ONE-STEP LACCASE IMMOBILIZATION ON PVDF (POLYVINYLIDENE DIFLUORIDE) MEMBRANES INTENDED FOR WASTEWATER TREATMENT

Vinicius C. Albuquerque[1*], Maikon Kelbert [1], Débora de Oliveira[1] & Sergio Y.G. González [1]

[1] *Chemical and Food Engineering Department, Federal University of Santa Catarina, Florianopolis, Brazil.*
** Corresponding author's email address: vacastro2007@outlook.com*

ABSTRACT

Enzymes, the catalysts of biochemical reactions, are pivotal in maintaining environmental equilibrium. Laccases (LC), a subset of the multicopper enzyme family, find extensive use in industry and academic research. However, LC requires further development of strategies to increase its stability. This study aims to improve LC stability through immobilization on membranes (PVDF) using Poly (vinylidene fluoride). Combining polyvinylidene fluoride (PVDF) membranes with polydopamine (PDA) for enzyme immobilization offers a robust and efficient solution for effluent treatment. PVDF membranes provide exceptional chemical resistance, thermal stability, mechanical strength, and high flux rates for versatile filtration applications. Integrating PDA further enhances this system by offering strong adhesion, versatile functionalization for effective enzyme immobilization, and improved enzyme stability against environmental stressors. PDA also boosts enzymatic activity, simplifies immobilization, resists fouling, and allows for enzyme reuse, all contributing to a cost-effective and scalable solution. PVDF-PDA-LC creates a synergistic effect that significantly enhances efficiency, stability, and overall performance. The immobilization process herein developed uses dopamine (DP) as a precursor, exploiting in-situ polymerization to produce PDA, achieving enzyme immobilization on PVDF in a single process (one step). The immobilization process was developed by assessing the effect of PD concentration, LC concentration, and time immobilization as the factors for the enzymatic activity response. The results disclose the immobilization conditions that enhance the enzymatic activity: 30 minutes with an LC concentration of 2 mg·mL-1 and a dopamine concentration of 0.25 mg·mL-1. This study showcases the faster and more straightforward approach for LC immobilization reported, simultaneously displaying the higher LC activity, highlighting the process feasibility and the application potential.

Keywords: Laccase, Polydopamine, PVDF, One-step immobilization.

1 INTRODUCTION

Enzymes maintain environmental balance by acting as catalysts for essential biochemical reactions in various ecosystems [1]. These biological molecules present a significant role in compound biodegradation by breaking down complex molecules into simpler components, making them more readily absorbable and recycled. Their efficiency, specificity, and ability to operate in diverse environmental conditions make them valuable tools to pursue sustainable solutions for contemporary environmental challenges [2]. Laccase (LC), EC 1.10.3.2, or p-diphenol: dioxygen oxidoreductase, are part of a larger group of enzymes termed the multicopper enzymes [3]. Enzymes catalyze the oxidation of many substrates, such as lignin, phenols, dyes, and aromatic compounds. Widely used in industry and biodegradation research due to their broad range of activity, enzymes require further research and immobilization techniques due to their low stability in terms of temperature, pH, storage, and reusability [4,5].

LC can be immobilized by physical (adsorption, entrapment, and encapsulation) or chemical (covalent bonding) 3. New research on simpler immobilization protocols using fewer chemicals is fundamental and attractive for industries, as it is typically carried out without chemical additives and preserves the native conformation of the enzyme [6]. Polydopamine (PDA) can adhere firmly to different materials and has thus been used for enzyme immobilization. Due to several reactive functional groups, such as amine, imine, quinone, and catechol, the PDA coating can bind enzymes to various materials [7]. Among membrane materials, poly (vinylidene fluoride) (PVDF), a semi-crystalline polymer, has been prominently highlighted in recent years for membrane production due to its numerous advantageous characteristics[8]. PVDF exhibits excellent membrane-forming ability, thermal stability, chemical resistance, mechanical strength, and antioxidation activity. These outstanding physical and chemical properties, combined with superior thermal stability, have triggered its use[9]. Its potential applications, such as in membrane enzymatic reactors, further underscore its importance.

The one-pot process for an enzymatic system involves a single reactor that allows all reactions to co-occur. This setup enables other compounds to act in the middle of the process, maintaining low concentrations and reducing inhibitions that occur in the process or catalyzing the ongoing reactions. Additionally, it reduces operational costs by eliminating other stages typically used in enzymatic reactors, such as purification and separation. The only limitation is the need for studies to balance and optimize the reaction conditions, such as medium, temperature, pH, and catalyst stability, to achieve maximum productivity [10,11].

Laccase-assisted dopamine polymerization (PDAL) is an innovative material resulting from rapid and efficient dopamine (DP) polymerization in an acidic medium catalyzed by LC. This method provides a stable structure, giving PDAL a uniform and compact morphology and high chemical stability in acidic and alkaline environments. Additionally, PDAL supports secondary reactions, such as interaction with amine groups, making it a versatile platform for various biotechnological applications [12]. However, to the best of our knowledge, there are no reports regarding the retained activity of LC after being used as a catalyst in DP polymerization. In this sense, the present study aimed to develop and evaluate the one-step DP polymerization and LC immobilization process and the formation of a biocatalyst on a membrane surface and assess the conditions that influence the immobilization process.

2 MATERIAL & METHODS

2.1 Materials and Reagents

Dopamine hydrochloride and ABTS (5,5′-dithiobis 2nitrobenzoic acid, DTNB), laccase from *Trametes versicolor* (≥0.5 U mg^{-1}), and 3-ethylbenzthiazoline-6-sulfonic acid (ABTS ≥ 98%) were obtained from Sigma Aldrich Brazil. Hydrochloric acid (≥ 37%), Citric acid (≥ 98%), and dibasic sodium phosphate (≥ 98%) were purchased from Neon (Brazil). Ultrapure water was used in all experiments (Millipore simplicity system). Microfiltration Poly(vinylidene fluoride) (PVDF) membranes with a pore size of 0.2 µm were obtained from MICRODYN-NADIR® (NADIR® MV020, USA).

2.2 Measurement of laccase and biocatalyst activity

The LC activity was performed by analyzing the activity of the developed biocatalyst. For that, 0.3 mL of an ABTS standard solution(5mM) and 2.7 mL of phosphate-citrate buffer were added to a 24-well plate that contained the membrane with immobilized LC. The solution was incubated for 10 minutes at 30° and 150 rpm. After that, the reaction was halted by adding hydrochloric acid (37%) at a ratio of 50 µL per milliliter of the total solution. The activity was measured in a spectrophotometry UV-Vis Femto Cirrus-80-Pr at a wavelength of 420nn. The activity was calculated using Equation 1.

$$\frac{U}{L} = \frac{\Delta abs \cdot V}{\varepsilon \cdot d \cdot g \cdot t}\ [UL^{-1}] \qquad (1)$$

One unit of LC activity (expressed in $U \cdot g^{-1}$) is defined as the amount of enzyme necessary to catalyze 1 µmol of ABTS per min. Δabs is the variation of absorbance. V is the reactional volume (mL). ε is the molar extinction coefficient (for ABTS = $3.6 \times 10^4\ M^{-1} \cdot cm^{-1}$). d is the cell path length (cm). g is the biocatalyst weight (mg), and t is the reaction time (min).

2.3 Immobilization procedure

Immobilization assays were conducted to assess the process variables and behavior trends. Three factors that significantly influenced the process were identified and evaluated: time, LC concentration, and DP concentration. The assays were performed testing different immobilization times (0.25, 0.5, and 1 h), LC concentrations (1, 2, and 4 $mg \cdot mL^{-1}$), and DP concentrations (0.25, 0.5, and 1 mg·mL-1) to observe their influence on the immobilization process and to determine the optimal parameters. In a 24-well plate, a membrane weighing 20 mg was added to a solution of 0.5 mL of DP with the concentrations to be studied, prepared with phosphate-citrate buffer (0.1 M, pH 5.5). Additionally, 0.5 mL of enzyme solution was added (prepared with phosphate-citrate buffer 0.1 M, pH 5.5). The plate containing the membranes was placed under orbital agitation at 150 RPM in a shaker incubator at 30°C for the chosen evaluated times. Afterward, the membranes were taken from the plate and washed with 50 mL of ultrapure water. The enzymatic activity was measured according to the methodology described above.

3 RESULTS & DISCUSSION

The use of LC as a catalytic agent for the polymerization of DP and the formation of polydopamine for various applications such as biosensors or biocatalysts has been documented in numerous articles [13,14,15]. However, the present work focuses on the role of the simultaneous DP polymerization and LC immobilization with the optimal operational parameters. The analysis of the ideal immobilization conditions co-occurred using the parameters of the immobilization process. The parameters of time, concentration of DP, and LC concentration were used as the basis for this analysis, revealing the ideal conditions for a more effective and efficient immobilization process (Figure 1).

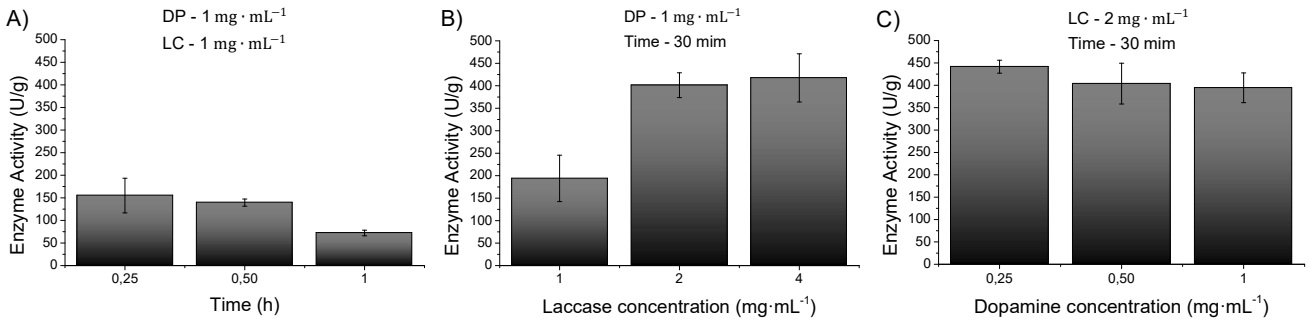

Figure 1 Screening to evaluate LC immobilization: A) immobilization time, B) laccase concentration, and C) dopamine concentration.

Figure 1A shows that the ideal working point for the experiment is 30 minutes. After this time, approximately half of the enzymatic activity decreases. Regarding LC, there is an increase in the enzymatic activity with an increase in the enzyme concentration depending on the LC concentration, reaching the maximum at 2 $mg \cdot mL^{-1}$ (Figure 1B). The results also disclose a slight decrease in enzymatic activity as a function of DP concentration; therefore, lower quantities of DP result in a membrane with a higher potential for enzymatic activity. The analysis revealed potential in the immobilization process, particularly with the utilization of DP as a catalyst for the enzyme immobilization reaction. This process demonstrated remarkable efficiency compared to most immobilization methods found in the literature regarding the increased enzyme activity obtained during immobilization[6,11] both in

terms of reaction rate and its characteristic as a one-pot process. Notably, it offers straightforward operating conditions without requiring complex equipment or procedures. The efficiency and straightforward manufacture of the enzyme-functionalized membrane underscore its feasibility and applicability for the degradation of phenolic compounds and its potential for use in effluent treatment reactors [12,16].

4 CONCLUSION

This study demonstrates promising results for biotechnological and industrial applications. This method offers an innovative and practical approach to stabilizing and enhancing the catalytic properties of these enzymes, leveraging the adhesive and polymeric properties of DP to anchor the LC onto different supports. These positive outcomes underscore the potential of this approach to drive significant advancements in green biotechnology and the development of more sustainable and efficient processes.

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