 1: Cycles of reuse of Novozym 435 for 2-ethylhexyl palmitate production. Experimental conditions: acid to alcohol molar ratio of 1:5.5, stirring rate of 150rpm, 70°C, enzyme concentration of 10.5wt% and 6 hours of reaction in a solvent-free system. Results are expressed by mean and respective standard error (bars) and different letters (for each response) indicate values that differ statistically ($p<0.05$) by analysis of variance followed by Tukey's test.

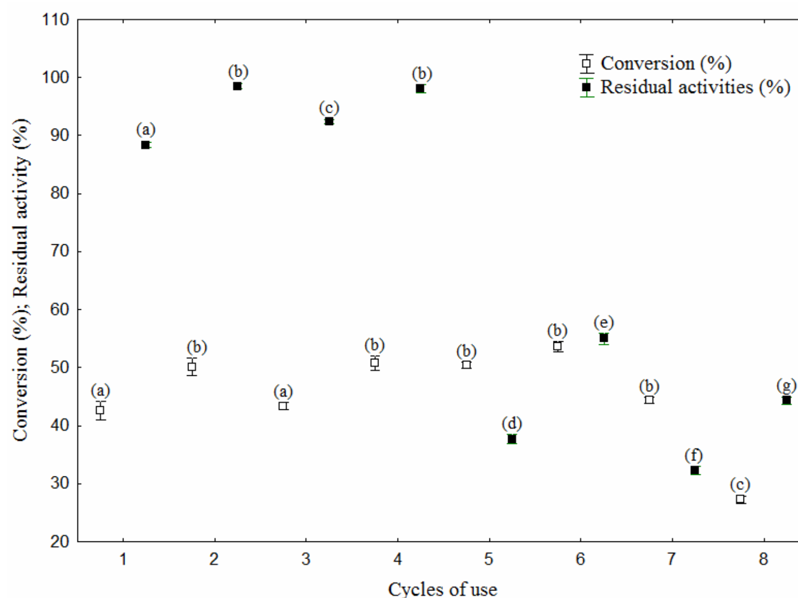

Figure 2: Cycles of reuse of Novozym 435 for ascorbyl palmitate production. Experimental conditions: ascorbic acid to palmitic acid molar ratio of 1:9, stirring rate of 150rpm, 70°C, enzyme concentration of 5wt% at 17 hours of reaction, using tert-butanol as solvent. Results are expressed by mean and respective standard error (bars) and different letters (for each response) indicate values that differ statistically ($p<0.05$) by analysis of variance followed by Tukey's test.

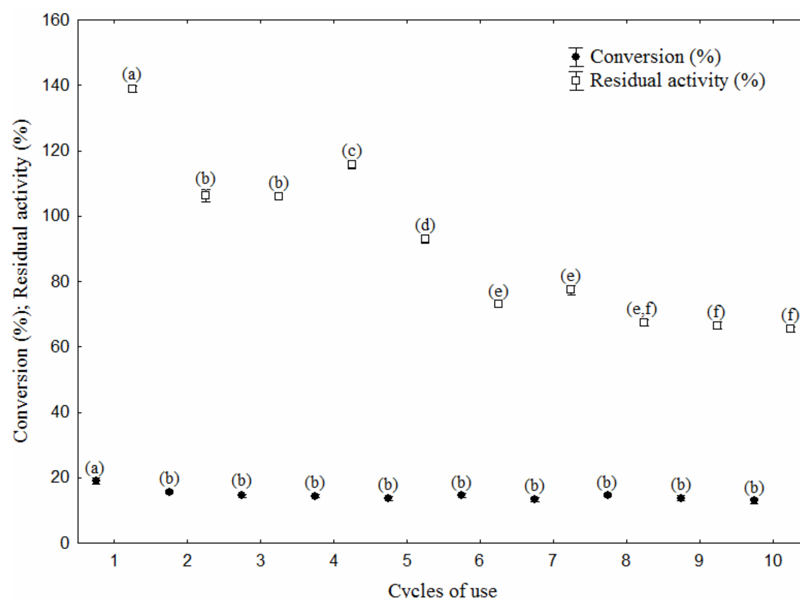

Figure 3: Cycles of reuse of Novozym 435 for 1-glyceryl benzoate production. Experimental conditions: methyl benzoate to glycerol molar ratio of 1:1, stirring rate of 150rpm, 50°C, enzyme concentration of 10wt% at 24 hours of reaction, using 2-propanol as solvent. Results are expressed by mean and respective standard error (bars) and different letters (for each response) indicate values that differ statistically ($p<0.05$) by analysis of variance followed by Tukey's test.

From the results presented in this work, it is possible to conclude that the residual enzyme activity and the product conversion are directly dependent on the substrates and solvents employed for the execution of the reaction.

The use of organic solvents as reaction media for enzymatic reactions provides numerous industrially attractive advantages compared to traditional aqueous reaction systems, such as enhancement of the solubility of the substrates, the reversion of the thermodynamic equilibrium, the suppression of water-dependent reactions and the elimination of microbial contamination. However, some disadvantages can be cited, such as inactivation of the enzymes, labor and cost-intensive preparation of biocatalysts in form of covalently-modified systems, mass-transfer limitations in the case of heterogeneous systems or of viscous solvents, and water activity control needed for processes involving condensation reactions (Doukyu and Ogino, 2010). The step of selecting an appropriate organic solvent is a crucial factor in enzymatic catalysis in non-aqueous media, due to the direct interference of this choice on activity, stability and specificity of the enzyme. In general, hydrophobic solvents are less harmful to the enzymes and the water necessary to maintain the structure of the enzyme. Hydrophilic solvents tend to strip the essential water from the proteic structure, resulting in loss of enzyme activity (De Paula et al., 2005). An ideal organic solvent will also dissolve the substrate well, favoring the progress of the reaction without affecting the enzymatic activity (Tsukamoto, 2006).

The specialized literature points out that, of all the usual parameters such as dielectric constant, dipole moment, hydrogen bonding, or polarity, the logarithm of the ethanol-water partition coefficient (log P) gives the best correlation with the enzyme activity (Yang and Robb, 1994). In general, for the optimization of biocatalytic systems in organic solvents, log P is considered to be a good quantitative parameter since it represents the polarity of the solvent. The following classification is recommended for choosing the most adequate organic solvents for enzymatic catalysis: (i) solvents with $\log P<2$, due to their high polarity, can cause the denaturation of the catalyst; (ii) solvents with $2<\log P<4$ are considered to be moderate; and (iii) solvents with $\log P>4$ can be considered to be the most appropriate since they are non-polar

(Tsukamoto, 2006). Tsukamoto (2006) thus affirms that solvents with log P<2 are not appropriate for biocatalytic purposes, while those with log P>4 (apolar) can be considered ideal.

In spite of the relevance of determining the number of cycles of utilization of a biocatalyst before scale-up of the process, very few works concerning this subject were found in the literature. As an example, Hajar et al. (2009) evaluated the cycles of reuse of Novozym 435 for the methanolysis of canola oil in a solvent-free system. The reaction was conducted for 5 successive cycles or a total of 432 hours and conversion remained at about 97%. The authors did not determine the residual activity of the biocatalyst.

Ghamgui et al. (2006) studied the cycles of reuse of a lipase from *Staphylococcus simulans* immobilized on sodium carbonate. After 4 cycles, no significant difference was observed in the production of isoamyl acetate at 37°C. After 10 cycles, a reduction of about 50% in the conversion was observed and the residual activity was 76%.

Brígida et al. (2008) evaluated the use of coconut fiber residue as a support for immobilization of lipase from *Candida antarctica* type B (CALB). The authors studied the cycles of reuse of the immobilized enzyme and compared the results with those obtained for Novozym 435. After three cycles of hydrolysis, the immobilized lipase presented residual activity lower than 50%. Novozym 435, under the same experimental conditions, retained more than 70% of its activity after ten cycles. However, for the synthesis of butyl butyrate, the CALB lipase immobilized on coconut fiber showed good stability compared to Novozym 435, keeping 80% of its initial activity after 6 successive cycles of utilization. The authors concluded that the stability of the enzyme depends on the reaction system under investigation.

The work of Gomes et al. (2004) evaluated the influence of the reaction temperature and substrate polarity on non-conventional biocatalysis by *Candida rugosa* lipase immobilized on chitin functionalized with hexamethylenediamine followed by glutaraldehyde activation. Production of butyl esters was found to be dependent on the substrate partition coefficient, which was the greatest for the system butanol and butyric acid.

CONCLUSIONS

New experimental data on residual esterification activity and product conversion after 10 successive cycles of utilization of a commercial immobilized lipase (Novozym 435) are reported in this worh for three important reactional systems, information relevant for the scale-up of processes using this commercial enzyme. The esterification of 2-ethyl hexanol and palmitic acid for 2-ethylhexyl palmitate production in a solvent-free system showed that, after 10 cycles, the enzyme retained 90% of its activity. Considering the system consisting of ascorbic acid, palmitic acid, Novozym 435 and tert-butanol, a reduction in enzyme activity was accompanied by a reduction in reaction conversion. The production of 1-glyceryl benzoate in 2-propanol showed that the reduction in enzyme activity did not affect the product conversion.

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The authors dedicate this work to the memory of Professor Octavio Augusto Ceva Antunes (and his wife and little boy), whose lives were too prematurely taken away from our existence one year ago, in the Air France flight 477 tragedy. Professor Antunes will remain a reference as scientist, an unforgettable friend, and an example of courage, always enlightening us with his experience and creativeness.

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