DEVELOPMENT OF MODIFIED ENZYMATIC BIOCATALYSTS FOR APPLICATION IN BIODIESEL PRODUCTION

Isabela O. Costa[1]*, Luciana R. B. Gonçalves[2], Everaldo S. dos Santos[1] & Nathália S. Rios[1]

[1] *Departamento de Engenharia Química, Universidade Federal do Rio Grande do Norte, Natal, Brasil.*
[2] *Departamento de Engenharia Química, Universidade Federal do Ceará, Fortaleza, Brasil.*
** costaisabela@hotmail.com*

ABSTRACT

The demand for eco-friendly technologies in industries has led to the rise of enzymatic technology, which has been gaining prominence for replacing traditional chemical methods. To optimize large-scale applications, enzyme immobilization approaches have been developed, enhancing biocatalyst qualities and optimizing the process efficiently and economically. For biodiesel, immobilization emerges as a potent strategy due to its ability to create modified and multienzymatic biocatalysts capable of taking highly heterogeneous substrate (oils and fats) with greater efficiency. In this study, biocatalysts utilizing lignocellulosic residues as supports for immobilization and employing modifying agents were developed to facilitate applications in biodiesel synthesis. Three lipases were chosen and immobilized on green coconut fiber. It was followed by modifications (with polyethyleneimine (PEI) and glutaraldehyde (GA)) to enhance their resistance and facilitate the formation of layers in co-immobilized biocatalyst construction. Thermal stability tests revealed that modifications were advantageous for all enzymes, since the half-lives for biocatalysts modified with GA and PEI were above 48 hours of incubation at high temperatures. These alterations also minimized enzyme desorption. The maximum enzyme load was able to be determined (CALB: 5 mg/g; PFL and EVERSA: 10 mg/g). These positive results further strengthen the viability of their application in biodiesel production.

Keywords: Biodiesel. Lipases. Immobilization.

1 INTRODUCTION

Environmental pollution from fossil fuels is increasing, leading many countries to turn to renewable energy sources like biofuels [1]. Biodiesel is one such renewable, clean, and eco-friendly liquid fuel derived from lipids, mainly through transesterification, in the presence of a suitable catalyst [2]. Enzymatic catalysis by lipases provides an alternative route to the environmental impact caused by chemical routes. With high conversion efficiency, enzymes offer a cleaner technology due to easier separation of the product and glycerol (byproduct), elimination of side reactions, and operation at lower temperatures, consuming less energy and contributing to pollution reduction [3].

The decisive factor in the application of lipases is their specificity where they act on the ester bonds of the triglyceride molecule (broad specificity, specific for positions 1 and 3, or specific for position 2) [4]. This characteristic is interesting in the production of biodiesel since the raw materials used are often highly heterogeneous, then being interesting the combination of various types of lipase to enhance the efficiency of the production process [5]. For the present study, the following lipases will be employed: Lipase B from *Candida antarctica* (CALB), lipase from *Pseudomonas fluorescens* (PFL), and Eversa® Transform 2.0 lipase. As a way of improving, an enzyme immobilization strategy can be applied to improve enzyme stability, facilitate its recovery and reuse, and provide protection against the inhibitory effects of alcohol and glycerol produced in biodiesel synthesis [3]. Immobilization typically occurs on a solid support, including lignocellulosic residues such as green coconut fiber, a renewable waste resource.

Thus, this work will aim to develop biocatalysts using green coconut fiber as a support for immobilization and modified with polyethyleneimine (PEI) and glutaraldehyde (GA) to investigate which biocatalyst is better suited for biodiesel synthesis results. This may enable the reuse of enzymes in the reaction, and the use of residues in this process contributes to the circular economy context.

2 MATERIAL & METHODS

Biocatalysts production: A solution of each lipase 0,05 mg/mL in 5 mM sodium phosphate buffer, at pH 7.0 was put in contact with the green coconut fiber (CF) (pre-treated with steam explosion 210 °C, 20 bar 10 minutes) to perform the immobilization (24h/48h, at 24 rpm and 25°C).

Biocatalysts modifications: Firstly, a coating was carried out with a solution of Polyethyleneimine (PEI) (10% w/v, pH 7, at 4°C, 130 rpm for 18h). It was also produced by first crosslinking it with glutaraldehyde (GA) solution (1% v/v, 50 mM sodium phosphate buffer, pH 7, 25 °C, 24rpm for 1h) and then coating it under the conditions already presented.

Biocatalysts evaluation: Lipase activity was spectrophotometrically measured by the hydrolysis of p-nitrophenylbutyrate (pNPB) at 348 nm with a reaction time of 1,5 min. The thermal stability assay was performed using Tris-HCl buffer, pH 7.0, 50 mM at 60 °C, 75 °C and 80°C. Desorption assay was performed by incubating the biocatalysts in NaCl solution (1M in 50 mM Tris-HCl

buffer, pH 7 for 2h) and Triton X-100 (1% v/v in 50 mM Tris-HCl buffer, pH 7 for 4h). The maximum enzyme load test was carried out with different lipase immobilization loading (1m/g, 2mg/g, 5mg/g, 10 mg/g, and 20 mg/g) and performed in the ideal immobilization conditions settled previously.

3 RESULTS & DISCUSSION

The lipases were sucessuly immobilized and reached an immobilization yield of 87,62 ± 4,34%, 82,01 ± 2,02% and 77,47 ± 1,88% for CALB, PFL and EVERSA, respectively. With immobilization, it is expected that enzymes become more resistant to temperature effects, delaying enzyme structure denaturation. This resistance can be compared by the enzymatic decay curve of the biocatalyst with the free enzyme (Figure 1). The initial activity is set as 100%.

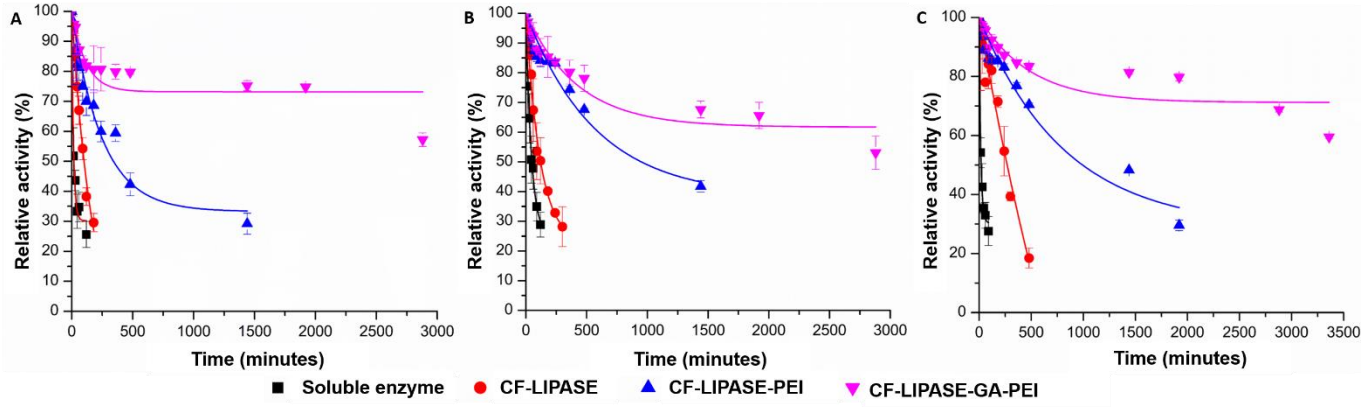

Figure 1 Thermal stability assay. (A. CALB; B. PFL; C. Eversa ®). The thermal stability assay was performed using Tris-HCl buffer, pH 7.0, 50 mM at 60 °C (CALB), 75 °C (EVERSA) and 80°C (PFL).

It is possible to observe that all lipases immobilized by adsorption showed greater resistance (red line), meaning the activity took longer to decrease compared to soluble enzymes (black line). This difference becomes more evident when treatments are added (blue and pink lines). The comparison can also be through half-life time, which is the time required to reach half of the initial activity, as shown in Table 1.

Table 1 Half-life of biocatalysts obtained from thermal stability assay.

Lipases	Soluble enzyme	CF-LIPASE	CF-LIPASE-PEI	CF-LIPASE-GA-PEI
CALB (60 °C)	18 min 36 sec	1 h 41 min	5h 43 min	> 48 h
PFL (80 °C)	49 min 07 sec	2h 01 min	15h 52 min	> 48 h
EVERSA (75 °C)	19 min 28 sec	4h 29 min	16h 29 min	> 56 h

For CALB, the increase in half-life time for immobilization by adsorption was 5.4 times, after treatment with PEI it increased by 18.5 times, and with the addition of glutaraldehyde, after 48 hours, the immobilized still maintained 57.2 ± 2.2% of its initial activity. Regarding PFL, immobilization provided a 2.5-fold increase, with PEI it had an improvement of 19.4 times, and in the presence of glutaraldehyde, it maintained 53 ± 5.6% after 48 hours. Immobilized EVERSA became 14 times more resistant and with PEI 51 times. 59.52 ± 0.6% of the initial activity of immobilized with GA and PEI remained after 56 hours.

From the desorption assay, it is possible to have a suggestion of the type of enzyme-support interactions. That is, a greater reduction in activity after incubation with Triton X-100 or NaCl indicates a predominance of hydrophobic or ionic interactions, respectively. The results of this assay are presented in Table 2.

Table 2 Activity retained after desorption assay.

Biocatalyst	Activity retained (%)	
	Triton (1% v/v, 4h)	NaCl (1M, 2h)
CF-CALB	77,98 ± 5,25	73,03 ± 5,00
CF-CALB-PEI	52,90 ± 2,33	55,81 ± 2,70
CF-CALB-GA-PEI	48,13 ± 1,20	96,57 ± 1,64
CF-PFL	74,36 ± 4,54	85,79 ± 1,66
CF-PFL-PEI	38,35 ± 1,44	68,88 ± 3,12
CF-PFL-GA-PEI	62,47 ± 0,28	96,16 ± 0,26
CF-EL	36,57 ± 1,36	83,70 ± 0,95
CF-EL-PEI	38,89 ± 0,28	69,28 ± 0,33
CF-EL-GA-PEI	94,12 ± 0,40	79,59 ± 0,58

For CF-PFL and CF-EL were observed a lower activity retention after incubation with Triton X-100 (1% v/v, 4h), 74,36 ± 4,54% and 36,57 ± 1,36%, compared to 85,79 ± 1,66% and 83,70 ± 0,95% after incubation with NaCl (1M, 2h), respectively. This indicates a slight predominance of hydrophobic interactions, mainly in the CF-EL sample. However, CF-CALB showed a similar behavior after the incubations (activity retention: 77,98 ± 5,25% and 73,03 ± 5,00%, after incubation with Triton X-100 or NaCl, respectively), indicating the predominance of the two types of interactions.

The results following treatment with PEI suggested that this agent might facilitate the desorption of immobilized enzymes. Therefore, covalent cross-linking treatment with glutaraldehyde was tested before modification with PEI. With this alteration, as expected, we noted a higher resistance to enzyme desorption, as the retained activity is greater or comparable to that of enzymes immobilized by adsorption. In summary, eight of the biocatalysts tested showed that hydrophobic or mixed interactions (CF-CALB)

are more predominant, with CF-CALB-GA-PEI being the only one with predominance for the ionic bonding character. These interactions have significant force since no samples without enzymatic activity were obtained after the desorption tests.

To assess the maximum enzyme load that can be immobilized on green coconut fiber, immobilization assays were conducted for different initial concentrations of lipases. This parameter is crucial for the production of co-immobilized biocatalysts and practical application. With increasing concentration, it is expected that the activity of the immobilized enzyme would also increase. However, as observed in Figure 2, beyond a certain load, the activity value ceases to be proportional to the increase in load.

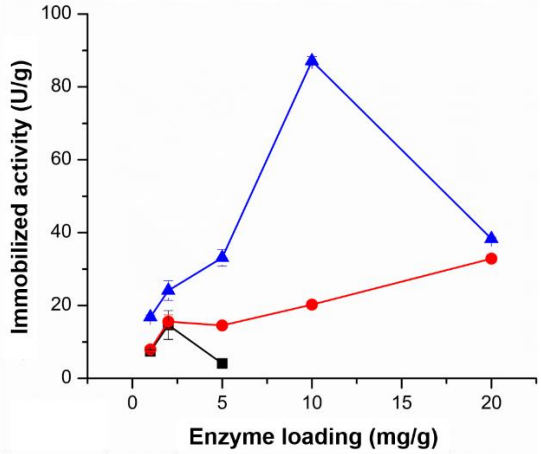

Figure 2 Immobilization potential in green coconut fiber. CALB (black line); PFL (red line); Eversa ® (blue line).

For CALB, at a load of 5 mg/g, a deviation from this expected proportionality is already evident, and the same behavior is observed for EVERSA at a load of 20 mg/g (the immobilized activity values can be observed in Table 3). For PFL, although there is a trend of increased activity of the immobilized enzyme at a load of 20 mg/g, it does not correspond to the expected increase. At a load of 1 mg/g, the activity of the immobilized enzyme was 7.92 ± 1.00 U/g. Therefore, for a load of 20 mg/g, a value approximately 20 times higher in activity was expected; however, it was 32.87 ± 0.29 U/g. This difference is likely due to diffusion issues, which is why higher loads were not tested.

Table 3 Immobilized activity (U/g) in different enzyme loadings.

Enzyme loading (mg/g)	CALB	PFL	EVERSA
1	7,43 ± 0,39	7,92 ± 1,00	16,84 ± 0,58
2	14,65 ± 3,93	15,85 ± 1,28	24,1 ± 2,67
5	4,11 ± 0,13	14,53 ± 0,25	33,14 ± 2,26
10	-	20,26 ± 0,75	87,13 ± 1,17
20	-	32,87 ± 0,29	38,31 ± 0,46

Based on the results obtained, the optimal loadings for CALB, EVERSA, and PFL catalysts have been determined. The maximum load for CALB was set at 5mg/g, while EVERSA and PFL have a load of 10mg/g each. These loadings will serve as the foundation for the application of these catalysts in the biodiesel production process.

4 CONCLUSION

In view of this, the technology of using modified enzyme biocatalysts has shown effective and favorable capacity in the conversion of oil into biodiesel by the positive results obtained in thermal stability and desorption tests after the modifications. These findings could aid in the development of multienzymatic systems featuring lipases immobilized on coconut fiber, being individually immobilized or co-immobilized. This novel approach and its application in biodiesel synthesis have not been previously documented, revealing its innovative potential.

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