CATALYTIC PERFORMANCE OF LIPASE EVERSA® TRANSFORM 2.0 IN HYDROESTERIFICATION REACTION AND SPECTROSCOPY STUDIES

Wagner C. A. Carvalho[1]*, Rayane A. S. Freitas[1], Adriana de J. Santos[1,2] Ranyere L. de Souza[1,2], Ernandes B. Pereira[3], Adriano A. Mendes[4], Elton Franceschi[1,2] & Cleide M. F. Soares[1,2]

[1] *Universidade Tiradentes (UNIT), Av. Murilo Dantas, 300, Aracaju, SE, Brazil*
[2] *Instituto de tecnologia e pesquisa (ITP), Av. Murilo Dantas, 300, Aracaju-SE, Brazil*
[3] *Universidade Federal de Alfenas (UNIFAL-MG), Instituto de Química, R. Gabriel Monteiro da Silva, 700, Alfenas, MG, Brazil*
[4] *Universidade Federal de Alfenas (UNIFAL-MG), Faculdade de Ciências farmacêuticas, R. Gabriel Monteiro da Silva, 700, Alfenas, MG, Brazil*
** Corresponding author's email address: wag.carlos@hotmail.com*

ABSTRACT

The objective of this study is to understand the catalytic potential of immobilized lipase Eversa® Transform 2.0 (ET2.0) via physical adsorption on silica-based heterofunctional supports (hybrid support = Bi.Hyb and inorganic support = Bi.Inor) in hydroesterification reaction (hydrolysis + esterification) of *Moringa oleifera* seed oil (MOSO). Their catalytic performance were compared with its free form. FTIR spectroscopy analysis was used to investigate changes in the secondary structure of both free and immobilized lipase. Free ET2.0 showed a maximum hydrolysis percentage of the oil around 40%, while both immobilized biocatalysts showed hydrolysis percentage around 30% after 180 e 240 min for Bi.Hyb and Bi.Inor h respectively . However, both immobilized biocatalysts achieved the highest free fatty acids (FFA) conversion percentage than free ET2.0 (74,95% conversion), along with a reduced time to reach the static phase, decreasing from 24 hours (above 82%) after 4 and 8 h for Bi.Hyb and Bi.Inor h respectively. FTIR spectroscopy analysis revealed that β-sheet region increased after the immobilization process, making the biocatalysts more rigid and stable for application in hydrolysis and esterification reactions. The immobilized biocatalysts exhibited a reduction in α-helix, which could make the biocatalysts more active by exposing the active site to the reaction.

Keywords: Conformational change. Eversa® Transform 2.0. Immobilization. Hidroesterification

1 INTRODUCTION

The immobilization process of enzymes becomes advantageous due to the potential to mitigate the effects of using free enzymes. Immobilized lipases can gain greater thermal and mechanical stability, along with the possibility of recovering the immobilized biocatalyst from the reaction medium for reuse in multiple reaction cycles, making the process of using enzymatic reactions more advantageous and facilitating the transition from chemical to enzymatic routes[1].

The choice of support for the immobilization process to be effective ensures the step is performed properly and allows for possible enzyme activation. The suitability of the support varies concerning the immobilized lipases, as each can interact with different amino acid residues of the lipase with the functional groups present on the support surface. Heterofunctional supports are highlighted for their ability to promote different types of enzymatic interactions due to the presence of various functional groups, enabling interactions through physical adsorption, ionic, and/or covalent[2]. Supports can be organic, inorganic, or hybrid. Hybrid supports can be classified as heterofunctional supports due to the presence of different functional groups, as a hybrid support can have both an organic and an inorganic part. This fact allows for a reduction in biocatalyst costs and even the reuse of plant material that would otherwise be discarded for the incorporation of the organic part in the synthesis of a hybrid support[3].

Besides the choice of support, one of the variables that complicate the use of enzymes on an industrial scale is the choice of lipase is very lower production cost, such as lipase Eversa® Transform 2.0[1] [1]. FTIR spectroscopy was used to investigate changes in the secondary structure of the lipase. Structural changes can increase or decrease the stability of proteins and, consequently, promote their activation or inactivation[4]. Therefore, it is important to understand the effects of lipase immobilization techniques on different supports. Based on the issues discussed, this work study reports the catalytic potential of Eversa® Transform 2.0 (ET2.0) free and immobilized by physical adsorption on two different supports (Bi.Hyb and Bi.Inor) in a two-step hydroesterification reaction (hydrolysis + esterification), along with the elucidation of possible changes in the secondary structure of the lipase after the immobilization process by deconvolution using Fourier Transform Infrared (FTIR) spectra.

2 MATERIAL & METHODS

Mature seeds of *Moringa oleifera* Lam. (MOSO) were provided by the Brazilian Agricultural Research Corporation (EMBRAPA), separated by specific particle sizes, and used for oil extraction. The organic matter remaining after extraction was then separated into specific particle sizes (100-150 mesh) for use in preparing the hybrid support.

Initially, two supports were prepared via Tetraethoxysilane (TEOS) polycondensation, one silica-based support (inorganic support) and the other using silica together with the residue from the extraction of *Moringa oleifera* Lam. seed oil (hybrid support) using the sol-gel technique according to methodology described in the literature[6]. The immobilization stage was carried out according to the methodology established in the literature with minor modifications[5]. The Eversa® transform 2.0 was kept in a 5mM sodium phosphate buffer solution, pH 5.0together with the support for the synthesis of the two biocatalysts used in this work, the inorganic support named Bi.Inor and the hybrid support named Bi.Hyb. The enzyme loading used was 5 mg of protein per g of support. The suspension was kept under stirring at 200 rpm on an orbital shaker for 24 hours. The prepared heterogeneous biocatalysts were filtered under vacuum in a Buchner funnel with Whatman filter paper no. 41, washed thoroughly with distilled

water and stored at 4ºC for 24 hours and the biocatalysts were characterized by the Bradford method and the immobilization yield of Bi.Inor was 71% and Bi.Hyb was 82%.

Free and immobilized ET2.0 (Bi.Inor, Bi.Hyb) were evaluated in the hydrolysis reaction of MOSO using an initial protein loading of 0.8 mg of protein/g of oil under reaction conditions described by BARBOSA[5]. For total conversion of MOSO into free fatty acid (FFA) to be used as raw material, a non-specifc lipase from *Candida rugosa* was used as biocatalysts. The complete hydrolysis was conducted at of 37°C, 1000 rpm, 25% oil, 75% water, and 550 U/g oil for 75 min, following the methodology of BARBOSA[4].

The esterification of the resulting FFAs with isoamyl alcohol catalyzed by immobilized ET2.0 on was conducted according to the methodology described in the literature[5]. The reactions were carried out in a glass reactor under experimental conditions of 300 rpm and 40°C, with molar ratios of 1:1 FFA:alcohol and an enzymatic loading of 4.5 mg of protein in the reaction medium.

The supports and biocatalysts from the functionalization/activation of silica were characterized by scanning electron microscopy (SEM) (JEOL JSM - IT 200) through the dehydration of samples and coatings of particles for analysis. After, Fourier transform infrared spectroscopy (FTIR) spectra of supports and immobilized biocatalysts were obtained using a Cary 630 FTIR spectrometer (Agilent Technologies, Germany) with an ATR accessory. Spectra were collected in the range of 4000 to 650 cm^{-1} with a spectral resolution of 4 cm^{-1}, and 32 scans were averaged for each spectrum. Secondary structure analysis was performed on spectra ranging from 1700 to 1600 cm^{-1}. Analysis of secondary structure changes involved secondary derivative and peak deconvolution using Origin software version 8.5.

3 RESULTS & DISCUSSION

A two-step hydroesterification reaction (hydrolysis + esterification) of MOSO catalyzed by free and immobilized ET2.0 on heterofunctional support was conducted in order to evaluate their catalytic activities. The reaction conditions in the hydrolysis *Moringa oleifera* Lam seed oil followed those described by described by Barbosa[5], with some modifications outlined in Figure 1A

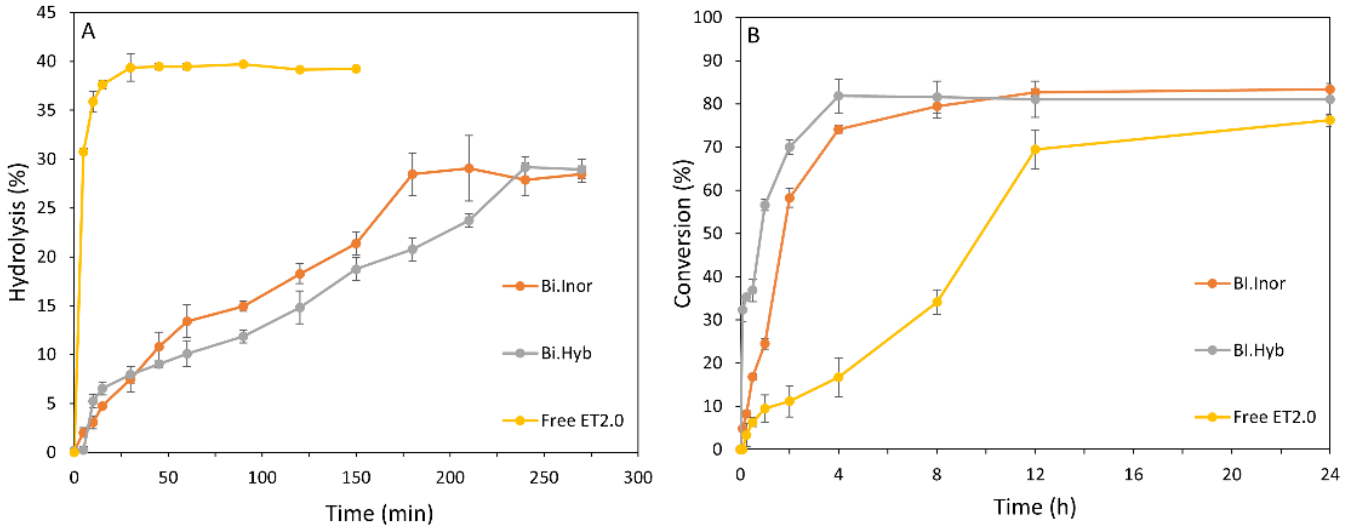

Figure 1. Hydrolysis activity of free and immobilized ET2.0 in *Moringa oleifera* Lam. oil. Reaction conditions: mass ratio 25% oil, 75% water, 37°C, 1000 rpm, enzymatic loading of 0.8 mg of protein/g of oil for free and immobilized ET2.0 in the reaction medium (A). Catalytic activity of free and immobilized ET2.0 in the esterification reaction of FFA from *Moringa oleifera* Lam. oil with isoamyl alcohol. Reaction conditions: molar ratio (1:1) acid:alcohol, 40°C, 300 rpm, enzymatic loading of 5 mg of protein for each reaction (B).

Free ET2.0 exhibited a maximum conversion of 39.36 ± 1.39% within 30 min of reaction (Fig. 1A). After verifying the potential of the free enzyme, the immobilized biocatalysts were prepared and tested under the same reaction conditions. On the other hand, the two immobilized biocatalysts showed a lower hydrolysis percentage than the free enzyme using the same amount of protein in all assays (around 30% in approximately 3 h). The hydrolysis reaction time for the immobilized biocatalysts was longer than the free enzyme due to possible problems with diffusive effects[1].

These results corroborate studies reporting that ET2.0, a genetically modified TLL expressed in *Aspergillus oryzae*, is a sn-1,3 specific lipase[8]. Therefore, a non-specific lipase from *Candida rugosa* was used to fully convert MOSO into FFAs to be used as raw material in esterification step with isoamyl alcohol

The catalytic potential of free and immobilized ET2.0 on different heterofunctional supports was evaluated in esterification reaction, as shown in Figure 1B. Free ET2.0 achieved a maximum yield of 76.21 ± 1.42% in 24 hours. All immobilized biocatalysts showed better catalytic performance than the free enzyme. Bi.Inor achieved a conversion of 79,46 ± 2,68% in 8 hours, and Bi.Hyb achieved 81.84 ± 3.95% in 4 hours. The immobilization process on the supports may have promoted interfacial activation by physical adsorption, which could have led to greater exposure of the enzyme's active site, thereby enhancing its catalytic activity[1]. The difference in time to reach the stationary stage of the reaction between the supports could be due to the possibility of additional interactions between the enzyme and the support beyond physical adsorption.

The use of Bi.Hyb in the esterification reaction reduced the time by 6-fold compared to the free enzyme. This indicates that using a support where part of it is eco-friendly and derived from biological material is suitable for the immobilization process, thus reducing the cost of biocatalyst synthesis[1]. ET2.0 exhibited good catalytic efficiency in the esterification reaction in organic medium, which aligns with its initial purpose, which was to be used in transesterification reactions for biodiesel synthesis[8].

Barbosa[9] evaluated of the same esterification reaction proposed in this work was conducted using CRL for the immobilization process. It was found that approximately 30% of the total cost of the process is related to the cost of the biocatalyst. Reducing the cost of the biocatalyst could promote greater economic viability for the process. The proposed work uses a genetically modified enzyme (ET2.0) with a much lower cost than CRL, which could facilitate the transition from chemical to enzymatic routes.The biocatalysts were characterization 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, where it

indicates possible changes in the secondary structure of proteins[4]. This range was adjusted using Gaussian curves to identify the contents of the α-Helix (1650-1658 cm-1), β-Sheet (1620-1640 cm-1), β-Turn (1660-1680 cm-1), and random coil (1640-1650 cm-1) by area percentage, as shown in Table 1[10].

Table 1 Percentage composition of secondary structure of Eversa® Transform 2.0 immobilized on Bi.Inor and Bi.Hyb

Suport	β-Sheet	Random Coil	α-Helix	β-Turn
Bi.Hyb	23,60	31,12	15,44	17,59
Bi.Inor	24,15	41,15	15,80	13,34
Free	19,48	42,81	16,5	11,22

The immobilization process promoted an increase in the β-sheet region for both biocatalysts evaluated in this study, compared to the free enzyme. The β-sheet is associated with rigidity; the larger the region, the less prone it is to conformational changes in the reaction medium4. These values corroborate the literature, indicating that the immobilization process aims to produce more rigid biocatalysts, and this increased rigidity can make biocatalysts more stable when applied in chemical reactions. The α-helix is associated with the lid region of the lipase; the smaller its value, the greater the interaction with the lid region, which can facilitate substrate access to the enzyme's active site, resulting in higher catalytic activity[10,11]. For the α-helix, the immobilization step led to a reduction in the area of this structure for both immobilized biocatalysts, corroborating the results obtained in the occurrence of esterification.

4 CONCLUSION

This study shows that ET2.0 immobilized on a hybrid support (Bi.Hyb) produced the best biocatalyst for esterification (81.84 ± 3.95% conversion in 4 hours), allowing for a substantial reduction in the cost of biocatalyst synthesis and time, resulting in higher productivity. Additionally, it utilizes a byproduct of Moringa oleifera Lam. that would otherwise be discarded for support preparation. This work opens up possibilities for further studies on this biocatalyst, including thermal and reaction stability, enzyme loading test, desorption test, as well as the possibility of using the organic support as a means of enzyme immobilization. The immobilization process yielded more rigid biocatalysts, with an increase in the β-sheet region and greater interaction in the lipase lid region due to the decrease in α-helix, facilitating substrate access to the enzyme's active site. ET2.0 needs to be used in conjunction with other lipases for its use in hydrolysis reactions to be advantageous and to achieve a conversion close to 100%.

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