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EVERSA® TRANSFORM 2.0 LIPASE IMMOBILIZATION ON CHITOSAN-BASED SUPPORTS CROSSLINKED WITH SUSTAINABLE ALDEHYDES

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Abstract

Enzymes are biocatalysts used industrially for producing high-value products such as biofuels, flavors, fragrances and pharmaceuticals. However, their low stability and complex reusability in soluble form limit their large-scale application. Enzyme immobilization offers an alternative to enhance their industrial use, enhancing its stability and promoting their reutilization. Despite numerous studies on optimizing support functionalization to maximize immobilization efficiency, the reagents typically used are often toxic and environmentally unfriendly, such as glutaraldehyde, which, while effective, is neither eco-friendly nor biocompatible. Thus, there is a pressing need for sustainable protocols in this context. This study aimed to evaluate the immobilization of Eversa® Transform 2.0 lipase on a chitosan-based support chemically crosslinked with different aldehydes: glutaraldehyde, octanal, and dodecanal. The immobilization process involved suspending the enzyme in 5mM sodium phosphate buffer (pH 7.0) for 1 hour at 25°C under agitation. Enzymatic activity was assessed using p-nitrophenyl butyrate (ρNPB), soybean oil, and olive oil. The results showed varying outcomes depending on the aldehyde used, with octanal-functionalized biocatalysts exhibiting higher activity (35.4 U/mL) compared to glutaraldehyde for the hydrolysis of olive oil. This study highlights more environmentally friendly alternatives for support functionalization and biocatalyst production.

Keywords: Lipase immobilization, Support funcionalization, Chitosan hydrogel beads, Octyl and dodecyl aldehydes.

1. Introduction

Enzymes are important alternatives to traditionally applied chemical catalysts. They tend to perform efficient and more environmentally friendly catalysts, being applicable in food, pharmaceuticals, and energy industries. Lipase are among the most industrially applied enzymes, especially in the production of fatty esters of industrial interest, by the hydrolysis of vegetal oils, for the production of fragrances and biodiesel [1].

Though efficient, enzymes usually present lower stability than chemical catalysts, as they are more sensitive to temperature and pH changes, and present lower reusability in their soluble form, hindering their industrial applications. Thus, the immobilization of enzymes is a commonly used method, which results in the aggregation of enzymes into solid surfaces, usually by adsorption, and entrapment of covalent bindings, resulting in stabler and easier to recover biocatalysts.

However, glutaraldehyde, one of the most applied crosslinking agents for enzyme immobilization, presents low ecological appeal, as it presents high potential for water contamination, high acute toxicity for aquatic organisms (median lethal concentration of 7 mg/L), especially algae (<1 mg/L) [2]. Other aldehydes have been applied as crosslinking agent for enzyme immobilization, such as glyoxal and glyceraldehyde, due to their lower toxicity in comparison to glutaraldehyde, however, it has presented lower efficiency [3]. Thus, new research must seek to evaluate the applicability of other less toxic and more effective cross-link agents.

In this context, this work aims to evaluate the potential of octanal and dodecanal as cross-linking agents for lipase immobilization. After the immobilization essays and biocatalyst

characterization, its potential for vegetal oils hydrolysis was also evaluated and compared to glutaraldehyde cross-linked ones. The production of these octyl or dodecyl-aldehyde cross-linked biocatalysts presents new perspectives over lipase immobilization, resulting in safer methods, which are inclined to the green chemistry and sustainable practices.

2. Methodology

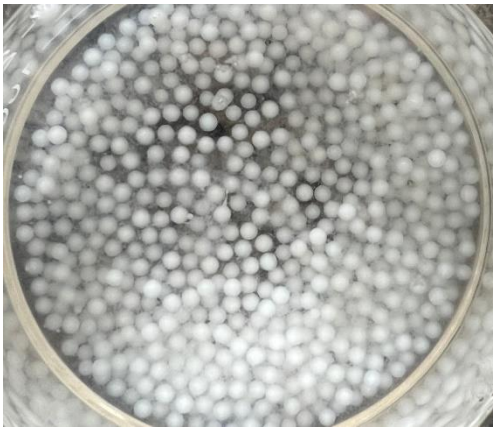
2.1 Materials

Eversa Transform 2.0® (Lipase from *Aspergillus oryzae*, EC 3.1.1.3), Chitosan, *p*-nitrophenyl butyrate (*p*-NPB) octanal and dodecanal were obtained from Sigma-Aldritch® (Saint Louis, MO, USA). Sodium tripolyphosphate (TPP) was purchased from Synth® (Diadema, SP, Brazil). Acetic acid was obtained from Dinamica Química Contemporânea ® (Indaiatuba, SP, Brazil). All reagents were of analytical grade and all essays were performed in triplicate.

2.2 Chitosan Beads Production

A chitosan solution was produced by solubilizing 2.5% (w/v) of low molecular weight chitosan (LMWC) in a 1% (v/v) acetic acid solution. Then, 50 mL of the solution were slowly dripped in a 3% TPP solution under agitation for 5 min and kept immersed under the solution for 90 min (Fig. 1). Finally, the beads were filtered, stirred with distilled water (three times), and with acetone (once). The beads were further stored at 4 ºC.

Figure 1. Chitosan hydrogel beads (CHB) immersed in TPP solution.



2.3 Functionalization of CHB

The beads activation was performed as an adapted methodology from Palla *et al.* 2011 [4]. Thus, 1g of CHB were added to 10 mL of an aldehyde (octyl of dodecyl aldehyde) solution (1g/ 28 mL of ethanol) under agitation, protected from the light and then kept submerged in the solution for 30 minutes. The control was produced by adapted methodology from Rodrigues *et al.* 2008 [5]. Thus, 1 g of CHB was added to 10 mL of a 5 mM phosphate buffer solution (pH 7.0) containing 5% (v/v) of glutaraldehyde. All the beads were filtered, stirred with distilled water three times and with acetone once, and then stored at 4 ºC.

2.3 Enzyme immobilization and vegetal oils hydrolysis

For the immobilization, 0.5 g of the functionalized support was added to a 15 mL-Tube containing a 5 mL of 5 mM phosphate buffer (pH 7.0) and 0.5 mg of enzyme, and then kept under gentle agitation for 90 min.

Then, the produced biocatalysts were applied for the hydrolysis vegetal oils. Thus, an emulsion (27.5% v/v) of vegetal oil (soy or olive oil) was prepared using a solution of arabic gum (7% m/v), then 55 mL of phosphate buffer (100 mM, pH 7.0) was added to the solution and mixed in turrax until emulsion formation. After that, 150 mg of biocatalyst were added to 100 mL-Erlenmeyer containing 9 mL of the emulsion and incubated at 160 rpm, for 10 minutes at 37 ºC. The reaction was stopped by adding 10 mL of acetone-ethanol solution (1:1), and the hydrolysis was evaluated by the titration of the fatty acids produced by the reaction using 25 mM KOH solution.

2.4 Analytical methods

2.4.1 Enzymatic activity determination

The enzymatic activity of the free and immobilized enzyme was performed as adapted methodology from Rios *et al.* 2022 [6]. Thus, a 50 mM *p*-NPB solution (in pH 7.0, 25 mM phosphate buffer) was used as substrate. For the free enzyme, 50 µL of the enzyme as added to 2.55 mL of the substrate solution, and 0.02 g of derivate was used for biocatalyst activity evaluation. The *p*-NPB

degradation was evaluated by spectrophotometer at 448 nm. A unity of lipolytic activity (U) was determined as the amount of enzyme necessary to hydrolyze 1 µmol of *p*-NPB per minute.

2.4.2 Total protein determination

The total protein determination content was performed by Bradford (1976) essays. Thus using 100 µL of protein solution in 1 mL of Bradford's reagent, then left in the absence of light for 10 minutes. The total protein concentration was evaluated in spectrophotometer as 595 nm and compared with calibration curve previously determined.

3. Results and discussion

After immobilization, though both the biocatalyst produced using octyl (CB-Octyl) and dodecyl aldehyde (CB-Dodecyl) presented lower immobilization yield than the control, all the biocatalyst presented similar retained activity in all essays (Table 1), which is an indicative of similar stabilization for octyl and dodecyl aldehyde in comparison to glutaraldehyde (CB-Glu).

Table 1. Immobilization profile of Eversa Transform 2.0® using different cross-link agents

Cross-link agent	Theoretical activity	Immobilization yield (%)	Biocatalyst activity (U/g)	Retained activity (%)	Activity yield (U/mg $_{enzyme}$)
Glutaraldehyde	132.60	91 ± 2	30,1 ± 3	22.7 ± 4	32.97
Octyl aldehyde	85.17	58 ± 2	18.0 ± 7	21.1 ± 6	31.03
Dodecyl aldehyde	94.26	63 ± 3	23.0 ± 5	24.4 ± 6	36.50

Additionally, although both CB-Octyl and CB-Dodecyl presented lower final biocatalyst activity in comparison to the control, the similar activity yield, presented in U/mg$_{enzyme}$, was similar, which indicates that though CB-Glu may have incorporated more enzyme to the biocatalyst, it has similar activity per mg of enzyme immobilized, thus indicating that both octyl and dodecyl aldehydes does not have deactivated or influenced negatively the enzyme performance.

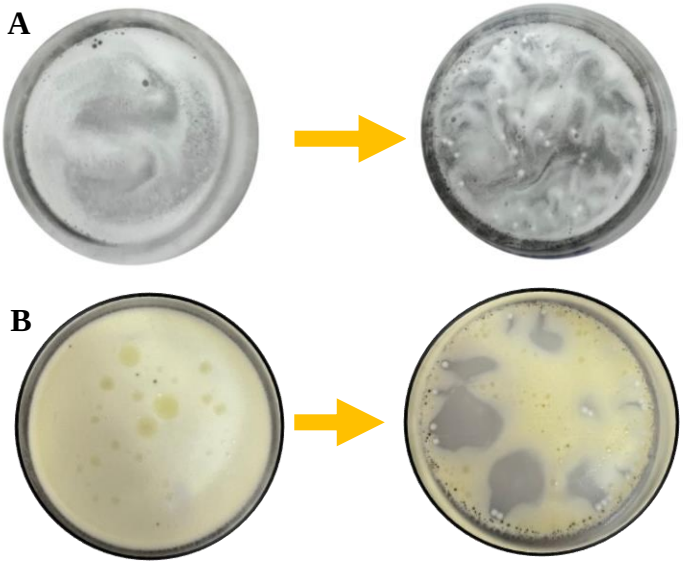
However, the CB-Octyl and CB-Dodecyl have presented a higher potential for the hydrolisys of vegetal oils (Table 2).

Table 2. Lipolytic activity of the produced biocatalyst over vegetal oils.

Biocatalyst	Activity over soy oil (U/mL)	Activity over olive oil (U/mL)
CB-Glu	11.7^{a}	17.3^{a}
CB-Octyl	15.5^{b}	35.4^{b}
CB-Dodecyl	26.2^{b}	21.9^{c}

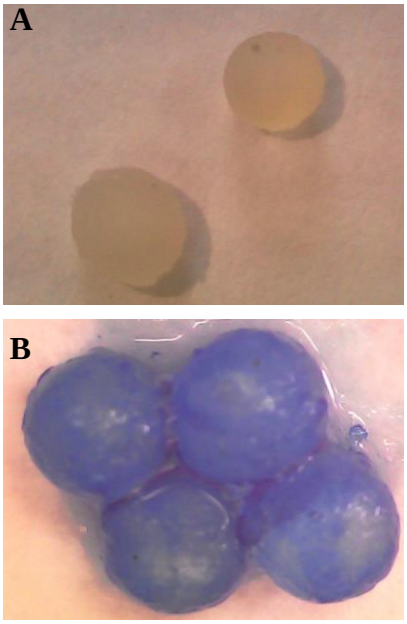
Both CB-Octyl and CB-Dodecyl presented higher lipolytic activity over soy oil and olive oil than CB-Glu, which is an indicative of, though it presented a minor enzyme immobilization yield, octyl and dodecyl aldehydes have presented a higher activity over non-synthetic substrates. Also, though similar against soy oil, CB-Octyl has presented higher activity yield over olive oil than CB-Dodecyl, which may indicate a higher potential to produce biocatalyst for the synthesis of biofuels than using glutaraldehyde or dodecyl-aldehyde, thus octyl-aldehyde was selected for further analysis (Figure 1).

Figure 1. Hydrolisis of (A) soy oil and (B) olive oil by CB-Octyl biocatalyst



The Octyl-aldehyde has presented high potential for the immobilization of Eversa Transform 2.0® (Figure 2), as show by the bradford coloration essay, which revealed the immobilization of the lipase by the blue coloring present in the surface of the hydrogels.

Figure 2. CB-Octyl (A) before and (B) after bradford coloring essay.



Thus, the present results indicate the high potential of these aldehydes for usage as alternatives for glutaraldehyde. Especially, Octyl-aldehyde have presented high potential for the immobilization of Eversa Transform 2.0® and for its further usage in the production of high-value esters for the energy, food and pharmaceutical industries.

Conclusion

The present work has reported the high potential of octyl and dodecyl aldehydes for functionalization of chitosan hydrogel beads. Both aldehydes have presented high retained activity in comparison to glutaraldehyde. Also, CB-Octyl presented higher potential for hydrolysis of vegetal oils, thus indicating a high potential to produce esters for usage as biodiesel or fragrances. These foundings also presented new perspectives to produce greener biocatalysts, enhancing the green industry, and promoting more sustainable practices, by reducing the usage of toxic compounds.

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