

Research Paper

# Agroindustrial wastes as a support for the immobilization of lipase from *Thermomyces lanuginosus*: synthesis of hexyl laurate

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**Abstract:** As a consequence of intense industrialization in the last few decades, the amount of agro-industrial wastes has increasing, where new forms of valorization are crucial. In this work, 5 residual biomasses from Maranhão (Brazil) were investigated as supports for immobilization of lipase from *Thermomyces lanuginosus* (TLL). The new biocatalysts BM-TLL (babaçu mesocarp) and RH-TLL (rice husk) showed immobilization efficiencies >98% and hydrolytic activities of 5,331 U.g<sup>-1</sup> and 4.608 U.g<sup>-1</sup> respectively against 142 U.g<sup>-1</sup> by Lipozyme® TL IM. High esterification activities were also found, with 141.4 U.g<sup>-1</sup> and 396.4 U.g<sup>-1</sup> from BM-TLL and RH-TLL against 113.5 U.g<sup>-1</sup> by TL IM. Results of porosimetry, SEM and BET demonstrated BM and RH supports are mesoporous materials with large hydrophobic area, allowing a mixture of hydrophobic adsorption and confinement, resulting in hyperactivation of TLL. These biocatalysts were applied in the production of hexyl laurate, where RH-TLL was able to generate 94% conversion in 4 h. Desorption with Triton X-100 and NaCl confirmed that new biocatalysts were more efficient with 5 times less protein than commercial TL IM. All results demonstrated that residual biomass was able to produce robust and stable biocatalysts containing immobilized TLL with better results than commercial preparations.

**Keywords:** lipases, *Thermomyces lanuginosus*, agroindustrial waste, hexyl laurate, lipase immobilization, biomass valorization, green chemistry, biocatalysis

## 1. Introduction

Due to the increase in the life expectancy, the world population has been growing alarmingly, bringing as immediate consequences the increase in industrial and agricultural activities as a way to supply the energy and food demands [1]. Besides the unsustainability of fossil fuels requires a shift towards the use of renewable sources to meet future energy and chemical needs as a way to balance economic and environmental aspects through reducing emissions of greenhouse gases which cause climate change, global warming, and health issues, severely threatening the current environment [2].

As a direct consequence of this development, around 200 million tons of agro-industrial wastes have been generated worldwide, alarming the concern about the environmental impact caused and the development of new approaches for waste valorization [3]. In this trend, the integration between technologies developed by researchers and industry has been crucial to the search for short-term solutions where the reuse of waste and the search for new (bio)catalysts are essential to obtain more selective processes with higher yields in biorefineries in the future [4-6]. To this purpose, the only

solution to effectively resolve the challenge is to discover advanced technologies for the sustainable development and production from eco-friendly biomass feedstock [7].

Biomass is currently the largest source of renewable carbon-based energy. Lignocellulosic biomass, consisting of cellulose (40–50%), hemicellulose (15–25%) and lignin (15–30%) is the most abundant source of carbon-based molecules [8]. This fact is often overlooked, as most of this biomass is used non-commercially for energy and heating, which is inefficient in terms of productivity, in addition to generating more pollution. Due to its chemical and structural complexity, biomass can be used in a better way through the synthesis of bio-based fuels or value-added chemicals [9, 10].

Brazil has one of the greatest biodiversity on the planet, where agricultural extraction is highly economically important. Among the states that stand out in this activity, Maranhão is responsible for supplying more than half of the Northeast, North and Southeast regions of the country due to climatic and soil conditions conducive to the cultivation of several agricultural genres [11]. As a result of this extractive activity, a high range of lignocellulosic by-products with a high concentration of organic material are generated, where many of them are still discarded without appropriate treatment in the soil or directed to serve as animal feed, destinations that a priori do not generate considerable economic advantages for the industry, besides representing logistical and environmental issues [12-14]. In this context, a better valorization of lignocellulosic biomass can be an auspicious source of new added value compounds, since it is abundant and diversified. In addition, due to its varied morphologies and appreciable hydrophobicity and rigidity, lignocellulosic biomass can also be applied as a support for the immobilization of enzymes, making it a renewable, abundant and low-cost support for obtaining new biocatalysts [15, 16]. Among the enzymes with notable industrial importance, lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) have received great prominence in recent years [17]. Although the natural reaction of this class of enzymes is the hydrolysis of triacylglycerols, lipases are also capable of catalyzing synthesis reactions by recognition a high range of substrates, showing activity in organic solvents, in addition to acting with high regio-, enantio- and chemo selectivity, factors that, together with the absence of co-factors, make these enzymes highly attractive in several industrial segments [18-22]. However, the applications of lipases in industrial processes are often hindered due to their low long-term stability and gradual decrease of activity during storage, expensive and/or inefficient recovery and subsequent reuse [23]. These limitations can be overcome by applying appropriate immobilization techniques to improve their stability against denaturing agents and proteolysis, facilitate their recovery from the reaction media at the end of the processes for subsequent reuse with concomitant reduction of costs in downstream processes, avoid enzyme aggregation, develop processes with multienzymatic cascade reactions and increase the flexibility of reactor designs [24, 25]. The search for supports able to confer important interactions with enzymes and provide robust processes is still a field of intense study. However, the high cost of this technology still limits the application of immobilized enzymes on an industrial scale. In this context, lignocellulosic biomass, being abundant and multifunctional, can be an interesting and accessible alternative as a support for enzymatic immobilization [26-29].

In this work, residual biomass from sugarcane bagasse (SC), rice husks (RH), corn cobs (CC), babassu mesocarpus (BM), coconut bark (CB) and coffee grounds (CG) from the industrial from Maranhão (Brazil) were investigated as supports for immobilization of the commercial lipase of *Thermomyces lanuginosus* (TL 100L- Novozymes®) with subsequent physico-chemical biocatalysts characterization and application in the hexyl laurate biosynthesis.

## 2. Materials and Methods

### 2.1. Materials

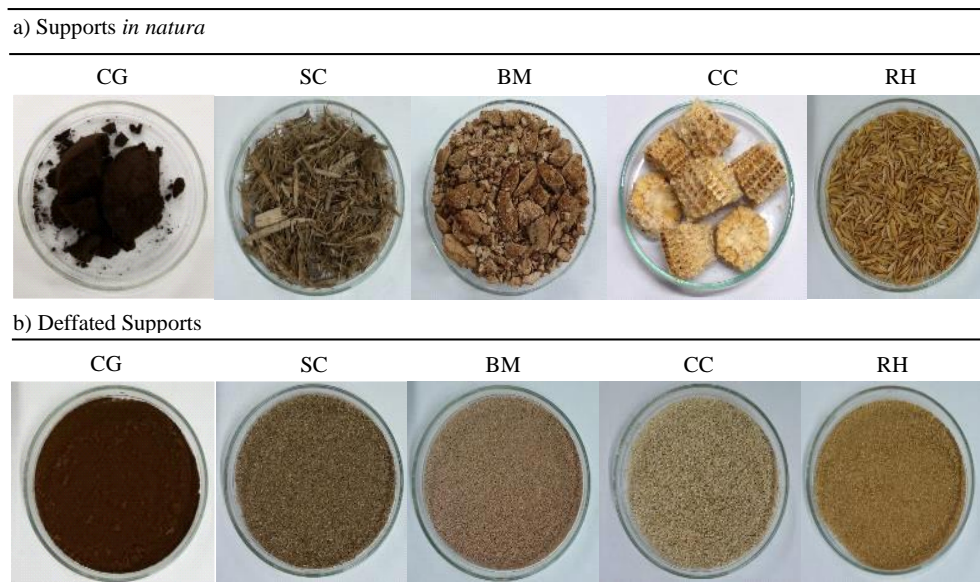
Lignocellulosic wastes from sugarcane bagasse (SC), rice husks (RH), corn cobs (CC), babassu mesocarpus (BM), coconut bark (CB) and coffee grounds (CG) used as support were collected in supply and processing centers in the State of Maranhão (Brazil). Free lipase from *Thermomyces lanuginosus* (Lipozyme TL100L - Novozymes®) with 22.8 mg. mL<sup>-1</sup> of protein and respective corresponding immobilized on silicate support (Lipozyme TL IM- Novozymes®) were purchased by LNF-LatinoAmericana (Bento Gonçalves, Brazil). Bovine serum albumin (BSA), Coomassie Brilliant Blue G250 dye, ethyl alcohol anhydrous and 95% and orthophosphoric acid 85%, oleic acid, acetone and, lauric acid and n-hexanol, used in the enzymatic activity and protein quantification stages, and the Pink Cane dye was purchased from Sigma-Aldrich (St. Louis, MO, USA). Arabic Gum and olive oil (Gallo®) with low acidity used as a substrate in the hydrolytic activity test were purchased from Synth (São Paulo, SP, Brazil). All reagents used were of analytical grade.

### 2.2 - Production of Biocatalysts

#### 2.2.1 - Preparation of Supports

Initially, *In-natura* agro-industrial residues of coffee grounds (CG), corncobs (CC), sugar cane (SC), babassu mesocarp (BM) and rice husks (RH) were washed with distilled water, dried for 24h at greenhouse (Figure 1.a), crushed, sieved (28 - 35 mesh), and then washed with ethanol (99.8% PA) at room temperature [30, 31]. Approximately 10 g of each washed support was subjected to degreasing in a Soxhlet extractor for 4 hours with 200 mL of ethanol at 50 °C. After extraction, the sample present in the extraction cartridge was removed and dried in an oven at 60° C for 6h (Figure 1.b). The defatted biomasses were applied in the immobilization protocols.

**Figure 1.** Agro-industrial wastes used to support the immobilization of TLL. a) Supports *In natura*, b) Defatted Supports. CG- coffee grounds; SC - sugarcane bagasse; BM - babassu mesocarp, CC - corn cobs and RH - rice husks.



#### 2.2.2 - Immobilization of TLL lipase

TLL was immobilized by adsorption to the hydrophobic surface on defatted biomasses according to the methodology described by [32]: 1 g of each support was added to 10 mL of hexane and stirred at room temperature for 2 h. Then, 10 mL of enzymatic solution (containing 5 mg of protein per g of support) in sodium phosphate buffer solution

pH 7.0 (0.025M or 0.1M) was added to the medium under stirring for more 2 h following by conservation under static conditions at 4°C for 3 to 24 hours, where aliquots of the supernatant were taken in order to quantify the immobilization efficiency through the concentration of proteins and residual hydrolytic activity. At the end of the process, the resulting biocatalysts were filtered under vacuum and washed three times with 10 mL of hexane.

The efficiency of the adsorption process ( $E_{ads}$ ) was determined by Equation (1) [24]:

$$E_{ads} = \frac{C_0 - C_f}{C_0} * 100 \quad (1)$$

where:  $C_0$  and  $C_f$  are the initial and residual protein concentration in the immobilization supernatant (mg. mL<sup>-1</sup>) respectively;

The lipase activation ratio in the new immobilized biocatalysts in terms of activity ( $N_{act}$ ) was determined by Equation (2) [33]:

$$N_{act} = \left( \frac{A_i}{A_0} \right) * 100 \quad (2)$$

where:  $A_i$  is the specific activity of the immobilized biocatalyst (U. g<sup>-1</sup>) on hydrolytic activity assay and  $A_0$ , the hydrolytic activity offered at the beginning of the immobilization (U. g<sup>-1</sup>).

### 2.2.3- Determination of protein concentration

The concentration of proteins from the enzyme extract of free TLL and supernatants during the immobilization process was carried out according to the method of Bradford where the protein content was estimated by the average of a calibration curve obtained using albumin bovine serum (BSA) as standard [34].

### 2.2.4 - Determination of hydrolytic activity

The activities of free and immobilized lipases were determined as follows [28]: 1 mL of extract and 10 mg of each supported enzyme were added to 19 mL of an emulsion prepared with olive oil (5% w/v) and Arabic gum (10% w/v) in sodium phosphate buffer (100 mM, pH7.0). The reactions were carried out under agitation (200 rpm) at 35 °C for 30 min. The reactions were then stopped by the addition of 20 mL of the acetone–ethanol mixture (1:1 v/v), and the fatty acids produced were extracted under agitation (200 rpm) for 10 min and titrated until end point (pH 11.0) with NaOH solution (0.04 N). The blank assays were performed by adding the extract just after the addition of the acetone–ethanol solution to the flask. One unit of lipase activity (U) was defined as the amount of enzyme that catalyzes the release of 1 μmol of fatty acids per minute, under the assay conditions.

### 2.2.5 - Determination of esterification activity

The esterification activity (U. g<sup>-1</sup>) of the new biocatalysts and the commercial lipase TL IM (positive control) was quantified through the reaction between oleic acid and ethanol in n-heptane (1: 1, 100mM) [35, 36]. The reaction was carried out at 40 °C, 160 rpm and 5% (w / v) of biocatalyst for 40min, followed by the addition of 20mL of acetone-ethanol for the extraction of residual fatty acids. The amount of acid consumed was determined by titration with 0.04 M NaOH and using phenolphthalein as an indicator. 1 unit of activity was defined as the amount of enzyme that consumes 1 μmol of oleic acid per minute and calculated using Equation 5 [37]:

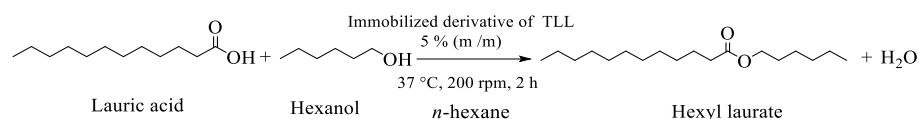
$$EA = \frac{(V_b - V_a) \times M \times V_f \times 1000}{t \times m \times V_c}$$

(5)

where:  $V_a$  is the volume of NaOH titrated in the sample (mL);  $V_b$  is the volume of NaOH titrated in the control sample (mL);  $M$  is the molar concentration of NaOH solution (mol. L<sup>-1</sup>);  $V_f$  is the final volume of reaction medium (mL);  $t$  is the reaction time (min);  $m$  is corresponding to the mass of the sample (g of derivative or immobilized commercial enzyme);  $V_c$ , volume of the aliquot removed from the reaction medium (mL).

## 2.4. Hexyl Laurate Synthesis

The new biocatalysts were applied in the synthesis of hexyl laurate (Scheme 1) through the esterification of lauric acid n-hexanol in n-hexane. The amount of each biocatalyst was adjusted to 100 U based on the data obtained from the hydrolysis assay. Solutions (10 mL) of lauric acid in n-hexanol/heptane (1:1, 0.1 M) were added to 25 mL conical tubes in the presence of the biocatalysts and stirred under for 2 h at 200 rpm and 40 °C in shaker.



**Scheme 1.** General reaction conditions for lipase-catalyzed production of Hexyl laurate.

Aliquots of 20  $\mu$ L were collected from 5 to 120 min to study the time course reaction. All analyses were done in triplicate. The samples were derivatized with 20  $\mu$ L of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and graded to 1.0 mL of heptane. Subsequently, the samples were analyzed in a gas chromatography unit equipped with a mass spectrometry detector (GC-MS). Analyses were performed in a GC-MS system (Shimadzu CG2010, DB 5 capillary column). Samples were prepared by dissolving 10  $\mu$ L of the final product in 980  $\mu$ L of heptane and 10  $\mu$ L of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA). The injector and detector temperatures were 250 °C, and the oven temperature was constant at 60 °C for 1 min and then increased by 10 °C per min to 250 °C, when it was held constant for 3 min.

## 2.5. FT-IR Analysis

Confirmation of hexyl laurate in the best reaction condition was also qualitatively determined by Fourier transform infrared spectroscopy (FTIR), on a Thermo-Nicolet Magna® spectrophotometer (IR 760), in the 400 - 4000 cm<sup>-1</sup> spectral range. The experiments consisted of 200 readings with a resolution of 2 cm<sup>-1</sup>.

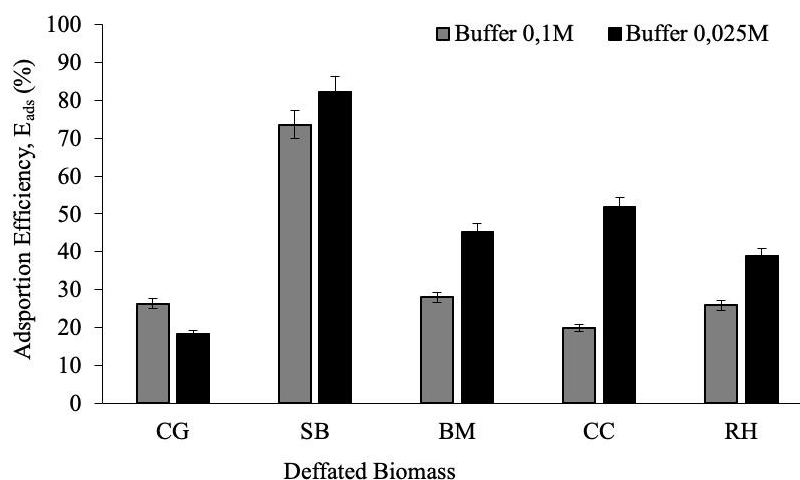
## 2.6. Support Desorption

The new biocatalysts were subjected to protein desorption assays according to the methodology previously described [38,39]: 1 g of immobilized biocatalyst was poured into 50 mL of 25 mM phosphate buffer, pH 7.0, containing 2.5% (v/v) of Triton X-100 or NaCl 0.8 % (w/v). The supports immersed in the desorption solution were kept under constant stirring at 100 rpm for 4h at room temperature. At the end of this period, the particles were separated from the supernatant (vacuum filtered), the supernatant being subjected to protein quantification assay, and the particulate material applied in the hexyl laurate esterification reaction.

### 3. Results and Discussion

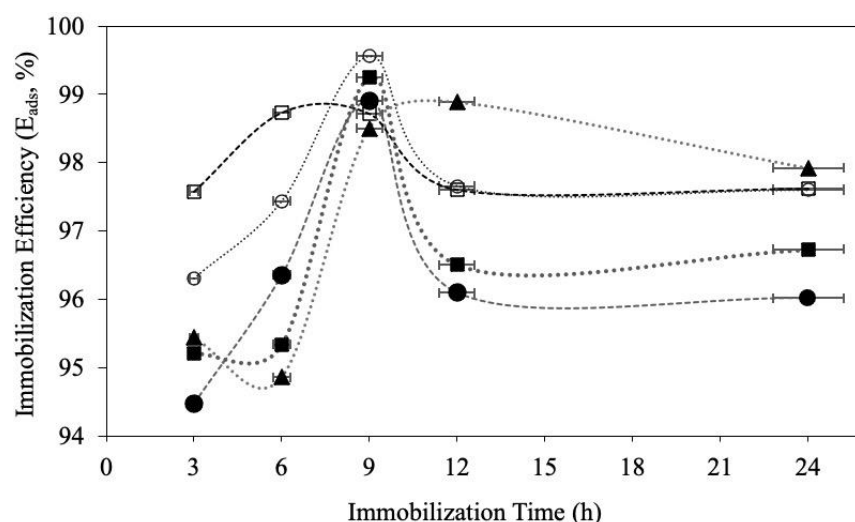
#### 3.1. Investigation of TLL adsorption as a function of ionic strength and time

For the construction of a robust biocatalyst, the selection of the appropriate immobilization method is a crucial step, and it is related to the knowledge of the properties both of enzyme and supports [40]. In this work we proposed a TLL immobilization strategy based on the adsorption on the surface of the previously dehydrated residual biomass from Maranhão, avoiding further functionalization or derivatization steps. Initially, the influence of the ionic strength of sodium phosphate buffer pH 7.0 in two concentrations was investigated: 0.025 M (low ionic strength) and 0.1 M (high ionic strength) on the adsorption efficiency ( $E_{ads}$ ) of TLL in the different supports, with results are shown in Figure 2.



**Figure 2.** Adsorption efficiency ( $E_{ads}$ ) of TLL on CG, SC, BM, CC and RH supports in function of low (■) and high (▒) ionic strengths. Immobilization conditions: protein offered: 5 mg/g of support; 25 °C for 4 h and 4 °C for 24 h; pH 7.0; Ionic strength: 0.025 and 0.1 M.

In general, the highest  $E_{ads}$  were found in the lowest ionic strength, where the best value was obtained for the SB derivative with 82.24% in 0.025 M and 72.54% in 0.1 M phosphate buffer. At low ionic strengths, lipase is solvated with the lowest ion charge possible, enabling greater hydrophobic interaction with the support, without protein aggregation [41], since at neutral pH, the amino acids of TLL surfaces are mostly at non-ionized form [42,43]. In order to observe the effects of immobilization at low ionic strength over time, all deffated biomasses were subjected to a 24h kinetic test, in order to find the shortest immobilization time (Figure 3).



**Figure 3.** Kinetic profile of immobilization efficiency ( $E_{ads}$ , %) of TLL on supports CG-TLL (●), SC-TLL (□), BM-TLL (▲), CC-TLL (○) e RH-TLL (■) as a function of time. Immobilization conditions: Protein load offered: 5 mg / g of support; Temperature: 25 °C for 4 h and 4 °C for 3, 6, 9, 12, 24 h; pH 7.0; Ionic strength: 0.025 M.

As a result, a saturation of TLL immobilization in 9h could be observed by all supports, with  $E_{ads} > 98\%$ . This immobilization time is much lower than that reported by [44] when immobilizing lipase of *Candida rugosa* by adsorption, where 28h of immobilization were necessary. This contact time was also reported by [45], when immobilizing the lipase of *Burkholderia cepacia* on pretreated corn cob. Such results show a high affinity between enzyme and support, and therefore 9h of immobilization and 0.025M of ionic strength were adopted for subsequent studies.

### 3.2. Selection of Biocatalyst

In order to choose the best immobilized biocatalyst for hexyl laurate synthesis, the activities of hydrolysis and esterification in addition to the enzyme activation index in the final derivatives were determined (table 1).

**Table 1.** Catalytic properties of the new home-made immobilized biocatalysts containing TLL.

Catalytic Properties					
Biocatalyst	$E_{ads}$ (%) <sup>a</sup>	Hydrolytic Activity (U.g <sup>-1</sup> )	Esterification Activity (U.g <sup>-1</sup> )	$N_{act}$ <sup>b</sup> (%)	Standard Error (%)
CG-TLL	98	1,715	N.D	108	0.09
SC-TLL	98.6	700	N.D	44	0.03
BM-TLL	99	5,331	141.4	335	0.07
CC-TLL	98.4	618	26	39	0.12
RH-TLL	99	4,608	396	290	0.15
TL IM <sup>®</sup>	-	142.3	113.5	-	0.09

Immobilization conditions: 5 mg of protein per g of support; 25 °C for 4 h and 4 °C for 9 h; Ionic strength: 0.025 M pH 7.0. <sup>a</sup>adsorption efficiency, <sup>b</sup>Lipase activation index ( $N_{act}$ ). N.D – Not detected.

As a result, the immobilized biocatalysts BM-TLL, RH-TLL and CG-TLL presented the most promising hydrolytic activities, where  $5,331 \text{ U g}^{-1}$  was obtained by BM-TLL, which corresponds to an activation index of 335% of the TLL taking into account the initial activity offered of free TLL before the immobilization process ( $318.5 \text{ U. g}^{-1}$ ). For RH-TLL and CG-TLL, high activation rates were also obtained, being 290 and 108% respectively. It is important to note that all hydrolytic activities found were significantly superior to those found by the commercial biocatalyst TL IM ( $142.3 \text{ U. g}^{-1}$ ), which reveals promising results.

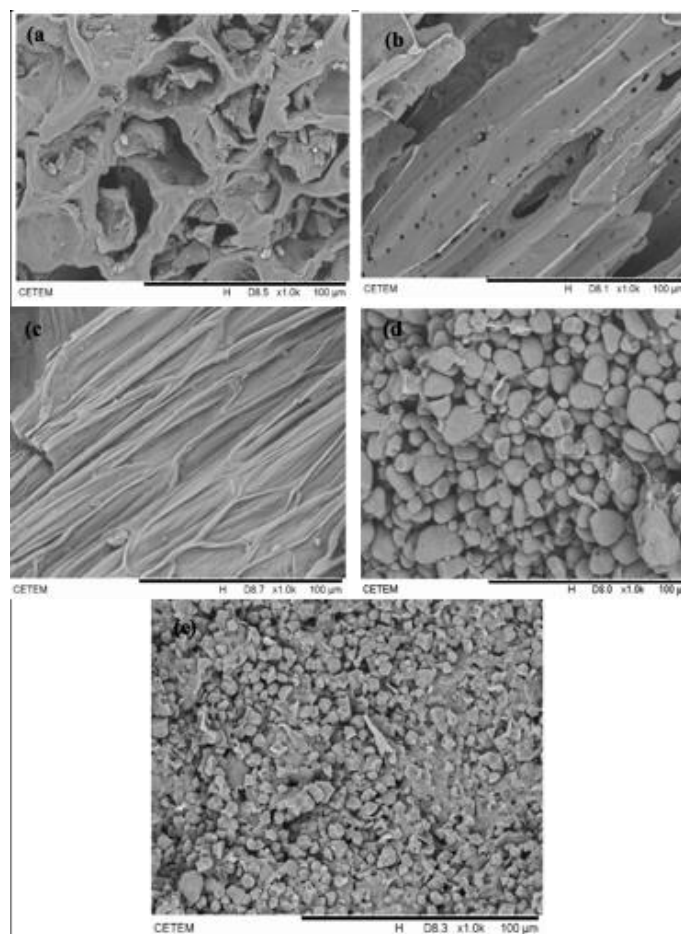
The high results of hydrolytic activity obtained by the new derivatives can be justified by a possible hyperactivation of TLL in the presence of hydrophobic surfaces of the support [46, 47]. In the absence of hydrophobic surfaces or emulsions, the TLL has its conformational balance shifted to the closed and inactive form, where a hydrophobic lid containing an alpha-helical polypeptide chain covers the active site, hindering its catalytic activity [48]. However, in the presence of hydrophobic interfaces, such as the residual biomasses applied in this work, the open conformation of the lid is favored and stabilized by several hydrophobic interactions of its outer face with the support during the adsorption process, generating distortions and exposure of the catalytic site, increasing intensively the catalytic activity [49, 50]. Our results were superior of those obtained by [51-53] whose previously reported the immobilization and stabilization of TLL in several different organic supports.

The esterification activity of the new biocatalysts was also investigated through ethyl oleate synthesis (Table 1). Surprisingly, the biocatalysts CG-TLL and SC-TLL did not show any esterification activity. Although they have shown high hydrolysis activity, as porous supports, other interactions between enzyme and biomass may have caused diffusion limitations, where the enzyme can be stocked in compartments of difficult access, limiting the substrate access [54]. In addition, due to the fact that TLL requires interfacial activation, this phenomenon is disturbed in the presence of organic solvents, which makes the lipase structure more rigid and hinders its agility, resulting in lower esterification yields, as reported in previous studies by [48, 55]. On the other hand, BM-TLL, CC-TLL and RH-TLL derivatives showed esterification activities far superior to the commercial preparation TL IM ( $113.5 \text{ U. g}^{-1}$ ).

These promising results must also be explained taking into account the previously performed physical-chemical characterizations of defatted supports before immobilization. The degreasing process leads to the formation of new pores in the materials, forming new potential points of interaction and enzyme entrapment. In the CNHS elementary analysis, the lignocellulosic nature of the materials studied was confirmed (table 1 of SI), with are in accordance to those already reported in the literature [56,57]. The high amount of carbon demonstrated in addition to the percentage of lignin denoted a high hydrophobic surface, which together with the large number of pores in the materials, favored multiple interactions with the enzyme.

The consequences of the comminution and degreasing process can be seen in figure 4, which shows the SEM analysis performed with each defatted support, where it is possible to observe the irregular and porous nature of these materials, characteristic of lignocellulosic residues. For this reason, TLL probably was immobilized on these supports not only by adsorption to the surface, but also by confinement in the pores of these materials, characterizing multiple interactions.





**Figure 4.** Scanning electron microscopy of the supports (a) CG, (b) SC, (c) BM, (d) CC, (e) defatted RH, before the TLL immobilization process.

Due to be heterogeneous supports, other physical-chemical characteristics need to be analyzed. Table 2 shows the values found for hydrophobicity by the fixation test of the cane pink dye, in addition to the measurements of the surface area, volume and pore diameter of the defatted supports.

**Table 2.** Hydrophobicity and morphological characteristics of defatted biomasses used as supports for TLL immobilization.

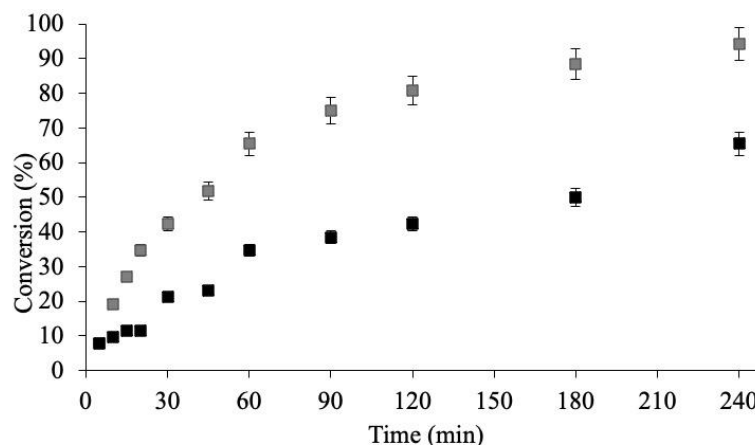
Support	Hydrophobicity ( $\mu\text{g} \cdot \text{g}^{-1}$ )	superficial Area ( $\text{m}^2 \cdot \text{g}^{-1}$ )	Porous Volume ( $\text{cm}^3 \cdot \text{g}^{-1}$ )	Porous Diameter ( $\text{\AA}$ )	Standart Error (%)
CG	12,03	0.30	$2.72 \times 10^{-4}$	30.2	0.003
SC	2600	0.42	$1.22 \times 10^{-4}$	11.7	0.005
BM	1298	0.35	$3.16 \times 10^{-4}$	36.1	0.002
CC	14,782	0.79	$3.82 \times 10^{-4}$	19.3	0.006
RH	724	1.42	$23.21 \times 10^{-4}$	65.4	0.002

Support hydrophobicity is an important property to be considered when immobilizing TLL, once interfacial activation is necessary due to the presence of a hydrophobic lid. In these cases, immobilization by surface adsorption is an interesting strategy to immobilize

the lipase in its open conformation [46,47,58]. According to table 2, the RH support showed the lowest hydrophobicity ( $724 \mu\text{g}\cdot\text{g}^{-1}$ ), followed by BM, SG, CC and CG. However, in heterogeneous supports, the interaction with the enzyme is not only carried out in a hydrophobic way, since the greatest hydrolysis activity was found by the biocatalyst BM-TLL ( $5331 \text{ U}\cdot\text{g}^{-1}$ , table 1), whose support presented the second lowest hydrophobicity (table 2). In addition to hydrophobicity, the surface area, volume and the pore diameter are characteristics of particular importance on lipase immobilization. The surface area of the studied supports obtained by the BET method varied from 0.2 to  $1.4 \text{ m}^2\cdot\text{g}^{-1}$ . This parameter reveals the actual specific surface area available for the interaction with the enzyme [59]. In this sense RH support proved to be quite interesting, as its surface area ( $1.42 \text{ m}^2\cdot\text{g}^{-1}$ ) was approximately seven times greater than the value found for SC ( $0.30 \text{ m}^2\cdot\text{g}^{-1}$ ), and twice as much the surface area of the CC support ( $0.79 \text{ m}^2\cdot\text{g}^{-1}$ ). Qi et al. (2015) [60] when applied defatted and chemically modified rice bran as supports in the immobilization of lipases, observed values much lower than those reported in this work, obtaining  $0.68 \text{ m}^2\cdot\text{g}^{-1}$  and  $0.67 \text{ m}^2\cdot\text{g}^{-1}$  respectively. In addition, RH was the support with the largest pore volume ( $23.21 \times 10^{-4} \text{ cm}^3\cdot\text{g}^{-1}$ ). The pore volume of a support can influence the immobilization process, since it can limit the amount of enzyme that can be lodged inside it or direct the protein adsorption for only on the surface [61]. Regarding the pore diameter, the values obtained ranged from 11.6 to  $65.38 \text{ \AA}$ , with the SC ( $11.670 \text{ \AA}$ ) and CC ( $19.318 \text{ \AA}$ ) supports classified as microporous and CG, BM and RH classified as mesoporous [62]. Porous supports can be responsible for hosting large amounts of protein, influencing catalytic properties of biocatalysts, such as immobilization yield, selectivity and specificity and molecular diffusion (substrates and/or products) in the biocatalyst microenvironment [26,63]. In addition, the TLL structure has been described as of spherical shape and diameter of  $35 \times 45 \times 50 \text{ \AA}$ , that is ideal for immobilization on supports containing pores within the range of mesopores (20 -  $500 \text{ \AA}$ ) [48]. Thus, the biocatalysts BM-TLL and RH-TLL, which presented the best results of hydrolysis and esterification activities, were able to form a set of important interactions that turn TLL more active and stable under the conditions studied, combining the hydrophobic and porous effects in the activation and enzyme entrapment. These were selected for the application stage in the synthesis of hexyl laurate.

### 3.4. Synthesis of hexyl laurate

In this step, the biocatalysts BM-TLL and RH-TLL were submitted to a kinetic study for the formation of hexyl laurate in n-hexane (Figure 8). As a result, the RH biocatalyst showed conversions of 65.3% in 4 h of reaction against 94.2% demonstrated by BM-TLL. When comparing these results to those obtained by [64] for the synthesis of hexyl laurate catalyzed by commercial TL IM, which reported a conversion of 92.2% using 45.5% (w/v) of commercial biocatalyst, the results presented in this work are promising as it was obtained higher conversions with only approximately 5mg of immobilized enzyme per g of support, 5% (w/v) of BM-TLL (table 1) in order to contemplate 100U of standardized activity.



**Figure 8:** Kinetics of hexyl laurate synthesis catalyzed by home-made biocatalysts BM-TLL (■) and RH-TLL (■). Reactional conditions: 5% (w/v) of biocatalyst, lauric acid and n-hexanol 1:1(100mM), at 40°C and 150 rpm. Conversions determined by titration methods and confirmation by GC-MS.

Analysis by GC-MS (Figure S1 of supporting information) and FT-IR spectra on KBR disk (Figure S2 of SI) confirmed high selectivity of BM-TLL for hexyl laurate formation as well as purity of starting materials.

### 3.5. Desorption of TLL by BM-TLL biocatalyst

In order to confirm the type of the predominant interaction in the BM-TLL biocatalyst, desorption tests with Triton X-100 and NaCl were performed. The results were compared with the commercial enzyme TL IM (table 4).

**Table 4** – Desorption results of BM-TLL and TL IM with Triton X-100 and NaCl.

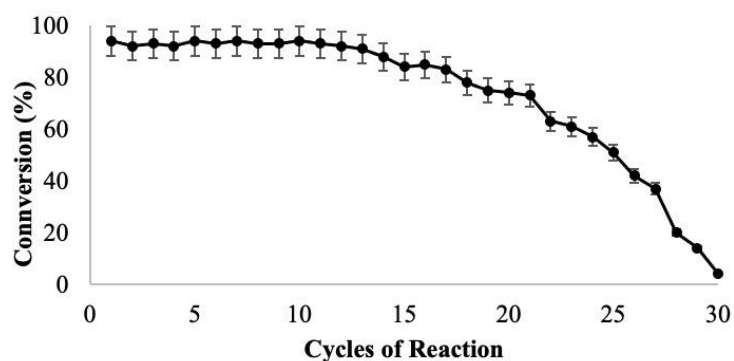
Biocatalyst	% of Dessorbed Protein	
	Triton X-100	NaCl
BM-TLL	99 (4.9mg)*	41(2mg)*
TL-IM	25 (5mg)*	88 (17,6%)*

Reactional conditions: 1g of biocatalyst on 50mL of phosphate buffer 0.025mM, pH 7.0 containing 2.5 % (v/v) of Triton X-100 or NaCl 0.8 M. \*Protein concentration determined by Bradford method on supernant.

Triton X-100 and NaCl were applied in order to break the hydrophobic and electrostatic interactions, respectively. As noted, almost all the protein present in the BM-TLL biocatalyst was adsorbed by Triton X-100, which competes with lipases for the hydrophobic regions of the support, causing desorption by molecular exclusion [58]. In addition, the amount of protein detected in the supernatant corroborates the data previously obtained on immobilization procedures. The commercial lipase TLIM presented the highest percentage of desorbed proteins when treated with NaCl, which reduces the negative charges present in the support, resulting in less ionic interaction between the lipase molecules and the support, corroborating an immobilization through ionic adsorption, differently interactions found in the BM-TLL biocatalyst. In addition, these studies demonstrated a quantity of proteins in TLIM that was much higher than that found by the home-made biocatalyst, demonstrating that the enzyme immobilized in the

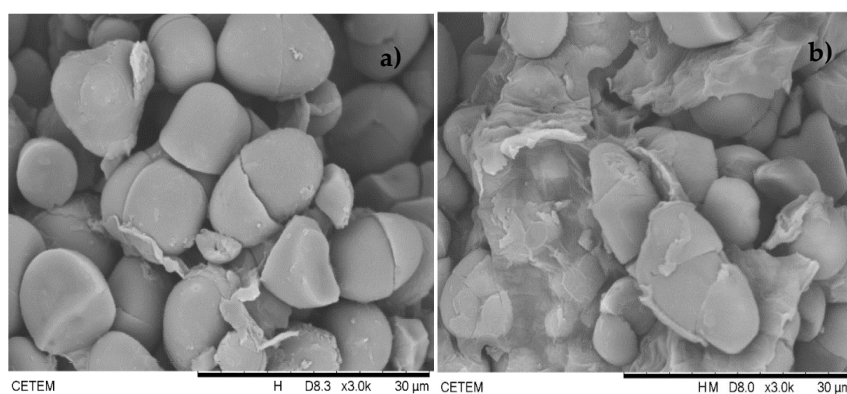
lignocellulosic residue did indeed present intense hyperactivation and stability, since it presented superior catalytic activities with lower amounts of protein.

The reusability of the new biocatalyst BM-TLL was tested in subsequent cycles of hexyl laurate synthesis (Figure 9), where after approximately 11 cycles, the enzyme continued to show the same conversion values.



**Figure 9.** Recycles of BM-TLL on hexyl laurate synthesis

Subsequent conversion drops were observed, where 50% conversion was detected after 25 recycles. These results may be a consequence of the escape of proteins into the reaction medium, as a result of the degradation of the support with the advance of the cycles of use of the biocatalyst. This effect can be highlighted on Figure 10 that shows the SEM analysis of the BM-TLL biocatalyst before (Fig 10 a) and after (10 b) the reaction cycles. It is possible to observe that after the recycles, the surface has undergone small changes, making it possible to more easily observe the presence of proteins, probably due to the escape effect. Even so, these results show high robustness and stability of home-made immobilized enzyme.



**Figure 10 – SEM analysis of BM-TLL before (10 a) and after (10 b) recycles.**

#### 4. Conclusions

In this work we demonstrated the application of 5 residual biomasses from Maranhão (Brazil) as supports for TLL immobilization as a way of valorization of these

wastes. The babaçu mesocarp (BM) offered the best physical-chemical characteristics for TLL hydrophobic adsorption and entrapment, resulting in a competitive home-made biocatalyst (BM-TLL) that showed higher hydrolytic and esterification activities than the commercial Lipozyme® TL IM with 4 times lower protein amounts. On hexyl laurate synthesis BM-TLL it was possible to acquire 94% of conversion in 4h of reaction and with high recyclability of the home-made biocatalyst, demonstrating to be a robust and renewable alternative to commercial biocatalyst.

**Supplementary Materials:** The following are available online at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), Figure S1: GC-MS analysis of hexyl laurate catalyzed by BT-TLL biocatalyst at 240 min; Figure S2: FTIR spectrum of hexyl laurate catalyst by BM-TLL; Table S1: Chemical composition of agro-industrial wastes studied.

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## References

1. Banerjee, A.; Dick G. R.; Yoshino T. and Kanan M.W. Carbon dioxide utilization via carbonate-promoted C–H carboxylation. *Nature*, **2016**, *531*, 215–219.
2. Wojcieszak, R.; Santarelli, F.; Paul, S.; Dumeignil, F.; Cavani, F. and Gonçalves, R.V. Recent developments in maleic acid synthesis from bio-based chemicals. *Sustainable Chem.Processes*, **2015**, *3*, 9.
3. Sarsaiya, S.; Jain, A.; Kumar, S.; Awasthi, S.K.; Duan, Y.; Awasthi, M.K.; Shi, J. Microbial dynamics for lignocellulosic waste bioconversion and its importance with modern circular economy, challenges and future perspectives. *Bioresource Technology*, **2019**, *291*, 121905.
4. Madhavan, A.; Sindhu, R.; Binod, P.; Sukumaran, R.K.; Pandey, A. Strategies for design of improved biocatalysts for industrial applications. *Bioresource Technology*, **2017**, *245 (B)*, 1304–1313
5. Sheldon, R. A.; Woodley, J. M. Role of Biocatalysis in Sustainable Chemistry. *Chemical Reviews*, **2018**, *118 (2)*, 801–838.
6. Jiang, L-Q.; Fang, Z.; Zhao, Z-L.; Zheng, A-Q.; Wang, X-B.; Li, H-B. Levoglucosan and its hydrolysates via fast pyrolysis of lignocellulose for microbial biofuels: A state-of-the-art review. *Renewable Sustainable Energy Rev.*, **2019**, *105*, 215–229.
7. Rover, M. R., Aui A., Wright, M. M., Smith R.G. and Brown, R.C. Production and purification of crystallized levoglucosan from pyrolysis of lignocellulosic biomass. *Green Chem.*, **2019**, *21*, 5980–5989.
8. Climent, M. J.; Corma A.; and Iborra, S. Conversion of biomass platform molecules into fuel additives and liquid hydrocarbon fuels. *Green Chem.*, **2014**, *16*, 516–547.
9. Wang, Z.; Shen, D.; Wu, C.; and Gu, S. State-of-the-art on the production and application of carbon nanomaterials from biomass. *Green Chem.*, **2018**, *20*, 5031–5057

10. Itabaiana Jr, I., Nascimento, M. A., de Souza, R.O. M.A., Dufourd, A. and Wojcieszak, R. Levoglucosan: a promising platform molecule? *Green Chem.*, **2020**, *22*, 5859-5880
11. CONAB. Acomp. safra bras. grãos, v. 6 - Safra 2018/19 - Décimo segundo levantamento. **Companhia Nacional de Abastecimento**, v. 6, n. 12, p. 1-126, 2019.
12. Galford, G. L.; Peña, O.; Sullivan, A.K.; Nash, J.; Gurwick, N.; Pirolli, G.; Richards, M.; White, J.; Wollenberg, E. Agricultural development addresses food loss and waste while reducing greenhouse gas emissions. *Science of The Total Environment*, **2020**, *699*, 134318.
13. Singh, A. Managing the uncertainty problems of municipal solid waste disposal. *Journal of Environmental Management*, **2018**, *240*, 259-265
14. Goldemberg, J.; Coelho, S. T. Renewable energy – traditional biomass vs. modern biomass. *Energy Policy*, **2004**, *32*, 711-714.
15. BILAL, M.; IQBAL, H.M.N. Naturally-derived biopolymers: Potential platforms for enzyme immobilization. *International Journal of Biological Macromolecules*, **2019**, *130*, 462-482.
16. Ravindran, R.; Hassan, S.S.; Williams, G.A.; Jaiswal, A.K. A Review on Bioconversion of Agro-Industrial Wastes to Industrially Important Enzymes. *Bioengineering* **2018**, *5*, 93.
17. Patel N., Rai, D., Shivam, Shahane S, Mishra U. Lipases: Sources, Production, Purification, and Applications. *Recent Pat Biotechnol.* **2019**, *13(1)*, 45-56.
18. Guerrand, D. Lipases industrial applications: focus on food and agroindustries. *OCL*, **2017**, *24 (4)*, D403.
19. Avelar do Nascimento, M.; Ester Gotardo, L.; Miguez Bastos, E.; C. L. Almeida, F.; A. C. Leão, R.; O. M. A. de Souza, R.; Wojcieszak, R.; Itabaiana, I., Jr. Regioselective Acylation of Levoglucosan Catalyzed by *Candida antarctica* (CaLB) Lipase Immobilized on Epoxy Resin. *Sustainability* **2019**, *11*, 6044.
20. Nascimento, M.A.; Gotardo, L.E.; Leão, R.A.C.; De Castro, A.M.; De Souza, R.O.M.A. and Itabaiana, I. Enhanced Productivity in Glycerol Carbonate Synthesis under Continuous Flow Conditions: Combination of Immobilized Lipases from Porcine Pancreas and *Candida antarctica* (CALB) on Epoxy Resins. *ACS Omega*, **2019**, *4*, 860-869.
21. Ferraz C. A.; do Nascimento M. A.; Almeida R.F.O.; Sergio, G.G.; Junior A.A.T., Dalmônico G., Caraballo R.; Finotelli, P.V., Leão, R.A.C., Wojcieszak, R., de Souza, R.O.M.A. and Itabaiana Jr, I. Synthesis and characterization of a magnetic hybrid catalyst containing lipase and palladium and its application on the dynamic kinetic resolution of amines. *Molecular Catalysis*, **2020**, *493*, 111106.
22. JAEGER, K.-E.; DIJKSTRA, B.W.; REETZ, M.T. Bacterial Biocatalysts: Molecular Biology, Three-Dimensional Structures, and Biotechnological Applications of Lipases. *Annual Review of Microbiology*, **1999**, *53 (1)*, v. 53, 315-351.
23. Sarmah N, Revathi D, Sheelu G, Yamuna Rani K, Sridhar S, Mehtab V, Sumana C. Recent advances on sources and industrial applications of lipases. *Biotechnol Prog.* **2018**, *34(1)*, 5-28.
24. Carvalho, T.; Pereira A.S.; Bonomo, R.C.F.; Franco, M.; Finotelli, P.V.; Amaral, P.F.F. Simple physical adsorption technique to immobilize *Yarrowia lipolytica* lipase purified by different methods on magnetic nanoparticles: Adsorption isotherms and thermodynamic approach. *International Journal of Biological Macromolecules*, **2020**, *160*, 889-902.
25. GUTARRA, M.L.E.; MIRANDA, L.S.M.; DE SOUZA, R.O.M.A. *Enzyme Immobilization for Organic Synthesis*. **2016**. Organic Sy ed. [s.l.] Elsevier Inc..
26. GIRELLI, A.M.; ASTOLFI, M.L.; SCUTO, F.R. Agro-industrial wastes as potential carriers for enzyme immobilization: A review. *Chemosphere*, **2020**, *244*, 1-12.
27. Bilal, M.; Asgher, M.; Iqbal, H.M.N, Hu, H.; Zhang, X. Biotransformation of lignocellulosic materials into value-added products – A review. *International Journal of Biological Macromolecules*, **2017**, *98*, 447-458.
28. Brígida A.L., Pinheiro A.D., Ferreira A.L., Gonçalves, L.R. Immobilization of *Candida antarctica* lipase B by adsorption to green coconut fiber. *Appl Biochem Biotechnol.* **2008**, *146(1-3)*, 173-87.
29. PEREIRA, A. DA S.; FONTES-SANT'ANA, G. C.; AMARAL, P. F. F. Mango agro-industrial wastes for lipase production from *Yarrowia lipolytica* and the potential of the fermented solid as a biocatalyst. *Food and Bioproducts Processing*, **2019**, *115*, 68-77, 2019.
30. BRUM, A.A.S.; ARRUDA, L.F.DE; REGITANO-D'ARCE, M. A. B. Métodos de extração e qualidade da fração lipídica de matérias-primas de origem vegetal e animal. *Química Nova*, **2009**, *32 (4)*, 849-854.
31. IUPAC. Standard methods for the analysis of oils, fats and derivatives. 6ª Ed. ed. Pergamon Press Oxford: 1979.
32. Soares, C.M.F., De Castro, H.F., De Moraes, F.F. et al. Characterization and utilization of *Candida rugosa* lipase immobilized on controlled pore silica. *Appl Biochem Biotechnol*, **1999**, *79*, 745-757.
33. Okura N.S., Sabi G.J., Crivellenti M.C., Gomes R.A.B., Fernandez-Lafuente R., Mendes A.A. Improved immobilization of lipase from *Thermomyces lanuginosus* on a new chitosan-based heterofunctional support: Mixed ion exchange plus hydrophobic interactions. *Int J Biol Macromol*, **2020**, *163*, 550-561.
34. BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **1976**, *72*, (1-2), 248-254.
35. Bernardes, O.L., Bevilaqua, J.V., Leal, M.C.M.R. et al. Biodiesel fuel production by the transesterification reaction of soybean oil using immobilized lipase. *Appl Biochem Biotechnol*, **2007**, *137*, 105-114.
36. Silva M.F., Freire D.M., de Castro A.M., Di Luccio, M., Mazutti, M.A., Oliveira, J.V., Treichel, H., Oliveira, D. Production of multifunctional lipases by *Penicillium verrucosum* and *Penicillium brevicompactum* under solid state fermentation of babassu cake and castor meal. *Bioprocess Biosyst Eng.* **2011**, *34(2)*, 145-52.

37. Reinehr, C. O., Rizzardi, J., Silva, M. F., Oliveira, D., Treichel, H., Colla, L. M. Produção de lipases de *Aspergillus niger* e *Aspergillus fumigatus* através de fermentação em estado sólido, avaliação da especificidade do substrato e seu uso em reações de esterificação e alcoólise *Química Nova*, **2014**, 37(3), 454-460.
38. Cabrera Z., Fernández-Lorente G., Fernández-Lafuente R., Palomo J.M., Guisán J.M. Novozym 435 displays very different selectivity compared to lipase from *Candida antarctica* B adsorbed on other hydrophobic supports. *J. Mol. Catal. B: Enzym.* **2009**, 57, 171-176.
39. Costa, I.C. R., Leite, S. G. F., Leal, I. C. R., Miranda, L. S. M., & Souza, R. O. M. A. Thermal effect on the microwave assisted biodiesel synthesis catalyzed by lipases. *Journal of the Brazilian Chemical Society*, **2011**, 22(10), 1993-1998.
40. Mohamad, N. R., Marzuki, N. H., Buang, N. A., Huyop, F., & Wahab, R. A. An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. *Biotechnology, biotechnological equipment*, **2015**, 29(2), 205-220.
41. Mogensen, J. E.; Sehgal, P.; Otzen, D. E. Activation, inhibition, and destabilization of *Thermomyces lanuginosus* lipase by detergents. *Biochemistry*, **2005**, 44 (5), 1719-1730.
42. Matte, C.R., Bordinhão, C., Poppe, J. K., Benvenuti, E. V., Costa, T.M.H., Rodrigues, R.C., Hertz, P. F., & Ayub, M.A.Z. Physical-Chemical Properties of the Support Immobead 150 Before and After the Immobilization Process of Lipase. *Journal of the Brazilian Chemical Society*, **2017**, 28(8), 1430-1439.
43. Manoel, E. A., dos Santos, J.C.S.; Freire, D.M.G.; Rueda, N.; Fernandez-Lafuente, R. Immobilization of lipases on hydrophobic supports involves the open form of the enzyme. *Enzyme and Microbial Technology*, **2015**, 71, 53-57.
44. Soares, C.M., De Castro, H.F., De Moraes, F.F., Zanin, G.M. Characterization and utilization of *Candida rugosa* lipase immobilized on controlled pore silica. *Appl Biochem Biotechnol.* **1999**, (77-79), 745-57.
45. Costa, D.M.; Castro, R.S.S.; Padilha, R.Y.C.; Ruzene, D.S.; Silva, D.P.; Lima, A.S.; Soares, C.M.F. Aplicação De Sabugo De Milho Como Suporte Para Imobilização De Lipase. XX Congresso Brasileiro de Engenharia Química, *Blucher Chemical Engineering Proceedings*, **2015**, 1, 2532-2539.
46. Moreno-Perez, S.; Ghattas, N.; Filice, M.; Guisan, J. M.; Fernandez-Lorente, G. Dramatic hyperactivation of lipase of *Thermomyces lanuginosa* by a cationic surfactant: Fixation of the hyperactivated form by adsorption on sulfopropyl-sepharose. *Journal of Molecular Catalysis B: Enzymatic*, **2015**, 122, 199-203.
47. Zaak, H.; Siar, E-H.; Kornecki, J.F.; Fernandez-Lopez, L.; Pedrero, S.G.; Virgen-Ortiz, J.J.; Fernandez-Lafuente, R. Effect of immobilization rate and enzyme crowding on enzyme stability under different conditions . The case of lipase from *Thermomyces lanuginosus* immobilized on octyl agarose beads. *Process Biochemistry*, **2017**, 56, 117-123.
48. Fernandez-Lafuente, R. Lipase from *Thermomyces lanuginosus*: uses and prospects as an industrial biocatalyst. *Journal of Molecular Catalysis B: Enzymatic*, **2010**, 62, 197-212.
49. Mateo, C.; Palomo, J.M.; Fernandez-Lorente, G.; Guisan, J.M.; Fernandez-Lafuente, R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and Microbial Technology*, **2007**, 40(6), 1451-1463.
50. Schmid, R. D.; Verger, R. Lipases: Interfacial Enzymes with Attractive Applications. *Angewandte Chemie International Edition*, **1998**, 37 (12), 1608-1633.
51. MENDES, A.A.; CASTRO, H.F. DE; GIORDANO, R.D.L.C. Triagem de suportes orgânicos e protocolos de ativação na imobilização e estabilização de lipase de *Thermomyces lanuginosus*. *Química Nova*, **2013**, 36, (2), 245-251.
52. Miranda, J.S.; Silva, N.C.A.; Bassi, J.J.; Corradini, M.C.C.; Lage, F.A.P.; Hirata, D.B.; Mendes, A.A. Immobilization of *Thermomyces lanuginosus* lipase on mesoporous poly-hydroxybutyrate particles and application in alkyl esters synthesis: Isotherm, thermodynamic and mass transfer studies. *Chemical Engineering Journal journal*, **2014**, 251, 392-403.
53. Vescovi, V.; Giordano, R.L.; Mendes, A.A.; Tardioli, P.W. Immobilized Lipases on Functionalized Silica Particles as Potential Biocatalysts for the Synthesis of Fructose Oleate in an Organic Solvent/Water System. *Molecules*, **2017**, 22(2), 212.
54. Salis A., Svensson I., Monduzzi M., Solinas V., Adlercreutz P. The atypical lipase B from *Candida antarctica* is better adapted for organic media than the typical lipase from *Thermomyces lanuginosa*. *Biochim Biophys Acta*. **2003**, 1646(1-2), 145-51.
55. Stergiou, P-Y.; Foukis, A.; Filippou, M.; Koukouritaki, M.; Parapouli, M.; Theodorou, L.G.; Hatziloukas, E.; Afendra, A.; Pandey, A.; Papamichael, E.M. Advances in lipase-catalyzed esterification reactions. *Biotechnology Advances*, **2013**, 31(8), 1846-1859.
56. Obruca, S.; Benesova, P.; Kucera, D.; Petrik, S.; Marova. I. Biotechnological conversion of spent coffee grounds into polyhydroxyalkanoates and carotenoids. *New Biotechnology*, **2015**, 32 (6), 569-574.
57. Prasad, S.; Singh, A.; Joshi, H. C. Ethanol as an alternative fuel from agricultural, industrial and urban residues. *Resources, Conservation and Recycling*, **2007**, 50(1), 1-39.
58. Palomo, J.M., Guisan, J.M. Different strategies for hyperactivation of lipase biocatalysts. *Methods Mol Biol.* **2012** (861), 329-41.
59. Li, Y., Gao, F.; Wei, W.; Qu, J-B.; Ma, G-H.; Zhou, W-Q. Pore size of macroporous polystyrene microspheres affects lipase immobilization. *Journal of Molecular Catalysis B: Enzymatic*, **2010**, 66 (1-2), 182-189.
60. Qi, J.; Li, Y.; Yokoyama, W.; Majeed, H.; Masamba, K.G.; Zhong, F.; Ma, J. Cellulosic fraction of rice bran fibre alters the conformation and inhibits the activity of porcine pancreatic lipase. *Journal of Functional Foods*, **2015**, 19 (Part A), 39-48.
61. Lima L.N., Oliveira, G.C., Rojas, M.J., Castro, H.F., Da Rós, P.C., Mendes, A.A., Giordano, R.L., Tardioli, P.W. Immobilization of *Pseudomonas fluorescens* lipase on hydrophobic supports and application in biodiesel synthesis by transesterification of vegetable oils in solvent-free systems. *J Ind Microbiol Biotechnol.* **2015**, 42(4), 523-35.

62. Thommes, M., Kaneko, K., Neimark, A., Olivier, J., Rodriguez-Reinoso, F., Rouquerol, J. & Sing, K.. Physisorption of gases, with special reference to the evaluation of surface area and pore size distribution (IUPAC Technical Report). *Pure and Applied Chemistry*, **2015**, 87(9-10), 1051-1069.
63. Alves, M,D.; Aracri, F.M.; Cren, E.C.; Adriano A. Mendes Isotherm, kinetic, mechanism and thermodynamic studies of adsorption of a microbial lipase on a mesoporous and hydrophobic resin. *Chemical Engineering Journal*, **2017**, 311, 1–12.
64. Chang, S-W.; Shaw, J-F.; Yang, K-H.; Shih, I-L; Hsieh, C-H.; and Shieh, C-J. Optimal lipase-catalyzed formation of hexyl laurate. *Green Chemistry*, **2005**, 7 (7), 547–551.