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Immobilization of Amano Lipase from Pseudomonas Fluorescens on Octyl-Agarose Support: The Impact of Aldehyde Cross-Linking on Stability against Desorption

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Abstract

Lipases can have their stability and activity enhanced when immobilized by adsorption in octyl-agarose. However, this type of immobilization is susceptible to leakage of enzymes by desorption and this problem can be minimized by aldehyde crosslinking. This work aimed to study the glutaraldehyde and dextran aldehyde coating of amano lipase from *Pseudomonas fluorescens* (PFL) immobilized in octyl-agarose for desorption reduction. Two biocatalysts were obtained by crosslinking PFL previously adsorbed to octyl-agarose with glutaraldehyde or dextran aldehyde, realizing another subsequent immobilization to prevent substrate's diffusional limitations through the crosslinking agents, but the simply octyl-agarose adsorbed biocatalyst were also considered for comparison. The immobilization parameters yield, offered activity, derivative activity and recovered activity were calculated, and the biocatalysts were submitted to Electrophoresis, desorption essay in Triton X-100 and operational stability essay for milk hydrolysis. The biocatalyst obtained with dextran aldehyde crosslinking has similar immobilization parameters to the glutaraldehyde one, even surpassing its recovered activity, both presenting the values 92,9% and 87,5%, respectively. Both aldehydes promoted higher resistance to desorption against Triton X-100 than the simply adsorbed one, especially glutaraldehyde, but reached full desorption with 2 h of incubation. Also, the crosslinked biocatalysts presented higher operational stabilities for milk fat hydrolysis, however only kept their activity until the second hydrolysis cycle. This work seems to indicate that octyl-agarose based biocatalyst has a great potential for modification with aldehydes for desorption reduction.

Keywords: lipase adsorption; hydrophobic support; coating

1. Introduction

Specific 1,3-sn lipases, such as *Pseudomonas fluorescens* (PFL) amano lipase, have been used in several reactions to obtain high value-added products, such as biodiesel and food [1]. Due to the modifications caused by the reactional conditions, such as pH and temperature, the lipases are susceptible to loss of activity and this problem can be overcome through immobilization [2].

There are physical and chemical immobilization methods, with adsorption being one of the fastest and most versatile physical methods. Due to their hydrophobic nature, lipases adsorb to the octyl-agarose support by interfacial activation, promoting high activities for the biocatalysts obtained [3]. However, weak interactions allow desorption of the enzyme when in contact with hydrophobic substances that overlap the enzyme-support interaction [4].

Covalent cross-linking protocols with aldehydes have been developed to offer greater protection against desorption. Glutaraldehyde is a strong bifunctional agent and as most aldehydes promotes the formation of Schiff's base between amine and aldehyde groups [5]. However, its toxicity has stimulated the search for substituents, such as dextran aldehyde, obtained by the oxidation of the polymer dextran [5].

Thus, this work sought to evaluate the influence of the aldehyde coating of PFL immobilized in octyl-agarose on the desorption process of the enzyme from its support.

2. Materials and Methods

The amano lipase from *Pseudomonas fluorescens* (≥20,000 U/g), dextran from *Leuconostoc* sp. (450-650 kDa) and glutaraldehyde grade II, 25% in water, were purchased from Sigma Aldrich®. The octyl-agarose resin was acquired from the GE Healthcare®. Whole milk powder (Ninho®) was purchased in local shops. Other materials were acquired of analytical grade from diversified brands.

2.1 PFL immobilization in octyl-agarose

PFL was immobilized on octyl-agarose with an offered protein load of 2 mg/g under agitation at (25±2) °C for 1 h [6]. Subsequently, centrifugation was followed by washing the biocatalyst with distilled water and 5 mM sodium phosphate buffer (SPB) (pH 7.0) and vacuum filtering to obtain the Oct-PFL coded biocatalyst.

2.2 Oct-PFL coating with aldehydes

For cross-linking with glutaraldehyde, 1.0 g of Oct-PFL was added to 10 mL of 1%$_{v/v}$ glutaraldehyde solution (Glu) in 50 mM SPB (pH 7.0), remaining under agitation at (25±2) °C for 1 h [7].

The dextran aldehyde solution (DexA) was produced with the [8] method adaptation. For crosslinking, 1.0 g of Oct-PFL was offered to 10 mL of SPB added to 5 mL of dextran aldehyde solution in a 1:5 ratio, Oct-PFL mass: volume of DexA, determined through preliminary tests, stirring the mixture at (25±2) °C for 1 h.

After crosslinking, the biocatalysts were washed with distilled water and SPB and vacuum filtered. To circumvent diffusion limitations, a second immobilization was performed above the crosslinking layer [9], performing the same process presented in section 2.1 and obtaining: Oct-PFL-Glu-PFL and Oct-PFL-DexA-PFL.

2.3 Hydrolytic activity and Calculation of immobilization parameters

The hydrolytic activity was determined using p-nitrophenyl butyrate (p-NPB) and 348 nm [10].

The immobilization parameters were calculated using the [11] methods. The parameters consist of immobilization yield (Y), offered activity (At_O), derivative activity (At_D), which is biocatalyst's activity, and recovered activity (At_R), that is the ratio between At_D and theoretical activity.

2.4 Evaluation of enzyme-support interaction by SDS-PAGE Electrophoresis and Desorption Assay on Triton X-100 detergent

Electrophoresis was performed on polyacrylamide gel according to [12] method. The samples were subjected to β-mercaptoethanol buffer under 100 °C for 10 minutes before running.

The desorption essay was adapted from the [13] method and a Triton X-100 4% detergent solution was prepared in deionized water. Then, the biocatalysts were incubated in the solution under agitation at (25±2) °C for 2 hours. Activity measures were performed periodically as described in section 2.3 and were expressed as residual activity.

2.5 Operational stability for milk hydrolysis

This assay was performed according to the adaptation of [14] procedures. Beginning with mixing 13.75 g of powdered milk with 55 ml of distilled water added to 100 mM SPB (pH 7.0). The milk's hydrolysis was performed with 50 mg of biocatalyst at 120 rpm and 37°C for 20 min. After the reaction, the samples were centrifuged, and the supernatant was collected for titration with 0.1 M NaOH. The remaining content was washed with 5 mM SPB (pH 7.0) and centrifuged again, repeating the process 6 times for the addition of the biocatalyst in the sample of the next cycle. The results were expressed as residual activity.

3. Results and Discussion

Table 1 presents the results of PFL immobilization yield on octyl agarose support. Because Oct-PFL is a precursor of Oct-PFL-Glu-PFL and Oct-PFL-DexA-PFL, the yield of Oct-PFL is relative to the first immobilization layer, while that of the other biocatalysts is relative to the second layer. The Oct-PFL-DexA-PFL showed lower immobilization yield than Oct-PFL-Glu-PFL, which may be associated with structural differences between the crosslinking agents. While the glutaraldehyde molecule has a chain containing only 5 carbons, bringing the molecules it connects closer together, dextran can have 18 carbons in its chain.

Table 2 shows other immobilization parameters for heterogeneous biocatalysts, including theoretical activity, derivative activity, and recovered activity. The theoretical activity of the biocatalysts was

28,9±1,59, 57,4±0,97 and 55,8±0,29 U/g for Oct-PFL, Oct-PFL-Glu-PFL and Oct-PFL-DexA-PFL, respectively. It influenced the AtR values together with the immobilization yields. The possible hyperactivation caused by the hydrophobicity of dextran aldehyde may have contributed to the higher AtR value of Oct-PFL-DexA-PFL.

Table 1. Immobilization yields for the octyl-agarose based biocatalysts.

Biocatalyst	Y (%)
Oct-PFL	92,5±0,961 [ab]
Oct-PFL-Glu-PFL	94,8±1,31 [a]
Oct-PFL-DexA-PFL	90,8±1,77 [b]

*Different letters in the same column indicate a significant difference at a level of 5% by Tukey's test.

Table 2. Immobilization parameters for the octil-agarose based biocatalysts with a At_O of 30 U/g.

Biocatalyst	At_D (U/g)	At_R (%)
Oct-PFL	37,4±2,78 [b]	129,5±4,6 [a]
Oct-PFL-Glu-PFL	50,3±2,99 [a]	87,5±3,86 [b]
Oct-PFL-DexA-PFL	51,8±1,29 [a]	92,9±2,76 [b]

*Different letters in the same column indicate a significant difference at a level of 5% by Tukey's test.

Figure 1 presents the SDS-Page Electrophoresis for enzyme solution, immobilization supernatants and derivatives. The PFL monomer is identified at the 33 kDa position, while its dimer is found with approximately the double of size (66 kDa).

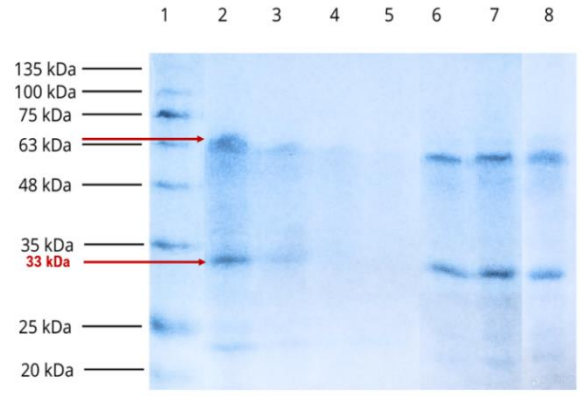

Fig. 1. SDS-Page Electrophoresis. Lanes: marker (1), enzyme solution (2), immobilization supernatants: Oct-PFL (3), Oct-PFL-Glu-PFL (4) and Oct-PFL-DexA-PFL (5), derivatives: Oct-PFL (6), Oct-PFL-Glu-PFL (7) and Oct-PFL-DexA-PFL (8).

Between the lanes 3 to 5 is possible to see the low quantity of protein in the supernatants, indicating the great yield of PFL immobilization in both lipase layers of the biocatalysts. The lanes 6 to 8 indicates the fragile or non-existent of strong covalent bonding between lipases and the aldehydes, since the enzymes were release after the interaction with β-mercaptoethanol and the 100 °C temperature. However, the qualitative character of this analysis does not allow quantifying the release of proteins, so it is possible that only the second layer of enzymes was released from the crosslinked biocatalysts.

Figure 2 shows the results for the desorption essay with 4% Triton X-100. And it indicates the full desorption of lipases from Oct-PFL within 30 min, while it took 2 hours for the total desorption of the lipases of the cross-linked biocatalysts. It is possible that the first layer of PFL was exposed close to 90 min of incubation, since the activities of Oct-PFL-Glu-PFL and Oct-PFL-DexA-PFL has increased.

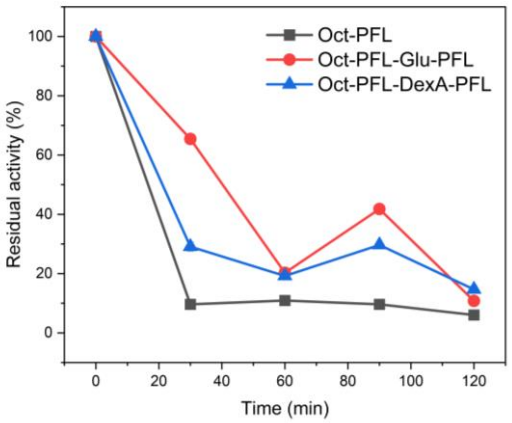

Fig. 2. Desorption in Triton X-100 4%. Oct-PFL has initial activity of 37.4 U/g (■), Oct-PFL-Glu-PFL of 50.3 U/g (●) and Oct-PFL-DexA-PFL of 51.8 U/g (▲).

Within the fact that the detergent was in high concentration, the almost total desorption of lipases from the octyl-agarose support suggest that the aldehydes, especially glutaraldehyde, had contributed for the biocatalysts resistance to desorption but also permitted the support recovery.

Figure 3 presents the operational stability of the biocatalysts for milk fat hydrolysis. Oct-PFL presented higher initial activity, and this is associated with diffusional limitations caused by the aldehydes in the coated biocatalysts, with dextran aldehyde representing a greater barrier to substrate diffusion through biocatalyst.

While Oct-PFL couldn't keep its activity upper to 50% to the cycle number 2, the two other

biocatalysts kept their average activity above 70% in the second cycle. It demonstrates the higher capability of the crosslinked biocatalysts to reduce the lipases desorption. When comparing Figures 2 and 3 it is possible to assume that the enzyme leaking was the main cause of the biocatalyst's hydrolytic activity reduction in the third reaction cycle.

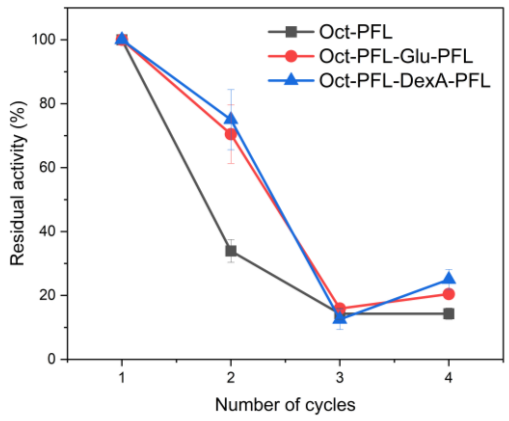

Fig. 3. Residual activity of milk hydrolysis at 37°C in cycles. Oct-PFL has initial activity of 129.44 U/g (■), Oct-PFL-Glu-PFL of 97.85 U/g (●) and Oct-PFL-DexA-PFL of 92.46 U/g (▲).

4. Conclusion

Dextran aldehyde has shown capacity to reach the immobilization parameters obtained with glutaraldehyde. Both aldehydes promoted higher resistance to desorption against Triton X-100, especially glutaraldehyde, but couldn't prevent it to happen. Also, the crosslinked biocatalysts presented higher operational stabilities, however only kept their activity until the second hydrolysis cycle because of desorption. Besides the interesting results, these biocatalysts can be enhanced in future studies.

Acknowledgements

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