IMMOBILIZATION OF ENDOGLUCANASE AND XYLANASE FROM COMMERCIAL PREPARATION ON MAGNETIZED GRAPHENE OXIDE

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ABSTRACT

Hydrolyzing cellulose and hemicellulose-rich byproducts into monomeric sugars for bioproduct conversion is a key industrial process. Traditional chemical and physical methods are often costly and generate toxic by-products, making enzymatic hydrolysis an environmentally friendly alternative. However, the economic feasibility of using enzymes industrially can be limited by their cost, difficulty in reuse, and stability maintenance. Enzyme immobilization on solid materials, such as magnetized graphene oxide (MGO), offers a solution by potentially enhancing enzyme stability and reusability. This study aimed to immobilize endoglucanase and xylanase on MGO and assess their catalytic activity and immobilization efficiency. Ideal protein loading ranged from 55-65 mg per gram of support, achieving a 68% immobilization yield with about 42 mg of protein immobilized per gram of support. The optimal activity conditions for free and immobilized endoglucanase and xylanase were identified at pH 4-5 and temperatures between 50-70 °C. The study highlighted the regular yields (25.6% for endoglucanase and 40% for xylanase) and high efficiencies (70.9% and 84.4%, respectively), demonstrating the potential of MGO as a support for enzyme immobilization in industrial settings.

Keywords: Magnetized graphene oxide (MGO). Cellulolytic enzymes. Immobilization of enzymes. Immobilized enzyme characterization.

1 INTRODUCTION

Cellulases and hemicellulases are enzymes that hydrolyze lignocellulosic materials, primarily composed of cellulose, hemicellulose, and lignin, into simpler sugars and oligosaccharides that are easier to metabolize[1]. Brazil, one of the world's largest agricultural producers, generates significant amounts of agro-industrial byproducts from the processing of crops like sugarcane, rice, corn, and soy [2]. These byproducts, mainly derived from the sugar-alcohol, pulp and paper, and food industries, are rich in cellulose, hemicellulose, and lignin, presenting substantial potential for reuse in both economic and environmental contexts [3]. Enzymatic hydrolysis of cellulose and hemicellulose is a critical step for releasing fermentable sugars for industrial bioproducts. However, the economic viability of enzymes on an industrial scale is challenged by high costs and difficulties in maintaining enzyme stability. Enzyme immobilization, particularly covalent binding on solid materials, emerges as a solution by enhancing enzyme stability, allowing reuse, and facilitating product separation[4]. Graphene oxide (GO), with its large surface area, functional groups, and water dispersibility, is identified as a promising support material for immobilizing commercially valuable enzymes[5].

2 MATERIAL & METHODS

Synthesis of Graphene Oxide (GO): GO was synthesized using a modified Hummers and Offeman method.[6] Graphite (1 g) was mixed with 60 mL of H_2SO_4 (96%) in a 500 mL flask and stirred for 15 min. Then, 100 mL of a $KMnO_4$ solution (35 mg/mL) was added, and the mixture was stirred for 120 min at 25 °C. The flask was cooled again, and 100 mL of distilled water was added. About 10 mL of H_2O_2 (30%, v/v) was added until the bubbling stopped. After 12 h, the solid was vacuum filtered and washed with distilled water, 10% aqueous HCl, ethanol, and more distilled water then dried at 50°C in a vacuum oven.

Synthesis of Magnetized Graphene Oxide (MGO): MGO was prepared by adding 1.755 g of $FeCl_3 \cdot 6H_2O$ and 0.645 g of $FeCl_2 \cdot 4H_2O$ to 100 mL of a 3% (v/v) acetic acid solution. Then, 10 mL of GO dispersion was added, the temperature was raised to 80 °C, and 20 mL of NH_4OH (25%, v/v) was added, stirred for 15 min, and the reaction was halted. The MGO was collected with a magnet, washed with water and methanol, and dried at 50 °C.

Covalent Immobilization of Enzymes on MGO: A 20 mL MGO suspension (0.5 $mg.mL^{-1}$) in sodium acetate buffer (pH 4.8) was sonicated. 20 mg of N-Hydroxysuccinimide (NHS) was added, and sonicated for 15 more min, then 24 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added and stirred for 2 h. The MGO was separated, washed, and resuspended in the same buffer at 1 $mg.mL^{-1}$. Commercial enzyme cocktail Celluclast (Novonesis) as added and stirred for 12 h, and the immobilized material was collected, washed with buffer, and resuspended in buffer. Enzymatic activity was assessed under optimal conditions to determine the effective protein load. The protein content in the supernatant was determined using the Bradford reagent.[7] To confirm successful enzyme immobilization, the immobilization yield, efficiency, and recovered activity were determined.[8] Figure 1 presents a schematic representation of the synthesis of MGO and immobilization of enzymes.

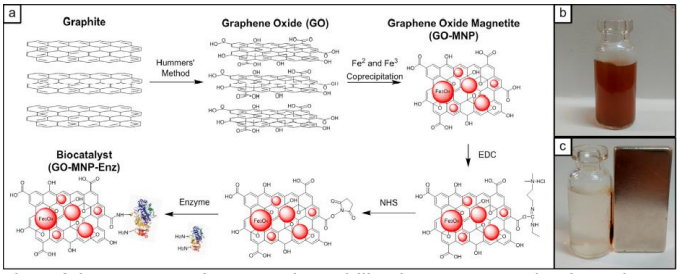

Figure 1 (a) Schematic representation of the process of enzyme immobilization on magnetized graphene oxide functionalized with EDC and NHS and (b, c) dispersion of the immobilized catalyst. [9]

Enzyme activity assays: Endoglucanase activity was measured by hydrolyzing carboxymethylcellulose (CMC), 0.9 mL of 0.44% CMC (m/v) in 0.05 M sodium acetate buffer (pH 4) was combined with 0.1 mL of diluted enzyme (Celluclast-Novonesis) and incubated at 50 °C for 60 min.[10] The reaction was halted by adding 1.5 mL of dinitrosalicylic acid (DNS) reagent and heating at 100 °C for 5 min. Absorbance was read at 540 nm. Xylanase activity was measured by hydrolyzing 0.9 mL of 1% (m/v) Birchwood xylan in 0.05 M sodium acetate buffer (pH 5) with 0.1 mL diluted enzyme (Celluclast-Novonesis) and incubating at 50 °C for 5 min.[6] The reaction was stopped with 1.5 mL of DNS reagent and heated at 100 °C for 5 min. Absorbance was taken at 540 nm.

3 RESULTS & DISCUSSION

The enzyme immobilization process using varying protein loads on a constant MGO mass was assessed for immobilization efficiency by monitoring protein content (Figure 2). Optimal protein quantity was determined for subsequent studies based on enzyme activities. High immobilization yields occurred with lower initial protein amounts (7.5–17.4 mg), achieving 100% immobilization. Protein immobilization was saturated at around 60 mg, beyond which additional protein did not bind effectively, leading to reduced immobilization yields. The optimal protein load, considering enzyme activities of xylanase and endoglucanase in its immobilized form, stabilized at 60 mg, with average activities of 330 $U.g^{-1}$ and 180 $U.g^{-1}$, respectively. With this protein load, the immobilization yield was 68% (42 mg of protein per gram of support). This optimal load was used for subsequent studies on immobilization conditions and parameters.

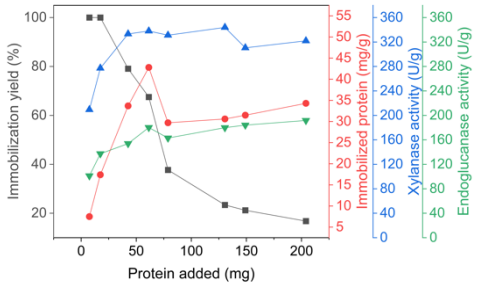

Figure 2 Immobilization yield (%; black), immobilized protein ($mg.g^{-1}$; red), xylanase activity ($U.g^{-1}$; blue), and endoglucanase activity ($U.g^{-1}$, international unit per gram of support, MGO; green) in relation to the amount of protein (mg) added at the beginning of the immobilization (x-axis).

This study evaluated the immobilization process based on the activities of individual enzymes in the Celluclast complex, determining parameters of yield, efficiency, and recovered activity (Table 1). Immobilization yields were 25.6% for endoglucanase and 40.0% for xylanase, indicating suboptimal enzyme-support interaction. Efficiency showed good results at 70.9% for endoglucanase and 84.4% for xylanase, despite not reaching the ideal 100%, likely due to mass transfer limitations and enzyme conformational changes. Recovered activity was 18.1% for endoglucanase and 35.5% for xylanase. Compared to literature[9], which used the same support and immobilization route but different enzymatic preparations, our endoglucanase showed lower yield but higher efficiency, while xylanase parameters were superior in our study. Enzyme activities were 191.7 ± 7.6 $U.g^{-1}$ for endoglucanase and 337.5 ± 58.0 $U.g^{-1}$ for xylanase. These were lower for endoglucanase but significantly higher for xylanase compared to literature[9], highlighting variations due to different enzymatic preparations and methodologies.

Table 1 Immobilization yield, efficiency, and recovered activity in the immobilization of endoglucanase and xylanase from commercial cocktail (Celluclast - Novonesis) on magnetic graphene oxide.

Enzyme	Immobilization yield (%)	Efficiency (%)	Recovered activity (%)	Activity ($U.g^{-1}$)*
Endoglucanase	25.6 ± 0.9	70.9 ± 4.1	18.1 ± 0.7	191.7 ± 7.6
Xylanase	40.0 ± 8.0	84.4 ± 9.6	35.5 ± 6.6	337.5 ± 58.0

**International unit per gram of support (MGO).*
*Immobilization yield = (Initial activity of supernatant – Final activity of supernatant) / (Initial activity of supernatant)*100%.*
*Efficiency = Immobilized catalyst activity / (Initial activity of supernatant – Final activity of supernatant) *100%.*
*Recovered activity = Immobilized catalyst activity / (Initial activity of supernatant)*100%.*

The ideal temperature for immobilized xylanase remained at 60 °C after immobilization, with an activity of 337.5 $U.g^{-1}$. The ideal pH was confirmed at 5, even though the immobilized enzyme showed considerably lower activity at pH 6 compared to the free form. The ideal temperature for immobilized endoglucanase shifted to 50 °C, showing an average activity of 206.0 $U.g^{-1}$. The ideal pH was maintained at 4 for endoglucanase, with a higher relative activity observed at pH 5 and 6 compared to the free enzyme, although activity sharply decreased above pH 7.

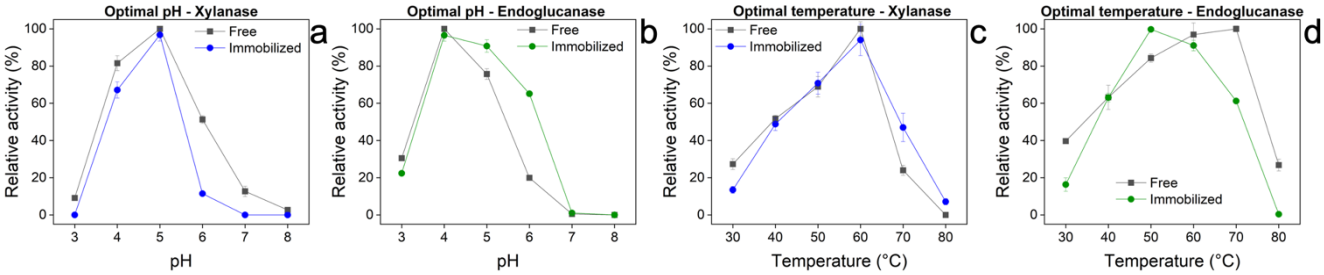

Figure 3 Optimal pH of free and immobilized (a) xylanase and (b) endoglucanase. Optimal temperature of free and immobilized (c) xylanase and (d) endoglucanase.

Table 2 shows that the optimal pH and temperature ranges for xylanase and cellulase activity are generally 4-6 and 40-60 °C, respectively, and these conditions are mostly maintained after enzyme immobilization. Exceptions, like the study reported in the literature[7] that found more alkaline pH values optimal for xylanases from *Thermomyces lanuginosus*, are minor. Variations in optimal conditions are linked to the support used, the type of immobilization, and the enzyme's nature. Immobilization aims to enhance enzyme stability under pH and temperature conditions, improve reusability, and ease recovery. Further studies are needed to evaluate these parameters and any changes post-immobilization compared to the free enzyme form.

Table 2 Optimal temperature and pH of xylanase and endoglucanase free and immobilized in different supports.

Enzyme	Support	Immobilization pathway	Optimal activity (Free / Immobilized)		Reference
			pH	Temperature (°C)	
Endoglucanase	OGM	EDC-NHS	4 / 4	60-70 / 50	This work
Xylanase	OGM	EDC-NHS	5 / 5	60 / 60	This work
Xylanase	OGM	PEGA	6.5 / 7.5	60 / 60	11
Total cellulases	OGM	4arm PEG NH2	5 / 4	40 / 50	12
Total cellulases	NPM	EDC	4 / 5	50 / 50	13

4 CONCLUSION

The synthesis of MGO was successfully conducted, and the material exhibited the expected magnetic properties. Ideal conditions for endoglucanase and xylanase activity were confirmed within the typical pH range of 4-6 and temperature range of 50-70 °C. The immobilization process on MGO did not alter the optimal conditions for xylanase activity, which remained at pH 4 and 60 °C. However, it shifted the ideal temperature range for free endoglucanase from 60-70°C to a lower optimal temperature of 50°C when immobilized. The optimal initial protein loading range for immobilization was 55-65 mg per gram of support, aligning with support saturation and stabilization of enzymatic activity, and the immobilization yield was 68%, equating to about 42 mg of protein immobilized per gram of support. The immobilization process via the EDC-NHS route showed regular yields (>50%) and the immobilized catalyst maintained good enzymatic activity compared to theoretical values derived from initial activities and supernatant measurements. This study confirms that GO is a promising material for the immobilization of cellulases and xylanases, serving as an effective solid support, and can be synthesized with relative ease and high yield.

5 REFERENCES

1. Wilson, D. B. Cellulases and biofuels. *Curr Opin Biotechnol* **20**, 295–299 (2009).
2. Dos Santos, F. K. F. *et al.* High Added-Value by-Products from Biomass: A Case Study Unveiling Opportunities for Strengthening the Agroindustry Value Chain. *Biomass* **4**, 217–242 (2024).
3. Singh, N. *et al.* Global status of lignocellulosic biorefinery: Challenges and perspectives. *Bioresource Technology* vol. 344 Preprint at https://doi.org/10.1016/j.biortech.2021.126415 (2022).
4. Sirisha, V. L., Jain, A. & Jain, A. Enzyme Immobilization: An Overview on Methods, Support Material, and Applications of Immobilized Enzymes. *Adv Food Nutr Res* **79**, 179–211 (2016).
5. Adeel, M., Bilal, M., Rasheed, T., Sharma, A. & Iqbal, H. M. N. Graphene and graphene oxide: Functionalization and nano-bio-catalytic system for enzyme immobilization and biotechnological perspective. *Int J Biol Macromol* **120**, 1430–1440 (2018).
6. Hummers, W. S. & Offeman, R. E. Preparation of Graphitic Oxide. *J Am Chem Soc* **80**, 1339–1339 (1958).
7. Bradford, M. M. A Rapid and Sensitive Method for the Quantitation Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal Biochem* **254**, 248–254 (1976).
8. Sheldon, R. A. & van Pelt, S. Enzyme immobilisation in biocatalysis: why, what and how. *Chem Soc Rev* **42**, 6223–6235 (2013).
9. Paz-Cedeno, F. R. *et al.* Magnetic graphene oxide as a platform for the immobilization of cellulases and xylanases: ultrastructural characterization and assessment of lignocellulosic biomass hydrolysis. *Renew Energy* (2020) doi:10.1016/j.renene.2020.09.059.
10. Tanaka, M., Taniguchi, M., Matsuno, R. & Kamikubo, T. Purification and Properties of Cellulases from Eupencillium javanicum : Studies on the Re-utilization of Cellulosic Resources(VII). *Journal of fermentation technology* **59**, 177–183 (1981).
11. Mehnati-Najafabadi, V., Taheri-Kafrani, A. & Bordbar, A. K. Xylanase immobilization on modified superparamagnetic graphene oxide nanocomposite: Effect of PEGylation on activity and stability. *Int J Biol Macromol* **107**, 418–425 (2018).
12. Han, J. *et al.* Preparation and characterization of Fe3O4-NH2@4-arm-PEG-NH2, a novel magnetic four-arm polymer-nanoparticle composite for cellulase immobilization. *Biochem Eng J* **130**, 90–98 (2018).
13. Jordan, J., Kumar, C. S. S. R. & Theegala, C. Preparation and characterization of cellulase-bound magnetite nanoparticles. *J Mol Catal B Enzym* **68**, 139–146 (2011).

6 ACKNOWLEDGEMENTS

São Paulo Research Foundation (FAPESP) - Process number 2021/07058-0.
National Council for Scientific and Technological Development (CNPq) - Process number 302946/2023-3.
São Paulo State University (UNESP) – Notice 06/2024 PROPe.
School of Pharmaceutical Sciences (FCF) - Multi-User Lab.