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EVALUATION OF FUNCTIONALIZATION STEPS FOR LACCASE IMMOBILIZATION ON POLYACRYLONITRILE SUPPORT

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ABSTRACT

This study is focused on modifying polyacrylonitrile (PAN) particles to allow an efficient laccase immobilization. Sequential steps were performed to chemically alter PAN, including treatment with alkaline and acidic solutions, amination, and cross-linking with glutaraldehyde. Fourier transform infrared spectroscopy (FTIR) confirmed a change in PAN chemical structure. This modification improved the laccase's ability to adhere to PAN compared to untreated PAN. They achieved a yield of 99.48% in enzymatic immobilization with modified PAN, while the immobilization yield of untreated PAN was 14.29%. PAN's ability to form functionalized groups through reactions was crucial to creating strong bonds between the enzyme and the support. This research demonstrated that the modified PAN is effective in immobilizing laccase.

Keywords: Laccase; *Trametes versicolor*; functionalized polyacrylonitrile particles; enzyme immobilization.

1 INTRODUCTION

Laccases are low-specificity enzymes that can convert recalcitrant compounds into less harmful molecules to the environment. The laccase application, according to industrial requirements, needs improvement and stability. The most investigated strategy to meet these requirements is enzyme immobilization, in which the studied protein adheres to a solid surface to provide greater thermal and chemical resistance for the enzyme.

Synthetic polymers have been used as immobilization supports due to their ease of production and low synthesis cost. Furthermore, the presence of functional groups or groups capable of being functionalized in the structure of these polymers allows the formation of more robust interactions between the support and the enzyme.[1] Polyacrylonitrile (PAN) is a synthetic polymer known for its resistance to organic solvents and microbiological corrosion and its good mechanical and thermal stability properties.[2]

The immobilization of enzymes in synthetic polymers is commonly addressed in the scientific literature. Moreover, it is essential to analyze existing groups in the polymeric structure that can be functionalized, especially when the immobilization process requires a covalent bond between the enzyme and the support. In general, synthetic polymers have inert structures and do not have reactive groups for such interactions with the enzyme. Therefore, specific reactions are carried out to activate functional groups and allow interactions with amino acid residues located in the three-dimensional structure of the enzyme. It is important to mention that in immobilization processes, the aim is to avoid the interaction of the support with residues present in the active site, which can cause steric hindrance and make it impossible for the enzyme to contact the substrate.

The effect of functionalization of polyacrylonitrile particles on the immobilization of laccase from *Trametes versicolor* was analyzed in this study. Millimeter particles ($1.2 \leq Dp \leq 1.7$ mm) underwent a four-step chemical modification process: alkaline hydrolysis, acid hydrolysis, amination, and crosslinking with Glutaraldehyde (Glu). Subsequently, the treated particles were immersed in a containing laccase solution for immobilization. The same process was carried out on non-functionalized particles as part of the comparative study.

2 MATERIALS & METHODS

After synthesis (adapted from Gurgel *et al.* 2024),[3] drying, and separation, 100 mg of PAN particles were weighed and functionalized in four steps. For alkaline hydrolysis, the particles were immersed in 50 mL of 1 mol·L^{-1} NaOH in an incubator, maintained at 40 ºC for different times (1, 2, and 3 h). After this time, the samples were washed with distilled water and sent to the second stage, where they were immersed in 50 mL of 10% v/v HNO_3/H_2SO_4 solution (50:50 v/v) for 2 h at 25 °C. The two first steps' main objective was forming –COOH groups and the protocol was adapted from Taheran *et al.* 2017.[4] After being washed with distilled water, the particles were immersed in 50 mL of 1 mol·L^{-1} ethylenediamine (pH 4.7)/0.1 mol·L^{-1} carbodiimide solution at 25 °C for 1.5 h to generate free primary amino groups on the polyacrylonitrile-based support.[5] A new wash was carried out before the fourth stage, which consisted of immersing the particles in a solution containing different amounts of glutaraldehyde (2, 4, 6, 8, and 10% v/v) for 1.5 h at 25 ºC. Furthermore, samples were embedded in potassium bromide pellets and analyzed by FTIR to determine the modifications in the polymer chain achieved by support activation in NaOH.

The functionalized PAN was treated with 8 mL of laccase in acetate buffer solution (0.1 mol·L^{-1}, pH 4.5) (0.5 mg·mL^{-1}) for up to 6 h. The authors developed the protocol to immobilize the laccase in a single step, considering the complexity of functionalizing

the support. The heterogeneous catalysts were then removed and thoroughly rinsed. This same treatment with laccase solution (0.5 mg·mL^{-1}) was tested with PAN without any prior functionalization step to evaluate the support ability to immobilize the enzyme. The immobilization yield was determined from the decay of enzymatic activity in the supernatant after 6 hours of immobilization.

3 RESULTS & DISCUSSION

According to the results obtained by FTIR (Figure 1), the PAN samples showed an absorption peak corresponding to the stretching vibration in the range of 2243 cm^{-1}, which indicates the clear presence of the nitrile group (C≡N), and another peak at 2939 cm^{-1}, which represents the C–H bond of –CH_2 present in acrylonitrile, distinctive characteristics of this polymer.[6,7]

Regarding the functionalization of PAN, as shown in Figure 1, a gradual decrease was observed, over the reaction time, in the relative intensity of the peaks associated with the –CN groups and the CH bond of –CH_2, visible in the peaks close to 2243 cm^{-1} and 1456 cm^{-1}, respectively. This reduction becomes more evident after 2 hours of reaction. Furthermore, the increase in peak intensity around 3450 cm^{-1} strongly suggests the formation of the –OH radical in the sample. However, there is no clarity regarding the formation of the carboxylic group, as the characteristic C=O stretching peak was not detected in the range of 1710-1780 cm^{-1}.[8]

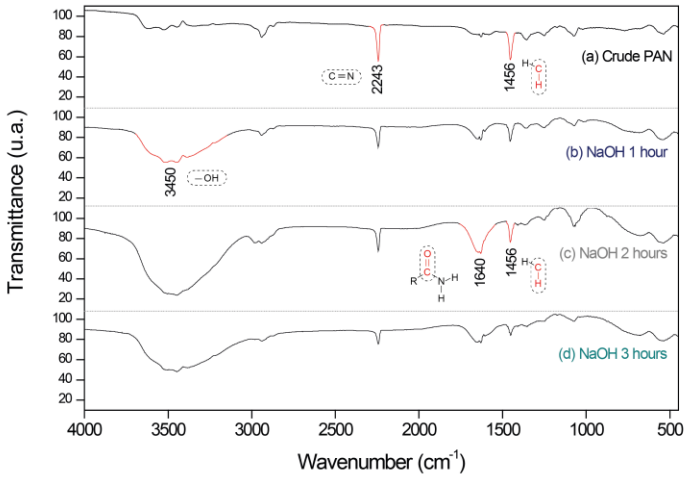

Figure 1 Fourier Transform Infrared Spectra (FTIR) of PAN particles crude and after different times of reaction with NaOH (1; 2; 3 h)

The same bond can be observed in the range from 1680 cm^{-1} to 1630 cm^{-1}, referring to the C=O of the primary amides. The same elongation in the range of 3,500 cm^{-1} to 3,100 cm^{-1} may indicate the formation of NH_2. It is unclear whether the strong and broad peak could be an overlap between the -OH and -NH_2 peaks of the amide, which would explain the first step of conversion of the -CN group by reaction with NaOH. The spectra presented at 2 and 3 hours were chemically similar, without major changes in the intensity or location of the main peaks. The activation time of 2 hours was considered ideal for alkaline hydrolysis of PAN with NaOH. Hydrolysis in an alkaline medium by NaOH involves a sequence of reactions, that culminate in the formation of imidic acid and poly(acrylamide).[9]

The acid hydrolysis of PAN leads to the formation of poly(acrylonitrile-co-acrylamide). The association of the amino group of the amide with the water in the solution forms oxiammonium groups, which are more electropositive than the ammonium cation. They release hydronium in the second phase of the reaction, forming carboxylic acid.[10] The resulting ion exchange is responsible for the carboxylic group formation necessary for subsequent amination.

These carboxylic groups, in turn, could be used to establish a link between the support and the enzyme, facilitated by glutaraldehyde. It is worth noting that the aqueous ethylenediamine solution is highly alkaline due to the cationic properties of the amino group.[11] To reduce the pH to 4.75, it is necessary to add 0.1 mol/L carbodiimide and titrate it with HCl. This decrease in pH was crucial for the process of PAN functionalization and enzymatic immobilization. This is because, when using only EDA solution for amination, as suggested by other authors,[12] laccase immobilization does not occur.

When testing different concentrations of glutaraldehyde in the fourth functionalization step, it was observed that this variable had an insignificant effect on the immobilization yield (Figure 2a). Both concentrations tested demonstrated yields close to 99%. Glutaraldehyde is discussed in the literature as an efficient and cheap cross-linking agent, but its chemical composition presents high toxicity and dangerous handling.[13] In this sense, the glutaraldehyde concentration selected as ideal for this study was 2% (v/v). It is possible to assume that, based on the research data, this concentration is sufficient for the reagent to form the Schiff base, that is, the glutaraldehyde molecule will serve as a spacer arm between the functionalized PAN and the laccase.[14]

The overall impact of four-phase functionalization was assessed using laccase immobilization. The data obtained demonstrated a significant difference in the immobilization yield between functionalized and non-functionalized particles, reaching 99.48% and 14.29%, respectively (Figure 2b). This demonstrated the need for functionalization to obtain better immobilization performance.

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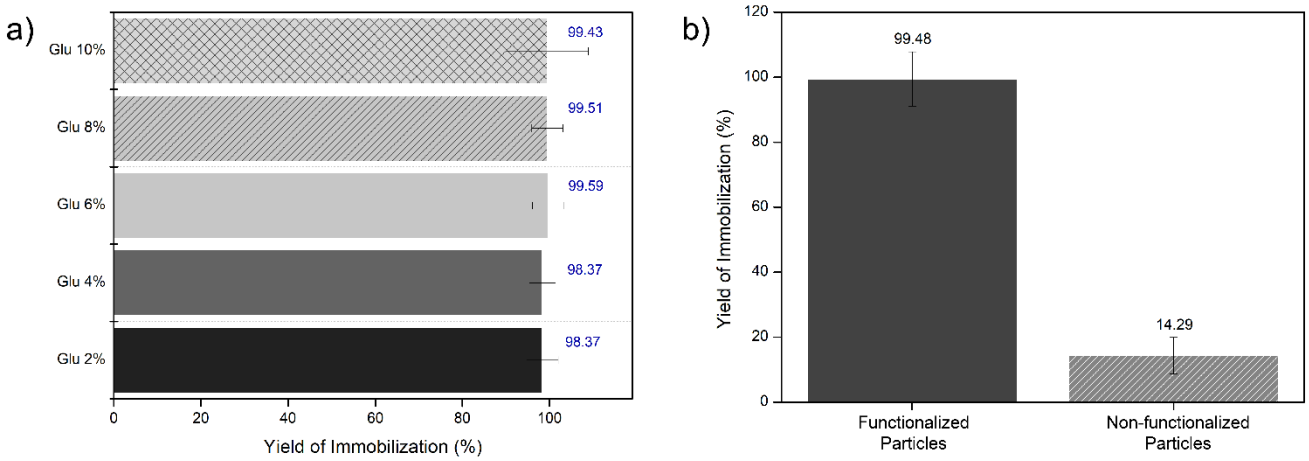

Figure 2 a) Comparison of laccase immobilization yield on PAN particles with different glutaraldehyde concentrations (2; 4; 6; 8; 10 % v/v). Temperature and pH set at 25 °C and 4.5 in 0.1 mol/L acetate buffer solution; **b)** Laccase immobilization on PAN particles by adsorption (non-functionalized PAN) and by covalent bonding (functionalized PAN) Temperature and pH set at 25 °C and 4.5 in 0.1 mol/L acetate buffer solution. In 0.5 mg/mL of enzyme solution.

4 CONCLUSION

Laccase from *Trametes versicolor* was immobilized on polyacrylonitrile particles after functionalization. Enzyme immobilization on functionalized particles resulted in significantly higher immobilization yields compared to non-functionalized PAN (99.48% and 14.29%, respectively). Therefore, it is correct to state that the four-step functionalization process (alkaline hydrolysis, acid hydrolysis, amination, and cross-linking) effectively facilitated immobilization. Variations in glutaraldehyde concentration did not impact the immobilization yield under the tested conditions. Although more tests are needed to identify what type of immobilization occurred between the enzyme and the support, as they are promising, the results presented in this work could serve as a basis for future studies.

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