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Biodegradation of Chlorolignin and Lignin-Like Compounds Contained in E_1-Pulp Bleaching Effluent by Fungal Treatment

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Abstract

The ligninolytic system from the fungi *Trametes villosa* and *Panus crinitus* can efficiently degrade all fractions of different molecular mass contained in E_1-bleaching effluent, but with different degradation rates. The lower-molecular-mass (MM) materials were better characterized when the elution in the size-exclusion high-performance liquid chromatography were monitored at 210 than at 280 nm, which indicates that these compounds may be ring cleavage byproducts from depolymerized chlorolignin. The biodegradation of E_1 effluent by both fungi was a multistage process, involving an initial chemical modification of the higher-MM compounds and concomitant oxidation of the lower-MM materials. A subsequent depolymerization of chemically modified polymeric lignin-like compounds also took place. Each stage may require one or several different enzymes. The results suggested that laccase was involved in the initial stage.

Index Entries: Effluent biodegradation; E_1-pulp bleaching effluent; basidiomycete fungi; laccase; ligninolytic enzymes; size-exclusion high-performance liquid chromatography; chlorolignin.

Introduction

In the conventional multistage bleaching process for pulp, the largest amount of material is dissolved from the pulp during stages C and E_1,

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which consist of a treatment of the pulp with elemental chlorine (C), and subsequent extraction of chlorinated pulp with alkali (E_1) *(1)*. The liquor from each of these stages contains a wide range of organic compounds that can be divided into several fractions of different molecular mass (MM) and chemical structures. From an environmental standpoint, the determination of the nature and amount of these organic materials as well as their resistance to biodegradation is of great interest. Previous results obtained, particularly with E_1 liquor, have shown that the major constituent fraction originates from residual pulp lignin *(2)*. In addition, about 95% of the organically bound chlorine is found in the higher-MM material, and this fraction contains increased acidic groups, lower content of aromatic nuclei, and lower relative MM distribution when compared to native lignin *(3)*. These lignin-derivative materials dissolved in E_1 liquor exhibit chromophoric structures that are responsible for the dark color of this liquor, which is of environmental importance since light-absorbing materials in receiving waters are a problem. Thus, lignin biodegradation studies, particularly by basidiomycetes, are important.

White-rot fungi degrade native wood lignin, industrially modified lignins such as kraft lignin, and chlorinated high-MM lignins from pulp bleaching *(4–6)*. Among the large number of enzymes produced by these organisms, lignin peroxidase (LiP), laccase (Lac), and manganese peroxidase (MnP) appear to be the most important enzymes involved in lignin biodegradation *(7–9)*. LiP and MnP are glycosylated, extracellular heme proteins that are secreted in several isoenzyme forms. Lac is also extracellular but is a copper-containing protein. Determining the role that each of the fungal enzymes plays in lignin biodegradation is an extremely difficult task. In particular, an opposite view between in vivo and in vitro conditions regarding the enzymatic mechanism of action has been pointed out *(10)*. These observed discrepancies can be owing either to methodological factors or to the complexity of factors involved in the regulation of the enzymes' production and ligninolytic activity. In addition, the interactions among the several enzyme activities, synergistic and/or antagonistic effects, have been poorly understood and only recently begun to be clarified *(11–15)*. Complete biodegradation (depolymerization and mineralization) of lignin by cell-free enzyme systems has not yet been demonstrated. An approach to study the relative importance for each enzyme involved in lignin biodegradation by fungi is an attempt to establish correlations between their level of the extracellular ligninolytic activities and the lignin biodegradation rates.

In previous studies, *Trametes villosa* and *Panus crinitus* were shown to promote extensive decolorization of E_1 effluent *(16)*, and the decolorization rate was faster than the uptake rate of total lignin-like compounds, which was measured by using two different methods. The ligninolytic enzyme patterns were quite similar. LiP and Lac were the major extracellular ligninolytic enzymes secreted by both fungi, and MnP extracellular activity was not detected under the conditions employed *(17)*. On the other hand,

these fungi exhibited differences in their individual behavior regarding their LiP:Lac activity ratio, β-glucosidase activity levels, overall peroxidatic activity levels, and the temporal profile of enzyme liberation into the extracellular medium. In these studies on the composition and fungal biodegradation of pulp bleaching effluent, ultraviolet (UV) spectroscopic methods *(16)* showed intensive intermediate carbonyl group formation during the initial stage (0–48 h) of the E_1 biodegradation process by the fungus *T. villosa* K10, monitored at 310 nm, and its subsequent disappearance with the concomitant increase in absorbance at 215–230 nm. This behavior was not observed for *Panus crinitus* M10.

The aim of the present study was to obtain a better understanding of the initial stage of the biodegradation process of E_1-pulp bleaching effluent, particularly of the chemical modifications of the highest-MM material by fungal treatment and its correlation with the decolorization process.

Materials and Methods

Fungal Strains

T. villosa K10 and *P. crinitus* M10 were obtained from the basidiomycetes culture collection maintained at the Mycological Laboratory, CCB, Universidade Federal de Santa Catarina, Brazil, and were donated by Dr. Clarice L. Leite. All strains were collected on Santa Catarina Island, Brazil.

Effluent

The effluent used represents the first alkaline extraction stage (E_1) (pH 11.0–13.0) from bleached kraft pulp (Eucalyptus). Samples were colected and stored at –4°C until use.

Culture Conditions

All culture strains were maintained on malt extract-agar medium. Biodegradation experiments were conducted using the submerged culture method, and no additional elements were added in the effluent. About 400 mg (wet wt) of mycelium (7- to 10-d old) was incubated with 125 mL of effluent (the initial absorption value was 0.80 at pH 7.6 at 465 nm) at pH 9.5 and 25°C under constant agitation.

Enzyme Assays

Enzyme activities were measured in the sample of effluent incubated with a fungal culture. LiP activity was measured by UV spectroscopy of the veratrylaldehyde formed by oxidation of veratryl alcohol *(19)*. Phenoloxidase activity (Lac and peroxidase) was measured by using syringaldazine as a substrate in the absence and presence of H_2O_2 *(20)*. β-Glucosidase activity was measured by using *p*-nitrophenyl-β-D-glucopyranoside as substrate *(21)*. MnP was measured by oxidizing phenol red in the presence of H_2O_2 and Mn(II) *(22)*.

Analytical Assays

The measurement of decolorization of both fungi-treated and untreated effluent (E_1) was carried out by monitoring of the absorbance at 465 nm at pH 7.6. The total phenol content of the effluent was measured by the standard APHA *(23)* and 4-aminoantipyrine (4-AAP) methods *(18)*. Fifty microliters of sample was mixed with 0.5 mL of 0.1 *M* glycine buffer, pH 9.7, containing 1% potassium ferricyanide. Then, 2.5 mL of 1% 4-AAP in 0.1 *M* glycine buffer, pH 9.7, was added to give a red color to the resulting solution, which was monitored at 505 nm. The protein content was determined spectrophotometrically at 595 nm with Coomassie blue *(24)*.

Molecular-Size Distribution Analysis

Size-exclusion semipreparative chromatography of treated and untreated E_1 effluent was performed by high-performance liquid chromatography (HPLC) using a Shimadzu model chromatograph, equipped with an SPD-10A UV-VIS detector, and monitored concomitantly at 210 and 280 nm. A size-exclusion Asahipak column was utilized and the eluent, H_2O/0.05 *M* NaOH, was run at a flow rate of 1.0 mL/min. The effluent sample was centrifuged at 12,300 g for 7 min prior to HPLC analysis. Blue dextran, bovine serum albumin, carbonic anhydrase, bradykinin, angiotensin, tyrosine, and dioxane lignin were used as MM markers. The fractions of different molecular mass contained in the effluent sample were classified as high MM (MM > 15,000), middle MM (15,000 > MM > 1000) and low MM (MM < 1000).

Results and Discussion

To obtain a broader understanding of the action of ligninolytic enzymes of basidiomycete fungi, we carried out a comparative study of the patterns of E_1-pulp bleaching effluent degradation with two different fungi—*T. villosa* and *P. crinitus*—in order to correlate their effluent degradation patterns to the expression of their ligninolytic system. Our purpose was not only to evaluate the pattern of degradation of the several MM fractions, particularly high-MM material contained in the E_1-pulp bleaching effluent, but also to verify the possible chemical changes in polymeric lignin-related materials prior to the depolymerization reaction. In this context, in addition to the decolorization measurement of E_1-pulp bleaching effluent monitored at 465 nm, studies of changes in the molecular-size distribution during the effluent incubation period with the fungi were conducted using size-exclusion HPLC, and the elution was concomitantly monitored at 210 and 280 nm. Significant differences could be verified in this case. It was observed that the two elution profiles at 210 and 280 nm were different (Fig. 1) and that the formation of lower-MM intermediate compounds during the biodegradation process could be better characterized at 210 than at 280 nm (Fig. 2B). The reason for this behavior is discussed in the next section. The E_1-bleaching effluent analyzed contained predomi-

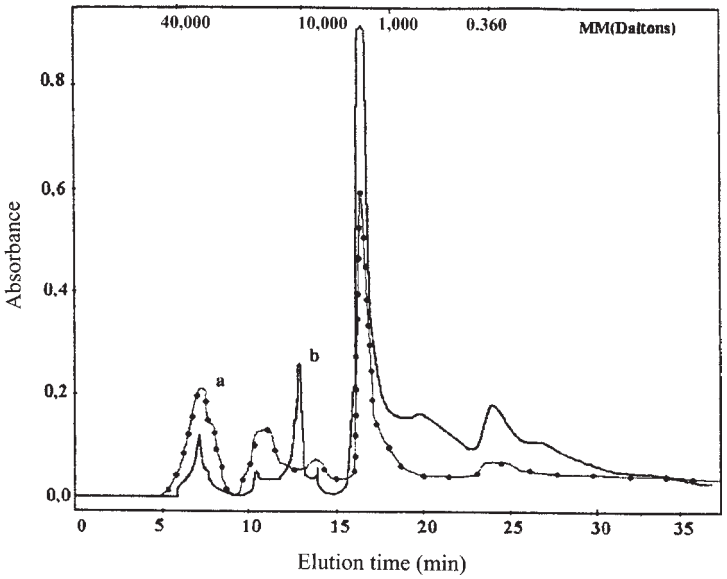

Fig. 1. Size-exclusion HPLC chromatograms of untreated E_1-pulp bleaching effluent obtained using aqueous 0.05 *N* NaOH as the eluent and 1.0 mL/min as the flow rate, monitored with a UV detector a, 280 nm; b, 210 nm.

nantly compounds that presented middle MM, 52.0% at 210 nm or 57.2% at 280 nm, as shown in Table 1, and low-MM compounds, 43.6%, if one takes into account the 210-nm data or 41.0% for the 280-nm data.

The area of each peak in the size-exclusion HPLC chromatograms (Figs. 1–3) was integrated, and the total area (sum of individual area of each peak) in the chromatograms was calculated, which represents the total absorbance of the analyzed effluent sample at 280 and/or 210 nm measured with an UV detector in the HPLC analysis. The relative content (in percentage) of each fraction (low, middle, and high MM) in Tables 1 and 2 was determined by the ratio between the individual area of each fraction and the total area.

Comparative Study Between T. villosa *and* P. crinitus

Changes in Molecular-Size Distribution

As can be seen from Figs. 2 and 3, the ligninolytic system from both *T. villosa* and *P. crinitus* can efficiently degrade all the fractions of different molecular masses. However, the individual degradation rate for each fraction was different. For *T. villosa*, the lower-MM material was biodegraded rapidly and intensively in the initial 48-h incubation stage, as shown in Table 1. The middle-MM fraction did not present the same behavior and its relative content increased. However, the total absorbance at 280 nm (sum of all fractions) decreased during this period. The increase in the relative content of middle-MM fraction (15,000 > MM > 1000) can be partially owing to the slower uptake of these fractions relative to the lower-MM (MM < 1000)

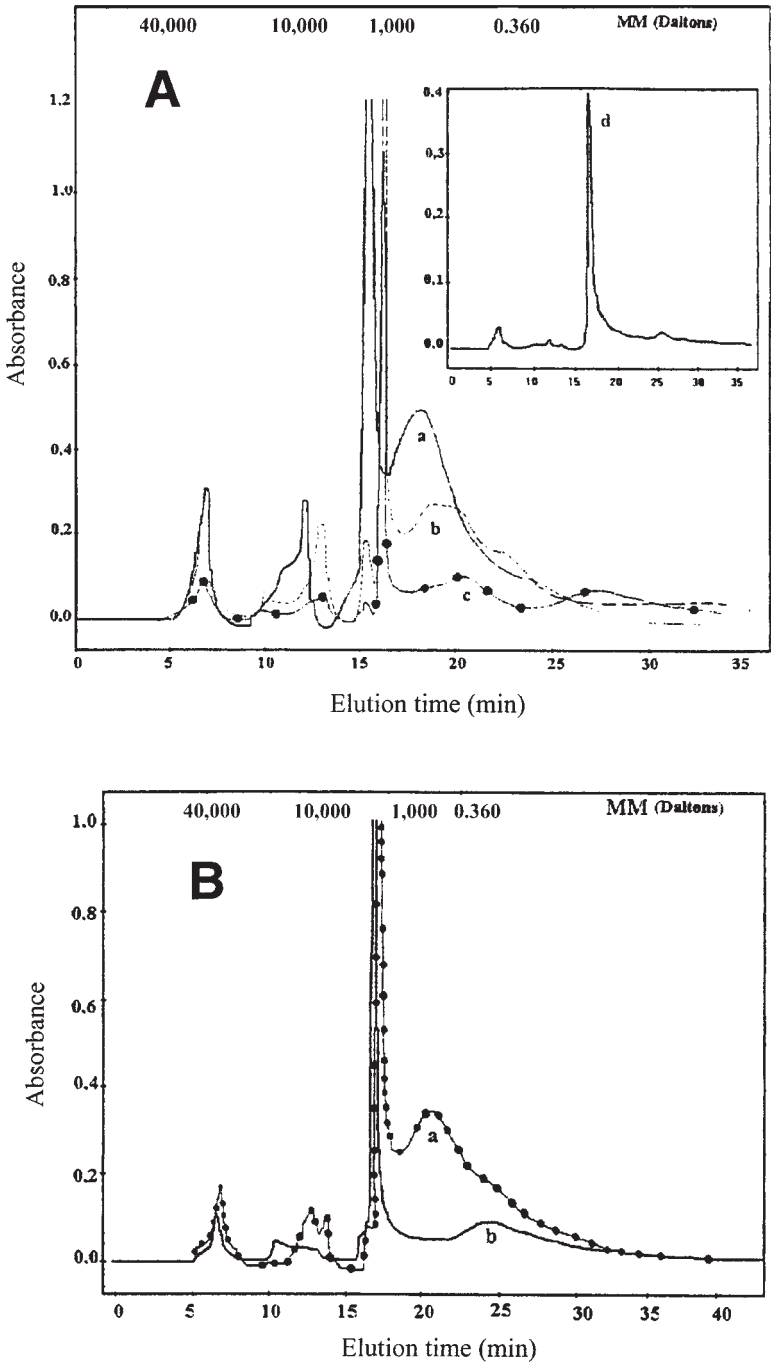

Fig. 2. Size-exclusion HPLC chromatograms of E_1-pulp bleaching effluent during the biodegradation process by *T. villosa* fungus obtained using aqueous 0.05 *N* NaOH as the eluent and 1.0 mL/min as the flow rate, monitored with a UV detector. **(A)** Incubation time: a, 12 h; b, 48 h; c, 120 h; d, 144 h (monitored at 210 nm. **(B)** Comparison between the molecular-size distribution curves obtained at different λ: a, 210 nm; b, 280 nm (incubation time of 24 h).

Table 1
Changes in Relative MM Distribution During E_1-Pulp Bleaching Effluent Biodegradation Process by Fungal Treatment with *T. villosa*, Performed by Size-Exclusion HPLC Using a UV Detector at 210 and 280 nm

	Relative content of fraction (%)								
	Incubation time (h)[b]								
Fraction[a]	λ (nm)	Ef	A_1/A_2	24	A_1/A_2	48	A_1/A_2	72	A_1/A_2
Σ fraction	210	4.0	0.09	10.7	0.72	8.5	0.52	10.5	0.76
Higher-MM	280	41.0		14.7		16.1		13.8	
Σ fraction	210	52.0	0.90	61.7	0.72	32.1	0.38	41.8	0.58
Middle-MM	280	57.2		84.9		83.1		71.9	
Σ fraction	210	43.6	24.22	27.5	152.72	59.4	98.9	47.6	3.25
Lower-MM	280	1.8		0.18		0.6		14.2	

[a]Σ fraction is the overall area obtained by addition of each fraction, i.e., $\Sigma\,\text{lower} - \text{MM} = L_1 + L_2$
[b]EF, untreated effluent; A_1, $A_{210\,nm}$; A_2, $A_{280\,nm}$

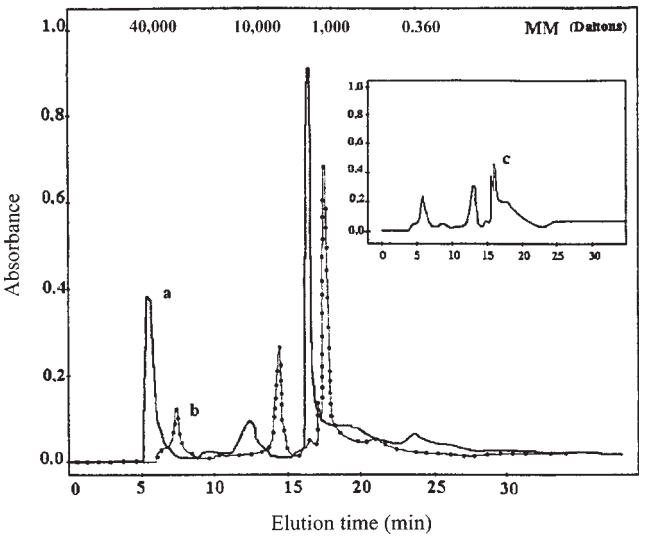

Fig. 3. Size-exclusion HPLC chromatograms of E_1-pulp bleaching effluent during the biodegradation process by *P. crinitus* fungus obtained using aqueous 0.05 *N* NaOH as the eluent and 1.0 mL/min as the flow rate, monitored with a UV detector at 210 nm. Incubation time: a, 24 h; b, 48 h; c, 144 h.

Table 2
Changes in MM Distribution During E_1-Pulp Bleaching Effluent Biodegradation Process by Fungal Treatment with *P. crinitus*, Performed by Size-Exclusion HPLC Using a UV Detector at 210 nm

	Relative content of fraction (%)			
	Incubation time (h)			
Fraction	EF	24	48	72
Higher-MM	4.0	21.9	9.4	13.80
Middle-MM	52.0	44.6	89.6	77.89
Lower-MM	43.6	33.8	0.3	7.80

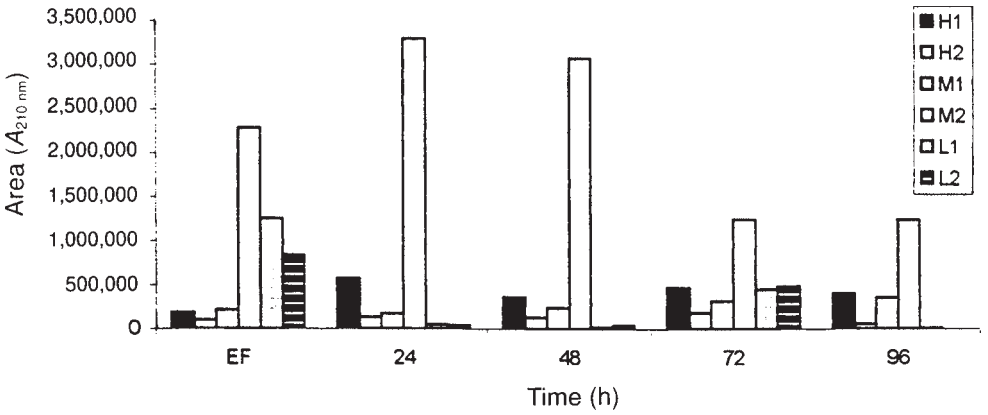

Fig. 4. Molecular-size distribution during the E_1-pulp bleaching effluent biodegradation process by *T. villosa*, showing the evolution of the individual area of each fraction in the chromatograms, performed by size-exclusion HPLC, eluted with aqueous 0.05 *N* NaOH and monitored at 210 nm. H_1 and H_2, high-MM fractions; M_1 and M_2, middle-MM fractions; L_1 and L_2, low-MM fractions; EF, original effluent (untreated by fungus) or time = zero.

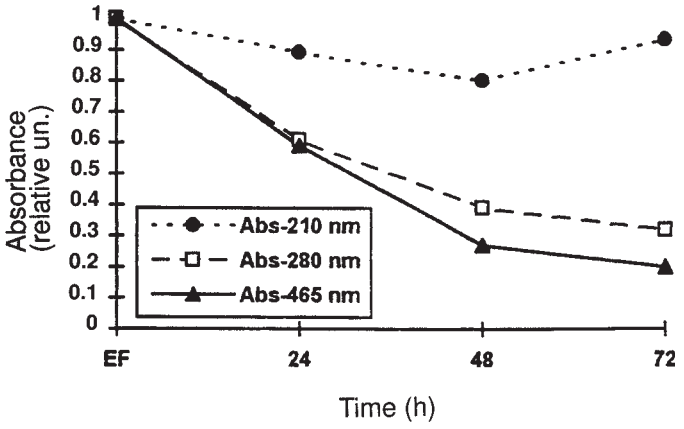

Fig. 5. Time course of E_1-pulp bleaching effluent biodegradation by *T. villosa*, monitored at different absorbance (Abs) λ; EF, original effluent (untreated by fungus) or time = zero; un, unit.

and higher-MM (MM > 15,000) fractions. The *P. crinitus* biodegradation rate of the lower-MM material was slower than that of *T. villosa* in the initial phase of the biodegradation process and increased in the subsequent period. The disappearance of the lower-MM compounds in the biodegradation process by both fungi may be owing either to their complete oxidation or, alternatively, to polymerization reactions. In the *T. villosa* case (Figs. 2 and 4), although undesirable intermediary polymerization reaction could be observed, the former reaction seems to be the major process, since all fractions presented decreased areas at the 72-h incubation period (Fig. 4) when monitored at either 210 or 280 nm (Fig. 5). However, as clearly indi-

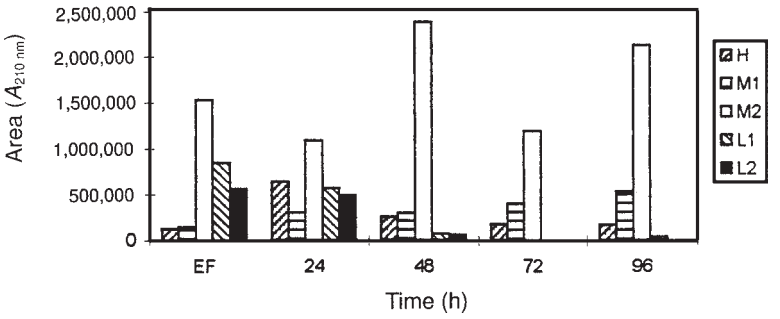

Fig. 6. Molecular-size distribution during the E_1-pulp bleaching effluent biodegradation process by *P. crinitus*, showing the evolution of the individual area of each fraction in the chromatograms, performed by size-exclusion HPLC, eluted with aqueous 0.05 *N* NaOH and monitored at 210 nm. H, high-MM fraction; M_1 and M_2, middle-MM fractions; L_1 and L_2, low-MM fractions; EF, original effluent (untreated by fungus) or time = zero.

cated by the molecular-size distribution curves (Figs. 3 and Fig. 6), the fungus *P. crinitus* catalyzed the polymerization of lignin-like compounds of low-MM in the initial incubation stage particularly at 24 h. In the subsequent stage, the depolymerization reaction was the preferential mechanism with this fungus. Therefore, the differences between the respective biodegradation patterns, as clearly shown by the molecular-size distribution curves (Figs. 2–6), seem to indicate that both the depolymerizing and polymerizing abilities for each fungus were dependent on incubation time, which varies from fungus to fungus. Note that H_1 and M_1 fractions in Figs. 4 and 6 may contain some contaminant compounds released into the medium by the fungi. However, they should not represent >10% of the area of each fraction.

Correlations Between Enzymatic Activities and Changes in Molecular-Size Distribution

The data in Table 3 indicate that both major enzymes, LiP and Lac, detected in the effluent medium incubated with these fungi, may catalyze polymerizing or depolymerizing reactions. In the LiP case, data have been reported where both depolymerizing and polymerizing capabilities have been found *(7,8,25,26)*. In addition, Lac has been referred to as an enzyme in which the polymerizing ability is predominant in an isolated system, not when fungi is present. There is agreement that the catalytic mode of action of peroxidases or phenoloxidases, such as LiP and Lac, is via initial formation of a cation radical or phenoxy radical intermediates, which can undergo many subsequent reactions, including either their oxidative degradation or coupling to produce polymeric materials *(27)*. Obviously, both processes compete and therefore the conditions that favor the depolymerizing pathway must inhibit polymerization. Our present results indicate that the depolymerization occurred to a greater extent in the *T. villosa* and *P. crinitus* cases, even though Lac activity is the major

Table 3
Summary of Changes in E_1-Pulp Bleaching Effluent Characteristics During Fungal Treatment

	Enzyme activities					Chemical parameter		
Incubation time (h)	Lac (U/L)	Peroxidase (U/L)	β Glucosidase (U/L)	LiP (U/L)	MnP (U/L)	Total protein (mg/L)	Residual phenol (%)	Residual color (%)
Biodegradation by *P. crinitus*								
24	235.3	17.9	17.8	21.2	0.0	12.3	87	76
48	276.9	23.9	24.8	28.1	0.0	14.1	91	48
72	239.9	23.0	28.5	32.5	0.0	27.2	77	29
144	230.0	21.2	31.0	29.3	0.0	31.6	65	28
Biodegradation by *T. villosa*								
24	295.3	18.4	35.0	19.2	0.0	21.2	86	59
48	295.2	15.6	32.3	26.3	0.0	23.4	62	27
72	239.9	4.6	14.0	21.2	0.0	29.9	47	20
144	147.6	5.0	0.8	20.8	0.0	21.6	41	19

enzymatic activity observed for both fungi. The kinetics of the depolymerizing ability of *T. villosa* was different from that of *P. crinitus*. This may be an important factor in elucidating the role of enzyme in this process. In this regard, among the hypotheses proposed for other fungal systems, three in particular seem more conceivable to us based on the following considerations:

1. The interactions of the phenoloxidase system with other enzymatic systems, such as oxalate oxidase/oxalate *(28,29)*, can be operative. This postulate is justified by the experimental observations by HPLC analysis (data not shown) that the fungus *T. villosa* secretes organic acids, particularly oxalic acid, into the medium, which results in a marked decrease in the pH of the medium. The same drastic effect on pH was not observed for *P. crinitus*. Oxalic acid has been considered an inhibitor of LiP activity *(30)*.
2. The interactions of the phenoloxidase system with cellobiose: quinone reductase *(9)* and/or glucose oxidase enzymatic systems *(11)* can be operative. The *T. villosa* β-glucosidase activity levels are higher than the respective *P. crinitus* levels, which may indicate the possible involvement of carbohydrates in the E_1-effluent biodegradation process. The ability of these carbohydrate:reductase enzymatic systems to reduce quinone and phenoxy radicals has been suggested to favor the depolymerization during the ligninolytic process *(9)*.
3. Concentrations of H_2O_2, as low as 10^{-6} *M*, and the presence of veratryl alcohol as an intermediate in the reaction medium can be found with *T. villosa* (data not shown). These conditions have also been mentioned as favoring depolymerizing conditions in the LiP-catalyzed reaction of synthetic lignin *(31)*.

Furthermore, the detailed analysis of Table 3 shows that in the initial stage of incubation, the LiP:Lac activity ratio of *P. crinitus*, which exhibited a higher polymerizing ability, was higher than the respective *T. villosa* ratio, whereas Lac, in quantitative terms, is the major enzyme detected for both fungi.

In addition, intermediate carbonyl group formation, in the *T. villosa* biodegradation process, exhibited a close temporal correlation to the evolution of the Lac activity level *(17)*. The formation of the α-carbonyl group during degradation of polymeric lignin in wood has been observed *(32,33)*, and several investigators have pointed out that the presence of this carbonyl group may favor the depolymerization process *(10)*.

Another interesting observation is the fact that the M_1-middle-MM fractions, which show a certain recalcitrance, were more effectively degraded by *P. crinitus* than *T. villosa* at the longer-term incubation time (14.4 h) (Fig. 2A, curve d; Fig. 3, curve c). This finding may be owing to the maintenance of the higher Lac activity level after 72-h of incubation by *P. crinitus*, as shown in the Table 3.

These results seem to indicate that for both fungi, Lac may play an important role. Although the depolymerizing ability of Lac has been questioned, earlier findings have demonstrated its possible role in lignin biodegradation. Some investigators *(34,35)* have pointed out that in the presence of a mediator such as 2,2-azinobis-3-ethylbenzenethiazoline-6-sulfonate, Lac can efficiently depolymerize lignin. In this case, it seems to us that the E_1-pulp bleaching effluent may contain some compound that may act as a mediator during effluent biodegradation. Progress is being made in the isolation and characterization of these metabolites.

Furthermore, the synergistic/antagonist effect between the enzymes can not be excluded. Our results show that slight differences in enzymatic composition or, more specifically, in the ratio of enzymatic activities can determine a different pattern of biodegradation, which was evident not only by the differences between the profiles of molecular-size distribution, but also by the analysis of the final products of the biodegradation process by gas chromatography-mass spectrometry methods (data not shown). In addition, the involvement of enzymes, other than the ones assayed, seems to be irrefutable.

Chemical Changes During Biodegradation Process

The data in Table 1 provide further evidence about changes among the different fractions (H-MM, M-MM, and L-MM) of E_1-pulp bleaching effluent during biodegradation by *T. villosa*. By comparing the percentage of each fraction obtained at 210 to the one at 280 nm, as shown in Table 1, we detected chemical changes owing to fungal treatment. We verified that the higher-MM fraction at all incubation times showed similar contents when the detection was performed at 210 to those obtained at 280 nm, whereas in the untreated E_1-pulp bleaching effluent, the difference between the respective contents was larger. These findings seem to indicate that structural modifications have taken place. By contrast, the middle-MM fraction exhibited a partially different behavior. In the untreated effluent sample, this fraction showed a similar value at 210 compared with 280 nm (52.0 and 57.2%, respectively), and then, during the first 48-h incubation period, the difference between the relative contents (percentage), increased slowly from 52.0/57.2 to 32.1/83.1. This fraction absorbed preferentially at 280 nm ($A_1/A_2 < 1$). However, at the 72-h incubation period, a difference was observed between the relative contents, 41.8 and 71.9%, at 210 and 280 nm, respectively. These results clearly indicate that changes in spectral characteristics of this fraction are related to structural changes in the molecules. One must pay attention to the fact that the change in the variation of the relative contents (from increasing to decreasing) at 48 h correlates with the intensive depolymerization reaction of this fraction, as shown in Fig. 4. The lower-MM fraction produced during the biodegradation process presented quite different absorption at 210 and 280 nm, during all incubation times and absorbed preferentially at 210 nm, with highly variable differences between the different wavelengths. This is expected

because of the great diversity of lower-MM compounds produced by the biodegradation process and to the rapid change in the relative composition of this MM fraction.

In our previous studies using UV spectroscopy *(16)*, we observed that several different MM fractions of the original (untreated) E_1-pulp bleaching effluent exhibited differences in the UV-spectral characteristics. The lower-MM fraction (MM < 1000), which is rich in acidic groups, presents higher absorptivity in the 210- to 230-nm region than the higher-MM fraction (MM > 15,000), which exhibits a higher absorptivity in the 280- and 310- to 370-nm regions owing to the higher content of aryl conjugated carbonyl groups. Based on these observations, several facts seem evident from the present findings. First, the lower-MM compounds produced in this case exhibited a higher absorptivity at 210 than at 280 nm, since the A_1:A_2 ratio was >1.0 (Table 1). This probably indicates these compounds are acid ring-cleavage byproducts from degradation of lignin-like polymeric compounds, particularly from the middle-MM fraction. Second, the middle-MM fraction, apparently, was the most chemically modified fraction by the enzymatic system of the fungus prior to depolymerization. As with the lower-MM fraction, the structural modifications shifted the absorption maximum from the 215- to 230-nm region to the 280-nm region (A_1:A_2 ratio change from 0.9 to 0.38), and since in our previous studies *(16)* the formation of aryl conjugated carbonyl (and/or carboxyl) groups appeared evident, we can conclude that ring cleavage as a C_α-oxidation reaction may have taken place in the polymeric form. In addition, the fact that the decolorization process (monitored at 465 nm, pH 7.6) was faster than uptake of total lignin-like compounds (monitored by the Folin method) and that about 70% loss of color occurred in the 0- to 48-h incubation period *(24)*, as shown in Table 3, seem also to confirm this postulate. Third, the higher-MM fraction was degraded at a slower rate than the lower-MM fraction. However, the more complex structural modification/molecular-size relationship makes the detection of chemical modifications by simple UV-spectroscopic methods difficult.

Another interesting finding was that the biodegradation or, more specifically, the decolorization process, promoted concomitantly the destruction of the UV chromophores contained in E_1-pulp bleaching effluent together with the visible-chromophore destruction, since the decrease in total absorbance monitored at 465 nm correlated with that at 280 nm (Fig. 5).

Conclusion

The biodegradation of E_1-pulp bleaching effluent by *T. villosa* and *P. crinitus* is a multistage process that seems to involve the initial chemical modification of the higher-MM material contained in the E_1-pulp bleaching effluent and concomitant oxidation of the lower-MM material. Subsequent depolymerization of chemically modified polymeric lignin-like material

must take place. Each stage may require one or several different enzymes. The decolorization process seems to be better correlated with the initial chemical modification observed, and Lac may be involved in this initial stage.

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