Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Molecular Catalysis B: Enzymatic 39 (2006) 117–123
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Influence of compressed fluids treatment on the activity of *Yarrowia lipolytica* lipase

Débora de Oliveira [a], Andresa C. Feihrmann [a], Cláudio Dariva [a], Aline G. Cunha [b], Juliana V. Bevilaqua [c], Jaqueline Destain [d], J. Vladimir Oliveira [a], Denise M.G. Freire [b,*]

[a] *Department of Food Engineering, URI–Campus de Erechim, Av. Sete de Setembro, 1621, Erechim 99700-000, RS, Brazil*
[b] *Instituto de Química, Centro de Tecnologia, UFRJ, Rio de Janeiro 21945-970, RJ, Brazil*
[c] *Petrobras Research and Development Center—CENPES, Petrobras, Brazil*
[d] *Centre Wallon de Biologie Industrielle, Universite de Liege, Gembloux, Belgium*

Available online 2 March 2006

Abstract

This work investigated the influence of temperature, pressure, exposure times and depressurization rate on the activity of a non-commercial immobilized lipase from *Yarrowia lipolytica* (YLL) submitted to compressed carbon dioxide, propane and *n*-butane. A high-pressure cell was employed in the experiments, in the pressure range of 10–280 bar, varying the temperature from 35 to 75 °C, exposure times from 1 to 6 h, and adopting distinct decompression rates. Results showed that significant activity losses were obtained when the treatment was conducted in carbon dioxide, while negligible losses were observed in both propane and *n*-butane. For the treatment with carbon dioxide, within the range studied, the decompression rate affected positively enzyme activity, while the exposure time and temperature presented an opposite effect on the non-commercial immobilized lipase from *Y. lipolytica* (YLL). Additionally, the performance of two commercial immobilized lipases (Lipozyme IM and Novozym 435) and the immobilized YLL in the three solvents was compared. Immobilized YLL has shown to be more suitable than Lipozyme IM for enzyme-catalyzed reactions using compressed propane and *n*-butane as solvents, but with inferior performance compared to Novozym 435 treated in these solvents.

Keywords: Lipases; Enzyme immobilization; Compressed gases; Enzyme activity; Supercritical fluids

1. Introduction

Microbial lipases (acylglycerol acylhydrolase, EC 3.1.1.3) have been increasingly employed in a broad range of applications. Furthermore, biocatalysis has been considered as the most efficient way of producing fine chemicals in a near future [1,2]. Lipases are generally effective biocatalysts due to high-substrate specific activity, low impact on the environment, functional group and stereoselectivities. Lipase immobilization offers unique advantages in terms of better process control, enhanced stability, enzyme-free products, predictable decay rates and improved economics [3–5]. Adsorption is the most usual method for lipase immobilization as it is a low cost method and presents minor deleterious effects on enzyme activity and selectivity [5]. Polymeric resins such as microporous polypropylene Accurel® MP 1000, and materials containing hydrophobic groups as octyl-agarose are examples of carriers suitable for lipase immobilization by adsorption [4–6].

For a long time enzymes were believed to work efficiently only in aqueous solutions. Consequently, their utilization in organic synthesis was rather scarce, as the low water solubility of many substrates represented a serious obstacle [7]. This disadvantage, nevertheless, stimulated the search for systems based on the use of non-aqueous solvents in order to increase the solubility of hydrophobic substrates, and as a consequence, non-aqueous biocatalysis has rapidly becoming a standard approach in the development of strategies for organic synthesis [8–11].

Since the first reports on enzyme-catalyzed reactions in supercritical fluids [12–15], much attention has been paid to the use of dense gases, mainly supercritical carbon dioxide, as potential alternatives to conventional organic solvents. The advantages of

* Corresponding author. Tel.: +55 21 2562 7360; fax: +55 21 2562 7266.
E-mail address: freire@iq.ufrj.br (D.M.G. Freire).

1381-1177/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2006.01.016

using compressed fluids over organic liquid solvents as reaction media are well discussed in the literature [1,16–18].

Several studies have shown that many reactions can be conducted in liquid or supercritical carbon dioxide, and, in some cases, rates and selectivities achieved are greater than those obtained in normal liquid or gas phase reactions [19–21]. Undoubtedly, compared to other gases, carbon dioxide has been the most widely studied fluid as solvent medium for enzyme-catalyzed reactions [1,22]. Nonetheless, a serious drawback of those applications may arise from the non-polarity of carbon dioxide, which means non-proper dissolution of both hydrophobic and hydrophilic compounds [1]. Furthermore, the hydrophilic characteristics of carbon dioxide over wide pressure ranges may affect negatively the activity of the enzyme, as the water partitioning between the enzyme and reaction mixture may be a key factor for conducting enzymatic reactions [1,23].

Nevertheless, other compressed gases seem also to be adequate for biocatalysis [18]. Actually, the comparable dielectric constant of propane and *n*-butane to carbon dioxide [8,24], and the higher pressure phase transition values generally found in systems formed by carbon dioxide with high molecular weight (e.g. triglycerides) compounds when compared to the use of propane and *n*-butane support a firm belief that propane and *n*-butane may also be suitable as reaction media for enzyme-catalyzed bioconversions [25–27]. Besides, compared to higher homologue hydrocarbons, liquid solvents at ambient conditions, the use of propane and *n*-butane offers the advantages of low separation costs, and also solvent-free products.

In order to accomplish lipase-catalyzed reactions at high pressures, the enzyme behavior in compressed fluids is of primary importance as the loss of enzyme activity may lead to undesirable poor reaction rates and low yields of target products. Enzyme stability and activity may depend on the enzyme species, characteristics of compressed fluid, water content of the enzyme/support/reaction mixture, and process variables manipulated. As aforementioned, while there is relatively abundant data regarding activity and stability of enzymes in carbon dioxide, there is a lack of corresponding experimental information for propane and *n*-butane.

Though at present the high cost of enzyme production may be the major obstacle to commercialization of enzyme-catalyzed processes, recent advances in enzyme technology, such as the use of solvent-tolerant lipases and immobilized lipases—making catalyst re-utilization possible, have been made to develop cost-effective systems [28]. Furthermore, the possibility of using a non-commercial lipase obtained from relatively low-cost and renewable raw materials renders additional importance towards conducting studies on enzymatic activity in non-conventional fluids.

In this context, this work investigates the influence of temperature, pressure, exposure time and decompression rates on the activity of an immobilized, non-commercial lipase from *Yarrowia lipolytica* (YLL). A comparison between immobilized YLL with two commercial immobilized lipases (Lipozyme IM and Novozym 435) as submitted to supercritical carbon dioxide and compressed propane and *n*-butane media at the same experimental conditions is provided.

2. Experimental

2.1. Materials

2.1.1. Enzymes

Lipase from *Y. lipolytica* was produced in a 2000 L fermentor containing (w/v) 1% of glucose, 3% of whey powder, 0.8% of ammonium sulphate, 1% of cornsteep syrup and 0.5% of olive oil. After 30 h of fermentation, the culture broth was centrifuged and the supernatant was dried by lyophilization [29].

Commercial immobilized lipases were kindly supplied by Novozymes Brazil (Araucária, PR, Brazil): *Mucor miehei* (Lipozyme IM) immobilized on a macroporous anion exchange resin, and *Candida antarctica* (Novozym 435) immobilized on a macroporous anionic resin. According to the manufacturer, the optimum activity is achieved at 40 °C for Lipozyme IM and 70 °C for Novozym 435 [30].

2.1.2. Chemicals

Lauric acid, ethanol, acetone and other chemicals (analytical grade) were obtained from Merck. Carbon dioxide (99.9%), propane (99.5%) and *n*-butane (99.5%) were purchased from White Martins S.A.

2.2. Apparatus and experimental procedure

2.2.1. Lipase immobilization

The lyophilized preparation was solubilized in phosphate buffer (0.05 M pH 7.0) and submitted to preferential immobilization by physical adsorption on hydrophobic support (Accurel® MP 1000) [6].

Carrier preparation was performed by adding 10 mL of ethanol to 1000 mg of the carrier. After 30 min, the supernatant was poured out and the carrier was washed repeatedly with distilled water until ethanol was completely removed. Enzyme immobilization was performed at a relation soluble enzyme (mL): support (mg) of 1:25 and the activity was in the range of 30–260 U_H/mL. Immobilization was performed with magnetic stirring in an ice cooler and aliquots were sampled periodically (0, 1, 5, 10, 15, 20, 30, 60, 90, 120 min) for protein content assay. The supernatant and carrier with enzyme were also assayed for enzyme activity. Lipase activity was determined as described previously [31]. One lipase activity unit (U_H) was defined as the amount of enzyme that produces 1 μmol of fatty acids per minute, under the assay conditions.

2.2.2. Lipases esterification activity

The enzyme activity was determined as the initial rates in esterification reactions between lauric acid and propanol at a molar ratio of 3:1, temperature of 60 °C and enzyme concentration of 5 wt% in relation to the substrates. At the beginning of the reaction, samples containing the mixture of lauric acid and propanol were collected, and the lauric acid content was determined by titration with 0.04 M NaOH. After the addition of the enzyme to the substrates, the mixture was kept at 60 °C for 15 min. Then, the lauric acid consumption was determined. One lipase activity unit (U_E) was defined as the amount of enzyme

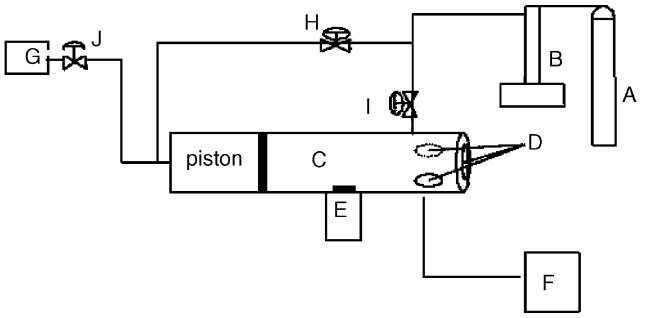

Fig. 1. Schematic diagram of the high-pressure apparatus for enzyme activity tests. A, solvent reservoir; B, syringe pump; C, equilibrium cell; D, sapphire windows; E, magnetic stirrer; F, white light source; G, pressure transducer; H, ball valve; I, micrometering valve; J, relief valve.

necessary to consume 1 μmol of lauric acid per minute at the established experimental conditions presented previously. All enzymatic activity determinations were replicated at least three times.

2.2.3. High-pressure enzyme treatment

The equipment used in all experiments consists basically of a solvent reservoir, a 20 mL view cell with three sapphire windows for visual observations, an absolute pressure transducer (Smar LD 301), with a precision of ±0.30 bar, a portable programmer (Smar, HT 201) for the pressure data acquisition, and a syringe pump (ISCO 260D). The equilibrium cell contains a movable piston, which permits the pressure control inside the cell. The experimental apparatus, built to conduct the experiments up to 300 bar and 80 °C, is schematically presented in Fig. 1.

The lipases (approximately 1.0 g) were charged into the cell and the temperature established in the experimental design was set. Afterwards, the system was pressurized and then maintained at constant temperature and pressure during preestablished exposure time. Typically, the pressure come up time was less than 0.5 min and accordingly it was not included in the pressure holding time because of its relatively small time compared to longer holding times. Then, at the decompression rates defined: 10–200 kg m^{-3} min^{-1} for CO_2 and in the range of 2–50 bar min^{-1} for propane and *n*-butane, the system was depressurized and the lipase activity was measured. The change in the lipase activity was defined as the difference between the activity at the beginning and at the end of the process.

2.3. Experimental design

A Plackett–Burman semi-factorial experimental planning with two levels and four variables (temperature, exposure time, decompression rate and reduced density or pressure) was adopted to establish the experimental conditions. The experimental planning was conceived to cover the variable ranges commonly used for enzyme-catalyzed reactions in compressed fluids. The experiments were accomplished randomly, and duplicate runs were carried out at the central point of the experimental design to allow estimation of standard deviation. The influence of the process variables on the enzyme activity was assessed by an empirical modeling technique. In order to obtain independent parameters (effects) and to allow a direct comparison of each variable effect, the independent variables were normalized in the range of −1 to +1, according to:

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

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

The "−1" level represents the lower limit, while the "+1" level represents the upper limit of each variable. A statistical modeling technique was used to obtain an empirical model able to reproduce the experimental data. Empirical models were built by assuming that all variable interactions were significant, the parameters related to each variable interaction and main variable effects were estimated, and the meaningless parameters were discarded considering a confidence level of 95%, by using the Student's "*t*" test. The objective of using the Student's *t*-test is to evaluate whether the parameters were significantly different from zero. This test takes into account the standard error of each parameter according to a well-known procedure available in many textbooks [32]. The parameters were estimated using the Statistica® 5.5 software [33].

3. Results and discussion

Table 1 presents YLL immobilization at different initial protein contents and results obtained as hydrolytic activities (U_H) and protein immobilization yields. The YLL immobilization procedure was completed within 2 h for all initial protein contents, with protein and hydrolytic activity immobilized yields in the range of 58–83% and 7.9–44.5%, respectively (Table 1).

Table 1
Immobilization of lipase from *Yarrowia lipolytica* at different initial protein contents

Protein content (mg/g carrier)	Immobilized protein (mg/g carrier)	Protein immobilized yield (%)	Hydrolytic activity (U_H/g carrier)	Immobilized specific activity (U_H/mg protein)	Hydrolytic activity yield (%)
4.0	2.3	58	18	7.8	36.4
8.1	4.9	60	47	9.6	44.9
20	13	65	68	5.2	24.3
42	35	83	58	1.7	7.9

Hydrolytic specific activity equal to 21.4 ± 0.1 U_H/mg.

Table 2
Experimental design for *Yarrowia lipolytica* activity measurements in pressurized CO_2

Experiment	T (°C)	t (h)	R (kg m^{-3} min^{-1})	RD	Enzyme activity (U_E/g)		Activity loss (%)
					Initial	Final	
1	35 (−1)	1 (−1)	10 (−1)	0.5 (−1) 71.5[a]	342	307	10.2
2	35 (−1)	1 (−1)	200 (+1)	1.6 (+1) 110.4	338	297	12.1
3	35 (−1)	6 (1)	10 (−1)	1.6 (+1) 110.4	390	306	21.5
4	35 (−1)	6 (1)	200 (+1)	0.5 (−1) 71.5	385	322	16.3
5	75 (+1)	1 (−1)	10 (−1)	1.6 (+1) 276.4	390	346	11.2
6	75 (+1)	1 (−1)	200 (+1)	0.5 (−1) 100	387	342	11.6
7	75 (+1)	6 (1)	10 (−1)	0.5 (−1) 100	360	257	28.6
8	75 (+1)	6 (1)	200 (+1)	1.6 (+1) 276.4	382	288	24.6
9	55 (0)	3.5 (0)	105 (0)	1.05 (0) 117.9	370 383	324 338	12.1[b]

[a] Second line along this column provides pressure values (bar) estimated from the Angus et al. [34] equation.
[b] Standard deviation: ±0.4. R is the decompression rate (kg m^{-3} min^{-1}) and RD is the reduced density (dimensionless).

An increase in the initial protein content led to an enhancement of protein immobilization yields. However, immobilized hydrolytic activity yields enhanced until 8.1 mg of initial protein content (44.9%), decreasing afterwards. Immobilized YLL activity (U_H/g) was smoothly improved up to 20 mg protein per g carrier (maximum activity of 68 U/g carrier). Nevertheless, higher initial protein contents have not increased the hydrolytic activity neither the hydrolytic activity yields probably due to the fact that at this value the carrier Accurel® MP1000 had achieved maximum enzyme load and higher protein contents caused carrier overcrowding. Immobilized YLL preparation immobilized with 20 mg of initial protein content (68 U_H/g) was selected for further experiments to evaluate storage stability, esterification activity and stability under high pressure conditions.

Immobilized biocatalyst presented storage stability higher then 90% during about 1 month of storage at room temperature (25 °C) in comparison to only 1 day for soluble YLL.

The biocatalyst esterification activity was found to be 350 U_E/g. It is worth mentioning that the soluble YLL preparation did not show any esterification activity, so that this immobilization procedure is essential for enzyme utilization in non-hydrolytic reactions.

The esterification activity of immobilized YLL after been submitted to supercritical carbon dioxide and compressed propane and *n*-butane are presented in Tables 2–4. In these tables, real and coded levels (±1) are given on columns 2–5, while the last three columns show the enzyme activity values before and after treatment along with the computed percent change on

Table 3
Experimental design for *Yarrowia lipolytica* activity measurements in compressed propane

Experiment	T (°C)	P (bar)	t (h)	R (bar min^{-1})	Enzyme activity (U_E/g)		Activity loss (%)
					Initial	Final	
1	35 (−1)	30 (−1)	1 (−1)	2 (−1)	336	336	0
2	35 (−1)	250 (+1)	1 (−1)	50 (+1)	315	312	0.9
3	35 (−1)	250 (+1)	6 (+1)	2 (−1)	322	319	0.9
4	35 (−1)	30 (−1)	6 (+1)	50 (+1)	331	329	0.6
5	75 (+1)	250 (+1)	1 (−1)	2 (−1)	350	345	1.4
6	75 (+1)	30 (−1)	1 (−1)	50 (+1)	331	329	0.6
7	75 (+1)	30 (−1)	6 (+1)	2 (−1)	325	320	1.5
8	75 (+1)	250 (+1)	6 (+1)	50 (+1)	391	387	1.0
9	55 (0)	140 (0)	3.5 (0)	26 (0)	389 364	385 358	1.3[a]

[a] Standard deviation: ±0.3. R is the decompression rate (kg m^{-3} min^{-1}) and RD is the reduced density (dimensionless).

Table 4
Experimental design for *Yarrowia lipolytica* activity measurements in compressed *n*-butane

Experiment	T (°C)	P (bar)	t (h)	R (bar min^{-1})	Enzyme activity (U_E/g)		Activity loss (%)
					Initial	Final	
1	35 (−1)	10 (−1)	1 (−1)	2 (−1)	322	319	0.9
2	35 (−1)	250 (+1)	1 (−1)	50 (+1)	339	334	1.4
3	35 (−1)	250 (+1)	6 (+1)	2 (−1)	390	387	0.7
4	35 (−1)	10 (−1)	6 (+1)	50 (+1)	330	328	0.6
5	75 (+1)	250 (+1)	1 (−1)	2 (−1)	395	392	0.7
6	75 (+1)	10 (−1)	1 (−1)	50 (+1)	355	351	1.1
7	75 (+1)	10 (−1)	6 (+1)	2 (−1)	364	360	1.0
8	75 (+1)	250 (+1)	6 (+1)	50 (+1)	349	347	0.5
9	55 (0)	130 (0)	3.5 (0)	26 (0)	317	314	0.7[a]
					318	316	

[a] Standard deviation: ±0.1. R is the decompression rate (kg m^{-3} min^{-1}) and RD is the reduced density (dimensionless).

enzyme activity. Additional runs (last rows of these tables) were carried out at the central point, coded 0, to estimate the overall curvature effect.

The experiments were accomplished in the temperature (T) range of 35–75 °C and exposure times (t) of 1–6 h for all solvents. For carbon dioxide, the decompression rates (R) covered the range 10–200 kg m^{-3} min^{-1} with reduced density (RD) varying from 0.5 to 1.6. For this solvent, densities were estimated from the Angus et al. [34] equation. For propane and *n*-butane, due to their nearly incompressible nature within the temperature and pressure ranges investigated, the lower (slow) and upper (fast) depressurization (R) levels of 2 and 50 bar min^{-1} were adopted, and the system pressure (P) as process variables (30–250 bar for propane and 10–250 bar for *n*-butane). Results for immobilized YLL can be better visualized in Fig. 2, which shows that exposure to carbon dioxide led to the highest activity losses (up to ≈29%), and then followed in a much lesser extent by propane (maximum of 1.5%) and by *n*-butane (maximum of 1.4%).

Table 5 presents the results obtained in the statistical modeling for immobilized YLL. The values of the parameters shown in this table reflect the enzyme activity losses as a result of changing each variable from the lower to the upper level. In this sense, positive values of a specific parameter mean that the change of a process variable from lower to upper level leads to an increase in the response variable (enzyme activity loss). The absolute value of each variable indicates how much the variable influences the enzyme activity loss. For example, concerning the results for carbon dioxide, a change of temperature from 35 °C (−1) to 75 °C (+1) causes a decrease of 1.9% in the enzyme activity compared to the untreated enzyme. The independent parameter indicates the mean value of the enzyme loss when all independent values are located in the average values.

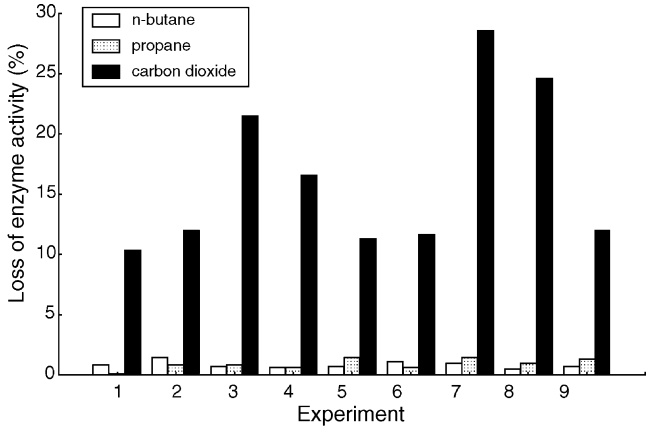

Fig. 2. Loss of immobilized YLL activity after treatment in compressed carbon dioxide, propane and *n*-butane. Experimental conditions presented in Tables 2–4.

Table 5
Regression results for the activity of *Yarrowia lipolytica* in compressed CO_2, propane and *n*-butane

Effect	Parameter value	Parameter uncertainty
Carbon dioxide: activity loss = $a_0 + a_1T + a_2t + a_3R + a_4T\mathrm{RD} + a_5\mathrm{RD}R + a_6$ quadratic, correlation coefficient = 0.999		
Independent	$a_0 = 12.0$	0.59*
Temperature (T)	$a_1 = 1.9$	0.21*
Exposure time (t)	$a_2 = 5.7$	0.21*
Decompression rate (R)	$a_3 = -0.8$	0.21*
TRD	$a_4 = -1.3$	0.21*
RRD	$a_5 = 1.8$	0.21*
Quadratic	$a_6 = 5.0$	0.63*
Propane: activity loss = $a_0 + a_1T + a_2P + a_3t + a_4R + a_5TR + a_6tR + a_7$quadratic, correlation coefficient = 0.999		
Independent	$a_0 = 1.2$	0.05*
Temperature (T)	$a_1 = 0.2$	0.02↑
Pressure (P)	$a_2 = 0.1$	0.02*
Exposure time (t)	$a_3 = 0.1$	0.02*
Decompression rate (R)	$a_4 = -0.1$	0.02*
TR	$a_5 = -0.2$	0.02*
tR	$a_6 = -0.1$	0.02*
Quadratic	$a_7 = -0.3$	0.05*
n-Butane: activity loss = $a_0 + a_1T + a_2t + a_3R + a_4TP + a_5$quadratic, correlation coefficient = 0.986		
Independent	$a_0 = 0.6$	0.05*
Temperature (T)	$a_1 = 0.1$	0.02↑
Exposure time (t)	$a_2 = -0.1$	0.02*
Decompression rate (R)	$a_3 = -0.1$	0.02*
TP	$a_4 = -0.1$	0.02*
Quadratic	$a_5 = 0.1$	0.06*

Superscripts * and ↑ denote, respectively, significant and non-significant parameter at a 95% confidence level. Quadratic term: indicates possible non-linear behavior of some variable within the experimental range investigated.

One should note from this table that the treatment in all solvents promotes a decrease in enzyme activity, especially in compressed carbon dioxide (see the significant value of the independent parameter). In general, the decompression rate is affected negatively by the enzyme activity while the temperature showed to be relevant only for carbon dioxide and the exposure time did not demonstrate to be a relevant variable for propane and *n*-butane. Note also that some cross-interaction effects, like temperature-reduced density or temperature-pressure, on enzyme activity should not be neglected.

Apart from the results obtained in three experimental conditions (3, 7 and 8) for carbon dioxide, activity losses for the immobilized YLL can be considered low when compared to the values reported in the work of Habulin and Knez [8], who studied the activity of some lipases through the esterification reaction of butyric acid and ethanol in supercritical carbon dioxide and propane at 40 °C and 100 bar. It is interesting to mention that these authors also verified that the enzyme activity was improved in propane.

Fig. 3 presents a comparison between the immobilized YLL with two commercial immobilized lipases, Lipozyme IM and Novozym 435, at the same experimental condition (central point of the experimental design—condition 9 of Tables 2–4). The treatment in carbon dioxide also had a deleterious effect on the activity of the commercial Novozym 435 and Lipozyme IM, though in a less degree compared to immobilized YLL. On the other hand, it is worth noticing that treatment of Novozym 435 in compressed propane and *n*-butane improved enzyme activity for this experimental condition, with resulting activity gains around 10%. For these two compressed gases however activity losses were also observed for Lipozyme IM.

Results reported in this work for all enzymes treated in carbon dioxide are in complete agreement with those reported by some authors [1,8,17,18,35–39], but opposite to the ones reported by Gieβauf and Gamse when dealing with porcine pancreatic lipase [40]. Furthermore, a series of enzyme-catalyzed reactions conducted in both conventional and supercritical fluid media has shown that while no loss of enzyme activity was experimentally observed for the conventional medium [41,42], the same was no longer valid for supercritical carbon dioxide containing systems [19,20].

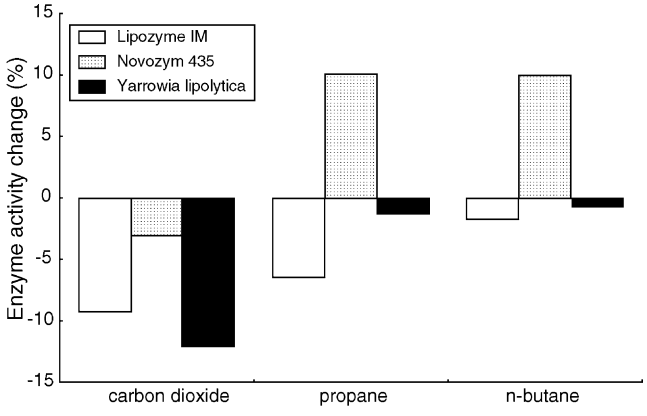

Fig. 3. Comparison of activity changes between lipase from *Yarrowia lipolytica* with two commercial lipases after treatment in compressed carbon dioxide, propane and *n*-butane at experimental condition 9 of Tables 2–4.

It has been argued that the decrease of lipase activity in supercritical carbon dioxide may be attributed to enzyme–solvent interactions, leading to the formation of covalent complexes with the free amino groups on the surface of the enzyme to form carbamates, which would result in charge removal at histidine residues, and thus could contribute to the loss of enzyme activity [1,8,22,43].

According to the log *P* hydrophobicity factor, defined as the logarithm of the partition coefficient in a standard octanol–water two-phase system, activities are low in relatively hydrophilic solvents having $\log P < 2$, are quite variable for solvents having $\log P$ between 2 and 4, and is high in hydrophobic solvents for which $\log P > 4$ [3]. The reason is that solvents having $\log P < 2$ strongly distort the essential water–biocatalyst interactions, thereby inactivating or denaturing the biocatalyst [3].

Recent results show that pressurized carbon dioxide is rather hydrophilic; with $\log P$ values lower than 2 in a wide pressure range and hydrophilicity increasing with a decrease in pressure, though the water solubility decreases simultaneously [23]. Accordingly, supercritical carbon dioxide could strip the essential water from the enzyme microenvironment, thus causing enzyme deactivation with the unfavorable water partitioning between the enzymatic support and the solvent [1,8,22]. Hence, differences in water partitioning may have also contributed to the higher activity values found for the examined lipases in propane and in *n*-butane compared to those in carbon dioxide [8].

This factor may have a crucial role when considering a rapid release of carbon dioxide dissolved in the bound water [18]. Steinberger et al. [38] have pointed out that the stability of an enzyme in supercritical carbon dioxide depends on both its tertiary structure and on several parameters during exposure to high-pressure carbon dioxide. They argued that high temperatures, water content in carbon dioxide and pressurization/depressurization steps can cause enzyme inactivation.

Regarding treatment in propane and in *n*-butane, it has been mentioned that solvents with low dielectric constant favor interfacial activation thus opening the lipase lid [1,8]. Furthermore, because of the hydrophobic characteristics of propane and *n*-butane and the fact that they behave like a conventional liquid in the present study, and possibly due to affinity of the enzyme particles for these solvents, relatively lower activity losses as compared to treatment in carbon dioxide or even significant activity improvements were experimentally observed.

4. Conclusions

The results obtained in this work showed that this new biocatalyst (YLL immobilized in hydrophobic carrier) presented good storage stability and high stability under high pressure conditions, enabling its utilization in many biotransformation processes of raw materials in compressed and supercritical fluids. We concluded that the magnitude of pressure (or reduced density), temperature, decompression rate and exposure time affected lipase activity in different grades, depending on the nature and the source of enzyme and, mainly, whether the

enzyme is in its native or immobilized form. It seems also that the use of non-commercial, "home-made", enzymes may be a feasible alternative to the development of cost-effective enzymatically catalyzed processes.

Acknowledgements

The authors thank CNPq, FAPERJ, FAPERGS and ANP/FINEP/PETROBRAS for the financial support of this work and scholarships.

References

[1] Z. Knez, M. Habulin, J. Supercrit. Fluids 23 (2002) 29.
[2] E. Cernia, C. Palocci, S. Soro, Chem. Phys. Lipids 93 (1998) 157.
[3] C. Laane, S. Boeren, R. Hilhorst, C. Veeger, in: C. Laane, J. Tramper, M.D. Lilly (Eds.), Biocatalysis in Organic Media, Elsevier Science Publishers, Amsterdam, The Netherlands, 1987, p. 65.
[4] W. Tischer, V. Kasche, Trends Biotechnol. 17 (1999) 326.
[5] M. Persson, E. Wehtje, P. Adlercreutz, Biotechnol. Lett. 22 (2000) 1571.
[6] B. Al-Duri, Y.P. Yong, Biochem. Eng. J. 4 (2000) 207.
[7] E. García-Junceda, J.F. García-García, A. Bastida, A. Fernández-Mayoralas, Bioorg. Med. Chem. 12 (2004) 1817.
[8] M. Habulin, Z. Knez, J. Chem. Technol. Biotechnol. 76 (2001) 1260.
[9] W. Kaewthong, A. H-Kittikun, Enzyme Microb. Technol. 35 (2004) 218.
[10] D. Oliveira, M. Di Luccio, C. Faccio, C. Dalla Rosa, J.P. Bender, N. Lipke, S. Menoncin, C. Amroginski, J.V. Oliveira, Appl. Biochem. Biotechnol. 115 (2004) 771.
[11] D. Oliveira, I.N. Filho, M. Di Luccio, C. Faccio, C. Dalla Rosa, J.P. Bender, N. Lipke, C. Amroginski, C. Dariva, J.V. Oliveira, Appl. Biochem. Biotechnol. 121 (2005) 231.
[12] T.W. Randolph, H.W. Blanch, J.M. Prausnitz, C.R. Wilke, Biotechnol. Lett. 7 (1985) 325.
[13] D.A. Hammond, M. Karel, A.M. Klibanov, Appl. Biochem. Biotechnol. 11 (1985) 393.
[14] K. Nakamura, Y.M. Chi, Y. Yamada, T. Yano, Chem. Eng. Commun. 45 (1986) 207.
[15] Y.M. Chi, K. Nakamura, T. Yano, Agric. Biol. Chem. 52 (6) (1988) 1541.
[16] Z. Knez, M. Habulin, V. Krmelj, J. Supercrit. Fluids 14 (1998) 17.
[17] M. Habulin, M. Primozic, Z. Knez, J. Supercrit. Fluids 33 (2005) 27.
[18] F.-J. Kao, S.A. Ekhorutomwen, S.P. Sawan, Biotechnol. Tech. 11 (12) (1997) 849.
[19] J.V. Oliveira, D. Oliveira, Ind. Eng. Chem. Res. 39 (2000) 4450.
[20] D. Oliveira, J.V. Oliveira, J. Supercrit. Fluids 19 (2001) 141.
[21] P.E. Savage, S. Gopalan, T.L. Mizan, C.J. Martino, E.E. Brock, AIChE J. 41 (1995) 1723.
[22] S.V. Kamat, E.J. Beckman, A.J. Russell, Crit. Rev. Biotechnol. 15 (1995) 41.
[23] H. Nakaya, O. Miyawaki, K. Nakamura, Enzyme Microb. Technol. 28 (2001) 176.
[24] H.-Q. Chen, J.-C. Yang, Proceedings of the Fifth International Symposium on Supercritical Fluids, Atlanta, USA, 9–11 April, 2000, p. 1.
[25] M. Lanza, P.M. Ndiaye, F.W. Tavares, D. Oliveira, C. Dariva, J.V. Oliveira, J. Supercrit. Fluids 34 (2005) 215.
[26] P.M. Ndiaye, M. Lanza, F.W. Tavares, C. Dariva, D. Oliveira, J.V. Oliveira, Braz. J. Chem. Eng., in press.
[27] P.M. Ndiaye, E. Franceschi, D. Oliveira, C. Dariva, F.W. Tavares, J.V. Oliveira, J. Supercrit. Fluids 37 (2005) 29.
[28] H. Fukuda, A. Kondo, H. Noda, J. Biosci. Bioeng. 92 (2001) 405.
[29] J. Destain, D. Roblain, P. Thonart, Biotechnol. Lett. 19 (1997) 105.
[30] Novo Nordisk, Technical Report A-05948 (1992).
[31] D.M.G. Freire, E.M.F. Teles, E.P.S. Bon, G.L. Sant'Anna Jr., Appl. Biochem. Biotechnol. 63 (1997) 409.
[32] D.M. Himmelblau, Process Analysis by Statistical Methods, John Wiley & Sons, New York, 1970.
[33] Statistica for Windows, 5.5, Statsoft (2000).
[34] S. Angus, B. Armstrong, K.M. Reuck (Eds.), International Thermodynamic Tables of the Fluid State, Pergamon Press, Oxford, UK, 1976.
[35] A. Gieβauf, W. Magor, D.J. Steinberger, R. Marr, Enzyme Microb. Technol. 24 (1999) 577.
[36] M.C. Almeida, R. Ruivo, C. Maia, L. Freire, T.C. Sampaio, S. Barreiros, Enzyme Microb. Technol. 22 (1998) 494.
[37] M. Lanza, W.L. Priamo, J.V. Oliveira, C. Dariva, D. Oliveira, Appl. Biochem. Biotechnol. 113 (2004) 181.
[38] D.-J. Steinberger, T. Gamse, R. Maar, Proceedings of the Fifth Conference on Supercritical Fluids and Their Applications, Verona, Italy, 13–16 June, 1999, p. 339.
[39] M. Castellari, L. Matricardi, G. Arfelli, P. Rovere, A. Amati, Food Chem. 60 (1997) 647.
[40] A. Gieβauf, T. Gamse, J. Mol. Catal. B: Enzym. 9 (2000) 57.
[41] D. Oliveira, T.L.M. Alves, Appl. Biochem. Biotechnol. 77 (1999) 835.
[42] D. Oliveira, T.L.M. Alves, Appl. Biochem. Biotechnol. 84 (2000) 59.
[43] S. Kamat, G. Critchley, E.J. Beckman, A.J. Russell, Biotechnol. Bioeng. 46 (1995) 610.