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Covalent Immobilization of Lipase in Residual Yerba Mate Stick (*Ilex paraguariensis* A. St.-Hil.)

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Abstract: The objective of this study was to immobilize Eversa® Transform 2.0 lipase on residual yerba mate stick. The stick went through an alkaline pre-treatment and different activation treatments (APTS/glutaraldehyde and sodium metaperiodate). Immobilization was performed using hexane solvent and ammonium nitrate buffer. Support characterization, esterification activity, immobilized enzyme characterization, and operational stability were performed. Characterization by SEM demonstrated that the activation treatments were efficient. The immobilization of lipase on APTS/glutaraldehyde activated support showed a yield of 225.52 % and metaperiodate 162.76 %, using hexane as solvent. Good operational stability of the immobilized lipase was observed both in support activated with APTS / glutaraldehyde (8 recycles) and in support activated with metaperiodate (5 recycles), maintaining the activity of 65.62% and 52.00% in concern to the activity initial, respectively. The optimal reaction temperature was 40 ºC for the free and immobilized enzyme. K_m and $V_{máx}$ values were 16.55 μmol.g^{-1} and 5555.56 μmol.g^{-1}.min^{-1} for free enzyme; 33.52 μmol.g^{-1} and 4761.9 μmol.g^{-1}.min^{-1} for immobilized enzyme, respectively. The parameters of thermal inactivation confirmed a better thermostability of the lipase in free form.

Keywords: alkaline pre-treatment; APTS; glutaraldehyde; organic support; sodium metaperiodate.

1. Introduction

Biocatalysis represents a crucial strategy in sustainable processes, where enzymatic synthesis is typical in several industrial processes. Due to the wide versatility of reactions that catalyze and their specificity in relation to the substrate, lipases (EC 3.1.1.3) are the most used enzymes in biocatalytic processes, being produced by several organisms, such as animals plants, fungi, and bacteria [1-3].

Lipases are enzymes that catalyze a series of reactions such as esterification, hydrolysis of triacylglycerols, transesterifications, and interesterification of oils and lipids. They present essential industrial applications, such as the food industry (for flavor modification), in pharmaceutical products (cosmetics and digestion of fats), elaboration of fine chemicals (synthesis of esters), waste treatment, detergents, among others [4].

Most commercial lipases are single-use and difficult to recover when used in a homogeneous environment, resulting in high operating costs. As an alternative to overcome

this limitation, enzymatic immobilization stands out. An incorporating enzyme in solid support facilitates recyclability and allowing an increase in activity, specificity, and selectivity of the biocatalyst [5-8].

The enzyme immobilization can generally be carried out by three different methods: covalent bonding on given support, adsorption of enzyme molecules on a support material, and entrapment or encapsulation of the enzyme in polymers [9]. Among these, the methodologies based on the use of covalent bonds stand out, where there is a strong interaction between the enzyme and the support providing immobilized derivatives with greater stability in relation to the leaching of the enzyme from the support [10].

Choosing the immobilization technique and support is an important step. The support must have thermal and microbiological resistance and characteristics compatible with the enzyme to be immobilized [11]. The use of natural supports can be a good alternative, with the use of agro-industrial residues being valid, which present significant economic benefits and reduce disposal problems. Other characteristics of these supports are the large surface area and high porosity that allow the use of high enzymatic loads and the protection of the reaction medium's catalytic site [2].

The yerba mate industry (*Ilex paraguariensis* A. St.-Hil.) has stuck as an agro-industrial residue. According to the Brazilian Institute of Geography and Statistics (IBGE), Brazil produced around 346 thousand tons of this product in 2016 and many residual sticks, which correspond to approximately 2 % of mass production. These sticks are composed of 34.85 % α-cellulose, 24.77 % hemicellulose, 25.78 % lignin, 10.11 % extractives, and 4.49 % ash. These factors emphasize the need for alternative uses for this waste to give it a more dignified destination [12].

Due to the lignocellulosic characteristics of yerba mate that make it resistant to biological and chemical treatments, a pre-treatment capable of destroying lignin and hemicellulose is necessary, allowing better accessibility cellulose. In this case, the use of alkaline pre-treatment has stood out as a favorite for its selectivity in removing lignin and solubilizing carbohydrates, in addition to being able to be carried out in milder conditions than acid pre-treatments [13].

In this context, the present study aimed to evaluate the residual sticks generated from the processing of yerba mate (*Ilex paraguariensis* A. St.-Hil.) as a support for the immobilization of the Eversa® Transform 2.0 lipase. In response to the process's efficiency, the immobilization yield and the inactivation parameters of the free and immobilized enzyme were evaluated. Although the literature presents a wide variety of studies with enzymatic immobilization on organic supports, no work has been found using the yerba mate stick as a support, making this research innovative.

2. Materials and Methods

2.1. Enzyme.

Eversa® Transform 2.0 lipase (Novozyme) was donated by an oil company located in the north of the state of Rio Grande do Sul.

2.2. Yerba mate stick (Ilex paraguariensis A. St.-Hil.).

The residual yerba mate stick (*Ilex paraguariensis* A. St.-Hil.) was donated by the herb company Barão Erva Mate e Chás, located in the north of the state of Rio Grande do Sul.

2.3. Support functionality.

2.3.1. Pre-treatment of the support with NaOH.

The pre-treatment of the residual yerba mate stick (*Ilex paraguariensis*) followed the methodology described by Santos *et al*. [14], with modifications. The process was carried out with 5 g of the stick holder in a conical flask containing 250 mL of a 0.5 M NaOH solution (Vetec). The system was kept under constant agitation (150 rpm) using a magnetic stirrer (Fisatom - Mod. 752A) for 24 h at room temperature (25 ºC). Subsequently, the support was washed with distilled water until neutral pH, filtered, and dried in an oven (Crhomos) at 100 ºC until reaching constant mass.

2.3.2. Silanization and activation of support with APTS/glutaraldehyde.

The support was silanized according to the methodology described by Queiroz *et al*. [15], with modifications, using 12 mL of a solution of γ – aminopropyltriethoxylan (APTS) (Sigma-Aldrich, 98 %) 0.5 % (v/v) in heptane (Sigma-Aldrich) and 1 g of a residual stick of yerba mate treated with 0.5 M NaOH. The system was kept under constant agitation (40 rpm) at 75°C for 3 h. Subsequently, the system was filtered. The support was washed with 3 aliquots (10 mL) of hexane (P.A. - Química Moderna) to remove excess APTS. The washed stick was kept in an oven (Crhomos) at 100 °C for 15 h for complete drying.

Then, the silanized stick was activated with 10 mL of a 2.5 % (v/v) aqueous glutaraldehyde solution (Vetec, 25 %). The mixture was kept for 1 h at room temperature (25 °C). Afterward, it was filtered, washed 3 times with 10 mL of distilled water, and dried in an oven at 100 ºC for 1 h to remove excess water.

2.3.3. Activation of support with sodium metaperiodate.

Activation with sodium metaperiodate followed the methodology described by Coradi *et al*. [16], with modifications, where 1 g of the support treated with 0.5 M NaOH was activated with 10 mL of 0.1 M sodium metaperiodate. The system remained static, protected from light, for 48 h to form aldehyde groups. After it was filtered, washed with distilled water 3 times (100 mL each) and kept in an oven (Crhomos) at 100 °C for 1 h to remove moisture.

2.4. Support characterization-scanning electron microscopy (SEM).

SEM analyzes were carried out at the Integrated Regional University of Alto Uruguai e das Missões – URI Erechim, using a Zeiss microscope, model EVO LS25. To cover the surface of the sample with gold, a Quorum metallizer, SC 7620 was used. The micrographs were obtained at a voltage of 30 kV. The analyzed samples were the following: in natural support; support pretreated with NaOH and activated with APTS/glutaraldehyde; support pretreated with NaOH and activated with sodium metaperiodate.

2.5. Immobilization.

The immobilization of the lipase in the functionalized yerba mate sticks (APTS/glutaraldehyde or sodium metaperiodate) was performed according to the methodology proposed by Queiroz *et al*. [15], where 1 g of the functionalized stick was suspended in 10 mL of solvent (hexane or ammonium nitrate buffer pH 10 (NH_4NO_3)). The systems were kept under mechanical agitation, at room temperature, for 15 min. Then, 0.3 g of the enzyme was added,

which corresponds to 30 % (m/m) of the support mass, keeping the system under agitation (100 rpm) for 3 h at 25 °C for homogenization, followed by storage in a static condition, at 4 °C, for 24 h.

Subsequently, the support containing the immobilized enzyme was filtered and washed twice with 10 mL of solvent (hexane or ammonium nitrate buffer) to remove bound non-adsorbed enzymes. After vacuum filtration (MARTE - mod. 131), the immobilized derivative was kept in a desiccator for 24 h to remove excess moisture and stored in the refrigerator until the time of analysis.

2.6. Esterification activity.

The enzymatic activity was evaluated using the esterification method using the methodology described by Hildebrand *et al*. [17], with modifications, where lauric acid and ethanol were used as a substrate, with a 1:1 molar ratio, free of solvent. According to previous, the reaction was conducted using 0.3 g of the free enzyme and/or the immobilized derivative at 40 °C, 150 rpm, for 5 minutes the free enzyme, and 10 minutes the immobilized enzyme tests. Aliquots of 150 µL of the reaction medium were diluted in 2 mL of acetone-ethanol solution (1:1 v:v) in a conical flask and analyzed volumetrically using 0.1 M NaOH with the titrant and phenolphthalein as an indicator.

For white, the procedure was the same but was not added to an enzyme (free or immobilized derivative).

The activity of esterification of the yerba mate stick holder was carried out before the immobilization process to verify that there would be no interference in the activity measure.

A unit of esterification activity ($U.g^{-1}$ of free or immobilized enzyme) was defined as the amount of lauric acid (µmol) consumed per min, per enzyme mass (g), defined by Equation 1.

$$Ea = \frac{(V_{NaOH\ white} - V_{NaOH\ sample}) \cdot 0.1}{t.m} . 1000 \quad (1)$$

Where: Ea: esterification activity ($U.g^{-1}$); $V_{NaOH\ White}$: Volume of NaOH spent with the blank test (mL); $V_{NaOH\ sample}$: N_{aOH} volume spent after the esterification reaction of each sample (free or immobilized enzyme) (mL); t: Time (minutes); m: Mass of free enzyme or immobilized enzyme used (g).

The immobilization yield {Y (%)} was calculated according to equation 2.

$$Y(\%) = \frac{m_{(IE)} \cdot Ea_{(IE)}}{Ea_{(F)} \cdot m_{(E)}} . 100 \quad (2)$$

Where: Y (%)= Yield obtained from immobilization; $m_{(IE)}$ is the mass of the immobilized enzyme used for esterification, in g; $Ea_{(IE)}$ is the esterification activity ($U.g^{-1}$) of the immobilized enzyme; $Ea_{(F)}$ is the esterification activity ($U.g^{-1}$) of the free enzyme; $m_{(E)}$ is the mass of the free enzyme offered in immobilization, in g.

2.7. Operational stability (reuse).

The reuse of the lipase immobilized on a residual yerba mate stick was determined using 0.3 g of the immobilized derivative, in successive cycles, in batch, for the esterification of lauric acid/ethanol (1/1 (m/m)) at 40 ºC for 10 min. After each batch, the samples were removed from the reaction medium, filtered through a vacuum pump (MARTE - mod. 131), and added to a new lauric acid/ethanol solution. The activity of the first cycle was considered to be 100 %. The efficiency of reuse was calculated as presented in Equation 3.

$$ER\ (\%) = \frac{Lipolytic\ activity_{\ cycle\ n}}{Lipolytic\ activity_{\ cycle\ 1}} .100 \quad (3)$$

Where: ER (%) = efficiency of reuse; Lipolytic activity cycle n = lipolytic activity of each cycle; Lipolytic activity cycle 1 = initial lipolytic activity.

2.8. Characterization of the immobilized enzyme.

2.8.1. Reaction temperature evaluation.

To verify the reaction temperature effect, experiments were conducted with the free lipase and the derivative immobilized on the yerba mate stick. As a model reaction, the determination of esterification activity was used at 35, 40, 45, 50, 55 and 60 ºC, under the agitation of 150 rpm, for 5 min for the free enzyme and 10 min for the immobilized enzyme, as previous tests, because above 5 min of reaction the free enzyme starts to lose activity.

2.8.2. Determination of the kinetic parameters constant of affinity (K_m) and maximum reaction speed ($V_{máx}$).

In determining the maximum reaction velocity ($V_{máx}$) and affinity constant (K_m) of the free enzyme and the immobilized derivative, enzyme activity assays were conducted using different ethanol concentrations (0.668 to 3.9 M) in lauric acid/ethanol substrate. The initial reaction speed (V_0) was determined for each substrate concentration [S], setting the reaction time variables at 5 min for the free enzyme and 10 min for the immobilized enzyme and the temperature maintained at 40 ºC. The calculation of the kinetic parameters ($V_{máx}$ and K_m) was performed according to the Michaelis-Menten kinetic model, presented in Equation 4 with the Lineweaver-Burk linearization (Equation 5), and the catalytic efficiency was calculated according to Equation 6.

$$V_0 = \frac{V_{máx} \cdot [S]}{K_m + [S]} \quad (4)$$

$$\frac{1}{V_0} = \frac{K_m}{V_{máx}} . \frac{1}{[S]} + \frac{1}{V_{máx}} \quad (5)$$

Where: V_0 = initial reaction speed; $V_{máx}$ = maximum reaction speed; K_m = Michaelis-Menten kinetic constant; [S] = substrate concentration.

$$CE = \frac{V_{máx}}{K_m} \quad (6)$$

Where: CE = catalytic efficiency; $V_{máx}$ = maximum reaction speed; K_m = Michaelis-Menten kinetic constant.

2.8.3. Thermal stability profile.

Samples of the free enzyme and the immobilized derivative were incubated in closed glass flasks in an oven with controlled temperatures (40, 50, 60 and 70 ºC) for up to 24 h. Periodically, for each temperature, aliquots were removed from each sample and subjected to the activity measure. For each evaluated time, new samples were used, which after the activity dosage were discarded.

Residual activities were calculated as Equation 7.

$$R\ (\%) = \frac{U_F}{U_I} .100 \quad (7)$$

Where: R (%) = is the residual activity; U_I = initial activity; U_F = activity after incubation at the evaluated temperature.

2.8.4. Estimation of kinetic parameters of inactivation.

The influence of temperature (40, 50, 60, and 70 ºC) on the enzyme inactivation rate constant (Kd) was analyzed by Arrhenius' law, represented in Equation (8). Ed parameters were calculated by non-linear regression using Excel software.

$$Kd = Ae^{\left(\frac{-Ed}{RT}\right)} \quad (8)$$

Where: Kd = thermal inactivation speed constant; A*e* = Arrhenius pre-exponential constant; Ed = enzymatic deactivation energy; R = universal gas constant ($8.314x10^{-3}$ kJ.mol^{-1}.K^{-1}); T = absolute temperature (K).

The half-life ($t_{1/2}$) and the decimal reduction value (D), whose values correspond to the times necessary to obtain a 50 and 90 % reduction in the initial activity at a given temperature, were calculated using equations 9 and 10, respectively.

$$t_{1/2} = \frac{Ln\,(2)}{Kd} \quad (9)$$

Where: $t_{1/2}$ = half-life; Ln (2) = natural logarithm; Kd = deactivation constant.

$$D = \frac{Ln\,(10)}{Kd} \quad (10)$$

Where: D = decimal reduction; Ln = natural logarithm; Kd = deactivation constant.

The Z value, specified as the temperature variation required for the D value to be reduced by a logarithmic cycle, was estimated by the inverse of the line's slope on the log (D) versus temperature (°C) graph.

2.8.5. Estimation of thermodynamic parameters.

The thermodynamic parameters enthalpy (ΔH^o; kJ.mol^{-1}), Gibbs free energy (ΔG^o; kJ.mol^{-1}) and entropy (ΔS^o; kJ.mol^{-1}.K^{-1}) of the lipase denaturation process, free and immobilized, were determined using equations 11, 12 and 13, respectively:

$$\Delta H° = Ed - RT \quad (11)$$

$$\Delta G° = -RTLn\,\frac{K_d h}{K_b h} \quad (12)$$

$$\Delta S° = \frac{(\Delta H - \Delta G°)}{T} \quad (13)$$

Where: Ed = enzymatic deactivation energy; R = universal gas constant ($8.314x10^{-3}$ kJ.mol^{-1}.K^{-1}); T = absolute temperature (K); Kd = denaturation rate constant; h = is the Plank constant ($1.84x10^{-40}$ kJ.h^{-1}); Kb = Boltzmann constant ($1.38x10^{-26}$ kJ.K^{-1}).

2.9. Statistical analysis.

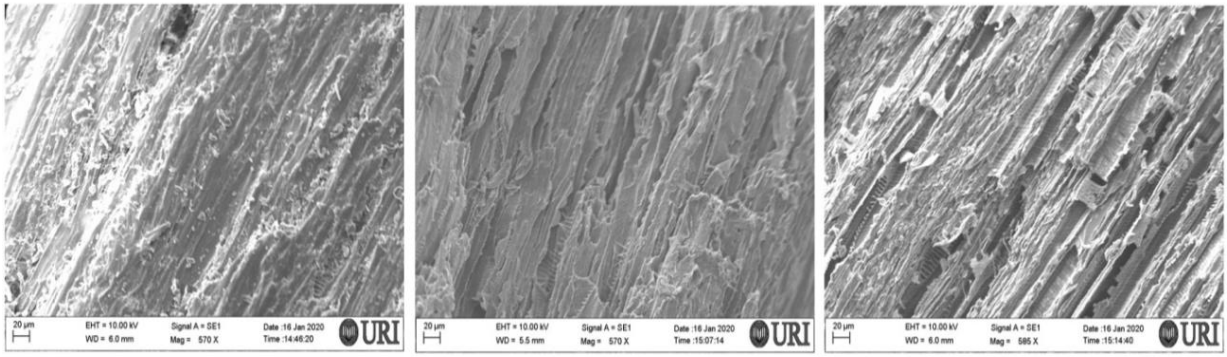
All experiments were carried out in duplicate of reaction and titration triplicate; from this, the results obtained through statistical analysis using the Excel software, version 2010, were averaged.

3. Results and Discussion

3.1. Physical characterization of the support.

When analyzing Figure 1, it can be seen that the fresh stick has a more rigid/smooth structure compared to the activated sticks, which had open grooves in their structures. Of these, the sample activated with sodium metaperiodate ($NaIO_4$) showed a greater structural change compared to that activated with APTS/glutaraldehyde. This trend was expected and was linked

to the different activation mechanisms to which the supports were subjected, more specifically, to the degradative character of the reagents used in each assay. Both supports were pretreated with NaOH, a powerful alkaline agent; however, activation with APTS/glutaraldehyde is less aggressive compared to activation with sodium metaperiodate, a powerful oxidizing agent.



(a) (b) (c)

Figure 1. (a) Visual aspect of the residual yerba mate stick; (b) stick activated with APTS/glutaraldehyde; (c) stick activated with sodium metaperiodate.

3.2. Immobilization of the Eversa® Transform 2.0 lipase on a residual yerba mate stick.

The performance results of the Eversa® Transform 2.0 lipase immobilization process on a residual yerba mate, stick activated with different methodologies, considering the use as ammonium nitrate buffer pH 10 and hexane as a solvent, are shown in Table 1.

Table 1. Yield of the Eversa® Transform 2.0 lipase immobilization process.

Enzyme	Solvent	Total mass (g)	U.g$^{-1(1)}$	U$^{(2)}$	Yield (%)
Free		0.300	941.7	282.5	-
Immobilized APTS/Glut.	NH_4NO_3 Buffer	1.485	190.6	283.1	100.2
	Hexane	1.288	494.6	637.1	225.5
Immobilized Metaperiodate	NH_4NO_3 Buffer	1.020	-	-	-
	Hexane	1.063	432.5	459.8	162.7

(1) U.g^{-1}= Enzymatic activity

(2)U= Total activity offered at the derivative

The supports before immobilization did not show any activity for the esterification reaction, demonstrating that they do not interfere in the results obtained with immobilized derivatives.

The free lipase used in the immobilization showed an esterification activity of 941.7 U.g^{-1}. Of the 4 immobilized derivatives synthesized, only one showed no activity. For the 3 immobilized derivatives that showed activity, the values varied between 283.1 and 637.1 U.g^{-1}. Despite the lower activity values observed for immobilized derivatives, concerning free enzyme, the masses of synthesized immobilized derivatives (between 1.06 and 1.48 g), which are at least 3 times higher than that of the free enzyme (0.3 g), suggest a beneficial effect of the immobilization process, providing immobilization yields (calculated considering the total activities (U) of the free enzyme and derivatives), between 100 and 225 %.

Between the two reviews, pre-treatment, regardless of the solvent from the reaction medium, activated with APTS/glutaraldehyde resulted in the generation of immobilized derivatives with higher activity, probably due to more effective generation of reactive sites, more specifically aldehydes end groups for anchoring enzymatic by covalent bonding.

According to Melo *et al*. [18], APTS silanization process, using the reaction of its radical with the support silanol group, leading to the formation of an organic bridge with an amine termination, which is subsequently activated with glutaraldehyde, incorporating a necessary aldehyde terminal group for enzyme-support binding, as shown in Figure 2.

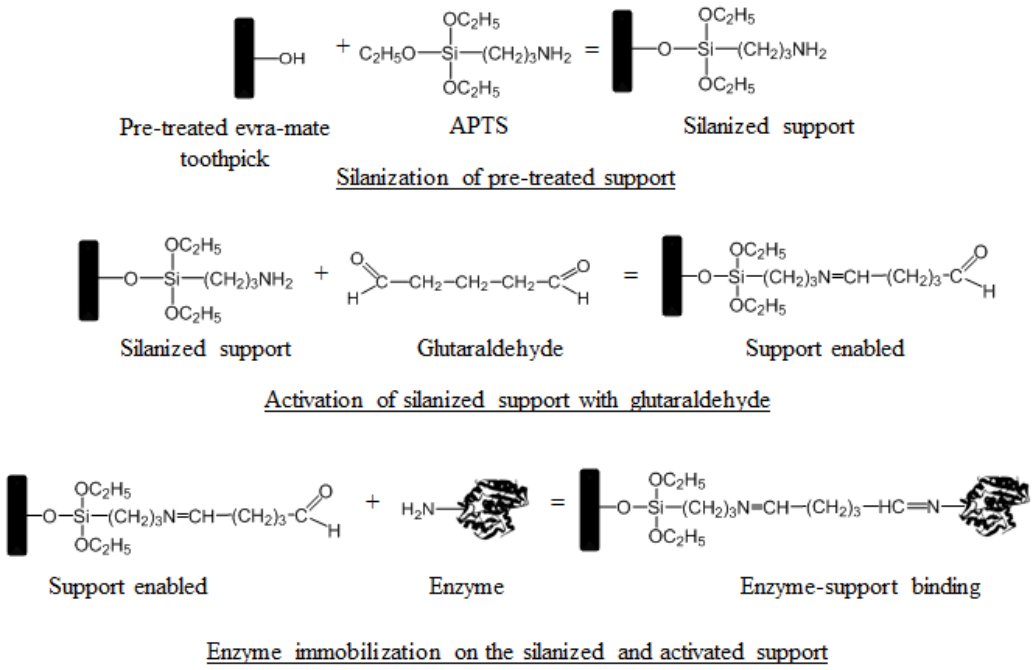


Figure 2. Signaling and activation process, using APTS/glutaraldehyde, of the residual yerba mate stick for enzymatic immobilization.

In the activation with sodium metaperiodate the suggested mechanism is via chemical oxidation of the hydroxyls exposed in the lignocellulosic material in the alkaline pre-treatment to aldehyde groups. The fact that the hydroxyls are not found in terminal carbons, that is, primary, in cellulose, makes the process of generating reactive aldehyde groups on the surface of the support more complex, the efficiency of the process is directly linked to the generation of terminal hydroxyls, by the rupture of C-C bonds in cellulose, for later oxidation to aldehyde groups (Figure 3).

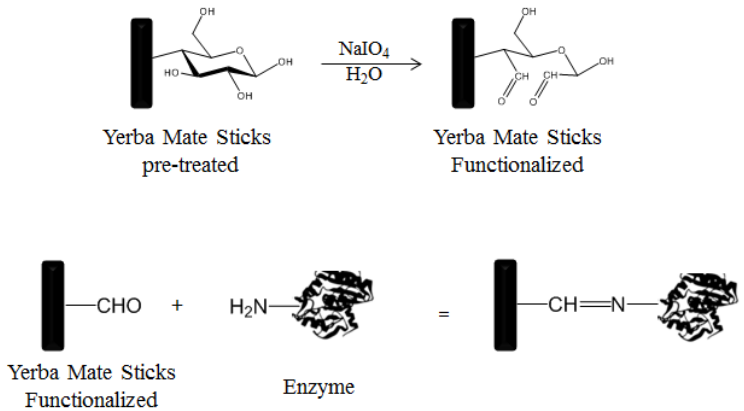


Figure 3. Activation process using sodium metaperiodate on the residual yerba mate stick for enzymatic immobilization.

Another effect observed is related to the reaction medium. The immobilized derivatives synthesized using hexane as a reaction medium, independent of the pre-treatment, activity

values much higher (2-3 x) than the values observed for the immobilized derivatives synthesized using an aqueous solution of NH_4NO_3, pH 10 as a solvent.

Boudabbous *et al*. [19] report that in the aqueous medium, the polar groups of proteins are usually located outside the globule in contact with water. However, the enzyme's nonpolar groups are located inside the globule in a protein's hydrophobic nucleus. This can generate biomolecular aggregates with low catalytic activity. These particles can be dissociated by solvents' action, promoting an increase in enzymatic activity due to unimolecular aggregates' formation. The nonpolar solvents have transported nonpolar hydrophobic residues promoting the interface, stabilizing the lipases in their open conformation, and causing these enzymes' hyperactivation [20]. This trend is linked to the maintenance capacity, the hydration layer necessary to maintain the catalytic activity [18]. All of these factors end up resulting in a high conversion rate of the product and viability of the process [21].

In this case, hexane may have caused an effect due to hydrophobic interactions between the solvent and the enzyme, leading to greater catalytic site exposure, which would facilitate catalysis [22]. Another factor is that the nonpolar solvent can break up enzyme aggregates, which normally occur by hydrophobic interaction between protein molecules, improving their dispersion in the reaction medium and supporting enzyme interaction essential for covalently binding. This allows biocatalytic transformations of water-insoluble reagents, opens up new reaction pathways, and can lead to altered selectivities [7].

3.3. Operational stability (reuse).

Different trends are observed between the two immobilized derivatives, with the functionalized with APTS/glutaraldehyde presenting a more pronounced loss in the first cycle (Figure 4 (a)), corresponding to 30 %, remaining stable, with ≅ 70 % of the residual activity, until the seventh cycle, followed by a further reduction in activity to 50 % of residual activity between the ninth and eleventh cycles. The immobilized derivative activated with metaperiodate presents a less intense loss of activity in the first cycles (with activities of 90 and 84 % for the first and second, respectively). However, this loss of activity is constant, presenting a 50 % loss of activity in the fifth cycle, demonstrating that the immobilization process conducted using the APTS/glutaraldehyde functionalized support leads to the formation of a more stable immobilized derivative.

Duman, Tufan and Kaya [23] explain that recycling the immobilized enzyme can weaken the binding force between the matrix and the immobilized enzyme, which leads to loss of enzyme activity. Also, the active site's conformation can be altered due to the repeated encounter of the substrate with the active site of the immobilized enzyme.

Regarding the 11 cycles with residual activity greater than 50 % observed for the immobilized derivative obtained with functionalized support with APTS/glutaraldehyde, they are superior to those reported by Ding *et al*. [24], who observed a total of 5 recycles with residual activity of approximately 55 % for the lipase *Candida rugosa* immobilized covalently in nanoparticles functionalized with APTS. These results are also superior to those found by Ficanha *et al*. [25], which obtained 6 recycles with residual activity of approximately 51 % using CALB immobilized on air gel modified silica. According to the results, it was decided to use the support activated with APTS/glutaraldehyde and immobilized in a solution containing the hexane solvent to continue the studies concerning the optimal reaction temperature, kinetic parameters K_m and $V_{máx}$, thermodynamic parameters, and thermal inactivation.

3.4. Evaluation of the optimal reaction temperature.

The results regarding the optimal reaction temperature for free and immobilized enzymes (Figure 4 (b)), demonstrated that this lipase, in both situations, can act and have its greatest esterification activity at a temperature of 40 °C, which is consistent with the literature [26-28], which report an optimum range between 40 and 60 °C for the temperature.

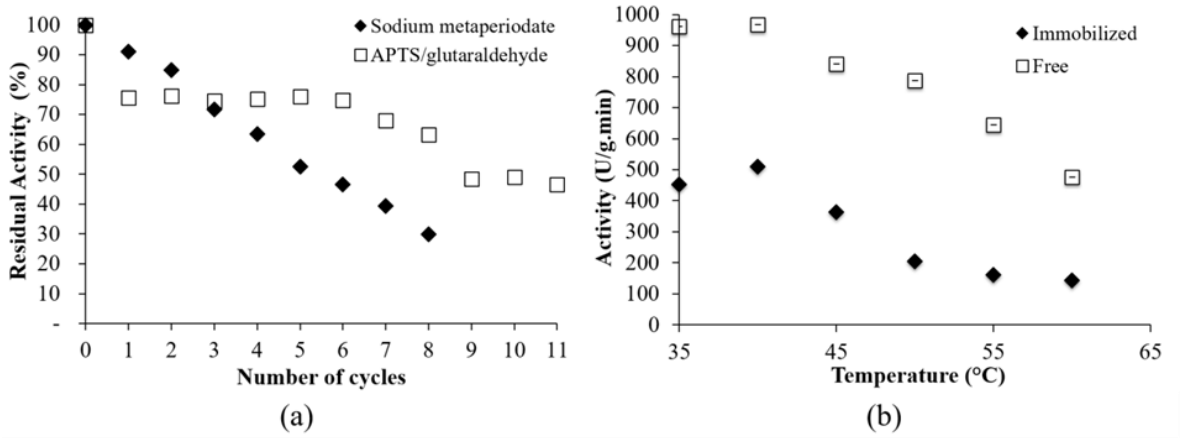


Figure 4. (a) Capacity/cycles of reuse of the immobilized derivative in the support functionalized with APTS/glutaraldehyde and in the functionalized support with sodium metaperiodate; (b) the effect of the reaction temperature for a free and immobilized enzyme in the support functionalized with APTS/glutaraldehyde.

3.5. Determination of the kinetic parameters $V_{máx}$ and K_m.

The K_m value allows us to dimension the type of interaction between the enzyme and its substrate and varies inversely with affinity; in other words, the smaller the K_m, the greater the affinity. $V_{máx}$ is defined as the maximum speed of the total amount of enzyme participating in the reaction. This measure is theoretical and has an approximate value because, at a given moment, it would require that all enzymatic molecules be strongly linked to their substrates [29].

Table 2 presents the values of K_m and $V_{máx}$ obtained in this study and the catalytic efficiency values ($V_{máx}/K_m$). Concerning K_m, the lowest value was observed for the free enzyme (16.55 μmol.g^{-1}), compared to immobilized, with 33.52 μmol.g^{-1}, suggests a greater affinity of the free enzyme for the substrate, which reflects in the greater reaction speed ($V_{máx}$), with 5555.56 μmol.g^{-1}.min^{-1} and better catalytic efficiency (($V_{máx}/K_m$), with 335.6 min^{-1}, observed for the free enzyme compared to immobilized, with 4761.90 μmol.g^{-1}.min^{-1} e 142.0 min^{-1}, respectively.

Table 2. Constant kinetic parameters of affinity (Km) and maximum reaction speed (Vmax) of the free enzyme and immobilized on the functionalized support with APTS/glutaraldehyde.

	K_m (μmol.g^{-1})	$V_{máx}$ (μmol.g^{-1}.min.$^{-1}$)	$V_{máx}/K_m$ (min^{-1})	Linear model	R^2
Free enzyme	16.55	5555.56	335.6	0.00298x + 0.00018	0.98932
Immobilized enzyme	33.52	4761.90	142.0	0.00704x + 0.00021	0.99291

This trend between enzymes was linked to the system's homogeneity when using the free enzyme, which favors the contact of the enzyme with the substrate with the heterogeneous medium when using the immobilized enzyme.

Possible changes in the three-dimensional conformation of the immobilized enzyme may also be contributing, which may be affecting its active sites, as well as the properties of

the support, such as its hydrophilic or hydrophobic nature of the presence of fixed charges that affect the mode of action of the enzyme, in addition to the largest enzyme mass in the free system with the immobilized one, which has in its composition approximately 20 % of the equivalent in free enzyme [30].

Similar behaviors are reported in the literature for lipases immobilized on other supports. Wang *et al*. [31] used *Aspergillus oryzae* lipase immobilized on amphiphilic montmorillonite (Mt) functionalized with 3-aminopropyltriethoxysilane (APTS) and found K_m values of 0.357 mM and 3.406 mM, and catalytic efficiency ($V_{máx}/K_m$) of 178.4 min^{-1} and 91.7 min^{-1}, for free and immobilized lipases, respectively. Kirtikumar and Badgujar [32], when evaluating lipase immobilized in a biocompatible copolymer of polyvinyl alcohol and chitosan, found $V_{máx}$ values of 50000 $\mu mol.g^{-1}.min^{-1}$ for the free enzyme and 36360 $\mu mol.g^{-1}.min^{-1}$ for the immobilized enzyme.

Tomke and Rathod [4] evaluated the K_m and $V_{máx}$ of free and immobilized lipase on activated carbon generated from mixed agro-industrial residues of coconut and peanut shell and obtained values for K_m of 0.57 mg/mL and 0.64 mg/mL and $V_{máx}$ of 4.62 and 4.71 $\mu mol.g^{-1}$ for free and immobilized lipases, respectively. The authors link this behavior to a decrease in the conformational flexibility of the immobilized enzyme structure.

For Cal B lipase immobilized in chitosan, Simões *et al*. [30] found $V_{máx}$ values of 12050 $mmol.g^{-1}.min^{-1}$ and 1409 $mmol.g^{-1}.min^{-1}$ and for K_m of 534 mM and 851 mM for free and immobilized lipases, respectively. According to the authors, this behavior suggests a change in the enzyme's affinity for the substrate due to incorporating the enzyme to support the immobilization process.

3.6. Estimation of thermodynamic and inactivation parameters.

Considering that the optimum reaction temperature range obtained experimentally, which is consistent with the literature [27-28], was between 40 and 60°C, the thermostability tests for free and immobilized enzymes were evaluated between 40 and 70°C.

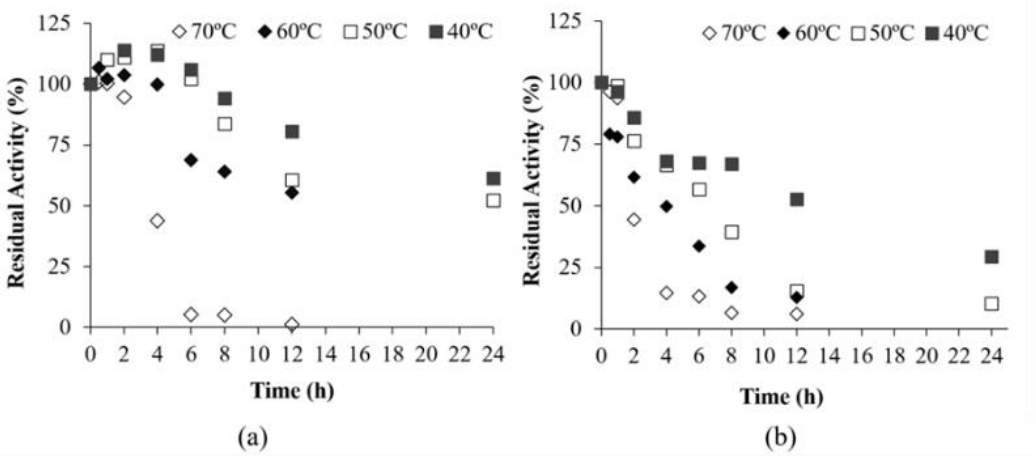


Figure 5. Thermostability of free lipase (a) and immobilized on the functionalized support with APTS/glutaraldehyde (b) at temperatures of 40, 50, 60, and 70 ° C.

For both enzymes (free and immobilized), all temperatures evaluated show the same trend, with loss of residual activity as a function of time (Figure 5). This loss of activity varies linearly with temperature, with the tests at 40 and 70 °C showing the lowest and highest loss of activity, respectively. Among the enzymes, the immobilized activity showed more marked

losses of activity concerning the free, indicating a negative effect of the immobilization concerning the enzyme's thermostability.

According to Table 3, temperature accelerates the process of thermal inactivation of enzymes, both free and immobilized. The speed at which inactivation of immobilized lipase, represented by the deactivation constant (K_d), is greater compared to free lipase, indicating the absence of thermal protection due to immobilization. Comparing the K_d obtained at temperatures of 40, 50, and 60 °C, it appears that the K_d of the immobilized lipase was 2.73; 3.69 and 3.48 times greater than those determined for free lipase, respectively.

Chiou Wu [33], associate the increase in the thermal stability of immobilized enzymes with the fixation in the multipoint support. There was no fixation in the present study's multipoint system, but in a single enzyme-support bonding bridge, reducing thermal stability in concern to the free enzyme.

Another possibility is related to the structural rearrangement and less flexibility of the enzyme in its immobilized form due to the probable lack of multipoint fixations to the support [34].

Table 3. Thermodynamic and thermal inactivation profile of free and immobilized lipase.

T (°C)	T (K)	K_d	R^2	t½ (h)	D (h)	Z (°C)	Ed (kJ.mol^{-1})	ΔG (kJ.mol^{-1})	ΔS (kJ.mol^{-1})	ΔH (kJ.mol^{-1})
Free enzyme										
40	313.15	0.02	0.97	35.72	118.69	48.08	81.26	10.03	0.097	-30.36
50	323.15	0.03	0.99	25.96	86.23			9.51	0.095	-30.88
60	333.15	0.05	0.97	13.72	45.59			8.08	0.097	-32.31
70	343.15	0.36	0.96	1.91	6.37			2.83	0.109	-37.56
Immobilized enzyme										
40	313.15	0.05	0.98	13.27	44.11	37.03	54.17	7.51	0.149	-46.65
50	323.15	0.10	0.98	7.18	23.86			6.14	0.148	-48.03
60	333.15	0.17	0.98	3.97	13.21			4.73	0.148	-49.44
70	343.15	0.34	0.98	2.04	6.77			3.01	0.149	-51.16

The half-life (t½) and the D value are values that represent the time required for a reduction of 50 % and 90 %, respectively. They are important economic parameters in many industrial applications. The higher the values, the greater the thermostability of the enzyme [35]. The free lipase was more stable to thermal inactivation than in the immobilized form, presenting t½ in the range from 35.72 to 1.91 h and D values from 118.69 to 6.37 h (Table 3).

The Z values were 48.08 °C and 37.03 °C for the free and immobilized enzyme, respectively; that is, when the temperature was changed up or down by the Z value, the D value was changed in a logarithmic cycle. The Z value indicated that large temperature variations are necessary to considerably affect the enzyme's stability, especially concerning it in its free state.

The higher value of deactivation energy (Ed) of the free lipase (81.26 kJ.mol^{-1}) compared to the immobilized one (54.17 kJ.mol^{-1}) suggests that the free one requires a greater amount of energy to occur the protein unfolding and initiate the process of thermal inactivation, concerning the immobilized enzyme, suggesting that the support was not efficient for the protection of the enzyme against thermal degradation.

Gibbs' free energy measures the spontaneity of the reaction, and protein instability is directly related to ΔG values, which, when elevated, indicate greater thermal stability of the enzyme. The positive values of ΔG^o for all enzymatic derivatives studied suggest that the lipase's thermal inactivation process is thermodynamically non-spontaneous [36]. In addition, the highest values of ΔG^o recorded for free lipase demonstrated that the state assumed by the

enzyme, after heat treatment, has greater available energy and the original structural conformation remained more active compared to the immobilized derivative.

The enthalpy (ΔH) and entropy (ΔS) parameters provide the number of broken non-covalent bonds and the change in enzyme/solvent disorder associated with the formation of the transition state, where positive ΔS values suggest that the enzyme split can be a decisive step towards irreversible thermal inactivation of the enzyme. Low ΔS values suggest an insignificant disorder in the system and can also be highly influenced by several factors, including the effect of the solvent and structure [37]. In this study, the ΔS values were positive for both cases, showing that the reaction is entropically favorable, however, the ΔS variation was small. For the immobilized enzyme, the values of ΔS (0.148 to 0.149 $kJ.mol^{-1}$), were higher than those observed for the free enzyme (0.095 to 0.109 $kJ.mol^{-1}$), and the temperature was not a factor that influenced this parameter.

Regarding the enthalpy (ΔH), all values were negative, both for the free enzyme and for the immobilized enzyme, indicating that the thermal inactivation reaction is exothermic, with a greater release of energy into the medium for the immobilized enzyme (-46.65 a -51.16 $kJ.mol^{-1}$) concerning free enzyme (-30.36 to -37.56 $kJ.mol^{-1}$).

Similar results were observed by Antunes [38] for the CALB lipase immobilized in xerogel employing polyvinyl alcohol (PVA) as an additive, presenting higher K_d values and lower t½ with the free enzyme, for the evaluated temperature range (40 to 80 °C). According to the author, the lower thermal stability may be related to the functionality of the support, combined with the temperature, interfering negatively with the enzyme's stability, leading to its loss of activity and, consequently, potentiating the thermal inactivation of the immobilized enzyme with free.

4. Conclusions

The proposal to immobilize the Eversa® Transform 2.0 lipase in yerba mate stick residue showed promise for obtaining a derivative immobilized by covalent bonding with good residual activity and an optimum mild reaction temperature. The kinetic parameters observed suggest a better affinity for the substrate from the free enzyme, one of the factors that may justify this is the fact that the immobilized derivative has in its composition approximately 20 % of the equivalent in a free enzyme. Compared with the free enzyme, the immobilized enzyme did not show such an efficient thermostability, which may be related to the support's functionality, combined with the temperature, interfering negatively with the enzyme's stability.

However, immobilized derivatives showed excellent behavior in relation to reuses, demonstrating that there is the possibility of using supports from agro-industrial residues, which would be discarded in the environment, making it a great alternative to reduce the costs involved in the process of enzyme immobilization.

The results obtained can be a reference base for further research to optimize the process of immobilizing lignocellulosic waste and its subsequent application.

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Conflicts of Interest

The authors declare no conflict of interest.

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