

Modification of Lignin and Lignin Rich Fibers via Oxoreductase Enzymes

Oxidation of Isolated Lignin with Laccase

Lignin preparations from either pulping processes or from known isolation techniques have been treated with laccase relatively infrequently. This may be due to the media in which the lignin is isolated, since lignin is not water-soluble and laccase enzymes function optimally in aqueous environments.¹ The reaction of laccase with insoluble lignin would be inefficient compared to activity with dissolved lignin due to improved accessibility of the substrate in solution. Nevertheless, there have been a few studies involving milled wood lignin (MWL), black liquor lignins (BLL), lignosulfonates and organosolv lignin. In addition to revealing the effects of laccase with lignin substrates, these experiments have been performed in an effort to find potential uses for lignin waste products from pulp and paper manufacture. In the following discussion, systems where laccase in solution was reacted with insoluble lignin will be referred to as heterogeneous systems and systems where all the components were in solution will be referred to as homogenous systems.

Reactions with Lignin In Heterogeneous Systems

Konishi and Inoue treated milled wood lignin with laccase enzymes from *T.versicolor*.² These authors found that laccase treatments increased the water solubility of the MWL by increasing in the number of carboxylic groups. Konishi and Inoue subsequently analyzed the increase in carboxylic groups in MWL by laccase treatment of model compounds and subsequent infrared analysis. This work verified that laccase treatment oxidizes the phenolic hydroxyl groups on lignin to carboxylic groups.

¹ Chakar, F.S. and Ragauskas, A.J. Laccase-Lignin Reactions. in Sixth European Workshop on Lignocellulosics and Pulp. 2000. Brodeaux, France. p.53-56.

² Konishi, K., Inoue, Y., and Higuchi, T., *Decomposition of Lignin by Coriolus versicolor. V. Formation of Carboxyl Groups in the Lignin Treated with Fungal Laccase*. Mokuzai Gakkaishi, 1974. **20**(1): p. 26-30.

Ishihara and Miyazaki treated MWL with laccase and found that laccase increased the molecular weight.³ In addition to the polymerization, a small fraction of the MWL was also shown to be degraded to low molecular weight quinone structures. These authors concluded that laccase enzymes operated in both polymerization and depolymerization with the former dominating. Additional evidence in favor of lignin polymerization by laccase was revealed when the molecular weight of water insoluble lignin increased when it was added to cultures of the fungus *Fomes annosus*.⁴ Polymerization of lignin was prevented by the addition of a known laccase inhibitor (azide) to the medium.

Kantelinen et al. used laccases to treat black liquor lignin (BLL) and wood powder lignin (WPL).⁵ Both lignin samples were separated into water-soluble and insoluble fractions. With laccase treatment, a decrease and/or complete disappearance of monomeric compounds in the water soluble fraction of the BLL was observed; however, the molecular weight of water insoluble fraction remained the same. Unfortunately the molecular weight data for the treatment of the water-soluble lignin fractions was not shown. Since it is the by-product of milled wood lignin isolation, the WPL possessed a higher molecular weight than the black liquor lignin.⁶ Laccase treatment did not impart changes to the molecular weight of the solid fraction of the WPL, however, similar to the BLL, water-soluble components of the WPL disappeared during the reaction. The inability of laccase to increase the molecular weight of the solid fractions of BLL and WPL was most likely due to the lack of accessibility of the water-insoluble lignin to laccase.

³ Ishihara, T. and Miyazaki, M., *Oxidation of Milled Wood Lignin by Fungal Laccase*. Mokuzai Gakkaishi, 1972. **18**(8): p. 415-419.

⁴ Huttermann, A., Herche, C., and Haars, A., *Polymerization of Water-Insoluble Lignins by Fomes Annosus*. *Holzforschung*, 1980. **34**: p. 64-66.

⁵ Kantelinen, A., Hortling, B., Ranua, M., and Viikari, L., *Effects of Fungal and Enzymatic Treatments on Isolated Lignins and on Pulp Bleachability*. *Holzforschung*, 1993. **47**(1): p. 29-35;
Bjorkmann, A., *Studies of finely divided wood: extraction of lignin with neutral solvents*. *Svensk Papperstidning*, 1956. **59**(447-485)

Reactions with Lignin In Homogenous Systems

Ferm used laccase enzymes to treat methyl cellulose solutions of milled wood lignin (MWL) mixed with up to 50% of water.⁷ Laccase activity decreased 30-70% in the cellulose solution, but retained adequate activity to carry out significant reactions with the lignin. Analysis of the MWL by EPR (electron paramagnetic resonance) indicated the formation of phenoxy radicals during laccase treatment. The amount of radicals were shown to decrease after 20 min. This was most likely due to radical instability and subsequent coupling of the radicals. Free-radical formation was not observed in MWL treated with boiled laccase. Further work by these authors involved laccase treatment of water-soluble lignosulfonates. Not surprisingly, a browning of the lignosulfonate solutions combined with an increase in average molecular weight was observed. Phenoxy radicals have been shown to be formed during laccase treatment of lignin model compounds and isolated lignin. It appears that the formation of phenoxy radicals is the predominant mechanism of lignin oxidation by laccase.

Leonowicz observed laccase-catalyzed polymerization low molecular weight fractions and depolymerization of high molecular weight fractions.⁸ Further evidence for the depolymerization-polymerization “double function” of laccase is given in the work of Bae and Kim as well as the work of Hataaka et al.⁹ Bae and Kim used gel permeation chromatography (GPC) measurements to show that laccase enzymes depolymerized large lignosulfonates at pH 4.5, however, polymerization was shown to occur at pH 6. Unfortunately, these authors were unable to provide any explanation for the action of laccase. Hataaka et al. also treated lignosulfonates with laccase enzymes. These authors observed that laccase was able to depolymerize lignin preparations only if the molecular weight of the sample was less than 10,000 g/mol, however, polymerization was shown to occur with larger molecular weight fragments.

⁷ Ferm, R., Kringstad, K.P., and Cowling, E.B., Formation of free radicals in milled wood lignin and syringaldehyde by phenol-oxidizing enzymes. *Svensk Papperstidning*, 1972. **75**: p. 859-864.

⁸ Leonowicz, A., Szklarz, G., and Wojta-Wasilewska, M., *The Effect of Fungal Laccase on Fractionated Lignosulfonates* (Peritan Na). *Phytochemistry*, 1985. **24**(3): p. 393-396.

⁹ Bae, H.J. and Kim, Y.S. Degradation of lignosulfonates by Simultaneous action of laccase and Mn-peroxidase. in *Biotechnology in the pulp and paper Industry*. 1996. Vienna: ACS; Hataaka, A., Mettala, A., Toikka, B., Hortling, B., and Brunow, G. Modification of lignin by laccase and manganese peroxidase. in *Biotechnology in the pulp and paper Industry*. 1996. Vienna: ACS. p.333-340.

This contrasts the work of Leonowicz⁸ who observed depolymerization of large fragments and polymerization of smaller fragments.

Laccase treatment of organosolv lignin in dioxane-H₂O or passing the lignin solution through a column containing laccase on sepharose beads resulted in the immediate formation of color accompanied by increases in molecular weight. The rapid color formation upon laccase treatment should indicate the formation of quinone structures from phenolic hydroxyl groups. The observed increases in molecular weight were from an average of 3,200 to 20,000 g/mol.¹⁰ Huttermann et al. concluded that the most significant alterations of lignin during treatment with laccase enzymes include solubilization, demethylation, changes in phenolic and aliphatic hydroxyl content and changes in molecular weight distribution.

Poppius-Levlin et al. dissolved lignin from oxygen-delignified pine kraft pulps (O₂-PKP) and conventional pine kraft pulps (PKP) by alkaline extraction.¹¹ The alkali-soluble fractions from the PKP contained a larger number of free phenolic hydroxyl groups than the lignin sample isolated from the O₂-PKP. Laccase treatment of alkali-dissolved lignin resulted in an increase in molecular weight from 900 to 70,000 g/mol in the PKP lignin and 900 to 13,500 in the O₂-PKP lignin. This corresponds to the higher number of phenolic hydroxyl groups in PKP lignin available to react with laccase, compared to O₂-PKP. Chakar and Ragauskas¹ reacted laccase with lignin isolated by acidolysis in a 50:50 dioxane:water mixture with subsequent analysis by ³¹P and ¹³C NMR. Laccase treatment resulted in a five-fold increase in carboxylic acid groups (Figure) in the lignin with a concomitant decrease in non-condensed phenolic, condensed phenolic, and aliphatic hydroxyl groups (Figure 1). Similar to the results of Poppius-Levlin et al.,

¹⁰ Milstein, O., Huttermann, A., Ludemann, H.-D., and Schulze, K. *Enzymatic transformation of organosolv lignin*. in *Biotechnology in the pulp and paper industry*. 1996. Vienna: ACS. p.645-648; Huttermann, A., Milstein, O., Nicklas, B., Trojanowski, J., Haars, A., and Kharazipour, A. *Enzymatic Modification of Lignin for Technical Use*. in *Adhesives from Renewable Resources*. 1989: ACS.

¹¹ Poppius-Levlin, K., Wang, W., Tamminen, T., Hortling, B., Viikari, L., and Niku-Paavola, M.-L., *Effects of Laccase/HBT Treatment on Pulp and Lignin Structures*. *Journal of Pulp and Paper Science*, 1999. **25**(3): p. 90-94.

phenolic groups were shown to be the targets for modification of lignin by laccase. It has been established that laccase can react with lignin and phenolic model compounds causing significant changes in chemical structure and molecular weight. Further studies have investigated the ability of laccase to co-polymerize compounds with lignin.

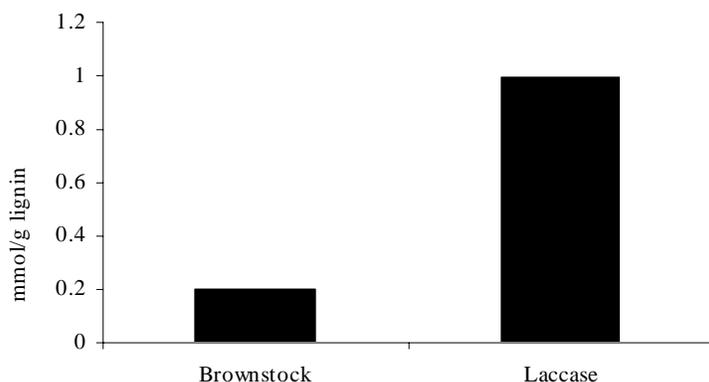


Figure 1. Carboxylic Acid Groups in Laccase-Treated Residual Lignin Isolated from Softwood High-Kappa (78) Pulp Measured by ^{31}P NMR.¹

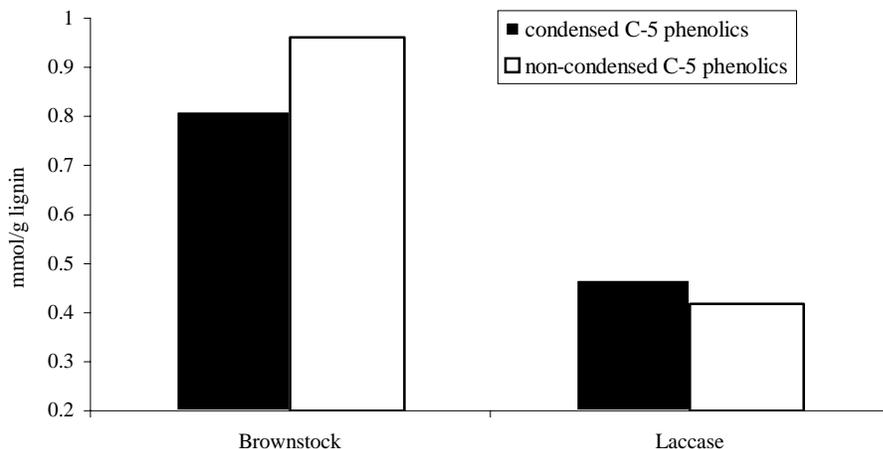


Figure 1: Condensed and Non-Condensed C5 phenolic units in Laccase-Treated Residual Lignin Isolated from Softwood High-Kappa (78) Pulp Measured by ^{31}P NMR.¹

Laccase and Peroxidase Treatments for the Co-polymerization of Compounds with Lignin

Peroxidase Treatments

Oxidoreductase treatments have been applied to evaluate the potential of increasing the value of lignin as a possible resin or alternative plastic material. Work in this area has been rare; therefore it is useful to discuss all oxidoreductase treatments rather than those that employed laccase exclusively. Peroxidases were first employed for their potential to increase the value of lignin as a resin by Popp et al. The study employed ^{14}C -p-cresol with kraft and milled wood lignin and monitored the reaction by gel-permeation chromatography in a dioxane:water solution (7:3). It was shown that the co-polymerization reaction did not occur with acetylated lignin or methylated lignin (phenolic groups blocked), indicating the necessity for the peroxidase to form phenoxy radicals with both the p-cresol and the lignin. Based on this observation, Popp et al. proposed a mechanism for the co-polymerization reaction. The proposed mechanism involves the coupling of phenoxy radicals created by peroxidase on lignin and on p-cresol (Figure 2).

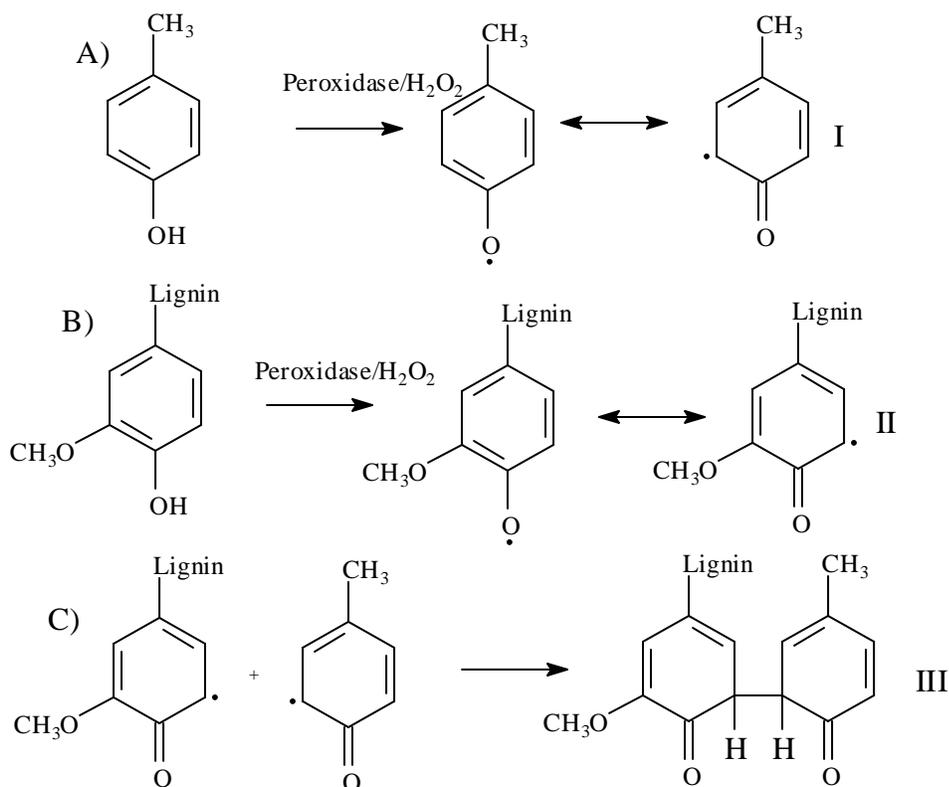


Figure 2: Proposed mechanism of Peroxidase-Catalyzed Co-Polymerization of *p*-Cresol with Lignin¹²

A second study by Blinkovsky and Dordick showed that a co-polymer can be created by the peroxidase-catalyzed reaction of lignin with *p*-phenylphenol.¹³ The co-polymer consisted of 80% lignin. The resulting polymer became insoluble in dimethylformamide presumably due to cross-linking within the structure. A remarkable decrease in glass transition temperature from 180°C (lignin) to 98°C (lignin *p*-phenylphenol) was also observed. It is interesting to note that the polymerization of the phenolic compounds with lignin decreased significantly when the proportion of water in the reaction mixture was greater than 50%. At a concentration of water

¹² Popp, J.L., Kirk, T.K., and Dordick, J.S., *Incorporation of p-cresol into lignins via peroxidase-catalyzed copolymerization in nonaqueous media*. Enzyme and Microbial Technology, 1991. **13**: p. 964-968.

¹³ Blinkovsky, A.M. and Dordick, J.S., *Peroxidase-catalyzed synthesis of lignin-phenol copolymers*. Journal of Polymer Science, Part A: Polymer Chemistry, 1993. **31**: p. 1839-1846.

greater than 50%, the lignin became markedly less soluble, indicating that the insoluble lignin was less reactive with peroxidase.

Laccase Treatments of Lignin

Milstein et al. used laccase to co-polymerize organosolv lignin (dioxane-H₂O) with a variety of compounds.¹⁴ Laccase treatments were the most effective in co-polymerization of lignin with aromatics containing carboxyl functional groups. Using ¹⁴C-labelled vanillic acid these authors showed that laccase was capable of decarboxylating this compound. However, upon the addition of organosolv lignin, the decarboxylation ceased and the radio-labeled vanillic acid was incorporated into the lignin to form a co-polymer].

In later work, Milstein et al. used laccase to co-polymerize lignin with acrylamide. Laccase facilitated co-polymerization was compared to polymerization initiated by chemicals.¹⁵ Milstein et al. found that laccase was able to polymerize lignin and acrylamide as indicated by the increase in molecular weight from 14,900 to 27,800 upon incubation with laccase. Unfortunately, the yield of polymerization of laccase with acrylamide was less than that achieved by chemical means. However, the proportion of the constituents and structure of the co-polymer showed no significant differences from the acrylamide-lignin co-polymer formed by chemical initiation. It was concluded that both the chemical and enzyme mediated initiation proceeded via a similar free radical mechanism.^{15, 16}

Mai et al. later showed that the addition of organic peroxides to the reaction of laccase with organosolv lignin and acrylamide significantly increased the yield of the acrylamide/lignin co-

¹⁴ Milstein, O., Huttermann, A., Frund, R., and Ludemann, H.-D., *Enzymatic co-polymerization of lignin with low-molecular mass compounds*. Applied Microbiology and Biotechnology, 1994. **40**: p. 760-767.

¹⁵ Milstein, O., Mai, C., and Huttermann, A. Enzymatic copolymerization of lignin with low molecular weight compounds. in Proceedings of the Sixth International Conference on Biotechnology in the Pulp and Paper Industry. 1996. Vienna. p.645-648.

¹⁶ Meister, J.J. and Patil, D.R., *Solvent effects and Initiation Mechanisms for Graft Polymerization on Pine Lignin*. Macromolecules, 1985. **18**: p. 1559-1564.

polymer.¹⁷ It was postulated that peroxides may act as laccase mediators, producing radicals upon reaction with acrylamide while phenoxy radicals from lignin are produced by laccase. The authors proposed that the acrylamide radicals combined with the lignin phenoxy radicals to produce the lignin-acrylamide co-polymer. Later studies by Mai investigated the effects of varying types of organic peroxides, and the use of lignosulfonates to improve the grafting reaction.¹⁸

Lund et al. reacted guaiacol sulfonate and vanillylamine with Indulin (kraft lignin).¹⁹ The reaction with vanillylamine and the Indulin in the presence of laccase formed a precipitate containing nitrogen indicating a co-polymerization, while the reaction with guaiacol sulfonate did not form a precipitate. In a later study, Lund and Ragauskas reacted kraft lignin in dioxane with 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, guaiacol sulfonate, and 4-hydroxybenzene sulfonic acid with subsequent characterization by potentiometric titration and NMR.²⁰ Guaiacol sulfonate (GS) and 4-hydroxyphenylacetic acid (HPA) were successfully incorporated into lignin. An increase in carboxylic groups in lignin was shown to be the result of the reaction with HPA, however, in the case of GS; the resulting co-polymer became water soluble at pH 2.4. This was an unprecedented result. These authors hypothesized that the methoxyl group on GS and the methylene group on the HPA acted as electron donating groups that stabilized phenoxy radicals enabling polymerization with lignin instead of self-condensation.

¹⁷ Mai, C., Milstein, O., and Huttermann, A., *Fungal laccase grafts acrylamide onto lignin in presence of peroxides*. Applied Microbiology and Biotechnology, 1999. **51**: p. 527-531.

¹⁸ Mai, C., Milstein, O., and Huttermann, A., *Chemoenzymatical grafting of acrylamide onto lignin*. Journal of Biotechnology, 2000. **79**: p. 173-183.; Mai, C., Schormann, W., and Huttermann, A., *Chemoenzymatically Induced Co-polymerization of Phenolics with Acrylate Compounds*. Applied and Microcal Biotechnology, 2001. **55**: p. 177-186.; Mai, C., Schormann, W., Huttermann, A., Kappl, R., and Huttermann, J., *The influence of laccase on the chemo-enzymatic synthesis of lignin graft-copolymers*. Enzyme and Microbial Technology, 2002. **30**: p. 66-72.

¹⁹ Lund, M., Felby, C., and Bjerrum, M. *Modification of kraft pulp and lignin by copolymerisation of phenolic compounds initiated by laccase*. in *7th International Conference on Biotechnology in the Pulp and Paper Industry*. 1998. Vancouver, Canada. p. C129-132.

²⁰ Lund, M. and Ragauskas, A., *Enzymatic Modification of Kraft Lignin Through Oxidative Coupling with Water Soluble Phenols*. Applied Microbiology Biotechnology, 2001. **55**: p. 699-703.

Laccase Treatment of Pulp Fibers

The use of laccase enzymes to improve the bonding between pulp fibers has been applied frequently compared to other oxidoreductase enzymes.²¹ The treatments usually involve the application of laccase enzymes to activate lignin on fibers (one-component system) or the addition of another component with laccase to act as a potential cross-linking agent (two-component system). Because laccase enzymes are too large to penetrate fibers (55-80 kDa) treatments should only result in a surface modification. Therefore, during treatments of fibers to increase paper/board strength, free phenolic groups on the fiber surface should act as potential reactive sites for laccase enzymes to create phenoxy radicals.

Laccase Treatments to Improve the Strength of Boards

Auto-adhesion has been described as a phenomenon whereby under a specific set of processing conditions, hot-pressed fibers or wood particles can adhere to each other. The factors suspected to affect auto-adhesion include the flow of lignin and hemicellulose.²² Unfortunately, the level of achievable auto-adhesion is insufficient to warrant the elimination of conventional adhesives. Another approach is the use of enzymes to enhance auto-adhesion. Early laccase treatments of fibers for the improvement of bonding strength involved the use of two-component systems employing laccase and an additional compound followed by hot-pressing. The goal of these treatments was to achieve cross-linking between lignin on adjacent fibers.

²¹ Haynes, K. and Ragauskas, A.J. Effects of Laccase Mediator Delignification on Fiber Properties. in International Pulp Bleaching Conference. 1998. Helsinki, Finland. p.355-358; Felby, C., Olesen, P.O., and Hansen, T.T. Laccase catalyzed bonding of wood fibers. in Biotechnology in the pulp and paper industry. 1996. Vienna: ACS. p.89-97; Lund, M. and C., F., Wet Strength Improvement of Unbleached Kraft Pulp Through Laccase Catalyzed Oxidation. *Enzyme and Microbial Technology*, 2001. **28**: p. 750-765; Kharazipour, A., Huttermann, A., and Luedmann, H.D., Enzymatic activation of wood fibers as a means for the production of wood composites. *Journal of Adhesion Science and Technology*, 1997. **11**(3): p. 419-427; Kharazipour, A., Huttermann, A., Luedmann, H.D., and Nonninger, K., Properties of fiber boards obtained by activation of the middle lamella lignin of wood fibers with peroxidase and H₂O₂ before conventional pressing. *Journal of Adhesion Science and Technology*, 1998. **12**(10): p. 1045-1053.

²² Back, E.L., *The Bonding Mechanism in Hardboard Manufacture*. *Holzforschung*, 1987. **41**(4): p. 247-257.

Huttermann et al.²³ hypothesized that laccase could be used in combination with spent sulfite liquor as an adhesive for particleboard. In addition to cross-linking, these authors claimed that the use of less polar milled wood lignin could increase the water resistance of boards in contrast to using more hydrophilic lignosulfonates.

Yamaguchi et al.²⁴ utilized laccase in a two-component system with vanillic acid, catechol, Mimosa tannin (*Acadia mollissima*), and tannic acid (m-galloylgallic acid). These compounds were polymerized by laccase and subsequently combined with thermomechanical pulp. The ply-bond strength of the paperboard made from hot-pressing the polymer-precipitated TMP was 2 to 4 times greater than the control. These authors suggested that laccase treatments loosened the rigid three-dimensional framework of lignin on the TMP fibers while simultaneously activating the lignin on the fiber surface. The activation of surface lignin would allow for cross-linking with the precipitated DHP.

Kharazipour et al.²¹ used laccase to treat mechanical pulp fibers that were later used for the formation of medium density fiberboard (MDF). The laccase treatment resulted in an increase in internal bond strength from 0.1 to 0.52 N/mm². Through the use of ¹³C-NMR these authors showed that laccase treatment resulted in the formation of carboxylic groups and a decrease in phenolic groups.. This indicated that the phenolic hydroxyl groups were oxidized to carboxylic groups. These results are similar to the observations made by Chakar and Ragauskas¹ and Konishi² in their reactions of laccase with lignin. Kharazipour et al. postulated that the high temperature fiberization of wood during the thermomechanical pulping process caused the lignin in the wood to flow and cover the fiber surface, facilitating accessibility to laccase. These authors believed that both the enzymatic activation of fibers and the hot-pressing stage were necessary to achieve increase in the strength of the MDF. Hansen et al. have patented a treatment of kraft pulp for the formation of linerboard using laccase treatment in buffer for 2

²³ Huttermann, A., Milstein, O., Nicklas, B., Trojanowski, J., Haars, A., and Kharazipour, A. *Enzymatic Modification of Lignin for Technical Use*. in *Adhesives from Renewable Resources*. 1989: ACS.

²⁴ Yamaguchi, H., Maeda, Y., Sakata, I.,, *Applications of Phenol Dehydrogenative Polymerization by Laccase to Bonding among Woody-Fibers*. Mokuzaigakkaishi, 1992. **38**(10): p. 931-937.

hours with subsequent dewatering.²⁵ However, this patent neglected to present any strength properties for their hypothetical linerboards. In a series of extensive studies, Felby attempted to reveal the mechanisms behind the strength improvements achieved during laccase treatment of mechanical pulp fibers.

The application of laccase to beech (*Fagus sylvatica*) thermomechanical pulp (TMP) fibers to enhance auto-adhesion during the formation of MDF has been studied by Felby. The dry-process boards made from laccase treated fibers had substantially improved strength properties over the control boards (Table 1).²⁶ The results show a marked decrease in water absorption. Measurements of contact angle of fibers with water revealed an increase from 23 degrees for the untreated pulp to 63 degrees for the laccase treated pulp. The increase in hydrophobicity with laccase treatment may be due to cross-linking of lignin at the fiber surface.

Table 1: Dry Process Boards^a Made From Laccase-treated Beech Fibers Showing Increases in Strength Properties

Treatment	Density g/cm ³	Modulus of Rupture (Mpa)	Modulus of Elasticity (Mpa)	Internal Bond Strength (Mpa)	Water Absorption (%)
Blank	0.906	27.4	3.61	0.95	122
Inactive Laccase ^b	0.850	25.3	3.42	0.91	143
Laccase	0.895	27.4	4.02	1.57	72

^a air-laid fibers pressed at 200°C for 5 min

^b laccase deactivated in an autoclave

²⁵ Hansen, T.T., Holm, H.C., and Franks, N.E., *Process For Production of Linerboard and Corrugated Medium*. 1997, Novo Nordisk A/S: United States Patent no. 5603804.

²⁶ Felby, C., Pedersen, L.S., and Nielsen, B.R., *Enhanced Auto-Adhesion of Wood Fibers Using Phenol Oxidases*. *Holzforschung*, 1997. **51**(3): p. 281-286; Felby, C., Nielsen, B.R., Oleson, P.O., and Skibsted, L.H., *Identification and Quantification of Radical Reaction Intermediates by Electron Spin Resonance Spectrometry of Laccase Catalyzed Oxidation of Wood Fibers from Beech*. *Applied Microbiology and Biotechnology*, 1997. **48**: p. 459-464.

Felby et al. also measured the amount of free radicals formed as a result of laccase treatments using electron spin resonance spectroscopy (ESR).²⁶ The ESR measurements made 24 hours after laccase treatment showed a five-fold increase in free-radical activity compared to the control. In fact, these radicals were found to have a half-life of 2 weeks in the pulp fibers. Free radical activity of control and laccase-treated samples obtained are shown in Table 2. It is evident from the data that laccase was effective in creating free radicals in the fibers.

Table 2: Free Radical Activity (arbitrary units) Measured by ESR-spectroscopy by Felby for Beech Thermomechanical Pulp Fibers Treated with Laccase.²⁶

Treatment	Wet Fibers (Free radical activity)	Dry fibers (Free radical activity)
Blank	78	87
Inactivated Laccase	81	82
Laccase ^a	537	488

^a laccase deactivated in an autoclave

In addition to the ESR studies, Felby performed differential scanning calorimetry on treated pulp fibers to show whether laccase treatment affected the glass transition temperature of lignin.²⁸ The DSC measurements showed little difference between the control and the laccase treated pulps. It should be noted that DSC scans for the glass transition of the heterogeneous mixture of polymers that constitute a lignocellulosic pulp fiber is difficult because of the low sensitivity of a DSC compared to other methods. The fibers should also show multiple transitions as a result of the onset of molecular motion from different components and their side groups.²⁷ Since the glass transition temperature did not change, Felby attributed the beneficial effect of laccase to an alteration of the fiber surface by laccase.²⁸

²⁷ Hatakeyama, H., Kubota, K., and Nakano, J., *Thermal Analysis of Lignin by Differential Scanning Calorimetry*. Cellulose Chemistry and Technology, 1972. **6**: p. 521-529.

²⁸ Felby, C., *Laccase Catalyzed Oxidation of Fibers from Beech (Fagus Sylvatica)*, in *Plant Fibre Laboratory*. 1997, Royal Veterinary and Agricultural University: Taastrup, Denmark. p. 151.

In a later study, Felby et al. focused on elucidating the mechanism behind the radical generation in the beech wood fibers by laccase. This study confirmed the formation of phenoxy radicals by laccase treatments.²⁶ In this study it was shown that the phenoxy radicals in the fibers decayed faster after removal of the fibers from the laccase reaction solutions. It appeared that contact between laccase and the fibers in the reaction solution was necessary for the continual steady-state concentration of radicals to be maintained. The authors attributed the necessity for fiber/enzyme contact to a cyclic radical flow mechanism between fiber lignin and colloidal lignin in solution, where the colloidal lignin functions as an enzyme mediator that enters the fiber matrix to oxidize lignin (Figure 3).

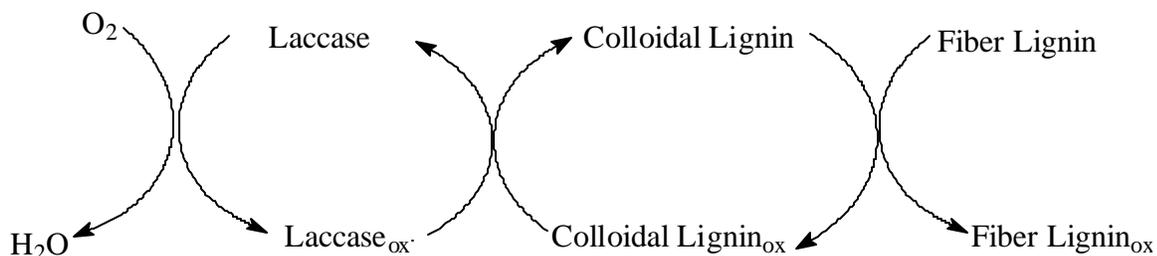


Figure 3: Felby's Proposed Mechanism for the reaction of Laccase with Beech Thermomechanical Pulp Fibers²⁶

Hassingboe and Felby used dialysis to remove the water-soluble extractives and colloidal lignin from a pulp slurry.²⁹ Subsequent reaction of the dialyzed pulp with laccase did not result in the strength improvements observed by Felby . The addition of ABTS (a laccase mediator) to the water extracted pulp restored the ability of laccase to increase the strength of MDF. These results suggested that colloidal lignin and extractives were acting as mediators during the

²⁹ Hassingboe, J., Lawther, J.M., and Felby, C. Influence of Extractives on Enzymatic Catalyzed Bonding of Norway Spruce TMP Fibers. in *Biotechnology in the Pulp and Paper Industry*. 1998. Vancouver, British Columbia. p.A125-A128.

reaction of pulp fibers with laccase similar to the mechanism illustrated in Figure 3. Barsberg³⁰ investigated the effects of laccase treatments on beech fibers using molecular modeling. This study used the rate of formation of laccase-generated radicals (measured by oxygen consumption) as a basis for a rather complex theoretical approach. Barsberg proposed that the initial rapid formation of laccase-generated radicals during reactions with pulp fibers reflects the reaction of the laccase enzyme at the fiber surface. The slower radical generation as the reaction progresses is due to radical propagation to the interior of the fibers.

Laccase Treatments to Improve the Strength of Paper Sheets

Studies employing laccase to improve the strength of paper sheets initially focused on employing two-component systems followed by hot-pressing in attempts to improve sheet bonding. Later studies shifted their focus toward treatments of high-yield pulp fibers with laccase and subsequent formation of wet-pressed handsheets.

Huttermann et al.³¹ combined kraft lignin pre-treated with laccase with spruce sulfite pulp. The resulting handsheets were press-dried at 105°C. These sheets had higher tear strength and wet strength than untreated controls, however, it should be noted that these authors did not show the results of their paper tests. Apparently, after 6 hours, the lignin was irreversibly bound to the fibers. This irreversible bond between lignin and cellulose facilitated by laccase was confirmed in a later study. Subsequent X-ray microanalysis in combination with transmission electron microscopy revealed that a high proportion of lignin was localized at contact areas between the fibers. The results indicate that lignin may have acted as a “glue” between the fibers to improve paper strength. It is interesting to note that in the successful findings of both Huttermann and

³⁰ Barsberg, S., Thygessen, L.G., and Felby, C. *Detection of Laccase Induced Modification of Beech and Spruce TMP Fibres by Luminescence Spectroscopy*. in *7th International Conference on Biotechnology in the Pulp and Paper Industry*. 1998. Vancouver, Canada. p.C123-126.

³¹ Huttermann, A., Kharazipour, A., Schindel, K., Fastenrath, M., Noetzold, S., and Schroeter, M. *Enzymatic Reactions of Activated Lignin with Nucleophiles as a Basis for the Design of Compound Materials with Lignin*. in *7th International Conference on Biotechnology in the Pulp and Paper Industry*. 1998. Vancouver, Canada. A207-A209.

Yamaguchi two-component laccase treatments systems were followed by an application of a “hot-pressing” step.³² The strength improvements may be due to enhanced bonding between fibers achieved with the combination of lignin “activated” by laccase and the hot-pressing step.

Viikari et al used laccase enzymes to treat softwood mechanical pulps and found dramatic increases in both wet and dry tensile indices. These authors attributed the strength improvements of laccase treatment to the polymerization of lignin by laccase.³³ Felby et al. has shown that treatment of beech TMP fibers with laccase and a ferulic acid-arabinoxylan dimer was able to impart wet strength (the controls disintegrated) and decrease the thickness to half of that of control pulps. Dry tensile also increased from 8.1 Nm/g to 47.0 N/m/g. The explanation given for the strength increases was the coupling of the ferulic acid to the pulp fiber with subsequent enhancement of fiber bonding by the arabinoxylan portion of the dimer. A patent by Pederson et al. also describes the attachment of phenolic compounds for increasing fiber charge, however the patent did not describe any paper strength results.³⁴

Wong et al. and Mansfield have both performed studies applying laccase treatments to mechanical pulps.³⁵ Wong et al. found strength increases of 5-10% during laccase treatment of thermomechanical pulp fibers. Mansfield impregnated wood chips with laccase prior to mechanical pulping resulting in improved refining efficiency and sheet properties. The most

³² Yamaguchi, H., Maeda, Y., Sakata, I., *Applications of Phenol Dehydrogenative Polymerization by Laccase to Bonding among Woody-Fibers*. Mokuzai Gakkaishi, 1992. **38**(10): p. 931-937; Yamaguchi, H., Maeda, Y., and Sakata, I., *Bonding among Woody Fibers by Use of Enzymatic Phenol Dehydrogenative Polymerization: Mechanism of generation of bonding strength*. Mokuzai Gakkaishi, 1994. **40**(2): p. 185-190.

³³ Viikari, L., Harkki, T., Niku-Paavlova, M.-L., Buchert, J., and Popplus-Levlin, K. *Oxidative Enzymes for Fibre Modification*. in *7th International Conference on Biotechnology in the Pulp and Paper Industry*. 1998. Vancouver, B.C. p. A121-A124.

³⁴ Pedersen, L.S., Munk, N., and Felby, C., *A Method for Increasing the Surface Charge of Lignocellulosic Fibers*, 1995, Novo Nordisk A/S: European Patent. no.879318.

³⁵ Wong, K.K.Y., Richardson, J.D., and Mansfield, S., *Enzymatic Treatment of Mechanical Pulp Fibers for Improving Papermaking Properties*. *Biotechnology Progress*, 2000. **16**: p. 1025-1029; Mansfield, S., *Laccase Impregnation During Mechanical Pulp Processing - Improved Refining Efficiency and Sheet Strength*. *Appita*, 2002. **55**(1): p. 49-53.

probable explanation for the improved refining and sheet properties is a softening of lignin within chips prior to mechanical pulping, resulting in lower refining energy consumption and less fiber destruction during the refining process.

