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EFFICIENT IMMOBILIZATION METHOD OF LACCASE ON δ-FeOOH MAGNETIC NANOPARTICLES

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ABSTRACT

Utilizing Magnetic Nanoparticles for enzyme immobilization provides a direct method to enhance enzymatic catalyst efficiency across diverse industrial sectors. Despite its straightforward synthesis and cost-effectiveness, Feroxyhyte (δ-FeOOH), an iron oxyhydroxide, remains underexploited as an enzyme immobilization support, rendering it a promising candidate for various applications. In this investigation, Laccase was covalently immobilized onto the surface of δ-FeOOH magnetic nanoparticles coated with tetraethoxysilane (TEOS) and 3-aminopropyltriethoxysilane (APTES). The optimal immobilization conditions including pH, temperature (°C), agitation (RPM), glutaraldehyde concentration (%), and Laccase amount (mg) were determined through PB design. Subsequently, the optimal immobilization condition (pH 7, temperature of 25°C, agitation at 400 RPM, 0.1% glutaraldehyde, and 1 mg of Laccase) was applied to evaluate the behavior of free and immobilized activity of laccase. Results showed no alterations in the maximum activity conditions for both forms, indicating that laccase can be utilized in either form without compromising its optimal activity conditions.

Keywords: δ-FeOOH. Magnetic Nanoparticles. Immobilized laccase.

1 INTRODUCTION

Enzymes, highly specific proteins, serve as efficient biocatalysts both in natural and industrial contexts. Their specificity and effectiveness have led to extensive research and widespread industrial applications. For instance, Laccase (oxygen oxidoreductase, EC 1.10.3.2) is a copper-containing oxidase enzyme recognized for facilitating the one-electron oxidation of aromatic compounds alongside a four-electron reduction of oxygen to water [1,2]. It is prevalent in numerous plants, microorganisms, and notably in fungi exhibiting white-rot. Boasting a wide substrate spectrum covering aromatic hydroxyl and amine groups, polyphenols, and methoxy-substituted monophenols, laccase is utilized across various sectors such as the breakdown of lignin in lignocellulosic biomass, generation of bio-products, clarification of wine, manufacture of detergents, and degradation of azo dyes [1,2,3].

Enzymes can achieve reusability and stability through immobilization techniques like enzyme cross-linking, enzyme attachment onto nanomaterials, and encapsulation of enzymes within beads. In comparison to various other immobilization supports, magnetic nanoparticles (δ-FeOOH) have garnered significant attention due to their simple synthesis, robust magnetic properties, affordability, low toxicity, and favorable physical attributes. δ-FeOOH provides ample surface area for the immobilization of bioactive molecules, allowing for multiple reuses facilitated by an external magnetic field. β-glucosidase, β-xylosidase, and xylose isomerase have been effectively immobilized on δ-FeOOH for ethanol production in fermentation processes[3,4,] Enzyme immobilization on δ-FeOOH demonstrates favorable catalytic performance, increased thermal and storage stability, rapid recovery, and reduced mass transfer limitations[4].

To the best of our knowledge, no study has been reported in the literature evaluating the use of laccase immobilized in magnetic nanoparticles (δ-FeOOH). Then, this study aimed to investigate the application of laccase immobilized in magnetic nanoparticles (δ-FeOOH) and evaluate the activity of the free and immobilized enzyme.

2 MATERIAL & METHODS

Plackett-Burman designs (PB) were used to screen and adjust the variables that may affect immobilization of laccase (Rodrigues and Iemma, 2014) [5]. For this, 0.0142 g of magnetic nanoparticles (δ-FeOOH) synthesized by process described by Guilherme et al. [3]. Next, the effect of eleven independent variables in the extraction process was evaluated in the PB design (Table 1). The variables pH, temperature (ºC), agitation (RPM), glutaraldehyde (%) and Lacase (mg). To ensure a sufficient degree of freedom to calculate the standard error, the design included twelve tests (seven more than the number of independent variables) and four central points (0), totalizing 16 experiments (Rodrigues and Iemma, 2014) [5,] These experiments were randomly performed to eliminate external effects. The statistical analyzes were carried out using the online software Protimiza Experimental Design (http://experimental-design.protimiza.com.br/). After determining the best laccase immobilization condition in PB design, the activity of free and immobilized laccase was studied using central composite rotatable design (CCRD) [5] with laccase activity determined according to Qiu et al. [6].

3 RESULTS & DISCUSSION

Plackett-Burman (PB) designs used employed to monitor and fine-tune variables influencing laccase immobilization on δ-FeOOH magnetic nanoparticles is presented in Table 1 and (Fig. 1A-B). For the effects of the independent variables in the immobilization of bound laccase (mg/g), glutaraldehyde levels and the amount of laccase (mg) were the variables that cause an increase in mg/g in ranged studied (Fig. 1A). In the immobilization activity (U/g), pH was the variable that cause an increase in U/g in ranged studied (Fig. 1B).

Table 1. Plackett-Burman (PB12) design with coded, real values and observed responses for the milligram of bound laccase (mg/g) and activity (U/g) on the δ-FeOOH magnetic support.

Run	pH	Temperature (ºC)	Agitation (RPM)	Gluteraldehyde (%)	Laccase (mg)	Bound laccase (mg/g)	Laccase activity (U/g)
1	7 (+1)	25 (-1)	1200 (+1)	0.1 (-1)	0.5 (-1)	21.10	198.18
2	7 (+1)	65 (+1)	400 (-1)	1.6 (+1)	0.5 (-1)	35.34	192.04
3	3 (+1)	65 (+1)	1200 (+1)	0.1 (-1)	1.5 (+1)	63.64	15.40
4	7 (+1)	25 (-1)	1200 (+1)	1.6 (+1)	0.5 (-1)	17.19	189.51
5	7 (+1)	65 (+1)	400 (-1)	1.6 (+1)	1.5 (+1)	27.92	206.36
6	7 (+1)	65 (+1)	1200 (+1)	0.1 (-1)	1.5 (+1)	51.46	140.87
7	3 (-1)	65 (+1)	1200 (+1)	1.6 (+1)	0.5 (-1)	35.21	0.84
8	3 (-1)	25 (-1)	1200 (+1)	1.6 (+1)	1.5 (+1)	51.88	5.98
9	3 (-1)	25 (-1)	400 (-1)	1.6 (+1)	1.5 (+1)	35.92	12.11
10	7 (+1)	25 (-1)	400 (-1)	0.1 (-1)	1.5 (+1)	58.07	267.15
11	3 (-1)	65 (+1)	400 (-1)	0.1 (-1)	0.5 (-1)	37.60	2.92
12	3 (-1)	25 (-1)	400 (-1)	0.1 (-1)	0.5 (-1)	32.16	0.47
13	5 (0)	45 (0)	800 (0)	0.85 (0)	1.0 (0)	32.08	41.57
14	5 (0)	45 (0)	800 (0)	0.85 (0)	1.0 (0)	27.24	41.65
15	5 (0)	45 (0)	800 (0)	0.85 (0)	1.0 (0)	26.00	42.10
16	5 (0)	45 (0)	800 (0)	0.85 (0)	1.0 (0)	26.02	42.16

As seen in the results in Fig 1A, only the gluteraldehyde content and the amount of laccase significantly affected the bound laccase. The highest amount of bound laccase (mg/g) was observed in run 3 (pH 3, Temperature of 65 ºC, Agitation 1200 rpm, 0.1% Glutaraldehyde and 1.5 mg of Laccase), approximately 63.64 mg/g and Laccase activity 15.40 U/g. Evaluating the results of the immobilization activity of laccase Fig 1B, only pH significantly affected the laccase immobilization activity. For the highest immobilization activity (U/g), was 267 (U/g) in run 10 (pH 7, Temperature of 25 ºC, Agitation of 400 rpm, 0.1% glutaraldehyde and 1.5 mg of Laccase) with bound laccase 58.07 mg/g. Although the best conditions observed for the immobilization of bound laccase and immobilization activity of laccase were not the same, an advantage was observed in using the best condition for immobilization activity (run 10). Since in this condition it is observed that 87.3% of the laccase is bound when compared with the best condition for immobilization of bound laccase. In order to validate the results observed in the best condition (run 10), experiments were carried in duplicate and the values observed the values of immobilization activity was 272.10±1.58 U/g and 53.41±0.68 mg/g for bound laccase. Overall, the results were within the expected range (10% relative error), with experimental values were close to run10 results.

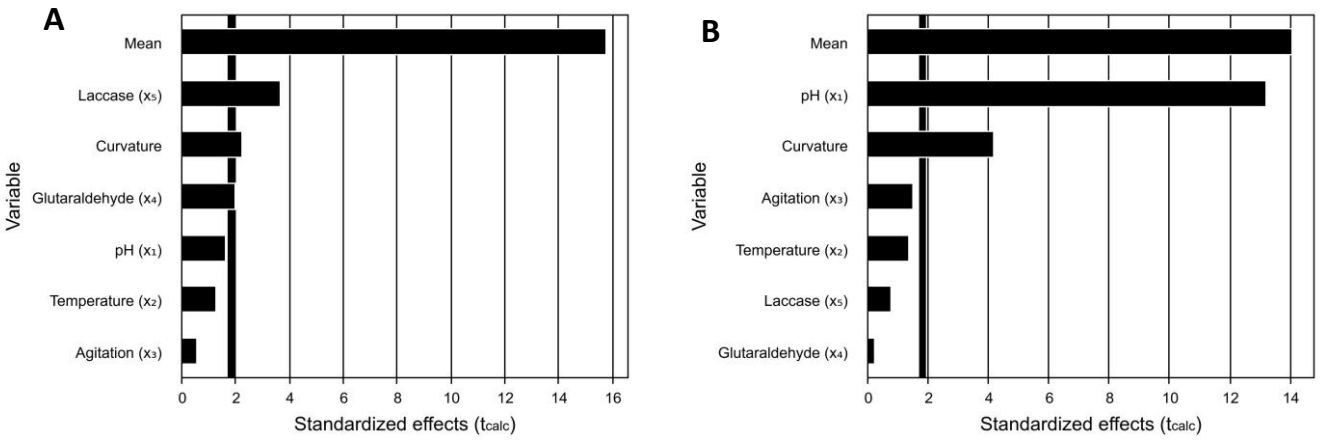

Figure 1: Pareto Chart with coded parameters variables pH (x1), temperature (x2), agitation (x3), glutaraldehyde (x4) and Lacase (x5) for bound of laccase (mg/g) (A) and immobilization activity of laccase (U/g) (B) at a significance level of 10% for Plackett-Burman (PB12) design.

After determining the best operational conditions for laccase immobilization by the Plackett-Burman (PB12) design, was evaluated the influence of pH and temperature on the activity of free and immobilized laccase in magnetic nanoparticles (δ-FeOOH) using a CCRD and the results are shown in Table 2 and Figure. 1C-D. The representative model was built for enzymatic activity considering a significance level of 5% ($p < 0.05$) for free laccase ($Y = 9.96 - 1.13\ x_1 - 4.58\ x_1^2 - 1.78\ x_2^2$; $R^2 = 95.29$; F_{calc}= 54 e F_{tab}= 3.59) and immobilized laccase ($Y = 285.19 - 36.60\ x_1 - 130.57\ x_1^2 - 66.10\ x_2^2$, $R^2 = 94.09$; F_{calc}= 42.1 e F_{tab}= 3.59). The results of models for enzyme activity for free and immobilized laccase were used to build the contour surfaces of this model are shown in (Fig 2 A-B).

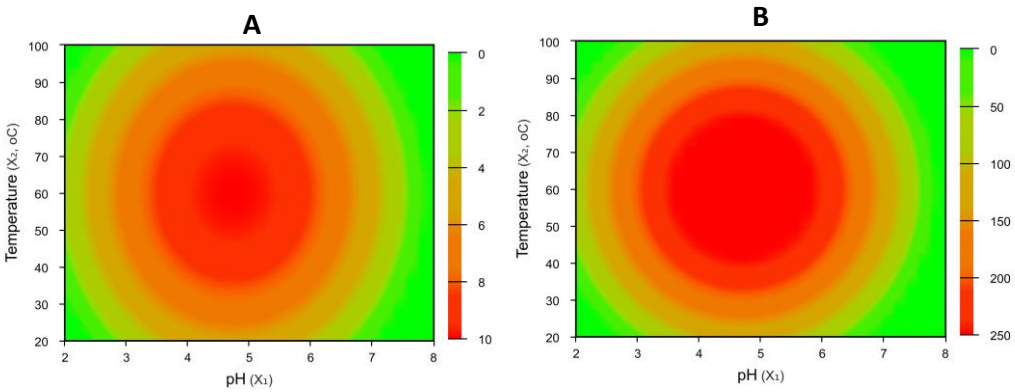

Figure 2: Contour curves for independent variables pH and temperature for activity of free (C) and immobilized laccase (D) with variables pH (x1) and temperature (x2)

The contour plot shows that the highest enzymatic activity yields are obtained in pH 4 to 6 for free enzyme and pH 3.5 to 6 for immobilized enzyme. The excellent temperature range of about 35 to 85 °C for free and immobilized enzyme. These results are interesting from the point of view of changes in maximum pH and temperature conditions on laccase activity. Since, no changes are observed in the best temperature conditions for laccase activity. When we observed the results in relation to pH, the difference observed was minimal, which did not affect the best conditions for its activity (pH 5 and 60ºC). Due to this information, pH 5.0 and temperature 60ºC were used to validate the models, with the values of 9.42±0.10 U/mg for free laccase and 269.88±11.49 U/g for immobilized laccase. The models were within the expected range (below of 10% relative error) and the predicted values were close to experimental results. The models were satisfactorily validated, demonstrating that they are adequate for predicting variations in laccase activity.

Table 2**.** Central composite rotatable design matrix used to study laccase activity compared between the free and immobilized.

Run	pH	Temperature (ºC)	Free laccase (U/mg)	Immobilized laccase (U/g)
1	2.89 (-1)	35.45 (-1)	6.30	162.65
2	7.11 (+1)	35.45 (-1)	2.12	53.96
3	2.89 (-1)	84.65 (+1)	5.59	158.26
4	7.11 (+1)	84.65 (+1)	1.80	7.85
5	2.02 (-1.41)	60.05 (0)	0.83	28.79
6	7.98 (+1.41)	60.05 (0)	0.09	4.99
7	5.00 (0)	25.26 (-1.41)	6.15	159.05
8	5.00 (0)	94.84 (+1.41)	5.95	132.62
9	5.00 (0)	60.05 (0)	10.21	278.77
10	5.00 (0)	60.05 (0)	9.90	284.63
11	5.00 (0)	60.05 (0)	9.89	290.87
12	5.00 (0)	60.05 (0)	9.83	286.48

This study presented a factor that is important to highlight, that although both free and immobilized laccase have maximum activity at more acidic pH, its optimal immobilization pH is more neutral. Another point that deserves to be highlighted is that in the temperature range there was no significant difference in the PB studied and did not affect the laccase activity in the free and immobilized forms.

4 CONCLUSION

The MNPs of δ-FeOOH was used to synthetized magnetic biocatalyst, by choosing the best immobilization conditions determined in the study using PB design. The best condition of laccase immobilization was used to evaluated behavior of free and immobilized laccase, where no changes were observed in the maximum activity conditions of both forms. Which demonstrated that in both forms laccase can be used without affecting the optimal activity conditions.

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