EVALUATING IONIC LIQUIDS FOR THE ENTRAPMENT OF FUNGAL LACCASE IN LIGNOCELLULOSIC BIOPOLYMERS

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ABSTRACT

The use of byproducts derived from 2G bioethanol production in the development of a solid matrix for enzyme immobilization represents a novel and sustainable alternative for valorizing materials that originate as waste from renewable natural sources. This strategy contributes to the projection of a biorefinery for transforming biomass into valuable products, aligned with the concept of a circular economy. Our previous research demonstrated that the imidazolium ionic liquids (IL) BMIM Ac and BMIM Cl can dissolve the solid residue obtained in bioethanol production from Eucalyptus spp biomass. The resulting mixture of biopolymers and IL served as the raw material for developing active hydrogels with entrapped laccase. In this work, two new imidazolium ILs were evaluated to improve the immobilization process: 1-ethyl-3-methylimidazolium acetate (EMIM Ac) and 1-ethyl-3-methylimidazolium ethyl sulfate (EMIM ES). Although the activity and stability of the laccase from *Dichostereum sordulentum* were higher in the presence of EMIM ES, no hydrogel formation could be achieved with this IL. The immobilization of laccase in lignocellulosic biopolymers using EMIM Ac yielded similar results to those previously obtained with BMIM Ac. This demonstrates the potential of EMIM Ac and the need to continue optimizing the hydrogel formation process.

Keywords: Laccase. Enzyme entrapment. Lignocellulosic biopolymers.

1 INTRODUCTION

Laccases (EC 1.10.3.2) stand out among the enzymes with the greatest field of applications because they do not require cofactors and have low specificity. The latter allows them to act on a wide range of phenolic compounds and oxidize other more complex substrates using redox mediators [1–4]. For example, laccases of fungal origin have proven to be effective in the degradation of endocrine disruptors, managing in many cases to reduce the associated toxicity [5–10]. Considering its industrial application, its immobilization represents an important tool that allows its reuse reducing process costs; reaction control by removing the immobilized enzyme from the reaction medium; design continuous processes and in many cases increase enzyme stability [11,12]. A wide variety of polymers have been used as matrix for the immobilization of laccases [7,12–15].

The use of lignocellulosic materials to immobilize biocatalysts has several advantages: their low price, their wide distribution, they are a renewable and biodegradable resource, and they have hydrophilic and hydrophobic regions capable of interacting with the enzyme. In different studies material composed of cellulose or lignin is used as a support source for the immobilization of proteins[16–18], however, the use of the solid material generated as byproduct in the production of second-generation bioethanol (B2G) represents a novel and sustainable alternative. The use of this material is aligned with the concept of circular economy and the development of biorefineries associated with biofuel production, to generate various products and materials with high added value, contributing to the economic viability of B2G production[19–21]. Eucalyptus spp wood is among the biomasses of greatest interest for the B2G production in our country. Due to its rapid growth and high quality of wood It is the most planted hardwood tree in the world and particularly in Uruguay[22,23].

Our research is focused on obtaining biopolymers from the lignocellulosic waste suitable for use in the immobilization of laccases. In previous work, it was demonstrated that biopolymers generated as a by-product of B2G production from Eucalyptus biomass were suitable for immobilizing laccase from the fungus *Dichostereum sordulentum*. This process resulted in the formation of porous, spherical hydrogel beads with a diameter of approximately 3 mm. [24]. An insoluble biocatalyst was obtained with good capacity to remove the endocrine disruptor ethinylestradiol. Furthermore, it was observed that the ionic liquid (IL) which was most suitable for dissolving the material with lesser impact on enzyme activity and stability was 1-butyl-3-methylimidazolium acetate (BMIMAc). Given that the insoluble biocatalyst showed low binding efficiency, alternatives should be explored to obtain a more active derivative. In this work it is thus proposed to test other two ionic liquids for laccase entrapment: 1-ethyl-3-methylimidazolium acetate (EMIM Ac) and 1-ethyl-3-methylimidazolium ethyl sulfate (EMIM ES).

2 MATERIAL & METHODS

Materials: Laccase from *Dichostereum sordulentum* produced at laboratory (lyophilized); 2,6-Dimethoxyphenol(DMP); buffer solution 0.1M sodium acetate pH 3.8; dimethyl sulfoxide (DMSO); Ionic liquids (ILs): 1-butyl-3-methylimidazolium chloride (BMIM Cl), 1-butyl-3-methylimidazolium acetate (BMIM Ac), 1-ethyl-3-methylimidazolium ethyl sulfate (EMIM ES), 1-ethyl-3-methylimidazolium acetate (EMIM Ac).

Lignocellulosic material: the solid by-product of B2G production from *Eucalyptus globulus* wood was obtained after a Steam explosion pretreatment (200ºC, 10 min residence time) in a semi-continuous pre-pilot reactor and the saccharification of the resulting solid cellulosic fraction. The material was lyophilized for this study. It was composed mainly of lignin, with lesser amounts of non-hydrolyzed cellulose and remnants of hemicellulose[24].
Laccase activity was measured with DMP 2.0 mM in 0.1 M sodium acetate buffer pH 3.8 (activity buffer) as substrate, by registration of absorbance at 477 nm. One enzyme unit (EU) was defined as the amount of enzyme that catalyzed the appearance of 1 µmol of product per minute at 25 °C and pH 3.8 [25].
*Enzyme activity in the presence of ILs***:** The laccase activity assay was carried out in the presence of 1, 5, 10 and 30% of the ILs BMIM Cl, BMIM Ac, EMIM ES and EMIM Ac.
*Enzyme stability in the presence of ILs***:** Aliquots of enzyme were incubated in 30% and 60% of BMIM Ac, EMIM ES and EMIM Ac, at 40ºC and pH 3.8 for 1 h. The residual activity was quantified as already described after removing the IL by gel filtration.
*Dissolution of lignocellulosic material in ILs and DMSO***:** One gram of EMIM Ac or EMIM ES was placed in a 20 mL vial with 100mg of lignocellulosic material and heated to 100 °C under stirring for 15 min. The mixture was cooled down and filtered through frits with pore size between 20 to 85 µm. For the assays with DMSO, 0.4g of IL and 0.6mL of the solvent were used. The filtrates were collected and dripped onto distilled water.
Beads formation and enzyme immobilization: Three EMIMAc:DMSO ratios were assessed in beads formation and laccase immobilization: 1.5:1.0 - 1.0:1.0 - 1.0:1.5. Proper amount of both solvents were placed in 20 mL vial and heated up to 100 °C under stirring. Then, the lignocellulosic material (100 mg) was added to the mixture and stirred until dissolution. For enzyme entrapment in the hydrogel beads, once the material was dissolved at 100ºC the mixture was cooled down to 40 °C, the lyophilized enzyme was added (approx. 30 EU), and the vial content was quickly transferred to a plastic syringe and dripped over 0.05 M acetate buffer pH 5.0. Laccase activity was measured in supernatant, washes, and beads.
The immobilized enzyme activity was assayed by incubating the beads with the substrate DMP 2.0 mM under magnetic stirring (100 rpm) and registering absorbance at 477 nm at 30 seconds intervals, keeping the volume of the reactive mixture unchanged[26].

3 RESULTS & DISCUSSION

Laccase activity decreased with increasing IL concentration in all cases (**Fig. 1**). EMIM ES caused the least inactivation of the enzyme; very similar trends were seen between BMIM Ac and EMIM Ac; while, as expected according to previous findings [24], the enzyme was strongly inactivated by BMIM Cl, even at very low concentration.

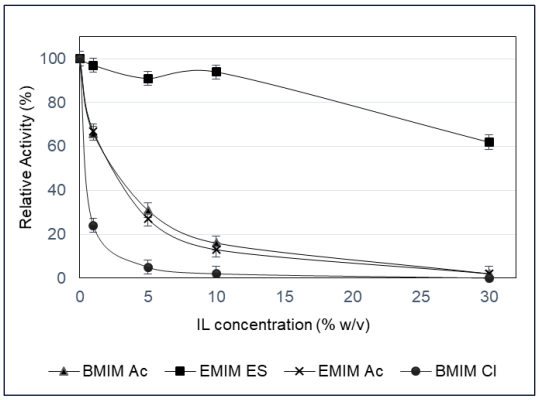

Figure 1. Laccase activity in the presence of Ionic Liquid.

The enzyme stability was almost unaffected at 30% concentration of the tested ILs, with remaining activity around 85% for all of them (**Table 1**). However, doubling the IL concentration during incubation completely inactivated the enzyme in BMIM Ac and EMIM Ac, but EMIM ES decreased the enzymatic activity only by 40%.

Table 1 Laccase stability in the presence of ILs (40ºC, pH3.8, 1h)

IL concentration (%)	Residual Activity (%)			
	Control	BMIM Ac	EMIM ES	EMIM Ac
30	88	79	78	88
60	85	0	61	0

When EMIM ES was used for dissolving the lignocellulosic material the resulting mixture was liquid and allowed filtering, both in the presence and absence of DMSO. However, the filter retained a significant amount of solids, and when dripped over water, the filtered fraction did not solidify but instead separated and formed a cloudy suspension when shaken. These observations indicate the presence of undissolved material, suggesting that this ionic liquid would not be useful for the formation of the solid matrix necessary for immobilization. Therefore, to improve the immobilization process, the focus of the work was on the use of EMIM Ac.

The mixture obtained with EMIM Ac without DMSO could barely be filtered and when the filtered fraction was put in contact with water, it did not disperse. The mixture with DMSO exhibited greater fluidity. In both cases the dissolution of the material with this IL was confirmed by the solidification of the filtered fraction when dripped into water.
The effect of the IL:DMSO ratio in the material dissolution was then assessed for EMIM Ac. Increasing DMSO concentration facilitates the dissolution of the lignocellulosic material and improves the fluidity of the mixture. Nevertheless, according to the

background of our work, it decreases the expressed immobilized activity.
For the three ratios of EMIM Ac and DMSO, spherical and firm beads with good mechanical resistance to agitation were obtained. The apparent expressed activity was similar in the three conditions, with some increase of EU expressed per gram of biopolymer when the ratio 1:1 was used (**Table 2**).

Table 2 Effect of the ratio of solvents used in laccase entrapment

	EMIM Ac : DMSO ratio		
	1.5 : 1	1 : 1	1 : 1.5
Apparent expressed activity (EU/g biopolymer)	3.0	6.4	3.9

4 CONCLUSION

The use of two ionic liquids was evaluated in the immobilization of laccase from *D. sordulentum* by entrapment in biopolymers derived from Eucalyptus biomass. In this process, the ionic liquid must be able to dissolve the lignocellulosic material to form the solid matrix, but it is also required that it does not affect the enzyme stability. It was found that the ionic liquid that least affected the stability of the enzyme (EMIM ES) was not able to dissolve the material under the tested conditions.
The immobilization of laccase in lignocellulosic biopolymers using EMIM Ac yielded similar results to those previously obtained with BMIM Ac. It is necessary to optimize this process to draw definitive conclusions about the effectiveness of this ionic liquid.

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ACKNOWLEDGEMENTS

This study has been supported by Agencia Nacional de Investigación e Innovación (ANII), project FCE_1_2019_1_156567, and Programa de Desarrollo de las Ciencias Básicas (PEDECIBA), Uruguay.