Acta Scientiarum
http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-8664
Doi: 10.4025/actascitechnol.v42i1.46582

Ultrasound affects the selectivity and activity of immobilized lipases applied to fatty acid ethyl ester synthesis

José Carlos Quilles Junior[1*], Ana Lucia Ferrarezi[2], Janaina Pires Borges[3], Jessika Souza Rossi[1], Daniela Alonso Bocchini[3], Eleni Gomes[2], Roberto da Silva[1] and Mauricio Boscolo[1]

[1]Departamento de Química e Ciências Ambientais, Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista "Julio de Mesquita Filho", Rua Cristóvão Colombo, 2265, 15054-000, São Paulo, São Paulo, Brazil. [2]Departamento de Biología, Universidade Estadual Paulista, "Julio de Mesquita Filho", São José do Rio Preto, São Paulo, Brazil. [3]Departamento de Bioquímica e Tecnología Química, Instituto de Química, Universidade Estadual Paulista "Julio de Mesquita Filho", Araraquara, São Paulo, Brazil. *Author for correspondence. E-mail: quillesjcj@gmail.com

ABSTRACT. Hydrophobic carriers can be used to improve the activity, stability and other properties of enzymes. Physical agents, like ultrasound, may also contribute to improving the dispersion and collision of the reagent molecules, decreasing the reaction time and intensifying the catalytic process. However, its effect on the enzyme activity and reaction selectivity is still not entirely understood. Here, enzyme modulation of immobilized lipases was studied under pulsed ultrasound irradiation in fatty acid ethyl ester (FAEE) synthesis for biodiesel production. Novozym 435® and two commercial lipases from *Thermomyces lanuginosus* and *Rhizomucor miehei,* immobilized on Octadecyl-Sepabeads were used as a biocatalyst in the transesterification reaction of vegetable oils and ethanol. The use of ultrasound associated with catalysis by the Novozym 435 increased the production of FAEE by about three times (from 8.9 to 26.4%) using soybean oil and changes were observed in the profile of the products. From the sonicated reaction, ethyl-palmitate production decreased from 23.4 to 11.7%, while the ethyl-linoleate content rose from 47.5 to 59.2%. On the other hand, the *T. lanuginosus* lipase was less affected by sonication with the overall production of FAEE increasing from 17.2 to 24.1%, with ethyl-palmitate and ethyl-linoleate content changing from 16.2 to 17.5% and 55.0 to 47.8%, respectively. Although the changes in the production yield are not too high, the main idea here was to show that ultrasound modulates the lipase activity as well as its respective selectivity. Thus, ultrasound, is responsible for changing the ethyl ester production, which can be applied to many other biochemical processes to improve or modulate their synthesis yield.

Keywords: ultrasound; biodiesel synthesis; immobilized lipases; hydrophobic immobilization; enzyme selectivity.

Received on February 2, 2019
Accepted on April 22, 2019

Introduction

Biodiesel is a mixture of fatty acid esters produced by a transesterification reaction between triacylglycerols from vegetable oil or animal fat with alcohol (Bouaid, El boulifi, Hahati, Martinez, & Aracil, 2014; Zhou, Chen, & Yan, 2015) and it has been investigated as a replacement for petrodiesel. Acid or basic homogeneous transesterifications are the most common routes in biodiesel synthesis due to their high yield, as well as their low catalyst cost, especially that of the alkaline catalyst (Schuchardt, Sercheli, & Matheus, 1998). However, this process may follow an undesirable reaction route if any water content is present, i.e., the saponification reaction, resulting in the deactivation of the catalyst, reducing the yield and requiring further steps for product purification (Xu, Yang, Yu, & Guo, 2008).

The transesterification of triacylglycerols catalyzed by lipases may be a method that overcomes this problem and could bring advantages over chemical catalysis (Amoah et al., 2016; Shimada et al., 1999) such as lower energy consumption and easier glycerol separation. Lipases used in this process are classified based on their regioselectivity for the position of unsaturated bonds of the triacylglycerol molecule: R1 and R3 (sn-1-3), R2 (sn-2) or nonspecific (Taher, Al-Zuhair, Al-Marzouqi, Haik, & Farid, 2011). However, the biotransesterification needs a longer reaction time than the acid/alkaline catalysis and the main problem with this process is the high cost of the catalyst (Balat & Balat, 2010). The immobilization of enzymes is needed to decrease the process cost and to improve the enzyme characteristics, such as stability and activity

(Boros et al., 2013), and also their capacity to be recycled (Aulakh, Prakash, & Prakash, 2013; Jiang et al., 2016).

Triacylglycerol lipases (E.C. 3.1.1.3) show a particular mechanism of activation by a hydrophobic amino acid chain (a lid), that covers up their active sites giving it two different conformations (active and inactive) depending on the medium conditions (Ferrarezi et al., 2014; Quilles et al., 2014). Due to the presence of the lid, the lipases have flexibility in their structure, allowing the modulation of catalytic properties by immobilization methods (Hernandez & Fernandez-Lafuente, 2011) and they are used in many biotechnology applications because of their ability to catalyze different reactions with triacylglycerol molecules, among them, hydrolysis, esterification and transesterification (Rupasinghe, 2016). Hyperactivation can improve the yield of these processes due to the stabilization of the active enzyme on the support (Bastida et al., 1998; Fernandez-Lorente et al., 2011; Fernández-Lorente, Palomo, Cabrera, Guisán, & Fernández-Lafuente, 2007; Palomo et al., 2002).

Sonication approaches have been used to improve the rates of many chemicals, physical and biological processes, mainly due to the resulting increase in the available catalytic surface (Fiametti et al., 2011; Yu et al., 2010) while respecting some green chemistry concepts (Rupasinghe 2016). Ultrasound irradiation (UI) creates, expands and implodes gaseous cavities in a liquid medium, leading to a better mixture of and collision between the reagent molecules improving the reaction yield (Lobo, Singh, Pinjari, Pandit, & Shankarling, 2013;). Some studies have shown an improvement in lipase properties caused by acoustic cavitation in different processes (Babicz, Leite, Souza, & Antunes, 2010; Badgujar, Pai, & Bhanage, 2016; Yu et al., 2010). The aim of this study was to evaluate the effect of UI on lipase selectivity and activity in an organic medium, as well as any increase in the reaction yield. Pulsed UI was used to intensify the catalytic process and its effect on the activity and selectivity of commercial lipases immobilized by hydrophobic adsorption when acting as biocatalysts in the transesterification of vegetable oils and ethanol was evaluated by comparing them to the lipase Novozym 435® (N-435).

Material and methods

Lipases from *Thermomyces lanuginosus* (TLL), *Rhizomucor mihei* (RML), and *Candida antarctida* fraction B (N-435), *p*-nitrophenyl butyrate *(p*-NPB), and the ester standards of gas-chromatography were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), Coomassie protein assay reagent from Thermo Fisher Scientific Inc. (Waltham, USA), Octadecyl-Sepabeads® (C18) from Mitsubishi Chemical Holdings Corporation (Tokyo, Japan). Vegetable oils (corn, soybean, and sunflower) were purchased in a local market.

Hydrolytic activity assay of soluble and immobilized lipase

Lipase activity was determined by the release of *p*-nitrophenol (*p*NP) from hydrolysis of 0.4 mM *p*NPB in 25 mM sodium phosphate buffer pH 7 at 25°C, detected by the increase in absorbance at 348 nm (Fernandez-Lafuente, Armisén, Sabuquillo, Fernández-Lorente, & Guisán, 1998). One international unit was defined as the amount of enzyme necessary to hydrolyze one µmol of *p*NPB per minute (IU) under the assay conditions. The enzymatic activity was calculated using Equation 1:

$$\text{Enzyme activity } \left(\frac{\mathrm{U}}{\mathrm{mL}}\right) = \frac{\mathrm{Abs}\cdot\mathrm{Vt}}{\mathcal{E}\cdot\mathrm{Ve}\cdot\mathrm{t}} \qquad (1)$$

where: the Abs is the absorbance from the *p*NP production measured at 348 nm, V_t is the total reaction volume, $\mathcal{E}$ is the molar extinction coefficient of the product (12.854), V_e is the enzyme volume, and t is the reaction time.

Total protein determination

Protein concentration was determined using a modified Bradford Method (Bradford, 1976). A standard curve was taken by adding different amounts of BSA (bovine serum albumin) to Coomassie protein reagent, with an end volume of 1.0 mL. After 10 min. of reaction between the BSA and the reagent, the absorbance was measured at 595 nm.

Immobilization of lipases by hydrophobic adsorption

Octadecyl-Sepabeads were used to immobilize the TLL and RML on their surface hydrophobically. The enzyme and support incubation was carried out in 10 mM sodium phosphate buffer at pH 7 with a protein loading of 20 mg of protein per gram of support. After the incubation time, the amount of immobilized protein was calculated by measuring the protein concentration as described in 2.3. The derivatives were washed with acetone, filtered under vacuum to remove any trace of the buffer and stored at 4°C. Immobilization parameters were determined by Equations 2 and 3 below:

$$\text{IY}\ (\%) = \left(\frac{A_{os} - A_{fs}}{A_{os}}\right) \cdot 100 \qquad (2)$$

where: the immobilization yield (IY) is calculated using the initial lipase activity in the immobilization supernatant (soluble enzyme: A_{os}) and the final lipase activity after the incubation time during the immobilization process (soluble enzyme not immobilized: A_{fs}).

After the immobilization, the loading protein (LP) on the derivative (immobilized lipases) was determined by Equation 3:

$$LP\ (mg \cdot g^{-1}) = \frac{\mathrm{V}_{enz} \cdot (\mathrm{C}_0 - C_f)}{\mathrm{m}_s} \qquad (3)$$

where: V_{enz} is the enzyme volume used for the immobilization process, C_o is the initial concentration of available enzyme for immobilization ($mg \cdot mL^{-1}$), C_f is the final enzyme concentration ($mg \cdot mL^{-1}$), and m_s is the support mass used for the immobilization (g).

Thermal properties of the support and free and immobilized lipases

Octadecyl-Sepabeads support with and without immobilized lipases (~3.0 mg) and the lyophilized free enzyme (~3.0 mg) were separately scanned from 25 to 200°C under N_2 at a rate of $1°C \cdot min.^{-1}$ in a hermetic stainless steel pan in a DSC 8000 differential scanning calorimeter (PerkinElmer, Waltham, USA). All the scans were done in duplicate. For the free and immobilized enzyme, the thermal transition midpoint (or melting temperature) (T_m) was determined, from which it was possible to evaluate the stabilization of the enzymatic structure by the immobilization process.

Adsorption isotherm models (total superficial area)

In this study, 100 mg of the Octadecyl-Sepabeads and the derivates containing immobilized lipases were submitted to the nitrogen adsorption process to determine the specific surface area using a Gemini VII 2390 porosimeter (Micromeritics Instrument Corporation, Norcross, USA) by the Brunauer, Emmett and Teller method (BET) (Fagerlund, 1973). Samples were heated to 90°C for one hour to eliminate any trace of water and submitted to the gas adsorption process using N_2 as the adsorbent gas.

FAEE production catalyzed by immobilized lipases and their reuse

FAEE were produced by an ethanolysis reaction performed with corn, soybean and sunflower oils used as the triacylglycerol source, at a fixed molar ratio of oil to alcohol (1:6) and 3 mg of biocatalyst (immobilized lipase) in 125 mL Erlenmeyer flasks. The final volume was ~45 mL. Hexane was used as the solvent, maintaining the enzymatic activity with a volume ratio of oil to solvent (2:3). This mixture was incubated for 72 h at 45°C and 150 rpm and aliquots were taken every 24 hours. The commercial lipase Novozym 435 was used as a control reference in this experiment. At the end of each period, the mixture was vacuum filtered and the recovered biocatalyst was washed with *n*-hexane. The effect of the UI was evaluated during the ethanolysis reactions with sonication (40 kHz) for 15 seconds every 15 minutes for a total time of 24 hours. The immobilized lipases were evaluated for their ability to be reused in five reaction cycles of 24 hours.

FAEE production by alkaline catalysis

Homogeneous alkaline transesterification was performed in a 125 mL erlenmeyer flasks and used as a control reference for FAEE production with 100 g of each oil, 30 mL of ethanol, and 2 g of NaOH for 4 hours under agitation at 60°C. After this reaction time, the mixture was decanted to separate the esters from the glycerol, the organic phase was washed with distilled water to remove the biocatalyst and roto-evaporation removed the excess ethanol.

Analysis of FAEE production by gas chromatography (GC)

The FAEE produced were diluted in *n*-hexane and 1 μL of the mixture was injected into a Clarus 680 FID gas chromatograph (PerkinElmer, Waltham, USA) coupled to a Biodiesel FAME column (30 m x 0.32 mm x 0.25 μm) (Agilent Technologies, Santa Clara, USA) using ethyl heptadecanoate (> 99%) as the internal standard. The analysis was performed as described in the literature (Torres, Fornari, Tenllado, Señoráns, & Reglero, 2008) in triplicate with the chemical reaction yield adopted as maximum production (100%) and the yield of enzymatic transesterification being calculated relative to the homogenous alkaline synthesis (Urioste, Castro, Biaggio, & Castro, 2008).

Statistical data analysis

All the data were analyzed using the Origin Pro, version 8.0 statistical software (OriginLab Corporation, Northampton, USA). The experiments were performed in quadruplicate in three distinct assays and the results represent the average of these assays. The standard deviation (SD) is based on these replicates and it is described for each result.

Results and discussion

Immobilization parameters and physical characterization

The immobilization yield of lipases from TLL and RML on Octadecyl-Sepabeads was about 98 and 87%, respectively. This difference in the protein-loading among the derivatives is almost negligible when compared to the difference in their activities. It was expected that the hydrophobic adsorption of the lipases would lead to hyperactivation of these enzymes, but the results for RML showed a significant loss of activity, with recovered activity lower than 8%, against approximately 30% for TLL. The results suggest that lipases from *R. miehei* are more sensitive to possible structure distortion by the hydrophobic interaction with the support, since it showed low recovered activity after immobilization. Due to the low activity of the derivative from RML, the physical properties and all the other assays were performed based on the TLL derivatives.

A critical enzyme parameter is its melting temperature (T_m) at which the protein denaturation occurs and, consequently, its activity is lost. Enzyme immobilization might lead to its thermal stability and improve its activity. A high T_m is seen for the soluble enzyme of approximately 155°C; it is known that enzymes from mesophilic organisms, in general, present a T_m between 60 and 80°C (Ahmad & Rao, 2009). However, the ability of lipases to form aggregates in the aqueous medium may affect their stabilization due to the formation of dimers. These lipase dimers stabilize the enzyme in its inactive conformation and improve its thermal stability. Besides that, after the hydrophobic immobilization on C18, the enzyme stability was improved, with undetectable T_m up to 200°C (Figure 1A). The specific surface area is also an important parameter for an inert material to be used as a support for enzyme immobilization. Carriers with a higher superficial area can load more protein units and, consequently, the biocatalyst could show more activity. After the immobilization process, a decrease from 66.8 to 8.9 $m^2 \cdot g^{-1}$ in the superficial area of the carrier was observed, which is an indication of the enzyme adsorption efficiency. Because of this, gas adsorption of the C18-TLL biocatalyst was dramatically less in comparison to the support without enzymes (Figure 1B). Almost no gas adsorption was observed in the C18-TLL derivative, while for C18 free of enzymes, the profile in the adsorption curve was favorable to adsorption, showing a curve with a tendency to adjust to a quadratic function. Using this parameter with those from the immobilization process and the enzyme thermal stability, the biocatalyst can be successfully prepared, improving the thermal stability and activity of the enzyme, as well as having a high capacity in the protein load of the support used.

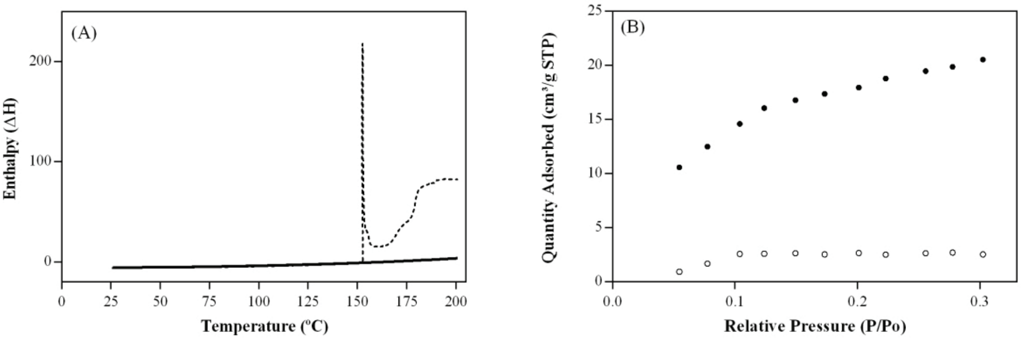

Figure 1. Physical characterization of the soluble lipase from *T. lanuginosus* (TLL) and immobilized on C18 resin (C18-TLL) analyzed by (A) differential screening calorimetry (DSC), in which TLL and C18-TLL are represented by a dot and straight lines, respectively. The analysis was performed from 25°C up to 200°C with a heat gradient of 1°C·min.$^{-1}$ under N_2 atmosphere. Also, (B) isothermal adsorption of N_2 at 77K of C-18 (black circles) and C18-TLL (white circles) was performed to determine the total superficial area occupied by the enzymes after the immobilization.

Fatty acids ethyl ester production by enzymatic transesterification

TLL and RML derivatives and the commercial immobilized lipase (N-435) were used for ethyl ester production using different fatty acid sources, such as soybean, sunflower and corn oils. The total FAEE production was quantified by gas chromatography as the sum of the palmitic, oleic, linoleic and linolenic esters produced, being the most common esters in the biodiesel blend (Figure 2A). Chemical transesterification using these oils was used as a reference for the complete ester production. Some reports in the literature show that lipases from *R. miehei* present low capacity for the transesterification of vegetable oil (Su, Zhang, Zhang, Gao, & Wei, 2007) and have lesser activity than lipases from *T.lanuginosus* (Mendes et al., 2011; Moreno-Perez, Filice, Guisan, & Fernandez-Lorente, 2013; Rodrigues, Mendes, Adriano, Gonçalves, & Giordano, 2008). These reports present results very similar to those obtained here, with no detectable amounts of ethyl ester produced by C18-RML. Besides that, this result may have been intensified by adsorption onto the C-18 resin based on the relative activity of this derivative (about 8%), evidencing the inferiority of this enzyme. For that reason, the FAEE production and preference for the type of substrate were tested for the C18-TLL and N-435 enzymes, with distinct results. C18-TLL showed a maximum yield of 25% after 72 hours using soybean oil as substrate (Figure 2B), while the N-435 had a different preference for the substrate, with the same production yield for corn oil (Figure 2C). The catalytic preference for the different vegetable oils was determined by statistical analysis, showing divergent substrate preference for the lipases. The low activity of N-435 in this reaction was also a curious result and could be explained by enzyme intolerance to the *n*-hexane used as the solvent in the reaction; something which has not been previously reported. Besides that, lipase stability and activity are also dependent on many other factors, such as the physical condition in the reaction medium (temperature, pH, pressure), lipase source and the enzyme-support interaction (Habulin & Knez, 2001).

Because of this, other studies need to be done to better investigate the low activity of this enzyme under the reaction conditions used here. However, this enzyme was only used as a control parameter since the biocatalyst prepared here (C18-TLL) is the focus of this work. Regarding the vegetable oil composition, all of them have high concentrations of unsaturated acids (Sundaram, Bukowski, Lie, Picklo, & Yan, 2016) but the lipase from *T. lanuginosus* showed a preference for soybean oil. This oil is a common substrate for lipases and one of the most used oils for biodiesel production (Moreno-Perez et al., 2013; Rodrigues et al., 2008; Ting, Huang, Giridhar, & Wu, 2008) and, for that reason, it was used for the next assays. The results showed the potential for TLL to be used in the production of biodiesel, converting triacylglycerol molecules into fatty acid ethyl esters. Another important point to be evaluated is the capacity of the biocatalyst for recycling. C18-TLL and the N-435 were submitted to reuse cycles of 24 hours in the transesterification reaction of soybean oil. C18-TLL derivatives produced higher FAEE yields than N-435 in all reuse cycles and no significant activity loss was observed during the first two reaction cycles (Figure 2D). However, in the third cycle, around 50% of the initial FAEE production yield remained, decreasing to 20 and 10% for the fourth and fifth cycles, respectively. Hydrophobic adsorption is an immobilization method in which the enzyme may be recovered in its soluble form due to the reversibility of the process. When the derivatives are washed, the immobilized enzymes may be desorbed from the support, making it a critical procedure for decreasing enzyme activity and stability. The use of immobilized enzymes on Octadecyl-Sepabeads for use in biocatalysis has the advantage of being an easy and inexpensive process but it is limited by the number of washes, where the immobilized enzymes can be desorbed from the support. This makes this step critical to the enzyme recycling process. Considering that both derivatives lost activity in the recycling, both the washes and the long period of the transesterification reactions (24h) may have contributed to the falls in activity and FAAE synthesis. To intensify this process, ultrasound was applied during the reaction and catalytic parameters were analyzed.

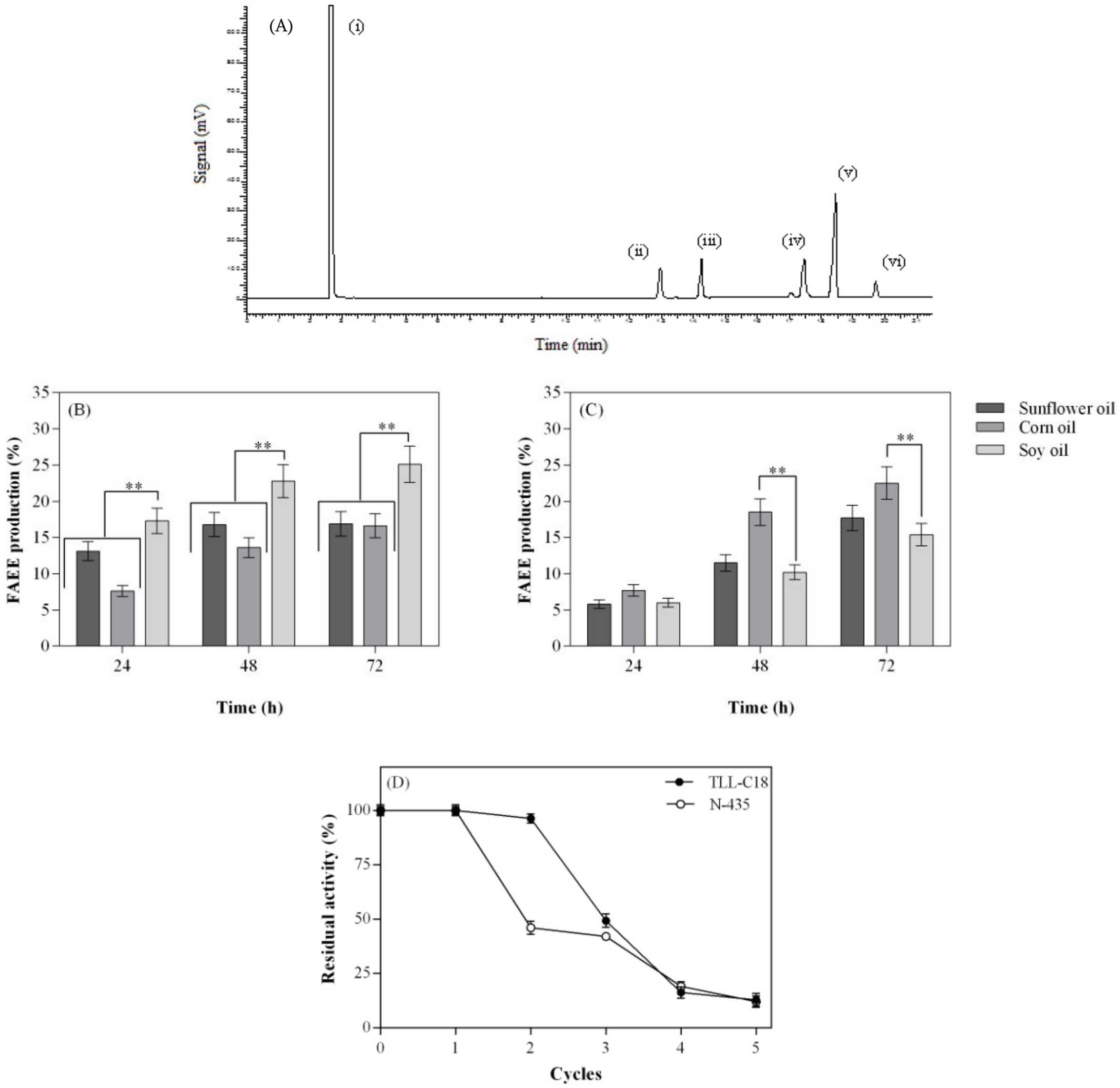

Figure 2. (A) Chromatogram of FAEE production from transesterification of the vegetable oil catalyzed by the C18-TLL derivative: (i): *n*-hexane; (ii): ethyl palmitate; (iii): ethyl heptadecanoate; (iv): ethyl oleate; (v): ethyl linoleate; (vi): ethyl linolenate. FAEE production course by biotransesterification using (B) TLL-C18 derivative and (C) the commercial immobilized lipase N-435. Two-way Analysis of Variance (ANOVA) was done to determine which vegetable oil is the best one to be converted to ethyl esters by the different lipases, considering $p < 0.05$. (D) Reuse cycles of TLL-C18 (black circle) and N-435 (white circle) derivatives during the transesterification of soybean oil. The experiments were performed in triplicate, and the error bars indicate the experimental error or uncertainty in a reported measurement.

FAEE production yield with ultrasound irradiation

UI is known as an alternative that can intensify the reaction conditions in organic media, mainly when the process uses immiscible liquids, promoting better mixing of the substrates (Suslick, 1989). UI increased the FAEE production for both the enzymes studied, mainly for N-435 with an increase of almost three times when compared to the same conditions in the absence of acoustic cavitation (Table 1). The use of ultrasound may promote extreme conditions and induces the formation of chemical species with different structures that are hardly formed under conventional conditions (Zheng et al., 2012). These species, more easily formed by the application of UI, can find the active side of the enzyme faster and promote higher product conversion than the same process without ultrasound. Furthermore, the reaction time is decreased, making the ultrasound irradiation an interesting process to aid transesterification catalyzed by immobilized lipases. However, the C18-TLL derivative was not strongly influenced by UI, with a small improvement in the FAEE production from 17.2 to 21.1%. Since UI is a physical process, in which bubbles are made to implode and increase the collisions between the substrate molecules (Suslick, 1989; Lobo et al., 2013), it may also promote the destruction of some derivative molecules, consequently, presenting no significant alteration in the reaction yield profile. Due to the immobilization method, the acoustic cavitation pulse must be carefully controlled to reduce enzyme desorption from any broken support and its inactivation by ultrasound irradiation. UI promoted physical changes in the medium without negatively affecting the properties of the N-435, showing a 188% improvement in the FAEE production yield. This demonstrates the importance of the immobilization reversibility concerning its successful application, in which the structural stability of enzymes immobilized by an irreversible method was higher than the reversible process.

Table 1. FAEE production by biotransesterification of soybean oil in the presence and absence of ultrasound irradiation.

Derivative	FAEE production*		
	No ultrasound irradiation	With ultrasound irradiation	Improvement
C18-TLL	17.2%	24.1%	40.1%
Novozym 435	8.9%	26.4%	188.8%

*FAEE production was based on the total production of esters after the reaction time. The experiments were performed in triplicate, as described in 2.6 with a standard error of less than 10%.

Influence of ultrasound irradiation on the enzymatic selectivity

Enzymatic catalysis selectivity depends mainly on the enzyme conformation and how it interacts with the substrate. The enzyme conformation is related to ionic and hydrophobic intramolecular interactions that maintain the enzymatic structure and it can be modulated by external physical and chemical factors. It was possible to change the selectivity of the lipases from *C. rugosa* using different pressures and temperatures, due to the disruption of the intramolecular electrostatic interaction and decreasing the enantiomeric excess (Herbst, Peper, Francisco, Ruck, & Niemeyer, 2014).

The selectivity of lipases was studied with ultrasound irradiation, considering 100% yield as the sum of the production of the most common ethyl esters in the composition of biodiesel (Table 2). This process shows a high FAEE yield due to the catalyst being soluble in the reaction medium, providing a large superficial surface for catalysis. The non-selectivity of the chemical catalysis can be seen and this is due to a specific yield for each FAEE produced and is related to the percentage of the acid precursors present in soybean oil. However, the results here show that the biochemical catalysis is more selective than the chemical catalysis, with different FAEE production. The main difference observed between the chemical and enzymatic catalysis was the increase in the production of ethyl palmitate ($C_{16:0}$) and linolenate ($C_{18:3}$), showing the high specificity of the enzyme to long-chain substrates. Comparing the biotransesterification using distinct lipases, the enzymes present particular selectivity. Lipases from different species may present some modification in their structure and it is crucial to determine the difference in the substrate preference (Herbst et al., 2014). C18-TLL preferred unsaturated fatty acids, such as linoleic acid, while N-435 produced the same ethyl ester in a higher amount but with a mix of other ethyl ester components of the biodiesel. The immobilization process is also an important factor in improving the enzyme properties (Rodrigues, Ortiz, Berenguer-Murcia, Torres, & Fernández-Lafuente, 2013). FAEE production by biotransesterification performed with ultrasound irradiation affected the enzymatic selectivity of the lipases. C18-TLL had its selectivity changed by UI, producing a mix of FAEE. Ethyl linoleate, produced in a higher amount, had its production decreased by UI while an increase in other esters was observed. N-435, on the other hand, was more selective, increasing its specificity for ethyl linoleate from 47.5 to 59.2% using ultrasound irradiation. Based on these results, ultrasound affects both enzyme structures, changing their conformation and substrate molecules, and also increasing the collisions within the mixture (Fiametti et al., 2011; Yu et al., 2010). The C18-TLL enzyme changed the ratio between the ethyl-linoleate and ethyl-linolenate esters from almost thirteen to five. On the other hand, ethyl-linolenate production was twice as much with UI than the reaction without it. This latter change shows that acoustic cavitation is an interesting approach to focus on the production of the minority esters.

Table 2. Yield of each FAEE produced by chemical and enzymatic catalysis.

FAEE	Content of the acid precursors (%)*	Yield of alkaline catalysis (%)	Enzymatic catalysis (%)			
			C18-TLL	C18-TLL UI	N-435	N-435 UI
Palmitate ($C_{16:0}$)	10.7	7.2	16.2	17.5	23.4	11.7
Oleate ($C_{18:1}$)	24.5	29.4	24.4	26.1	19.5	19.9
Linoleate ($C_{18:2}$)	56.7	62.4	55.0	47.8	47.5	59.2
Linolenate ($C_{18:3}$)	5.4	1.0	4.4	8.6	9.6	9.2

The experiments were performed in triplicate with a standard error of less than 5%. Total FAEE production of the four esters was considered as 100%.
*Percentual composition of the esters present in soybean oil.

Conclusion

An increase of four times in the FAEE production was achieved under UI, a feasible and easy factor to be applied to this reaction system. Alterations in the enzyme selectivity were also observed using UI, in which unsaturated fatty acids were preferred by lipases in the biotransesterification. In addition, the ultrasound irradiation was also interesting to be applied in biocatalysis systems, considering the possibility of greater selection among the esters produced. Though, some limitations of its use must be considered. However, the relevant effect on the lipase activity after sonication use is evident, highlighting the positive effects during FAEE synthesis.

Acknowledgements

The authors would like to thank the Brazilian National Council for the Improvement of Higher Education (CAPES) and the São Paulo Research Foundation (FAPESP 2008/58077-0; 2012/09054-3 and 2014/20504-6) for their financial support.

References

Ahmad, S., & Rao, N. M. (2009). Thermally denatured state determines refolding in lipase: Mutational analysis. *Protein Science*, *18*(6), 1183–1196. DOI: 10.1002/pro.126

Amoah, J., Ho, S.-H., Hama, S., Yoshida, A., Nakanishi, A., Hasunuma, T., ... Kondo, A. (2016). Lipase cocktail for efficient conversion of oils containing phospholipids to biodiesel. *Bioresource Technology*, *211*, 224-230. DOI: 10.1016/j.biortech.2016.03.062

Aulakh, S. S., Prakash, N. T., & Prakash, R. (2013). Transesterification of triglycerides by dried biomass of *Aspergillus* sp. *Journal of Oleo Science*, *62*(5), 297-303. DOI: 10.5650/jos.62.297

Babicz, I., Leite, S. G. F., Souza, R. O. M. A., & Antunes, O. A. C. (2010). Lipase-catalyzed diacylglycerol production under sonochemical irradiation. *Ultrasonics Sonochemistry*, *17*(1), 4-6. DOI: 10.1016/j.ultsonch.2009.07.005

Badgujar, K. C., Pai, P. A., & Bhanage, B. M. (2016). Enhanced biocatalytic activity of immobilized *Pseudomonas cepacia* lipase under sonicated condition. *Bioprocess and Biosystems Engineering*, *39*(2), 211-221. DOI: 10.1007/s00449-015-1505-5

Balat, M., & Balat, H. (2010). Progress in biodiesel processing. *Applied Energy*, *87*(6), 1815-1835. DOI: 10.1016/j.apenergy.2010.01.012

Bastida, A., Sabuquillo, P., Armisen, P., Fernandez-Lafuente, R., Huguet, J., & Guisan, J. (1998). A single step purification, immobilization, and hyperactivation of lipases via interfacial adsorption on strongly hydrophobic supports. *Biotechnology and Bioengineering*, *58*(5), 486-93. DOI: 10.1002/(SICI)1097-0290(19980605)58:5

Boros, Z., Falus, P., Márkus, M., Weiser, D., Oláh, M., Hornyánszky, G., ... Poppe, L. (2013). How the mode of Candida antarctica lipase B immobilization affects the continuous-flow kinetic resolution of racemic amines at various temperatures. *Journal of Molecular Catalysis B: Enzymatic*, *85-86*, 119-25. DOI: 10.1016/j.molcatb.2012.09.004

Bouaid, A., El boulifi, N., Hahati, K., Martinez, M., & Aracil, J. (2014). Biodiesel production from biobutanol. Improvement of cold flow properties. *Chemical Engineering Journal*, *238*(2014), 234-241. DOI: 10.1016/j.cej.2013.10.022

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248-254. DOI: 10.1016/0003-2697(76)90527-3

Fagerlund, G. (1973). Determination of specific surface by the BET method. *Matériaux et Constructions*, *6*(3), 239–245. DOI: 10.1007/BF02479039

Fernandez-Lafuente, R., Armisén, P., Sabuquillo, P., Fernández-Lorente, G., & Guisán, J. M. (1998). Immobilization of lipases by selective adsorption on hydrophobic supports. *Chemistry and Physics of Lipids*, *93*(1–2), 185–97. DOI: 10.1016/S0009-3084(98)00042-5

Fernandez-Lorente, G., Bolivar, J. M., Rocha-Martin, J., Curiel, J. a., Muñoz, R., de las Rivas, B., ... Guisan, J. M. (2011). Synthesis of propyl gallate by transesterification of tannic acid in aqueous media catalysed by immobilised derivatives of tannase from *Lactobacillus plantarum*. *Food Chemistry*, *128*(1), 214-217. DOI: 10.1016/j.foodchem.2011.02.057

Fernández-Lorente, G., Palomo, J. M., Cabrera, Z., Guisán, J. M., & Fernández-Lafuente, R. (2007). Specificity enhancement towards hydrophobic substrates by immobilization of lipases by interfacial activation on hydrophobic supports. *Enzyme and Microbial Technology*, *41*(5), 565-569. DOI: 10.1016/j.enzmictec.2007.05.004

Ferrarezi, A. L., Ohe, T. H. K., Borges, J. P., Brito, R. R., Siqueira, M. R., Vendramini, P. H., ... Gomes, E. (2014). Production and characterization of lipases and immobilization of whole cell of the thermophilic Thermomucor indicae seudaticae N31 for transesterification reaction. *Journal of Molecular Catalysis B: Enzymatic*, *107*(1), 106-113. DOI: 10.1016/j.molcatb.2014.05.012

Fiametti, K. G., Sychoski, M. M., De Cesaro, A., Furigo, A., Bretanha, L. C., Pereira, C. M. P., ... Oliveira, J. V. (2011). Ultrasound irradiation promoted efficient solvent-free lipase-catalyzed production of mono- and diacylglycerols from olive oil. *Ultrasonics Sonochemistry*, *18*(5), 981-987. DOI: 10.1016/j.ultsonch.2010.11.010

Habulin, M., & Knez, Ž. (2001). Activity and stability of lipases from different sources in supercritical carbon dioxide and near-critical propane. *Journal of Chemical Technology and Biotechnology*, *76*(12), 1260-1266. DOI: 10.1002/jctb.514

Herbst, D., Peper, S., Francisco, J., Ruck, W., & Niemeyer, B. (2014). Pressure effects on activity and selectivity of Candida rugosa lipase in organic solvents. *Journal of Molecular Catalysis B: Enzymatic*, *100*, 104-110. DOI: 10.1016/j.molcatb.2013.12.002

Hernandez, K., & Fernandez-Lafuente, R. (2011). Lipase B from *Candida antarctica* immobilized on octadecyl Sepabeads: A very stable biocatalyst in the presence of hydrogen peroxide. *Process Biochemistry*, *46*(4), 873-878. DOI: 10.1016/j.procbio.2010.12.007

Jiang, Y., Sun, W., Zhou, L., Ma, L., Hey, Y., & Gao, J. (2016). Improved performance of lipase immobilized on tannic acid-templated *Mesoporous silica* nanoparticles. *Applied Biochemistry and Biotechnology*, *197*(7), 1155-1169. DOI: 10.1007/s12010-016-2056-1

Suslick, K. S. (1989). The chemical effects of ultrasound. *Scientific American*, *260*(2), 80-86. DOI: 10.1038/scientificamerican0289-80

Lobo, H. R., Singh, B. S., Pinjari, D. V., Pandit, A. B., & Shankarling, G. S. (2013). Ultrasound-assisted intensification of bio-catalyzed synthesis of mono-N-alkyl aromatic amines. *Biochemical Engineering Journal*, *70*(1), 29-34. DOI: 10.1016/j.bej.2012.09.013

Mendes, A. a, de Castro, H. F., Rodrigues, D. D. S., Adriano, W. S., Tardioli, P. W., Mammarella, E. J., ... Giordano, R. D. L. C. (2011). Multipoint covalent immobilization of lipase on chitosan hybrid hydrogels: influence of the polyelectrolyte complex type and chemical modification on the catalytic properties of the biocatalysts. *Journal of Industrial Microbiology & Biotechnology*, *38*(8), 1055-66. DOI: 10.1007/s10295-010-0880-9

Moreno-Perez, S., Filice, M., Guisan, J. M., & Fernandez-Lorente, G. (2013). Synthesis of ascorbyl oleate by transesterification of olive oil with ascorbic acid in polar organic media catalyzed by immobilized lipases. *Chemistry and Physics of Lipids*, *174*(1), 48-54. DOI: 10.1016/j.chemphyslip.2013.06.003

Palomo, J. M., Muñoz, G., Fernández-Lorente, G., Mateo, C., Fernández-Lafuente, R., & Guisán, J. M. (2002). Interfacial adsorption of lipases on very hydrophobic support (octadecyl–Sepabeads): immobilization, hyperactivation and stabilization of the open form of lipases. *Journal of Molecular Catalysis B: Enzymatic*, *19*, 279-286. DOI: 10.1016/S1381-1177(02)00178-9

Quilles, J. C. J., Brito, R. R., Borges, J. P., Aragon, C. C., Fernandez-Lorente, G., Bocchini-Martins, D. A., … Guisan, J. M. (2014). Modulation of the activity and selectivity of the immobilized lipases by surfactants and solvents. *Biochemical Engineering Journal*, *93*, 274-280. DOI: 10.1016/j.bej.2014.10.009

Rodrigues, D. S., Mendes, A. a., Adriano, W. S., Gonçalves, L. R. B., & Giordano, R. L. C. (2008). Multipoint covalent immobilization of microbial lipase on chitosan and agarose activated by different methods. *Journal of Molecular Catalysis B: Enzymatic*, *51*(3-4), 100-109. DOI: 10.1016/j.molcatb.2007.11.016

Rodrigues, R. C., Ortiz, C., Berenguer-Murcia, Á., Torres, R., & Fernández-Lafuente, R. (2013). Modifying enzyme activity and selectivity by immobilization. *Chemical Society Reviews*, *42*(15), 6290-307. DOI: 10.1039/c2cs35231a

Rupasinghe, Z. H. P. V. (2016). Sonochemical enzyme-catalyzed regioselective acylation of flavonoid glycosides. *Bioorganic Chemistry*, *65*(1), 17-25. DOI: 10.1016/j.bioorg.2016.01.005

Schuchardt, U., Sercheli, R., & Vargas, R. M. (1998). Transesterification of vegetable oils: a review. *Journal of Brazilian Chemical Society*, *9*(1), 199-210. DOI: 10.1590/S0103-50531998000300002

Shimada, Y., Watanabe, Y., Samukawa, T., Sugihara, A., Noda, H., Fukuda, H., & Tominaga, Y. (1999). Conversion of vegetable oil to biodiesel using immobilized *Candida antarctica* lipase. *Journal of the American Oil Chemists' Society*, *76*(7), 789-793. DOI: 10.1007/s11746-999-0067-6

Su, E.-Z., Zhang, M. J., Zhang, J. G., Gao, J. F., & Wei, D. Z. (2007). Lipase-catalyzed irreversible transesterification of vegetable oils for fatty acid methyl esters production with dimethyl carbonate as the acyl acceptor. *Biochemical Engineering Journal*, *36*(2), 167-173. DOI: 10.1016/j.bej.2007.02.012

Sundaram, S., Bukowski, M. R., Lie, W. R., Picklo, M. J., & Yan, L. (2016). High-fat diets containing different amounts of n3 and n6 polyunsaturated fatty acids modulate inflammatory cytokine production in mice. *Lipids*, *51*(5), 571-582. DOI: 10.1007/s11745-015-4093-x

Suslick, K. S. (1989). The chemical effects of Ultrasound. *Scientific American*, *2,* 80-86.

Taher, H., Al-Zuhair, S., Al-Marzouqi, A. H., Haik, Y., & Farid, M. M. (2011). A review of enzymatic transesterification of microalgal oil-based biodiesel using supercritical technology. *Enzyme Research*, *2011*(1), 1-25 DOI: 10.4061/2011/468292

Ting, W. J., Huang, C. M., Giridhar, N., & Wu, W. T. (2008). An enzymatic/acid-catalyzed hybrid process for biodiesel production from soybean oil. *Journal of the Chinese Institute of Chemical Engineers*, *39*(3), 203-210. DOI: 10.1016/j.jcice.2008.01.004

Torres, C. F., Fornari, T., Tenllado, D., Señoráns, F. J., & Reglero, G. (2008). A predictive kinetic study of lipase-catalyzed ethanolysis reactions for the optimal reutilization of the biocatalyst. *Biochemical Engineering Journal*, *42*(2), 105-110. DOI: 10.1016/j.bej.2008.06.004

Urioste, D., Castro, M., Biaggio, F., & Castro, H. (2008). Síntese de padroes cromatográficos e estabelecimento de método para dosagem da composiçao de ésteres de ácidos graxos presentes no biodiesel a partir do óleo de babaçu. *Química Nova*, *31*(2), 407-412. DOI: 10.1590/S0100-40422008000200038

Xu, L., Yang, X., Yu, X., & Guo, Y. (2008). Preparation of mesoporous polyoxometalate–tantalum pentoxide composite catalyst for efficient esterification of fatty acid. *Catalysis Communications*, *9*(7), 1607-1611. DOI: 10.1016/j.catcom.2008.01.005

Yu, D., Tian, L., Wu, H., Wang, S., Wang, Y., Ma, D., & Fang, X. (2010). Ultrasonic irradiation with vibration for biodiesel production from soybean oil by Novozym 435. *Process Biochemistry*, *45*(4), 519-525. DOI: 10.1016/j.procbio.2009.11.012

Zheng, M.-M., Wang, L., Huang, F.-H., Dong, L., Guo, P.-M., Deng, Q.-C., Li, W.-L., Zheng, C. (2012). Ultrasonic pretreatment for lipase-catalyed synthesis of phytosterol esters with different acyl donors. *Ultrasonics Sonochemistry*, *19*(5), 1015-1020. DOI: 10.1016/j.ultsonch.2012.02.004

Zhou, G. X., Chen, G. Y., & Yan, B. B. (2015). Two-step biocatalytic process using lipase and whole cell catalysts for biodiesel production from unrefined jatropha oil. *Biotechnology Letters*, *37*(10), 1959-1963. DOI: 10.1007/s10529-015-1883-4