Manganese Peroxidase, Produced by *Trametes versicolor* during Pulp Bleaching, Demethylates and Delignifies Kraft Pulp

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Previous work has shown that *Trametes (Coriolus) versicolor* bleaches kraft pulp brownstock with the concomitant release of methanol. In this work, the fungus is shown to produce both laccase and manganese peroxidase (MnP) but not lignin peroxidase during pulp bleaching. MnP production was enhanced by the presence of pulp and/or Mn(II) ions. The maximum level of secreted MnP was coincident with the maximum rate of fungal bleaching. Culture filtrates isolated from bleaching cultures produced Mn(II)- and hydrogen peroxide-dependent pulp demethylation and delignification. Laccase and MnP were separated by ion-exchange chromatography. Purified MnP alone produced most of the demethylation and delignification exhibited by the culture filtrates. On the basis of the methanol released and the total and phenolic methoxyl contents of the pulp, it appears that MnP shows a preference for the oxidation of phenolic lignin substructures. The extensive increase in brightness observed in the fungus-treated pulp was not found with MnP alone. Therefore, either the MnP effect must be optimized or other enzymes or compounds from the fungus are also required for brightening.

The white rot fungus *Trametes (Coriolus) versicolor* delignifies and bleaches kraft pulp (36, 41). However, the process is rather slow compared with chemical bleaching (days instead of hours), and the cellulose in the pulp is also attacked. To overcome these drawbacks of the fungal process, we have attempted to determine the enzymology of fungal delignification and then to apply directly the relevant enzymes to pulp. Laccase, a copper-containing polyphenol oxidase (42) that is produced abundantly by the fungus during bleaching, delignifies kraft pulp, but this process could be demonstrated only in the presence of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), a commonly used assay substrate for the enzyme (12, 13). No naturally occurring analog of ABTS has been identified in the fungal pulp-bleaching system to date (13). Both the fungus and the combination of laccase and ABTS demethylate pulp before and during delignification; in the absence of ABTS, methanol release and delignification by laccase are greatly reduced.

Lignin peroxidase and manganese peroxidase (MnP) have both been implicated in lignin biodegradation by *Phanerochaete chrysosporium* and *T. versicolor* (14, 22, 24, 44). Lignin peroxidase from *P. chrysosporium* has been reported to bleach kraft pulp (15) and to facilitate subsequent chemical bleaching (5, 35). However, it seems unlikely that lignin peroxidase is involved in kraft pulp bleaching by *T. versicolor*, since the enzyme is not detected during bleaching (7, 25), the enzyme is strongly inhibited by fungal culture bleaching liquor, and fungal bleaching is not inhibited by metavanadate ions, inhibitors of lignin peroxidase (6).

MnP was first discovered in *P. chrysosporium* (26, 37), and there is now increasing evidence that this enzyme plays a role in lignin depolymerization (48). The Mn-induced enzyme oxidizes Mn(II) to Mn(III) which, in chelated form, is capable of oxidizing many simple phenolic compounds (9) and phenolic lignin substructures (45, 47). In the presence of thiols, nonphenolic lignin substructures are also oxidized (17, 49).

There are now several reports of lignin-degrading fungi that secrete laccase and MnP but not lignin peroxidase. These include *Dichomitus squaless* (38), *Lentinus edodes* (29), *Rigidopus lignosus* (18), and *Stereum hirsutum* (33). *D. squaless* and *S. hirsutum* have been shown to bleach kraft lignin (33). Recently, MnP was found to depolymerize synthetic lignin (48) and to degrade high-molecular-weight chlorolignin (27, 31). In this work, we show that MnP is produced by bleaching cultures of *T. versicolor*, that the peak production of the enzyme occurs at the same time as the maximum rate of fungal culture bleaching, and that manganese- and peroxide-dependent demethylation and delignification of kraft pulp occur in vitro.

**MATERIALS AND METHODS**

**Chemical and enzyme sources.** Catalase and glucose oxidase from *Aspergillus niger* were from Sigma Chemical Co. (St. Louis, Mo.). Soytone and malt agar were from Difco Laboratories (Detroit, Mich.). Diethylenetriamine pentaacetic acid (DTPA), pentasodium salt (40% in water), was from Eastman Kodak Co. (Rochester, N.Y.). All other chemicals were certified A.C.S. grade and were from Fisher Scientific Co. (Fair Lawn, N.J.).

**Enzyme assays.** MnP activity was determined by monitoring the formation of manganese(III) malonate at 270 nm (38). Enzyme was added to a solution of MnSO₄ (0.2 mM)-sodium malonate (50 mM, pH 4.5), and the reaction was initiated with hydrogen peroxide (0.1 mM) in a final volume of 3 ml. The initial increase in the A420 was determined. Laccase activity was determined by monitoring the oxidation of ABTS (50). The reaction mixture contained 0.5 mM ABTS, 0.1 M sodium acetate buffer (pH 5.0), and a suitable amount of enzyme. The oxidation of ABTS was monitored by measuring the increase in the A420 (ε₄₂₀ = 3.6 × 10⁴ M⁻¹ cm⁻¹). Enzyme activity was expressed in units (micromoles per minute). Lignin peroxidase activity was determined by

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monitoring the oxidation of veratryl alcohol to veratraldehyde as described previously (6).

Cellulose quinone oxidoreductase (CBQase) activity was determined by monitoring the reduction of 3,5-di-tert-buty1-1,2-benzoquinone (0.33 mM) in the presence of cellulose (0.67 mM) and ethanol (0.3 ml) at pH 4.5 (acetate buffer, 0.2 M). The total assay volume was 3.0 ml, and the reaction was run at room temperature under nitrogen to prevent reoxidation by laccase. The reduction of the quinone was monitored at 420 nm (4).

Glucose oxidase activity was determined by the peroxidase-coupled production of hydrogen peroxide in the presence of glucose (11). Catalase activity was determined from the rate of oxygen production from 20 mM H2O2 in 50 mM phosphate buffer (pH 7). Oxygen was determined with a Clark electrode (Rank Ltd., Cambridge, United Kingdom).

Production and purification of MnP. The T. versicolor strain used was a dikaryotic strain (52P) isolated in our laboratory (ATCC 20869). Disks (three with a 1-cm diameter) of vegetative mycelium from malt agar plates were added to 200 ml of culture medium in 500-ml polypropylene shake flasks containing mycological broth (glucose, 40 g/liter; Soytone, 10 g/liter), trace metals (30), MnSO4 (0.2 mM), and hardwood kraft pulp (0.25%), and the pH was adjusted to 5.0. MnSO4 and pulp were omitted for some experiments, as mentioned below. A glass marble was added to each flask to prevent pellet formation, and the flasks were shaken at 200 rpm and 25°C for 7 days. Solids were removed by centrifugation (6,000 x g). The combined filtrates were concentrated by a factor of 10 by ultrafiltration (Millipore Pellicon; 10,000-molecular-weight cutoff). The viscosity of the filtrate was reduced by freezing-thawing and centrifugation of the gelatinous precipitate.

The enzyme concentrate was dialyzed against 0.05 M bis-Tris buffer (pH 6.0) overnight and then fractionated on a Mono Q or Q-Sepharose fast-flow (Pharmacia) column with a salt gradient of 0 to 0.45 M NaCl. Fractions containing MnP activity were pooled, dialyzed against water, and frozen or lyophilized. MnP-enriched fractions were further separated by gel permeation on a Sephacryl S-200 (Pharmacia) column with 20 mM bis-Tris buffer (pH 5.5). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 8 to 25% gradient gels (Phast system; Pharmacia), and proteins were visualized by staining with Coomassie blue.

Pulp treatments. Hardwood kraft pulp (a mixture of various wood species) was obtained from an eastern Canadian mill. Black spruce and other kraft pulps were prepared in a pilot plant at the Pulp and Paper Research Institute of Canada. Fungal and enzyme treatments were performed on washed pulp (4 g) suspended in a total volume of 200 ml of solution in 500-ml glass Erlenmeyer flasks. For fungal treatments, the solution contained 30 ml of a 5-day-old T. versicolor liquid culture and 170 ml of deionized, distilled water. For enzyme treatments with MnP, the solution typically contained MnP (0.4 ΔOD/min/ml [ΔOD is the change in the optical density units]), glucose (2.5 mM), glucose oxidase (0.025 U/ml), MnSO4 (0.5 mM), and sodium malonate (50 mM, pH 4.5). Suspensions were autoclaved before inoculation, except for enzymes and glucose, which were filter sterilized. The flasks were shaken at 200 rpm and 25°C. Following the treatments, pulp was recovered on a polyethylene macrofilter (Spectrum; 290-μm pore size).

Pulp properties. Handsheets (i.e., sheets of paper produced from pulp samples in the laboratory) were prepared with a British standard handsheet machine (Noram) by use of 4 g of pulp that had been homogenized for 30 s in a Brookfield counter-rotating mixer. Each handsheet was then air dried on a blotter before it was tested for brightness and kappa number (see below).

Brightness was determined by measuring at 457 nm the reflectance of a handsheet section measuring 1.5 by 1.5 cm by use of a Perkin-Elmer λ3B spectrophotometer with a reflectance sphere attachment. A barium sulfate plate was used as a standard (100%).

The kappa number is defined as the amount (milliliters) of a 0.1 N KMnO4 solution consumed by 1 g of moisture-free pulp under standard conditions (standard T236 of the Technical Association of the Pulp and Paper Industry, Atlanta, Ga.). The kappa number is equivalent to approximately six times the lignin content (percent).

Methanol determination. The methanol concentration in the pulp treatment supernatants was determined by gas chromatography as described by Ni et al. (34). Aliquots from supernatants were clarified with activated charcoal, filtered with a 0.45-μm-pore-size filter, and analyzed on a Chromosorb 102-packed column. The injection, oven, and flame ionization detector temperatures were 130, 120, and 150°C, respectively, and the helium flow rate was 30 ml/min. Methanol standards were linear in a range of 2 to 50 mg/liter, and the detection limit was 1 mg/liter.

Methoxyl determination. Pulp samples were freeze-dried and ground to pass through a 20-mesh screen in a Wiley mill. The total methoxyl content was determined by a micro-Zeisel method (32). The number of methoxyl groups adjacent to a phenolic hydroxyl group was determined on the basis of periodate oxidation (28).

RESULTS

MnP production during fungal bleaching. Fungal cultures bleaching hardwood kraft pulp were monitored for MnP activity. Peak production of the enzyme occurred 3 days after fungal inoculation (Fig. 1), coincident with the maximum rate of bleaching. No lignin peroxidase was detected during the 7-day bleaching period. MnP production was also monitored during fungal inoculum growth prior to bleaching. MnP production in the inoculum was stimulated by the presence of pulp and/or Mn(II) (Table 1). The induction by pulp may have been due to the manganese present in pulp fibers (Table 2). Iron was also abundant in some pulps; soluble Fe(III) is known to inhibit MnP (19). MnP production

FIG. 1. Production of MnP by T. versicolor during demethylation and bleaching of hardwood kraft pulp.

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by monokaryons (52J and 52D) derived from the dikaryon used here (52P) is described in a companion paper (1).

Hydrogen peroxide destruction by catalase was used to determine whether the observed fungal demethylation and delignification were $\text{H}_2\text{O}_2$ dependent. The daily addition of catalase partially inhibited methanol release from the softwood kraft pulp (Fig. 2). The decrease in the kappa number caused by the fungus was also somewhat smaller in the presence of catalase. The results obtained with hardwood kraft pulp were similar (data not shown). The addition of catalase to the standard MnP assay completely inhibited the formation of Mn(III)-malonate by MnP. However, when hydrogen peroxide in the MnP assay was generated by glucose (10 mM) and glucose oxidase (0.025 U/ml), the addition of catalase only slowed Mn(III)-malonate formation by approximately fourfold. Thus, it is possible that MnP produced during bleaching can still function, although at a slower rate, in the presence of catalase if hydrogen peroxide is produced continuously by the fungus.

**Effect of fungal enzymes on bleaching.** Fungal enzymes secreted at day 7 of inoculum growth with 0.25% pulp and 0.2 mM MnSO$_4$ were centrifuged free of biomass and concentrated by ultrafiltration. The filtrate contained both MnP and laccase activities (Table 1). In the presence of Mn(II), malonate, glucose, and glucose oxidase, the concentrate produced demethylation and delignification of hardwood kraft pulp (Fig. 3). The brightness of the pulp, however, was only changed marginally. Fiber strength, as measured by zero-span breaking length, was maintained during the 5-day treatment (data not shown). Methanol release and kappa number reduction were manganese ion dependent and required a source of hydrogen peroxide (Table 3). Hydrogen peroxide was either generated from glucose and glucose oxidase or added as a dilute solution. However, hydrogen peroxide was inhibitory at a high concentration (Table 4). The enzymes produced methanol fastest in the presence of a continuously added low feed (2.5 mM/day) of hydrogen peroxide.

Mn(III) chelates, produced by the action of MnP, have a wide range of redox potentials dependent on both the structure and the concentration of the chelator (21, 46). Table 5 shows the effect of various chelator concentrations and species. The same methanol release and kappa number could be obtained whether malonate was present or not. Oxalate, EDTA, and DTPA all inhibited the enzyme action. Since unchelated Mn(III) is a powerful and unstable oxidant, it seems probable that one or more effective chelators must already be present in the pulp or in the crude enzyme preparation; the most likely is gluconic acid derived from the oxidation of glucose by glucose oxidase.

**Effect of purified MnP and laccase on pulp.** A concentrated supernatant from *T. versicolor* cultures was fractionated by Mono Q ion-exchange chromatography into separate peaks of laccase, MnP, and CBQase activities. MnP peak II, the second of two resolved peaks of MnP, was free of detectable laccase activity (Fig. 4) and was tested, alone and in combination with laccase peak I, in pulp bleaching. The results

<table>
<thead>
<tr>
<th>Kraft pulp</th>
<th>Mn (mg/kg)</th>
<th>Fe (mg/kg)</th>
<th>Kappa number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black spruce, pilot plant</td>
<td>41.5</td>
<td>15.2</td>
<td>32.0</td>
</tr>
<tr>
<td>White spruce, pilot plant</td>
<td>4.8</td>
<td>17.5</td>
<td>27.6</td>
</tr>
<tr>
<td>Aspen, pilot plant</td>
<td>5.1</td>
<td>13.3</td>
<td>26.1</td>
</tr>
<tr>
<td>Softwood, western interior Canadian mill</td>
<td>55.0</td>
<td>8.0</td>
<td>27.8</td>
</tr>
<tr>
<td>Hardwood, eastern Canadian mill</td>
<td>44.9</td>
<td>82.5</td>
<td>14.8</td>
</tr>
</tbody>
</table>

$^a$ The lignin content (percent) is approximately equal to the kappa number divided by six.

![FIG. 3. Effect of a culture filtrate containing MnP on demethylation and bleaching of hardwood kraft pulp. Reactions were performed at a 2% pulp consistency in the presence of MnSO$_4$, malonate, glucose, and glucose oxidase as described in Materials and Methods. A control without a culture filtrate yielded 3 mg of methanol per liter and pulp with a kappa number of 13.2 and a brightness of 34.4% after a 5-day treatment.](http://aem.asm.org/)

![FIG. 2. Inhibition of fungal demethylation of softwood kraft pulp by catalase. At day 10, the kappa numbers of the pulps were 18.5, 20.4, and 22.9 for the control, catalase at 50 U/ml, and catalase at 200 U/ml, respectively. Catalase was added daily at the concentrations shown.](http://aem.asm.org/)
TABLE 3. Effect of various initial Mn(II) and glucose concentrations on methanol release and kappa number of hardwood pulp

<table>
<thead>
<tr>
<th>MnSO4 (mM)</th>
<th>Glucose (mM)</th>
<th>Methanol (mg/liter)</th>
<th>Kappa numberb</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>2.5</td>
<td>2.6</td>
<td>12.7</td>
</tr>
<tr>
<td>0.05</td>
<td>2.5</td>
<td>4.6</td>
<td>12.1</td>
</tr>
<tr>
<td>0.1</td>
<td>2.5</td>
<td>7.1</td>
<td>12.2</td>
</tr>
<tr>
<td>0.2</td>
<td>2.5</td>
<td>9.4</td>
<td>11.9</td>
</tr>
<tr>
<td>0.2</td>
<td>10</td>
<td>11.5</td>
<td>11.7</td>
</tr>
<tr>
<td>0.2</td>
<td>25</td>
<td>13.8</td>
<td>11.8</td>
</tr>
</tbody>
</table>

a Experimental conditions were as follows: pulp (4 g) in 200 ml of 50 mM sodium malonate buffer (pH 4.5) was incubated at 25°C with MnP (0.43 ΔOD/min/ml), glucose oxidase (0.025 U/ml), MnSO4, and glucose for 24 h.

b Original kappa number for hardwood kraft pulp, 13.6.

Table 6 indicate that partially purified MnP alone produced most of the demethylation and delignification exhibited by the culture filtrate.

In studies with lignin substructure model compounds, it was found previously that MnP-Mn(III) chelate systems could only oxidize phenolic substituents (23) or aromatic rings containing three or four methoxyl substituents (39). To investigate the lignin substrate range of MnP in kraft pulp, we determined the total methoxyl and phenolic methoxyl contents of pulps before and after enzyme treatment. The results (Table 7) show that for hardwood pulp, MnP and, to a lesser extent, laccase removed methoxyl groups from the phenolic rings of the residual lignin, consistent with the reported ability of Mn(III) chelates to oxidize the adjacent hydroxyl and methoxyl groups and guaiacol (9). The total amount of methoxyl lost from the pulp was not significantly larger than the amount of methoxyl lost from the phenolic rings; thus, there is no evidence of demethylation of non-phenolic residues. The amount of methanol released from the pulp by the enzyme was smaller than the amount of methoxyl removed from the pulp. It is possible that some aromatic rings were removed from the lignin with their methoxyl groups still attached. The results for black spruce pulp were similar, although laccase alone was unable to demethylate the pulp under these conditions.

DISCUSSION

T. versicolor produces both laccase and MnP during bleaching of kraft pulp, while lignin peroxidase is usually not detectable. We showed previously (13) that laccase alone can produce limited demethylation of kraft pulp and that, in the presence of ABTS, extensive demethylation can occur. In addition, laccase in the presence of certain phenols can oxidize Mn(II) to Mn(III) (8). MnP-Mn(III) chelate systems can be more powerful oxidants than laccase, as judged by their oxidation of various methoxybenzenes (39). Since the demethylation of kraft pulp by crude culture filtrates of T. versicolor is enhanced by MnP and H2O2, we conclude that most of the in vivo demethylation is caused by MnP-mediated oxidation. Laccase has a small effect alone, consistent with some methanol production by the fungus in the presence of catalase. There appears to be no synergistic action of laccase and MnP (Table 6), in contrast to an earlier report on lignin degradation by Galliano et al. (18).

The concentrations of Mn(II), chelator, and hydrogen

TABLE 4. Effect of H2O2 addition rate and concentration on methanol release and kappa number of hardwood pulp

<table>
<thead>
<tr>
<th>H2O2 (mM)</th>
<th>Methanol (mg/liter)</th>
<th>Kappa numberb</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 initial</td>
<td>&lt;1.0</td>
<td>12.9</td>
</tr>
<tr>
<td>2.5 total over 24 h</td>
<td>9.6</td>
<td>12.3</td>
</tr>
<tr>
<td>25 total over 24 h</td>
<td>2.3</td>
<td>13.1</td>
</tr>
</tbody>
</table>

a Experimental conditions were as described in Table 3, footnote a, but with 0.2 mM MnSO4 and no glucose or glucose oxidase.

b Original kappa number for hardwood kraft pulp, 13.6.

c H2O2 was added continuously by a peristaltic pump.

TABLE 5. Effect of chelators on MnP oxidation of pulp

<table>
<thead>
<tr>
<th>Chelator (mM)</th>
<th>Methanol (mg/liter)</th>
<th>Kappa numberb</th>
<th>Brightness (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>17.3 ± 0.1</td>
<td>12.3 ± 0.2</td>
<td>33.5 ± 0.1</td>
</tr>
<tr>
<td>Malonate (0.5)</td>
<td>17.8 ± 0.1</td>
<td>12.1 ± 0.1</td>
<td>34.2 ± 0.6</td>
</tr>
<tr>
<td>Malonate (50)</td>
<td>17.5 ± 0.1</td>
<td>12.4 ± 0.1</td>
<td>33.7 ± 0.1</td>
</tr>
<tr>
<td>Oxalate (50)</td>
<td>8.4 ± 0</td>
<td>13.8 ± 0</td>
<td>33.1 ± 0.4</td>
</tr>
<tr>
<td>EDTA (50)</td>
<td>6.1 ± 0.3</td>
<td>13.3 ± 0.3</td>
<td>33.7 ± 0.7</td>
</tr>
<tr>
<td>DTPA (50)</td>
<td>5.4 ± 0.4</td>
<td>12.4 ± 0.1</td>
<td>34.0 ± 0.4</td>
</tr>
</tbody>
</table>

a Experimental conditions were as described in Table 3, footnote a, but with 2.5 mM glucose and 0.5 mM MnSO4.

b Original kappa number for hardwood kraft pulp, 13.6.

FIG. 4. (A) Ion-exchange chromatography (Mono Q) of a 7-day-old culture filtrate grown in mycelial broth with 0.25% pulp and 0.2 mM MnSO4, showing the separation of laccase (●), MnP (▲), and CBQase (■). (B) SDS-PAGE of molecular mass standards (from top to bottom, 94, 67, 43, 30, 20.1, and 14.4 kDa) (a), MnP peak II (b), and MnP peak II after Sephacryl S-200 gel permeation (c).
peroxide are all important in MnP-mediated oxidation. Manganeses is present in kraft pulps, probably close to the lignin substrate (10), and dicarboxylic acid chelators are abundantly produced during kraft pulp production (2). Also, white rot fungi produce hydrogen peroxide (16), and *T. versicolor* secretes a variety of Mn chelators in the presence of pulp (7, 43). The availability of these three components provides the potential for a renewable flux of Mn(III) chelates in vivo. However, the extensive delignification and brightening observed with the fungus were not achieved with isolated MnP. Even if the in vitro MnP effect can be optimized, it seems likely that other enzymes and substances, not active or stable in the crude culture supernatant under the conditions used here, are required for extensive brightening.

The rapid demethylation of residual lignin by MnP with no effect on fiber strength may be of practical interest. Also, the known substrate range of MnP can be extended with thiols (17, 49) and is substantially affected by the concentration, ratio, and nature of the chelator, which may allow enhanced delignification. On the negative side, the enzyme would be expensive to produce at present, although cloning studies are promising (3, 20). The sensitivity of the enzyme to hydrogen peroxide may also be a problem. However, the pulp and paper industry is very experienced in the application of both hydrogen peroxide and chelators and may eventually find a role for MnP in bleaching, as it has for other enzymes (40).

### TABLE 6. Effect of purified MnP and laccase on hardwood pulp demethylation and delignification

<table>
<thead>
<tr>
<th>Enzyme treatmentb</th>
<th>Methanol (mg/liter) at:</th>
<th>Kappa number at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>24 h</td>
</tr>
<tr>
<td>None</td>
<td>&lt;1.0</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>Purified laccase</td>
<td>&lt;1.0</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td>Purified MnP</td>
<td>3.2 ± 0.1</td>
<td>18.9 ± 0.1</td>
</tr>
<tr>
<td>MnP + laccase</td>
<td>5.6 ± 1.2</td>
<td>21.2 ± 0.2</td>
</tr>
<tr>
<td>Unfractionated culture supernatant</td>
<td>9.8 ± 0.5</td>
<td>22.8 ± 0.7</td>
</tr>
</tbody>
</table>

* Experimental conditions were as described in Table 3, footnote a, but with 0.5 mM MnSO₄ and 10 mM glucose. Data are averages of duplicates. 

### ACKNOWLEDGMENTS

We sincerely appreciate the skilled technical assistance of Sylvie Renaud.

### REFERENCES


### TABLE 7. Total methoxyl and phenolic methoxyl contents of kraft pulps and methanol released by purified enzymesa

<table>
<thead>
<tr>
<th>Kraft pulp and enzyme treatment</th>
<th>% MeOH recovered (±0.001)</th>
<th>% Phenolic methoxyl (±0.003)</th>
<th>% Total methoxyl (±0.03)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black spruce</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.018</td>
<td>0.261a</td>
<td>0.73a</td>
</tr>
<tr>
<td>Laccase</td>
<td>0.018</td>
<td>0.262a</td>
<td>0.74a</td>
</tr>
<tr>
<td>MnP</td>
<td>0.080</td>
<td>0.150b</td>
<td></td>
</tr>
<tr>
<td>Hardwood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.028</td>
<td>0.135c</td>
<td>0.30c</td>
</tr>
<tr>
<td>Laccase</td>
<td>0.035</td>
<td>0.092d</td>
<td>0.30c</td>
</tr>
<tr>
<td>MnP</td>
<td>0.075</td>
<td>0.075e</td>
<td>0.26c</td>
</tr>
</tbody>
</table>

* Experimental conditions were as described in Table 6, footnote a, except that laccase was in 0.05 M Na acetate buffer (pH 5). Data are means of two to four determinations. Within each column, numbers followed by the same letter are not significantly different at the 95% probability level, as determined by the Student-Newman-Keuls test.