Modification of Lignocellulosic Materials by Laccase

William Kenealy, John Klungness, Mandla Tshabalala, and Roland Gleisner, USDA Forest Service, Forest Products Laboratory, Madison, WI
Eric Horn and Masood Akhtar, Biopulping International, Madison, WI
Hilda Zulaica-Villagomez, University of Wisconsin, Madison, WI
Gisela Buschle-Diller, Auburn University, Auburn, AL

ABSTRACT

Altering the surface properties of pulp can enhance binding, increase paper strength, and decrease the cost of fiber. In this study, we modified lignocellulosic materials (bark and pulp) with laccase and selected substrates to change the nature of the pulp surface. Modified pulps were evaluated by the amount of methylene blue (a cationic dye) that would bind to the pulp and the acid–base and dispersive results of inverse gas chromatography (IGC) analysis. Combinations of laccase and substrates that altered various surface properties of the pulp were determined. Methylene blue binding increased for many of the substrates but did not correlate with the IGC-determined acid–base characteristics of the pulp, indicating involvement beyond cation binding. Substrates that altered the surface characteristics of loblolly pulp were used on a thermomechanical spruce pulp. The treated spruce pulps were analyzed for handsheet strength and binding characteristics. Modest strength improvements were observed in some of the treatments. Laccase treatments to alter pine bark were also explored.

INTRODUCTION

Surface characteristics of pulp fiber influence the strength of the paper produced from the pulp and often determine which products the fiber might be used for. Alteration of the fiber to contain carboxylic acid groups or other ionic groups and grafting of different polymers onto the pulp can change the range of products in which the fiber can be used. Aside from fiber length and morphology related issues, any fiber could be tailored for any purpose if we understood how to systematically modify the fiber. Altering the surface properties to enhance binding, increase the strength, and decrease the cost of fiber are identified as objectives in fiber engineering [1].

Enzymatic methods of altering fibers have been sought because these methods are catalytic, use less chemicals, are deemed more controlled, and are less likely to damage the fiber than chemical methods. Laccase, manganese peroxidase, and peroxidase have all been used to modify polymers by free radical based mechanisms. Manganese peroxidase generates Mn^{+++} from hydrogen peroxide. The enzyme requires Mn^{++}, a chelator and hydrogen peroxide, and can oxidize target molecules at a distance from the enzyme. The direct oxidation of a polymer by laccase or peroxidase requires contact with the polymer. To overcome this contact problem, laccase was reacted with soluble diffusible substrates (mediators) to generate long-lived radicals that can oxidize targets at a distance from the enzyme [2,3]. Laccase-mediator systems have been described that assist with delignifying and bleaching of pulps [4-6].

Several researchers have explored the use of laccase to modify the surface characteristics of lignocellulose [7-10]. Particle boards, liner boards, and composites have been made with laccase and phenolic-containing compounds as the adhesive [11-13]. Thermomechanical pulps (TMP) and high lignin pulps have been treated with laccase, which improved strength and decreased the need for bleaching chemicals [14-16]. Chandra and Ragauskas [17] reported strength increases by grafting laccase substrates onto linerboard softwood kraft pulp. They used guaiacol sulfonate, tyrosine, and 4-hydroxyphenylacetic acid with laccase, but there was strength improvement only with the addition of 4-hydroxyphenylacetic acid. The strength increase was attributed to the addition of carboxylic acids groups to the surface of the fiber.

Laccases have been purified from a variety of sources and have a vast substrate range [18,19]. The individual enzymes have different oxidative capabilities [19,20] and, depending on the amino acid sequence, will oxidize a greater variety of substrates than other enzymes [21,22]. The vast substrate range, the nature of free radical reactions, and the complexity of polymers like lignocellulose have made it impractical to predict the products that can be formed by these reactants. Only in carefully defined conditions where the substrates have relatively few resonance structures can a predictable product be obtained in high yield [23,24].

The vast substrate range of laccase can also be oxidized by manganese peroxidase. Manganese peroxidase is commercially available in much smaller amounts and has not yet been made as a commodity enzyme. While Mn^{+++} will also oxidize the same substrates as laccase, the generation of Mn^{+++} by manganese peroxidase has the additional requirements of a chelator and hydrogen peroxide addition or generation. There have also been reports of laccase generating Mn^{+++} if sufficient oxalate is present.

The purpose of this work is to survey an array of laccase substrates to determine if they can be grafted onto fiber with laccase to alter the surface characteristics of pulp fiber. Changes were determined empirically since the complexity of possible reactions is great. Once potential changes were identified, handsheet strength was used to determine if there is a benefit from the treatment. Further work will focus on conditions where beneficial changes have been observed.

Materials and Methods

Dry TMPs (mature loblolly pine (*Pinus taeda*)) were obtained through the U.S. Department of Agriculture at Pineville, Mississippi. Labels 4, 8, and 12 bar indicate the steam pressure used during the TMP process. Spruce (*Picea glauca*) chips were refined at 103 kPa followed by atmospheric refining to 350 or 44 mL Canadian Standard Freeness (CSF). Spruce pulps (350 mL CSF) were further refined after enzyme treatments using a PFI (Testing Machines Inc., New York) mill to reduce the freeness. Laccase NovoSample 51002 was obtained from Novozymes (Denmark).

PULP TREATMENTS

Dry loblolly pine pulp samples (2 g) were weighed into Erlenmeyer flasks, 75 mL deionized water was added, and the mixture was stirred for at least 3 h at room temperature to ensure complete wetting. Sodium tartarate buffer (5 mL, 200 m*M*, pH 5) and laccase (5µL, 1 unit) were added and stirred for 20 to 25 min before 1 mL substrate (0.3 *M*) was added. After approximately 25 min of stirring, another 20-µL laccase was mixed in. The samples were stirred for 1 to 2 h at room temperature and then loosely covered with foil and placed in the refrigerator for 15 to 17 h. After warming to room temperature, the supernatant solution was filtered off and the pulps washed three times with 1.5 L of distilled water. Finally, they were strained through a sieve and dried in the freeze-drier for at least 48 h. They were then stored in a dessicator until analyzed. Laccase activity was determined using a YSI oxygen meter with a temperature controlled stir cell.

Spruce pulps were treated at 12% consistency with 300 units manganese peroxidase per 120 g wet pulp. Malonate pH 5 buffer (50 m*M*) was used with 0.1 m*M* $MnSO_4$ and 0.8 m*M* H_2O_2 (added slowly with time) at 37°C for 24 h. The conditions described by Chandra and Ragauskas [17] were used to treat spruce pulp with 4-hydroxyphenylacetic acid and laccase. For the resorcinol, phloroglucinol, and catechol treatments, 315 g of delatent spruce pulp (27% solids) was weighed into plastic bags and mixed with 50 mL 200 m*M* tartarate buffer pH 4.6. Laccase (0.4 mL) was added to 50 mL of distilled water, and 150 mL more water was added. The solution was incubated at 37°C for 1 h. Substrates (50 mL water containing 0.7 to 1 g) were added at hourly intervals and the contents of the bag were mixed. After three substrate additions, the bags were incubated for 18 h, diluted to 2.2 L with hot water, and disintegrated in a handsheet disintegrator for 5,000 revolutions. The pulps were filtered and submitted for handsheet tests. A portion of the pulp was freeze-dried. The freeze-dried material was bleached (at a final consistency of ~10%) using 5% H_2O_2 (on dry pulp) in 0.2% $MnSO_4$ and 4% Na_2SiO_3 at pH ~11 for 2.75 hours at 70°C. The bleached pulps were suspended in hot water, disintegrated as above, and submitted for paper tests.

METHYLENE BLUE BINDING

Known weights of pulp (up to 150 mg) were placed in 50-mL centrifuge tubes with 5 mL 1 *M* borate buffer (pH 8.5) and five clean steel balls. The tubes were then mixed to break clumps. Aqueous methylene blue (35 mL of 1 m*M*) was added and tubes tumbled for 1 h. Samples (1.5 mL) were removed and centrifuged at 20,000×g for 5 min. Two dilutions of 0.5 mL into 5 mL H_2O + 0.125 mL 1 N HCl were made and mixed. Two (0.2 mL) samples from each dilution were analyzed at 610 nm on a Molecular Devices (Sunnyvale, CA) Spectra Max Plus microtiter plate reader. Micromoles of dye adsorbed by pulp equals $35\times(1 - A_{sample}/A_{blank})$. Determination of the binding of methylene

blue was by plotting the amount of dye bound compared with the weight of pulp and determining the slope of the line (with at least three points).

INVERSE GAS CHROMATOGRAPHY

Approximately 1 g of pulp was packed into stainless steel chromatographic columns of 0.5-m length and 0.63 mm in diameter. A Hewlett Packard (Palo Alto, CA) 5790A gas chromatograph was used with helium as carrier gas at a flow rate of 15 mL/min. The temperature of the flame ionization detector was set to 200°C and the injector at 150°C. Measurements were performed at five different oven temperatures from 40°C to 80°C in 10°C increments. For the reference line, five n-alkanes were used (n-pentane to n-nonane), and as polar probes tetrahydrofuran, acetone, and chloroform were selected as the basic, amphoteric, and acidic probe, respectively. Results for the dispersive component were obtained according to Felix and Gatenholm [25] by plotting $RT\ln V_n$ with $2Na(\gamma_l^d)^{1/2}$, with V_n the net specific retention volume, a the surface area occupied by the probe, γ_l^d the dispersive energy of the probe, R the gas constant, and T the temperature. The plot of $RT\ln V_n$ with $2Na(\gamma_l^d)^{1/2}$ gives a straight line. From the slope of this line, the surface energy of the substrate can be determined at each temperature. Polar probes deviate from the straight relationship due to specific interactions, including Lewis acid–base interactions. If the vertical distance of each of the polar probes is measured to the straight line, the free energy of sorption can be determined and plotted as a function of temperature. The slope of that line is related to the free enthalpy of adsorption–desorption. Donor (DN) and acceptor (AN) numbers of the polar probes are available in the literature. A plot of the free enthalpy of sorption with DN/AN allows the determination of acidity (K_A, slope) and basicity (K_D, intercept) of the substrate. Determinations were made for two independent samples and results averaged.

PAPER TESTING

Handsheets (60 g/m^2) were made according to TAPPI standards T 205 sp-95. Burst, tear, and tensile strength were determined according to TAPPI standards T 403 om-97, T 414 om-98, and T 494 om-96, respectively.

Bark Reactions

Southern pine bark was milled, extracted, activated, and reacted with bisaminopropylethylenediamine (BAPED). Laccase (10 μL) was reacted with 0.5 to 0.7 g bark in 5 mL of 40 m*M* tartarate buffer pH 5.0 for 30 min at room temperature. Phloroglucinol (1 mL of 0.3 *M* in ethanol) or catechol (1 mL of 0.3 *M*) were added and incubated 3 h at room temperature, followed by 18 h at 5°C. The particles were rinsed and recovered by filtration and then freeze dried. They were tested for the ability to remove phosphate from solution.

Atomic Force Microscopy

Contact–friction images were created using silicon nitride cantilevers (Nanoprobes, Digital Instruments, Santa Barbara, CA, USA) of nominal 0.58 N/m spring constant mounted in a Nanoscope III MultiMode SPM (Digital Instruments) with an extender electronics module mounted on a pneumatic vibration isolation table. The SPM was equipped with a J vertical scanner with scan size of 125 by 125 μm and vertical range of 5 μm. All imaging was performed under controlled relative humidity (50±2% relative humidity) by flushing nitrogen through a humidity generator (Model RH1500, VTI, Miami, FL, USA) into the sample chamber, which was enclosed with a Humplug (BioForce Laboratory, Ames, IA, USA). A single pulp fiber was glued to a glass slide using a thin line of epoxy (clear 2-ton epoxy). The pulp fiber was then placed in the glue using a 20-μm platinum wire attached to a three-dimensional translator stage in a procedure similar to that reported previously [26]. The epoxy was allowed to cure for 24 h before imaging.

RESULTS AND DISCUSSION

In initial testing, loblolly pine fiberboard furnishes produced at 4-, 8-, and 12-bar steam pressure were used to evaluate laccase activity and alterations in surface properties. These furnishes were easily drained and rinsed, allowing for rapid evaluation of various laccase substrates. Laccase was incubated with a sample of each furnish at 37°C, and the rate and extent of oxygen consumption was measured. Oxygen consumption by laccase was fastest for the 12-bar furnish with 12.0 nmoles oxygen consumed per milligram furnish. The 4- and 8-bar furnishes had 4.0 and

8.4 nmoles oxygen consumed per milligram furnish. The majority of the testing work was performed with the 12-bar pulp.

Laccase reactions catalyzed a one-electron oxidation leaving a free radical that can further react with other compounds. Depending on the reactants, a variety of products can be produced that may contain anionic groups. Anionic groups can be estimated by conductometric titrations, or the binding of cations such as metal atoms or dyes (e.g., methylene blue) [27, 28]. Conductometric titrations are very time consuming, and the methylene blue adsorption correlated well with conductometric results using pulp [27]. Thus, we modified the method of methylene blue adsorption [28] to determine the amount of anions bound to the fiber.

Single measurements of methylene blue sorption on pulp fibers gave different results, depending on the reagent preparation and dyeing conditions. Therefore, the assay was adjusted so the sorption of methylene blue to a pulp sample could be determined reproducibly. The final form of the assay is described in the methods and uses at least three different samples of pulp with increasing weights, all of which adsorb less than half of the methylene blue in the assay.

We incubated 12-bar loblolly pine pulp in the presence of laccase with and without various laccase substrates as described in the methods. The results of the analysis of these fibers in the methylene blue adsorption assay are presented in Table I. Methylene blue is positively charged at pH 8.5, and adsorption can be due to ionic binding to negative charges on the pulp. It was expected that the laccase substrates containing acid groups would interact more than those without acid groups. Methylene blue binding did not directly correlate with acid content of the substrate. Methylene blue also has aromatic character and could be attached by other means. No discernable feature of ring substitution on the phenolic ring correlated with methylene blue binding. The presence of oxygen (a necessary substrate for laccase-catalyzed reactions) can also change the nature of the products and might be indicated by IGC analysis.

Table I. Methylene blue binding of laccase-treated pulps

Substrate used with 12-bar loblolly pine pulp	Methylene blue binding μMoles/g
2,5 dihydroxybenzoate	149
Control + laccase	161
Catechol	183
Control (no laccase)	188
Vanillic acid	192
Hydroquinone	199
4 hydroxy phenyl urea	204
Phenol	206
Isovanillic acid	208
Guaiacol	208
Phloroglucinol	209
3,4 dihydroxybenzoate	218
4 methyl catechol	231
4 hydroxy phenylacetic acid	241
1,2,3 trihydroxybenzene	247
1,2,4 trihydroxybenzene	276
Resorcinol	301

We analyzed many of these pulps by IGC to determine if there was an increase in donor or acceptor character for the pulp or if the dispersive characteristics of the pulp were changed in a manner that would be correlated to the methylene blue binding results. The average dispersive component (results at all temperatures averaged) determined for these pulps by IGC is shown in Figure 1.

The dispersive component does not correlate with the methylene blue binding of the pulps ($r^2 < 0.04$). The dispersive component did decrease with increasing steam pressure (4, 8, and 12 bar) used to treat the pulp. This

would be expected if the more intensive treatment increased the extractives coating the fibers. Laccase treatment without additional substrates decreased the dispersive component further, and with the exception of catechol, the addition of substrates also decreased the dispersive component compared with the controls.

Figure 2 shows the surface acid–base characteristics for the pulps analyzed by IGC. Although correlation of methylene blue binding with fiber surface K_A and K_D was not very strong, the trend showed a negative slope for the plot of methylene blue binding with K_A and a positive slope for the plot of methylene blue binding with K_D. All substrates other than resorcinol, 4-hydroxyphenylacetic acid, 1,2,3-trihydroxybenzene, and 1,2,4-trihydroxybenzene increased the K_A of the fiber.

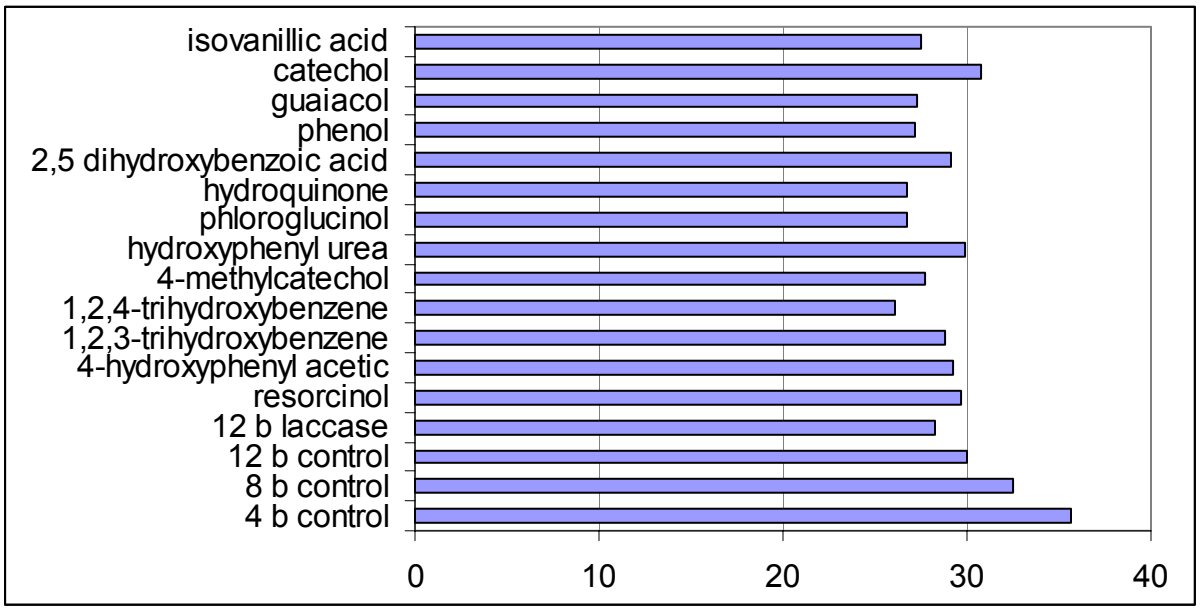

Figure 1. Average dispersive component for loblolly pine pulps determined by inverse gas chromatography.

Because methylene blue is a cationic dye, it can also be expected to behave as a Lewis acid. Therefore, we expected those fiber surfaces with K_A/K_D ratios greater than the control (~4) to exhibit a low affinity for methylene blue. However, that was not the case because most of the treatments that showed higher capacities for binding methylene blue also showed much higher K_A/K_D ratios than the control. In addition, resorcinol treatment with a K_A/K_D ratio equivalent to the control showed the highest binding capacity for methylene blue. These observations may be explained in one of two ways. The first is that methylene blue interacted with the treated fiber surfaces by means other than Lewis acid–base interactions. The other explanation is that the treatments resulted in fiber surfaces that had poorly developed Lewis acid–base characteristics compared with the control. If the former explanation is true, then methylene blue was not a very good choice for probing the acid–base characteristics of the treated fibers.

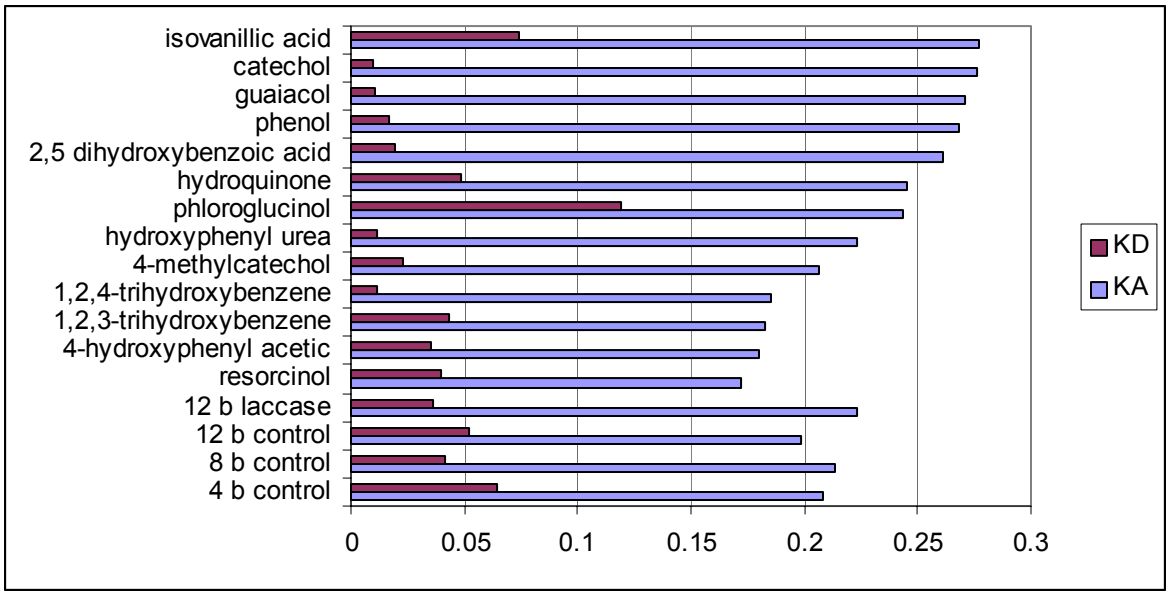

Figure 2. Surface acid-base characteristics, KA and KD for loblolly pine pulps. Substrate name indicates the results for 12-bar pulps treated with that substrate.

However, there have been cases where methylene blue has been used successfully to demonstrate the presence of anionic sites on thermomechanical pulps. Methylene blue binding might be useful as an indicator of other properties in a product derived from the pulp but these have not yet been investigated. The laccase- and resorcinol-treated loblolly pine pulps were further examined by atomic force microscopy to determine any structural differences.

The atomic force microscopy (Fig. 3) revealed many small crystalline structures on smooth regions of the laccase-resorcinol-treated fibers that were not present in the untreated control or the control treated with laccase. These crystalline regions were only observed in the laccase-resorcinol-treated material. While these regions might not contribute greatly to the overall surface characteristics of the fiber, they might reconcile the methylene blue binding results if they bound methylene blue to a greater extent than the rest of the fiber.

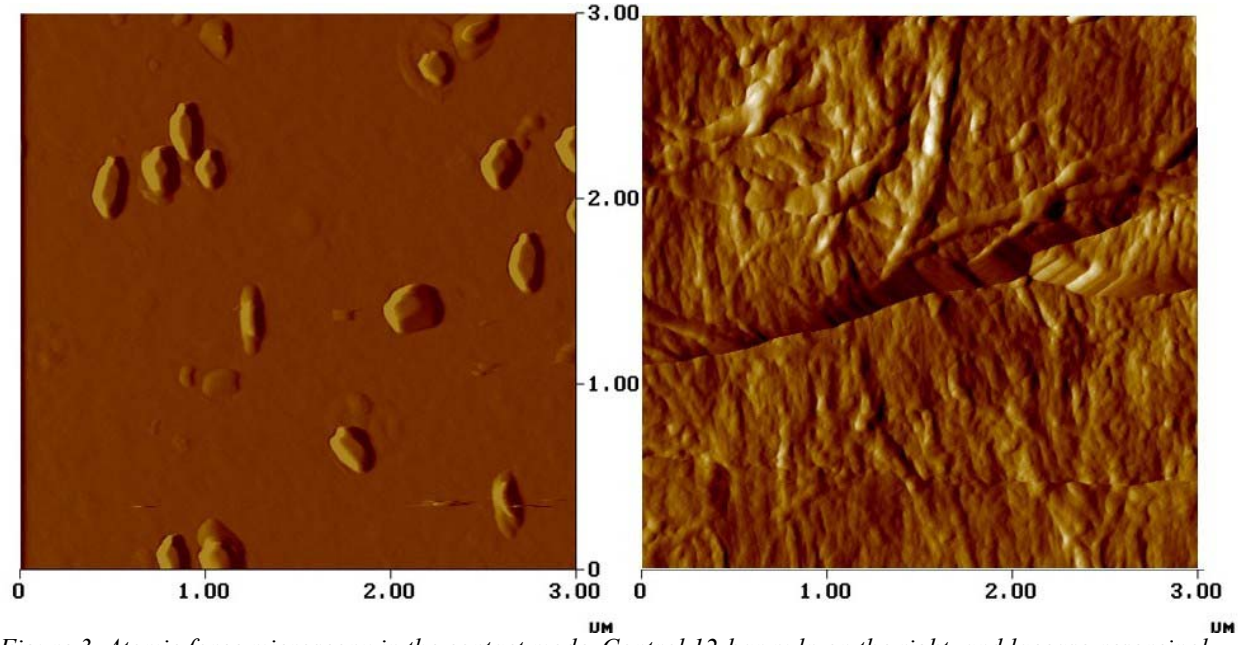

Figure 3. Atomic force microscopy in the contact mode. Control 12-bar pulp on the right, and laccase-resorcinol-treated 12-bar pulp on the left.

Several conditions were selected to test the effects of oxidative enzyme treatment on spruce pulps and determine if handsheet properties were altered by the treatment. Spruce pulp was treated with manganese peroxidase or laccase with 4-hydroxyphenylacetic acid. The pulp was then refined in a PFI mill, and handsheets were made. The results of strength testing on handsheets made from these pulps are shown in Table II.

Table II. Handsheet strength properties of treated spruce TMP after refining by PFI mill.

Pulp treatment	Tear index[a] (mN m^2/g)	Burst index[a] (kN/g)	Tensile index[a] (N m/g)	Canadian Standard Freeness (mL)
Control	3.52	1.54	33.5	65
MnP + H_2O_2 + Mn + buffer	3.80	1.64	34.1	69
H_2O_2 + Mn	3.51	1.52	33.5	70
Buffer	3.56	1.50	33.4	71
Laccase + 4-hydroxyphenylacetic acid	3.61	1.59	32.3	69

[a]Average value for 10 handsheets.

Manganese peroxidase treatment increased the burst index by 6%, the tear index by 8%, and the tensile index by 2%. The increases were due to the presence of the enzyme; the substrates and buffer alone had no effect. The manganese peroxidase treated pulp required less refining to obtain the same degree of freeness, which indicates potential energy savings. The laccase treatment with 4-hydroxyphenylacetic acid brought about an increase of 3% for the burst and tear index and a decrease of 4% for the tensile index. Chandra and Ragauskas [17] reported an 18% increase in the burst index, an 11% increase in the tear index, and about an 8% increase in the tensile index for the softwood linerboard pulp treated with laccase and 4-hydroxyphenylacetic acid.

A commercial spruce pulp was obtained and tested for altered properties after treatment with laccase and substrates. This pulp was stored at 5°C. The latency was removed. It was treated with enzymes and washed. A portion was then bleached with alkaline hydrogen peroxide, and then handsheets were made. The results of handsheet testing of these treatments are compared with those for mill-produced handsheets in Table III. The mill strength tests were the highest of all the measurements. It is quite possible that application of an enzyme and substrate would give substantially different results when applied in a commercial setting where there is little or no washing of the pulp and extractives are available for additional reactions by laccase.

Compared with the untreated control, there were small increases in the burst and tensile index for the phloroglucinol-treated material without bleaching. Improvements for the tear index after bleaching were noted for laccase-treated material and laccase-catechol-treated material. Prior to bleaching, the laccase-catechol-treated pulp was very dark. Upon bleaching, most of the color was observed in the rinse water.

Laccase modification of other lignocellulosic materials was also investigated. Southern pine bark treated with BAPED is effective at removal of phosphorous from aqueous solutions. The bark was treated with laccase and also with phloroglucinol and catechol to try to increase the number of sites on the bark where BAPED could be bound. The laccase-treated material decreased the capacity of the bark to remove phosphorous by 31%. The catechol and phloroglucinol treatments decreased the capacity by 38% and 45%. Further characterization is necessary to determine if the reactions reduced the nitrogen content of the treated bark or if phenolics were bound to the amines via free radical additions.

Table III. Handsheet strength properties of treated and untreated commercial spruce TMP.

Pulp treatment	Tear index[a] ($mN\ m^2/g$)	Burst index[a] (kN/g)	Tensile index[a] (Nm/g)	Canadian Standard Freeness (mL)
Unbleached pulps				
Mill tests on pulp	8.00	3.87	53.6	44
Control	6.41	2.98	50.8	48
Laccase	6.12	2.97	51.4	54
Laccase + phloroglucinol	6.20	3.04	51.4	51
Laccase + resorcinol	6.11	2.83	49.3	52
Laccase + catechol	5.92	2.68	46.2	56
Bleached pulps				
Control	5.13	3.12	51.1	59
Laccase	5.41	3.15	51.0	58
Laccase + phloroglucinol	4.76	3.07	50.3	60
Laccase + resorcinol	4.89	2.60	45.2	63
Laccase + catechol	5.30	2.49	41.8	65

[a]Average value for 10 handsheets.

CONCLUSIONS

Laccase and manganese peroxidase are able to generate free radicals in pulps and substrates. The results are influenced by the treatment of the pulp prior to enzyme treatment, how the pulp is treated with enzymes, and the substrates used. Laccase reactions alter the surface characteristics of the fiber. Some of these alterations resulted in increased strength properties or lower energy needs during refining. The reactions with laccase might also be useful in the further modifications of lignocellulosic materials.

ACKNOWLEDGMENTS

We thank Aimee Stephens of the USDA Forest Service, Forest Products Laboratory, paper test laboratory, for technical assistance.

REFERENCES

[1] Baum, G., and Ebeling K., "Fiber engineering", in *Setting the industry technology agenda*, TAPPI Press: Atlanta, GA. pp. 113–120 (2002).
[2] Bourbonnais, R., Paice, M. G., Freiermuth, B., Bodie, E., and Borneman, S., "Reactivities of various mediators and laccases with kraft pulp and lignin model compounds", Applied and Environmental Microbiology, 63, pp. 4627–4632 (1997).
[3] Li, K., Xu, F., and Eriksson, K. E. L., "Comparison of fungal laccases and redox mediators in oxidation of a nonphenolic lignin model compound", Applied and Environmental Microbiology, 65, pp. 2654–2660 (1999).
[4] Bourbonnais, R., and Paice, M. G., "Enzymatic delignification of kraft pulp using laccase and a mediator", Tappi Journal, 79, pp. 199–204 (1996).
[5] Bourbonnais, R., Rochefort, D., Paice, M. G., and Leech, D., "Development of stable redox complexes to mediate delignification of kraft pulp by laccase", in *Oxidative delignification chemistry*, Argyropoulos, D.S., (ed.), American Chemical Society, Washington, DC, pp. 391–399 (2001).
[6] Li, K., Prabhu, G. N., Cooper, D. A., Xu, F., Elder, T., and Eriksson, K.-E. L., "Development of new laccase-mediators for pulp bleaching", in *Oxidative delignification chemistry*, Argyropoulos, D.S., (ed.), American Chemical Society, Washington, DC, pp. 400–412 (2001).
[7] Lund, M., and Ragauskas, A. J., "Enzymatic modification of kraft lignin through oxidative coupling with water-soluble phenols", Applied Microbiology and Biotechnology, 55, pp. 699–703 (2001).
[8] Huttermann, A., Mai, C., and Kharazipour, A., "Modification of lignin for the production of new compounded materials", Applied Microbiology and Biotechnology, 55, pp. 387–394 (2001).
[9] Huttermann, A., Majcherczyk, A., Braun, L. A., Mai, C., Fastenrath, M., Kharazipour, A., Huttermann, J., and Huttermann, A. H., "Enzymatic activation of lignin leads to an unexpected copolymerization with carbohydrates", Naturwissenschaften, 87, pp. 539–541 (2000).

[10] Mai, C., Milstein, O., and Huttermann, A., "Fungal laccase grafts acrylamide onto lignin in presence of peroxides", Applied Microbiology and Biotechnology, 51, pp. 527–531 (1999).
[11] Felby, C., Olesen, P. O., and Hansen, T. T., "Laccase catalyzed bonding of wood fibers", in *Enzyme applications in fiber processing*, Paulo, A.C., (ed.), American Chemical Society, Washington, DC, pp. 88–98 (1998).
[12] Felby, C., Hassingboe, J., and Lund, M., "Pilot-scale production of fiberboards made by laccase oxidized wood fibers: Board properties and evidence for cross-linking of lignin", Enzyme and Microbial Technology, 31, pp. 736–741 (2002).
[13] Kharazipour, A., Schindel, K., and Huttermann, A., "Enzymatic activation of wood fibers for wood composite production", in *Enzyme applications in fiber processing*, Paulo, A.C., (ed.), American Chemical Society, Washington, DC, pp. 99–115 (1998).
[14] Wong, K. K. Y., Anderson, K. B., and Kibblewhite, R. P., "Effects of the laccase-mediator system on the handsheet properties of two high kappa kraft pulps", Enzyme and Microbial Technology, 25, pp. 125–131 (1999).
[15] Lund, M., and Felby, C., "Wet strength improvement of unbleached kraft pulp through laccase catalyzed oxidation", Enzyme and Microbial Technology, 28, pp. 760–765 (2001).
[16] Gronqvist, S., Buchert, J., Rantanen, K., Viikari, L., and Suurnakki, A., "Activity of laccase on unbleached and bleached thermomechanical pulp", Enzyme and Microbial Technology, 32, pp. 439–445 (2003).
[17] Chandra, R. P., and Ragauskas, A. J., "Laccase: The renegade of fiber modification", proceedings from the 2001 Tappi Pulping Conference, Atlanta, GA.
[18] Xu, F., "Oxidation of phenols, anilines, and benzenethiols by fungal laccases: Correlation between activity and redox potentials as well as halide inhibition", Biochemistry, 35, pp. 7608–7614 (1996).
[19] Eggert, C., Lafayette, P. R., Temp, U., Eriksson, K. E. L., and Dean, J. F. D., "Molecular analysis of a laccase gene from the white rot fungus pycnoporus cinnabarinus", Applied and Environmental Microbiology, 64, pp. 1766–1772 (1998).
[20] ten Have, R., and Teunissen, P. J. M., "Oxidative mechanisms involved in lignin degradation by white-rot fungi", Chemical Reviews, 101, pp. 3397–3413 (2001).
[21] Xu, F., Shin, W., Brown, S. H., Wahleithner, J. A., Sundaram, U. M., and Solomon, E. I., "A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability", Biochimica et Biophysica Acta, 1292, pp. 303–311 (1996).
[22] Xu, F., Berka, R. M., Wahleithner, J. A., Nelson, B. A., Shuster, J. R., Brown, S. H., Palmer, A. E., and Solomon, E. I., "Site-directed mutations in fungal laccase: Effect on redox potential, activity and ph profile", Biochemical Journal, 334, pp. 63–70 (1998).
[23] Mikolasch, A., Hammer, E., Jonas, U., Popowski, K., Stielow, A., and Schauer, F., "Synthesis of 3-(3,4-dihyroxyphenyl)-propionic acid derivatives by n-coupling of amines using laccase", Tetrahedron, 58, pp. 7589–7593 (2002).
[24] Pilz, R., Hammer, E., Schauer, F., and Kragl, U., "Laccase-catalysed synthesis of coupling products of phenolic substrates in different reactors", Applied Microbiology and Biotechnology, 60, pp. 708–712 (2003).
[25] Felix, J. M., and Gatenholm, P., "Characterization of cellulose fibers using inverse gas chromatography", Nordic Pulp and Paper Research Journal, 1, pp. 200–203 (1993).
[26] Ibrahim, T. H., Burk, T. R., Etzler, F. M., and Neuman, R. D., "Direct adhesion measurements of pharmaceutical particles to gelatin capsule surfaces", Journal of Adhesion Science and Technology, 14, pp. 1225–1242 (2000).
[27] Fardim, P., Holmbom, B., Ivaska, A., and Karhu, J., "Critical comparison and validation of methods for determination of anionic groups in pulp fibres", Nordic Pulp and Paper Research Journal, 17, pp. 346–350 (2002).
[28] Philipp, B., Rehder, W., and Lang, H., "The determination of carboxyl group content in dissolving pulps", Papier, 19, pp. 1–9 (1965).

2003 TAPPI Fall Technical Conference: Engineering, Pulping & PCE&I. Atlanta, GA: TAPPI Press, c2003. 1 CD-ROM