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RESEARCH ARTICLE

IMMOBILIZATION OF LACCASES ENZYMES FROM *PLEUROTUS SAJOR-CAJU* IN POLYAMIDE 6.6 MEMBRANES MODIFIED

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ABSTRACT

Polyamide-6.6 (PA-6.6) membranes were modified with glutaraldehyde, in a phase inversion process. Immobilization of the enzyme laccase from *Pleurotus sajor-caju* in the modified PA-6.6 film was quantified in the presence of the reducer 2,2'-azino-bis-3- ethylbenzoazoline-6-sulfonate (ABTS). Under these conditions, phenolic compounds present in the ABTS were degraded by an oxidative process. In the case of the immobilized enzyme, 110 mol of ABTS was degraded after 6 hours of reaction. The same amount of ABTS was degraded after 1 hour in the case of the free enzyme, since the oxidation process is decreased by the low diffusion of ABTS through the membrane containing the immobilized enzyme. This characteristic is relevant since the reaction kinetics may be controlled in immobilized systems. The kinetics of the thermal inactivation (Kd) of laccase activity and the half-life (t1/2) were determined. It was found that there is the possibility for the enzyme to be reused. The process described may be useful for the treatment of textile effluents.

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INTRODUCTION

The textile industry uses annually 0.7 million tonnes of synthetic dyes, approximately 20% of which are discharged in industrial effluents. Many of these dyes are stable under different conditions of light, temperature, specific microbial attacks and redox processes, making them resistant to conventional treatment [1]. Since the 1970s, white-rot fungi have been study for the treatment of bleaching effluents of the cellulose and paper industry, along with the decolorization of several dyes *in vivo* and *in vitro* [2]. The extracellular enzyme systems of white-rot fungi can degrade a wide variety of recalcitrant compounds, such xenobiotics, lignin, and various types of dyes [3]. Leonowicz et al. [2], concluded that cellulose and lignin polymers are degraded simultaneously by enzymatic systems involving demethylation reactions catalyzed by laccases induced by radicals and mediators of low molecular weight resulting from the initial action of lignin peroxidase (LiP) and manganese peroxidase (MnP)[4,6].

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Laccases can catalyze the oxidation of various polyphenols and methoxyphenols [5]. The immobilization of an enzyme in soluble and insoluble substrates increases its stability, facilitating its reuse and separation from the mixture [6]. Among the laccases which have been intensely studied are those produced by *Pleurotus sajor-caju*, a basidiomycete in which forms part of the human diet, classified as a white-rot fungus since it is an active decomposer of wood, degrading even lignin [7]. The synthesis of polymeric membranes from different polymers, for example, polyamide, has been studied at the Chemistry Department of the University of Caxias to Sul. Griebenow et al. [8] immobilized laccase by adsorption onto different supports, including polyamide 6.6 membranes. These authors observed that the laccase activity varied according to the support used and the best results were obtained with the polyamide 6.6 membranes.

MATERIALS AND METHODS

Microorganism: The fungal strain used in this study was *Pleurotus sajor-caju* PS2001 from the collection of the Biotechnology Institute of the University of Caxias do Sul.

Culture Media

The maintenance medium contained 2% (w/v) ground sawdust of Pinus sp., 2% (w/v) ground wheat bran, 0.2% (w/v) $CaCO_3$, and 2% (w/v) agar. The medium was sterilized by autoclaving and placed into Petri dishes. The strains were grown at 28°C for 14 days and then kept at 4°C (±2). For the production of laccases *Pinus sp.* sawdust (93% w/w), ground wheat bran (6% w/w), $CaCO_3$ (1% w/w), (NH4)2SO4 (1.3% w/v), MnSO4 (0.0015% w/v), CuSO4 (0.0015% w/v) and water were used. Quantities of 180g of the medium were placed in polypropylene bags, autoclaved for 1 hour and inoculated with a 1.5cm agar disk. The inoculated bags were maintained at 28°C (±2) at a moisture content of 70-80% for 15 days.

Preparation of enzymatic extract

The contents of the cultivation bags (180g) were mixed with 360mL of water in Erlenmeyer flasks, which then shaken at 160rpm for 20 min. The mixture was filtered and centrifuged at 5000g for 15min. The supernatant was then precipitated with ammonium sulfate (80% w/v) for 2 hours at 4°C. The precipitate was resuspended with half of the initial volume in 0.1 M McIlvaine buffer solution, pH 6.0, and dialyzed in the same buffer [7,8].

Enzyme assay

The laccase activity was determined using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma) as the substrate. The laccase reaction mixture contained 0.45 mM ABTS, 90 mM of 0.1M sodium acetate buffer and 1 mL of diluted supernatant to give a final reaction volume of 2.2 mL. The oxidation of the substrate (ABTS) was monitored by the increase in the absorbance at 420 nm, over 90 s at 30°C (±2°C), using ε=/3.6X10^4 cm^{-1}M^{-1} (Wolfenden & Wilson 1982). Enzymatic activity was expressed in the following units: 1U = 1 µmol of ABTS oxidized per min at 25°C (±1).
The amount of protein was determined by the Bradford method, using bovine albumin as the standard (Sigma) [9].

Thermo stability of the laccase extract

The analysis of the thermo stability of the enzyme was carried out maintaining the enzymatic solution at a constant temperature of 20°C, 30°C, 40°C and 50°C (±1), for a period from 24 hours, using ABTS as the reducing substrate. The laccase activity was measured as described in section 2.3.

Kinetics of thermal inactivation

The kinetics of the thermal inactivation (Kd) of the laccase activity and the half-life (t1/2) were studied at different temperatures between 20–50°C (±0.5°C), with magnetic stirring. The thermal inactivation constant (K*d*) of the free laccase was determined using Equation 1, where Ain is the residual activity after thermal treatment during the incubation period, and Ain0 is the initial enzymatic activity. The half-life time (t1/2) was determined using Equation (2) [10].

$$\ln \frac{Ain}{Ain0} = \frac{Kd \cdot t}{} \quad (1)$$

$$t1/2 = \frac{0.693}{Kd} \quad (2)$$

Immobilization of laccase

Initially the supports used were immobilized using glutaraldehyde (Merck) in 0.1M acetate buffer at pH 7.0. The enzymes were immobilized by covalent bonds between the –NH2 of the protein and the aldehyde group of glutaraldehyde [11]. The excess of glutaraldehyde was washed off with distilled water. The films were then suspended in the enzymatic solution, in 0.2M acetate buffer, pH 5.0, for up to 48h, at 20°C, 30°C and 40°C. The quantity of immobilized protein was measured after 2, 4, 6, 8, 10, 12, 24 and 48h.

Statistical analysis of the results

For the statistical analysis we used analysis of variance (one-way ANOVA), with the post-hoc Tukey test ($P<0.05$) using the software GraphPad Prism 3.0 for Windows (GraphPad Software, San Diego, EUA).

RESULTS AND DISCUSSION

Free laccase activity

In general, the optimum pH for laccase activity can vary depending on the microorganism studied. Bollag & Leonowicz [12] characterized extracellular laccases obtained from *Fomes annosus*, *Pholiota mutabilis*, *P. ostreatus*, *T. versicolor*, *Rhizoctonia praticola* and *Botrytis cinerea fungus.* They observed that the optimum pH in the case of *P. mutabilis* remained in the neutral region, while for the other fungi it varied between 3-5. The optimum pH found for the free enzyme was pH 4-5 in McIlvaine buffer. The results showed that the *P. sajor-caju* laccases are sensitive to temperature (Fig. 1). After 2 h there was a sharp decrease in the enzymatic activity of the laccases in the solutions maintained at 30°C (30%) and 40°C (90%), with a total loss for that maintained at 50°C. The values for the thermal inactivation constant (Kd) and the half-life (t1/2) can be observed in Fig. 2. From the data given in Fig. 2, it can be seen that the half-life of the enzyme is reduced significantly with the increase in temperature, and also the thermal inactivation constant is increased, possibly due to an increase in the denaturation of the enzyme.

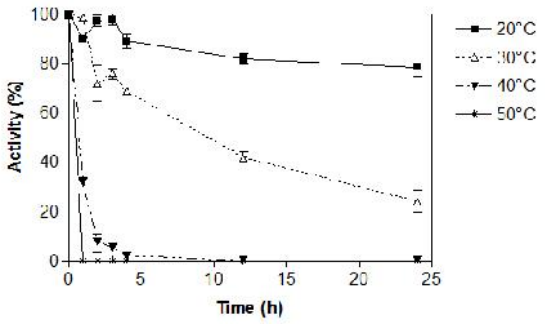

Fig. 1. Thermostability of the laccase extract at 20°, 30°, 40° and 50°C for 24 h, using ABTS as the substrate in acetate buffer pH 5

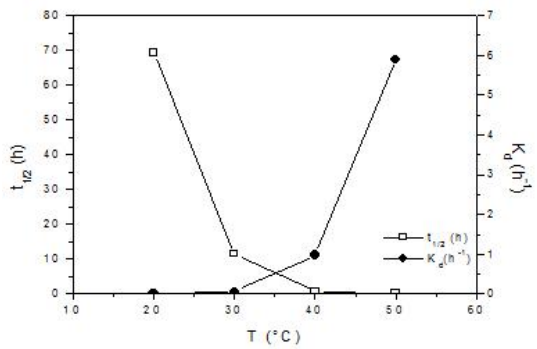

Fig. 2. Thermal inactivation constant (K_d) for laccase activity and half-life ($t_{1/2}$), using ABTS as the substrate reducer, at temperatures of 20° to 50°C

In Fig. 3 the data on the oxidation capacity of ABTS for the PA membranes with different immobilization times (2-24 h) and temperatures (20°C, 30°C and 40°C) are shown. As can be observed, the average values for the ABTS oxidation by membranes using an immobilization process of 6 h, at 30° and 40°C, are the highest. The low values obtained for ABTS oxidation at 2 h and 4 h may be due to the action of non immobilized laccases retained in the polymer which were liberated after 4 hours.

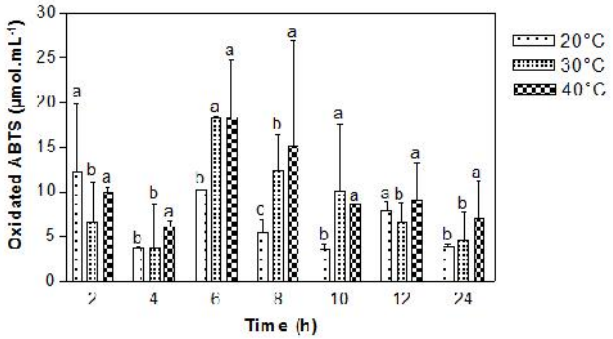

Fig. 3. Activity of laccase immobilized in PA membranes with different times and temperatures. Values (averages) with the same letters for the same period do not differ significantly according to the Tukey test (P > 0.05)

Conclusion

It was found that PA membranes can be used for the immobilization of laccases. However, a decrease in the optimum pH and in the oxidation rate occurred. These membranes also showed a potential for reuse of at least 4 times, with a retention of 50-70% of the ABTS oxidation activity.

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