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Research article

Preparation and characterization of new silica-based heterofunctional biocatalysts utilizing low-cost lipase Eversa® Transform 2.0 and evaluation of their catalytic performance in isoamyl esters production from *Moringa oleifera* Lam oil

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Abstract: Due to the need to replace lubricants derived from polluting processes and inputs, bioprocesses and raw materials such as vegetable oils have been used for the production of biolubricants. In this study, the synthesis of esters with lubricating potential was conducted through enzymatic hydroesterification. For complete hydrolysis of *Moringa oleifera* Lam. seed oil (MOSO), *Candida rugosa* lipase was applied under conditions already established in the literature. Subsequently, the synthesis of esters of industrial interest was carried out through esterification using a lipase (Eversa Transform 2.0 (ET2.0)) immobilized by different functional groups on heterofunctional silica-based supports: epoxy-silica (Epx), glyoxyl-silica (Gly), and amino-glutaraldehyde-silica (AmG). Two drying pre-treatment techniques were used to improve the immobilization yield of the ET2.0 lipase on different pre-treated supports: evaporation in a drying oven (with improvements ranging from 15%

to 46%) and pressure difference in a desiccator (with improvements ranging from 24% to 43%). The immobilizing supports and biocatalysts were characterized to verify their morphologies, structures, and topographies. Deconvolution was performed to evaluate the secondary structure of the ET2.0 lipase and showed increases in the α-helix and β-sheet regions for all biocatalysts after the immobilization process. In a solvent-free medium, the AmG-70h support performed best in the esterification reaction, at around 90% conversion, with a load of 1.65 mg of protein in the reaction. Moreover, it obtained a productivity around 4.45 times that of free ET2.0 lipase, maintaining its original activity until the fourth cycle. This work offers the opportunity to understand and synthesize new biocatalysts with a low-cost genetically modified lipase using a renewable raw material, opening new possibilities to fill gaps that still exist in the use of lipases for biolubricant production.

Keywords: heterofunctional supports; esterification; Eversa® Transform 2.0; immobilization; silica-based

1. Introduction

The exhaustive use of non-renewable raw materials to meet energy demands and the production of materials has aroused great environmental concern. Great efforts from researchers have been required to develop new processes that are based on the principles of green chemistry and that can meet demands with greater environmental friendliness. Replacing petroleum with animal fats, residual oils, microbial oil, and vegetable oils allows the production of environmentally friendly lubricants, known as "biolubricants" [1–3]. The synthesis of biolubricants from edible and non-edible vegetable oils can be achieved from more than 300 oilseeds, showing a large range of resources for their production. Moreover, these biolubricants can have tribological properties equal to or better than commercial lubricants based on petroleum, making them cheaper and more environmentally friendly [2].

An oleaginous matrix whose main fatty acid is oleic acid (around 70–80%) is *Moringa oleifera* Lam., a plant originating in northwest India, and has shown great application in the food and pharmaceutical industries due to its nutritional composition and pharmacological action [4]. Its seeds have a high oil content (35–45% by mass), w foi substituído em todo o texto conforme sugeridohich makes it advantageous for application in the production of biolubricants, and a report in the literature describes its use to obtain a hydraulic biolubricant [5].

The chemical route can produce biolubricants through reactions such as esterification, transesterification, epoxidation, and hydroesterification; however, the use of this route leads to long reaction times, high energy demand, formation of co-products, and the use of several purification steps to obtain of the final product [2]. The enzymatic route has been gaining prominence to overcome the limitations of the chemical route. It can reduce costs, be environmentally friendly, reduce reaction times, use mild temperatures, and obtain purer products using biological catalysts [5–7].

One of the strategies for the synthesis of biolubricants via the enzymatic route is hydroesterification, a two-step process (hydrolysis and esterification reactions) to obtain esters with lubricating properties. It can overcome the disadvantages of direct transesterification of triglycerides from vegetable oils with polyols, which requires high temperatures and reaction time due to the high viscosity of the medium [8]. Furthermore, the hydroesterification reaction prevents the adsorption of glycerol molecules that are generated in the transesterification reaction on the surface of the biocatalyst [9].

Furthermore, this reaction uses acids obtained by the hydrolysis (first stage) of residual and vegetable oils, avoiding the use of acids synthesized by a chemical route when compared to the direct esterification reaction, which requires an acid as one of the substrates [1].

Commonly used to synthesize biolubricants by enzymatic hydroesterification, the lipases (triacylglycerol acyl-hydrolases, E.C.3.1.1.3) the natural function is the hydrolyze of oils and fats to produce glycerol, free fatty acids, and monoglycerides or diglycerides. Moreover, they can catalyze different reactions, such as esterification, transesterification, acidolysis, amidations, etc. [1]. A lipase that has been gaining prominence for its versatility in different sectors of the industry is Eversa Transform 2.0 (ET2.0), a lipase from *Thermomyces lanuginosus* expressed in *Aspergillus oryzae*, a low-cost commercial lipase preparation initially prepared for use in free form for biodiesel production [2,9,10]. However, recent studies show ET2.0's ability to produce other products, such as biolubricant [11]; biogas [12]; monoesters, diesters, or triesters; and glycerol [13].

ET2.0 lipase can be applied to reactions in its free form, but its use in this form can lead to some disadvantages, such as low catalytic activity, the formation of lipase dimers which cause inactivation, lower thermal and mechanical stability, and difficulty in reuse. These adversities can be minimized when lipases are immobilized on supports, a technique that allows the reuse of the same biocatalyst for several cycles, thus reducing costs with this input. [14,15]. These enzymes have been immobilized using different techniques such as physical adsorption on hydrophobic or ion-exchange supports, covalent attachment, entrapment, and cross-linked enzyme aggregates (CLEAs) on different supports [16].

Supports can be functionalized by different functionalizing agents, which can incorporate a wide variety of functional groups and determine what type of interaction the enzyme will make with the support, such as (3-aminopropyl) triethoxysilane (APTES) and (3-glycidyloxypropyl) trimethoxysilane (GPTMS) [16,17]. The use of different functionalizing agents allows the preparation of biocatalysts that can carry out different types of interactions (physical, ionic, or covalent), allowing different amino acid residues of the enzyme to carry out interactions with the support, known as heterofunctional supports. Knowing which interactions, a support can perform with a certain enzyme makes it possible to improve the immobilization process, because each type of interaction requires different immobilization times for complete stabilization of the enzymes [18].

The synthesis of new, more efficient biocatalysts goes beyond the immobilization step and must take into account the steps that precede this process, such as the drying pre-treatment of the supports, which can promote the synthesis of more efficient heterofunctional supports in relation to enzymatic loading, as the presence of oxygen can prevent lipases from carrying out hydrogen bonding and/or physical adsorption [6]. However, there is a great lack of scientific research on pre-treatment and drying of pre-immobilization materials, as this process is generally not carried out, and when it is carried out, it is always for fixed times, without evaluation of the process.

A material commonly used to produce heterofunctional supports is silica, a material that can come from agricultural waste and that allows great possibility of modification on its surface, incorporating different functional groups for immobilizing lipases. Silica is also used in packed bed reactors, where this biocatalyst carries out several reactions and brings the advantage of reactions in a continuous mode, where the objective is to obtain products on an industrial scale [19].

The use of biocatalysts on an industrial scale still faces some difficulties in relation to implementation due to the cost of biocatalysts, which need to be better evaluated for application and better understanding of the enzyme-support interaction process. This problem can be solved with the use of biocatalysts with good operational stability results, which promotes greater economic prospects

for the process. Barbosa [15] reported that for the production of biolubricants using *Moringa oleifera* Lam. seed oil (MOSO), one of the main expense factors is not the energy consumption of the process but rather compounds that fit into other materials. these other materials (which represent around 52% of biolubricant production via enzymatic route), 60% is related to the cost of the biocatalyst, and its reduction can make the process more viable.

The objective of this work was to verify the catalytic ability of a lipase (ET2.0) immobilized in different functional groups on silica-based heterofunctional supports functionalized with different agents to produce industrial grade esters through esterification of MOSO, opening new possibilities for the synthesis of biocatalysts from a low-cost lipase with the possibility of use in continuous packed-bed reactors.

2. Materials and methods

2.1. Materials

In this work, ET2.0 lipase with 24.4 mg protein/mL, obtained from Novozymes (Araucária, Paraná), was used. The functionalizing silane agents APTES and GPTMS were obtained from Sigma-Aldrich (Missouri, USA). Silica gel (230–400 mesh) was obtained from Neon (São Paulo, Brazil). Isoamyl alcohol was obtained from Vetec (Rio de Janeiro, Brazil). To verify the protein concentration, a bovine serum albumin solution and *Candida rugosa* lipase obtained from Sigma-Aldrich (Missouri, USA) were used. All other reagents were of analytical grade.

2.2. Extraction of MOSO

The *Moringa oleifera* Lam. seeds were collected from the native population of the Caju Private Heritage Natural Reserve (RPPN), belonging to the Experimental Field of Embrapa Tabuleiros Costeiros, in the municipality of Itaporanga d'Ajuda, Sergipe, Brazil (latitude –11.116585°, longitude −37.186742°), from November to February to obtain oil with less acid content [20]. The seeds obtained were dried in an oven with hot air circulation at a controlled temperature of 45 °C until they reached a moisture content of approximately 5−7% (m/m). After that, they were crushed in a knife mill and then separated into the granulometry of 16–32 mesh. The samples were stored in a refrigerator (−4 °C) and protected from light until the moment of extraction. MOSO was extracted via Soxhlet extraction with a methodology described in the literature [21].

2.3. Enzymatic hydrolysis reaction of MOSO

Free fatty acids (FFA) were obtained in the enzymatic hydrolysis reaction of MOSO using free lipase from *Candida rugosa* (CRL) under conditions of 37 °C, 1000 rpm, 25% oil, 75% water, and 550 U/g of oil for 60 min, as described by the reported methodology of [21].

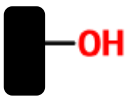
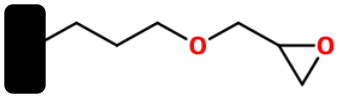
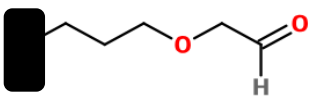
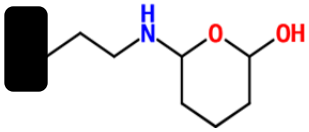
2.4. Silica drying pre-treatment

The silica gel was subjected to a pre-treatment of evaporation drying in an oven and a pressure difference in a desiccator for 8, 24, or 48 h before functionalization for the preparation of biocatalysts. The immobilization yield was the criterion for the evaluation of the influence of this treatment. Student's t test was used to assess the statistical significance of the data. Data interpretation was performed according to analysis of variance (ANOVA) results, and then the average values between the three biocatalysts were compared using Tukey's significant difference procedure. The significance level was considered $p \leqslant 0.05$.

2.5. Synthesis of different supports for immobilization of the lipase

For the synthesis of heterogeneous biocatalysts, it was necessary to prepare three different supports based on commercial silica gel with two functionalizing agents: GPTMS for the preparation of epoxy-silica (Epx) and glyoxyl-silica (Gly) supports and APTES for preparing the amino-glutaraldehyde-silica (AmG) support. Table 1 shows a representation of the possible supports synthesized to promote the immobilization of the lipase.

Table 1. Representations of the chemical structures of possible synthesized supports (created by eMolecules).

Support			
Silica surface	Epx	Gly	AmG
			

2.5.1. Synthesis of Epx and AmG

The synthesis of the support was carried out following the procedure reported in [22], with few modifications. A suspension containing 1 g of support and 20 mL of a solution of GPTMS in toluene (volume ratio GPTMS: toluene of 1: 10) was maintained at reflux for 4 h at 120 °C. The Epx functionalized support was vacuum filtered and washed with acetone, alcohol, and distilled water (200 mL each). After washing, the support was kept at 40 °C for 24 h in a drying oven. The AmG was prepared in the same way as Epx, changing only the functionalizing agent (APTES).

2.5.2. Synthesis of Gly

The preparation of the support was carried out according to the methodology reported in [22], with few modifications. The acid hydrolysis of the epoxy groups was carried out with a suspension containing 1 g of Epx in 30 mL of sulfuric acid (H_2SO_4) (0.1 mol/L) under reflux for 2 h at 85 °C. Subsequently, the support was washed with toluene and distilled water (200 mL each). After washing, the support was kept at 40 °C for 24 h in a drying oven. After drying, 1 g of the glyceryl support was kept in a thermostatic bath (25 °C) for 1 h with 10 mL of 0.1 mol/L sodium periodate ($NaIO_4$) solution.

The Gly was vacuum filtered and washed with 500 mL of distilled water. After washing, the support was kept at 40 °C for 24 h in a drying oven.

2.5.3. Immobilization of lipase on Epx

The immobilization of the ET2.0 lipase on the Epx support was carried out in 3 steps according to the reported methodology of [22]. In the first step, 1 g of Epx support was suspended in 15 mL of enzymatic solution with 5 mg/mL of the ET2.0 lipase, in 0.1 mol/L sodium phosphate buffer solution (pH 7.0). The suspension was maintained at 200 rpm in an orbital shaker for 8 h at 25 °C. The prepared heterogeneous biocatalyst was filtered under vacuum in a Büchner funnel with Whatman grade 41 filter paper and washed with distilled water (200 mL). In the second step, the biocatalyst was suspended in 15 mL of 0.1 mol/L sodium bicarbonate solution (pH 10.0), maintained at 200 rpm for 8 h at 25 °C. The biocatalyst was filtered and washed with 400 mL of distilled water.

In the last step, the biocatalyst was suspended in 4 mL of 3 mol/L glycine solution (pH 8.0) for 1 h under orbital stirring at 200 rpm at room temperature (25 °C) to block the remaining active groups on the surface of the support. After preparation, the supports were stored at 4 °C.

The protein content of the lipases was determined by the Bradford methodology at the first step of immobilization [23]. From the standard curve prepared with bovine serum albumin (BSA) in distilled water, the absorbance of the sample was read in a UV-vis spectrophotometer (Biochrom Libra S22) at 595 nm. The Bradford method applied defines the amount of immobilized lipase by comparing the enzyme solution before and after immobilization.

To determine the ET2.0 lipase protein content, an aliquot of 100 μL was removed from the enzymatic solution, deposited in bottles, and mixed with 5 mL of Bradford solution, maintained for 5 min. After that, the mixture was taken to the quartz cuvette, and the spectrum was analyzed.

2.5.4. Immobilization of lipase on Gly

The immobilization of the lipase on the Gly support was carried out in two steps according to a methodology reported in [22]. First, 1 g of Gly support was suspended in 15 mL of enzymatic solution with 5 mg/mL of ET2.0 lipase, prepared in 0.1 mol/L sodium bicarbonate buffer solution (pH 10.0). The suspension was kept stirring at 200 rpm on an orbital shaker for 8 h at 25 °C. After 8 h, the second stage began, where 15 mg of sodium borohydride was added to the suspension to perform the blocking stage. Finally, the prepared heterogeneous biocatalyst was filtered under vacuum in a Büchner funnel with Whatman n°41 filter paper and washed with distilled water (500 mL). After preparation, supports were stored at 4 °C. The protein determination step was performed in the first step of immobilization by the Bradford method, as described in section 2.5.3.

2.5.5. Immobilization of lipase on AmG

Before immobilizing on the AmG support, a support activation step was required, where 1 g of the support was suspended in 10 mL of 0.2% glutaraldehyde solution (v/v) prepared in 0.1 mol/L sodium phosphate buffer solution (pH 7.0) kept stirring in an orbital shakeat 200 rpm for 1 h at 25 °C. The activated support was vacuum filtered in a Büchner funnel with Whatman n°41 filter paper and washed thoroughly with distilled water (500 mL). Immediately after washing, 1 g of the support was

suspended in 15 mL of enzymatic solution with 5 mg/mL of ET2.0 lipase, 0.1 mol/L sodium phosphate buffer solution (pH 7.0), for 6 h at 25 °C. The prepared heterogeneous biocatalyst was filtered under vacuum in a Büchner funnel with Whatman n°41 filter paper and washed with distilled water (200 mL). The protein determination step was performed in the first step of immobilization by the Bradford method, as described in section 2.5.3.

2.6. Enzymatic esterification reaction of free and immobilized lipase with fatty acids of MOSO

The esterification of the FFA was carried out with isoamyl alcohol, under the conditions of 300 rpm, 40 °C, and molar ratios of 1: 1 and 1: 2 (oil: alcohol), using different concentrations of free ET2.0 lipase (5, 10, and 20% m/v), according to established methodology [5].

Immobilized ET2.0 lipase was used under the same conditions as free ET2.0 lipase, using 10% (m/m) of different heterogeneous biocatalysts (Epx, Gly, and AmG) of the total reaction medium (6 g). Aliquots (50 µL) from the reaction media with free and immobilized enzyme were withdrawn, added to 10 mL of an ethanol/acetone solution (volume ratio 1: 1), and titrated with NaOH (30 mM) using phenolphthalein to determine the conversion, as shown in Eq 1 [24].

$$Conversion\ (\%) = \frac{(F_{AO} - F_{Af})}{F_{AO}} \times 100 \quad (1)$$

F_{AO} and F_{Af} are the initial and residual FFA concentrations at a given reaction time t (mM).

The esterification activity was determined according to Eq 2:

$$Esterificationactivity\ (\mu mol/h.mg) = \frac{F_{AO}\ x\ CT}{t\ x\ m_{IP}} \quad (2)$$

where F_{AO} is the initial fatty acid (µmol), CT is the residual fatty acid concentration at a determined time of reaction, t is the reaction time (h), and m_{IP} iAn equation was added for a better understanding of the productivity calculation.s the mass of immobilized protein concentration (mg).

The productivity was determined according to Eq 3:

$$Productivity\ (\mu mol/h{\cdot}mg) = \frac{\frac{F_{AO}\ x\ CT}{t}}{C_{PRO}\ X\ m_{IP}} \quad (3)$$

F_{AO} is the initial fatty acid (µmol), CT is the residual fatty acid concentration at a determined time t of the reaction, t is the reaction time (h), C_{PRO} is the protein concentration (mg/g), and m_{IP} is the mass of immobilized protein concentration (mg).

Enzymatic esterification reactions of fatty acids from MOSO using the lipase (ET2.0), in both free and immobilized forms, follow the reaction mechanisms of hydrolysis (step 1) and esterification (step 2), as shown in Figure 1.

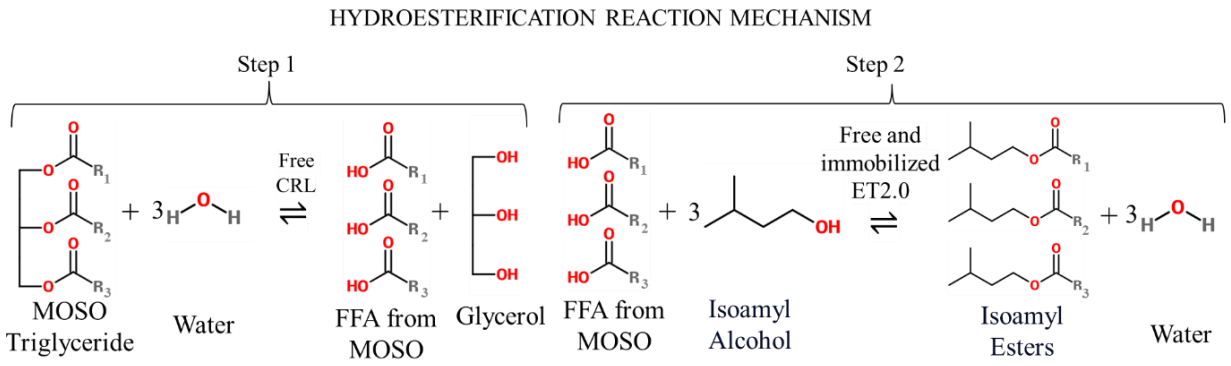


Figure 1. Enzymatic hydroesterification reaction mechanism of MOSO.

2.7. Determination of secondary structure by Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of supports and immobilized biocatalysts were recorded on a Cary 630 FTIR spectrometer (Agilent Technologies, Germany) equipped with an attenuated total reflectance (ATR) accessory. The spectra were measured in a spectral range from 4000 to 650 cm^{-1} and at a spectral resolution of 4 cm^{-1}. For each spectrum, 32 scans were added. For secondary structure analysis, the spectra were recorded from 1700 to 1600 cm^{-1}. Evaluation of secondary structure changes included second derivative and deconvolution of peaks using Origin software, version 8.5.

2.8. Characterization of biocatalysts

2.8.1. Scanning electron microscopy (SEM)

The supports and biocatalysts from the functionalization/activation of silica were characterized by SEM (JEOL JSM-IT 200) through the dehydration of samples and coatings of particles for analysis.

2.8.2. Atomic force microscopy (AFM)

AFM images were obtained using a SHIMADZU SPM-9700 microscope in dynamic operation mode. The supports and immobilized biocatalysts were applied to the surface of mica, which was earlier cleared by the mechanical removal of stripping. Finally, the materials were characterized at a 3 μm scale with an Econo-TESP tip under conditions of a resonance frequency of 300 kHz and an elasticity of 40 N/m.

2.9. Biocatalyst operational stability tests

These tests were performed under optimal conditions using 10% (m/m) ET2.0 lipase immobilized on AmG support of the total reaction medium (6 g) with FFA from MOSO in glass reactors, under conditions of 40 °C, an equimolar acid/alcohol ratio (1: 1), and stirring at 300 rpm. At the end of each reaction batch (30 h), the biocatalyst was recovered from the reaction mixture by filtration on a Büchner funnel under vacuum, washed with cold hexane to remove any reagent molecules retained on

the support, and kept overnight in a freezer at 4 °C under static conditions. Then, the used biocatalyst was resuspended in a new batch of substrate to start a new reaction cycle. The percentage of OH conversion was calculated at the end of each batch, as shown in Eq 1, as described in section 2.6.

2.10. Purification of esters

At the end of the esterification reaction, the reaction medium was centrifuged at 5000 rpm for 10 min to remove the biocatalyst. The organic phase was neutralized with 15% m/v Na_2CO_3 (1: 1 volume ratio) to remove any residual fatty acid, as described in the literature [5].

2.11. Characterization by FTIR of the bioprocess

Fourier transform infrared spectroscopy (ATR-FTIR) was used to investigate the compounds formed by the esterification reaction. The IR spectra were recorded using an Agilent Technologies Cary 630 FTIR spectrometer, equipped with a diamond single-bounce ATR accessory, in the range of 4000–400 cm^{-1}.

2.12. Statistical analysis

ANOVA was performed using an Excel data analysis tool add-in, Microsoft statistical analysis system. The level of confidence required for significance was defined as $p < 0.05$ with Tukey's test.

3. Results and discussion

3.1. Esterification of MOSO

The MOSO was subjected to an initial hydrolysis process using CRL, resulting in the production of free fatty acids. Subsequently, these free fatty acids were esterified in a solvent-free medium with ET2.0 lipase according to the established methodology [21]. CRL was selected due to the results obtained in the Barbosa [21] studie which evaluated seven different commercial lipases in the hydrolysis reaction of MOSO, and CRL was the lipase that presented the best performance and promoted complete hydrolysis of the oil in FFA. In this work, the confirmation of biotransformation of MOSO into FFAs in the same reaction and conditions described by Barbosa et al. [21] was confirmed by the FTIR spectrum shown in Figure 2, similar to the FFA profile found in the literature [25]. The FFA spectrum in the region from approximately 1750 to 1690 cm^{-1}, which represents carbonyl bonds (C=O), shows the variation of intensity for bioproduct (FFA = 1708 cm^{-1}), which provides evidence that biotransformation has been carried out.

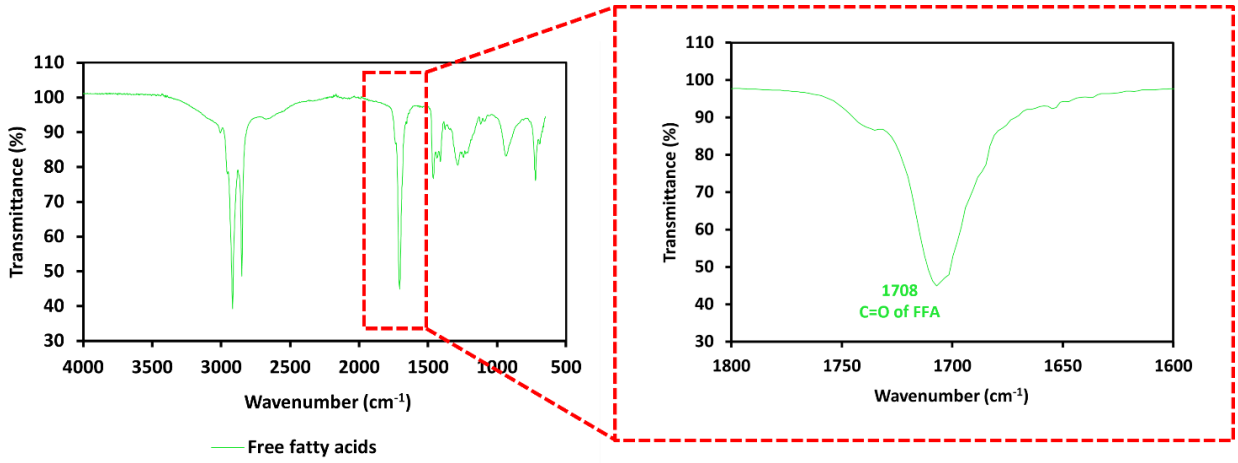


Figure 2. FTIR of the FFA of *Moringa oleifera* Lam.

Other regions that showed variation in the FTIR spectrum will be further discussed in section 3.7 below, adding the spectrum of the esterification product to the discussion and comparison between the three bioproducts used in this work.

After obtaining the FFA, the free ET2.0 lipase was utilized under the conditions previously outlined by Barbosa et al. [5] that yielded a hydraulic biolubricant (ISO VG 68 class) by esterification. Thus, the esterification reaction was carried out with three protein loadings, and two molar ratios were examined to observe the conversion profile over time. The catalytic efficacy of the free ET2.0 lipase is shown in Figure 3.

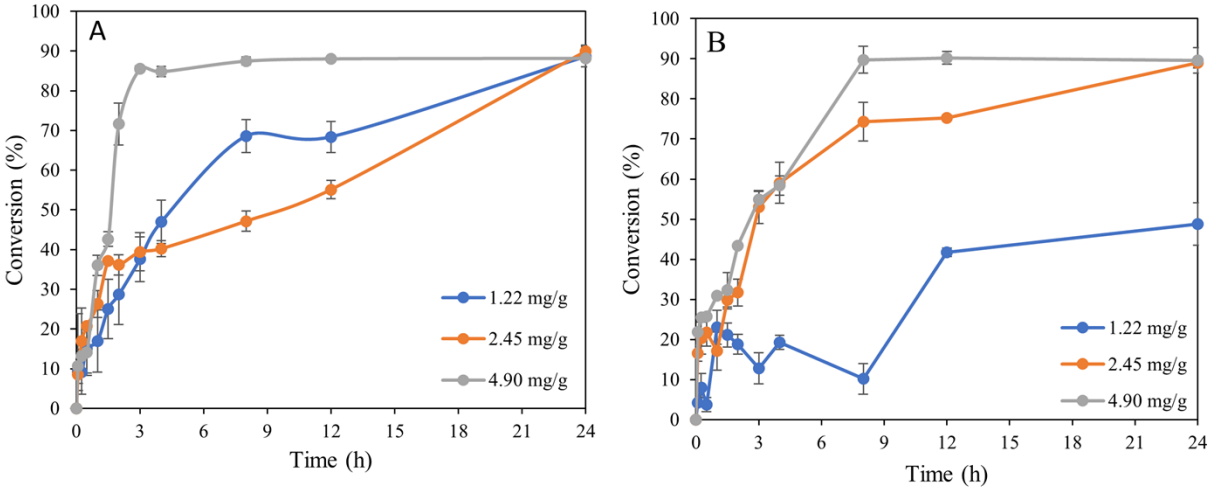


Figure 3. Esterification of *Moringa oleifera* Lam. FFA with isoamyl alcohol catalyzed by free ET2.0 lipase in two acid: alcohol molar ratios, (A) (1: 1) and (B) (1: 2), and three fractions of protein, 1.22, 2.45, and 4.9 mg/g, of the reaction mixture (which correspond to 5, 10, and 20% w/v of the reaction mixture). Reactions were conducted at 40 °C and 300 rpm for 24 h.

Figure 3A shows the catalytic profile of the ET2.0 lipase in the equimolar esterification reaction of FFA derived from MOSO and isoamyl alcohol, utilizing different enzyme concentrations. The best result was obtained at a concentration of 4.90 mg/g of the reaction mixture (about 90% conversion), and equilibrium was achieved within 4 h of the reaction. Conversely, reactions carried out with 1.22 and 2.45 mg/g of the reaction mixture necessitated 24 h to achieve similar conversion values. Figure 3B showcases the same reaction with a 1: 2 acid: alcohol molar ratio. The observed catalytic profile exhibited the maximal conversion only with the 4.90 and 2.45 mg/g reaction mixtures. Notably, the 1.22 mg/g of the reaction mixture achieved 50% conversion in 24 h. Based on the results obtained in Figures 3A,B, it can be observed that the concentration of the biocatalyst and the molar ratio directly influence the reaction time commonly reported in the literature [15,26].

The acid: alcohol molar ratio is a fundamental variable in the esterification reaction because it can shift the reaction equilibrium towards product formation, as it is thermodynamically controlled [1,2]. Carvalho et al. [6] investigated an esterification reaction employing refined soybean oil and cooking oil with TMP (2-ethyl-2-(hydroxymethyl)-1, 3-propanediol), utilizing ET2.0 lipase immobilized through physical adsorption. The results revealed that a small excess of fatty acids was effective in increasing catalytic activity due to change in reaction equilibrium. Amelia et al. [27] evaluated the effect of the molar ratio of methanol with FFA from palm oil on an esterification reaction; it was found that a high concentration of FFA achieved a maximum conversion of around 92% using free ET2.0 lipase in 4 h, a result commonly found in the literature. It is necessary to study this variable to promote a change in the equilibrium of a reaction that is thermodynamically governed. In this work, excess alcohol decreased the reaction speed and enzymatic activity, which may have occurred due to the change in the hydration layer of the enzyme with this excess substrate, reducing the catalytic activity [28].

Water is one of the major products of the esterification reaction and can shift the equilibrium of the reaction, promoting a cross-reaction [29]. This phenomenon manifested itself more visibly in the molar ratio of 1: 2 (FFA: alcohol), in which excess alcohol was detrimental to the efficiency of the biocatalyst in the reaction, increasing reaction time and possibly promoting effects due to substrate/product inhibition [30]. However, this limitation can be mitigated through the implementation of strategies capable of absorbing or eliminating the water generated throughout the reaction. Two solutions may be viable to avoid cross-reaction: the addition of a molecular sieve, a hydrophilic compound with the capacity to capture water molecules, and the use of an open reactor, approaches evaluated by Carvalho et al. [6] through their promising results in ester production. Zeng and Zhong [31], on the other hand, opted for a distinctive approach, employing evaporation via an N_2 gas stream with encapsulated lipases to remove the water particles generated during esterification. Araujo-Silva et al. [11] also used a quantity of molecular sieve (around 9%), achieving 70% conversion in the esterification reaction of soybean oil deodorizer distillate and isoamyl alcohol in 50 h employing free ET2.0 lipase. These works indicate that the capture of water molecules formed during the reaction helped to ensure that the equilibrium of the reaction was towards the production of esters and that the ET2.0 lipase has high activity in the FFA esterification reaction of MOSO.

3.2. Commercial silica gel drying pretreatment

To make the ester production process more advantageous through the enzymatic esterification reaction, three different silica-based supports were prepared for the immobilization of the ET2.0 lipase and to verify the catalytic profile of the heterogeneous biocatalysts. To improve the immobilization

yields of the ET2.0 lipase, two drying pre-treatment techniques were used before the support functionalization process, considering that humidity is a relevant factor in the activity and stability of immobilized biocatalysts [32]. In this work, two types of drying pre-treatment were used for three types of support (Epx, Gly, and AmG): evaporation in a drying oven and pressure difference in a desiccator. The supports were dried for different exposure times (8, 24, or 48 h). The effect of commercial silica drying pre-treatment is shown in Figure 4 for immobilization yield, with ANOVA and Tukey test results in the appendices (A1 and A2). Figure 4A presents the immobilization yield data of the evaporation pretreatment in an oven by air convection, and Figure 4B shows results for the pressure difference in a desiccator with a drying agent.

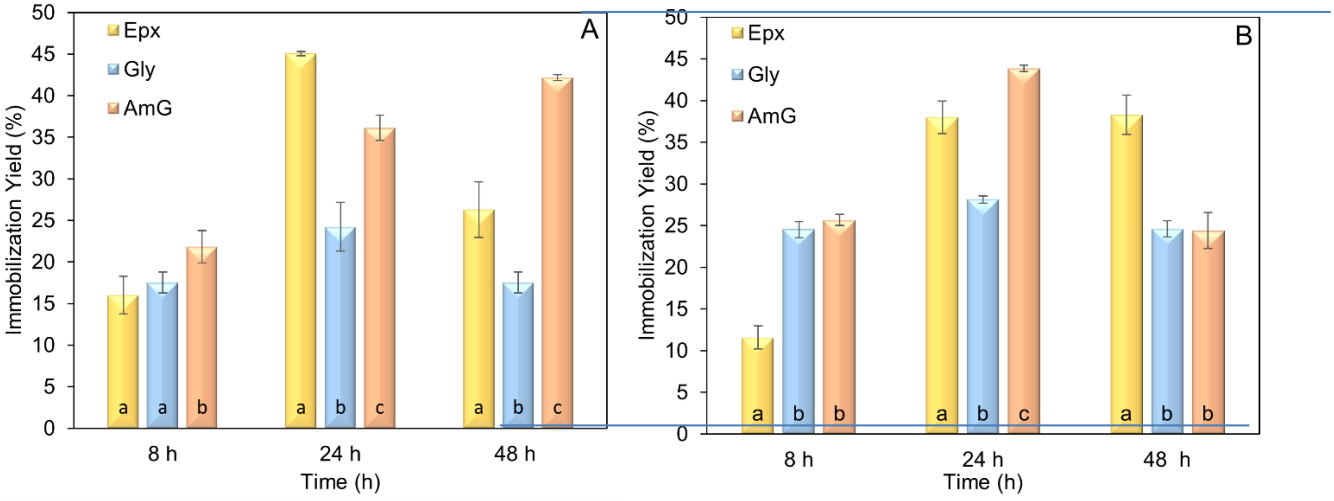


Figure 4. Immobilization yield of the ET2.0 lipase with drying pre-treatment (A) by evaporation in a drying oven and (B) by pressure difference, with the three types of support (Epx, Gly, and AmG) at different exposure times (8, 24, or 48 h).

The results obtained revealed that drying pre-treatment with commercial silica influenced the immobilization yield in both techniques used and for the different time intervals tested. The most significant yield values observed for each biocatalyst were 28% ± 0.43% for Gly in the desiccator, 45% ± 0.25% for Epx in the oven, and 43% ± 0.39% for AmG in the desiccator. However, studies evaluating commercial silica drying pretreatment techniques to identify differences in immobilization yield are currently absent from the literature.

The assessment of drying time can reveal that, for the shortest interval (8 h), there might be the presence of a certain amount of residual moisture hindering the hydrophobic interaction between the enzyme and the support. Conversely, an extended contact period (48 h) can also be detrimental, particularly in the case of the oven, as evidenced in Figure 4. Immobilization yield outcomes for the 24 h drying pre-treatment period for the AmG support corroborated literature findings that define a fixed drying time [22]. Prior research reports that Gly activated under vacuum at 200 °C achieved immobilization yields of 52.2% for *Candida antarctica* lipase B (CalB). Regarding the Epx functionalized with GPTMS and triethylamine (Et_3N), approximately 100% immobilized yield has been observed for three types of lipases (CalB, Thermomyces lanuginosus lipase (TLL), and Rhizomucor miehei lipase (RML)) [33]. The protocol outlined by Aghaei et al. [34] for immobilization

of CRL onto octyl-silica-epoxy, used in the synthesis of banana flavor and biodiesel production, employed centrifugation and vacuum drying as support treatment stages. In contrast, the Epx support was solely functionalized, excluding a silica drying pre-treatment stage, in the context of biodiesel production from palm oil, as described by Shahedi et al. [35], although the effect of drying time was not evaluated.

The primary rationale for biocatalyst immobilization revolves around functional groups that interact with enzymes. These groups vary in terms of spacer arm size, immobilization pH, density of reactive groups, and blocking stages [14]. The epoxy groups and the carboxylic acid of glyoxyl exhibit lower adsorption capacity due to the presence of oxygen that facilitates interaction with the enzyme [22]. The glutaraldehyde cycles in the AmG support impart a hydrophobic character, promoting ionic adsorption, followed by a covalent bond due to intramolecular interaction between the support's nucleophiles [14].

The size of the synthesized spacer arm differs for each support (Gly has 5 carbon atoms, Epx has 6, and AmG has 9, between the reactive group and the silica matrix). The spacer arm size is linked to stiffness and stability: With more rigidity, there is greater difficulty of mobility of the support to capture the proteins, leading to greater stability of the immobilized biocatalyst. Both Gly and Epx experience increased difficulty in movement due to their shorter spacer arms; nevertheless, this limitation permits heightened stability of the biocatalysts, a pattern that aligns with the existing literature [36].

GPTMS functionalized supports (Epx and Gly) involve a distinct number of immobilization steps. The Epx support necessitates an initial immobilization through physical adsorption at neutral pH, aiming for the potential immobilization of the ET2.0 lipase in its open and more active conformation through hydrophobic interactions via interfacial activation, where non-ionized primary amino groups favorably realize this first interaction [6]. A second step is requisite in alkaline medium (pH 10.0) to facilitate covalent attachment through nucleophiles on the surface of the ET2.0 lipase, such as secondary amino groups, ionic exchangers, and metallic chelates. This alkaline pH step can promote multipoint covalent binding, a phenomenon also observed by other authors [22]. The immobilization of the Gly support occurs in just one step at alkaline pH (10.0) and has the disadvantage of immobilization only with non-ionized amino groups, which can reduce the number of interactions, when compared to Epx [37].

The support functionalized by APTES (AmG) requires activation with glutaraldehyde reagent (crosslinking agent), followed by immobilization at neutral pH (7.0), which favors immobilization with non-ionized primary amino groups, in addition to stabilization of the bonds between the ionizable groups such as lysine and α-amino acids. The terminal amino groups of the enzyme are the most reactive to promote multipoint covalent immobilization with this support. These interactions with primary and terminal amino groups on the enzyme and support produce a heterofunctional support with the ability to perform physical and chemical interactions. [22,38]. The immobilization process promotes an easy first interaction with certain amino acid residues of the enzyme, as mentioned above; however other residues can carry out the interaction, but with greater difficulty, a fact that is governed by the density and reactivity of the enzyme's functional groups at the immobilization pH [37].

The literature lacks studies on the silica-based support drying pretreatment for lipase immobilization. In most processes, commercial silica is directly suspended in solutions containing functionalizing agents. As for silica derived from plant-based materials, only a standardized drying

treatment is observed, typically involving 24 h at a temperature of 40–60 °C. This practice is consistent with the preparation of supports containing functional groups based on epoxy, glyoxyl, and amino-glutaraldehyde [37]. This aspect is notable for its significant influence on immobilization yield, as extensively reported, underscoring the pressing need for a pre-drying treatment for lipase immobilization through the immobilization technique.

3.3. Secondary structure determination by FTIR-ATR

In the infrared spectrum, the vibrations of the amide region (A, B, I to IR) were determined in the region of the 1600−1700 cm^{-1} band, which is mainly correlated with the elongation of the C=O bonds of the peptide, indicating possible changes in the secondary structures of proteins [39]. This range was adjusted using Gaussian curves to identify the contents of α-helices (1650−1658 cm^{-1}), β-sheets (1620−1640 cm^{-1}), β-turns (1660−1680 cm^{-1}), and random coils (1640−1650 cm^{-1}) by area percentage, as shown in Table 2.

Table 2. Percentage compositions of secondary structures of ET2.0 lipase on Epx, Gly, and AmG.

Biocatalyst	α-Helices	β-Sheets	β-Turns	Random coils
ET2.0 lipase	16.50	19.48	11.22	42.81
AmG-6h	24.13	30.43	8.82	26.68
AmG-70h	21.61	28.85	34.42	6.34
Gly	26.35	23.85	30.03	7.28
Epx	26.64	20.37	36.09	7.00

The structure of the α-helix can indicate displacements of the lipase lid region, and low values indicate access of the substrate to the active site is favored [40]. The α-helix percentages in all biocatalysts with supports were greater than with the free enzyme, indicating a conformational change in the secondary structure in the lid region through immobilization, which can hinder access to the active site. These results corroborate the literature, where increases in α-helices occur after the process of immobilization, generally for covalent immobilization [40,41].

In the animo-6h and animo-70h biocatalysts, where their difference was the immobilization time, the biocatalyst with the longest time of immobilization had a decrease in α-helices, from 24.13% (AmG-6h) to 21.61% (AmG-70h). This was possibly due to the inactivation of the active groups remaining on the support after a greater time of immobilization, indicating a complete blocking step of the remaining active groups, allowing a better interaction of the primary and terminal amino groups of the ET2.0 lipase with the carboxyl groups of the AmG support, which has already been better discussed in section 3.2 [42].

The β-sheet region is related to the rigidity of the enzyme. The larger this region is, the more rigid and stable is the biocatalyst. The results obtained indicated that the process of immobilization increased the β-sheets for all biocatalysts, yielding more stable and rigid supports, which can provide greater stability, and biocatalysts with less claim for conformational change when applied under the reaction conditions of a specific reaction [41,43].

3.4. Characterization of the immobilized biocatalyst by AFM, SEM, and Moisture %

The morphological surfaces of the silica together with the prepared supports and biocatalysts were analyzed by scanning electron microscopy (SEM) and atomic force microscopy (AFM), as shown, respectively, in Figures 5 and 6. The micrographs of the supports and biocatalysts was evaluated for observation of the pre-treatment alterations functionalization and post-immobilization. In all images of Figure 5, it is possible to observe morphological patterns, which is to be expected, since all supports are silica-based, both in format and in apparent roughness. For biocatalysts, the appearance or agglomeration of microparticles on the surface is notable and can be the ET2.0 lipase immobilized, causing greater homogenization of the surface [38].

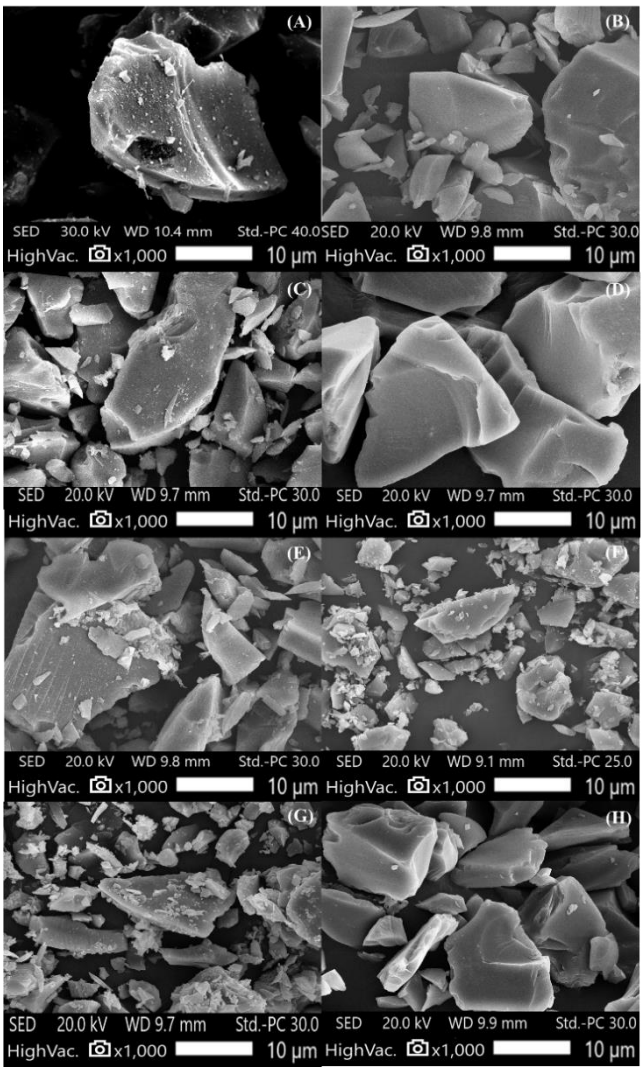


Figure 5. SEM images for (A) silica base; for supports (B) AmG, (C) Gly, and (D) Epx; and for biocatalysts (E) AmG 6h, (F) AmG 70h, (G) Gly, and (H) Epx.

The Epx support (Figure 5D) is the support that has the smoothest and most homogeneous surface of the particles among the supports, and it is also the one that requires the fewest steps for its synthesis. Furthermore, the functionalization process can promote the removal of residues due to the washing

steps or addition of other chemical modification steps, which may be present in the silica (Figure 5A). The Gly (Figure 5C) support, on the other hand, presents less agglomeration of particles, which may be remnants of the preparation processes, where acid hydrolysis and oxidation take place, which can degrade it.

The AFM images (Figure 6) show that the silica treatment steps cause changes to the surface, when evaluated in a certain region, as shown by all the supports presented. The supports Gly and Epx showed the greatest changes, according to Figure 6C,D, due to the number of steps for the synthesis of the final support, as discussed in section 3.2. The roughness increased for all biocatalysts, as shown in Table 3, which may indicate a preferential immobilization of the ET2.0 lipase on the external surface of the support, corroborating the results obtained in Figure 5, in which smaller particles appeared in the images after the immobilization step. Rapid immobilization on the external part is commonly found in immobilizations on mesoporous materials, which is the case with silica-based supports, which can preferentially promote physical adsorption and/or hydrophobic interaction on the external part of the support [18,44].

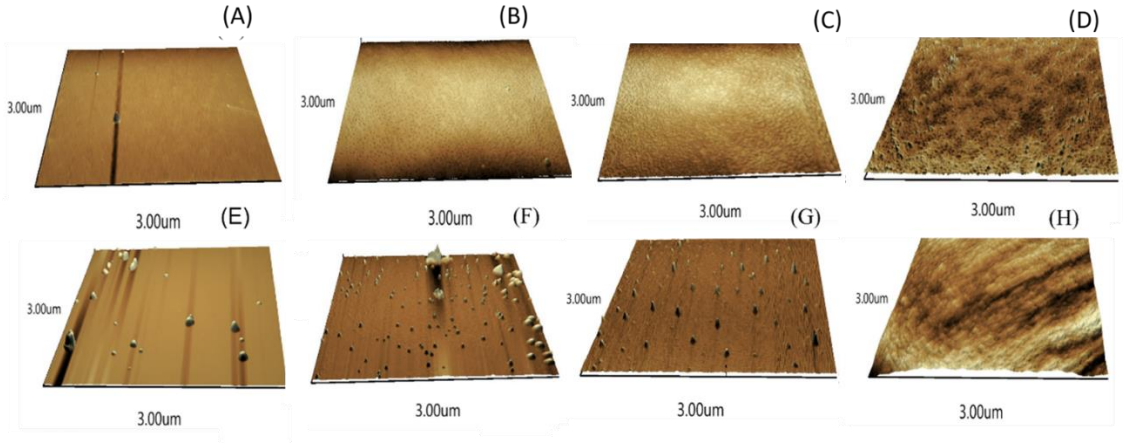


Figure 6. 3D AFM images of (A) silica; all supports, (B) AmG, (C) Gly, and (D) Epx; and Eversa Transform 2.0 lipase immobilized on supports (E) AmG-6h, (F) AmG-70h, (G) Gly, and (H) Epx.

Table 3. Moisture and roughness of biocatalysts pre- and post-immobilization.

Support	Moisture (%) (m/m)		Roughness	
	Pre-immobilization	Post-immobilization	Pre-immobilization	Post-immobilization
silica	-	-	0.86	-
AmG-6h	5.06	5.7	1.03	1.23
AmG-70h	5.15	6.12	1.21	2.09
Gly	0.93	2.67	0.89	2.34
Epx	1.09	3.82	0.49	2.92

Moisture contents are presented in Table 3. The supports functionalized with APTES (AmG-6h and AmG-70h) presented higher moisture contents (around 5%), whereas the supports functionalized with GPTMS (Gly and Epx) presented values around 1%. The supports likely have good microbial resistance due to the low water content, which is increased when the enzyme is incorporated in the

support, due to this molecule having its hydration layer, that presented values between 1 and 6%, results that are in line with those reported in the literature for silica-based mesoporous supports [45,46].

3.5. Esterification with immobilized ET 2.0 lipase

The use of the ET2.0 lipase in its free form in the esterification reaction (section 3.1) was carried out as a preliminary exploration to obtain relevant information about its behavior in the context of the esterification reaction itself to apply and evaluate the catalytic profiles of heterogeneous biocatalysts (ET2.0 lipase immobilized on Epx, Gly, and AmG). Figure 7 shows the catalytic profiles of the biocatalysts over time, with the amount of biocatalysts being set at 10% of the reaction medium for all biocatalysts to reduce diffusional effects and avoid an increase in viscosity of the medium during the reaction, which is common with heterogeneous reaction media [47,48].

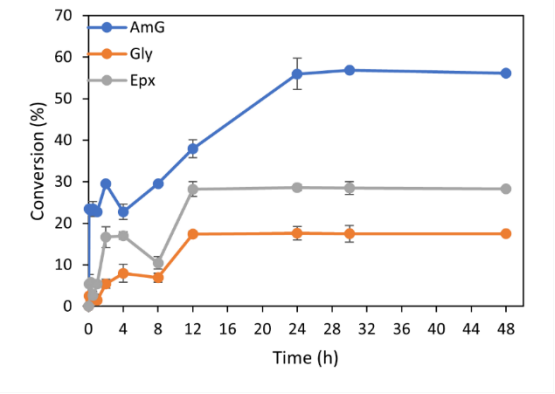


Figure 7. Esterification of *Moringa oleifera* Lam. FFA with isoamyl alcohol catalyzed by ET 2.0 lipase immobilized on Epx, Gly, and AmG (0.22, 0.14, and 0.21 mg of immobilized protein per gram of reaction mixture) supports in equimolar ratio (1: 1) acid: alcohol and with 10% w/w of biocatalyst in solvent-free reaction medium. Reactions were conducted at 40 °C, 300 rpm.

The biocatalysts exhibited maximum performance achievements and distinctive final time spans in the esterification reaction. The Gly and Epx supports reached maximum conversions of 17% and 28%, respectively, within 12 h. Moreover, AmG achieved a conversion of 56% in 24 h, as shown in Figure 7. The discrepancy in the catalytic profiles of each support could be attributed to the quantity of enzyme immobilized on each one (Gly: 28%, Epx: 45%, and AmG: 43%) since the amount of biocatalyst was fixed at 10% of the reaction medium. Biocatalysts with higher immobilization yields promoted superior conversion results, as evidenced in the cases of Epx and AmG [14]. The spacer arm revealed yet another crucial factor, as the two supports, Epx and AmG, each showing similar immobilization yields, exhibited divergent values in the conversion of the esterification reaction (28% and 57%, respectively). It is worth mentioning that supports characterized by longer spacer arms tend to favor substrate capture, an approach that was discussed in section 3.1 [14,49]. Another crucial factor is the orientation in which the enzyme is immobilized on the support, since the two supports (Epx and AmG) have different preferential immobilizations, where Epx can promote interfacial activation on immobilization stage, where amino acids from the lid region can promote immobilization and AmG

by ionic bonding on immobilization stage, where primary and terminal amino acid residues around of enzyme can perform immobilization, which can leave the active site more exposed to the reaction medium [18].

The immobilized ET2.0 lipase presented a different catalytic profile, where at one point in the reaction, the conversion had a decrease in ester production. Several kinetic studies of enzymatic reactions have been performed to explain possible effects and improve the yields of ester production, as is the case of the Michaelis-Menten and Ping Pong bi-bi kinetic models, which explain with good reliability the enzymatic reactions in multiphase systems [1,28,30]. A kinetic model that could well explain the results obtained in Figure 7 is the Ping Pong bi-bi model with inhibition by product, in which, in the course of the reaction, the presence of water formed in the esterification reaction promotes a shift in the equilibrium of the reaction, causing the ET2.0 lipase to perform a hydrolysis reaction, reducing the conversion into esters due to the formation of substrate (acid) and increasing the reaction time [50,51].

The immobilization time is another parameter to adequately stabilize the interactions of the amino acid residues present in the enzyme with the functional groups of the support in the immobilization step, which can promote greater immobilization yield and even better catalytic activities because it affects the stability of the biocatalyst [14,37,52]. The best biocatalyst in the esterification reaction (AmG) was chosen to verify whether the immobilization times of 6 h and 70 h would promote changes in the immobilization yield and consequently in the catalytic profile of the biocatalyst in the esterification reaction for 30 h, as shown in Figure 8.

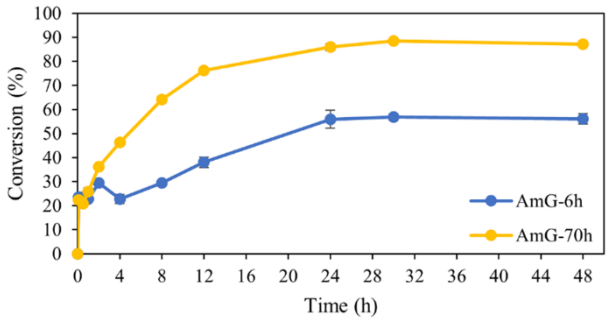


Figure 8. Evaluation of the immobilization time of Eversa Transform 2.0 lipase immobilized on the AmG support (6 h and 70 h) (0.21 and 0.29 mg of immobilized protein per gram of reaction mixture) for the esterification reaction of FFA from MOSO with isoamyl alcohol under equimolar acid:alcohol ratio, 10% w/w biocatalyst, 40 °C, and 300 rpm.

Figure 8 shows that an increase in the immobilization period had a positive impact on the conversion of esters, with the AmG-6h support reaching a maximum conversion of 57% in 30 h, while the AmG-70 h support reached the maximum conversion achieved with the use of free enzyme (approximately 90%). Furthermore, the amount of immobilized protein was quantified, and the immobilization yield increased from 43% to 58% with Amg-70h. These findings support the results evidenced by Serrano et al. [51], in which a prolonged period of immobilization generated an increase in enzymatic stability and an improvement in the catalytic profile, a result that was also achieved in this study.

One factor that may justify the difference in immobilization yield and catalytic activity between the AmG-6h and AmG-70h biocatalysts is the reactivity of the ET2.0 lipase amino acid residues in the immobilization process. The reactivity of the residues is linked to the chemical nature of the enzyme, the microenvironment, and the ionization at the immobilization pH [53]. In this hypothesis, the most reactive amino acid residues may be present in low density on the surface of the enzyme, which may hinder their interaction [14]. The longer immobilization time may be favoring both the higher density residues and the more reactive ones to carry out the interaction, thus stabilizing the remaining residues [53].

The productivity of free and immobilized ET2.0 lipase was evaluated for comparative purposes between the results and is presented in Table 4. In Table 4, the productivity of free lipase E2.0 was selected in the reaction with 2.45 mg/g of enzymatic load, in the molar ratios FFA: Alcohol (1: 1) and (1: 2), obtaining a productivity of 74.8 and 59.8 μmol/h.mg respectively. All immobilized biocatalysts offered for esterification reactions had around 10 times less protein (0.14, 0.22, 0.21, and 0.29 mg/g of immobilized protein per gram of reaction mixture for Gly, Epx, AmG-6h, and AmG-70h, respectively) than that offered in reactions with free ET2.0 lipase.

Table 4. Productivity of ET2.0 lipase, free and immobilized (reactions conducted at 40 °C, 300 rpm, acid/alcohol molar ratios of 1: 1 and 1: 2 for heterogeneous biocatalysts) on different supports with 10% w/w biocatalyst of the total reaction medium considering 24 h of reaction.

Biocatalyst	Productivity (μmol/h·mg)	Conversion at 24 h (%)	Conversion maximum (%)
Free-MR (1: 1)	74.84 ± 1.34	89.88 ± 1.62	89.88 ± 1.62
Free-MR (1: 2)	59.84 ± 0.83	88.95 ± 1.23	88.95 ± 1.23
Gly	141.40 ± 13.38	17.61 ± 1.67	17.61 ± 1.67
Epx	142.76 ± 5.09	28.57 ± 0.72	28.57 ± 0.72
AmG-6h	282.80 ± 26.66	55.93 ± 3.73	57.03 ± 1.54
AmG-70h	333.04 ± 8.30	85.91 ± 1.52	88.56 ± 0.38

ET2.0 lipase immobilized on AmG-70h obtained a productivity of 333.04 μmol/h·mg, showing an improvement of 4.45 times that of free ET2.0 lipase, showing that the immobilization process is advantageous for producing esters coming from MOSO and that the stabilization of the lipase on the support using the immobilization technique considerably improved the catalytic activity, requiring a very low amount of protein to obtain maximum conversion (around 90%) [22]. Barbosa et al. [5] evaluated the productivity of free and immobilized CRL in the same reaction proposed by this work, and the productivity results were around 190 μmol/h.mg, with no variation between the free and immobilized enzyme.

3.6. Operational stability of Eversa Transform 2.0 lipase immobilized on AmG support

The AmG-70h biocatalyst was used to study the operational stability of the esterification reaction proposed in this work. Figure 9 shows that the biocatalyst was effective in the reaction and without significant losses in two cycles, while the third and fourth yields fell by around 10%, reaching 77%, and in the fifth cycle the conversion fell by half, obtaining a conversion of 41% in esters. The biocatalyst remained in reaction for 150 h (four cycles) without losing catalytic activity. Barbosa et al. [5],

in the same reaction proposed in this work, reached an operational stability of 8 cycles with the maximum conversion without significant difference with CRL immobilized by physical adsorption. When the biocatalyst washing/drying step is not performed properly, the active sites of the enzymes can be filled with substrate or reaction product, which can cause a reduction in catalytic activity and ultimately inactivation of the biocatalyst [6,54].

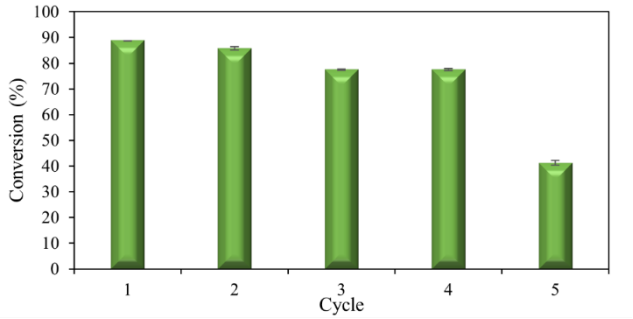


Figure 9. Operational stability of ET2.0 lipase immobilized on AmG-70h support in the equimolar acid: alcohol esterification reaction at 40 °C, 300 rpm, with 10% w/w biocatalyst in the reaction medium for 30 h each cycle in solvent-free systems.

The choice of enzyme used (ET2.0 lipase) plays a pivotal role in this context, which is confirmed by Remonatto et al. [55], where the stability of ET2.0 was higher in higher chain alcohols in the transesterification reaction. The selection of the enzyme can influence how the solvent interacts with the active sites of the ET2.0 lipase, affecting catalytic efficiency and stability throughout the reactions. This consideration for the ET2.0 lipase solvent interaction may contribute to explaining possible variations in the results obtained, as observed in this study. The combination of solvent selection and the inherent properties of the ET2.0 lipase may have played a synergistic role, impacting the stability of the enzyme and the performance during reaction cycles. Results similar to those obtained in this work were found by Remonatto et al. [56] reported that ET2.0 lipase immobilized on Sepabeads-C18 maintained a high ester content in the first cycles (98 and 95%) on the third cycle, the biocatalyst achieved only 20% on the conversion of ethyl esters on reaction transesterification. This result represents the need for further studies with immobilized lipase ET2.0 to improve operational stability, as was obtained in this study.

3.7. Characterization by FTIR of esters obtained by hydroesterification reaction

According to the IR (infrared) spectrum, the main spectroscopic profiles of MOSO, the hydrolysis product, and the purified esters of this work were observed and are represented in Figure 10. The FTIR technique can present the spectroscopic profile of the material that is subjected to analysis. The technique allows one to obtain information on which types of interactions are present in the sample according to certain wavelengths, as expressed by excitations in these regions [57]. An area of interest to explain the changes between the extraction, hydrolysis, and esterification steps is the region 3500–3300 cm^{-1}, a band that is related to the presence of hydroxyl groups (O–H), and a decrease in transmittance (band increase) can be noted when comparing the spectra of the oil and the fatty acid due to the possible presence of water in the hydrolysis product. As for the esterification product, the

profile is similar to that of oil, as the product was purified and only the organic phase [58]. The band in the region from 3000 to 2800 cm^{-1} is represented by the symmetric and asymmetric stretching of the C–H bond, indicating the presence of methyl (CH_3), methylene (CH_2), and methine (CH) groups, which in the products obtained present the presence of this interaction [57].

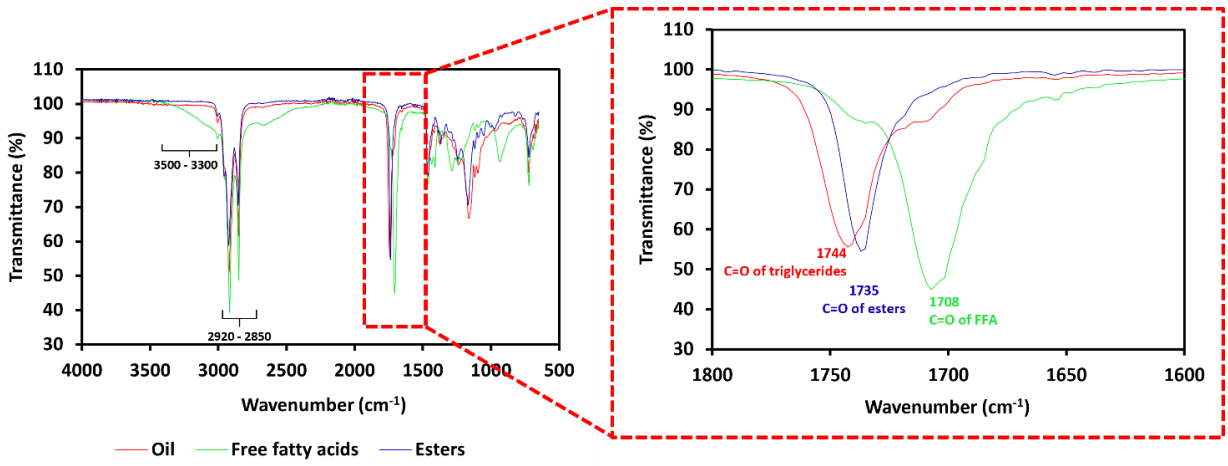


Figure 10. FTIR of the biotransformation of *Moringa oleifera* Lam. seed bioproducts.

The region from approximately 1750 to 1690 cm^{-1} represents carbonyl bonds (C=O). This bond was present in all the products evaluated (oil, hydrolysis product, and esterification product). However, the stretching band for each has a different intensity (oil = 1744 cm^{-1}, AGL = 1708 cm^{-1}, and esters = 1735 cm^{-1}), with an intensity increase from oil to FFA and a decrease from FFA to esters, resulting from the biotransformation between the extraction, hydrolysis, and esterification stages of MOSO, with their respective purification stages [59,60]. Thus, by means of the FTIR spectrum of the products obtained, it has become possible to prove the biotransformation of the MOSO. Initially, the oil is transformed into FFA, through the enzymatic hydrolysis reaction and later, into esters with potential for the application of possible biolubricants [5], biodiesel [61], between others.

4. Conclusions

The main objective of the present study was to obtain esters from MOSO through enzymatic biocatalysts ET2.0 lipase, a low-cost enzyme, immobilized on new silica-based biocatalysts modified by different functionalizing agents through esterification. The drying pretreatment showed significant differences in relation to the immobilization yield for silica-based supports, which has not yet been reported in the literature. The study of free ET2.0 lipase aimed to obtain initial working conditions for the application of immobilized biocatalysts, with AmG-70 being the support that had the best conversion of around 90% in 30 h among the biocatalysts evaluated, and its productivity was around 4 times greater than the free enzyme. The AmG-70 support had a longer immobilization time to better stabilize the enzyme on the support and completely carry out the blocking step of the other active groups, also promoting a greater immobilization yield, obtaining a biocatalyst with high catalytic activity until the fourth reaction cycle. This work aims to fill gaps in the production of biolubricants synthesized by an environmentally friendly route, using vegetable oil and showing the

applicability of new biocatalysts, opening possibilities for new studies for a better understanding of the types of interactions that occur between heterofunctional supports and enzymes. This work also aims to open the possibility of using other lipases in the immobilization process such as the use of combilipases, while also allowing the use of other vegetable oils for the production of esters for other purposes, as well as the use of biocatalysts on an industrial scale in continuous mode, as these processes generally have silica in packaged bed reactors to promote biocatalysis and bioproduct generation.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Conflict of interest

The authors declare no conflict of interest.

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Author contributions

The corresponding author declares the contributions of individual authors in the article: Conceptualization, Cleide M. F. Soares and Elton Franchesch; methodology, Wagner C. A. Carvalho and Rayane A. S. Freitas; formal analysis, Adriano A. Mendes and Milson S. Barbosa; investigation, Cleide M. F. Soares and Adriano A. Mendes; resources, Cleide M. F. Soares, Wagner C. A. Carvalho, and Rayane A. S. Freitas; data curation, Cleide M. F. Soares, Wagner C. A. Carvalho, and Rayane A. S. Freitas; writing—original draft preparation, Wagner C. A. Carvalho and Rayane A. S. Freitas; writing—review and editing, Cleide M. F. Soares, Adriano A. Mendes, Milson S. Barbosa; visualization, Ernandes B. Pereira and Ariela V. Paula; supervision, Cleide M. F. Soares and Adriano A. Mendes; project administration, Cleide M. F. Soares; funding acquisition, Cleide M. F. Soares. All authors have read and agreed to the published version of the manuscript.

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