# LACCASE IMMOBILIZATION ON MONTMORILLONITE CERAMIC SUPPORT

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## ABSTRACT

In recent years, enzymes have gained prominence due to their advantages, such as substrate specificity, generation of purer products and being environmentally friendly. However, they face limitations when used in their free form. Laccase is a promising enzyme in several fields (e.g., food industry and wastewater treatment), but it has low stability in solution and at acidic operational pH. Immobilizing laccase can increase its stability and expand its operating conditions. In this sense, this study aims to immobilize laccase on a ceramic support to improve its stability and allow it to be reused. Montmorillonite clay was used as a support, the surface of which was modified by ion exchange of $Na^+$ for $Cu^{2+}$, and the laccase was immobilized by chelate formation. The results showed that montmorillonite, even without functionalization, had an immobilization efficiency of 86%. After functionalization with $Cu^{2+}$, the efficiency reached 100%. Despite this, it is suggested that clay might be an excellent support. Furthermore, the use of clay might allow the laccase reuse in various industrial applications and making it a promising technology for various segments.

**Keywords:** Enzymatic degradation; Support development; Immobilization of enzymes; emerging pollutants.

## 1 INTRODUCTION

The high discharge of pollutants, promoted by industrial progress, has created a need to develop and improve new effluent treatment strategies, since sewage treatment plants, wastewater treatment plants and drinking water treatment plants are still inefficient at removing compounds originating from the widespread use of pharmaceutical and agro-industrial chemicals, among others. This class of compounds is called emerging pollutants [1]. They are found in ever-increasing concentrations and more frequently in treatment plants, but there is no characteristic legislation for their treatment, and their long-term effect on organisms is little known [2].

Due to the urgence on development of effective treatments to remove these pollutants, studies focused on this area are emerging, like advanced oxidation processes, with electrochemical and photocatalytic based in nanomaterials [3], [4], [5]. Other proposals explored are the biocatalytic process, involving enzymes. There are many advantages in front of physical and chemical process for degradation, like low energy cost, capacity to remedy low and high concentrations and high removal efficiency, but some disadvantages are included too, like high enzyme cost and recovery [6].

Oxidoreductases are an enzyme group that can degrade many compounds. Laccase is an enzyme capable of degrading phenolic compounds, while to reduce $O_2$ to $H_2O$, also, the enzyme presented low substrate specificity, allowing it to degrade a wide range of compounds [7], [8]. These characteristics turn the Laccase interesting in environmental remediation. to overcome the problem of reusing enzymes, immobilization techniques are employed to fix the enzyme on solid support. There are many methods, chemical and physical to immobilize. Chelate formation is a method that combines physical and chemical techniques for stronger adhesion than physical adsorption, preventing enzyme detachment. The mild immobilization conditions also protect enzyme activity and conformation [9]. In this project we made the ionic exchange of a mineral clay, Montmorillonite (MMT), to be a support for immobilization of Laccase for chelate formation.

## 2 MATERIAL & METHODS

Ionic exchange was carried out by replacing of $Na^+$ to $Cu^{2+}$ into the montmorillonite structure in two steps, acid activation and copper modification [10], [11]. In the first step the mineral clay was deposited in hydrochloride acid solution. Three concentrations are tested: 0,05M (I); 0,25M (II) and 0,5M (III), with ratio 10g/75mL (clay weight/acid solution). The mixture was stirred for 24h at room temperature, after, the material was neutralized with ultrapure water and oven dried at 80°C. For the next experiment the activated clay (AC) was ground to a particle size of less than 300 mesh.

In the second step, the activated clay was used, AC (I), AC (II) and AC (III) for second ionic exchange using $CuSO_4$ 0,05M solution. Using 5g/100mL for clay/solution ratio, the mixture was stirred for 6h at 60°C. After, the material was neutralized with ultrapure water and oven dried at 80°C. For immobilization the three modified clay (CuC) was used particle size of less than 300 mesh.

The immobilization process was realized with support/enzyme ratio of 1 (g/mg). The support was added in the laccase solution, prepared in citrate-phosphate buffer (pH 6). The mixture was incubated at 30°C with constant stirring for 5 hours, then centrifuged and the supernatant was used to measure the residual activity (which corresponds to the enzyme not immobilized on the support). The enzyme activity was measured by protocol described in other works by the group [12] Briefly, the procedure consists of adding to a quartz cuvette: 2.4mL of phosphate-citrate buffer solution (pH 6); 0.3mL of 5mM ABTS solution and 0.3mL of enzyme solution. Equation 1 was used to calculate the enzyme activity.

$$\frac{U}{L} = \frac{\Delta_{abs}.V}{\varepsilon.d.v.t} \quad (1)$$

The unit $U.L^{-1}$ expresses the amount of laccase needed to degrade 1 μmol of ABTS per minute. Δabs is the change in absorbance. V is the reaction volume (mL). ε is the molar extinction coefficient (ABTS = 3.6.104 $L^{-1}.M^{-1}.cm^{-1}$). d is the step length of the cuvette (cm). v is the volume of LAC solution (mL) and t is the reaction time.

The enzyme solution varied according to the activity to be carried out. Free laccase solution was used to determine the activity of the enzyme, and samples of the supernatant from the immobilization process on the supports were used to determine the residual activity. The enzyme activity was taken as 100% for comparison with the residual activities.
For the basic characterization of the support, XRD (Rikagu) was carried out at the Interdisciplinary Laboratory of Nanostructures (Linden) at the University of Santa Catarina (UFSC), the parameters were 2θ: (2°-70°), step size: 0.05° and scan speed 5°/min. The results obtained were analyzed based on the literature.

## 3 RESULTS & DISCUSSION

Residual activity (RD) are useful parameters for assessing the loading of the support after the immobilization process. The immobilization efficiency (1 – RD) was also to evaluate the process. The results showed high loading rates, in different ways. The results indicate that MMT was able to act, even in pure form, as a support, through surface and interlamellar immobilization mechanisms, also due to the hydrophobic nature of the clay surface and the replacement of the interlamellar $Na^+$ ions of the original MMT by LAC molecules, respectively [13]. The data reguardingimmobilization in all modified and non-modified clays are presents in Table 1.

**Table 1** Residual activity at first immobilization.

| Suport | Residual Activity (%) | Immobilization efficiency (%) |
|---|---|---|
| MMT | 14,0 ± 1,0 | 86,0 ± 1,0 |
| AC (I) | 2,9 ± 0,4 | 97,1 ± 0,4 |
| AC (II) | 2,0 ± 1,0 | 98,0 ± 1,0 |
| AC (III) | 2,0 ± 1,0 | 98,0 ± 1,0 |
| CuC (I) | 1,0 ± 2,0 | 99,0 ± 2,0 |
| CuC (II) | 0,0 ± 0,0 | 100,0 ± 0,0 |
| CuC (III) | 0,0 ± 0,0 | 100,0 ± 0,0 |

The immobilization was evaluated in pure montmorillonite showed an index of 86%, indicating a good percentage of the untreated clay to act as a support. Both ionic substitutions were able to reduce residual activity, generating clues that the enzyme was fixed to the support. To the develop support CuC, the main mechanisms involved may be related to the presence of larger ions, increasing the exchange potential and the entry of the enzyme,into lamellar structure, as well as chelate formation [11], [13].

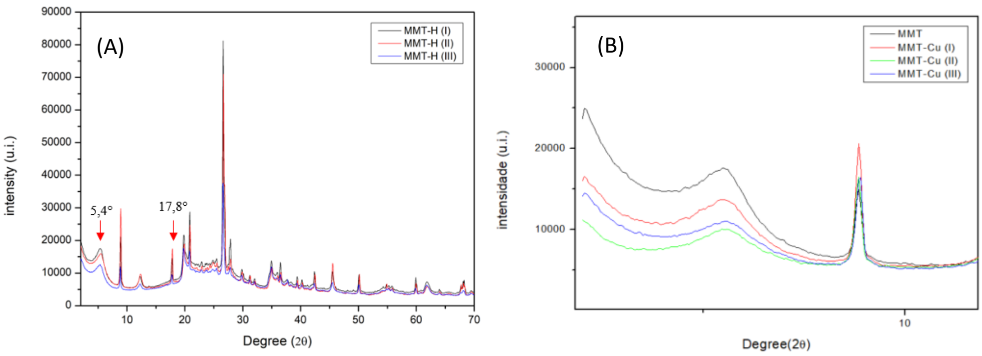


**Figure 1. DRX for both ionic exchange**

The XRD results allow to verify the modification of the montmorillonite structure (Fig 1.A and Fig 1.B), through the loss of diffraction patterns characteristic of this clay mineral. The decrease in these patterns was proportional to the concentration of acid used, with 0.5M being the most modified (Fig 1.A) [14].

## 4 CONCLUSION

This report presents the data from the laccase immobilization on montmorillonite. Pure MMT showed a good adsorption capacity, with low residual activity, and with ion exchange, an almost total decrease in enzyme activity. According to literature, ceramic support can be used in many conditions due their chemical and mechanical resistance, showing a good potential to development of a compact support model for immobilization.

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