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BIOTRANSFORMATION OF A PAPER AND CELLULOSE FACTORY EFFLUENT IN THE PRESENCE OF LACCASE AND HORSERADISH PEROXIDASE

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ABSTRACT

The biodegradation of effluent from an industrial paper mill was monitored in relation to the parameters total phenols, low molar mass phenols and color during its incubation with laccase (Novozymes), laccase-HBT and peroxidase (Merck). The results revealed that the laccase reduced the effluent color by 37% and the peroxidase by 43%. Also, the enzymes had a great efficiency in the reduction of color, total phenols and high molar mass compounds contained in the effluent, indicating that depolymerization occurred.

KEY WORDS: Phenolic compounds, lignolytic enzymes, depolymerization.

RESUMO

BIOTRANSFORMAÇÃO DOS EFLUENTES DE UMA INDÚSTRIA DE PAPEL E CELULOSE NA PRESENÇA DE LACASE E PEROXIDASE. A biodegradação dos efluentes de uma indústria de papel e celulose foi monitorada em relação aos parâmetros de fenóis totais, fenóis de baixa massa molar e cor durante a sua incubação com lacase (Novozymes), lacase-HBT e peroxidase (Merck). Os resultados revelaram que a lacase reduziu a cor do efluente em 37% e a peroxidase em 43%. Também foi possível observar que as enzimas possuiram grande eficiência na redução de cor, compostos fenólicos totais e compostos fenólicos de alta massa molar contidos no efluente, indicando, desta forma, que a despolimerização ocorreu.

PALAVRAS-CHAVE: Compostos fenólicos, enzimas lignolíticas, despolimerização.

INTRODUCTION

The paper and cellulose industry produces great volumes of effluents, whose characteristics vary depending on the manufacturing process used. The most toxic effluents are generated in the pulp bleaching stage. Of the pulping (pulp production) technological processes, the most used is that which employs sulfide in alkaline medium, more commonly known as the Kraft process (Thompson et al., 2001). The pulp produced by the Kraft process is said to be non-bleached, since a small percentage of lignin remains in it, due to oxidation reactions, and it has a darker color.

The many substances identified in effluents include lignins of low molar mass (MM) and their degradation products, such as: chlorophenols, chololignins, aromatic carboxylic acids, chlorides and non chlorides, and also high molar mass compounds, which can be biotransformed by some organisms, such as fungi and bacteria, especially fungi of the class Basidiomycetes (Soares; Durán, 2001). Several studies have shown that the biotransformation and biodegradation of wood by these fungi depend mainly on their capacity to produce extracellular lignolytic enzymes (Hatakka, 1994; Soares; Durán, 2001). The most notable lignolytic enzymes produced are laccases and peroxidases, belonging to the phenoloxidases group.

The laccases (1.10.3.2) are produced mainly by fungi and plants. They have molar masses in the region of 60-100 kDa, they are generally present in the form of 2 or 3 isoenzymes and they catalyze oxidation reactions through the extraction of one electron of the phenolic substrate generating a phenoxyl radical. However, these enzymes can oxidize non-phenolic compounds in the presence of mediators such as 1-hydroxy-benzotriazole (HBT) (Durán et al., 2002; Claus, 2004; Couto; Herrera, 2006; Dwived et al., 2011). The peroxidases (1.11.1.7) can be found in plants and animals (blood plasma, milk, yeasts, etc.). Their molar masses are in the region of 38-43 kDa and they catalyze the oxidation of phenolic and non-phenolic aromatic compounds (Nicell, 1994). Within this context, the objective of this study was to evaluate the possible chemical modifications caused

by laccase, laccase in the presence of a mediator and peroxidase in effluent samples from a paper and cellulose factory in Santa Catarina.

MATERIAL E METHODS

The effluent used was collected from a paper and cellulose factory in Santa Catarina, a producer of non-bleached pulps and had the following physico-chemical characteristics: pH = 8.1; conductivity =1.225 mS; chemical oxygen demand (COD) = 1,748 mg/L; biological oxygen demand (BOD) = 750 mg/L. The laccase used was donated by Novozymes. The peroxidase and the HBT were acquired from Sigma.

The characterization of the enzymes was carried out through the determination of enzymatic activity and through the determination of MM. The determination of enzymatic activity in different buffers (tartrate pH: 3.0, 3.5, 4.0, 4.5 and 5.0 and phosphate pH: 6.0, 6.5, 7.0 and 7.5) were carried out using the method of Eriksson (Ander; Eriksson, 1976). This method is based on the oxidation of syringaldazine to its quinone form which absorbs at 525 nm (ε = 65000 M^{-1} cm^{1}). The data were obtained with the aid of a Hewlett-Packard spectrophotometer. The molar mass of each enzyme was determined by High Performance Liquid Chromatography (HPLC), using a Shimadzu chromatograph, equipped with an SPD-10A UV-Vis detector and with a Shimadzu Diol-150 column. The eluent used was Tris (pH = 9.0) buffer, with a flow of 1 mL/min. The protein standard used was type MW-GF-200 kit; Sigma.

The decolorization of the effluent was studied through UV-Vis spectrophotometry after the effluent had been submitted to a period of incubation with the enzymes for 45 minutes. The total concentration of low molar mass phenols of the non-treated effluent and effluent treated with enzymes was verified through the 4-aminoantipyrine (4-AAP) method and the total phenol concentrations of the treated and non-treated effluent were verified through the Folin method (American Public..., 1989).

The molar mass distribution analysis of the treated and non-treated effluents was carried out through semi-preparative size-exclusion HPLC, using the equipment cited above. The column used was an Assaypack-Waters and the eluent H_2O/ 00.5 M NaOH, with a flow of 1.0 mL/min. The effluent samples analyzed were centrifuged at 6400 rpm for 10 minutes. The values for relative content were calculated based on the relation between the areas of each fraction and the total area (sum of the areas of all fractions) measured on each chromatogram. The samples were monitored at the following wavelengths: 240, 280 and 310 nm.

RESULTS AND DISCUSSION

In relation to the characterization of the enzymes, measurements of the kinetic reaction of the enzymes with syringaldazine in different buffers were initially carried out to determine the best pH conditions for the activity. The results obtained from the kinetic studies (Fig. 1) showed a better activity at pH 4.0 for both enzymes. The unit of enzyme activity (U) was defined as: the quantity of enzyme necessary to oxidize 1 mM of substrate per second.

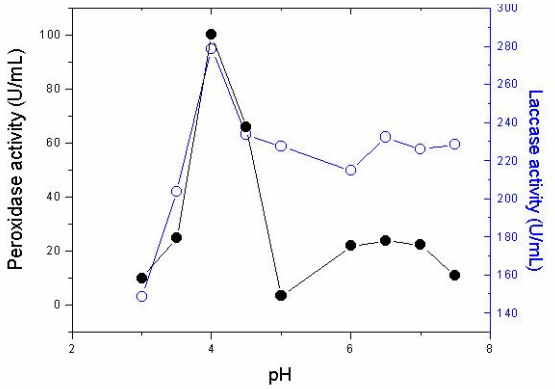

Fig. 1 - Analysis of the enzymatic activity (U/mL) of laccase (○) and peroxidase (●) front the different solutions with pH(s).

The molar mass of each enzyme was determined by HPLC. Through the chromatograms, and comparing with the standard used (MW-GF-200 kit; Sigma), it was observed that the MM of laccase was in the region of 91 kDa and 65 kDa and of peroxidase was 45 kDa. All of the results for the characterization of laccase and peroxidase revealed that they have the same characteristics, in relation to the activity x pH and molar mass, as the laccases and peroxidases cited in the literature, produced by fungi (Hublik; Schinner, 2000; Courteix; Bergel, 1995).

The decolorization of the effluent was monitored through the reduction in absorbance at several wavelengths in the visible-ultraviolet region, after 45 minutes of incubation of the effluent with laccase and peroxidase. A decolorization of the effluent was observed for the two enzymes tested. Laccase was responsible for a decolorization of the effluent of up to 37% and peroxidase of 43% (Figs. 2 and 3).

After the effluent treatment with laccase, a spectral modification was observed with a shift in the absorption band from 240-270 nm to 267-310 nm, indicating changes in the structures contained in the effluent. These changes increased proportionally with an increase in laccase concentration. When the same effluent was treated with peroxidase, no type of spectral change was observed, only a reduction in the concentration of total compounds with an increase in the enzyme concentration, observed through a

reduction in absorbance in the region of 240-270 nm. Overall, the results obtained revealed that peroxidase was more efficient in the decolorization of the effluents. The peroxidase (HRP) used in this experiment has many similarities with lignin peroxidase (LiP), which is also extensively used in biotechnological applications. Several studies with model substances have revealed that LiP is able to oxidize both phenolic structures and non-phenolic aromatic structures, showing a great flexibility in relation to the structure of substrates (Martínez, 2002). In view of the fact that peroxidase has the ability to oxidize a greater variety of compounds than laccase, may leads it to being more efficient in the degradation of effluent compounds and consequently in their decolorization.

Together with the process of effluent decolorization, the concentration of total phenols was measured by two methods, the Folin and the 4-aminoantipyridine methods.

The results obtained using these methods revealed a decrease in the total amount of phenolic compounds in the effluents treated with the two enzymes (Figs. 4 and 5).

The results obtained with the 4-AAP method showed that the two enzymes degrade efficiently the total and low MM phenolic compounds. The fraction of low MM compounds is that which contains chemical agents responsible for the toxicity of the effluents. The main substances indicated as being of high toxicity are chlorophenols, which are products of the partial degradation of lignin, notably its derivatives: di- and trichloroguaiacol; tri, tetra and pentachlorophenol, and others. The high MM fractions are considered inert and non-toxic, since they are not able to penetrate the cells of living organisms, due to their size. On the other hand, some studies have shown that some of these compounds may be degraded under environmental conditions generating low MM compounds. The high MM phenolic compounds were also efficiently degraded by the enzymes, and particularly by lacasse with HBT. Also, some studies have demonstrated a reduced oxidation capacity of laccase in relation to laccase + HBT or laccase-mediator (Cantorella et al., 2003).

The MM distribution analysis of the effluent components, during the period of incubation with the enzymes, indicated that the enzymes cause an intense degradation of the organic material contained in the effluent, as observed by a notable reduction in the total area obtained on the chromatograms measured at 240 and 280 nm for peroxidase and at 240, 280 and 310 nm for laccase. From the chromatograms it was possible to obtain a relative concentration of the different compound fractions in relation to molar mass, at the different wavelengths analyzed (Table 1).

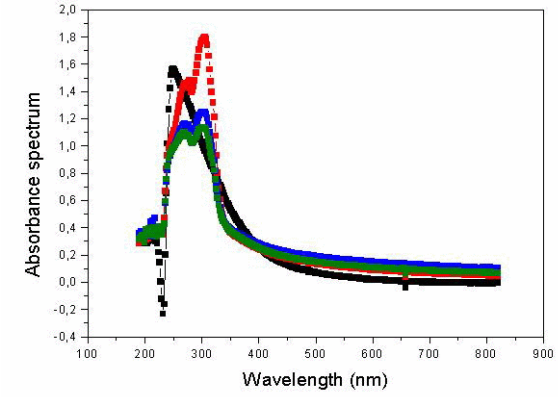

Fig. 2 - Absorbance spectrum of effluent sample before (■) and after 45 minutes of incubation with 50 µ L of laccase (■), 100 µL of laccase (■) and 150 µL of laccase (■).

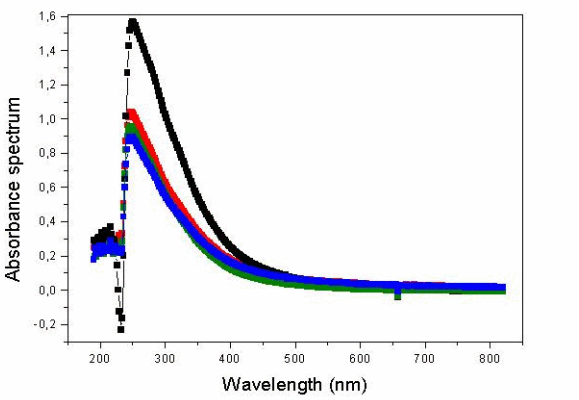

Fig. 3 - Absorbance spectrum of effluent sample before (■) and after 45 minutes of incubation with 50 µL of peroxidase (■), 100 µL of peroxidase (■) and 150 µL of peroxidase (■).

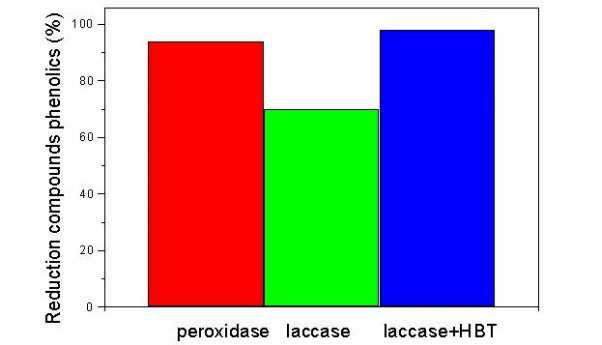

Fig. 4 - Total phenol degradation of effluent after 45 minutes of incubation with enzymes.

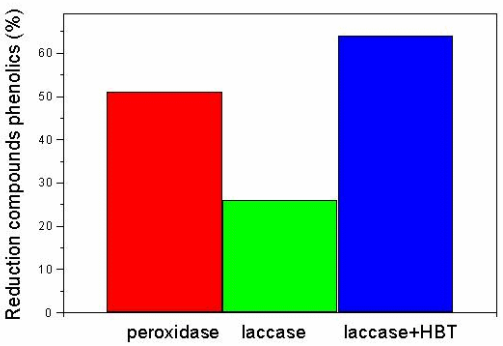

Fig. 5 - Degradation of low MM phenolic compounds of effluent after 45 minutes of incubation with enzymes.

Table 1 - Relative concentrations (molar mass, MM) of the composite fractions in the effluent obtained by HPLC.

Fractions[1]	Effluent			Effluent treated with laccase + HBT			Effluent treated with peroxidase	
	Relative concentration (%)							
	240nm	280nm	310nm	240nm	280nm	310nm	240nm	280nm
High MM	-	45.5	-	11.5	16.5	10.5	4,1	1,1
Medium MM	100	54.3	100	88.5	51.5	75.2	95.8	98.9
Low MM	-	-	-	-	31.7	13.5	-	-

[1]Composites of high MM (retention time: 0-11 minutes), composites of average MM (retention time: 12-21 minutes) and composites of low MM (retention time > 21 minutes).

Through the results showed in Table 1 it was possible to observe a decrease in the relative concentration of high MM compounds which absorb at 280 nm after treatment with laccase. Also, low MM compounds appeared which absorb at 310 nm.

For the set of fractions contained in the effluent without treatment, values for $Abstotal_{280}/Abstotal_{310}$ of 7.25 and $Abstotal_{280}/Abstotal_{240}$ of 1.95 were observed, revealing a greater number of compounds which absorb in the 280nm region than in the 310 nm or 240 nm regions.

The fractions contained in the effluent after treatment with laccase gave values for $Abstotal_{280}/Abstotal_{310}$ of 2 and $Abstotal_{280}/Abstotal_{240}$ of 0.72, which indicates a shift in the absorption of these compounds, possible due to structural changes. Therefore, the results obtained with the chromatograms are in agreement with the results obtained through the UV-Vis spectra and showed that laccase + HBT act on the effluent, depolymerizing high MM compounds.

The fractions contained in the effluent after treatment with peroxidase, had a value for $Atotal_{280}/Atotal_{240}$ of 0.24, which indicates a greater quantity of compounds which absorb in the 240 nm region in relation to laccase which had a value for $Atotal_{280}/Atotal_{240}$ of 0.72. Through Table 1 it is possible to observe that the non-treated effluent (monitored at 240 and 280 nm) contained a great quantity of medium MM compounds. After treatment with peroxidase this value remained almost unchanged. The fraction of medium MM compounds, which appear to remain unchanged, may suffer structural modifications, as indicated by the different percentages, when monitored at different wavelengths, as shown in Table 1.

CONCLUSIONS

The enzymes laccase and peroxidase showed a great efficiency in the reduction of color, total phenols and consequently of high MM compounds contained in the effluent, indicating that a depolymerization of the compounds occurred. Therefore, the use of enzymes in the procedures for the bioremediation of industrial effluents is shown to be an attractive alternative, mainly due to the good results obtained.

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