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ORIGINAL PAPER

Assessment of two immobilized lipases activity and stability to low temperatures in organic solvents under ultrasound-assisted irradiation

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Abstract Both stability and catalytic activity of two commercial immobilized lipases were investigated in the presence of different organic solvents in ultrasound-assisted system. In a general way, for Novozym 435, the use of ethanol as solvent led to a loss of activity of 35% after 10 h of contact. The use of iso-octane conducted to a gradual increase in lipase activity in relation to the contact time, reaching a maximum value of relative activity of 126%. For Lipozyme RM IM, after 5 h of exposure, the enzyme presented no residual activity when ethanol was used as solvent. The solvents tert-butanol and iso-octane showed an enhancement of about 20 and 17% in the enzyme activity in 6 h of exposure, respectively. Novozym 435 and Lipozyme IM presented high stability to storage after treatment under ultrasound-assisted system using *n*-hexane and tert-butanol as solvents.

Keywords Lipases · Enzyme activity · Ultrasound system · Enzyme stability

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Introduction

Lipases, triacylglycerol hydrolases, comprise an important group of biotechnologically relevant enzymes and they find huge applications in food, dairy, detergent and pharmaceutical industries. Lipases can be produced from microorganisms, and specifically bacterial lipases play a vital role in commercial ventures [1, 2]. Lipases are also defined as glycerol ester hydrolases that catalyze the hydrolysis of triglycerides to free fatty acids and glycerol. Lipases are also able to catalyze esterification, interesterification, acidolysis, alcoholysis and aminolysis in non- and micro-aqueous media [3–5]. The major advantages of using lipases are their wide range of substrates in many instances with high chemo-, regio- and stereoselectivity as well as the very mild reaction conditions that can be used [6].

The ability of enzymes being active in the presence of organic solvents has received a great deal of attention over the decades. In an organic solvent environment, some enzymes evidence enhanced thermostability, and it is possible to conduct reactions that are suppressed in water environments [7].

The use of organic solvents as reaction media for enzymatic reactions provides numerous industrially attractive advantages compared to traditional aqueous reaction systems, as enhancement of solubility of the substrates, reversion of thermodynamic equilibrium, and suppression of water-dependent reactions and elimination of microbial contamination. However, some disadvantages can be cited, as inactivation of enzymes, labor and cost-intensive preparation of biocatalysts in covalently modified systems, mass-transfer limitations in the case of heterogeneous systems or viscous solvents/substrates, water activity control needed for processes involving condensation reactions [8]. High stability of enzymes in the organic environment significantly

expands opportunities of their application in biotechnology. Data available in the literature in this subject concerned mainly proteolytic enzymes since their share among biocatalysts used in various industries reaches 60% [2, 9].

The step of selecting an appropriate organic solvent is a crucial factor on enzymatic catalysis in non-aqueous medium, due to the direct interference of this on the activity, stability and specificity of the enzyme. In general, hydrophobic solvents are less harmful to the enzymes significantly with the water necessary to keep of the structure of the enzyme. Hydrophilic solvents tend to strip the essential water of the protein structure, conducting to loss of enzyme activity [10]. Besides, an ideal organic solvent can dissolve considerably the substrate, favoring the progress of the reaction without affecting the enzymatic activity [11].

In a general way, for optimization of biocatalytic systems in organic solvents, the log P is considered a good quantitative method since it represents the polarity of the solvent. The following classification is recommended for choosing the most adequate organic solvents for enzymatic catalysis: (1) solvents with $\log P < 2$, due to its high polarity can cause the denaturation of the catalyst, (2) solvents with $2 < \log P < 4$ are considered moderate, and (3) solvents with $\log P > 4$ can be considered the most appropriate since they are non-polar [11].

Ultrasound as an environmentally benign method has found many interesting applications in organic chemistry [12–14]. The chemical and physical effects of ultrasound arise from the cavitational collapse which produces extreme conditions locally and thus induces the formation of chemical species not easily attained under conventional conditions, driving a particular reactivity [12]. When cavitation bubbles collapse near the phase boundary of two immiscible liquids, the resultant shock wave can provide a very efficient stirring/mixing of the layers. As a consequence, the cavitation phenomenon can increase reaction rates in heterogeneous reactions and hence may become a useful tool in enzymatic reactions [15, 16]. Although the application of ultrasound irradiation to enzymatic reactions is not well explored [17–20], it can be used to reduce mass transfer limitations in enzymatic reactions [15, 21]. Ultrasound is also known to perturb weak interactions and to induce conformational changes in protein structures [22, 23].

The effects of ultrasound on enzymatic reactions, however, have been less extensively studied [17, 21, 24–27]. The few studies available in the literature can be categorized into two main groups: the first approach makes use of ultrasound as an enzymatic pretreatment to reduce particle size. This is especially relevant when using enzyme powders to catalyze reactions in organic media [28–30]. In such cases, reduction in particle size and consequent increase in the catalytic surface area are useful to reduce mass transfer limitations. The second approach involves the use of ultrasound throughout the reaction. Here, the cavitation energy is thought to accelerate the reaction rate, yet the mechanism by which this occurs is unclear. Perhaps, by increasing the movement of liquid molecules, the substrate's access to the active site is increased. Other mechanisms have also been suggested [24].

While it has been shown that the second approach can accelerate enzymatic reactions [17, 21, 24], other reports have demonstrated the occurrence of enzyme inactivation [25–27]. In general, enzymes are known to be more stable in nearly anhydrous organic solvents [28–30]; therefore, it is not surprising that all reported cases of rate enhancements resulting from ultrasonic treatment are those involving enzymatic catalysis in organic media [17, 21, 24].

To conduct lipase-catalyzed reactions in ultrasound-assisted system, the enzyme behavior 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, the characteristics of the organic solvents, the water content of the enzyme/support/reaction mixture and the manipulated process variables. Inspection of the literature cited reveals that there is a lack of corresponding experimental information for the behavior of the enzyme activity and stability after treatment in organic solvents under ultrasound-assisted systems.

Thus, with the aim at understanding the potential of ultrasound application to enzyme processes, to help elucidating the reaction mechanism as well as for a rational reactor design, this work investigates the esterification activity and the stability to low temperatures of two commercial immobilized lipases under ultrasound-assisted irradiation using different organic solvents. Later, the analysis of these materials included studies on the morphology of treated and non-treated enzymes by scanning electron microscopy (SEM). From our knowledge, this is the first time that this kind of study is presented in the literature.

Here, it may be opportune to mention that the approach presented in this work comprises, in fact, a new attempt made by our research group to overcome mass transfer limitations when immobilized lipases are, for example, used as catalysts in organic systems, mainly when glycerol and/or triglycerides are used as substrates for biodiesel and emulsifiers production.

Materials and methods

Materials

Two 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 [31]. Ethanol (99%), tert-butanol (99.5%), hexane (99%), iso-octane (99%) and iso-propanol (99.5%) were used as organic solvents. The esterification activity of immobilized lipases was determined using lauric acid (Vetec) and *n*-propanol as substrates.

Equipment

Experiments were carried out in a reactor with thermostatic water bath (temperature accuracy of ±0.5 °C). The experimental setup consists of an ultrasonic bath (Unique Inc.-model USC 1800A, Brazil, BR) equipped with a transducer having longitudinal vibrations. The ultrasonic unit has an operating frequency of 37 kHz and a maximum-rated electrical power output of 132 W. The ultrasonic transducer (surface area of 282.2 cm^2) is fitted at the bottom of the bath horizontally along the length of bath. The advantage of using such a system is that it offers much larger effective cavitational area compared to the conventional immersion-based axial transducers and hence results in uniform cavitational activity distribution in the ultrasonic bath.

Lipase esterification activity

The enzyme activity was determined as the initial rates in esterification reactions between lauric acid and *n*-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 *n*-propanol were collected and the lauric acid content was determined by titration with NaOH (0.04 N). After the addition of the enzyme to the substrates, the mixture was kept at 60 °C for 15 min. The lauric acid consumption was then determined. One unit of activity (U) was defined as the amount of enzyme necessary to consume 1 μmol of lauric acid per minute [32]. All enzymatic activity determinations were replicated at least thrice. The relative esterification activity (relative activity, %) was calculated as $(A_f/A_i) \times 100$, where A_f is the (final) enzymatic activity, after treatments with organic solvent under ultrasound-assisted system (U/g) and A_i is the initial enzymatic activity (U/g).

Instrumentation

Scanning electron microscopy (SEM) was performed to check possible changes in the morphological properties of the enzymes after treatment with each organic solvent under ultrasound-assisted system. Micrographs of the immobilized enzymes were obtained using a scanning electron microscope Philips (XL-30 ESEM) Superscan equipment. Samples of lipases were displaced in metallic plates and submitted to a previous treatment of atomization with gold to improve the image definition.

Influence of organic solvents and ultrasound energy on the esterification activity

The treatment of the lipases under ultrasound system was carried out in the equipment described before (Unique Inc. model USC 1800A) in the presence of different organic solvents: ethanol ($\log P = 0.24$), tert-butanol ($\log P = 0.80$), *n*-hexane ($\log P = 3.50$), iso-octane ($\log P = 4.50$) and iso-propanol ($\log P = 0.28$) at 50 °C for Lipozyme IM and 70 °C for Novozym 435, without mechanical agitation. For each experiment, 0.5 g of enzyme and 5 mL of solvent (10% v/wt) were used. Before the conduction of the experiment, the enzyme was kept in oven at 40 °C for 60 min. The experiments were conducted for 10 h, with samples withdrawn each 1 h. After, enzymes were kept in oven at 40 °C for some minutes and then samples were placed in desiccator for at least 18 h. The esterification and relative activities were then determined, as described in "Lipase esterification activity".

Stability of commercial lipases submitted to ultrasound energy and organic solvents to low temperatures

After the treatment in ultrasound-assisted system, samples of non-treated and those using ethanol, *n*-hexane and tert-butanol as solvent were stored at 4 °C and the stability to low temperature was determined each 7 days until 100 days of storage.

Results and discussion

Influence of the solvent and ultrasound energy on the esterification activity of the immobilized lipases

Figure 1 presents the relative activity of Novozym 435 in different organic solvents after the treatment in ultrasound-assisted system during 10 h of contact time. From this figure, one can observe that the use of ethanol led to a relative activity of 55% after 10 h of contact. The solvents iso-propanol, tert-butanol and *n*-hexane led to an increase in the esterification activity of about 20%, keeping this activity after 10 h of contact. The use of iso-octane conducted to a gradual increase in lipase activity in relation to the exposure time, reaching a maximum value of relative activity of 126%.

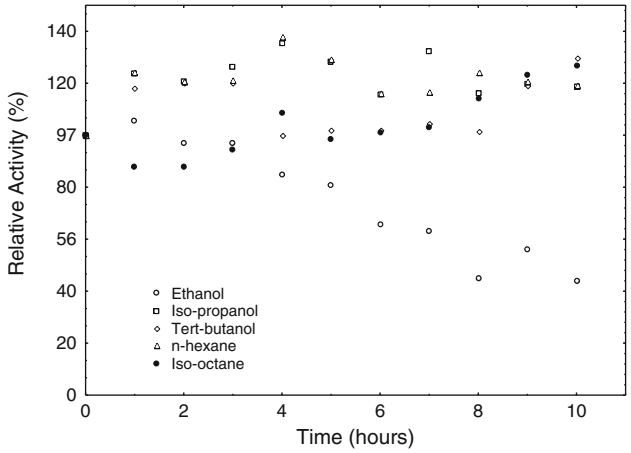

Fig. 1 Relative activity of Novozym 435 in different organic solvents treated in ultrasound-assisted system

Figure 2 depicts the relative activity of Lipozyme IM in different organic solvents treated in ultrasound-assisted system. After 5 h of the exposure, the enzyme lost all of its activity when ethanol was used as solvent. On the other hand, the use of iso-propanol led to a reduction of about 15% in the esterification activity after 6 h. The solvents tert-butanol and iso-octane promoted an enhancement of about 20 and 17% in the enzyme activity in 6 h of exposure, respectively. Based on the results showed above, it can be inferred that the solvent exerts direct influence on the activity, stability and specificity of the enzyme [10]. For Novozym 435 and Lipozyme IM, control experiments were carried out without the addition of organic solvents, only under the action of ultrasound device. The esterification activity of both lipases showed a residual activity of 100%. This information made us possible to conclude that the effects obtained along the work were caused only by the organic solvent, as will be discussed later.

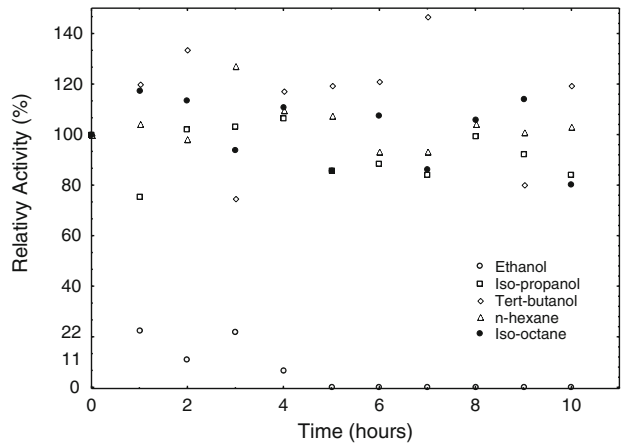

Fig. 2 Relative activity of Lipozyme IM in different organic solvents treated in ultrasound-assisted system

Novozym 435 treated with different organic solvents (2-propanol, iso-octane, tert-butanol and *n*-heptane demonstrated that the use of tert-butanol led to the highest stability until 48 h in this solvent. The solvents 2-propanol and *n*-heptane provoked decay in residual activity after 8 h and the use of iso-octane showed a great variation in enzyme activity along the period of investigation, due to the instability of Novozym 435 in these solvents [33].

Doukyu and Ogino [8] pointed out that several organic solvent-tolerant lipolytic enzymes are presented in the literature. Besides this statement, specialized literature pointed out that of all the various parameters, such as the dielectric constant, dipole moment, hydrogen binding, polarity, the logarithm of partition coefficient (log *P*) gives the best correlation with the enzyme activity [8, 34]. Taking into account the results obtained for Lipozyme IM and Novozym 435, the solvents that lead to an enhancement in the lipase activity were tert-butanol (log $P = 0.8$) and *n*-hexane (log $P = 3.50$). Tsukamoto [11] affirms that solvents with log $P < 2$ are not appropriate for biocatalytic purposes while those with log $P > 4$ (apolar) can be considered ideal. This behavior, however, was not observed in this work, since the best results were reached with solvents of extreme values of log *P*. Ceni et al. [33] also showed that the residual esterification activity of Novozym 435 after treatment with 2-propanol (log $P = 0.05$) for 6 h under ultrasound-assisted system in a reaction medium containing glycerol and methyl benzoate presented an enhancement of about 50% compared to its initial value. As verified for other works from the literature, in this case the enzyme activity was measured after a reaction, not only after direct contact of the enzyme to the organic solvent in ultrasound-assisted system. In another reaction system constituted by glycerol and ascorbic acid, Lerin et al. [35] obtained higher residual activities for Novozym 435 and Lipozyme IM when tert-butanol (log $P = 0.80$) was used as solvent.

Based on these aspects, one should expect that other parameters might be influencing the results obtained here, such as the functional groups of the solvents [36]. Liu et al. [37] showed that the activity of lipases in organic solvents is not only dependent on the hydrophobicity (log *P*) but also the functional group of the solvents. As an example, tert-butanol has a functional group –OH and *n*-hexane–C–C. The work by Secundo and Carrea [38] corroborates this fact. The authors demonstrated that Novozym 435 presented different activities when treated with organic solvents with different functional groups. A possible explanation can be attributed to the variation of water retained in the microenvironment of the catalytic active site, which is necessary to maintain the dynamical properties of the enzyme.

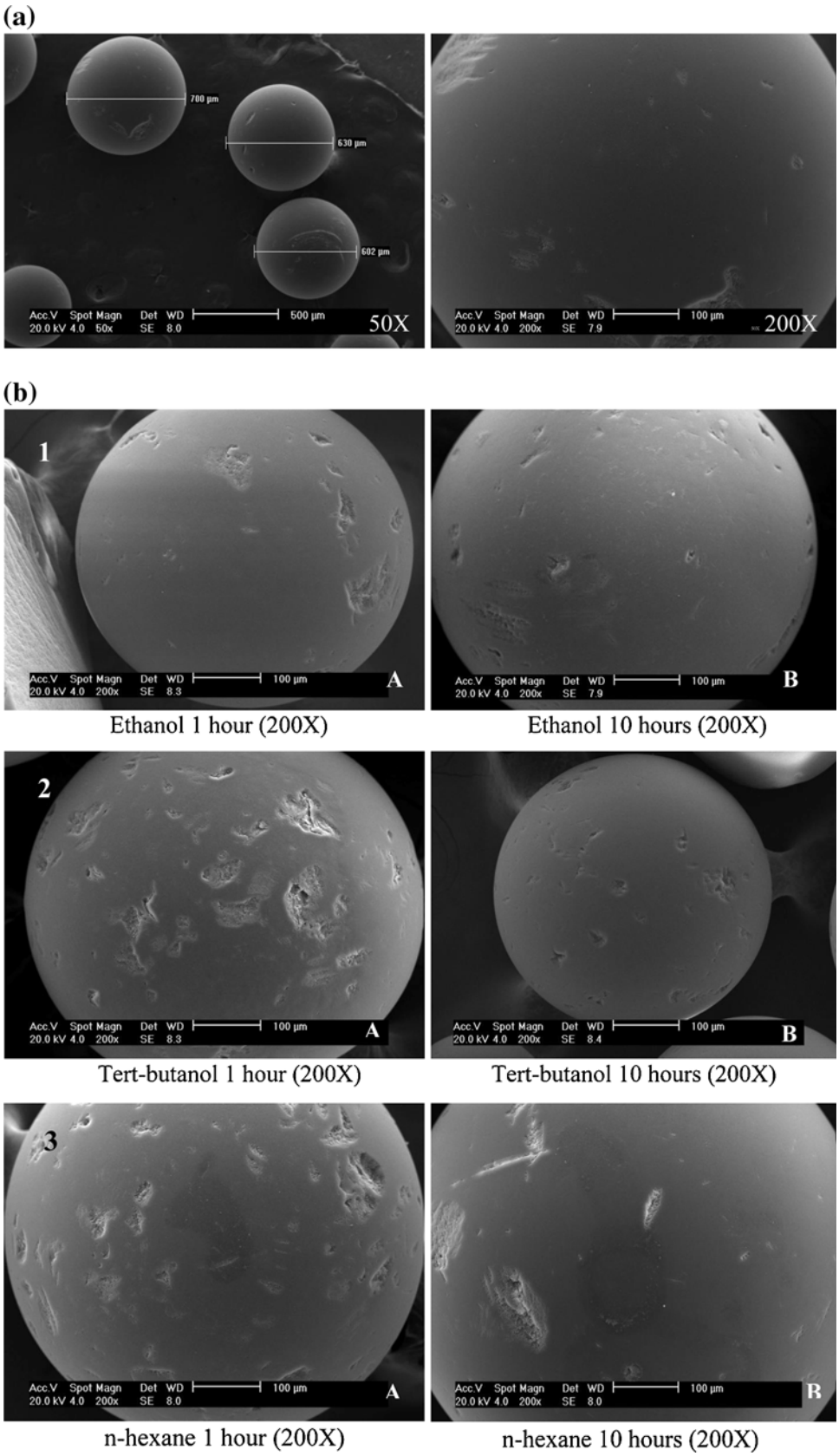

Fig. 3 SEM of Novozym 435 non-treated (**a**) and treated in ultrasound-assisted system (**b**) using different organic solvents and contact times

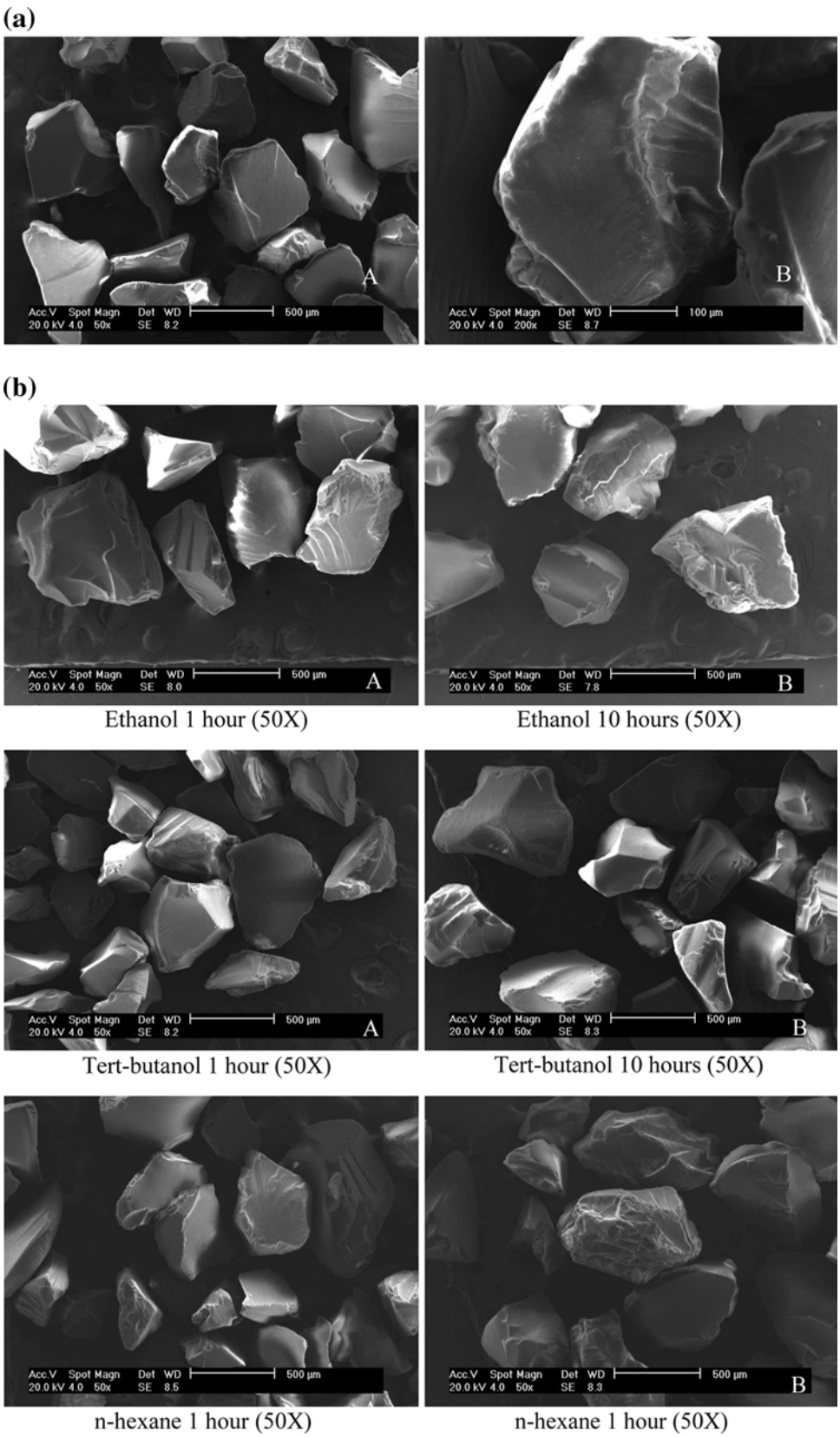

Fig. 4 SEM of Lipozyme IM non-treated (**a**) and treated in ultrasound-assisted system (**b**) using different organic solvents and contact times

Figures 3 and 4 present the SEM of treated and non-treated Novozym 435 and Lipozyme IM in ultrasound-assisted system, respectively, using different solvents and contact times.

From Fig. 3, it can be seen that the solvents and the ultrasound power seem to attack the support used for the lipase immobilization, while not affecting the enzyme activity can be checked in Fig. 1. Related to Lipozyme IM (Fig. 4) no change was observed on the enzyme surface and the enzyme activities increased their values after treatment in tert-butanol and *n*-hexane. After this evaluation, one can infer that some modifications at structural level seem to be occurred since almost no alteration could be observed after SEM analyses.

Stability of the immobilized lipases to the storage at low temperatures

Figure 5a, b presents the stability to low temperatures of Novozym 435 and Lipozyme IM using different organic solvents under ultrasound-assisted system during storage at 4 °C. Activities were monitored periodically until 100 days. Novozym 435 and Lipozyme IM presented high stability to storage after treatment under ultrasound-assisted system and *n*-hexane and tert-butanol as solvents. The treatment of both commercial immobilized lipases with ethanol caused complete loss of activity after 10 days of storage.

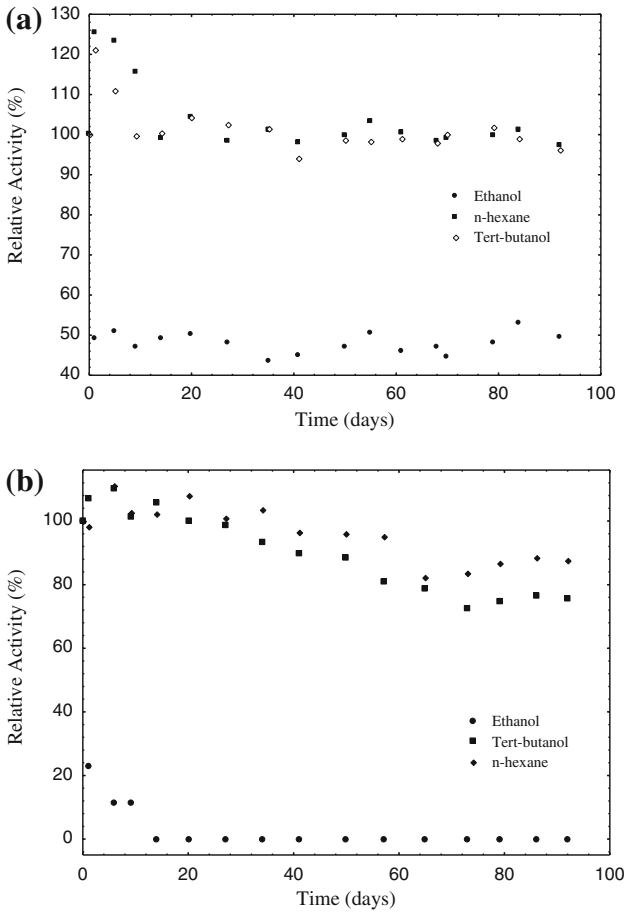

Fig. 5 Stability to low temperatures of Novozym 435 (**a**) and Lipozyme IM (**b**) using different organic solvents under ultrasound-assisted system

Conclusions

New experimental data on stability and catalytic activity of immobilized lipases in the presence of different organic solvents in ultrasound-assisted system is reported in this work, showing a perspective of the technique to overcome mass transfer limitations arising from the use of immobilized lipases in organic reaction systems. The use of ethanol as solvent led to a relative activity of 55% for Novozym 435, but the solvents iso-propanol, iso-octane, tert-butanol and *n*-hexane promoted an increase in the esterification activity. For Lipozyme, the enzyme lost all of its activity when ethanol was used as solvent, whereas the use of iso-propanol led to a reduction of about 15% in the esterification activity after 6 h. The solvents tert-butanol and iso-octane showed an enhancement of about 20 and 17% in the enzyme activity in 6 h of exposure. Novozym 435 and Lipozyme IM presented high stability to storage after treatment under ultrasound-assisted system and *n*-hexane and tert-butanol as solvents.

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