Black liquor lignin biodegradation by *Trametes elegans*

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Abstract

The white rot fungus *Trametes elegans* was used for direct treatment of spent black liquor from pulping processes with the aim to degrade solubilized lignin which is the primary organic by-product from the chemical digestion of lignocellulosic raw materials. The enzymatic activity of *T. elegans* and the resulting degradation was confirmed by UV/VIS spectrometric and size-exclusion chromatography (SEC) measurements on incubated and control samples of both industrial and synthetic black liquors.

Opposing polymerization and depolymerization reactions occurred after treatment of the black liquor with *T. elegans* in liquid media.

It is noteworthy that these effects, which originate from ligninolytic enzyme systems, occur after the direct treatment of the black liquor even if no nutrients are added. The effect of the suspension pH and incubation time on the lignolytic action of *T. elegans* is also presented.

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1. Introduction

Lignin is a complex aromatic polymer that gives strength and rigidity to the tissues of vascular plants. This macromolecule is highly ramified and forms a three-dimensional structure, which depends not only on the type but also on the growing conditions of plant. Lignin consists of phenyl-propane units linked together by ether or carbon–carbon bonds making up a structure with an average molecular weight over the range of 8000–11,000 (Browning, 1975; Sarkanen and Ludwig, 1971; Sjöström, 1981).

Wood and non-wood raw materials are used in the production of cellulosic pulps. However, the presence of residual lignin negatively affects some properties of the manufactured pulp and paper products. Therefore, lignin is selectively removed during pulping without significant degradation of cellulose fibers. The extent of lignin removal depends on both the pulping process and the raw material utilized. Among the alternatives for cellulose pulp production, the so-called kraft and soda digestion processes are the most common ones. In these processes a considerable volume of contaminant effluents (black liquors) are generated. The black liquor is characterized by high alkalinity and high dissolved solids content, mainly dissolved alkali–lignin and polysaccharide degradation by-products. If the black liquor is not incinerated in recovery units, it becomes a significant effluent. The environmental impact of black liquor results not only from its chemical nature, but also from its dark coloration that reduces oxygen availability and negatively affects aquatic fauna and flora (Jones, 1973).

Minimization of contaminant sources and effluents is a main concern in pulp and paper industries, and many efforts have been made in improving pulping, bleaching and papermaking processes. In the case of chemical pulping processes, the most commonly applied treatment involves the concentration and combustion of black liquors so that inorganic chemicals can be recovered and simultaneously energy can be generated from chemical oxidation of the organic fraction. Since the recovery process involves the combustion of lignin, many potential high-value derivatives of this polymer are lost.

White rot wood decay fungi have a unique capacity for degrading wood and its basic constituents, cellulose and lignin. These fungi use the cellulose fraction as a source of carbon...
and have the ability to completely degrade the lignin to have access to the cellulose molecule. Basidiomycetes species have been extensively studied due to their high degradation capacity (Crawford, 1981; Hatakka, 2001; Higuchi, 1993; Miyamoto et al., 2000; Vicuna, 2000).

The broad list of compounds that white rot fungi are able to degrade includes many pesticides, polyaromatic hydrocarbons, PCBs and other halogenated aromatics (including dioxins), some dyes, TNT and other nitroexplosives, and other toxic chemicals such as cyanides, azide, carbon tetrachloride, and pentachlorophenol (Aust and Benson, 1993). Interest in the application of white rot fungi for the bioremediation of hazardous wastes sites is growing because their application is expected to be relatively economical (Aust and Benson, 1993).

Several white rot fungi are recognized for their ability to bleach and delignify Kraft pulp. Their lignin-degrading capacity is attributed to extracellular oxidative enzymes that function together with low molecular weight cofactors (De Jong et al., 1994; Tekere et al., 2001a,b). Although this ability has been recognized for many years, only recently have investigators begun to understand the mechanisms by which degradation is accomplished (Aust and Benson, 1993).

Recent developments in new technologies and/or improvement of existing ones for the treatment of effluents from the pulp and paper industries include the use of the white rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor* (Mehna et al., 1995). Very limited experience is available on the possibility of direct degradation of highly contaminant black liquors by fungi.

*Trametes elegans* is commonly found in the tropics, in Central and South America. Its biodegradative capabilities have been little studied and only a few reports are available (Tekere et al., 2001b). In Venezuela, Holmquist et al. have identified the highly efficient ligninolytic capabilities of this specie (Holmquist, personal communication; Cervantes, 1993). The *T. elegans* shares several characteristics with *P. chrysosporium* and *T. versicolor*, both of which have been studied for their lignin degradation processes (Evans and Palmer, 1983; Hatakka and Uusi-Rauva, 1983; Kamaya and Higuchi, 1984; Kawai et al., 1985).

This study reports on the effectiveness of *T. elegans* for the biodegradation of both industrial (residual) and synthetic black liquors by investigating relevant properties of degradation by-products as measured by spectrophotometric and chromatographic techniques.

### 2. Materials and methods

#### 2.1. Materials

All reagents were of the highest quality available. Agar malt extract was purchased from HIMEDIA (India). Indulin C, a commercial sodium salt of kraft lignin, was obtained from Westvaco Corp. (Charleston, SC). All solutions were prepared with Millipore-quality water (Milli-Q plus, Ultra-pure water system, 18 MΩ cm).

#### 2.2. Microorganism

The white rot fungus *T. elegans* was provided by the Laboratory of Forest Pathology (Universidad de Los Andes, Mérida, Venezuela). *T. elegans* was maintained on malt agar at 25°C for 15 days in a stationary culture. The mycelium, grown in these conditions, was collected and mixed for about 30 s in order to obtain a homogeneous suspension (Cervantes, 1993; Rondon, 1999).

#### 2.3. Black liquors

The industrial black liquor was obtained from Smurfit-Mocarpel pulp mill (Yaracuy, Venezuela) where *Pinus caribaea* is digested in a continuous process (240 ton/day capacity) that uses low-sulfidity Kraft liquor and anthraquinone as catalyst. The waste liquor formed in the digestion operation consists of an alkaline aqueous solution that contains organic and inorganic solids and has a distinctive dark coloration. The samples were collected in the recovery plant, after dilution with water of a concentrated black liquor solution from the recovery boiler. Suspended solids were separated from the black liquor by filtration with a Whatman filter No. 1. The solid content of the black liquor was determined by weight loss in an oven at 100°C overnight (see Table 1 for related properties). Synthetic black liquors were also obtained by dissolution of the respective amounts of Indulin C in water.

The pH of the samples was adjusted with 0.1 N H₂SO₄ before inoculation with the *T. elegans* mycelium suspension. Preliminary experiments were carried out in order to measure the change in pH of the suspension after inoculation and incubation of the black liquor samples. The maximum change in pH (less than one pH unit increment) was observed after 15 days incubation of samples with an initial pH of 6. In all the other cases the pH variation was less important.

#### 2.4. Ligninolytic treatment

After the completion of growing period, 80 ml of milli-Q water was added to the mycelium suspension and stirred

### Table 1
Kraft black liquor characterization

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total solids (%)</td>
<td>32.7</td>
</tr>
<tr>
<td>Lignin content (%)</td>
<td>17.8</td>
</tr>
<tr>
<td>pH</td>
<td>12.5</td>
</tr>
<tr>
<td>Density (g/ml)</td>
<td>1.15</td>
</tr>
</tbody>
</table>

*Lignin content with respect to total solids.*

...
with a conventional blender for 10 min. This suspension was considered ready for inoculation. Then 10 ml of black liquor or synthetic black liquor (Indulin C) was inoculated with 40 ml of the mycelium suspension.

Each experiment was conducted in triplicate. Since light degradation may occur in the samples, the jars were covered with aluminum foil, and the inoculated suspensions were kept in capped jars in the sterile room at 25°C. After the respective time elapsed, the samples from the jars were filtered and kept under refrigeration at 4°C.

2.5. UV/Vis spectrophotometric analysis

For UV/Vis spectrophotometric analysis, a Lambda 3B (Perkin-Elmer®) was employed. In a typical run, 3 µl of the sample was dissolved in 5 ml of 0.1 N NaOH solution. In the case of Indulin C, a sample volume of 150 µl was used. The solutions were scanned over the wavelength range from 200 to 600 nm using PECSS program (Perkin-Elmer®, Computerized Spectroscopy Software, version 3.2). The absorbance maximum of Kraft lignin was measured at 280 nm (Janshekar et al., 1981).

2.6. Size-exclusion chromatography (SEC)

The high-performance SEC of black liquor lignin was performed on a high-performance liquid chromatograph with a size-exclusion column (310 × 10 mm id; Superose® 12HR 10/30; Pharmacia Biotech). For the chromatographic analysis, a UV/VIS detector (LC 295; Perkin-Elmer®), a binary pump (LC PUMP 250; Perkin-Elmer®) together with a personal integrator (PE NELSON; model 1022, Perkin-Elmer®) were used. The optimized conditions for the column operation were as follows: mobile phase was an aqueous 0.1 M NaOH; flow rate was 0.4 ml/min; injection volume was 20 µl; the sample concentration was 0.04% (w/v), and spectrometric detection at 280 nm.

Sodium poly(estyrenesulfonate) standards (Polysciences Inc., Warrigtom, USA) were used to obtain the calibration curve that relates the retention time ($T_r$, minutes) and the molecular weight ($M_w$). The fitted expression corresponds to:

$$\log(M_w) = 8.149 - 0.132T_r. \tag{1}$$

The average molecular weight distribution (both in weight, $M_w$ and number, $M_n$) and polymer dispersity, $D$, were obtained after standard numerical correlations.

3. Results and discussion

3.1. Effect of pH on the lignolytic action of T. elegans

The decrease in the absorbance of lignin after incubation with fungi has been considered as a criterion for the extent of lignin biodegradation (Janshekar et al., 1981). Therefore, we monitored the changes in absorbance during the treatment of black liquor with $T. elegans$.

Initially, the pH of black liquor (33% w/v) was adjusted to 6, 7, and 8 by acidification with 0.1 N H$_2$SO$_4$, and then inoculated with mycelium (experimental group) or with H$_2$O (control group), and incubated for 10 days. After the incubation, two characteristic features could be observed. Firstly, the shape of the spectra is similar showing that the basic structure of the lignin polymers is conserved, and secondly, a reduction in the 280-nm peak of the samples suggests ligninolytic action of $T. elegans$ (Fig. 1).

Fig. 2 illustrates representative chromatograms of the samples incubated at different pHs as compared to the corresponding control samples. A distinctive displacement is observed in the elution peaks of the treated black liquor samples, with respect to the peaks for the control samples. Therefore, a change in the size of the molecules occurred as judged by changes in the elution time. Note that SEC retention time is inversely related to the molecular weight of the diffusing fraction (see Eq. (1)).

In Fig. 2(a) for experiments at pH 6, the 35-min intensity peak, which corresponds to relatively high molecular-weight lignin fractions, is displaced towards higher elution times. This corresponds to a decrease in the molecular weight of the respective lignin molecules. At pH 7 (Fig. 2b) there is no noticeable variation in the position of this peak. However, at pH 8 (Fig. 2c) there is a distinctive displacement towards lower elution times. This change indicates that there is an increase in the molecular weight of the respective lignin fractions under this condition.

The changes noted before are accompanied by a displacement of the 40-min elution time peaks towards higher retention times, i.e., the relatively low molecular weight lignin fractions (that correspond to these peaks) undergo further reduction in molecular mass.

Most probably ligninolytic enzyme systems are responsible for the observed biodegradation as has been proposed...
by other authors in the case of graft copolymers of lignin and poly styrene (Milstein et al., 1994). It has been demonstrated that laccase plays an important role in this type of degradation (Eggert et al., 1997).

The use of laccase in the treatment of substrates which are rich in phenolic groups, such as milled wood lignin and soluble lignosulfonates, involves both oxidation and polymerization reactions (Ishihara, 1980; Leonowicz et al., 1985). Our experimental observations are also in agreement with polymerization reactions as has been reported to occur in the treatment of Kraft lignins with Trametes cingulata (Nutsubidze et al., 1998).

Bourbonnais and Paice (1990) verified that laccase, like other phenol-oxidizing enzymes such as peroxidases (Haemmeril et al., 1986; Huttermann et al., 1980), preferentially polymerizes lignin by coupling the phenoxy radicals produced from oxidation of lignin phenolic groups. It has been suggested that laccase possesses both polymerization and depolymerization activities on some preparations of lignosulfonates (Leonowicz et al., 1985).

Furthermore, experiments aimed at reproducing natural biodegradation in vitro showed that the biocatalysts lignin peroxidases and laccase were able to depolymerize high molecular weight lignins but that low molecular weight products accumulated and simultaneously recondensed (Himmel and Sopher, 1983; Leonowicz et al., 1985). This was related to the increase in the molecular weight observed in high-molecular-weight lignin fractions because these could condense either with the degradation products or with small lignin molecules originally present in the black liquor.

3.2. Ligninolytic treatment for different incubation times

Fig. 3 shows UV spectra of black liquor samples at pH 7.0 inoculated with the mycelium of T. elegans and incubated during different periods of time (5, 10 and 15 days). A reduction in the absorbance at 280 nm is observed as the incubation time is increased. Once again, it can be hypothesized that changes in the molecules, owing to the ligninolytic reactions, are taking place. Similar results occurred with the synthetic black liquor (Indulin-C solution) (see Fig. 4). The addition of the fungus suspension by itself could not explain these observations because as shown in Fig. 5, the spectrum of a sample immediately after inoculation did not show any substantial change in the absorbance scan.

It is interesting to note that there is a direct relationship between the incubation time and the displacement of both 35- and 40-min elution peaks. Fig. 6 shows the chromatograms for black liquor samples of 33% solids content at pH 7 immediately after inoculation and with different incubation times. As the time of incubation increases, the
35-min peak displaces towards lower elution times (higher molecular weight) whereas the 40-min peak displaces towards higher elution times (lower molecular weights). These tendencies can be better visualized in Table 2 where the retention times \( (T_r) \) for the different incubation periods are summarized along with \( \Delta T_r \), i.e., the difference of the final elution time with respect to the one initially observed.

In Table 3, a summary of the calculated average molecular weights (both weight and number) as well as the molecular weight dispersity for the investigated black liquors is included. The black liquor samples incubated with the suspension of \( T. \ elegans \) show a reduction in lignin \( M_n \) as the number of days of incubation is increased. This is consistent with a degradation process (Srebotnik and Hammel, 2000). However, it should be pointed out that the analysis of an average molecular weight could be misleading since the changes in the molecules occur in opposing directions (depolymerization/polymerization) and it is better to assess the results with the use of the \( M_w \) distribution.

In Fig. 7, dispersity values \( (D) \) are plotted against the incubation time. It can be observed that dispersity remains constant for 5 and 10 days. However, after 15 days there is a large increase in \( D \). For this incubation time, \( D \) is unexpectedly high as compared to conventional lignin samples (Glasser, 1993; Sarkanen and Ludwig, 1971).

Fig. 8 shows chromatograms for commercial lignin solutions (synthetic black liquor) after 5 and 15 days of incubation at pH 7. The changes that occur due to ligninolytic action are evident if one compares the incubated samples with the control samples. Furthermore, lignin molecules in the commercial sample are subject to similar ligninolytic
Table 2

<table>
<thead>
<tr>
<th>Days of incubation</th>
<th>$T_{r1}$ (min)</th>
<th>$T_{r2}$ (min)</th>
<th>$\Delta T_{r1}$ (min)</th>
<th>$\Delta T_{r2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>35.19</td>
<td>38.88</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>35.07</td>
<td>39.02</td>
<td>+0.122</td>
<td>−0.14</td>
</tr>
<tr>
<td>10</td>
<td>34.99</td>
<td>39.46</td>
<td>+0.202</td>
<td>−0.58</td>
</tr>
<tr>
<td>15</td>
<td>34.37</td>
<td>39.72</td>
<td>+0.822</td>
<td>−0.84</td>
</tr>
</tbody>
</table>

Subscripts 1 and 2 refer to the 35-min and 40-min peaks, respectively. $\Delta T_{r(+)} = sample eluted before the respective retention time for the control (i.e., sample has a higher $M_w$). $\Delta T_{r(-)} = sample eluted after the respective retention time for the control (i.e., sample has a lower $M_w$).

Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Days of incubation</th>
<th>$M_n$</th>
<th>$M_w$</th>
<th>$D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Industrial black liquor</td>
<td>0</td>
<td>5465</td>
<td>9032</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4125</td>
<td>7782</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3815</td>
<td>7729</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2388</td>
<td>7698</td>
<td>3.2</td>
</tr>
<tr>
<td>Indulin C (synthetic black liquor)</td>
<td>0</td>
<td>3409</td>
<td>12758</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3945</td>
<td>26005</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3129</td>
<td>23854</td>
<td>7.6</td>
</tr>
</tbody>
</table>

4. Conclusions

The ligninolytic activity of $T. elegans$ cultures when inoculated directly to industrial black liquors is evidenced despite the fact that very little or no nutrients were added to the substrate. Noticeably, these changes occur in opposing directions, i.e., lignin molecules are polymerized and depolymerized upon treatment. Different chemical or environmental treatments should be realized if a selective shift of the cultural functioning towards depolymerization (or polymerization) is required.

The dispersity of the fractions obtained after ligninolytic activity and the complexity of the lignin molecular structure call for further studies in order to elucidate the role of the different enzymes secreted by $T. elegans$. However, the presented results open the possibility for the use of biotechnology in the treatment of spent black liquors from pulping processes. In the case of small-scale mills this alternative becomes more interesting since installation and operation costs associated to conventional units for chemical recovery are very high.

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References


Tekere, M., Zvauya, R., Read, J.S., 2001b. Ligninolytic enzyme production in selected subtropical white rot fungi under different culture conditions. Journal of Basic Microbiology 41, 115.