J. of Supercritical Fluids 107 (2016) 170–178

Contents lists available at ScienceDirect

The Journal of Supercritical Fluids

journal homepage: www.elsevier.com/locate/supflu

# Activity of immobilized lipase from *Candida antarctica* (Lipozyme 435) and its performance on the esterification of oleic acid in supercritical carbon dioxide

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ARTICLE INFO

*Article history:*
Received 27 May 2015
Received in revised form 18 August 2015
Accepted 19 August 2015
Available online 20 August 2015

*Keywords:*
Lipozyme 435
Enzyme activity
Half-time
Esterification
High pressure

ABSTRACT

The factors influencing the stability of a catalyst are crucial information for the catalytic processes. The main objective of this work was to evaluate the activity of the enzyme Lipozyme 435 in processes with supercritical carbon dioxide (SC-$CO_2$) as reaction medium. The effects of temperature (40–60 °C), pressure (10–20 MPa), exposure time (1–6 h) and depressurization steps (1–3) on the activity of the enzyme were evaluated. The kinetic data of inactivation and thermodynamic parameters were also determined. Infrared spectroscopy (FT-IR) analyses and field scanning electron microscopy (FESEM) were carried out to investigate the structure of Lipozyme 435. The results showed that the activity of Lipozyme 435 decreased with the increase of pressure, temperature, exposure time and the number of pressurization/depressurization cycles. The thermodynamic parameters showed the stability of the immobilized lipase under the tested conditions, and the kinetic data of inactivation revealed a half-life of 11 h for the lipase exposed to SC-$CO_2$ (40 °C/10 MPa) for 1 h. FT-IR analyses suggested a change in the secondary structure of the immobilized lipase, considering the first amide band, while the FESEM images did not present morphological alterations on the macroporous anionic resin used as a support for the enzyme that could affect its activity. The study of the esterification of oleic acid with methanol showed that high yields ($Y$, $g/g \times h$) and esterification rates ($X$, %) can be obtained under certain process conditions (10 MPa and 40 °C). Moreover the esterification percentage in supercritical $CO_2$ was 67% higher than in *n*-hexane medium.

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## 1. Introduction

Lipases (glycerol ester hydrolases, EC 3.1.1.3) belong to the hydrolase group and are responsible for catalyzing the hydrolysis of glycerol esters and long-chain fatty acids, producing alcohol and acid [1]. They are also useful in esterification, transesterification and lactonization (intramolecular esterification). Most of these reactions are carried out in aqueous or organic solvent media [2].

However, several studies have recently shown that lipases are stable in pressurized fluids, which increased their potential use in reactions of esterification, interesterification, transesterification and aminolysis [3]. Among the supercritical fluids used in industrial processes, carbon dioxide ($CO_2$) is the most common, due to its advantages, such as low cost, nontoxicity, non-flammability, inertness, full recovery and moderate critical properties ($P_c$ = 7.38 MPa, $T_c$ = 304.2 K), when compared to other green solvents. Therefore, reactions in supercritical $CO_2$ can be carried out with low energy cost for pressurization, and at temperatures that do not damage the enzymes [4–6].

The main advantage of using a supercritical fluid as a reaction medium is the ability to tune its properties, such as density and the viscosity, by changing its pressure and/or temperature, thus favoring the mass transfer of the substrate to the enzyme active sites. Furthermore, the change in temperature and pressure may improve the separation of the products at a later reaction stage. These attributes provide flexibility in the use of supercritical fluids as media for enzyme catalyzed processes [3,7].

As in all catalytic processes, the stability of the catalyst is a critical parameter, and therefore it is important to determine the factors that affect it. The enzymes used as biocatalysts require strict operating conditions. Thus, reactions in supercritical media at high pressures and temperatures, as well as in high pH, can lead to denaturation and enzyme inactivation [3]. According Gießauf et al. [8], the stability and the activity of an enzyme exposed to supercritical

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http://dx.doi.org/10.1016/j.supflu.2015.08.011
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$CO_2$ depend on the enzyme type, water activity in the enzyme and reaction medium, pressure and temperature conditions. However, the most important factor in enzyme deactivation is probably the depressurization process, since enzymatic activity decreases with the increase in the number of pressurization and depressurization cycles.

The critical temperatures of most supercritical fluids used as reaction media are close to those of higher stability and activity of the enzymes. Generally, these temperatures are not harmful to thermally unstable solutes. On the other hand, supercritical water (critical pressure 22.1 MPa) cannot be used as a medium for biocatalytic reactions due to the high temperatures involved, which are greater than 647.1 K [9] and would inactivate completely the enzymes [3]. Another important factor in the catalyst stability is the pressure of the process, which can lead to denaturation and enzyme inactivation, through direct and/or indirect effects. The direct effect of pressure on the inactivation is small, since the structure of the enzyme is just slightly altered, with small changes occurring at specific locations. According to da Cruz Francisco, Gough and Dey [10], pressures up to 600 MPa can cause irreversible effects on the enzyme structure, leading to its inactivation. On the other hand, the indirect effect of the pressure is more significant and occurs due to the change of the properties of the supercritical solvent, which affect the reaction rate and the solubility of the reagents [3].

The objective of this work was to investigate the application of enzymes in reactions using supercritical carbon dioxide and to evaluate the influence of process conditions on the activity of a commercial lipase (Lipozyme 435). Moreover, the half-time, decimal reduction times and thermodynamic parameters were calculated. Infrared spectroscopy analysis (FT-IR) and field emission scanning electron microscopy (FESEM) analyses were performed to assess the possible structure changes on the support of immobilized enzyme after treatment under supercritical conditions.

## 2. Materials and methods

The work was carried out in the Laboratory of High Pressure in Food Engineering–DEA/UNICAMP (LAPEA) located in Campinas-SP/Brazil.

### 2.1. Materials and chemicals

The commercial lipase from *Candida antarctica* (Lipozyme 435, food grade [11]) immobilized on a macroporous anionic resin was kindly supplied by Novozymes Brazil (Araucária-PR/Brazil). The reagents oleic acid, ethanol, methanol, acetone, sodium hydroxide, arabic gum and other chemicals (analytical grade) were obtained from Êxodo Científica (Campinas-SP/Brazil). Olive oil (Gallo, Abrantes/Portugal) with low acidity (maximum acidity ≤ 0.5%) was acquired in a local market in Campinas-SP/Brazil. Carbon dioxide (99.9%) was purchased from White Martins S.A. (Campinas-SP/Brazil).

### 2.2. Lipase activity

The enzymatic activity was measured through the volumetric method, which is based on the titrimetric determination of the free acids released from triacylglycerols by lipase catalyzed hydrolysis [12,13]. About 5 ml of olive oil emulsion, water and arabic gum (7%), 2 ml of phosphate buffer pH 7.0 (0.1 M) and 10 mg of enzyme were incubated for 1 h in a shaker incubator (TE-421, Tecnal, Piracicaba-SP/Brazil) at 40 °C and 140 orbitals per minute (OPM). The hydrolysis reaction was stopped by adding 15 ml of acetone/ethanol (1:1, v/v) and the released free acids were titrated with a 0.05 M KOH solution, using phenolphthalein as an indicator. A unit of activity (U) was defined as the amount of enzyme needed to release 1 μmol of free acids per minute. All lipase activity experiments were replicated at least three times.

### 2.3. Apparatus and experimental procedure

#### 2.3.1. Lipase treatment

The experimental homemade apparatus used in all treatments of enzyme with supercritical $CO_2$ consists basically of a $CO_2$ pump (Maximator M-111, Zorge/Germany), a solvent reservoir, a cooling (SOLAB SL152/18, Êxodo Científica, Hortolândia/SP, Brazil) and a heating thermostatic bath (Marconi S.A., Campinas-SP/Brazil), manometers (Zurich, São Paulo-SP/Brazil), a magnetic stirrer (IKA, RCT Basic, Staufen/Germany), thermocouples, control valves (Autoclave Engineers, Erie/PA, USA), a micrometric valve (Autoclave Engineers, Erie/PA, USA) and a stainless steel vessel of 100 ml Fig. 1 shows the schematic flow diagram of the high-pressure stirred-bath reactor unit.

An amount of 0.4 g of immobilized lipase was introduced inside the high-pressure stirred-bath reactor and the temperature was adjusted. Next, the reactor was pressurized with $CO_2$ at a rate 10 MPa $min^{-1}$ and pressure and temperature were maintained constant for a pre-established exposure time. After the end of exposure time, the system was depressurized at a rate of 1 MPa $min^{-1}$, and the lipase activity was measured as described in Section 2.2. The process variables evaluated were pressure (10 to 20 MPa), exposure time (1 to 6 h) and number of pressurization/depressurization cycles (1 to 3). According to the results reported by Oliveira et al. [14] for Novozyme 435 and Melgosa et al. [11] for Lipozyme 435 and RM IM, an increase of temperature in a supercritical reaction process causes a higher decrease on the residual activity of enzyme. Based on the literature survey, experiments were carried out at temperatures from 40 to 60 °C. The residual activity (%) in the lipase was defined as the ratio between the activity of the untreated enzyme ($U_0$) and that of the lipase treated with supercritical $CO_2$ ($U$), as stated in the following equation:

$$\text{Residual Activity}\,(\%) = \left(\frac{U}{U_0}\right) \times 100 \quad (1)$$

#### 2.3.2. Catalytic tests

Many lipase-catalyzed esterification reactions in supercritical $CO_2$ have been studied [15–17]. Therefore, a simple esterification reaction was selected as model to evaluate the activity of Lipozyme 435 in supercritical $CO_2$: the esterification of oleic acid with methanol. First, the reaction mixture formed by oleic acid and methanol was introduced in the batch reactor, at molar ratio of 1:1 (equimolar) and 3:1 (alcohol:acid). Then, a determined amount (160 mg) of immobilized lipase was added. Finally, $CO_2$ was pumped into the batch reactor up to the working pressure and stirred at 600 rpm After the end of reaction time, the system was depressurized at a rate of 1 MPa $min^{-1}$, and the total amount of oleic acid was measured.

The amount of consumed oleic acid was measured through the volumetric method, using a 0.05 N KOH solution in water with phenolphthalein as an indicator [18]. All experiments were replicated at least three times. From the residual amount of oleic acid, the mass balance and the reaction stoichiometry, it was possible to determine the esterification rate or conversion ($X$, %), which was calculated by the molar ratio between the products ($n_s$) and substrates ($n_s$), using the following equation:

$$X\,(\%) = \left(\frac{n_s}{n_p}\right) \times 100 \quad (2)$$

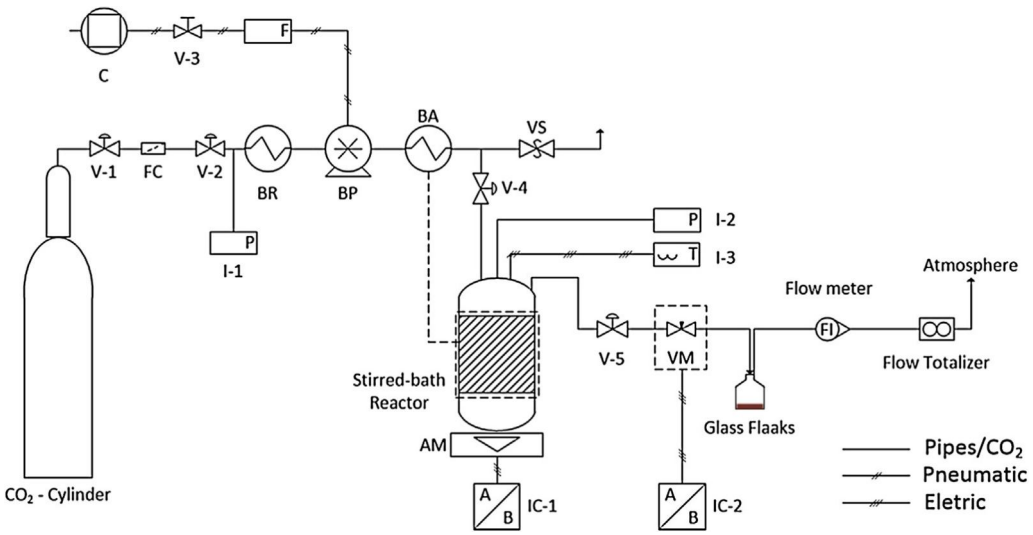


**Fig. 1.** Diagram of the homemade unit for high-pressure stirred-batch reactor containing: V-1, V-2, V-3, V-4 and V-5—control valves; VM—micrometer valves; VS—safety valve ($P_{max}$ = 30 MPa); C—compressor; F—compressed air filter; FC—$CO_2$ filter; BR—cooling bath; BP—pump (booster); BA—heating bath; I-1 and I-2—pressure indicators; I-3—temperature indicators; IC-1—indicators and controllers of magnetic stirrer; IC-2—indicators and controllers of temperature of micrometer valve; AM—magnetic stirrer.

The reaction yield ($Y$) was expressed as the mass ratio between the product ($m_p$) and the catalyst ($m_c$) per unit time ($t$), as shown in the following equation:

$$Y\left(g/g \times h\right) = \frac{m_p}{m_c \times t} \tag{3}$$

The esterification of oleic acid was also performed in *n*-hexane for comparison with the process in supercritical $CO_2$. Oleic acid and methanol were dissolved in 100 ml of hexane. The reaction was carried out at atmospheric pressure and temperature, stirring rate and substrate molar ratio (0.1 M—equimolar) equal to those used in the reactions in supercritical $CO_2$. The amount of consumed oleic acid was measured by the volumetric method, using a 0.05 N KOH solution in ethanol with phenolphthalein as indicator.

### 2.4. Estimation of kinetic data and thermodynamic parameters

The kinetic data of the lipase inactivation was calculated as described by Weemaes et al. [19], considering that in the range of experiments the inactivation kinetics behaves as a 1st-order model, as shown in Eq. (4). According to Naidu et al. [20] and Ortega et al. [21], it is assumed in this model that the enzyme activity decreases log-linearly with time.

$$\frac{dU}{dt} = -k_d \times [U] \tag{4}$$

Integrating Eq. (4) leads to:

$$\frac{U}{U_0} = \exp\left(-k_d \times t\right) \tag{5}$$

The experimental $k_d$ values were determined by the slope of the plot of $-\ln(U/U_0)$ against the sample treatment time, wherein $U$ is the enzyme activity at each experimental time and $U_0$ is the activity of the untreated enzyme, as described previously [22–24].

The half-life ($t_{1/2}$) of the lipase was calculated taking into account the time needed for the activity to be reduced to half of its original value, according the following equation:

$$t_{1/2} = \frac{\ln(2)}{k_d} \tag{6}$$

The decimal reduction time ($D$), which is defined as the treatment time needed for 90% inactivation of initial activity at a given condition, was calculated with the following equation:

$$D = \frac{2.3026}{k_d} \tag{7}$$

The enzyme deactivation energy ($\Delta E^{\#}$) was calculated from the Arrhenius equation, which relates the dependence of the constant rate of inactivation with temperature. The value of deactivation energy was obtained from the regression of the logarithm of the constant rate ($k_d$) versus the inverse of the absolute temperature ($1/T$) [25].

$$k_d = k_0 \exp\left(-\frac{\Delta E^{\#}}{RT}\right) \tag{8}$$

where the constant $k_0$ represents the probability of a reaction to take place and comprises components for the collision frequency and the orientation of the colliding particles, and R is the gas constant (8314 J mol$^{-1}$ K$^{-1}$) [26].

The energies and entropies of deactivation were estimated from the absolutes rates ($k_d$) using the following equation [20]:

$$k_d = \frac{\kappa T}{h} \times \exp\left(\frac{\Delta S^{\#}}{R}\right) \times \exp\left(-\frac{\Delta H^{\#}}{RT}\right) \tag{9}$$

where $h$ is the Plack constant ($6.6262 \times 10^{-34}$ J s), $\kappa$ is the Boltzmann constant ($1.3806 \times 10^{-23}$ J K$^{-1}$), $\Delta S^{\#}$ and $\Delta H^{\#}$ are the activation entropy and enthalpy, respectively. The values of $\Delta H^{\#}$ and $\Delta S^{\#}$ were obtained from the slope and the intercept of the regression of $\ln(k_d/T)$ versus the absolute temperature ($1/T$), respectively. The values of Gibbs free energy of deactivation ($\Delta G^{\#}$) were calculated using the following equation [21]:

$$\Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#} \tag{10}$$

### 2.5. Fourier transform infrared spectroscopy (FT-IR)

Infrared spectroscopy analysis was carried out to evaluate possible chemical alterations in the peptide bond of the lipase caused by the exposure to supercritical $CO_2$. Fourier transform-infrared spectroscopy was carried out using a CARY 630 FTIR Spectrometer Agilent Technologies in the 400–4000 cm$^{-1}$ range and with a resolution of 4 cm$^{-1}$. Untreated and treated immobilized Lipozyme 435

samples were mixed with KBr in pellets, which were prepared by pressing in a mold. The equipment was available at the Institute of Chemistry (IQ/UNICAMP) located in Campinas-SP/Brazil.

The normalization of IR spectra was carried out by dividing each spectral intensity by the square root of the sum of squares intensities on the corresponding spectrum, as shown in Eq. (11) [27]. The MATLAB software (2014Ra, MathWorks, Natick, MA, USA) was used for normalization.

$$SN_{i,j} = \frac{S_{i,j}}{\sqrt{\sum_{j=1}^{n}\left(S_{i,j}\right)^2}} \quad j = 1, 2, 3, \ldots, n. \tag{11}$$

where: $n$ is the number of wavenumbers in the scanning spectral region; $S_{i,j}$ is the spectral absorbance of sample $i$ at the $j$th wavenumber and $SN_i$ is the normalized spectral absorbance of sample $i$ at the $j$th wavenumber.

The *toolbox* in MATLAB routine developed by the Department of Analytical Chemistry and Pharmaceutical Technology (FABI)—Vrije Universiteit Brussel [28] was used for signal processing and baseline corrections by the Multiplicative Scatter Correction method (MSC) [29,30].

### 2.6. Field emission scanning electron microscopy (FESEM)

The morphology of the immobilized lipase before and after the SC-$CO_2$ treatment was analyzed using a scanning electron microscope equipped with a field emission gun (FESEM - FEI Quanta 650). Prior to analysis, the samples were coated with gold in a SCD 050 sputter coater (Oerlikon-Balzers, Balzers, Liechtenstein). Both equipment were available at the National Laboratory of Nanotechnology (LNNano) located in Campinas-SP/Brazil. The analyses of the samples were performed under vacuum, using a 10 kV acceleration voltage and a large number of images was obtained on different areas of the samples (20–25 images per sample) to guarantee the reproducibility of the results.

### 2.7. Statistical analysis

The results were statistically evaluated by analysis of variance (ANOVA), applied using the software Statistica for Windows 6.0 (Statsoft Inc., USA) in order to detect significant differences in the enzyme activity, esterification rate and yield. The significant differences at level of 5% ($p \leq 0.05$) were analyzed by the Tukey test.

## 3. Results and discussion

### 3.1. Effect of supercritical carbon dioxide on the lipase activity

The experimental enzyme activities of Lipozyme 435 treated in supercritical $CO_2$ are presented on Table 1. It can be observed that the enzyme activity decreased with the increase of pressure at the same treatment times. The lowest residual activity (about 30%) was obtained at 20 MPa and 6 h. Instead, the treatment condition with lowest influence on the enzymatic activity was the one carried out at 10 MPa for 1 h, in which 90% of the initial activity was preserved at the end of the process.

The enzymatic activity generally decreases after treatment with supercritical $CO_2$ at different pressures and exposure times. The results obtained in this work are consistent with data reported by other authors [11,14,31,32]. According to Monhemi and Housaindokht [33], supercritical $CO_2$ causes irreversible changes in the structure of enzymes, resulting in their inactivation. Furthermore, the decrease of the lipase activity treated with supercritical $CO_2$ has been related to the interactions between enzyme and solvent, which lead to the formation of covalent complexes with the free amino groups forming carbamates on the enzyme surface [14,34,35]. According to Oliveira et al. [14] and Kamat, Barrera and Beckman [35], these carbamates are possibly responsible for the removal of the histidine residues present at the active site of the enzyme, thus resulting in a decrease of its activity or even in its complete inactivation.

**Table 1**
Final and residual activities of the immobilized lipase Lipozyme 435 after treatment in supercritical $CO_2$ at constant temperature (40 °C).

| Enzyme activity (U g$^{-1}$) | | | |
|---|---|---|---|
| Initial activity 14.8 ± 1.0[a] | | | |
| Pressure (MPa) | Time (h) | Enzyme activity (U g$^{-1}$) | Residual activity (%) |
| 10 | 1 | 13.4 ± 1.3[ab] | 90.3 |
| 15 | 1 | 10.7 ± 0.5[abc] | 72.1 |
| 20 | 1 | 7.9 ± 0.8[bcd] | 53.3 |
| 10 | 3.5 | 11.1 ± 0.1[abc] | 75.1 |
| 15 | 3.5 | 8.0 ± 1.7[bcd] | 54.0 |
| 20 | 3.5 | 6.6 ± 1.0[cd] | 44.4 |
| 10 | 6 | 9.9 ± 1.5[bcd] | 67.1 |
| 15 | 6 | 6.0 ± 0.2[cd] | 40.7 |
| 20 | 6 | 4.8 ± 0.9[d] | 32.3 |

Results are expressed as mean ± standard deviation of the values performed in triplicates; time (h)—exposure time; residual activity is defined as absolute value of (final activity/initial activity) × 100; different indexes (a, b, c, d) in the same column indicate that the means differ significantly by Tukey's test ($p \leq 0.05$).

Another possible cause for the decreased lipase activity in supercritical $CO_2$ has been assigned to the hydrophilicity of the solvent, which would distort the essential interactions of water with the biocatalyst, thus promoting the inactivation or the denaturation of the enzyme. Recent results have shown that supercritical $CO_2$ has a hydrophilic nature, which can be increased with decreasing pressure [36]. Therefore, supercritical $CO_2$ removes the water present in the enzyme microenvironment, causing its inactivation [14]. Habulin et al. [37] showed that the water amount in the lipase decreased from 1.44% (before the treatment) to 0.88% after the treatment with supercritical $CO_2$. According to Silveira et al. [38], a small amount of water in the lipase can bind to specific protein sites, protecting it from the adsorption of $CO_2$ molecules, which is the main cause of enzyme inactivation under dry supercritical $CO_2$.

Some authors also suggested that the stability of a biocatalyst in supercritical $CO_2$ depends on intrinsic characteristics of the enzyme and of the process parameters during its exposure to $CO_2$ at high pressure [14,39]. According to Steinberger, Gamse, and Maar [39], high temperatures and cycles of pressurization/depressurization may cause enzyme inactivation. Considering this results, an investigation of the enzyme stability at different process temperatures (40, 50 and 60 °C) and undergoing different numbers of pressurization/depressurization cycles was carried out, keeping pressure (10 MPa) and exposure time (1 h) constant. These results are presented on Tables 2 and 3.

It can be observed in Table 2 that the activity of the enzyme decreased with increasing temperature, therefore the lowest residual activity was observed at 60 °C. At milder conditions, 40 and

**Table 2**
Final and residual activities of the immobilized lipase Lipozyme 435 after treatment in supercritical $CO_2$ at different temperatures.

| Pressure (MPa) | Temperature (°C) | Enzyme activity (U g$^{-1}$) | Residual activity (%) |
|---|---|---|---|
| 10 | 40 | 13.4 ± 1.3[a] | 90.3 |
| | 50 | 11.0 ± 0.5[ab] | 74.3 |
| | 60 | 8.4 ± 0.5[b] | 56.7 |

Results are expressed as mean ± standard deviation of the values performed in triplicates; time (h)—exposure time; residual activity is defined as absolute value of (final activity/initial activity) × 100; different indexes (a, b) in the same column indicate that the means differ significantly by Tukey's test ($p \leq 0.05$).

**Table 3**
Activity of Lipozyme 435 undergoing pressurization and depressurization cycles in supercritical $CO_2$.

| Number of cycles | Enzyme activity (U $g^{-1}$) |
|---|---|
| 1 | 13.4 ± 1.1[a] |
| 2 | 6.6 ± 1.0[b] |
| 3 | 4.9 ± 1.0[b] |

Results are expressed as mean ± standard deviation of the values performed in triplicates; Different indexes (a, b) in the same column indicate that the means differ significantly by Tukey's test ($p \leq 0.05$).

50 °C, the residual enzyme activity was not different at the level of 5% of significance. The highest value of residual activity was obtained at the lowest temperature. Investigating the influence of temperature on the activity of two commercial immobilized lipases submitted to supercritical $CO_2$, Oliveira et al. [14] observed that at higher temperatures, up to 70 °C, the activity reductions were at least 8%. Liu et al. [13] evaluated the effect of sub and supercritical $CO_2$ treatment, including pressure, exposure time and temperature on the residual activity of two commercial enzymes (*C. antarctica* Lipase B (CALB) and lipase PS in solution). Differently from this work, the authors observed an increase of activity for both lipases after treatment with sub- and supercritical $CO_2$, in which the highest residual activities obtained were 105% and 116% for CALB and lipase PS, respectively, at the same conditions (10 MPa, 40 °C and 30 min of exposure time). According to the authors, the improvement of the activity may be due to changes in the tertiary structure promoted during the exposure of the enzyme to supercritical $CO_2$.

Data on Table 3 show that the enzyme activity decreases continuously with the increasing number of cycles, and after three cycles the enzymatic activity decreased to about 64% of that obtained with only one cycle of depressurization/pressurization. An advantage of immobilized enzymes over non-immobilized enzymes is the possibility to reuse them many times. However, as any catalyst, their catalytic ability decreases with utilization cycles [40], as confirmed by the data on Table 3. The number of tested cycles is not large enough to obtain a definitive conclusion about the influence of the number of cycles on residual enzyme activity. However, the results are consistent with those found by Oliveira et al. [14], who obtained a decrease of about 10% in the residual activity after 5 cycles of depressurization/pressurization and Melgosa et al. [11], who reported a decrease of 22% after 3 cycles. Knez et al. [40], studying the esterification of lactic acid and *n*-butanol with a commercial lipase (Novozym 435), showed that after one cycle of pressurization/depressurization, 67.8% of conversion was achieved, while after three cycles the conversion rate decreased to approximately 11.4%. Besides, according to Knez [3], the pressurization step has small influence on the enzyme activity, but depressurization is usually the step that most affects the residual activity, in which the high expansion rate of the fluid can cause changes in the structure and even the inactivation of the enzyme.

In general, a decrease in the residual activity of lipase treated with supercritical $CO_2$ was observed in all the experiments, wherein the main factors were pressure and the number of pressurization and depressurization cycles. Based on these results, the half-life and the decimal reduction time of lipase treated with supercritical $CO_2$ were calculated at three different pressures, and these values are presented in Table 4.

As can be noted, a reduction of 48% in the half-life of the enzyme is achieved from 10 to 15 MPa The same behavior was observed for the decimal reduction values, for which the time necessary to obtain a 90% reduction in the enzyme activity at 10 MPa was 38 h, while for samples under other conditions the decimal reduction time was approximately 20 h. All regression coefficients ($R^2$) were greater than 0.95, indicating a good linear fit [24].

**Table 4**
Experimental values of half-life and decimal reduction time for the lipase Lipozyme 435 treated with supercritical $CO_2$ at 40 °C and three different pressures.

| Pressure (MPa) | $t_{1/2}$ (h) | $D$ (h) | $R^2$ (%) |
|---|---|---|---|
| 10 | 11.6 | 38.4 | 98.1 |
| 15 | 6.1 | 20.2 | 99.0 |
| 20 | 6.8 | 22.6 | 97.7 |

Where: $D$: decimal reduction (h) and $t_{1/2}$: half-time (h).

**Table 5**
Estimated thermodynamic parameters for the lipase Lipozyme 435 treated with $CO_2$ at 40–60 °C and 10 MPa.

| Thermodynamic parameter | Values |
|---|---|
| $\Delta E^{\#}$ (kJ $mol^{-1}$)[a] | 7.90 |
| $\Delta H^{\#}$ (kJ $mol^{-1}$)[b] | 5.22 |
| $\Delta G^{\#}$ (kJ $mol^{-1}$)[c] | 39.36–41.54 |
| $\Delta S^{\#}$ (J $mol^{-1}$ $K^{-1}$) | −109.02 |

[a] Regression coefficients ($R^2$) of 99.79%.
[b] Regression coefficients ($R^2$) of 99.97%.
[c] The temperature range was 40–60 °C.

The thermodynamic parameters were determined by irreversible deactivation in two-stage theory, as shown by Ortega et al. [21] and Tran and Chang [25]. According to these authors, at moderate temperatures the rate-limiting step for the irreversible heat deactivation of enzymes is the formation of an unfolded enzyme state ($U$), as the reaction of the following equation:

$$N \leftrightarrow T_n^{\#} \leftrightarrow U \rightarrow I \quad (12)$$

where $N$ is the native conformation of the lipase (folded), $T_n^{\#}$ is the transition state to irreversible inactivated state of lipase. Thus, $\Delta H^{\#}$ and $\Delta G^{\#}$ are the heat and the entropy change for the $N \leftrightarrow T_n^{\#}$ reaction, respectively.

Table 5 shows the thermodynamic parameters associated with the formation of the transition state ($T_n^{\#}$). It can be noted that the values obtained for entropy ($\Delta S^{\#}$) were negative. A possible explanation for this behavior is the compaction of the enzyme and the support. According to Naidu et al. [20], Ortega et al. [21] and Dannenberg and Kessler [41], negative values of $\Delta S^{\#}$ can appear due to the aggregation of charged particles around the enzyme molecule during the exposure of proteins to high temperatures. Moreover, the values obtained for $\Delta S^{\#}$ were lower than those reported by Tran and Chang [25], which found an entropy range from −28.22 to −25.11 J $mol^{-1}$ $K^{-1}$ for the immobilized lipase from *Burkholdeira* sp.; The enthalpy for the change $N \leftrightarrow T_n^{\#}$ reaction ($\Delta H^{\#}$) was also lower than the ones obtained by other research papers, such as Tran and Chang [25] and Owusu et al. [42].

According to Kristjansson and Kinsella [43], the Gibbs free energy ($\Delta G^{\#}$) can be related to the forces to stabilize the native structural state of the protein, such as the hydrophobic interaction, hydrogen bonds, disulfide bridges and electrostatic interactions, wherein high values indicate a greater stability. The values obtained for $\Delta G^{\#}$ in this work were lower than those reported by Tran and Chang [25] (88.35 to 90.13 kJ $mol^{-1}$), which were estimated for immobilized lipase from *Burkholdeira* sp. at temperature ranges from 45 to 85 °C. In turn, the deactivation energy ($\Delta E^{\#}$) obtained to the immobilized lipase treated with SC-$CO_2$ at 10 MPa and 40–60 °C was 7.90 kJ $mol^{-1}$. This value is also lower than the obtained by Chiou and Wu [44] for immobilized lipase from *Candida rugosa*, which was between 36.82 and 24.69 kJ $mol^{-1}$ for free, wet and dry lipase. According to Tran and Chang [25], the higher values of deactivation energy imply that a larger temperature change is required to deactivate the enzyme. Therefore, observing the behavior of $\Delta G^{\#}$ and $\Delta E^{\#}$ and comparing them with literature data, it can be stated

**Table 6**
Infrared band of amide regions for the enzyme Lipozyme 435 untreated and treated with supercritical $CO_2$.

| | Amide I ($cm^{-1}$) | Amide II ($cm^{-1}$) | Amide III ($cm^{-1}$) |
|---|---|---|---|
| Range* | 1600 to 1700 | ≈1560** | 1200 to 1400 |
| Untreated | 1648 ± 1 | 1551 ± *n* | 1387 ± *n* |
| 10 MPa and 1 h | 1648 ± 1 | 1551 ± 7 | 1387 ± 1 |
| 20 MPa and 6 h | 1648 ± *n* | 1551 ± 8 | 1388 ± 1 |

Results are expressed as mean ± amplitude of the values performed in duplicate; *n*—there was not variation between samples.
* Range of amide bands cited in literature for the lipase.
** Around 1560 $cm^{-1}$.

that the immobilized lipase treated with SC-$CO_2$ in this work is less stable than those obtained by other works with lipases [25,44,45]. This instability was described by many researches through molecular dynamic simulation [33,38,46,47].

### 3.2. Structural analysis

In order to analyze changes in the enzyme structure or in the support (macroporous anionic resin) due to the application of supercritical $CO_2$, FT-IR analysis and field scanning electron microscopy (FESEM) were carried out. The infrared spectroscopy spectra of Lipozyme 435 before and after treatment under two different pressure conditions are shown in Fig. 2, and the infrared band of the amide regions of Lipozyme 435 are shown on Table 6.

Analyzing the wavenumber range between 1900 and 1200 $cm^{-1}$, which are mainly assigned to the peptide group vibrations [48,49], two distinct amino bands can be observed, as shown in Fig. 2(b) and on Table 6. The first amide band (amide I), which is observed from 1600 to 1700 $cm^{-1}$, is associated to the carbonyl stretching of the peptide [48]. The second amide band (amide II) is responsible for the N—H bending with a contribution from the C—N stretching vibrations around 1550 $cm^{-1}$ [48]. A third amide band was also observed at 1387 $cm^{-1}$. According to Collins et al. [48], the amide III bands (observed between 1200 and 1400 $cm^{-1}$) are due to in-phase combination of the N—H deformation vibration with C—N, including a minor contribution from C—O to C—C stretching. In general, after the pre-processing of the spectra (baseline correction and normalization), a significant difference between the intensities of the amide I characteristic peak from the immobilized lipase before and after treatments with supercritical $CO_2$ at 10 and 20 MPa was observed. In other words, the treatment with supercritical $CO_2$ probably caused a conformational change in the secondary structure of the immobilized lipase in the amide I band, by the formation of covalent complexes with the free amino groups on the peptides forming carbamates on the enzyme, consequently decreasing the activity of the immobilized lipase.

The main objective of FESEM analysis was to investigate possible morphological changes on the support of immobilized lipase caused by the treatment with supercritical $CO_2$. Changes such as increase in porosity or the presence of obstructed pores could affect the efficiency of the enzyme. Fig. 3 shows the micrographs from Lipozyme 435 before and after treatments at different magnifications (scale bars from 300 μm to 1 μm).

At lower magnification (first line in Fig. 3) it is possible to observe that the resin particles acting as a support for Lipozyme 435 are almost perfect spheres, both before and after the treatment with supercritical $CO_2$. This suggests that the high pressures applied inside the reactor did not cause morphological modifications in the macroporous anionic resin of Lipozyme 435. Higher magnification images (second line in Fig. 3) show scratched areas on the particle surfaces due to abrasive wear. These scratched areas are equally distributed in all the analyzed samples, untreated or treated, possibly due to collisions among the particles during transport, manipulation and the flow inside the reactor. These changes cannot be assigned to the supercritical $CO_2$ treatment, since the scratches were already observed in untreated particles. In the third line of Fig. 3, amplifications inside the scratched areas are presented (scale bar of 1 μm), and they show that the porosity pattern in the particle internal region (just beneath the surface) is also kept unchanged after the treatments with supercritical $CO_2$. Some authors affirm that the treatment with supercritical $CO_2$ can cause deformations such as plasticization and swelling [50], formation of cracks, holes and fissures [14,49] in the particle surfaces, leading to increased porosity [11,14]. For instance, Melgosa et al. [11] showed a rough and cracked surface with an apparent increase in porosity for Lipozyme 435 after the treatment. Morphological changes in the resin support were not observed in the present work.

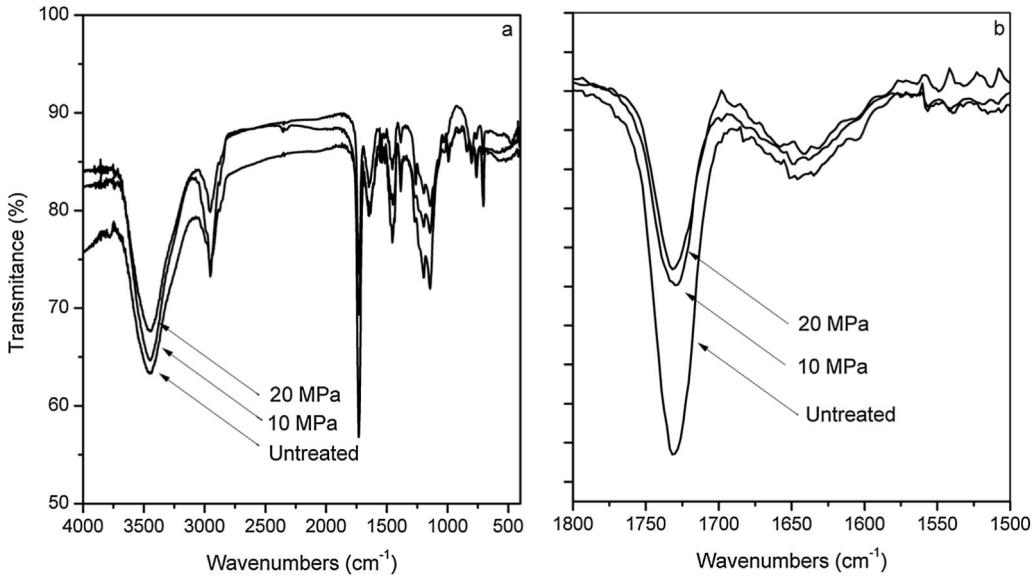


**Fig. 2.** FT-IR spectra of immobilized lipase (Lipozyme 435) untreated and treated with supercritical $CO_2$ at 10 MPa for 1 h and 20 MPa for 6 h: (a) complete IR spectra and (b) Normalized IR spectra in the 1800–1500 $cm^{-1}$ range. $CO_2$ temperature was kept constant at 40 °C.

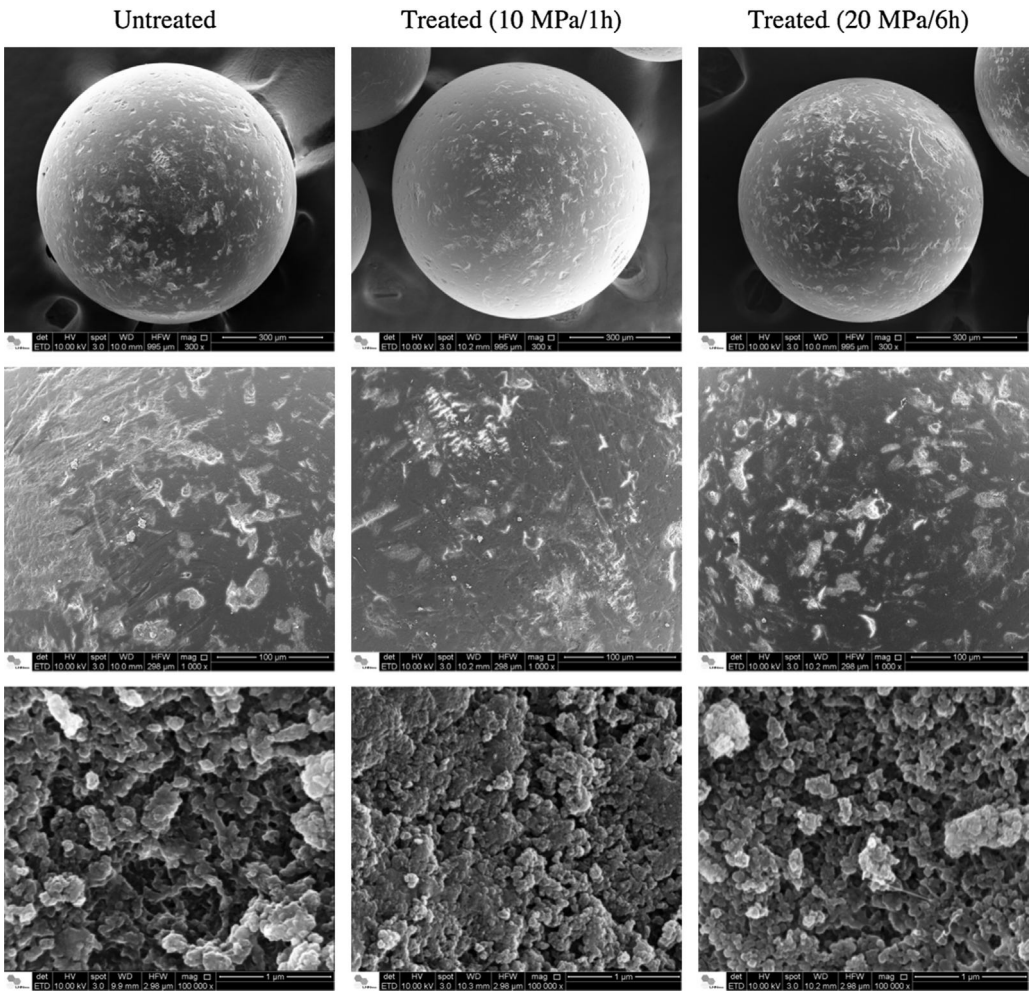


**Fig. 3.** FESEM micrographs of Lipozyme 435 untreated and treated with supercritical $CO_2$ at 10 MPa and 20 MPa for 1 and 6 h, respectively. First line: scale bar of 300 μm; Second line: scale bar of 100 μm; Third line: scale bar of 1 μm.

### 3.3. *Effect of supercritical $CO_2$ on the esterefication reaction*

In spite of the decreased residual activity of the immobilized lipase after the treatment with supercritical $CO_2$, the enzyme can still catalyze reactions in supercritical media efficiently, as demonstrated by several studies [40,51–55]. Therefore, the study of the esterification reaction of oleic acid (Fig. 4) using immobilized Lipozyme 435 as a catalyst was carried out in supercritical $CO_2$.

Fig. 4 shows the effect of pressure (Fig. 4(A)) and temperature (Fig. 4(B)) on the yield and rate of oleic acid esterification. It can be observed that an increase in pressure from 10 to 20 MPa caused a small increase in the esterification rate, from approximately 71% to 75%. The same behavior was observed for the esterification yield, which achieved the highest value at 20 MPa, approximately 7.0 kg (kg h)$^{-1}$. This behavior may be related to the higher solubilization of the substrate due to the increased density of supercritical $CO_2$ [40,56]. At the highest operating pressure (30 MPa), small decreases in the rate and in the esterification yield were noted. According to Knez et al. [40] and Knez et al. [57], these decreases may be assigned to dilution effects of the substrates due to the largest amount of $CO_2$ pumped into the reactor.

The increase of process temperature from 40 to 50 °C caused a decrease in the esterification rate and yield from approximately 71.5 to 67.7% and from 6.7 to 6.2 kg (kg h)$^{-1}$, respectively. The same behavior was observed when temperature was increased from 50 to 60 °C. These decreasing values can be explained by changes in the physical properties of the solvent, such as limitations in mass transfer, viscosity, surface tension and solvating power of the substrates (due to lower density) [3]. Moreover, this effect may not be related to enzyme denaturation because, according to Knez [3], most proteins denature at temperatures above 60 °C.

Esterification kinetics of oleic acid was performed in order to validate the results obtained in the best operating condition (40 °C and 10 MPa). In Fig. 4(C), a typical kinetic curve of an equimolar reaction is presented. The substrate consumption, which occurs simultaneously, increases the product concentration in such a way that the concentrations of both substrates were the equal at the same time, when it was possible to obtain about 74% esterification. Laudani et al. [18] studied the synthesis of n-octyl oleate by esterification of free fatty acid with 1-octanol using the immobilized lipase (Lipozyme RM IM) and found about 88% of esterification when the molar ratio between alcohol:fatty acid increased from 1:1 to 3:2. Based on this result, an esterification procedure was carried out with molar ratio alcohol:fatty acid of 3:1. It can be observed in Fig. 4(D) that the consumption of alcohol was not complete when the molar ratio was increased. On the other hand, all the oleic acid was consumed in the reaction. After 2 h, an esterification rate of 95% was achieved. In general, the results showed that the immobilized lipase Lipozyme 435 has good ability for the esterification of oleic acid in supercritical media, with values for esterification yield up to 74%.

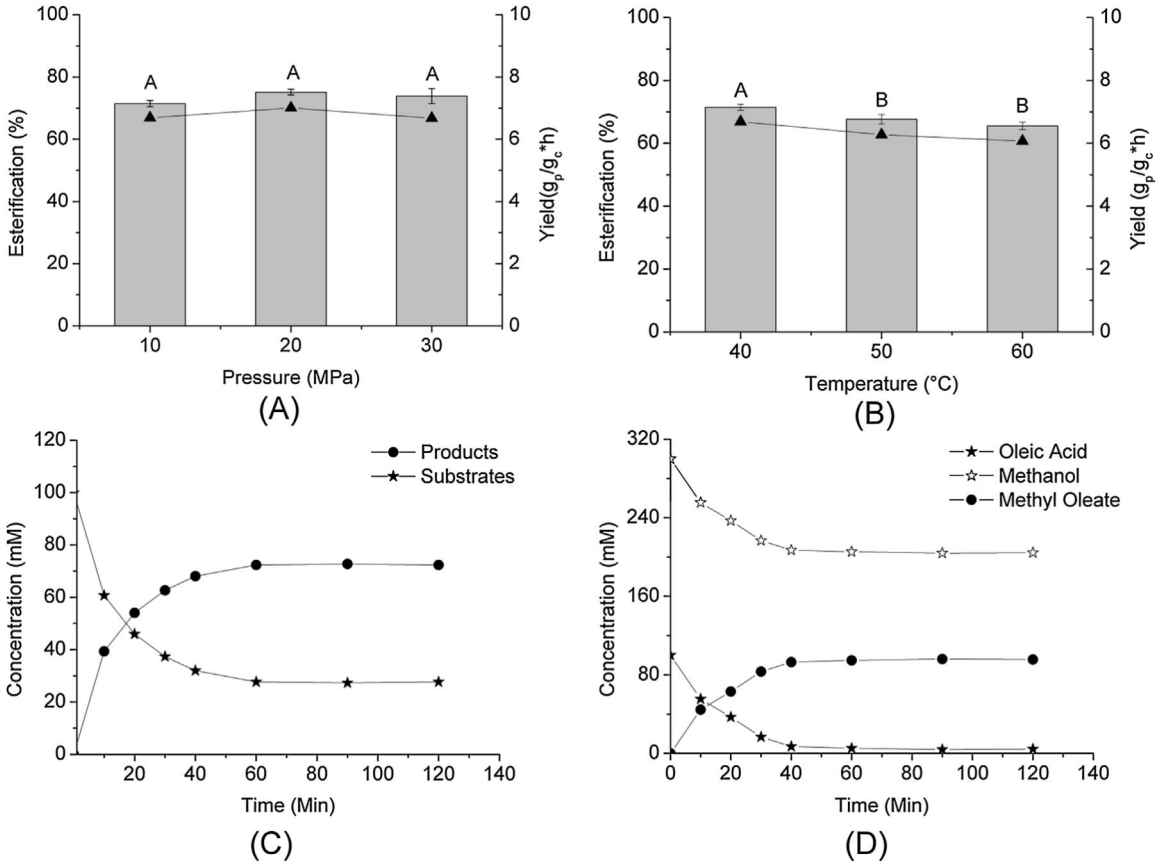


**Fig. 4.** Effect of pressure (A) and temperature (B) on the yield (▲) and esterification (bar chart) of oleic acid after 2 h of reaction using supercritical $CO_2$ as a reaction medium. Esterification kinetic of acid oleic at 40 °C and 10 MPa (C) and at molar ratio alcohol:acid 3:1; Reaction conditions: Equimolar reaction at 0.1 M (alcohol:acid) for (A), (B) and (C); 160 mg of enzyme; 600 rpm; 40 °C for different pressures and 10 MPa for different temperatures; (-) Empirically drawn lines; Different indexes on the bar chart indicate that the means differ significantly by Tukey's test ($p \leq 0.05$).

Finally, esterification reactions of oleic acid were carried out in *n*-hexane for comparison with the results obtained at supercritical conditions. The experiments were conducted with identical amount of enzyme, concentration of reagents and temperature (160 mg, 0.1 M (equimolar) and 40 °C, respectively). The comparison was made in the same reaction time, 1 h. The esterification percentage in *n*-hexane was 43.0 ± 1.5%, while in supercritical $CO_2$ (10 MPa and 40 °C) it was about 72%, achieving an increase of 67%. According to Laudani et al. [18], the higher diffusivity and lower viscosity and surface tension of supercritical $CO_2$ are responsible for the decrease of the interphase transport limitations, consequently increasing the reaction rate and esterification percentage of oleic acid.

## 4. Conclusions

The exposure of the lipase Lipozyme 435 to supercritical $CO_2$ under different process conditions reduces its residual activity. The best operating condition was 40 °C, 10 MPa and 1 h of exposure time, when the residual activity was about 90% of the original value. Higher half-life and lower decimal reduction time were also obtained at this condition. Furthermore, the residual activity of the lipase decreases with the increasing number of cycles of pressurization/depressurization. The infrared spectroscopy analysis showed that the treatment with supercritical $CO_2$ caused a conformational change in the secondary structure of the immobilized lipase in the first amide band, and the images obtained by FESEM demonstrated that high pressure treatment did not cause morphological alterations on the structure of the macroporous anionic resin support of Lipozyme 435.

The study of the oleic acid esterification with methanol was conducted to demonstrate that, although there is an inactivation of the enzyme with supercritical $CO_2$, it is still possible to obtain high yields and esterification rates under certain process conditions. The esterification percentage in supercritical $CO_2$ was 67% higher than in *n*-hexane media. Moreover, $CO_2$ is considered a green solvent and has the possibility to combine the reaction and separation of products at the end of process with a single stage. The results obtained by this work may be relevant in the selection of process conditions that cause lower activity losses for future studies with Lipozyme 435.

## Acknowledgments

The authors wish to thank CAPES, CNPq (Ph.D. fellowship: 142373/2013-3) and FAPESP (Project 2013/02203-6) for the financial support and LME/LNNano/CNPEM for the technical support during the electron microscopy work.

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