SONO-ENZYMATIC POLYMERIZATION OF CATECHOL

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Introduction

The potential of laccase enzymes for polymerizing, crosslinking and functionalizing various compounds was studied extensively and increasing interest has been focused on the application of this enzyme as a new biocatalyst in organic synthesis.[1-6] Laccases (EC 1.10.3.2) are a class of multi-copper-containing oxidoreductase enzymes able to catalyze the transformation of various aromatic compounds, specifically phenols and anilines, through the formation of a free cation radical after the transfer of a single electron to laccase. The radical can further react on non-enzymatic oxidation polymerizing various halogen, alkyl-, alkoxy-substituted anilines and phenols.[7-8] The phenolic derivatives resulting in the production of polymeric aggregates are usually less soluble and much stable than their parent compounds.[9,10] Unfortunately the relatively short catalytic lifetime of the laccases in the polymerization processes and the mass transfer limitations, restrict their applications. This effect can be attributed to the inactivation of the enzyme active site due to phenoxy radicals and polymers produced during enzyme treatment.[11]

To overcome this limitations the use of ultrasound, under proper conditions, has shown to enhance significantly the mass transfer as well as the structure stability, substrate binding, and activity of the enzyme.[12,13] Ultrasound alone or in combination with other methods is known to enhance a wide variety of chemical and physical processes, mainly due to the phenomenon known as cavitation in a liquid medium that is the growth and explosive collapse of microscopic bubbles.[14-16] These localized ‘‘hot spots’’ generate high local temperature and pressure rise, capable of decompose water to hydrogen atoms and hydroxyl radicals and of break several chemical bonds.[17,18]

Therefore in this work laccase from *Trametes villosa* was tested in combination with ultrasound to improve the radical polymerization of catechol. A solid-state “in situ” sono-enzymatic synthesis of poly(catechol) was also performed by coloration of wool. The results were analyzed by spectrophotometric and HPLC analyses.

Experimental part

Reagents. Laccase (EC 1.10.3.2) from *Trametes villosa* (5.3 mg protein/mL, 600 U/mL), was kindly provided by Novozymes (Bagsvaerd, Denmark). The wool fabrics (Albano Antunes Morgado Lda, Castanheira de Pera, Portugal) were previously washed with 1 g/L non-ionic surfactant Lutensol AT-25 (BASF, Ludwigshafen, Germany), in a bath ratio 1:20 at pH 9 ($NaHCO_3$ 0.1 M buffer), for 30 min at 40°C, in a Rotawash apparatus (MKII Series 7227, from Shirley Developments Limited, Stockport, England). After the washing procedure, the surfactant was removed from fabric with tap and distilled water. All other reagents were purchased from Sigma-Aldrich and used without further purification.

Ultrasound equipment 20 kHz. The experimental set used was composed of an electrical generator of frequencies of 20 kHz and intensity power from 7 W to 100 W supplying a piezoelectric transducer (Sonics & Materials, USA), with probe diameter of 13 mm. The reaction vessel was a not sealed glass cell (diameter 60 mm and height 200 mm), which contained 150 mL of sample solution. The sonochemical reactor was thermostated by a cooled water-jacket in order to maintain a constant temperature of 50ºC. Ultrasonic energy dissipated in the reactor was set at specific power using calorimetric method.[19]

Catechol polymerization with ultrasound and enzyme. Solutions of catechol (150 mL, 1 mM in 0.1 M acetate buffer pH 5) were treated with ultrasound and *Trametes villosa* laccase. The used concentration of the *Trametes villosa* laccase was 56 U/mL (100 µL of crude preparation in 150 mL of catechol solution). The ultrasound generator was set-up at 20 kHz and intensity powers of 7, 30 and 50 W. The temperature of treatment was 50ºC, the optimum temperature of the laccase, and the time ranged from 0 to 120 minutes. A control sample was reacted in a stirring system at the same condition without ultrasound. Catechol polymerization experiments, with and without ultrasound, were also performed with wool fabrics immersed in the same previously described solutions at 50ºC for 30 minutes.

HPLC analyses. The standard curve prepared for the HPLC assay for poly(catechol) determination contained samples from standard stock solutions at 6.5, 12.4, 29, 66, 150, 200, 430, 669, 2000 kDa. From the sonicated catechol solution (20 kHz at 50 W for 2 h) was taken a sample of 2 mL and it was dissolved in 1 mL of 0,5 M NaOH in a final volume of 3 mL. The same sample was prepared from the stirring control solution. The molecular weight distribution was determined by size-exclusion chromatography (SEC) on a 10 x 300 mm Superdex 200 HR 10/30 (Amersham Pharmacia Biotech) column eluted with phospate buffer at pH 6.5 (50 mM KH_2PO_4, 100 mM KCl). The flow rate was set up at 0.5 mL/min at room temperature. UV detection of the peaks following HPLC separation was performed with a Wellchrom K-2500 Spectrophotometer detector set at a 280 nm.[20]

K/S measurements. Color strength was evaluated in terms of K/S values and these were calculated using Kubelca-Munk's equation $(K/S= (1-R)^2/2R$, where R is the reflectance). The reflectance values were measured (five repletion for each sample) with a Datacolor apparatus at standard illuminant D65 (LAV/Spec. Incl., d/8, D65/10°). Before the measurement of the K/S values the samples were washed with tap and distilled water. The samples were left to dry at room temperature.

Results and discussion

The first step of the study was to analyze the relationship between the laccase catechol polymerization and the ultrasound intensity power in solution. A *Trametes villosa* laccase was used as bio-catalyst to produce poly(catechol) under different ultrasound intensity powers. Catechol was chosen because is a well knows substrate for laccase that polymerize forming poorly soluble products. Catechol is oxidized by laccase to aryloxy-radicals, which may undergo further non-enzymatic reactions originating colored dimeric, oligomeric and polymeric products. The formed alkoxy free radicals can also coupled in the ortho and para positions with the hydroxyl groups and it can form extended quinines.[21,22]

Usually the enzyme activity decreases significantly in presence of ultrasound with high intensity powers.[23,24] However, the results have been shown that the catechol enzymatic polymerization can be enhanced by ultrasound modulating the application time and the power intensity. In an ultrasound system in the same treatment conditions but without laccase, no catechol polymerization was observed. Figure 1 shows the linear time dependence of the catechol oxidation under different ultrasound power intensities at 20 kHz (7, 30 and 50 W) for 2 hours. The ultrasound enhancing effects were observed after 30 minutes of treatment in all experiments. This effect can be attributed to the hydroxyl radicals produced by ultrasound that reacts with the intermediate molecules previously produced by the enzyme, enhancing the catechol polymerization.[25-27] It is clear that the power intensity is the limiting factor for an efficient catechol bio-polymerization. In presence of laccase the catechol polymerization can be enhanced until to a maximum of 50 W. For higher power intensities the laccase activity decreases so rapidly that the enzymatic products cannot be efficiently formed, limiting the hydroxyl radical’s action.

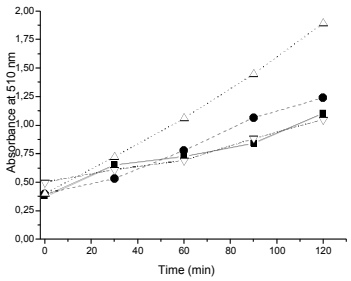

Figure 1 – Sono-enzymatic catechol polymerization with laccase from *Trametes villosa* at 50ºC in a ultrasound 20 kHz system with several power intensities: 7 W ■, 30 W ●, 50 W △, stirring system at 50ºC ▽.

The HPLC spectra of the polymers obtained in the conventional heated system at 50 ºC and in the sonicated 20 kHz system at 50 W and at the same temperature, revealed unexpected differences. The chromatogram of the sono-enzymatic system presents more two peaks (U2 and U4) than the stirred system (Figure 2). The polymers formed in presence of the ultrasound presented higher values of the average molecular weight (M_{Wp}) and the average polymerization degree (Dp_p) than the stirred system (Table1). The peaks U1 and U3 in the sonicated system are comparable respectively with the S1 and S2 peaks in the stirred system. These peaks showed similar values of M_{Wp}. However, higher Dp_p values in the sonicated system were observed, confirming the ultrasound enhancing effects in the polymerization degree.

The U2 and U4 peaks are not present in the stirring system. The U4 peak in particular showed the highest M_{Wp} and Dp_p values (22.3 kDa and 206.2 respectively). The formation of a so high molecular weight polymer can be explained due to the synergistic action of the enzyme and the ultrasound since the ultrasound alone is no able to perform the catechol polymerization. The enhancement of the physical diffusion processes, due to the ultrasound action, can promote the enzyme accessibility and it can have effects on the active site of the enzyme. At the same time the hydroxyl radicals produced by ultrasound can react with the enzymatic-oxidized catechol molecules promoting the polymer chain propagation.[28] The enzymatic polymerization of catechol without ultrasound was previously extensively studied.[29,30] However, low molecular polymers (0.8 kDa) were obtained, also using organic solvent to improve the chain propagation, limiting their application.[31]

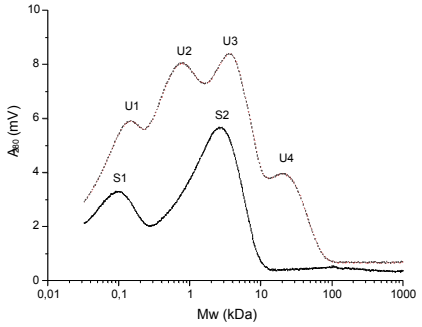

Figure 2 - HPLC chromatograms of the catechol polymerization with laccase from *Trametes villosa* at 50 ºC in a stirred system (straight line) and in an ultrasound 20 kHz system at 50 W (dot line).

Table 1 - The average molecular weights (M_{Wp}) and the average polymerization degrees (Dp_p) of the catechol polymers.

	Sonicated system				Stirred system	
Peaks	U1	U2	U3	U4	S1	S2
M_{Wp} (kDa)	0.1894	0.8664	4.0193	22.2660	0.1208	3.0937
Dp_p	1.75	8.02	37.21	206.17	1.11	28.65

To verify the polymerization in solid-phase an additional experiment was performed in the same previously described conditions adding in the medium a wool fabric. Recently various patents reported on coating achieved with laccase.[32-37] However, the knowledge about the combined ultrasound/enzymatic grafting polymerization is quite limited. The sono-enzymatic synthesized catechol polymer was "in-situ" adsorbed onto the wool providing darker brown coloration of the fabrics than the reported laccase-catechol wool coloration in absence of ultrasound.[38] In the figure 3 is presented the color strength of the fibers in term of K/S values, as indirect polymerization degree, in function of different intensity powers. These results confirm the previous evidence regarding the sono-enzymatic polymerization in solution. The grafting polymerization of the wool fabric surfaces increases with the increasing of the ultrasonic intensity power obtaining darker coloration in a short time of treatment. When the ultrasound is applied just with catechol in solution no coloration onto the fabrics was observed.

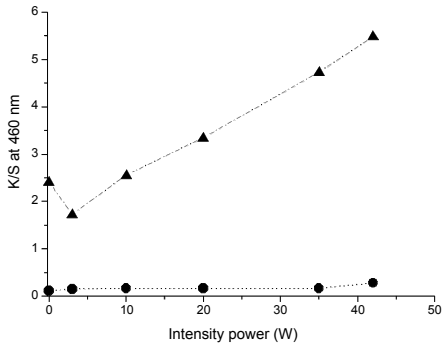

Figure 3 - Sono-enzymatic coating of wool with catechol at different power intensities with (▲) and without (●) laccase from Trametes villosa at 50ºC in an ultrasound 20 kHz system for 30 minutes.

Conclusions

This study has demonstrated that the ultrasonic low frequency waves (20 kHz), applied to the maximum power intensity of 50 W, improve the diffusion processes and may also have positive effect on the laccase active center structure. Moreover the hydroxyl radicals produced by ultrasound can react with the intermediate molecules produced by the enzyme, enhancing the enzymatic catechol polymerization. The sono-enzymatic polymerization provides higher molecular weight polymer (~22 kDa) than the conventional enzymatic polymerization (~1 kDa), offering the opportunity to produce a new class of enzymatic-synthesized polymers and coating techniques from phenols at mild condition of temperature and pH.

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