IMMOBILIZATION OF COMMERCIAL CELLULASE ON Fe_3O_4-CHITOSAN MAGNETIC PARTICLES

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ABSTRACT

Cellulases catalyze the hydrolysis of cellulose into glucose, crucial for producing sugar platforms. However, the economic viability of the cellulose hydrolysis process is impacted by the high costs associated with enzymes. Immobilizing enzymes offers a promising solution, enabling biocatalyst recovery and reuse, thus reducing costs. While alginate and chitosan matrices are efficient and cost-effective, recovering immobilized enzymes is laborious and costly, hindering large-scale applications. Magnetic supports offer an alternative, facilitating enzyme recovery with an external magnetic field, even in the presence of other solids. This study investigates the immobilization of the *Cellic®CTec2* cellulolytic complex on Fe_3O_4-chitosan magnetic nanoparticles. The particles were synthesized via coprecipitation and activated with glutaraldehyde (0.25 and 3.0% v/v). Immobilization was performed with 10 $mg_{protein}/g_{activated\ support}$ at pH 7. Results showed that the higher the glutaraldehyde concentration, the better immobilization efficiency (51% for 3% (v/v) glutaraldehyde and 19% for 0.25 % (v/v) glutaraldehyde). However, the enzymatic activity recovered was higher for the lower activator agent (90% for 0.25% (v/v) glutaraldehyde and 28% for 3 % (v/v) glutaraldehyde. Thus, future studies will explore the combined effect of glutaraldehyde, enzymatic concentration, and pH immobilization to maximize the immobilization yield, recovered activity, and stability.

Keywords: Enzymatic immobilization; Commercial cellulases; Magnetic particles; Second-generation ethanol

1 INTRODUCTION

Cellulases catalyze the hydrolysis of cellulose into glucose and are used to obtain sugar platforms. However, the high cost of enzyme complexes makes the process economically challenging[1]. Immobilization of enzymes is a promising solution, allowing the recovery and reuse of the biocatalyst in subsequent batches, thus reducing the cost of the process[2].

This technique involves binding the enzyme within a support material, allowing the substrate and product to diffuse through the matrix in which the enzyme is confined. Compared to free enzymes, immobilized enzymes may exhibit better thermal stability, pH stability, and resistance to inhibitory products present in the reaction medium, such as lignocellulosic hydrolysates[2].

Large particles of immobilized enzymes (larger than 100 µm) are often chosen because they facilitate the separation process. Thus, only the larger particles are recovered, and the larger the particles, the greater the diffusional effects present in the derivative. Therefore, the selective separation of the support can become laborious and costly through classical methods, making the process difficult on a large scale. These limitations could be overcome by using magnetic nanoparticle supports since they are easily recoverable using only an external magnetic field[3]. These nanoparticles offer high surface area, lower diffusional resistance, and improved thermal and pH stability, enhancing catalytic efficiency.

In this context, this present study aims to immobilize commercial cellulases on magnetic nanoparticle supports for practical bioprocess applications.

2 MATERIAL & METHODS

2.1 Materials

The commercial cellulase complex *Cellic®CTec2* from Novozymes Latin America was used for the immobilization assays. According to the manufacturer's catalog, the enzyme complex comprises a mixture of cellulases, with a high content of β-glucosidase and hemicellulases. Iron oxide (Fe_3O_4) magnetic particles coated with chitosan were used as carriers, while glutaraldehyde was the activation agent. Carboxymethylcellulose was used as the substrate to evaluate the immobilized enzyme's performance.

2.2 Synthesis of chitosan-coated magnetic particles

Fe_3O_4 magnetic particles were prepared using the conventional coprecipitation method with some modifications. In summary, a mixture of 0.04 M $FeCl_3 \cdot 6H_2O$, 0.02 M $FeSO_4.7H_2O$ (2:1), and 1 g of chitosan was dissolved in 800 mL of acetic acid (2% v/v) under nitrogen protection. The solution was stirred at 70°C for 1 hour, and its pH was adjusted to 10 by the dropwise addition of NH_4OH (22.5% v/v). Subsequently, the magnetite precipitates were separated from the reaction mixture using an external

permanent magnet, washed, and centrifuged with deionized water until the pH reached 7. Then, the particles were placed in an oven to dry for 24 hours. After drying, they were ground and stored at 4°C for immobilization.

2.3 Characterization of magnetic particles

X-ray diffraction analysis to identify the phase composition of the synthesized samples was performed at the Mechanical Laboratory Center (CLM) of FEI, using an XRD-7000 diffractometer (Shimadzu Inc.). The samples were scanned with a step of 0.015°, using a 1.54 Å (Cu-Kα) source operating at 40 kV and 35 mA. The collected data were processed using the HighScore Plus® software for phase identification and quantification of crystallinity parameters through the Rietveld method. The crystallographic records used in the analysis were 96-900-5843 and 96-901-0407 for magnetite and goethite, respectively.

2.4 Support activation and enzyme immobilization

Different concentrations of glutaraldehyde solution (0.25 and 3% v/v) were employed to activate the support and provide aldehyde groups for cellulase immobilization. 0.25 g of Fe_3O_4-chitosan particle support reacted with 7.5 mL of glutaraldehyde solution at diluted concentrations in 0.1 M phosphate buffer at pH 7. The reaction was conducted with agitation at 150 rpm, at 25°C for 120 minutes. After activation, the support was washed with deionized water ten times to remove unreacted glutaraldehyde.

For immobilization, 0.25 g of Fe_3O_4-chitosan particle support reacted with 2.5 mL of enzyme solution correspondent to 10 $mg_{protein}/g_{support}$. The immobilization occurred at 150 rpm, at 25°C for 24 h.

2.5 Immobilization performance

During immobilization, aliquots of the enzyme solution were quantified for Bradford protein content[4]. This analysis allows to determine the percentage of immobilized enzyme (I) on the carrier compared to the total amount of offered enzyme, expressed in equation 1:

$$I\ (\%) = 1 - \frac{C_f}{C_i}.100 \qquad eq.1$$

where C_i is the protein concentration of the cellulase solution before immobilization and C_f is the protein concentration in supernatant after immobilization.

The activity recovered (A_R) after immobilization was calculated according to Equation 2.

$$A_R(\%) = \left(\frac{A_d}{\frac{A_i * I(\%)}{100}}\right).100 \qquad eq.2$$

Where A_i is the free enzyme activity before immobilization, and A_d is the total activity observed in the derivative after immobilization.

The overall immobilization yield (O_y) was calculated according to Equation 3.

$$O_y(\%) = \left(\frac{A_d}{A_i}\right).100 \qquad eq.3$$

3 RESULTS & DISCUSSION

Figure 1 shows the obtained diffractogram, identifying the main crystalline facets of the prepared iron oxides. Semi-quantitative Rietveld analysis shows that 66.1% (mass basis) of the crystalline material is composed of iron oxide in the form of magnetite (Fe_3O_4), while 33.9% is identified as goethite (α-FeO(OH)). No peaks corresponding to chitosan were identified, suggesting its organization as an amorphous structure. Refinement showed that the magnetite particles have a diameter of 9.25 ± 0.56 nm, while the goethite particles have a size of 11.92 ± 0.16 nm.

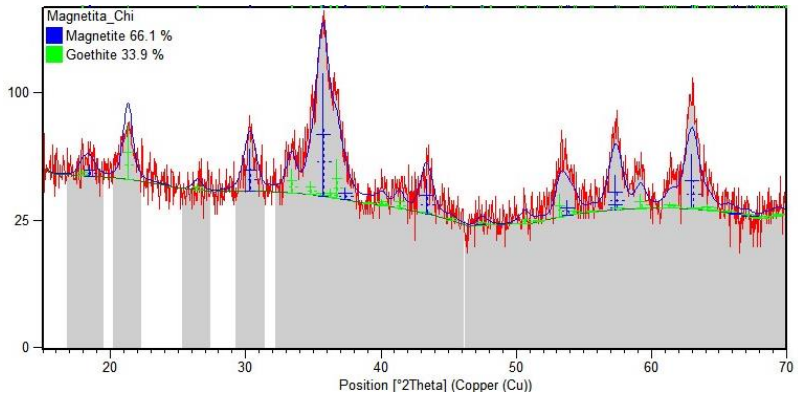
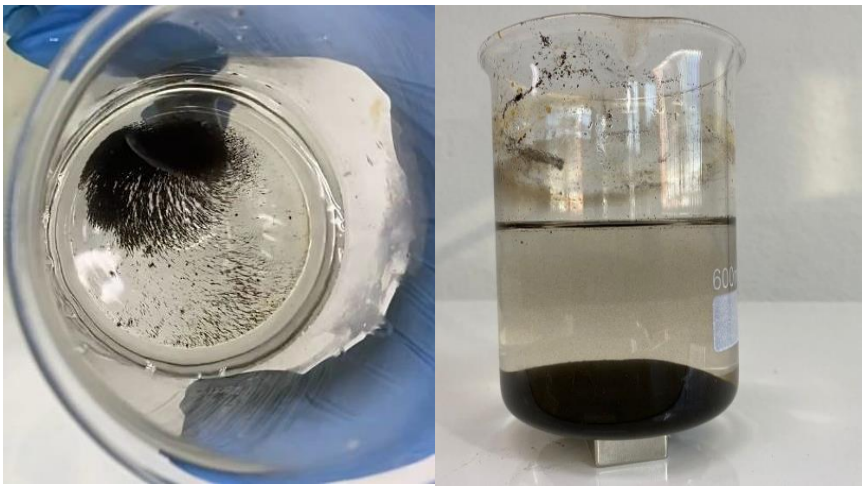


Figure 1 Diffractogram of Magnetic particles

The magnetic character of the particles can be seen in Figure2.



Figures 2. Magnetic character of the particles

The magnetic nanoparticles were activated with glutaraldehyde at two different concentrations (0.25% and 3% v/v) to evaluate its effect on the immobilization percentage. At 3% (v/v) glutaraldehyde, the immobilization yield was 2.6-fold higher. The higher the glutaraldehyde concentration, the more active groups are available to react with the enzyme, leading to an increased immobilization efficiency, as expected. On the other hand, the activity recovered for support activated with 0.25% (v/v) glutaraldehyde was 3.2-fold higher.

The lower enzymatic activity observed at 3% (v/v) glutaraldehyde is attributed to the strong interactions between the enzymes and the supports, which can induce structural distortions and diminish the immobilized enzymes' degree of freedom. Consequently, although using higher concentrations of crosslinking agents effectively prevents desorption, it also results in enzyme inactivation[3].

Despite the high recovered activity obtained in test 1, the overall immobilization yield was as low as in test 2. This behavior is due to the low immobilization percentage (19%), considerably reducing the overall process yield. Therefore, to achieve high overall yields, it is necessary to obtain a satisfactory immobilization percentage and a high level of recovered activity.

Table 1 Results of cellulase immobilization onto magnetic nanoparticles activated with glutaraldehyde at different concentrations.

Assay	Glutaraldehyde (% v/v)	Immobilization (%)	Activity recovered (%)	Overall immobilization yield (%)
1	0.25	19.1	89.58	17.1
2	3.0	50.7	28.32	14.3

4 CONCLUSION

The study shows that different concentrations of glutaraldehyde significantly influence the immobilization percentage, with better results at higher concentrations. However, a reduction in enzymatic activity was observed in this condition. Thus, future studies will evaluate the combination of enzyme extract concentration, activator agent, and immobilization pH to achieve higher immobilization efficiencies and enzymatic activities.

REFERENCES

[1] ELIAS, Andrew Milli. 2021. Retro-techno-economic-environmental analysis improves the operation efficiency of 1G-2G bioethanol and bioelectricity facilities. Applied Energy, v. 282, p. 116133.

[2] KHOSHNEVISAN, Kamyar. 2019. Recent advances of cellulase immobilization onto magnetic nanoparticles: an update review. Magnetochemistry, v. 5, n. 2, p. 36.

[3] KOPP, Willian. 2015. Synthesis and characterization of robust magnetic carriers for bioprocess applications. Materials Science and Engineering: B, v. 193, p. 217-228.

[4] Bradford, MM. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254. 1976.

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