Covalent immobilization of glucoamylase on polylactic acid carriers

Ilana Sessak[1], David Spressão de Lima Junior[1], Julian Paul Martinez Galan[2], Daniela Remonatto[3], Beatriz Bortoloti Teixeira[3], Mateus Vianna Ozorio[1], Murilo Daniel de Mello Innocentini[4,5], Ariela de Paula Veloso[3], Daniela Alonso Bocchini[1]*

[1] Institute of Chemistry, São Paulo State University (UNESP), Araraquara, SP, Brazil
[2] School of Nutrition and Dietetics, University of Antioquia (UdeA), Medellín, Colombia
[3] School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara, SP, Brazil
[4] University of Ribeirão Preto (UNAERP), Ribeirão Preto, SP, Brazil
[5]Centre for Regenerative design and Engineering for a NEt positive World (RENEW), University of Bath, UK
** Corresponding author's email address: daniela.bocchini@unesp.br*

ABSTRACT

Enzyme immobilization has emerged with the aim of improving existing industrial systems, by recovering the catalyst and easily separating it from the product, thus reducing process costs. Amylases are among the most important industrial enzymes used in the production of alcoholic beverages, bread making and starch processing. In this context, the present work aimed to study the covalent immobilization of a commercial fungal glucoamylase on powdered polylactic acid (PLA). The carriers were functionalized with hexamethylenediamine (HMDA) or ethylenediamine (EDA) and the influence of sodium borohydride on the functionalization step was evaluated. Functionalized carriers were activated with glutaraldehyde and the influence of pH and enzyme load on enzyme immobilization was evaluated. The conditions that resulted in the highest enzymatic activity in the immobilized derivative (9.03 U/g) were functionalization with HMDA in the absence of borohydride, immobilization at pH 7.0 and an enzyme load of 200 mg of protein per gram of carrier. The optimum pH for activity of the immobilized derivative was the same as that observed for the soluble enzyme (4.92). Regarding the optimum temperature for activity, the immobilized derivative was more active at 44 ºC.

Keywords: Glucoamylase. Covalent immobilization. Polylactic acid.

1 INTRODUCTION

Microbial enzymes are biocatalysts of relevant biotechnological importance, since they have been used in various industrial processes, fulfilling the assumption of "Green Chemistry", with high specificity and catalytic efficiency. However, the high cost of enzyme production and purification, their limited stability with respect to temperature, solvents, pH and ionic strength, as well as the impossibility of reuse are still the main disadvantages of using soluble enzymes in industry. Enzyme immobilization has proven to be an interesting strategy to overcome these obstacles, since it not only favors product recovery and enables catalyst reuse, but can also improve enzyme properties, including half-life, catalytic efficiency and stability, which is reflected in costs reduction[1].

Amylases represent more than 25% of the global industrial enzyme market and are used in the food, textile, paper, detergent, and pharmaceutical industries. Although the industrial processes in which these enzymes are used are traditionally well established, especially in the food industry, new application technologies are emerging to meet the demand for more efficient processes, in all application areas. Furthermore, specifically for glucoamylases, new applications have emerged in recent years[2] and different application strategies, such as immobilization, are important in this context.

The choice of carrier for enzymes immobilization represents an important step for the process to occur satisfactorily, and must ensure that the catalytic activity is maintained, as well as meet some requirements such as insolubility under reaction conditions, high affinity for the enzyme, thermal and chemical stability, presence of reactive functional groups, easy availability, low cost and, most importantly, being easily recovered and reused at the end of reaction[3].

The present work addresses the covalent immobilization of a commercial fungal glucoamylase in polylactic acid (PLA), an innovative and unprecedented carrier in the context of amylases, thermoplastic, biodegradable, low cost, obtained from renewable sources. The influence of functionalizing agent, presence of sodium borohydride in the functionalization step, enzyme loading, and immobilization pH were evaluated. The immobilized derivative was characterized in terms of optimal activity conditions, in order to establish a comparison with the characteristics of the soluble enzyme.

2 MATERIAL & METHODS

2.1. PLA carriers functionalization and activation

The polylactic acid carriers were obtained by crushing 3D printing residues and subsequent separation by granulometry using Tyler sieves (particles retained in the 65 mesh were used). For functionalization with HMDA, 1.0 g of carrier was added to 5.0 mL of HMDA solution (60 mg·mL^{-1} in isopropyl alcohol), for 2 hours, at 55 ºC, with gentle stirring. Subsequently, the carrier was washed and kept in distilled water on a roller bed for 24 hours. For functionalization with EDA, 35g of support were immersed in 200 mL of EDA solution (2.0 mol·L^{-1}) in isopropyl alcohol for 2 h at room temperature and under gentle stirring. After washing with distilled water, the functionalized carriers were dried using a vacuum pump. Functionalization was also performed in the presence

of sodium borohydride ($NaBH_4$) (0.057 g/g of carrier), for 4 hours, with gentle stirring. For activation, 1.0 g of carrier was immersed in 20% glutaraldehyde solubilized in 0.01 mol·L^{-1} phosphate buffer pH 7.5, for 15 h with gentle agitation. After this period, the carrier was washed with distilled water and dried using a vacuum pump.

2.2. Glucoamylase immobilization

Immobilization was performed with g 1g of carrier and 10 mL of solution consisting of enzyme (300 mg of protein/g of carrier) and buffer solution at 0.5 mol·L^{-1} (phosphate buffer for pH 7.0 and bicarbonate buffer for pH 10). The mixture was kept for 24 hours at room temperature under gentle stirring. After, carriers were washed with 80 mL of distilled water and dried in a vacuum pump for 12 minutes. Setting the best pH for immobilization, the influence of enzyme loading was evaluated, using 200, 300 and 400 mg of protein per gram of carrier.

2.3. Glucoamylase activity and protein quantification

The reaction was performed at pH 4.93 and 50 °C, for 10 min, and then stopped in an ice bath. Quantification of released glucose was performed by the DNS method[4]. One unit of glucoamylase activity was defined as the amount of enzyme required to release 1 µmol of glucose per minute, under assay conditions. Soluble and immobilized enzyme activities are expressed as U per mL and U per gram of carrier, respectively. Protein was quantified as described by Bradford[5] based on an analytical curve using bovine serum albumin.

2.4. Effect of temperature and pH on enzyme activity

The effect of pH and temperature on the activity of the immobilized derivative was carried out using a five-level Rotational Central Composite Design (RCCD) (Table 2). The evaluated response was the relative enzymatic activity, expressed as a percentage, where the highest value obtained was considered 100%.

3 RESULTS & DISCUSSION

3.1. Glucoamylase immobilization

The enzymatic activities of immobilized derivatives obtained by functionalization with HMDA or EDA, in the presence or absence of $NaBH_4$, and immobilization at neutral or alkaline pH are presented in Table 1.

Table 1. Influence of functionalizing agent, presence of $NaBH_4$ and pH on the immobilization of commercial glucoamylase using crushed PLA carriers.

Functionalizing agent	Immobilization pH	Using $NaBH_4$	Enzymatic activity (U/g)
EDA	7.0	No	3.17 (±0.15)
	10.0		3.09 (±0.29)
HMDA	7.0		**8.98 (±1.88)**
	10.0		2.72 (±0.80)
EDA	7.0	Yes	3.26 (±0.24)
	10.0		4.27 (±0.39)
HMDA	7.0		**9.03 (±0.19)**
	10.0		3.42 (±0.39)

Statistical analysis of the data was performed using ANOVA and Tukey's test (data not shown), which showed that the functionalizing agent and pH were significant ($p < 0.05$), while the presence of borohydride was not.

3.2. Influence of enzyme load on immobilization

Once the functionalization conditions and immobilization pH were established, the influence of enzyme loading on the immobilization was evaluated. The activity of the immobilized derivatives was 21.09 (± 0.68), 26.39 (± 0.94) and 27.15 (±0.69) U/g when using enzyme loads of 200, 300 and 400 mg of protein per gram of carrier, respectively. Statistical analysis performed using ANOVA and Tukey's test (data not shown) indicated that enzyme loads used were not statistically significant. Therefore, the enzyme load for subsequent assays was set at 200 mg of protein/g of support.

3.3. Effect of pH and temperature on the enzymatic activity of immobilized derivatives

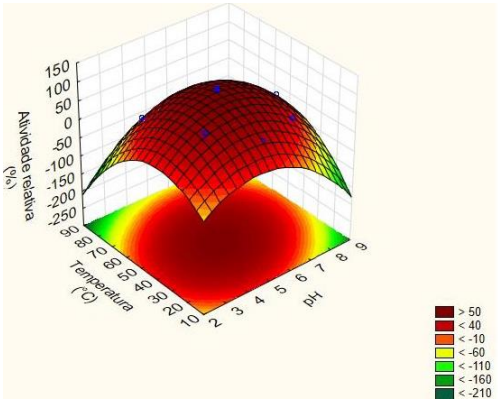
A Rotational Central Composite Design (RCCD) was performed to determine the optimal pH and temperature activity (Table 2). The results were analyzed by ANOVA, the model regression value was obtained by the Statistica 10 software (data not shown) and the coefficient of determination (R^2) was 0.90, indicating that the model is well suited. From the data obtained, a response

surface was generated for the effects of pH and temperature on the activity of the immobilized enzyme (Figure 1) and it can be inferred that the best results are obtained when the variables are used at their central points.

Table 2. Rotational Central Composite Design (RCCD), coded and real values of the variables and relative activity of the immobilized derivative.

Run	pH	Temperature (ºC)	Relative activity (%)
1	(-1) 3.5	(-1) 30	88.99
2	(+1) 7.5	(-1) 30	23.88
3	(-1) 3.5	(+1) 70	20.43
4	(+1) 7,5	(+1) 70	13.03
5	(0) 5.5	(0) 50	100
6	(0) 5.5	(0) 50	99.54
7	(0) 5.5	(0) 50	85.54
8	(0) 5.5	(0) 50	97.98
9	(-1.41) 2.67	(0) 50	10.36
10	(+1.41) 8.3	(0) 50	16.75
11	(0) 5.5	(-1.41) 21.7	37.46
12	(0) 5.5	(+1.41) 78.3	6.12

Figure 1. Response surface obtained for the effects of pH and temperature on the activity of the immobilized derivative.



The desirability function of the Statistica 10 software indicated 44 ºC and pH 4.92 as the optimal condition for the activity of the immobilized derivative. For soluble glucoamylase, optimal activity is obtained at pH 4.92 and 50 ºC.

4 CONCLUSION

The data obtained demonstrate the feasibility of using crushed PLA as a carrier for covalent immobilization of *A. niger* glucoamylase. Immobilization at pH 7.0 led to the best results, suggesting that single-point immobilization was more effective than multi-point immobilization (pH 10.0). The immobilized derivative practically maintains the physicochemical properties of the soluble enzyme with respectto the optimum pH and temperature of activity.

5 REFERENCES

[1]HETTIARACHCHY, N. S., FELIZ, D. J., EDWARDS, J. S., HORAX, R. 2018. The use of immobilizedenzymes to improve functionality. In Proteins in Food Precessing, 559–597.
[2]CRIPWELL, R.A., FAVARO, L., VILJOEN-BLOOM, M., VAN ZYL, W.H., 2020. Biotechnol. Adv. 42, 107579.
[3]Dias Gomes, M., Woodley, J.M., 2019. Molecules 24.
[4]MILLER, G. L. Analytical Chemistry.1959. 31(3), 426–428.
[5]BRADFORD, M. M. 1976. A ANALYTICAL BIOCHEMISTRY. [s.l: s.n.].

ACKNOWLEDGEMENTS

This study was financed by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES) through financial support (PROAP AUXPE grant no. 1765/2023), the São Paulo Research Foundation (FAPESP, grant no. 2020/09592–1) and the Brazilian National Council for Scientific and Technological Development (CNPq, grant no. 304399/2022-1).