

Dechlorination of high-molecular-weight chlorolignin by the white-rot fungus *P. chrysosporium*

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ABSTRACT *Dechlorination of chlorolignin in pulp bleaching wastewater was studied using the white-rot fungus P. chrysosporium immobilized on a rotating biological contactor. The total organic chlorine content of chlorolignin decreased almost by 50% during one day of treatment. The corresponding amount of inorganic chloride was liberated into the solution. Correlation studies suggested that dechlorination, decolorization, and degradation of chlorolignin (as COD decrease) are metabolically connected, although these processes have different rates. Size exclusion chromatography showed that polymerization took place in the early stage of the treatment. Low-molecular weight degradation products were not observed.*

KEYWORDS

Bleaching
Chemical analysis
Chlorolignins
Delignification
Effluent treatment
Environmental control
Polymerization

Most of the organic compounds in the wastewater of pulp mills producing bleached chemical pulp are chlorinated degradation products of lignin. During the chlorination stage—either C or (C+D)—the residual lignin is chlorinated and oxidized so it becomes soluble in alkali and can be extracted from the pulp in the subsequent alkali stage (E1). This chlorolignin is discharged with the other waste streams of the mill. Approximately 150,000 tons of chlorinated lignin derivatives, or about 15,000 tons of organic chlorine, are discharged annually in Finland (1) and 2.5×10^6 tons of derivatives, or about 250,000 tons of organic chlorine throughout the world (2).

Many organochlorine compounds, including some that are in the pulp mill effluents, have toxic effects on different forms of life (2). However, the fate and effects of high-molecular-weight chlorolignin (MW > 1000) in the environment are not known. The most common wastewater treatment processes used by the pulp and paper

industry—aerated lagoons and activated sludge plants—do not remove high-molecular-weight chlorolignin effectively, and the reduction of COD is low compared to the reduction of BOD (1). As measured by COD reduction, the methods that do remove chlorolignin—such as precipitation, ultraviolet irradiation, and ultrafiltration (3–5)—are not in common use, probably because of financial reasons or problems in depositing the concentrate.

White-rot fungi, like *P. chrysosporium*, can degrade lignin and chlorinated organic compounds (6–9). Ek and Eriksson (10) used fermentation with a white-rot fungus to remove chloroorganics from combined C- and E-stage effluents after oxygen prebleaching. Here, we report the ability of the white-rot fungus *P. chrysosporium* to dechlorinate and degrade high-molecular-weight chlorolignin in a rotating biological contactor.

Experimental procedures

Fungus and its culture conditions

P. chrysosporium Burds. (BKM F-1767) was cultured in Roux flasks on malt agar to obtain the conidial inocula. The concentration of the spore suspension was adjusted to give an absorbance of 0.5 at 650 nm, which corresponds to about 2.5×10^6 spores/mL. Typically, the spore suspension composed 5% of the total volume of the reactor, which was 2.1 L.

The fungus was allowed to grow for four days in solution in the rotating biological contactor before the effluent treatment experiments were started. This solution was replaced with fresh solution after 2 days' growth. It was composed of 890 mL of effluent, 60 mL of nutrient solution, 50 mL of spore suspension, and 10 g of glucose/L, at a pH of 4.5 at 39°C. The nutrient solution contained 33.33 g KH_2PO_4 , 8.33 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.67 g CaCl_2 , 1.93 g NH_4Cl , and 16.7 mg thiamine-HCl/L of distilled water.

During the decolorization stage, the

effluent solution contained: 820 mL effluent, 60 mL of the nutrient solution without NH_4Cl , 43.4 mg $(\text{NH}_4)_2\text{SO}_4$ (final NH_4 concentration 0.66 mM), 90 mL mineral solution, 0.84 g benzyl alcohol, and 1 g Tween 80 (11, 12). The mineral solution contained: 1.5 g nitrilotriacetic acid, 0.5 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.1 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g CoSO_4 , 0.1 g ZnSO_4 , 0.01 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.01 g $\text{AlK}(\text{SO}_4)_2$, 0.01 g H_3BO_3 , and 0.01 g NaMoO_4 per liter of distilled water. The initial pH was always adjusted with concentrated H_2SO_4 to 4.5; the reactors were maintained at 39°C and were flushed continuously with oxygen (about 0.2 L/min).

Effluent

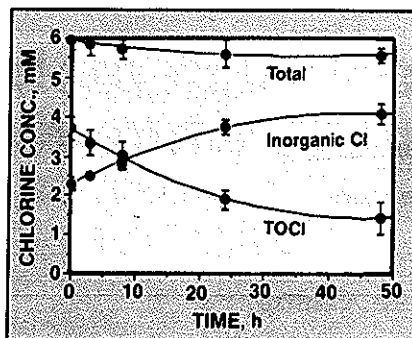
The effluent was collected at a commercial kraft pulp mill using the CEDED bleaching sequence. Only the first-alkali-stage effluent (E1) was used in the experiments. The effluent was ultrafiltered using a Proto-Sep III bench-scale unit and a spirally wound polysulfone membrane with a nominal cut-off at 3000 dalton (Koch Membrane Systems, Boston). The effluent was concentrated to 25% of its original volume.

The inorganic chloride was removed from the retentate by ultrafiltering it two more times after dilution with water back to the original volume. The dissolved solids were 9.48 g/L in the unfractionated effluent and 10.16 g/L in the retentate. The original effluent contained 43.0 mM of Cl^- and 7.50 mM of total organic chloride (TOCl), and the retentate contained 1.64 mM of Cl^- and 17.88 mM of TOCl. The retentate was diluted in all experiments with water at a ratio of 1:4 to obtain the same concentration of high-molecular-weight chlorolignin as in the original effluent.

Determination of TOCl

Total organic chlorine was determined by subtracting the amount of inorganic chloride from total chlorine, which was obtained using the Schöniger combustion method (13). For the Schöniger combustion, 2 mL of the effluent solution was applied in 4 or 5 portions on a piece of filter paper (4 × 4 cm; Whatman No. 41 ashless filter paper), which was dried at 120°C after each addition. After the final drying, the filter paper was

1. Dechlorination of chlorolignin by *P. chrysosporium*, where the horizontal bars show the standard deviation of four replicate incubations



burned in an oxygen atmosphere, and the liberated chloride was adsorbed into water (25 mL) over 2 h.

The chloride concentration of this solution and that of the original effluent sample was determined using a chloride selective electrode Model 94-17 (Orion Research, Boston). Separation of organic and inorganic chlorine was not necessary (14,15) since 95% of the inorganic chlorine was removed in the ultrafiltration. The method used for total chlorine was verified with freeze-dried, high-molecular-weight chlorolignin, for which the chlorine content was determined by neutron activation analysis.

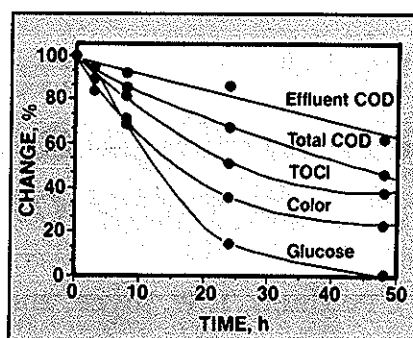
The recovery was 103.4% for a solid sample and 99.9% for a 5.00 mM solution. The coefficients of variation were 2.35 ($n = 6$) and 1.67 ($n = 5$), respectively.

Chromatography

Glucose concentration was determined with a Waters chromatography system, a Waters Carbohydrate column using 83% acetonitrile as the mobile phase, and a refractive index detector (Waters Associates, Milford, Mass.). Size-exclusion chromatography was performed with the same chromatograph equipped with a TSK G3000PW precolumn and a 30-cm analytical column (Toyo Soda Manufacturing Co., Tokyo).

A bicarbonate buffer was used as the mobile phase (16). UV-detection at 254 nm was used in this case. Poly (styrene sulfonate) standards were used to calibrate the column (Scientific Polymer Products, Ontario, N.Y.). Acetone was used as an internal standard and to check the total permeation volume (13.5 mL). The void volume was 6.1 mL (based on the

2. Kinetics of the studied parameters during the incubation of high-molecular weight chlorolignin



exclusion peak of chlorolignins), which corresponds to a molecular weight of over 116,000.

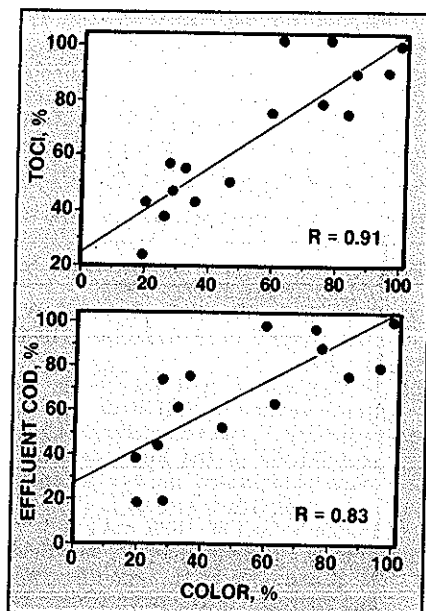
Results

The data indicated that 48.9% of the total organic chlorine was transformed to inorganic chloride in one day. Then, the dechlorination rate decreased considerably, so that the total dechlorination was 62.0% in two days (Fig. 1). The total chlorine concentration of the effluent was not constant, but it decreased by about 7% during the experiment. We first thought that this part of chlorolignin was adsorbed by the mycelium. However, the results of an experiment in sterile conditions suggest that the loss of total chlorine was caused by adsorption of inorganic chloride on the Plexiglas reactor walls. TOCl was unchanged in the sterile experiment (data not shown).

The rates of decolorization, dechlorination, and degradation of chlorolignin based on the COD decrease were different. In one day, color decreased by 65%, TOCl by 49%, and COD by 33% (Fig. 2). By correcting for the COD in the residual glucose, where 1 g of glucose = 1.067 g COD, the decrease of chlorolignin-related COD was only 14% in the one day. The difference in UV-spectra of the effluent samples before and after the incubation showed that carbonyl structures conjugated with an aromatic ring were cleaved faster than other chromophores (17). The changes at the wavelength range of 400–500 nm were smaller, although decolorization was extensive.

A simple correlation study suggested that the three processes—decolor-

3. Correlation of color removal and COD removal (top) and color removal and dechlorination (bottom)

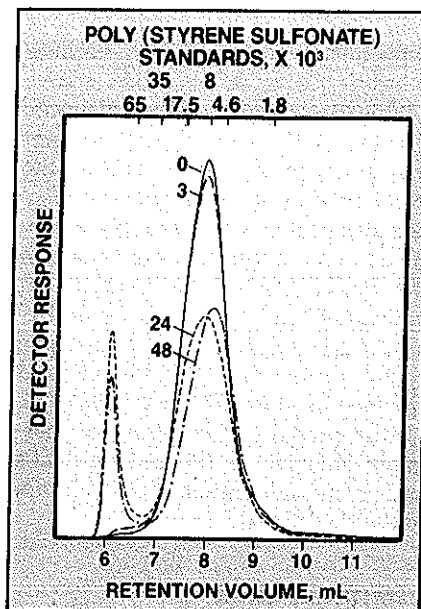


ization, dechlorination, and degradation of chlorolignin—are metabolically connected although the rates are different, as shown in Fig. 3. The correlation coefficients were 0.91 and 0.83, for color to COD effluent and color to TOCl, respectively. Effluent COD is defined as total COD minus the COD of the residual glucose. The correlation coefficient of color to total COD was higher, at 0.93.

The results of size-exclusion chromatography indicate that chlorolignin was partially polymerized during the first day of treatment and that the newly formed high-molecular-weight material started then to be degraded (Fig. 4). This polymerization likely reflects radical coupling of phenolic units. The ionization difference UV spectra of NaBH_4 -reduced samples showed that the number of phenolic hydroxyl groups decreased in the first day of the treatment and increased slightly during the second day. The addition of H_2O_2 into a sterile system did not lead to polymerization, indicating that a biochemical oxidant was involved. The chromatography system used was free of association effects (16).

Interestingly, no low-molecular-weight degradation products were found. In accordance with earlier reports (18, 19), the decrease in the size of the main peak suggests that

4. Size exclusion chromatograms of chlorolignin showing the polymerization during the first day of treatment. The numbers refer to the treatment time.



there were no major differences in the degradation rate of molecules of varying sizes. The position of the main peak corresponds to a molecular weight of 8,900 of poly(styrene sulfonate).

Discussion

The results presented here and those obtained earlier (8, 9, 20) show that *P. chrysosporium* is able to dechlorinate both low- and high-molecular-weight compounds in pulp bleaching effluents. This ability is important since the industry needs methods to reduce the discharge of TOCl in its wastewaters.

The mechanism of dechlorination is not presently known. We speculate that the process is an enzyme-initiated auto-oxidation, initiated by the fungal lignin peroxidases. On the other hand, it is also possible that there are other enzymes involved which catalyze dechlorination specifically. These enzymes could be intracellular and could work with depolymerized fractions of chlorolignin. Further research is needed to assess the mechanism of dechlorination.

Dechlorination of aliphatic compounds should be studied especially, because most of the chlorinated lignin from pulping is not aromatic (21, 22). Dechlorination may occur before

degradation of the chlorolignin structure. This conclusion may be reached from the rates of these two processes: dechlorination was 49% in one day, while chlorolignin-related-COD (effluent COD) decreased only by 14% in the same time.

An assumption was made in the calculation of effluent COD that all the glucose consumed was transformed to products which did not give rise to COD. Because this may not be true, the decrease of chlorolignin-related COD can be underestimated. However, the TOCl removal was faster even when no correction for residual glucose was made. (The decrease of total COD was 33% in one day). Apparently, high-molecular-weight chlorolignin was degraded slower than the unfractionated E1 effluent. In an earlier study (20), we estimated a 32% decrease in the effluent COD in one day; the corresponding figure for high-molecular-weight chlorolignin is only 14%.

The chlorolignin we used was ultra-filtered to remove organic low-molecular-weight compounds and inorganic chloride from the preparation. Inorganic chloride is normally present in E1 effluent in high concentrations (up to 2 g/L). This gives rise to a large error in the TOCl determination (14), because TOCl forms only a small fraction of the total chlorine concentration of the E1 effluent. High chloride concentration may also inhibit the fungal enzymes (23).

Polymerization of lignin by lignin-degrading fungi or by isolated lignin-degrading enzymes has been reported earlier (24–26). Polymerization is most likely due to the coupling of phenoxy radicals formed in the oxidation of phenols by peroxidases. It has been suggested that the fungus produces reducing enzymes, like cellobiose:quinone oxidoreductase (CBQ), to prevent radical coupling and thus polymerization. However, there is not much evidence for this mechanism (27), and polymerization does indeed take place.

Odier *et al.* (28) reported that CBQ does not prevent polymerization of lignin by lignin peroxidases, and no correlation was found by Roch *et al.* (29) between ligninolytic and CBQ activity. On the other hand, Faix *et al.* (19) and Chua *et al.* (18) did not report marked polymerization in their study with labeled lignins. Recently, Leisola

et al. (30) described a new reducing enzyme, NADP-dependent aryl alcohol oxidoreductase, which they suggest might be involved in lignin depolymerization.

Chua *et al.* (18) and Faix *et al.* (19) noticed accumulation of low-molecular-weight degradation products which were not found in our study. It is possible that the low-molecular-weight degradation products are nonaromatic and were not detected in the chromatography. UV detection at 254 nm was used in our study, whereas Faix *et al.* and Chua *et al.* used radioactive lignins. UV detection at this wavelength may not be good in the analysis of chlorolignin since, according to Lindström and Österberg (21), most of the material in E1 stage spent bleaching liquor is nonaromatic, and different chromophore systems have different extinction coefficients. The absence of low-molecular-weight compounds may also indicate that chlorolignin is a network structure that has to be extensively degraded to obtain small fragments.

Both Chua *et al.* (18) and Faix *et al.* (19) noticed that a large portion of lignin was bound to mycelium during the degradation process. However, such binding is less likely to have happened in our study here because chlorolignin is much more water-soluble than most other types of lignin. Furthermore, because the effluent was changed six times during the experiment, a steady state of absorption-desorption was probably established. The mycelium was brown in the beginning of the incubation, indicating some binding of chlorolignin to the mycelium, but it turned pale yellow in one day. The loss of total chlorine of about 7% in 2 days (Fig. 1) was caused by adsorption of inorganic chloride to the reactor walls rather than adsorption of chlorolignin to the mycelium.

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