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Evaluation of Different Heating Systems for the Hydrolysis of Residual Frying Oil Catalyzed by Free and Immobilized Lipase

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HIGHLIGHTS

- Studying alternatives for the enzymatic hydrolysis of residual frying oil catalyzed by free and immobilized lipase.
- The use of polyhydroxybutyrate, niobium oxide and chitosan as support for immobilization of lipases.
- Efficiency assessment of the cycle of immobilized lipases in residual oil hydrolysis.

Abstract: Large quantities of residual cooking oil are being increasingly generated in various sectors, as it is often improperly disposed of and causes serious environmental problems. A commonly used alternative is the use of lipases in enzymatic hydrolysis, since it is rather attractive to several sectors, in addition to allowing the use of products generated thereof, such as: free fatty acids, diacylglycerols, monoacylglycerols and free glycerol, moreover, it is capable of operating in mild temperature and pressure conditions. Therefore, the present study aimed to evaluate alternatives for the hydrolysis of residual frying oil by evaluating the efficiency of different types of supports and heating systems using free and immobilized forms of lipase from *Burkholderia cepacia* (BCL) and Porcine pancreas (PPL) as biocatalyst. Polyhydroxybutyrate (PHB), niobium oxide (Nb_2O_5) and chitosan (CHIT) were evaluated as support for immobilizing BCL and PPL lipases, and superior results were found regarding the hydrolytic activity of immobilization derivatives using PHB and Nb_2O_5. Among the heating systems under evaluation, the highest percentage of residual oil hydrolysis was found using the ultrasound system for both free and immobilized lipases, reaching 57.91% hydrolysis for lipases immobilized on Nb_2O_5 and 61.11% hydrolysis for the derivative immobilized on PHB. The operational

stability of both biocatalysts was evaluated using similar half-life time values for both. Thus, it was observed that the ultrasound system was efficient in improving lipase performance in the hydrolysis of free fatty acids, once this unconventional heating system is quite promising for accelerating such enzymatic reactions.

Keywords: Residual cooking oil 1; *Burkholderia cepacia* lipase 2; Porcine pancreas lipase 3; Immobilization 4; Hydrolysis 5.

INTRODUCTION

Vegetable oils are natural products extracted from seeds, grains or plants, which are soluble in organic solvents, but insoluble in water. They mainly consist of triacylglycerols formed from three fatty acid molecules and a glycerol molecule [1].

Vegetable oil extracted from soybean (*Glycine max*) is the one which is most commonly produced, and it is composed mostly of linoleic acid, lecithin and a variety of vitamins [2]. It is widely used as frying method in homes, restaurants, bakeries and industrial food processes, and it generates large amounts of residual oil [3].

In frying processes, it is subjected to high temperatures and it is used so often that multiple reactions occur as a result, such as those that change the composition of fatty acids, monoglycerides, diglycerides, in addition to polymerization, hydrolysis, isomerization and oxidation, thus tasting unpleasant, generating foam and changing its viscosity. The harmful compounds generated thereof makes it unsuitable for human consumption, and it may also cause environmental problems when discarded improperly [4].

Given the growing demand for fried products and an inadequate disposal of residual frying oil, concerns arise as for its disposal [5]. In the majority of cases, the final disposal of residual oil generated annually in Brazil is inadequate and causes serious environmental problems, such as clogging of sewage networks, direct contamination of rivers, lakes and soils, endangering fauna and flora and requiring further processes in sewage treatment plants [6].

In this context, technological advances have been proposed to reduce, reuse and recycle residual frying oil in order to generate valuable products to be used in various sectors [7]. The possibility of recycling residual oil is very attractive, once grounded in sustainable biological resources, environmental protection and economic considerations [8]. For instance, a gradual depletion of fossil fuel and a significant increase in human awareness about environmental protection, allowed biodiesel production using lipase to become an interesting alternative due to an improvement in its performance as it is a cleaner process.[9]

Lipases have the function of partially or totally catalyzing triacylglycerol through the hydrolysis of fats and vegetable oils, moreover, free fatty acids are generated as a result which can be used as main raw materials in biodiesel production. As reported in literature, biodiesel is a typical renewable and biodegradable energy source produced by hydroesterification of edible vegetable oils after hydrolysis [10, 11].

In addition, hydrolysis can generate other products: diacylglycerols, monoacylglycerols and glycerol, which can be applied in various industrial sectors, such as Food, Pharmaceutical, Cosmetic, Detergents, Wastewater treatment, Biodegradable Polymers [12], Biosurfactant [13]

Compared to acid chemical catalysis, due to mild reaction and low temperature and pressure conditions, enzymes have shown superior performance in transesterification, especially in the hydrolysis of oils, such as higher yield and the non-generation of undesirable by-products [14,15].

Enzymatic hydrolysis offers multiple advantages and can be performed in lower temperature and pressure conditions by the conventional chemical route, in addition to generating high quality products with greater yields.

Furthermore, lipases can be used in their immobilized form and bring some very promising advantages in the purification process of oil refining, given that it allows their reuse for further reactions under moderate conditions, in addition to representing breakthroughs as a green process and for the economy [16].Therefore, the use of lipases in their immobilized form is more advantageous due to high thermal stabilization, ease of recovery at the end of the process, cost reduction, and improvement in catalytic activity and specificity.[17]

Taking all these aspects into account and given the importance of investigating new technologies to recycle oil, this work aims to explore new alternatives for the hydrolysis of residual frying oil by evaluating the efficiency of different types of supports, such as niobium oxide, chitosan and polyhydroxybutyrate, in several types of heating systems: microwave, conventional and ultrasonic systems using free and immobilized forms of *Burkholderia cepacia* commercial lipase (BCL) and Porcine pancreas (PPL).

MATERIAL AND METHODS

Materials

Two commercial lipases *Burkholderia cepacia* and Porcine pancreas were acquired from Sigma-Aldrich (St. Louis, MO, USA) in crude form. Residual frying oil was collected from a restaurant at the Federal University of Alfenas (UNIFAL-MG). The oil was stored in dark plastic containers and kept at 20 °C for preserving its characteristics. All other reagents were of analytical grade, including arabic gum, monobasic potassium phosphate, monobasic sodium phosphate, bibasic sodium phosphate, all acquired from Dinâmica company™, sodium hydroxide, 70% ethyl alcohol (Vetec™); acetone (Neon™); phenolphthalein (Cinética™), Hexane, Nitric Acid, Polyhydroxybutyrate (PHB), γ aminopropiltrietoxylan (γ -APTS), Polyethylene Glycol (PEG), Glutaraldehyde 25%, Potassium Biftalate, and Olive Oil (Carbonell).

Synthesis of supports

Three supports for immobilizing BCL and PPL lipases were tested: niobium oxide, kindly provided by the Brazilian Metallurgy and Mining Company-CBMM and by the Chemistry Department of the Federal University of Technology at Paraná, Campus of Pato Branco/PR, and raw Chitosan and polyhydroxybutyrate (PHB) which were kindly provided by the Federal University of Ceará.

A commercial niobium oxide support was prepared according to the methodology described by Da SILVA [18]. Initially, the material was pretreated by immersion in nitric acid solution (HNO_3 at 1 %) and heated to 75 ºC under agitation for 1 h. Subsequently, the material was filtered and washed with distilled water until reaching neutral pH. The support was dried in a kiln at 105 ºC for 24 h. Then, pretreated niobium oxide was activated in two steps: silanization of the support in a trietoxylan γ-aminopropil (γ-APTS at 0.5 % v/v) solution followed by its activation in a glutaraldehyde solution (GA at 2.5 % in a hydrogen phosphate buffer 0.1 mol L^{-1}, pH 8).

The chitosan hydrogel (CHIT) support was prepared according to the methodology described by Mendes [19]. For each gram of chitosan powder, 20 mL of glacial acetic acid 5% (v/v) was added under mechanical agitation at 1000 rpm and 25 ºC. The solution was dripped in NaOH 0.5 mol L^{-1} in the ratio of 1:10 support: NaOH, under agitation at 100 rpm and 25 ºC for approximately 15 h. Then, the suspension was filtered, thoroughly washed with distilled water and stored in a 70% (v/v) ethanol solution at 4 ºC. Subsequently, chitosan hydrogel was activated using glutaraldehyde 2.5% (v/v) under agitation at 200 rpm and 25 ºC for 1 h.

The polyhydroxybutyrate (PHB) support was prepared based on a methodology adapted from Binhayeeding [20]. PHB powder was immersed in anhydrous ethanol for 4 h followed by filtration. The support activation was performed according to the activation steps regarding niobium oxide used as support described above.

Immobilization of lipases

The immobilization of lipases on supports was performed according to the methodology described by Da Silva [18], in which the activated support was immersed in hexane in a solid:liquid ratio of 1:10 and kept under mild agitation for 2 h. Then, for each gram of activated support, 100 µL of polyethylene glycol solution 5 mg mL^{-1} (PEG-1500) and 250 mg lipase were added in their free form. Suspensions containing the enzyme and support were kept under agitation for 2 h, followed by static contact for an additional period of 18 h until reaching 4 ºC. Then, the immobilized lipase was recovered by vacuum filtration and subjected to drying in a desiccator.

The process of immobilizing lipases supported on chitosan-based hydrogel was also carried out according to the methodology described by Mendes [19], in which 9 mL of enzymatic solution was used for each gram of support at 0.025 g mL^{-1} of lipase. The mixture was kept under agitation at 200 rpm for 18 h followed by filtration, washed with distilled water and stored at 4 °C for 18 h.

Determination of enzyme activity

Enzyme activity (U g^{-1}) was evaluated by hydrolysis of olive oil emulsion in a fixed ratio of oil/water 1:1. One unit (U) of enzyme activity was defined as the amount of enzyme that releases 1 µmol of free fatty acid per min under assay conditions. Its results were expressed in units of activity per gram of free enzyme [21].

Enzymatic hydrolysis of catalyzed lipases from residual cooking oil

Hydrolysis reactions were produced in different heating systems: conventional, ultrasound (Model USC 1800-A Ultrasonic Cleaner (Unique)) and microwave irradiation (Discover/ University-Wave, Cem Corporation model PN# CE-925SB235-SPB-PLUG), containing a substrate consisting of a 1:2 oil/buffer solution (0.1 mol L^{-1}, pH 7.0) and an emulsifier. The reactions under a conventional heating system were performed in 50 mL spherical glass reactor under mechanical stirring (500 rpm) at 40 °C for 4 h. The ultrasound heating reactions were performed under the same temperature and time conditions, and 100 W. Experiments carried out in the microwave reactor were maintained in a 100 mL spherical glass reactor, coupled with a reflux condenser under magnetic agitation at 200 rpm.

The proportions of biocatalysts were based on a work carried out by Souza [22], in which mixture planning was used to optimize the proportion of each biocatalyst in the process. Thus, the best established condition was 85% of BCL lipase and 15% of PPL.

In order to investigate the behavior of hydrolysis kinetics, 0.1 g aliquot samples were collected at various time intervals and analyzed by titration according to Da Rós [23]. Hydrolysis percentage (%) was calculated by equation 1 [23]. Hydrolysis (%) is defined as the percentage weight of free fatty acids in the sample divided by its maximum theoretical amount [24].

$$Hydrolysis\ (\%) = \frac{V_{KOH} \times M_{KOH} \times \overline{\mathrm{MW}}}{W \times f} \times 100 \quad (1)$$

Where: V_{KOH} is the volume of potassium hydroxide solution (KOH) required during titration; M_{KOH} is KOH molarity (0.0483 mol L^{-1}); $\overline{\mathrm{MW}}$ is the average molecular weight of fatty acids (g mol^{-1}); W is the sample weight; and f is the fraction of oil at the beginning of the reaction.

Immobilized lipase recovery

The resulting immobilized lipases at the end of each reaction were separated from the reaction medium and recovered by filtration, then washed with hexane in a Buchner funnel. Afterwards, the biocatalyst was kept in a desiccator for 2 h and stored at 4 °C for later use in hydrolysis reactions.

Operational stability of biocatalysts

After defining the best heating system for performing the reactions, the operational stability of immobilized systems was verified in hydrolysis reactions of residual frying oil in consecutive batches by reusing the immobilized system. Hydrolysis reactions were performed using the same operating conditions described in the hydrolysis process of residual frying oil. Between batches, immobilized lipase was washed and filtered with hexane for 3 h so as to remove the reagents and/or products found in the support. Afterwards, the recovered lipase was reused in another batch with the substrate at the same concentration as that used in the initial reaction. Deactivation constants (K_d, h^{-1}) and half-life ($t_{1/2}$, h) of biocatalysts were calculated by equation 1 and 2, respectively [25].

$$\ln A = \ln A_0 - K_d * t \quad (2)$$

$$t_{1/2} = \frac{Ln\ (2)}{K_d} \quad (3)$$

Where *A* (U/g) is the hydrolytic activity at time *t* and A_0 (U/g) is the initial hydrolytic activity.

RESULTS

Hydrolytic activity of lipases in their free and immobilized form

Table 1 presents the results found for the hydrolytic activities of BCL and PPL lipases in their free and immobilized form in different supports: niobium oxide (Nb_2O_5), polyhydroxybutyrate (PHB) and chitosan (CHIT), through olive oil hydrolysis.

Table 1. Hydrolytic activities of BCL and PPL lipases in their free form and immobilized on Nb_2O_5, PHB, CHIT.

Biocatalyst	Hydrolytic Activity (U/g)			
	Enzyme Free	Nb_2O_5	PHB	CHIT
BCL	8020.07	1428.50	1122.37	61.07
PPL	1201.06	798.70	205.31	48.66

The results presented in Table 1 reveal that BCL lipase of microbial origin presented higher hydrolytic activity (8020.07 U/g) compared to PPL lipase of animal origin (1201.06 U/g) both in their free form. According to literature, there are some factors differentiating these two origins of lipase, which may explain these results.

The high activity of lipases from *Burkholderia cepacia* is due to the fact that they present no regiospecificity, i.e. they are able to hydrolyze the fatty acids of triacylglycerols in any position [26]. Another relevant aspect of BCL is its high thermal stability. According to studies conducted by SÁNCHEZ; TONETTO; FERREIRA [27], BCL lipases at temperatures of 40 - 60°C do not denature and can therefore be applied in processes such as soybean oil hydrolysis at temperatures lower than the ones selected in traditional commercial processes. On the other hand, PPL lipase can reach excellent performance at temperatures ranging between 40 - 45°C [28], once it is a soluble enzyme secreted by porcine pancreas. In addition, PPL lipase is also capable of hydrolysing only the fatty acids of triglyceride positions 1,3 [29]. Thus, both evaluated lipases could be applied as combilipases (lipase mixture) in residual oil hydrolysis reactions in the same temperature range in subsequent stages.

Therefore, a combined use of lipases with different specificities in hydrolysis reactions is an innovative alternative in order to obtain an ideal catalyst, thus generating by-products such as free fatty acids (FFA) and glycerol, which are important precursors for the industrial sectors [22]. In addition, the use of combilipases favors an increase in reaction conversion rates and efficiency in the reaction process, compared to the use of a single lipase [30].

By analyzing the results of lipase activity in its immobilized form (Table 1), the significant values of hydrolytic activity were also found for BCL lipase immobilized on Nb_2O_5 (1428.50 U/g) and PHB (1122.37 U/g) supports when compared to chitosan immobilization, as lower results were achieved (61.07 U/g). Thus, given such lower result, the lipases immobilized with a chitosan support were not used in the hydrolysis tests of residual frying oil, since a low activity value would not represent efficiency in hydrolysis percentage. Despite the wide variety of immobilization methods, no method can be applied to all enzymes efficiently, i.e. there is heterogeneity between enzymes in relation to the composition and conformation of their structure, substrate, product properties and reaction medium [31].

Regarding the process PPL lipase immobilized on the two supported supports, results were inferior compared to those achieved by BCL immobilized on the same supports, i.e. Nb_2O_5 (798.70 U/g), PHB (205.31U/g) and CHIT (48.66 U/g). In addition to lower hydrolytic activity of PPL in its free form, it should be considered that when an amount of lipases are immobilized, the support is immobilized in such a way that its active site is less accessible to the substrate, consequently its reaction activity is lower [32].

Hydrolysis with residual frying oil using lipases from Burkholderia cepacia and **porcine pancreas** *in their free form*

Optimal enzymatic combination of lipases of microbial and animal origin were selected to study residual frying oil hydrolysis through an experimental design of lipase mixtures, as carried out by Souza [22], with the purpose of finding the most favorable hydrolysis conditions. The optimized hydrolysis results found by these authors were as follows: BCL lipases 0.4g (85%) and PPL 0.545g (15%). In the present study, the masses of immobilized enzyme are in agreement with the results found by Souza [22] regarding each lipase (BCL and PPL) used in microwave, conventional and ultrasound systems.

The use of a mixture of lipases having different specificities known as combilipases has become increasingly attractive to industrial sectors, as it allows the use of enzymes capable of reaching optimal hydrolysis conditions and resulting in high reaction yields and process productivity [33]. Rodrigues and Ayub [34] evaluated the hydrolysis of soybean oil using a mixture of two different types of lipases: *Thermomyces lanuginosus* and *Rhizomucor miehei*. Their results showed that the use of combilipases allowed achieving yields that were 15% higher than by using only one of these lipases, 95% hydrolysis was reached using a mixture of these two lipases. Thus, in view of these results, the use of a mixture of different lipases is an efficient and promising technology for an enzymatic synthesis and hydrolysis of vegetable oils. Figure 1 shows the percentage results of hydrolysis performed in a microwave system.

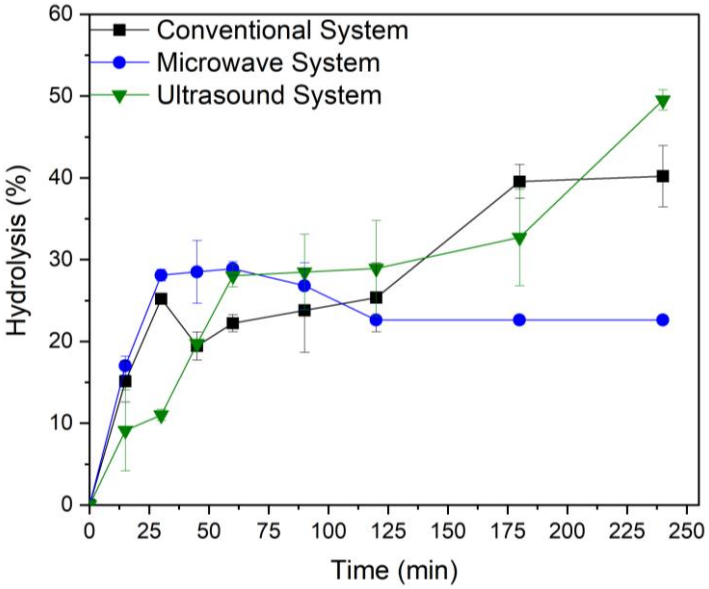

Figure 1. Enzymatic hydrolysis of a mixture of BCL and PPL lipases in their free form using ultrasound, microwave and conventional systems.

After 2 h of hydrolysis, the reaction stabilized and 22.61% hydrolysis was reached. According to Dalla Rosa [35], agitation is an important variable to be evaluated, as it can promote better homogenization of the medium during the reaction, minimize the separation of phases and allow adequate contact of enzyme/substrates. Therefore, magnetic agitation of 200 rpm was insufficient to maintain the emulsion intact, given that there is phase separation (water/oil), thus decreasing hydrolysis yields, since agitation is one of the fundamental factors for the hydrolysis process efficiency.

In conventional and ultrasound systems, a mechanical agitation of 500 rpm was employed, and its power was higher than that used in the microwave system. Therefore, the test reaching the highest percentage of residual frying oil hydrolysis was the ultrasound system, i.e. 49.51% within 240 minutes. In the conventional system, hydrolysis reached about 40.2% and was also superior to that found in the microwave system with magnetic agitation, but lower than that for the ultrasound system. In view of these results, agitation is not the only factor enhancing enzymatic hydrolysis, but ultrasound irradiation waves also provided an increase in reaction yields, in addition to providing the reaction medium (water/oil) with greater stability.

Marotti [36] evaluated the effect of the ultrasound system and the stability of a medium emulsified with mechanical agitation (300 rpm) in the process of hydrolysis of vegetable oils to assess the performance of *Penicillium* lipase, and found that mechanical agitation became the most effective reaction process, given that it provides interfacial stability (water/oil), increased diffusion of substrates with active sites of the enzyme, and resulting in increased hydrolysis yields.

Raizer [37] evaluated the influence of enzyme and temperature on the enzymatic hydrolysis of sunflower oil using phospholipase A1 lipase (Lecitase Ultra) in its free form assisted by the ultrasound system. The best hydrolysis conditions were 40°C and 1.7 m% of enzyme/substrate fraction, and it was found that the reaction medium (water/oil) acquires a homogeneous and opaque appearance using the ultrasound system, and stable emulsions were generated based on its results (95%), therefore the use of this system in enzymatic processes proved to be effective in enzymatic hydrolysis.

In search of new techniques to constitute an improvement in the performance of processes in various industrial sectors, the technology employing the ultrasound system has become increasingly efficient due to benefits, such as product quality, good catalyst reuse and reaction time minimization [38, 39].

Residual frying oil hydrolysis using a mixture of Burkholderia cepacia and porcine pancreas *lipases immobilized on a niobium oxide support (Combi-Nb_2O_5)*

Another important factor in the present study is the technique of enzymatic immobilization using combilipases, as it offers some advantages, such as possibility of reuse, ease of separating the catalyst from the product, high degree of substrate conversion and purity of the product, thus making it more commercially competitive, improving the enzyme operational stability and reducing production costs [32, 40].

Although the immobilization process can be performed using several methods, there is a constant search for simpler and more economical techniques. The most common methods for lipase immobilization are physical adsorption and covalent bound [41]. Given the results of superior hydrolytic activity of lipases immobilized on a niobium support (Combi-Nb_2O_5), they were selected for evaluation in ultrasound and conventional systems.

According to the results presented in Figure 2, the Combi-Nb_2O_5 derivative used in the ultrasound system proved to be efficient and reached hydrolysis percentage of 57.91% within 60 min, i.e. a better result than that obtained using BCL + PPL in their free forms (49.51%). As for the conventional method, approximately 34% of hydrolysis was reached within 60 min of reaction, and an enhanced result was also achieved if compared to that using combilipase (BCL + PPL) in its free form (22.23%).

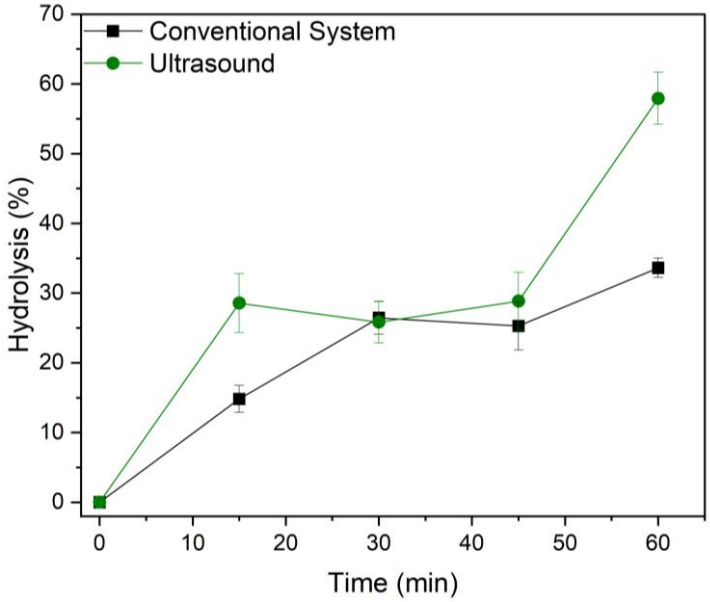

Figure 2. Combi-Nb2O5 enzymatic hydrolysis using conventional and ultrasound systems.

By comparing the results of heating systems, the ultrasound system promoted superior results. It may be attributed to the irradiation waves of the ultrasound system promoting greater emulsion interaction, thus increasing reaction rates and favoring the hydrolysis process. In literature, studies elucidating the ultrasound system can be found, in which its benefits in biotechnological reactions are reported [42].

Mello [43] also evaluated the use of ultrasound systems in the hydrolysis of crambe oil catalyzed by *Thermomyces lanuginosus* lipase in its free form. The authors reported that the use of ultrasound as heating system have led to an increase in the rate of hydrolysis, reaching up to 78% of crambe oil hydrolysis. It was also reported that the ultrasound waves provided greater stability in the process of emulsion during hydrolysis, but it does not occur in the separation of substrate phases (water/oil) and thus increases the interaction between lipases and the substrate.

Another factor worth mentioning is a combined use of several lipases with different specificities. This technique can be a way to obtain an ideal biocatalyst [34]. The concept of combilipase is considered an effective alternative to reduce reaction time and increase conversion rates in hydrolysis reactions, since combilipase acts briefly in different positions of triglycerides regarding the composition of the oil [44].

Alves [45] frames the concept of the biocatalyst as being combilipase for heterogeneous substrates, which is based on the fact that a biocatalyst composed of a mixture of different lipases is more effective while using heterogeneous substrates than a specific lipase.

Huang [46] used a mixture of two different lipases: a specific 1.3 lipase of *Rhizomucor miehei* and another non-specific lipase, namely *Penicillium cyclopium*. They were submitted to lard hydrolysis and, according to the authors, the obtained results reached up to 78.1% hydrolysis yield, moreover, they report that the use of combilipases having different specificities proved effective in the enzymatic production of AGL by using lipid substrates, such as lard.

Operational stability of a mixture of Burkholderia cepacia and* Porcine pancreas *lipases immobilized on a niobium oxide support

Biocatalyst stability is a parameter of fundamental importance when one intends to industrially use an immobilized enzyme [47]. Reusing immobilized lipases in several reaction cycles can cause partial or total loss of catalytic activity due to lipase desorption in the reaction medium.

The Combi-Nb_2O_5 derivative was used in 3 cycles for at least 180 min in the hydrolysis of residual frying oil, and it was recovered twice. The tests were submitted to the ultrasound system and better efficiency was achieved regarding the percentage of ultrasonic and conventional hydrolysis with mechanical agitation at 500 rpm and temperature of 40°C. Results of the ultrasound and conventional systems are presented in Figures 3 and 4, respectively:

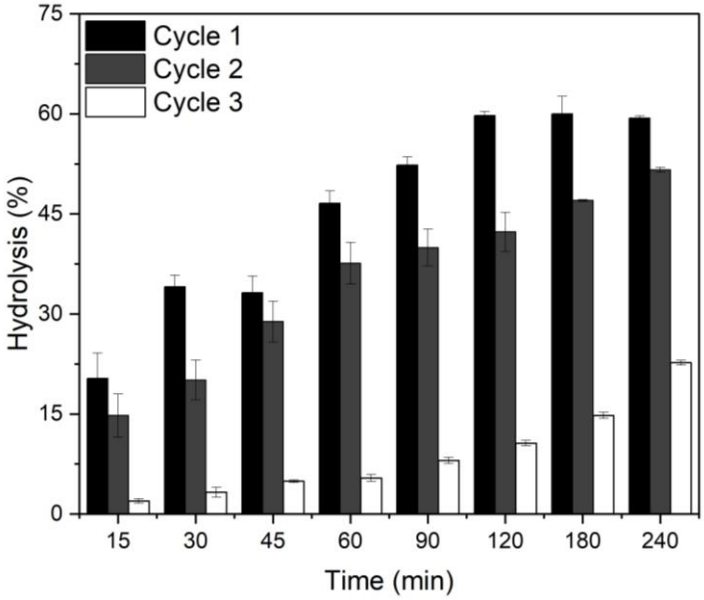

Figure 3. Combi-Nb_2O_5 recycle in the ultrasound system.

Figure 3 shows the results of using the ultrasound system from the Combi-Nb_2O_5 recycling. For the first reaction cycle, approximately 60% hydrolysis was reached in 120 min of reaction, which was maintained until 240 min. In the second cycle, there was a decrease by 13.06%, and 51.60% of maximum hydrolysis was reached in the third cycle, in addition to a more marked decrease by 61.77% and maximum hydrolysis yield of 22.69% within 240 min. These results might have been influenced by the biocatalyst recovery in each cycle, as 59.30% of immobilized derivative recovery was obtained in the second cycle and 7.71% in the third one.

Zenevicz [38] reported that using the ultrasound system in the enzymatic hydrolysis of soybean using 10% (m/m) of the immobilized commercial lipase Lipozyme TL IM allowed achieving results of 89% hydrolysis within 2h, thus indicating that the ultrasound system leads to increased hydrolysis percentage.

According to studies conducted by Waghmare and Rathod [39] while using Novozyme commercial lipase immobilized on macroporous polyacrylic resin granules in the hydrolysis of residual cooking oil under the influence of ultrasound irradiation, 74.19% of hydrolysis was achieved within two hours of reaction, thus ultrasound-assisted hydrolysis is rather efficiency in the process, mainly on account of reducing reaction time and reaching high yields of 80%.

Figure 4 shows results of using the conventional system in Combi-Nb_2O_5 recycling. For the first reaction cycle, a maximum percentage of 56% was reached within 180 min of reaction. In the second cycle, there was a decrease by 31.52% and a maximum percentage of 38.35% of hydrolysis was found within 180 min and a decrease by 92.52% and a low percentage of hydrolysis of 4.19% in 240 min in the third cycle. A pronounced decrease in both cycles and lower hydrolysis percentage than that reached by the ultrasound system might not only be due to the absence of ultrasound waves, but to the low biocatalyst recovery. On the other hand, only 31.21% of recovery was reached in the second cycle and 7.25% in the third one.

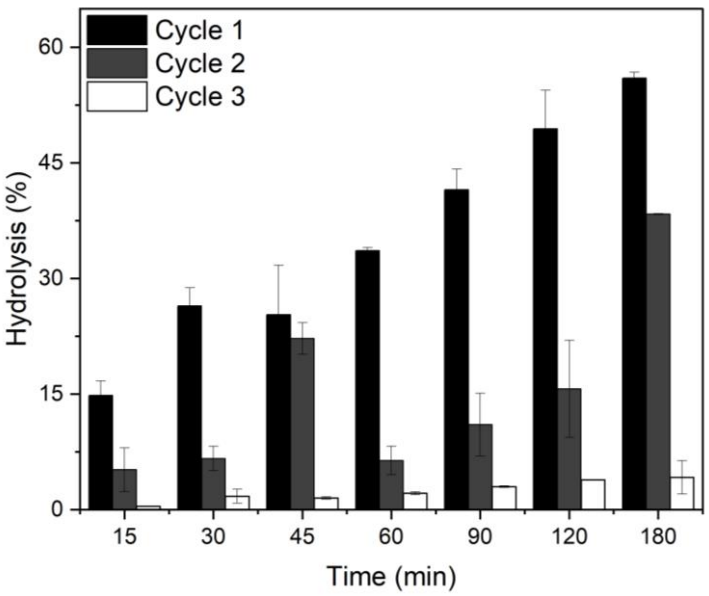

Figure 4. Enzymatic hydrolysis and recovery of Combi-Nb_2O_5 through the conventional system.

Operational stability of a mixture of Burkholderia cepacia and porcine pancreas *lipases immobilized on the PHB (Combi-PHB) support through the Ultrasound System*

Due to a high hydrolytic activity found for lipases immobilized on PHB (BCL, 1122.37U/g and PPL, 205.31U/g), the derivative of BCL and PPL lipases immobilized on PHB (Combi-PHB) was evaluated with the purpose of comparing its efficiency in the hydrolysis and recycle process to the results found for Combi-Nb_2O_5. The ultrasound system was selected to evaluate the application of Combi-PHB in the hydrolysis of residual frying oil, given that it proved to be more efficient in enhancing the hydrolysis process than the conventional system. Figure 5 shows the results obtained from the hydrolysis of residual frying oil by using combi-PHB through the ultrasound system within 360 min of reaction at 40°C.

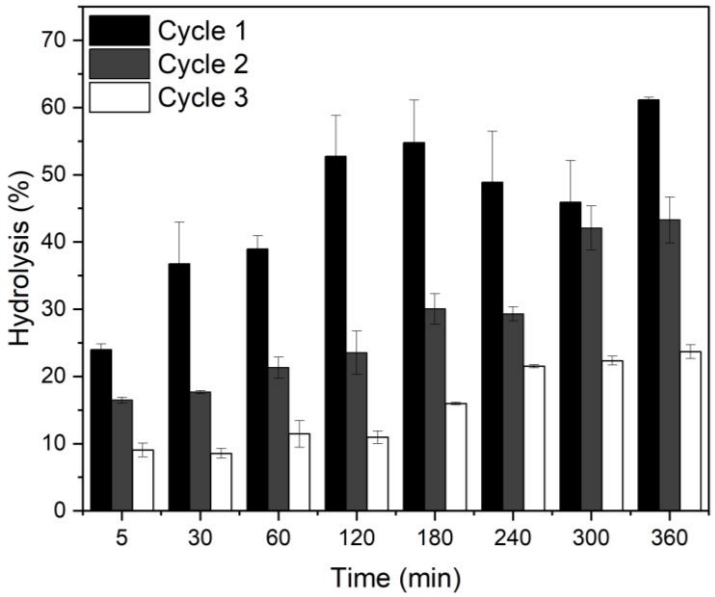

Figure 5. Enzymatic hydrolysis and recovery of Combi-PHB using the ultrasound system.

In the recycle assay using Combi-PHB, 61.11% of residual oil hydrolysis was found within 360 min in the first cycle, 43.21% in the second cycle and 23.64% in the third one. By making a comparison of recycling Combi-PHB and Combi-Nb_2O_5 derivatives within the same reaction time (240 min), the Combi-PHB derivative was less efficient, as it achieved 48.90% hydrolysis, i.e. a lower percentage than that found for the Combi-Nb_2O_5 derivative (59.35%). Furthermore, a greater decrease in hydrolysis percentage was also observed in the second cycle, i.e. a decrease by 40.10% in the first cycle and 56% for the third cycle. Some factors might

be attributed to this result, such as the immobilization efficiency found for each support, the initial activity of each biocatalyst, and the characteristics of each support being used [41].

Determination of operational stability parameters

Enzymatic immobilization allows reusing costly lipases, favoring improved stability and activity, increasing their productivity and bringing savings in reaction processes [48]. One of the main objectives of immobilization is to increase its time of use in relation to free lipase, thus the efficiency of several consecutive reactions remains unaltered [49]. The factor of operational stability is limited to the half-life time of an enzyme. This parameter is used to determine the effectiveness of immobilization, especially while using enzymes immobilized in industrial sectors [50]. In the present study, the operational stability of Combi-PHB and Combi-Nb_2O_5 combilipases was evaluated. The results obtained from lipase recycled in hydrolysis reactions allowed calculating half-life time ($t_{1/2}$) and thermal inactivation constants (Table 2). Half-life time is defined as the time required for achieving 50% reduction in enzyme initial activity.

Table 2. Operational stability parameters of Combi-PHB and Combi-Nb_2O_5 - Thermal inactivation and half-life times rate constants.

Support	k_d (h^{-1})	**Half-life time** (h)
Combi-PHB	0.11	5.99
Combi-Nb_2O_5	0.12	5.76

According to Table 2 results, k_d of 0.11 h^{-1} and half-life time of 5.99 h were observed for the Combi-PHB derivative, and k_d of 0.12 h^{-1} and half-life of 5.76 h were found for the Combi-Nb_2O_5 derivative. It is worth mentioning that half-life times were similar for both enzymatic derivatives, i.e. approximately 6h. Therefore, both prepared biocatalysts presented the same operational stability at 40 ºC in residual soybean frying oil hydrolysis.

CONCLUSION

In the present study, the influence of different heating systems (ultrasound, conventional and microwave systems) on the enzymatic hydrolysis of residual oil was evaluated, as well as the efficiency of the three different supports (Niobium oxide, PHB and CHIT) on the immobilization of BCL and PPL lipases. It was found that niobium oxide and PHB were efficient in immobilizing lipases. Moreover, the use of ultrasound systems leads to higher hydrolysis rates. The results obtained from the use of Combi-Nb_2O_5 proved to be more efficient than the combilipases in its free form, both in ultrasound and conventional systems. Regarding the operational stability of Combi-PHB and Combi- Nb_2O_5, half-life times were similar for both combilipases.

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