PRODUCTION OF MULTI-PURPOSE BIOCATALYST FOR INDUSTRIAL APPLICATIONS

Renata A. de Oliveira[1], Michelle M. dos Santos[1], Luana X. S. G. M. Fé[2,3], Gisela M. Dellamora-Ortiz[4], Eliane P. Cipolatti[5], Viridiana S. F. Leitão[6], Evelin A. Manoel[1,2,3]*

[1] *Postgraduate Program in Biochemistry (PPGBq), Institute of Chemistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil*
[2] *Pharmaceutical Sciences Course, Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil*
[3] *Postgraduate Program in Pharmaceutical Science (PPGCF), Faculty of Pharmacy, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil*
[4] *Pharmaceutical Sciences Course, Department of Pharmaceutical and Medicines, Faculty of Pharmacy, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil*
[5] *Departamento de Engenharia Química, Institute of Technology, Universidade Federal Rural do Rio de Janeiro, Rio de Janeiro, Brazil*
[6] *National Institute of Technology, Rio de Janeiro, Brazil*
* *Corresponding author's email address: biorecados@yahoo.com.br*

ABSTRACT

Immobilized lipases are biotechnological highlights, improving and enhancing enzyme applicability. Technologies like genetic engineering and enzyme immobilization enhance biocatalyst properties. Immobilization, which involves attaching enzymes to solid supports, offers benefits such as improved stability, reusability, and easier separation. Proper selection of supports and methods can optimize enzyme performance for various industrial applications, making processes more sustainable and cost-effective. In this study, lipase from *Candida antarctica* (CALB) and lipase from *Rhizomucor miehei* (RML) were immobilized using different matrices and distinct immobilization techniques, resulting in new biocatalysts. The top-performing biocatalysts underwent rigorous testing to evaluate their interactions with various substrates tailored for diverse applications. Consequently, they successfully synthesized key molecules pivotal to the cosmetic industry, such as ascorbic acid esters, essential for the pharmaceutical sector like ibuprofen esters, and example of compounds for the biodiesel industry, such as ethyl oleate. This resume highlights the advancements and challenges in biocatalyst immobilization.

Keywords: lipases, adsorption, covalent binding, ascorbyl esters, ibuprofen esters.

1 INTRODUCTION

Biocatalysts are enzymes which speed up biochemical reactions. They are distinguished by their specificity, efficiency, and ability to function under mild conditions compared to traditional chemical catalysts, as example capacity to operate at ambient temperatures and pressures, often in aqueous environments, reducing energy consumption and environmental impact. Biocatalysts can be obtained from animal, plant, or microbial sources, with the latter being the most useful for industrial enzyme production[1-3].

Different technologies can be employed to enhance the characteristics of biocatalysts. Molecular biology tools, for instance, can be used in genetic engineering to boost the production of a specific enzyme, improve its properties, or increase its enantioselectivity. Another effective strategy is the development of supports for enzyme immobilization, which can further improve the properties of the biocatalyst. Immobilization involves attaching enzymes to a solid support, which can be a natural or synthetic material (organic, inorganic or composite supports, through hybrid materials combining organic and inorganic components)[4,5].

The immobilization approach often offers several advantages, including easier separation of enzymes from the reaction mixture, improved enzyme stability, and the ability to reuse enzymes for multiple cycles. This technique enables the creation of multipurpose biocatalysts and the development of a library of biocatalysts tailored for various industrial applications. First, selecting the appropriate support; second, choosing the enzyme; third, deciding on a simple or combined method for immobilization; and fourth, optimizing the conditions for the immobilization process, mainly the mild-conditions used. By choosing appropriate supports and immobilization methods, industries can significantly improve the performance and applicability of enzymes in various sectors, leading to more sustainable and cost-effective production methods. On the other hand, the development of supports for enzyme immobilization is a critical advancement in biocatalysis, offering numerous benefits that enhance the practicality and efficiency of enzymatic processes[5,6].

Regarding the enzyme, each enzyme, with its unique 3D protein structure, may interact differently with various supports. Depending on the immobilization technique employed, structures with higher concentrations of certain amino acid residues may be more advisable. Alternatively, because these proteins can contain multiple reactive residues, there is a lack of control over the immobilization site and orientation. This can sometimes result in the random positioning of proteins on the solid support, which may decrease functionality by blocking the active site or, conversely, increase efficiency.

Biocatalysts were developed to facilitate the production of biotechnological products, particularly esters, utilized across various industrial sectors. Esters, with their diverse natural variations, play a pivotal role in industry applications. This overview delves

into the spectrum of immobilization engineering and the applications of innovative biocatalysts, emphasizing their advantages and potential hurdles.

2 MATERIAL & METHODS

Commercial lipase from *Rhizomucor miehei* (RML), and lipase B of *Candida antarctica* (CALB) were kindly donated by Novozymes and LNF Latino Americana. Accurel, made of polypropylene, was bought from Mem- brane GmbH (Germany). The stabilizer poly(vinyl alcohol) was also provided by Vetec Química Fina. Different epoxy, octadecyl and macroporous resins were purchased from LifeTech Purolite. All substrates and reagents used were purchased from Sigma, including ascorbic acid and ibuprofen.

The entire process involved: (I) Immobilization of CALB and RML on different supports: The immobilization of lipases on different supports were made according Fé et al.[6] and Jia et al.[7]; (II) Determination of the enzymatic activity: using p-NPL and p-NPB as substrates, and the determination of protein concentration were conducted according to Fé et al.[6]. The esterification activity was made according Manoel et al.[1]. Analysis of ascorbyl esters and esters of ibuprofen was made in HPLC and CG-MS according Manoel et al.[1]; (III) Antioxidant activity analysis: It was evaluated using DPPH (2,2-diphenyl-1-picrylhydrazyl) according Dudonné et al. (2009), and (IV) Biological characteristics of immobilized enzyme were made according Fé et al.[6] All analyzes in the present work were carried out in triplicate.

3 RESULTS & DISCUSSION

In this work, various biocatalysts were produced using different matrices such as mesoporous silica (SBA-15), polypropylene (Accurel), polystyrene, octadecyl, epoxy, and macroporous supports. After treating each support, the immobilization process was conducted using CALB and RML. The results were compared with the commercial versions of these biocatalysts. The main results are presented in Table 1.

Table 1 - Enzimatic activities of the main biocatalysts obtained from the lipase from CALB and RML immobilized on different matrices and different immobilization techniques. In all cases, the initial enzymatic activities was 4000 $U.g^{-1}$.

Biocatalyst	Immobilization technique	Hydrolytic activity[a] (U/g or U/mL for free lipase)	Hydrolytic activity [b] (U/g or U/mL for free lipase)	Esterification activity (U/g)
Free CALB	--	138.36±0.10	213.05±0.31	n.d.
Free RML	--	12.876,00±1.29	9.612,00±7.11	n.d.
SBA-CALB	Covalent binding	50.02±0.11	80.16±0.22	2887.16±0.56
SBA-RML	Covalent binding	850.17±0.19	912.03±0.61	27.443,21±1.10
Accurel-CALB	adsorption	49.41±0.06	27.22±0.04	995.36±0.87
Accurel-RML	adsorption	97.21±0.18	138.26±0.18	11.998,00±1.10
Oct-CALB	adsorption	12.08±0.03	321.16±0.11	708.03±0.19
Oct-RML	adsorption	109.03±0.22	704.16±0.13	22.761,00±0.52
Epo-CALB	Covalent binding	47.11±0.02	78.09±0.04	337.16±0.18
Epo-RML	Covalent binding	823.04±2.01	943.76±1.71	817.62±0.33
Novozym 435	Adsorption(interfacial activation)	47.08±0.10	96.48±0.23	2.998,56±1.88
RM-IM	Ionic binding	58.98±0.10	102.33±0.60	3.799,98±2.66

a: *p*-nitrophenyl laurate (*p*-NPL, 2.5) as substrate; b: *p*-nitrophenyl butirate (*p*-NPB, 2.5) as substrate; c: oleic acid and ethanol as substrate.

The best performance for hydrolysis reaction was found for EPO-RML, using *p*-nitrofenil butirate as substrate and SBA-RML when oleic acid an ethanol was used (esterification reaction). With respect to the two substrates for hydrolysis reaction, higher activities were generally obtained when *p*-NPB was used as the substrate. The exception occurred when Accurel-CALB was evaluated. Enzyme binding to supports can generally be achieved through physical means (adsorption) or chemical modification (covalent bonds). There are numerous protocols for covalently immobilizing proteins, involving different amino acid side chains of the enzyme and various activating groups in the supports[9].

As is well known, immobilization of enzymes produces biocatalysts with different characteristics of the free enzyme. Each procedure of immobilization produces a new biocatalyst. The calculation of kinetic parameters is used for evaluating and comparing the immobilization and affinity for the substrate[10] and it was used in this work. Results showed different values for Km and Vmax in relation to each biocatalyst (data not shown). As expected, the free enzyme shows a higher Vmax (417.08 µmol/min for CALB and 722.01 µmol/min for RML). The Km values of the biocatalysts showed similar to the free enzyme, which means that even the immobilization process is able to have a good interaction with the substrate. Some diffusional problems associated with the immobilization process may cause the maximum reaction rate to decrease, as observed for Oct-CALB (Vmax of 11.43 µmol/min), which can explain the low activity value. The best biocatalysts produced (EPO-RML, SBA-RML, Oct-RML and Accurel-RML) were selected to study the performance front of different esters production as ascorbyl oleate and ibuprofen esters. The results demonstrate 92% of *L*-ascorbyl oleate and 78% of *S*-ester-ibuprofen, when Accurel-RML and EPO-RML were used, respectively. For *L*-ascorbyl oleate, the antioxidant activity was evaluated and compared with ascorb acid. Surprisingly, the stability of ascorbyl ester was superior to ascorbic acid, maintaining its stability for more than 120 days, compared to 6 hours for the acid molecule.

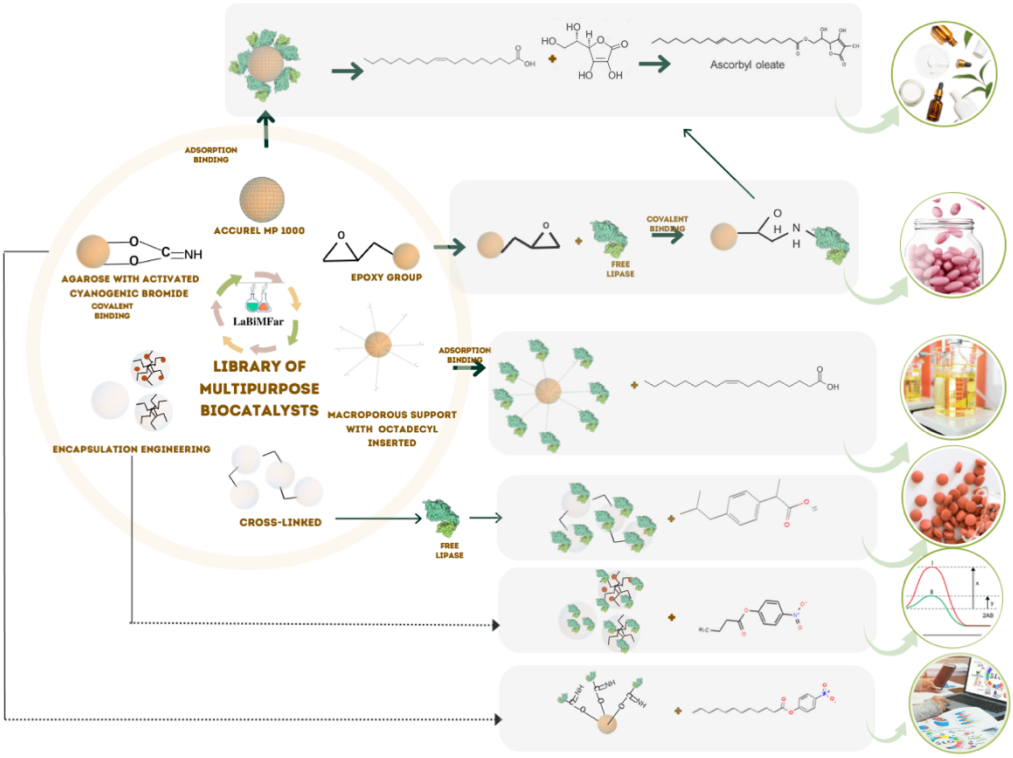

Figure 1. Main biocatalysts, substrates and products produced. From top to bottom: ascorbyl esters, ethyl oleate and ester ibuprofen.

4 CONCLUSION

The new biocatalysts have demonstrated significant potential for industrial applications, exhibiting higher enzymatic activity compared to commercial counterparts, as well as displaying high regio- and enantioselectivity in product synthesis.

REFERENCES

1 MANOEL, E. A., PINTO, M., dos SANTOS, J.C.S. , TACIAS-PASCACIO, V.G., FREIRE, D.M.G., PINTO, J. C., FERNANDEZ-LAFUENTE R. 2016. RSC Adv. 6, 62814–62824.

2 MATEO, C., PALOMO, J.M., FERNANDEZ-LORENTE, G., GUISAN, J.M., FERNANDEZ-LAFUENTE, R., 2007. Enzyme Microb. Technol. 40, 1451–1463.

3 TREICHEL, H., DE OLIVEIRA, D., MAZUTTI, M. A., DI LUCCIO, M., OLIVEIRA, J.V., 2010. Food Bioprocess Technol. 3, 182–196.

4 BILAL M., IQBAL H.M.N. 2019. Coord. Chem. Rev. 388, 1–23.

5 BOUDRANT J., WOODLEY J.M., FERNANDEZ-LAFUENTE R. 2020. Process Biochem 90, 66–80.

6 FÉ L. X.S.G.M., DOS SANTOS, M.M., COSTA C. S., PINTO, M. C. C., DE OLIVEIRA R. A., CIPOLATTI E. P., PINTO J. C., LANGONE M. L., DELLAMORA-ORTIZ G. M., MANOEL E. A. 2024. Process Biochemistry, 142, 136-148.

7 JIA D. X.,·XU H. P., SUN C.Y.,·PENG C., LI J. L., CHENG F., LIU Z. Q., XUE Y. P., ZHENG Y. G. 2020. Bioprocess and Biosystems Engineering, 43:1599–1607.

8 DUDONNÉ, S.; VITRAC, X.; COUTIÈRE, P.; WOILLEZ, M.; MÉRILLON, J.-M. 2009. Journal of Agricultural and Food Chemistry. [S. l.]: American Chemical Society (ACS).

9 TORRES-SALAS, P., DEL MONTE-MARTINEZ, A., CUTIÑO-AVILA, B., RODRIGUEZ-COLINAS, B., ALCALDE, M., BALLESTEROS, A. O., & Plou, F. J. 2011. Advanced Materials, 23(44), 5275–5282.

10 BANSODE, S.R., RATHOD, V.K., 2017. Ultrason. Sonochem. 38, 503–529.

ACKNOWLEDGEMENTS

The authors thank FAPERJ (Fundação Carlos Chagas Filho de Apoio a Pesquisa do Estado do Rio de Janeiro) for the scholarships and the financial support (Process number: E-26/201.366/2021).