Effect of process parameters in Laccase Mediator System delignification of flax pulp. Part II. Impact on effluents properties

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Abstract

Flax pulp was bleached by using an enzyme treatment with laccase (L stage) in the presence of HBT as mediator in order to replace chlorine-based pulp bleaching processes which produce toxic organochlorine compounds with biotechnological products, achieving an environmentally friendly process technology. Enzyme treatments were sequentially conducted according to a factorial design involving four variables: laccase dose, HBT dose, treatment time and oxygen pressure in the reactor. Their influence on the effluent properties (COD, color, toxicity, spectra and residual enzymatic activity) was examined to evaluate the impact of L stage on environment since no experimental data have been published concerning this matter.

Mathematical models accurately predicting effluent properties in terms of the previous four variables were developed. High COD levels were obtained as a result of using commercial laccase. Also, red color produced, especially at long treatment time, relates to formation of oxidation products from HBT. The residual enzymatic activity depends basically on mediator dose, and mainly activity loss is produced during the first 30 min of treatment. The toxicity of the effluents was below the limits set by the sewage regulations for Catalonia and can be ascribed to the combined effect of the laccase-mediator system.

Keywords

Bleaching, COD, color, Effluents, flax pulp, 1-hydroxybenzotriazole, laccase, mathematical model, oxygen pressure, residual enzymatic activity, sequential statistical plan, toxicity.

1. Introduction

Biotechnology possesses a high potential for cutting costs, improving end products and lessening the environmental impact of the pulp and paper industry, which has traditionally been regarded as a strong source of contamination [1]. Production of TCF pulp is growing steadily in response to environmental regulations seeking both to reduce organochlorine emissions and meeting the need to close bleaching plants via filtrate recycling circuits. Complete recycling is difficult with sequences using chlorine or its derivatives owing to their corrosiveness; this has promoted the use of TCF sequences, which allow most of the filtrate to be reused in the recovery cycle [2,3,4,5,6]. Although the effluents from TCF bleaching sequences contain no organochlorines, they must be checked for COD and color in order to facilitate circuit closure in future industrial biobleaching applications.

Laccase-mediator systems have facilitated the development of TCF sequences, the replacement of oxygen- and ozone-based delignification stages, and the efficient bleaching of pulp in addition to reducing kappa numbers and saving reagents. Based on the literature published recently, the use of a laccase in the presence of a mediator is a very promising choice for biobleaching pulp [7,8,9].

For a mediator to be industrially useful, it should be affordable and environmentally benign. The most efficient mediators for delignifying pulp are substances containing an –N(OH)- group (e.g. 1-hydroxybenzotriazole, HBT) [10,11]. However, HBT is expensive and potentially toxic by itself [12] or through its reactions products [13,14]. Effluents from laccase-mediator treatments have not seemingly been studied before; also, product safety sheets typically contain no information about their potential toxicity as assessed with the Microtox method. In addition, it is known that laccase can be inactivated by oxidized species of some mediators [15,16,17].

In previous studies, laccase-mediator systems were found to be efficient in the bleaching of flax pulp [18,19,20,21,22]. In a first part of the present study [23] the operating conditions for the laccase-HBT system were optimized and their influence on the pulp properties was examined, with the main objective of minimizing the reagent doses and the reaction time to make more suitable the industrial application. In this paper corresponding to a second part of the study, we examined the influence of process variables in a laccase-mediator treatment on the properties of the resulting effluents (COD, color, toxicity, residual

52 enzymatic activity and UV/vis absorbance spectra), being the first results obtained in this matter since no
53 data have been reported before.

54 **2. Materials and methods**

55 *2.1 Raw material*

56 The raw material used was unbleached flax pulp (*Linum usitatissimum*) subjected to acid washing. Its
57 properties are ISO brightness of 36.5 %ISO, kappa number of 10.5 and viscosity of 952 $mL \cdot g^{-1}$. The
58 commercial enzyme was laccase from *Trametes villosa* supplied by Novozymes® (Ref. NS-51002) with an
59 activity of 39.4 $U \cdot mL^{-1}$, and the mediator hydroxybenzotriazole (HBT) from Fluka (Ref. 54802).

60 *2.2 Experimental design*

61 Enzyme treatments were sequentially conducted according to a 2^4 factorial design with three replications at
62 the centre point and an 8-point star, being the four variables: laccase dose (X_1 from 1 to 20 $U \cdot g^{-1}$), HBT dose
63 (X_2 from 0.1 to 2 %odp), treatment time (X_3 from 0.5 to 6.5 h) and oxygen pressure in the reactor (X_4 from
64 0.2 to 0.6 MPa). The statistical analysis of pulp properties was based on the results of a planned sequence
65 of tests. The experimental design used to this end was conducted stage wise in each sequence. Thus, the
66 results obtained in each stage were used to decide whether the next was to be performed. More information
67 concerning the experimental design is reported in the first part of this study [23].

68 *2.3 Laccase mediator treatment (L stage)*

69 Enzymatic treatments were carried out in a pressurized reactor at 30 ºC and 3 %odp consistency using a 50
70 mM sodium tartrate buffering solution at pH 4. Control test was performed in the absence of enzyme and
71 mediator, using a treatment time of 6.5 h and oxygen pressure of 95 0.6 MPa, which were the respective
72 maximum values employed in the statistical plan [23].

73 *2.4 Effluent properties*

74 *2.4.1 COD and color properties*

The COD and color values of effluents from L stage were calculated in accordance with ASTM D1252-00 and ASTM D1029-00 standards, respectively. Absorbance data were taken at 600 nm for COD and 465 nm for color.

2.4.1 Toxicity values

Effluent toxicity was determined with the Microtox method, which measured the light reduction caused by the microbe *Vibrio fischeri* in contact with toxins (UNE-EN ISO 11348-3). One equitox·m^3 is defined as the reciprocal of the wastewater dilution (expressed in parts per one) resulting in 50 % inhibition within 15 min under typical biotesting conditions. Toxicity measurements were color-corrected as per the recommendations of Azur Environmental, the manufacturer of the MicrotoxOmni equipment used.

2.4.3 Spectrophotometric curves

The effluents from L stage were diluted to 1:20 and their absorbance measured between 200 nm and 400 nm in a UV-vis Shimadzu 1603 spectrophotometer.

2.4.4 Residual enzyme activity

The enzymatic activity of laccase was defined as the amount of enzyme needed to convert 1 µmol of the substrate ABTS per minute. Oxidation of ABTS was followed by an absorbance increase at 436 nm (ε_{436}=29300 $M^{-1}\cdot cm^{-1}$) in a UV-vis Shimadzu 1603 spectrophotometer. The reaction mixture contained 5 mM ABTS, 100 mM sodium acetate buffer at pH 5 and between 10 and 50 µL of enzyme.

3. Results and discussion

3.1 Statistical analysis

The four variables were normalized to three different values (-1, 0 and 1) for implementation of the factorial design. Table 1 shows the relationship between the process variables and their normalized values in the L stage. Table 2 shows the COD, color and residual enzymatic activity values in L stage effluents. The saturated model of the factorial design is showed in Eq. 1.

$$Y_L = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 + b_{123}X_1X_2X_3 + b_{234}X_2X_3X_4 + b_{124}X_1X_2X_4 + b_{134}X_1X_3X_4 + b_{1234}X_1X_2X_3X_4 \quad (1)$$

98 *3.1.1 Statistical analysis of the COD in L stage*

99 *Step 1. Study of factorial design.* The 2^4 design was established from tests L 1 to L 16 (see Table 2) and the
100 probability graph for the saturated model obtained (Eq. 1 and Fig. 1 a). Those coefficients falling on the right
101 of the significance line in the graph and departing from it were deemed significant. This allowed non-
102 significant coefficients for the saturated model to be discarded. The new model constructed was used to
103 determine the new coefficients for the kappa number and their significance. Only term X_1 in the model (Eq. 2)
104 was significant ($p < 0.05$).

$$Y_{COD\text{-}L} = 317 + 157X_1 \quad (2)$$

105 *Step 2. Study of the variance.* In order to detect potential curvature in a model, the variance might be
106 checked to verify if remains constant throughout the experimental field studied. By comparing the residual
107 mean square (RMS = 347) and variance for the three centre points (S_c^2 = 1010), the model for the kappa
108 number was found to be homoscedastic ($p = 0.22 > 0.05$), i.e. the variance was constant.

109 *Step 3. Is it influenced by quadratic terms?* In order to facilitate detection of potential curvature, the model
110 provided by the previous 2^4 factorial design was expanded with the 2 coefficients previously deemed
111 significant and that representing quadratic terms. The design matrix contained the central tests L 17 to L 19
112 (Table 2) and a 2^4 design with 3 central responses. Based on the statistical analysis for curvature, the
113 coefficient for the quadratic terms was not significant ($p = 0.4$).

114 *Step 5. Verification of the final model.* COD was measured in tests L 20 to L 23 (Table 2); although these
115 tests were unnecessary to construct the model, they were used in its analysis. The final model was
116 constructed from 23 responses and fitted the equation Eq. 3. The coefficient of determination (R^2) was 0.98
117 and the probability associated to F_{calc} 10.4. Table 3 shows the coefficients of the model and their
118 significance.

$$Y_{COD\text{-}L} = b_0 + b_1 X_1 \quad (3)$$

Residuals distributed according to no well-defined pattern as a function of the estimated responses. In addition, the COD responses predicted by the model, and the experimental responses, distributed around a straight line, the differences between the two ranging from - 42 to 27 $kg \cdot t^{-1}$ (data not shown). Also, the points in the normal probability graph Z:N(0,1) vs. residuals fitted a straight line (Fig. 1 b). Based on these results, the model was deemed statistically accurate.

3.1.2 Statistical analysis of the color in L stage

Step 1. Study of factorial design. The 2^4 design was established from tests L 1 to L 16 (see Table 2) and the probability graph for the saturated model obtained (Eq. 1 and Fig. 2 a). The graphical analysis of the saturated model allowed non-significant coefficients to be discarded. Those coefficients falling on the right of the significance line and departing from it were deemed significant and were used to test the model and determine its significance. All terms in the model thus obtained (Eq. 4) were significant ($p < 0.05$).

$$Y_{color\text{-}L} = 15.8 + 10.8X_1 + 14.7X_2 + 6.9X_3 + 10.7X_1X_2 + 5.4X_1X_3 + 6.8X_2X_3 + 5.3X_1X_2X_3 \quad (4)$$

Step 2. Study of the variance. Detecting curvature in a model requires checking whether the variance remains constant with time throughout the experimental range studied. A comparison of the RMS (3.19) and variance at the three centre points (1.00) provided $p > 0.05$; therefore, the model was homoscedastic (i.e. the variance was constant).

Step 3. Is it influenced by quadratic terms? Potential curvature in the model obtained by using the previous 2^4 factorial design was examined by expanding it with the 8 coefficients previously deemed significant and that representing quadratic terms. The design matrix encompassed the central tests (L 17 to L 19) (Table 2) and a 2^4 design with 3 central responses, and was used to search for potential curvature in the model. Based on the results of the statistical analysis, the representative quadratic term was significant ($p = 0.00$) and had a coefficient of -3.2. Because the central tests did not allow the specific factor influencing the response in a quadratic manner to be identified, the statistical study had to be repeated with additional tests.

Step 4. *Which factors have a quadratic effect?* In order to identify the factors with a quadratic effect on the response, the study was expanded with the star points (tests L 20 and L 22 to L 26) in order to deconvolute quadratic terms (Table 2). The model obtained by using the 2^4 factorial design with three central tests and the eight star points was examined.

Step 5. Verification of the final model. The final model was obtained from 25 responses and fitted the equation Eq. 5; the coefficient of determination (R^2) was 0.99 and the probability associated to F_{calc} 277. Table 4 shows the coefficients of the model and their significance.

$$Y_{color\text{-}L} = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 + b_{11}X_1X_1 \quad (5)$$

The residuals distributed according to no well-defined pattern with respect to the estimated responses. In addition, the color responses predicted by the model and their experimental counterparts clustered around a straight line (data not shown), and differences between the two ranged from 3 to 4 kgPtCo·t^{-1}. Also, the normal probability graph, in the form of Z:N(0,1) vs. residuals, fitted a straight line (Fig. 2 b). Based on these results, the model was deemed statistically accurate.

3.1.3 Statistical analysis of enzymatic residual activity

Step 1. Study of factorial design. The 2^4 design was established from tests L 1 to L 16 (see Table 2) and the probability graph for the saturated model obtained (Eq. 1, and Fig. 3 a). The graphical analysis of the saturated model allowed non-significant coefficients to be discarded. Those coefficients falling on the right of the significance line and departing from it were deemed significant and were used to test the model and determine its significance. All terms in the model thus obtained (Eq. 6) were significant ($p < 0.05$).

$$Y_{residual\ activity\text{-}L} = 4.1 + 4.1X_1 - 2.1X_2 - 0.3X_3 - 2.1\ X_1X_2 - 0.3X_1X_3 - 0.5X_2X_3 - 0.5X_1X_2X_3 \quad (6)$$

Step 2. Study of the variance. Detecting curvature in a model requires checking whether the variance remains constant with time throughout the experimental range studied. A comparison of the RMS (0.20) and the variance (0.03) at the three centre points provided $p = 0.27 > 0.05$; therefore, the model was homoscedastic (i.e. the variance was constant).

Step 3. Is it influenced by quadratic terms? Potential curvature in the model obtained by using the previous 2^4 factorial design was examined by expanding it with the 8 coefficients previously deemed significant and that representing quadratic terms. The design matrix encompassed the central tests (L 17 to L 19) (Table 2) and a 2^4 design with 3 central responses, and was used to search for potential curvature in the model. Based on the results of the statistical analysis, the representative quadratic term was significant ($p = 0.00$) and had a coefficient of 1.8. Because the central tests did not allow the specific factor influencing the response in a quadratic manner to be identified, the statistical study had to be repeated with additional tests.

Step 4. Which factors have a quadratic effect? In order to identify the factors with a quadratic effect on the response, the study was expanded with the star points (tests L 20, L 22 to L 26) in order to deconvolute quadratic terms (Table 2). The model obtained by using the 2^4 factorial design with three central tests and the eight star points was examined. The final model was obtained from 25 responses and fitted the equation Eq. 7; the coefficient of determination (R^2) was 0.98 and the probability associated to F_{calc} 140. Table 5 shows the coefficients of the model and their significance. The quadratic coefficient of the model was 1.7, similar to the previously calculated representative quadratic term.

$$Y_{\text{residual activity -L}} = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 + b_{22}X_2X_2 \quad (7)$$

Step 5. Verification of the final model

The residuals distributed according to no well-defined pattern with respect to the estimated responses. The enzymatic residual activity responses predicted by the model and their experimental counterparts clustered around a straight line (data not shown), and differences between the two ranged from -1.0 to 1.4 $U \cdot g^{-1}$. Also, the normal probability graph, in the form of Z:N(0,1) vs. residuals, fitted a straight line (Fig. 3 b). Based on these results, the model was deemed statistically accurate.

3.2 Effluent property model

Firstly, some control tests were carried out (Table 6) in order to establish the individual contribution of each reagent and operational conditions to the effluent properties. The control test was performed in the absence

of enzyme and mediator, using a treatment time of 6.5 h and oxygen pressure of 0.6 MPa, to evaluate the effect of buffer, temperature, oxygen pressure and pulp washing. The results show that no effect was found in color and toxicity, but 155 $kg \cdot t^{-1}$ of COD was obtained due to sodium tartrate buffer. Two additional controls (control-HBT and control-laccase) were carried out, applying 2 % of HBT or 20 $U \cdot g^{-1}$ of laccase, respectively. The most interesting result was the high COD value (352 $kg \cdot t^{-1}$) obtained with the control-laccase, suggesting the commercial laccase will be the main responsible for the high COD levels in L-treatments. HBT and laccase have no influence on color and toxicity properties.

3.2.1 Model for the COD in L stage

The model relating the COD of the effluent after the L stage to the process variables fitted the equation Eq. 8 and predicted COD from 160 to 477 $kg \cdot t^{-1}$. Based on the model, the sole variable affecting COD was X_1 (laccase dose), which influenced it in a linear manner. The other three variables [viz. mediator dose (X_2), treatment time (X_3) and oxygen pressure inside the reactor (X_4) had no effect on the COD response.

$$Y_{COD\text{-}L} = 318 + 158X_1 \quad (8)$$

where X_1=(L-10.5)/9.5, L= laccase dose ($U \cdot g^{-1}$)

COD increased with increasing dose of laccase (Fig. 4 a). The combined COD for the reagents when used at their highest doses and the control (Table 6) was 521 $kg \cdot t^{-1}$. This value is similar to that obtained in tests involving the highest laccase dose. Therefore, the high COD levels of the effluents were a result of that contributed by the commercial enzyme preparation (specifically, by the additives used to maintain its activity), in fact commercial laccase represents 74 % of total COD in L stage. It would be interesting to recommend the use of new enzyme formulations of lower COD in order to facilitate industrial application of the laccase-mediator system.

3.2.2 Color model for the L stage

The color responses of the effluents after the L stage ranged from 1 to 77 $kg \cdot t^{-1}$. The model relating effluent color after L and the process variables conformed to Eq. 9 and predicted color values of 4 to 81 $kg \cdot t^{-1}$. Based on the model, the variables influencing color were the laccase dose (X_1), mediator dose (X_2) and treatment

time (X_3). On the other hand, the oxygen pressure, X_4, had no effect on color. The variable most strongly influencing effluent color was the HBT dose (X_2), with a coefficient of 14.8. In addition, the factor laccase dose, X_1^2, had a quadratic influence on the response.

$$Y_{color\text{-}L} = 20.4 + 10.9X_1 + 14.8X_2 + 7.2X_3 + 10.7X_1X_2 + 5.4X_1X_3 + 6.8X_2X_3 + 5.3X_1X_2X_3 - 4{,}5X_1^2 \quad (9)$$

where X_1=(L-10.5)/9.5. L= laccase dose ($U \cdot g^{-1}$); X_2=(M-1.05)/0.95; M=HBT dose (%odp); X_3=(t-3.5)/3; t=reaction time (h).

The lowest color level provided by the plan was 4 $kg \cdot t^{-1}$ and corresponded to the lowest levels of variables, $X_1 = X_2 = X_3 = -1$ (viz. a laccase dose of 0.5 $U \cdot g^{-1}$, an HBT dose of 0.1 %odp and a treatment time of 0.5 h). On the other hand, the highest color level provided by the plan was 81 $kg \cdot t^{-1}$ and corresponded to $X_1 = X_2 = X_3 = 1$ (viz. a laccase dose of 20 $U \cdot g^{-1}$, an HBT dose of 2 %odp and a treatment time of 6.5 h). Therefore, the highest effluent color was obtained with the highest laccase dose, HBT dose and time studied in the plan.

Fig. 4 (b) shows the response surfaces of the color model as a function of the process variables. Effluent color with a low laccase or mediator doses was very low (less than 16 $kg \cdot t^{-1}$), even at long time reaction. Therefore, the color of the effluents was not due to the initial color of the reagents (Table 6), but rather developed during the laccase-mediator treatment -which must have caused the dissolution or formation of colored compounds in the liquid phase.

When both laccase and HBT doses have the high level ($X_1 = X_2 = 1$), color values are also higher even at short time. The more the time reaction is longer, the more higher the color is as the laccase and HBT increases. The increased color of the effluents obtained after the laccase-mediator treatment can be ascribed to the formation of colored oxidation products of the mediator and also to an increased content of degraded lignin in the effluents. Kappa number in L stage does not correlate with effluents color (Fig. 5) so color is mainly due to oxidation products from the mediator. HBT in a laccase-mediator system is partially converted into benzotriazole (BT) [8,21,22], which might be responsible for the increased red color of the effluents at long treatment times.

3.2.3 Model for residual enzyme activity after the L stage

Table 2 shows the residual activity responses obtained after the L stage and the respective operational conditions used in the laccase-mediator treatment (L). It was only determined the residual activity in two of the tests involving the lowest laccase dose (0.5 $U \cdot g^{-1}$, L 5 and L 9) and found it to be 0.6 and 0.9 $U \cdot g^{-1}$, respectively. Although the activity was seemingly retained or even increased after the treatment, the differences were very small and the average activity was similar to the detection limit of the assessment method used. For this reason, the model was constructed under the assumption that the residual activity after the tests involving the low enzyme dose (0.5 $U \cdot g^{-1}$; L 1, L 3, L 5, L 7, L 9, L 11, L 13 and L 15) would be 0 $U \cdot g^{-1}$. The activity loss was calculated from Eq. 10. The responses ranged from 0 to 18 $U \cdot g^{-1}$.

$$\text{activity loss}\,(\%) = \frac{\text{initial laccase dose - residual activity}}{\text{initial laccase dose}} \qquad (10)$$

Analysis of the mathematical model for the residual enzymatic activity

The model relating residual activity in the pulp after the L stage to the process variables conformed to Eq. 9 and predicted residual activity values from 13.1 to -1.9 $U \cdot g^{-1}$. Although some predicted values were negative, they were taken to be 0 $U \cdot g^{-1}$. Based on the model, the variables influencing residual activity were the laccase dose, (X_1), mediator dose (X_2) and treatment time (X_3). On the other hand, the oxygen pressure inside the reactor (X_4), had no influence on the residual activity response. The model factor mediator dose, X_1^2, exhibited a quadratic influence on the response.

$$Y_{\text{residual activity-L}} = 2.4 + 4.0X_1 - 2.2X_2 - 0.4X_3 - 2.1X_1X_2 - 0.5X_2X_3 - 0.6X_1X_2X_3 + 1.7X_2^2 \qquad (11)$$

where X_1=(L-10.5)/9.5. L= laccase dose ($U \cdot g^{-1}$); X_2=(M-1.05)/0.95; M=HBT dose (%odp); X_3=(t-3.5)/3; t=reaction time (h).

Fig. 6 (a) shows the residual activity response as a function of the variables of the plan. The responses corresponding to low laccase doses were excluded as they were close to the detection limit of the method used to determine residual activity. The highest residual activity was 13 $U \cdot g^{-1}$ and corresponded to X_1 = 1 and

254 X_2 = -1 (viz. a laccase dose of 20 $U \cdot g^{-1}$ and an HBT dose of 0.1 %odp). The lowest residual activity provided
255 by the plan corresponded to X_1 = -1 and X_2 = 2 (viz. 1 $U \cdot g^{-1}$ for laccase and 2 %odp for HBT). Therefore, the
256 enzyme can lose virtually its whole activity during the laccase-mediator treatment depending on the
257 particular reagent doses used. Residual activity after the laccase-mediator treatment therefore essentially
258 depends on the laccase and mediator doses used. By contrast, the variable treatment time has little
259 influence; residual activity decreases with increasing time, but the effect is only significant with a high
260 mediator dose.

261 A study of the activity loss as the difference between the initial and residual activity revealed that the
262 absolute value of the loss at a given mediator dose was greater in the tests involving an enzyme dose of 20
263 $U \cdot g^{-1}$ (X_1 = 1) than in those involving a dose of 10 $U \cdot g^{-1}$ (X_1 = 0), even though the relative activity was
264 independent of the initial laccase dose (Fig. 6 b). Using the lowest HBT dose, 0.1 %odp, in combination with
265 a treatment time of 0.5 h sufficed to reduce the enzyme activity considerably. The residual activity with an
266 initial laccase dose of 20 and 10 $U \cdot g^{-1}$ was 13 and 6 $U \cdot g^{-1}$, respectively. At a fixed laccase dose, increasing
267 the mediator dose decreased the residual activity. At the highest HBT dose studied, 2 %odp, the residual
268 activity fell below 3 $U \cdot g^{-1}$. This decrease with increase in mediator dose was substantial even when the dose
269 was raised from 0.1 to 1 %odp. Higher mediator doses resulted in rather low residual activity, so further
270 increasing the HBT dose caused no substantial change in residual activity (Fig. 6 a).

271 The residual enzymatic activity was found to depend essentially on the laccase and mediator doses; by
272 contrast, the treatment time had little effect on it -in fact, most of the enzyme activity was lost within the first
273 30 min of treatment. The relative residual activity was essentially a function of the mediator dose. Although
274 low mediator doses sufficed to decrease the residual activity by 40 %, an HBT dose of 2 %odp reduced the
275 activity by up to 90 %. HBT radicals formed during the treatment inactivate laccase by oxidizing aromatic
276 amino acids on its protein surface [24]. In the presence of pulp, laccase-mediator systems exhibit reduced
277 inactivation of the enzyme [16,17, 25] by effect of the free radicals formed reacting with the pulp and
278 delaying inactivation of laccase as a result. Under these conditions, the loss of enzyme activity as the
279 mediator dose is increased can be ascribed to an increased formation of oxidation radicals at an early stage
280 of the process that react with the pulp, but also facilitate inactivation of the enzyme.

281 *3.4 Toxicity analysis*

282 The results of the toxicity analysis of the effluents from the L stage are shown in Table 7. Toxicity responses
283 ranged from undetectable to 12 equitox·m^{-3}. Tests which involved the highest enzyme and mediator doses
284 (20 U·g^{-1} and 2 %odp, respectively), were those providing the highest toxicity values.

285 As per Spain's Water Bill (RDL 1/2001), qualitative and quantitative restrictions on the composition of
286 sewage are imposed by the competent hydraulic authority; by exception, sewage disposed of at any point in
287 the public network or through collectors managed by regional or local governments must be authorized by
288 the competent local or regional body. Decree 130/2003, which passed the regulations on public sewage
289 services currently in force in Catalonia Spain), set the limit for discharges of inhibitory matter (IM) at 25
290 equitox·m^{-3}. The toxicity values obtained in this study fell below such a limit. In any case, toxicity levels
291 changed with the particular treatment conditions (see Table 7).

292 The presence of color and/or turbidity can be the source of interferences and overestimated toxicity in some
293 effluents [26]. Therefore, as recommended in the Microtox instruction manual, our toxicity values were
294 corrected against absorbance measurements of the samples. Such corrections reduced the initially
295 estimated toxicity levels for the most strongly colored samples by about 20 %. The effluent from the control
296 treatment, which involved treating the pulp with sodium tartrate buffer containing no enzyme or mediator,
297 exhibited no toxicity (Table 7). Nor did the effluent from treatment L1, which used low reaction doses and a
298 short time. A HBT solution containing 620 mg·L^{-1}, which was the mediator concentration providing a 2 %odp
299 dose at 3 % consistency in the absence of pulp, resulted in an effluent toxicity value of 3 equitox·m^{-3}. An
300 identical solution additionally containing the amount of laccase used to obtain an enzyme dose of 20 U·g^{-1}
301 provided an identical residual activity; therefore, the enzyme introduces no toxicity in the effluent. The toxicity
302 levels obtained by using the laccase-mediator system in the presence of pulp (L stage) were higher than
303 those of the initial toxicity exhibited by the mediator. As can be seen from Table 7, increasing the laccase
304 and/or mediator dose increased effluent toxicity. Therefore, the toxicity of the effluents from L cannot be
305 exclusively ascribed to the mediator (HBT) as it can also result from HBT oxidation and degradation by-
306 products (e.g. benzotriazole), which are more toxic than the mediator itself.

307 *3.5 UV/vis absorbance spectra for the effluents*

Fig. 7 shows the spectra for the effluents from the laccase-mediator treatment as obtained following 1:20 dilution. The control effluent, obtained in the absence of enzyme and mediator, exhibited absorbance below 245 nm that was due to the sodium tartrate buffer used (control curve). A HBT solution containing 620 $mg \cdot L^{-1}$, which was the mediator concentration providing an HBT dose of 2 %odp at 3 % consistency in the absence of pulp, exhibited an absorbance peak at 305 nm and another at 280 nm (HBT curve). An enzyme solution containing the concentration needed to obtain a dose of 20 $U \cdot g^{-1}$ exhibited no appreciable absorbance (laccase curve). The spectral signals observed over the wavelength range 200 to 290 nm may correspond to the mediator, dissolved lignin, and HBT degradation and oxidation products. Because some signals were overlapped and due to various products, the effluents from the laccase-mediator treatment precluded determining the amount of dissolved lignin from absorbance measurements as described in Tappi 222 om-02. For this reason, the absorbance signals could only be used for qualitative purposes.

As can be seen from Fig. 7, the control signal exhibited the same spectral profile as in the treatments involving a low mediator dose (0.1 %odp), irrespective of the particular laccase dose and treatment time used. With a mediator dose of 1 %odp, the spectrum contained three peaks; those at 280 and 305 nm can be assigned to the mediator –an the HBT solution exhibited both-; on the other hand, the signals between 240 and 300 nm can be assigned to dissolved lignin, degradation products and mediator oxidized forms -in fact, the signal decreased with decreasing laccase dose and time. The absorbance signals obtained in the treatments using an HBT dose of 2 %odp were higher than those provided by lower mediator doses. The peaks at 280 and 305 nm were always lower than those exhibited by the HBT solution. Based on these two signals, the laccase-mediator treatment reduced the concentration of HBT.

Fig. 8 shows the spectra from effluents of L treatments at 2 %odp of HBT and different doses of laccases and reaction time. The signal at 305 nm was higher with a low laccase dose and a short treatment time. On equal times, a high laccase dose (20 $U \cdot g^{-1}$) resulted in decreased absorbance values. Therefore, increasing the laccase dose reduced the concentration of HBT, possibly through more marked oxidation of the mediator by the enzyme. On the other hand, the signals between 240 and 290 nm were stronger with a high enzyme dose; this may have been the result of an increased concentration of lignin, and HBT degradation and/or oxidation products. With a laccase dose of 20 $U \cdot g^{-1}$, the signal at 305 nm peaked at a treatment time of 0.5 h; therefore, increasing the time to 6.5 h might reduced the concentration of HBT in the system. The signals

between 240 and 290 nm were stronger in the long treatment (6.5 h); therefore, prolonging the treatment increased the concentrations of HBT degradation and/or oxidation products.

Acknowledgements

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References

[1] P. Bajpai, P.K. Bajpai, Biotechnology in the pulp and paper industry, Pira International, Leatherhead, Sussex, UK, 1998.

[2] M.C. Barascud, J.C. Jacquart, S. Nivelon, M. Pichon, Objectif zero rejet, Revue ATIP 49 (1995) 88-92.

[3] W.R. Kenealy, T.W. Jeffries, Enzyme processes for pulp and paper: A review of recent developments. In Wood deterioration and preservation: Advances in our changing world. Ed. Goodell, B., Nicholas, D. D. and Schultz, T. P. American Chemical Society. 2003, 210-239.

[4] D.H. Cates, C. Eggert, V. Ynag, K.-E. Eriksson, Comparison of effluents from TCF and ECF bleaching of kraft pulps, Tappi J. 78 (1995) 93-98.

[5] N.G. Johansson, F.M. Clark, Developing technologies open door to future closure of bleach plant, Pulp and Pap. 69 (1999) 71-75.

[6] M.R. da Silva, A.C. de Brito, J.L. Colodette, A seqüencia de branqueamento ideal para um processo em circuito fechado, O Papel. 3 (1997) 35-45.

[7] P. Bajpai, Application of enzymes in the pulp and paper industry, Application of enzymes in the pulp and paper industry, Biotechnol. Prog. 15 (1999) 147-157.

359 [8] R. Bourbonnais, M.G. Paice, B. Freiermuth, E. Bodie, S. Borneman, Reactivities of various mediators and
360 laccases with kraft pulp and lignin model compounds, Appl. and Environ. Microbiol. 63 (1997) 4627-4632.

361 [9] F.S. Chakar, A. Ragauskas, The kismet of residual lignins during LMS delignification of high-kappa kraft
362 pulps, Holzforschung. 54 (2000) 647-653.

363 [10] X. Geng, K. Li, F. Xu, Investigation of hydroxamic acids as laccase-mediators for pulp bleaching, Appl.
364 Microbiol. and Biotechnol. 64 (2004) 493-496.

365 [11] F. Xu, J.J. Kulys, K. Duke, K. Li, K. Krikstopaitis, H.-J. Deussen, E. Abbate, V. Galinyte, V, P.
366 Scheneider, Redox chemistry in laccase-catalyzed oxidation of N-hydroxy compunds, Appl. and Environ.
367 Microbiol. 66 (2000) 2052-2056.

368 [12] M. Amann, A. Candussio, R. Müller, V. Frey, In situ generation of mediator - a new concept for laccase-
369 catalyzed delignification of kraft pulp, in: TAPPI Pulping Process and Product Quality Conference, 2000.

370 [13] C. Johannes, A. Majcherczyk, Natural mediator in the oxidation of polycyclic aromatic hydrocarbons by
371 laccase mediator systems, Appl. and Environ. Microbiol. 66 (2000) 524-528.

372 [14] S. Camarero, D. Ibarra, M.J. Martínez, J. Romero, A. Gutiérrez, J.C. del Río, Paper pulp delignification
373 using laccase and naturals mediators, Enzyme Microb. Technol. 40 (2007) 1264-2171.

374 [15] D. Rochefort, D. Leech, R. Bourbonnais, Electron transfer mediator systems for bleaching of paper pulp,
375 Green Chem. 6 (2004) 14-24.

376 [16] A. Fillat, J. Colom, T. Vidal, Exploring enzyme stability during delignification of flax pulp by laccase and
377 natural mediators, in: 10th International Congress on Biotechnology in the Pulp and Paper Industry.
378 Integrating biology with processes. Abstracts, 2007, pp. 86-87.

379 [17] D. Ibarra, J. Romero, M.J. Martínez, J. Martínez, S. Camarero, Exploring the enzymatic parameters for
380 optimal delignification of eucalypt pulp by laccase-mediator, Enzyme Microb.Technol. 39 (2006) 1319-1327.

381 [18] S. Camarero, O. García, T. Vidal, J. Colom, J.C. del Río, A. Gutiérrez, J.M. Gras, R. Monje, M.J.
382 Martínez, A. T. Martínez, Efficient bleaching of non-wood high-quality paper pulp using laccase-mediator
383 system, Enzyme Microb. Technol. 34 (2004) 113-120.

384 [19] S. Camarero, O. García, T. Vidal, J. Colom, J.C. del Río, A. Gutiérrez, Flax pulp bleaching and residual
385 lignin modification by laccase-mediator systems, Biotechnology in the Pulp and Paper Industry 21 (2002)
386 213-221.

387 [20] J. Colom, O. García, S. Camarero, U. Fillat, A.T. Martínez, M.B. Roncero, A.L. Torres, M.J. Martínez,
388 M.J. Gras, T. Vidal, Application of enzymes in the bleaching processes of high quality pulps, in: COST E-23
389 Action on: Biotechnology for Pulp and Paper Industry. 2005 Conference for Biotechnology for Pulp and
390 Paper Manufacture: From Tailor-Made Biocatalysts to Mill Application. Abstracts, 2005, pp. 49-50.

391 [21] U. Fillat, O. García, A.L. Torres, T. Vidal, S. Camarero, J. Colom, A.T. Martínez, J.M. Gras, M.B.
392 Roncero, Is the laccase mediator system comparable to the ozone bleaching?, in: 2005 International Pulp
393 Bleaching Conference. SPCI A: Proceedings. 2005, pp. 233-235.

394 [22] O. García, S. Camarero, J.F. Colom, A.T. Martínez, M.J. Martínez, R. Monje, T. Vidal, Optimization of a
395 laccase-mediator stage for TCF bleaching of flax pulp, Holzforschung 57 (2003) 513-519.

396 [23] U. Fillat, M. B. Roncero, Effect of process parameters in Laccase Mediator System delignification of flax
397 pulp. Part I. Pulp properties, Chem. Eng. J., submitted 2009.

398 [24] M.I. Amann, The Lignozym(R) process coming closer to the mill, in: SWPC - 9Th International
399 Symposium on Wood and Pulping Chemistry - Oral Presentations, 1997, pp. F41-F45.

400 [25] K. Li, F. Xu, K.-E. Eriksson, Comparison of fungal laccases and redox mediators in oxidation of a
401 nonphenolic lignin model compound, Appl. Environ. Microbiol. 65 (1999) 2654-2660.

402 [26] E. Correia, B.J. Treves, R.J. Snook, Water toxicity monitoring using Vibrio fischeri: a method free of
403 interferences from color and turbidity, J. Environ. Monitor. 6 (2004) 97-102.

404 **FIGURE CAPTIONS**

405 Figure 1. Probability graphs of the model for COD in the L stage. (a) Semi-normal graph of the saturated
406 model. (b) Residuals vs. Z in the normal law.

407 Figure 2. Probability graphs for the color model in the L stage. (a) Semi-normal graph of the saturated model.
408 (b) Residuals vs. Z in the normal law.

409 Figure 3. Probability graphs for the residual enzymatic activity in the L stage. (a) Semi-normal graph of the
410 saturated model. (b) Residuals vs. Z in the normal law.

411 Figure 4. Effluent properties, COD (a) and color (b), as a function of the variables of the statistical plan for
412 the L stage.

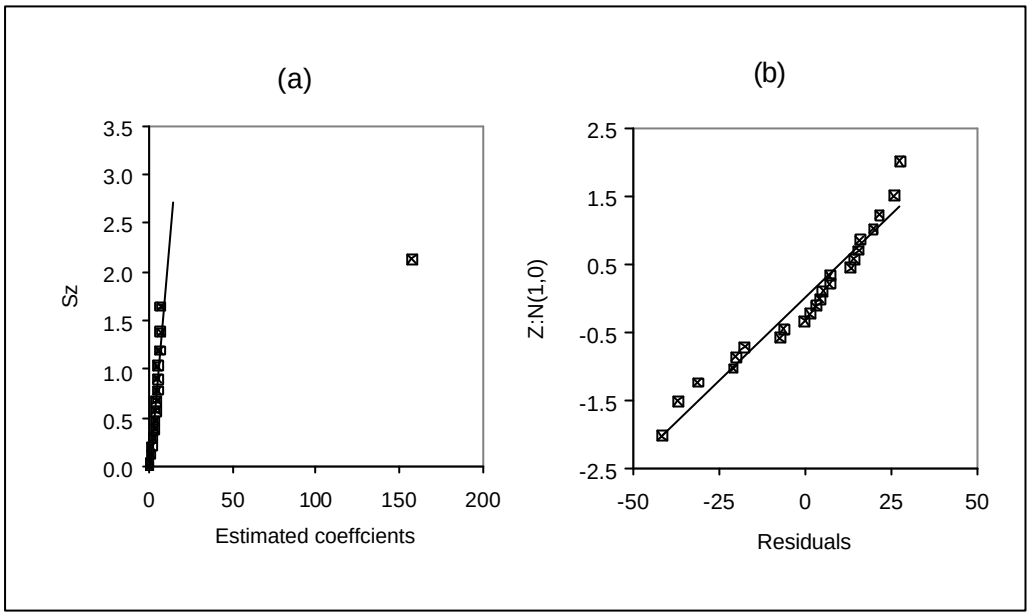
413 Figure 5. Relationship between kappa number and effluents color in L stage.

414 Figure 6. Residual activity as a function of the variables of the statistical plan for the L stage.

415 Figure 7. Spectra for an HBT solution, a laccase solution and the effluents (following 1:20 dilution) from the L
416 stage. Control curve: spectra from effluent from control treatment (without enzyme and mediator). HBT
417 curve: spectra from HBT solution (2 %odp, 3 % consistency). Laccase curve: spectra from laccase solution
418 (20 $U \cdot g^{-1}$). L curves: spectra from L treatments at 0.1 %, 1 % or 2 % HBT.

419 Figure 8. Spectra for an HBT solution and the effluents (following 1:20 dilution) from the L stage at an HBT
420 dose of 2 %odp, and different laccase doses (1 or 20 $U \cdot g^{-1}$) or reaction time (0.5 or 6.5 h).

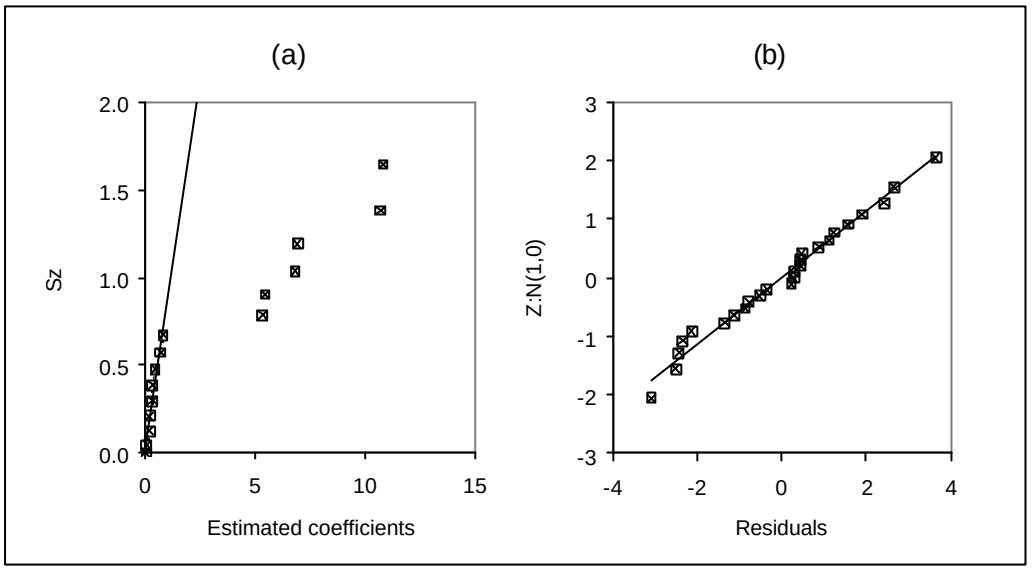
421 **FIGURES**



422

423 Figure 1.

424



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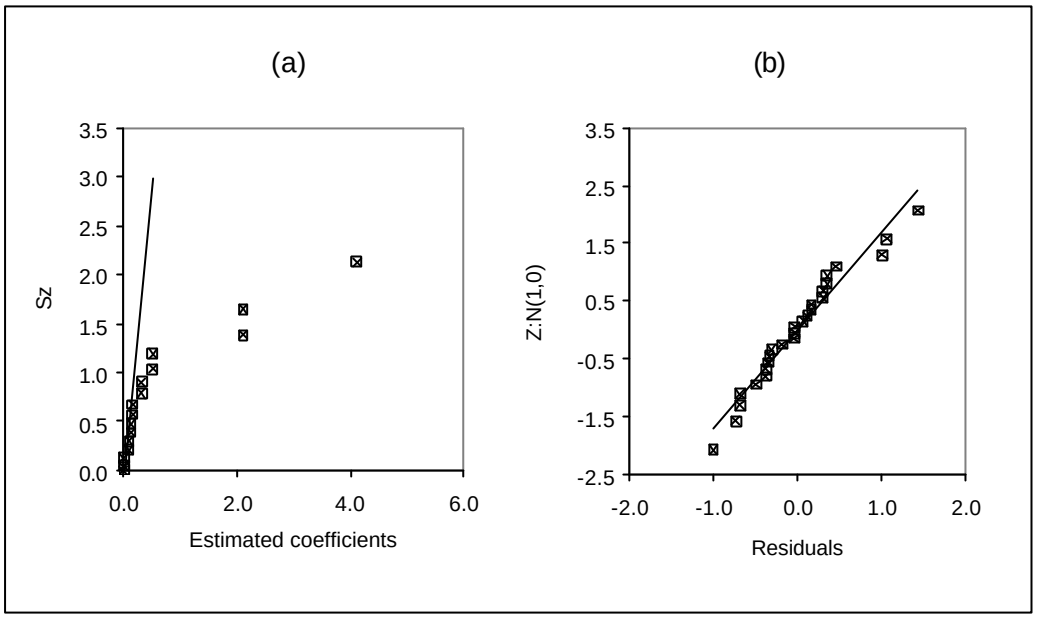
(a)
(b)
Sz
Estimated coefficients
Z:N(1,0)
Residuals
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.5
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-2.0
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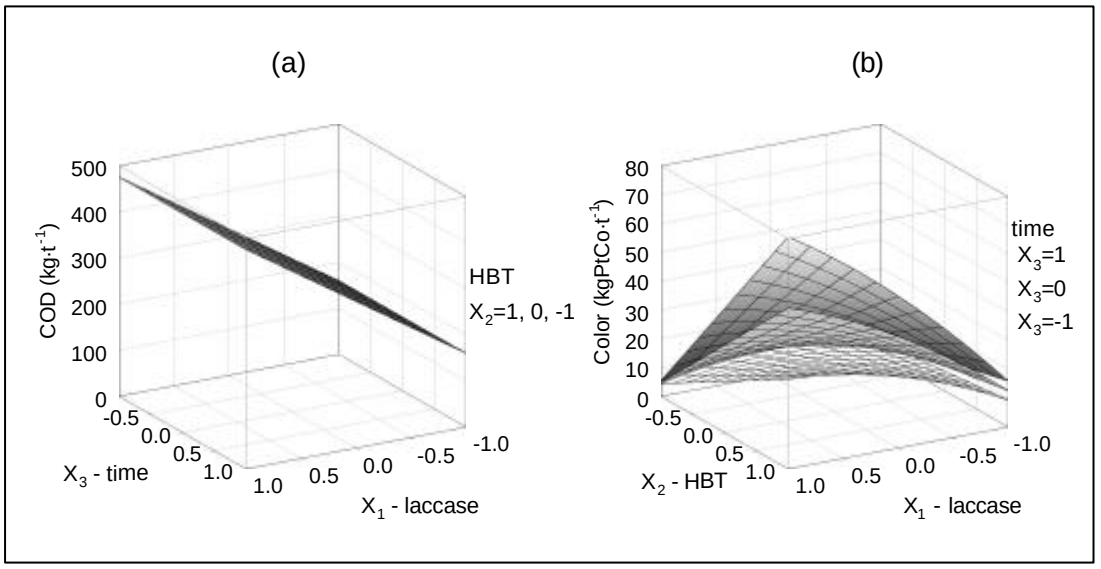
0.0
1.0
2.0
4.0
6.0

Figure 3.

430



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432 Figure 4.

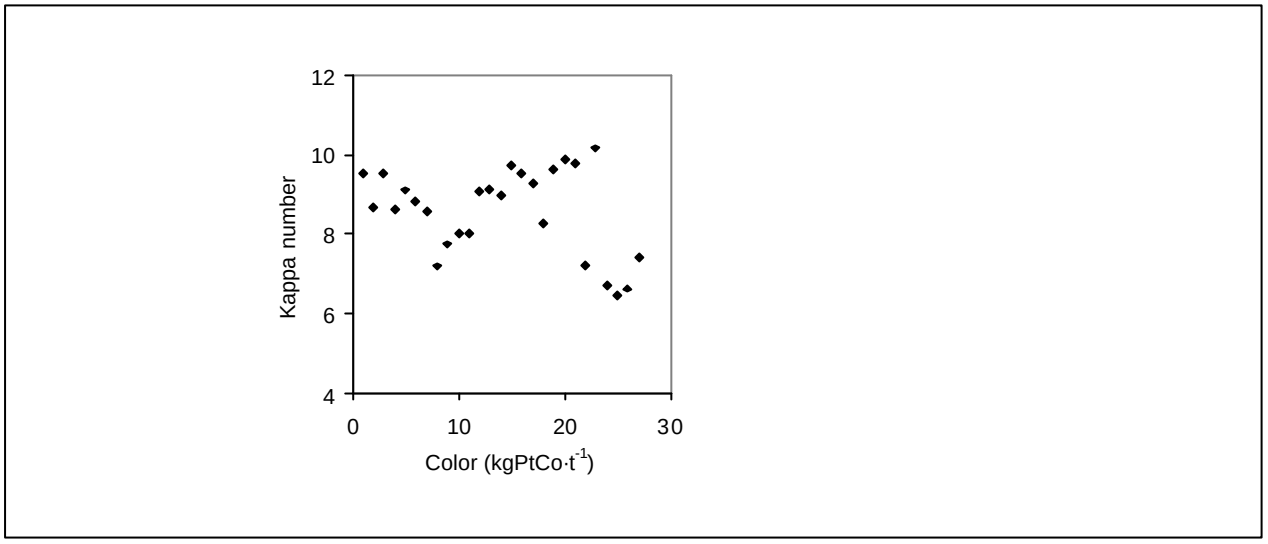
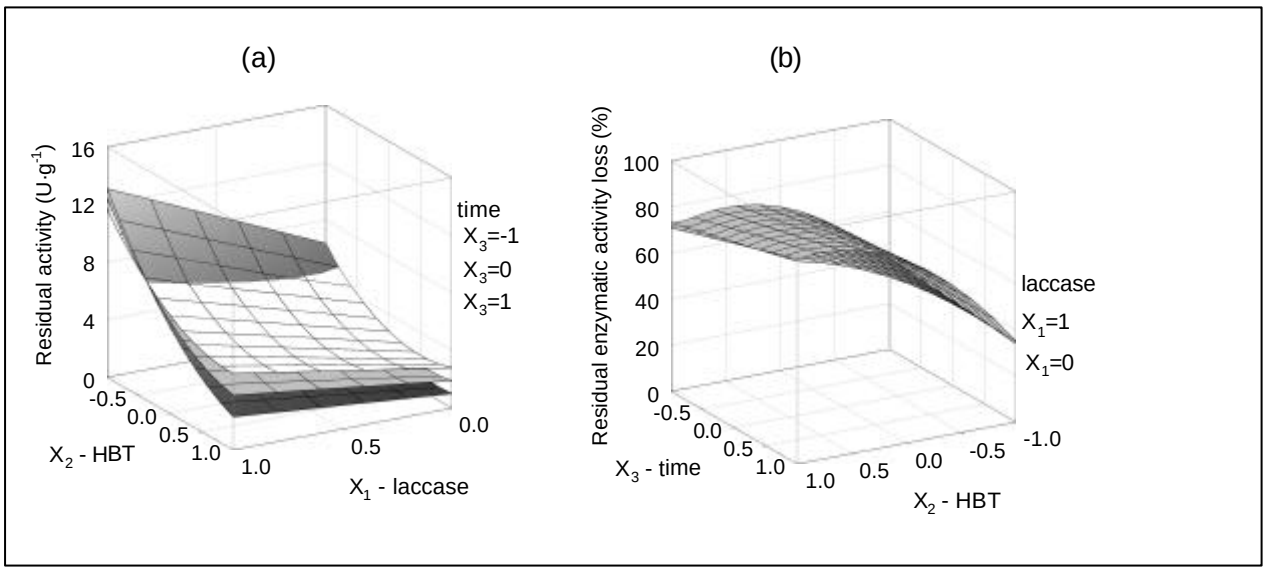


Figure 5.

436



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438 Figure 6.

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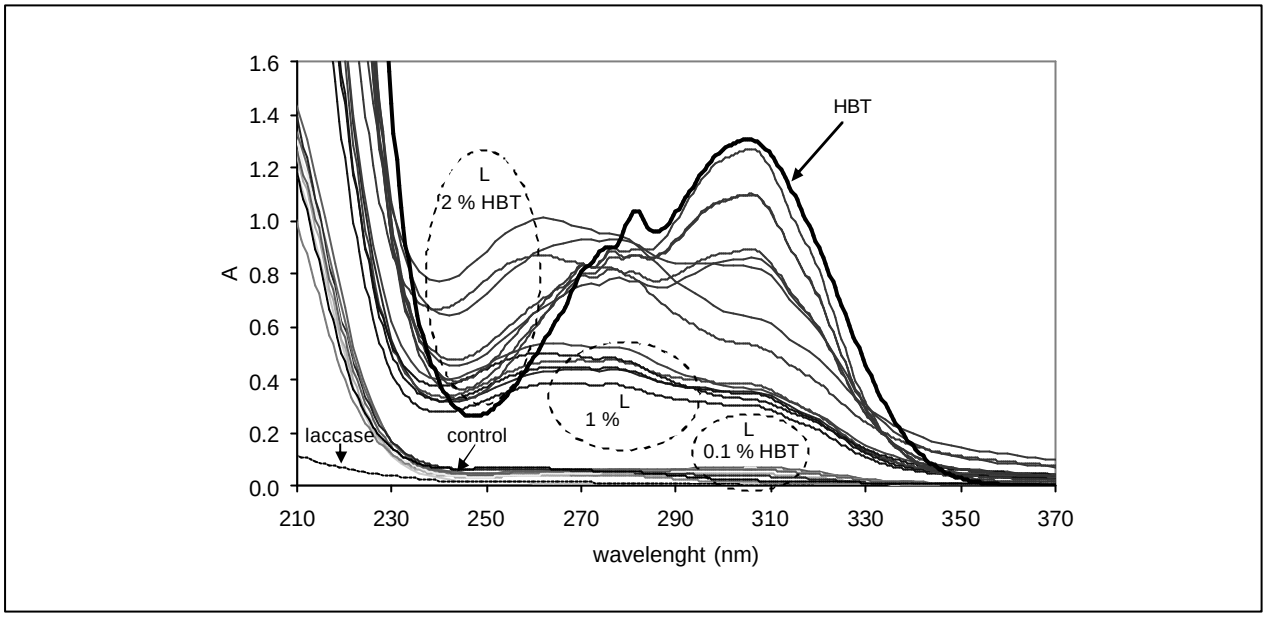

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441 Figure 7.

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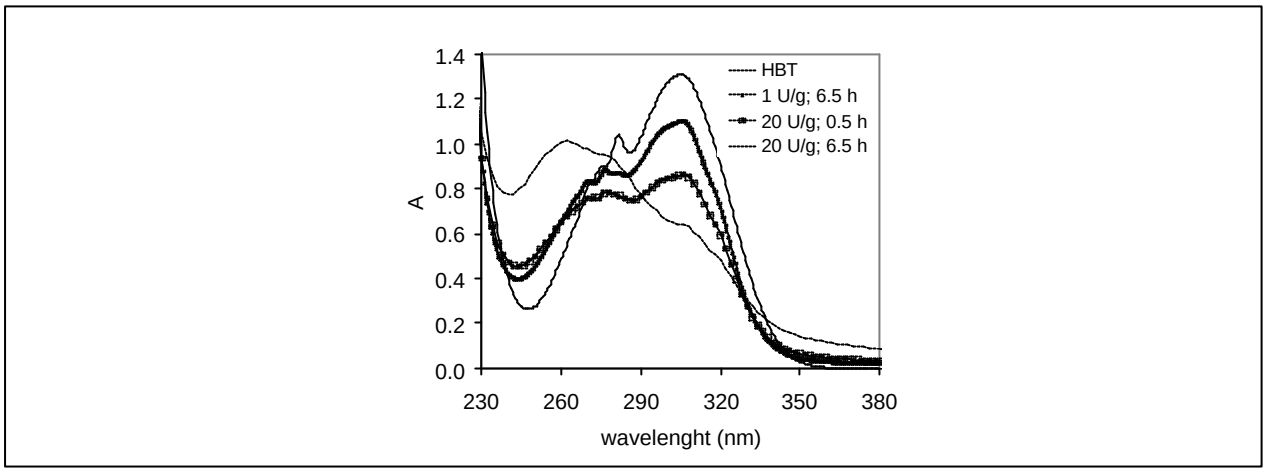

443

444 Figure 8.

445 **TABLES**

446 Table 1. Normalized values of the process variables in L stage.

447

		Normalized values		
Variables	**Factors**	**-1**	**0**	**1**
Laccase dose ($U_{lac}g^{-1}$)	x_1	1	10.5	20
HBT dose (%odp)	x_2	0.1	1.05	2
Time (h)	x_3	0.5	3.5	6.5
Oxygen pressure (MPa)	x_4	0.2	0.4	0.6

448

449 Table 2. COD, color and residual enzymatic activity of effluents after L stage.

x_1	x_2	x_3	x_4	Ref.	COD ($kg \cdot t^{-1}$)	Color ($kgPtCo \cdot t^{-1}$)	Residual enzymatic activity ($U \cdot g^{-1}$)
-1	-1	-1	-1	L 1	180	1	0
1	-1	-1	-1	L 2	484	1	12.3
-1	1	-1	-1	L 3	154	7	0
1	1	-1	-1	L 4	482	27	6.4
-1	-1	1	-1	L 5	129	0	0
1	-1	1	-1	L 6	456	1	12.4
-1	1	1	-1	L 7	153	10	0
1	1	1	-1	L 8	490	74	2.5
-1	-1	-1	1	L 9	140	1	0
1	-1	-1	1	L 10	480	1	11.8
-1	1	-1	1	L 11	176	5	0
1	1	-1	1	L 12	484	28	4.9
-1	-1	1	1	L 13	186	2	0
1	-1	1	1	L 14	440	2	13.2
-1	1	1	1	L 15	160	14	0
1	1	1	1	L 16	481	79	2.2
0	0	0	0	L 17	340	20	2.4
0	0	0	0	L 18	301	19	2.1
0	0	0	0	L 19	277	18	2.4
1	0	0	0	L 20	491	28	4.9
0	1	0	0	L 21	346	-	-
0	0	1	0	L 22	320	30	1.7
0	0	0	-1	L 23	334	24	2.6
0	-1	0	0	L 24	-	3	7.4
0	0	-1	0	L 25	-	11	4.3
0	0	0	1	L 26	-	23	2.6

450

451

452 Table 3. Coefficients of the COD model (Eq. 3) in the L stage.

Coefficients	Estimated coefficients	Standard error	t value	Significance
b_0	319	4.22	75.4	$4.7 \cdot 10^{-27}$
b_1	158	4.91	32.2	$2.3 \cdot 10^{-19}$

453

454

455 Table 4. Coefficients of the final color model (Eq. 5) in L stage.

Coefficients	Estimated coefficients	Standard error	t value	Significance
b_0	20.4	0.76	26.8	0.00
b_1	10.9	0.52	20.9	0.00
b_2	14.8	0.52	28.5	0.00
b_3	7.2	0.50	14.3	0.00
b_{12}	10.7	0.54	20.0	0.00
b_{13}	5.4	0.54	10.2	0.00
b_{23}	6.8	0.54	12.7	0.00
b_{123}	5.3	0.54	9.9	0.00
b_{11}	-4.5	0.92	-4.9	0.00

456

457

458 Table 5. Coefficients of the final model (Eq. 7) for enzymatic residual activity in the L stage.

Coefficients	Estimated coefficients	Standard error	t value	Significance
b_0	2.4	0.24	10.1	$7.7 \cdot 10^{-9}$
b_1	4.0	0.17	24.4	$3.0 \cdot 10^{-15}$
b_2	-2.2	0.16	-13.7	$5.7 \cdot 10^{-11}$
b_3	-0.4	0.16	-2.7	$1.6 \cdot 10E^{-2}$
b_{12}	-2.1	0.17	-12.4	$2.9 \cdot 10^{-10}$
b_{23}	-0.5	0.17	-3.0	$8.0 \cdot 10^{-3}$
b_{123}	-0.6	0.17	-3.4	$2.9 \cdot 10^{-3}$
b_{22}	1.7	0.29	5.9	$1.4 \cdot 10^{-5}$

459

460

461 Table 6. Effluent properties of control treatment after L stage. Individual contribution of laccase and HBT.

Ref.	COD ($kg \cdot t^{-1}$)	Color ($kgPtCo \cdot t^{-1}$)	Toxicity ($equitox \cdot m^{-3}$)
Control	155	No significative	No detectable
Control - HBT (2 %odp)	14	No significative	3
Control - Laccase (20 $U \cdot g^{-1}$)	352	No significative	No detectable

462

463

464 Table 7. Toxicity of HBT alone, combination of HBT and laccase and the effluents from the L stage.

	x_1	x_2	x_3	x_4	Ref.	Toxicity (UT - equitox·m^3)
Reagents	-	1	-	-	HBT	3
	1	1	-	-	HBT + laccase	3
L stage	-	-	-	-	control	Not detectable
	-1	-1	-1	-1	L 1	Not detectable
	-1	1	1	-1	L 7	6
	0	0	0	0	L 18	8
	0	0	1	0	L 22	7
	1	0	0	0	L 20	9
	0	1	0	0	L 21	8
	1	1	1	-1	L 8	12
	1	1	-1	-1	L 4	12

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