Improvement of ethyl ester synthesis via lipase immobilization on $NiFe_2O_4$ nanoparticles

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ABSTRACT

Enzyme immobilization is a particularly enabling technique in developing efficient and sustainable catalytic processes, facilitating the reuse and operational stability of the catalysts. Among various supports for immobilization, spinel ferrite has received attention due to its unique properties, especially the easy enzyme recovery. This work explores the capacity of nickel ferrite magnetic nanoparticles ($NiFe_2O_4$) as a support for the immobilization of lipase B from *Candida antarctica*. The effect of protein concentration on lipase immobilization was investigated, and concentrations above 0.5 mg mL^{-1} were unsuitable for lipase adsorption onto $NiFe_2O_4$. The highest immobilization efficiency by physical adsorption was low (21.3 %), and CalB was covalently immobilized after functionalization of $NiFe_2O_4$ with APTMS and activation with glutaraldehyde (GA) ($NiFe_2O_4$-APTMS-GA-CalB), with higher immobilization efficiency (62.9 %). The ethyl ester synthesis was successfully achieved, with an oleic acid conversion of 37.3 ± 1.0% and 62.1 ± 0.2% using $NiFe_2O_4$-APTMS-GA-CalB and $NiFe_2O_4$-CalB, respectively. $NiFe_2O4$-CalB maintained 92% of its initial activity after 4 cycles, showing good reusability. Nickel ferrite magnetic nanoparticles form an efficient heterogeneous catalyst with lipase, showing potential in esterification reactions like ethyl oleate synthesis.

Keywords: CalB. physical adsorption. covalent binding. esterification.

1 INTRODUCTION

The development of green chemistry has advanced the use of enzymes as biocatalysts in various industrial processes. Enzymatic biocatalysts are non-toxic and exhibit high activity, specificity, and selectivity, usually under mild environmental conditions, compared to chemical catalysts. Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) are among the most used enzymes in biocatalysis, showing high versatility and great stability, being applied in a variety of industry applications, such as in food, dairy, chemical, detergent, pharmaceutical, and biodiesel production [1]. To enable a large use of lipase as a biocatalyst, an immobilization process is necessary to reduce the limitations of free enzyme application. Immobilization can improve enzyme features and facilitate recovery and reuse [2]. Physical adsorption and covalent binding are widely used techniques applied for immobilizing enzymes. While adsorption is a simple and cheap technique, covalent binding provides strong interactions between enzyme and support, preventing enzyme leaching. However, enzymes can suffer conformational changes during immobilization, reducing their activity[3].

Recently, magnetic nanoparticles have received attention for enzyme immobilization due to their large surface area, lower mass transfer resistance, low porosity, mechanical stability, and presence of hydroxyl groups on their surface, facilitating further functionalization[4]. Applying an external magnetic field promotes an easy and fast separation of magnetic material from a suspension, enabling an effective recovery of the immobilized enzyme [4]. Immobilization of lipases on magnetic nanoparticles has been described for different purposes. Mehrasbi et al.[5] immobilized lipase B from *Candida antarctica* (CalB) on Fe_3O_4 core coated with a silica shell and functionalized with 3-(glycidoxypropyl) trimethoxylsilane (GPTMS) for biodiesel production from waste cooking oil. A fatty acid methyl ester (FAME) yield of 96 % was achieved using 100 mg of immobilized enzyme.

In this work, we investigated the application of the nickel ferrite magnetic nanoparticle ($NiFe_2O_4$) as a support for lipase B from *Candida antarctica* (CalB). The enzyme was immobilized by physical adsorption and covalent binding, and its potential as a biocatalyst was evaluated in ethyl ester synthesis through esterification reactions. The reusability of the obtained immobilized derivatives was also investigated in the ethyl oleate synthesis.

2 MATERIAL & METHODS

Novozymes Latin America Ltda (Araucária, Brazil) kindly donated the lipase B from *Candida antarctica* (CalB). A lipase solution with different concentrations was prepared by diluting CalB in a 5 mmol L^{-1} sodium phosphate buffer (pH 7). For immobilization by physical adsorption, 15 mL of enzymatic solution were mixed with 0.15 g of $NiFe_2O_4$ nanoparticles and gently stirred for 2 h at room temperature (25 ±1 °C). At the end of immobilization, the obtained derivative ($NiFe_2O_4$-CalB) was recovered using an external magnetic field, washed with 10 mL of 5 mmol L^{-1} phosphate buffer, and stored at 4 °C.

For immobilization by covalent binding, the support was functionalized with 3-aminopropyl trimethoxysilane (APTMS) reagent and activated with glutaraldehyde (GA). For the functionalization with APTMS, 100 mL of ethanol and 100 mL of distilled water were added to 0.1 g of nickel ferrite, followed by ultrasonication for 5 min. 10 mL of the APTMS solution were added to the mixture and

kept under agitation (150 rpm) at 60 °C for 24 h. The modified nickel ferrite nanoparticles ($NiFe_2O_4$-APTMS) were separated by applying an external magnetic field and washed with distilled water and ethanol. For the activation and immobilization of CalB, 2.5% v/v of GA and an adequate volume of CalB solution (final concentration of 0.1 mg mL^{-1}) were mixed with a 5 mmol L^{-1} phosphate buffer (pH 7). 15 mL of this solution were added to 0.15 g of nickel ferrite nanoparticles functionalized with APTMS and stirred for 24 h at 4 °C. The immobilized derivative ($NiFe_2O_4$-APTMS-GA-CalB) was separated by an external magnetic field, washed with 10 mL of 5 mmol L^{-1} sodium phosphate buffer, and dried in a desiccator. Immobilization was accompanied by determining the protein concentration in the supernatant obtained during the process.

Esterification reactions for ethyl oleate synthesis were carried out for 24 h using 15 mmol of oleic acid and 15 mmol of ethanol (molar ratio of 1), followed by the addition of the biocatalyst (CalB, $NiFe_2O_4$-CalB, and $NiFe_2O_4$-APTMS-GA-CalB). The amount of biocatalyst utilized in the experiments was adjusted to ensure the same protein content and activity units. A magnetically stirred and thermostated closed batch reactor was used for ethyl oleate synthesis. Samples were taken at fixed intervals (50 µL), and 40 mL of acetone-ethanol-water solution (1:1:1) was added to stop the reaction. The residual oleic acid was analyzed by volumetric neutralization with a sodium hydroxide 0.02 mol L^{-1} solution using a Mettler Toledo T50 Titrator. The blank tests were performed by adding $NiFe_2O4$ and $NiFe_2O_4$-APTMS to the reaction mixture (without enzyme). Reusability tests were conduct in the same conditions mentioned above, at 30°C for 24 h, after washed the biocatalysts with 4 mL of *tert*-butanol.

3 RESULTS & DISCUSSION

CalB was immobilized by physical adsorption by incubating $NiFe_2O_4$ nanoparticles with an enzyme solution under gentle stirring at room temperature (25 °C) for 2 h. The adsorption method offers several advantages, including straightforward operation, a mild enzyme immobilization process that preserves the enzyme's native structure, cost-effectiveness, and widespread applicability. To study the effect of lipase content on the immobilization efficiency, various CalB solutions with different protein concentrations were prepared, and then $NiFe_2O_4$ nanoparticles were added to these solutions. The immobilization efficiency and the amount of adsorbed protein (mg g^{-1} of support) according to the different protein concentrations tested after immobilization for 2 h are depicted in Figure 1. The increase in CalB concentration led to a gradual increase in protein adsorption in $NiFe_2O_4$ nanoparticles until they became stable, probably due to support saturation. Conversely, the immobilization efficiency decreased according to the rise in the protein concentration, meaning that immobilization on nickel ferrite at concentrations above 0.5 mg mL^{-1} (10 mg g-1) is not advantageous. Due to this, a concentration of 0.1 mg mL^{-1} was chosen for further experiments.

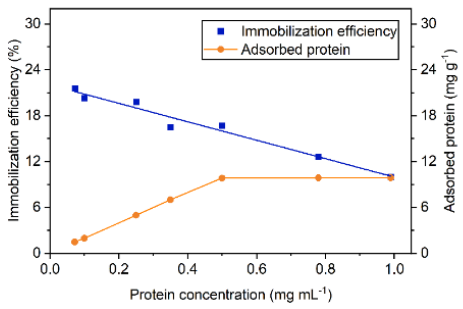

Figure 1 Effect of lipase loading on the immobilization process using nickel magnetic nanoparticles as support.

Table 1 shows the results for CalB immobilization by physical adsorption and covalent binding. Comparing both methods, the functionalization of $NiFe_2O_4$ nanoparticles with APTMS and activation with GA led to a 3-fold increase in the adsorbed enzyme. Lipase can be covalently attached to the magnetic nanoparticles due to a Schiff base linkage formed between the amino-terminal lipase and the aldehyde group of GA[6]. This crosslinking agent can react with the support, allowing multipoint covalent immobilization, and can improve enzyme rigidity due to the promotion of intramolecular or intermolecular crosslinking, which decreases enzyme release from $NiFe_2O_4$ nanoparticles [6].

Table 1 Immobilization efficiency (IE) and amount of immobilized protein (IP) obtained for CalB (0.1 mg mL^{-1}) immobilization on $NiFe_2O_4$ nanoparticles.

Biocatalyst	IE (%)	IP (m g^{-1})
$NiFe_2O_4$-CalB	21.3 ± 0.3	2.3 ± 0.3
$NiFe_2O_4$-APTMS-GA-CalB	62.9 ± 1.7	6.0 ± 0.6

The biocatalysts $NiFe_2O_4$-CalB and $NiFe_2O_4$-APTMS-GA-CalB were used in esterification reactions to synthesize ethyl oleate. The results of the conversion of oleic acid to ethyl oleate during 24 h are shown in Figure 2. The amount of biocatalyst (CalB, $NiFe_2O_4$-CalB, and $NiFe_2O_4$-APTMS-GA-CalB) utilized in the experiments was adjusted to ensure the same protein content (Figure 2). The two immobilized derivatives could synthesize ethyl oleate via esterification, with more excellent production at 24 h of reaction. The performance achieved with lipase physically adsorbed to nickel ferrite ($NiFe_2O_4$-CalB) surpassed that of the derivative obtained through covalent immobilization ($NiFe_2O_4$-APTMS-GA-CalB). Therefore, the physical adsorption binding strategy exhibited lower surface loading but higher catalytic activity than covalent immobilization. Covalent immobilization can cause a decrease in enzyme activity that can be attributed to protein denaturation due to the coupling process and diffusion limitation after enzyme immobilization [5]. Free CalB could convert only 19.2 % of oleic acid with the same content of lipase protein as the $NiFe_2O_4$-CalB after 24 h. These results demonstrate that $NiFe_2O_4$-CalB increased conversion about 3.3-fold compared to liquid CalB.

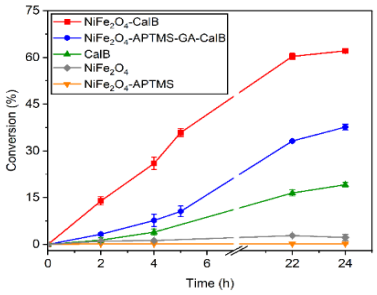

Figure 2 Kinetics of esterification reaction for ethyl oleate synthesis of $NiFe_2O_4$-CalB, $NiFe_2O_4$-APTMS-GA-CalB, and CalB at 30 °C for 24 h.

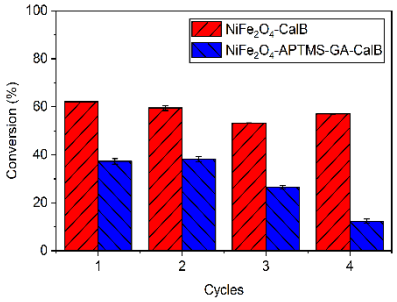

Figure 3 Evaluation of the reuse of the of $NiFe_2O_4$-CalB and $NiFe_2O_4$-APTMS-GA-CalB in the conversion of oleic acid in the esterification reaction.

The ethyl oleate synthesis was also evaluated using immobilized derivatives and free CalB in different concentrations while mantaining the same activity units (5 U) in each esterification reaction. Results were similar to those obtained when the same amount of protein was used, reaching an oleic acid conversion at 24 h of 54.3%, 37.5%, and 15.8% for $NiFe_2O_4$-CalB, $NiFe_2O_4$-APTMS-GA-CalB and CalB, respectively. The performance of immobilized CalB described in the literature significantly varies according to the nature of support, immobilization techniques applied, and reaction conditions. Chiaradia et al. [7] studied the immobilization of CalB in magnetic poly(urea-urethane) nanoparticles and used it to synthesize ethyl oleate, geranyl oleate, and geranyl propionate. In all esterification reactions, the production of esters by the biocatalyst was higher than 85%. In another work, Rios et al.[8] immobilized CalB and *Yarrowia lipolytica* lipase in mesoporous silica (SBA-15) by physical adsorption. After 24 h of reaction, CalB converted 60% oleic acid at 37 °C.

The reusability results of $NiFe_2O_4$-CalB and $NiFe_2O_4$-APTMS-GA-CalB are shown in Figure 3. It was observed that both immobilized derivatives catalyzed the synthesis of ethyl oleate effectively and could be reused. The biocatalyst derived from covalent immobilization maintained oleic acid conversions around 40% after 2 batches. The third batch could convert about 26% of oleic acid, with a more significant drop in the fourth. In contrast, the lipase immobilized by physical adsorption achieved a conversion of approximately 60% in the first batch and showed only a 5% decrease in conversion on its third reuse (4th cycle). Therefore, the operational stability of $NiFe_2O_4$-CalB was higher than that of covalently bound lipase ($NiFe_2O_4$-APTMS-GA-CalB).

While the adsorption method offers a simpler and faster immobilization process, it's important to note the potential risks it poses. Under specific conditions, there is a risk of enzyme desorption, which could lead to operational inactivation of the biocatalyst and product contamination. However, in the case of lipase immobilized through adsorption, no significant leaching effect was observed. The conversion rate decreased by approximately 5%, indicating that the enzyme remained active after four 24-hour reactions.

4 CONCLUSION

This study demonstrated that magnetic nickel ferrite nanoparticles can effectively be used as support for immobilization of lipase B from *Candida antarctica*. While physical adsorption resulted in lower immobilization efficiency (<22%) compared to covalent immobilization (62.9%), the biocatalyst produced via physical adsorption exhibited superior catalytic activity. It converted 62.1% of oleic acid into ethyl oleate and maintained 92% of its initial activity over four consecutive batches. $NiFe_2O_4$-CalB was easily recovered from the reaction mixture and demonstrated the potential to be applied in esterification reactions to synthesize ethyl oleate.

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