

IPST Technical Paper Series Number 621

Residual Lignin Studies of Laccase Delignified Kraft Pulps

J. Sealey and A.J. Ragauskas

August 1996

Submitted to
4th European Workshop
Stresa, Italy
September 7–11, 1996

Copyright© 1996 by the Institute of Paper Science and Technology

For Members Only

Residual lignin studies of laccase delignified kraft pulps

J. Sealey and A.J. Ragauskas

Institute of Paper Science and Technology, Atlanta, GA, 30318

Keywords: Laccase, biobleaching, delignification, lignin

Introduction

The removal of lignin from wood is a key operation in the manufacturing of high-value paper products. Although most lignin is removed from wood during pulping, the last vestiges must be removed using a series of oxidation bleaching reactions. Historically, this was accomplished using hypochlorous acid or chlorine.¹ Recently, environmental concerns have lead to the development of alternative bleaching agents, including chlorine dioxide, ozone, and hydrogen peroxide.² The enormous potential of applying biological treatments to displacement chemical bleaching stages has not been ignored, and several research groups have been active in this field. Until recently, few biological treatments could claim to have the bleaching efficiency of chemical agents. Call's recent patent of laccase and N-hydroxybenzotriazole as an efficient bleaching stage has dramatically altered the near-term bleaching potential of biological-based bleaching technologies.³

The role of laccase in biological systems to catalyze the polymerization and depolymerization of lignin has been extensively investigated.⁴ The use of laccase to delignify kraft pulps has been historically limited since the enzyme can not diffuse into the fibers due to size limitations.⁵ Attempts to circumvent these limitations have focused on the use of mediators, which are believed to be oxidized by the enzyme and then undergo oxidative reactions with lignin in the pulp fiber. Prior to Call's patent, the best available mediator was 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), which was shown to delignify kraft pulps by 27% over a period of 4 hours.⁶ In comparison, the use of laccase and N-hydroxybenzotriazole leads to 50-60% delignification of kraft pulps over a period of 4 hours.⁷ Control experiments demonstrated the need for both the mediator and laccase to be present in the reaction vessel during the biobleaching process.⁷ Call has demonstrated that pulps biobleached with laccase/N-hydroxybenzotriazole can be readily bleached to high brightness values and that this methodology could be incorporated into modern bleaching practices.⁸

Our own research group has been active in applying biotechnology to the field of pulp and paper,⁹ and we have recently begun to investigate the fundamental principles involved in the laccase/N-hydroxybenzotriazole bleaching system. This paper presents some of the first reported results summarizing the effects of laccase and N-hydroxybenzotriazole on the structure residual lignin during biobleaching.

Materials and Methods

Materials

N-hydroxybenzotriazole, p-dioxane, 1.0 N HCl, pyridine, and deuteriochloroform was commercially purchased and used as received. 1,3,2-dioxaphosphonayl chloride was prepared following Zwierzak's method.¹⁰

Bleaching studies employed two industrial, never-dried, softwood kraft pulps. The first pulp (pre-O₂) sample was collected after continuous, extended kraft delignification, and the second was acquired after oxygen delignification of pulp 1 (post-O₂). Physical properties for both pulps are summarized in Table 1. Prior to using the pulps for the studies reported in this paper, the extractives were removed following TAPPI method T264.¹¹ The extractives were removed from the pulp to simplify subsequent lignin characterization studies.

Laccase, isolated from a *Polyporus* fungi, was maintained at -20°C until use. Once thawed, the activity of the enzyme was measured, and the proper dose was added to the pulp.

Methods

Laccase assay.

The activity of the laccase was measured by monitoring the rate of oxidation of syringaldazine. The change in $A_{530\text{nm}}$ of 0.001 per minute per mL of enzyme solution in a 100 mM potassium phosphate buffer (2.2 mL) and 0.216 mM syringaldazine in methanol (0.3 mL) was set to one Unit of activity. This test was performed at 23°C.

General laccase/N-hydroxybenzotriazole biobleaching stage.

The biobleaching trials were performed in a pressurized vessel with 10 bar (145 psi) O₂ pressure for 24 hrs at 45°C. The pulp was added to the preheated vessel, and the mediator was added and stirred for 3 min. The pH was then adjusted to 4.5, and the enzyme solution was added (pH remained ~ 4.5). After treatment, the mixture was filtered, and an alkali extraction was performed with a 2% NaOH charge for 1 hr at 70°C.

Physical and chemical characterization of kraft pulps.

The lignin content of the kraft pulps was determined by KMnO₄ titration of the pulp following standard TAPPI method T-236 and expressed as a "kappa number." The viscosity of the pulp was measured employing a capillary viscometer as described in TAPPI method T-230.¹¹

General lignin isolation procedure.

Air-dried pulps were extracted with a 9:1 dioxane/aqueous acid solution (0.1 N HCl). The extraction was performed at 4% consistency and refluxed for 2 hrs under an argon atmosphere. The mixture was then filtered, and the filtrate was filtered through celite. The solution was neutralized and concentrated. After the volume was reduced to less than 400 mL, 200 mL of distilled water was added three separate times and reconcentrated. The lignin

solution was then acid precipitated by lowering the pH to 2.5 with HCl. The acid-precipitated lignin was then washed several times with distilled water and freeze-dried.

Lignin NMR studies.

All spectra data were acquired with a DMX 400 MHZ, Bruker NMR spectrometer. Quantitative ^{13}C -NMR experiments were performed following standard literature methods.¹² In brief, 100 mg of residual lignin was dissolved into ca. 0.6 ml of DMSO- d_6 and filtered into a 5-mm NMR tube. All spectra were recorded at 50°C. Typical recording conditions employed a 90° pulse, 11 sec pulse delay, 25,000 Hz sweep width with an inverse gated decoupling pulse sequence requiring approximately 15,000 transients for acceptable signal-to-noise. ^{31}P NMR spectra were acquired after phosphorylating residual lignin samples with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane following the procedure developed by Argyropoulos.¹³

Results and Discussion

The effects of the laccase/N-hydroxybenzotriazole biobleaching system was initially explored with an industrial softwood kraft pulps isolated before and after oxygen delignification. In each case, the pulp was treated with a laccase mediator system for 24 hours at 45°C and 10 bar of O_2 pressure. Following the initial biobleaching stage, the pulp was subsequently extracted with aqueous 2% NaOH solution for 1 h at 70°C. Table 1 summarizes the results of these studies.

Table 1. Laccase/N-hydroxybenzotriazole delignification of kraft pulps.

Pulp	Initial		Post-bleaching	
	Lignin Content (kappa #)	Viscosity (cP)	% Delignification	% Viscosity Loss
Pre- O_2	25.5	30.7	32	20
Post- O_2	17.2	26.8	53	31

As previously reported, the laccase/N-hydroxybenzotriazole system provided a significant degree of delignification of the pulps and with optimal results being achieved with the post- O_2 kraft pulp. The reductions in viscosity values for the biobleached pulps suggested that the laccase-mediator system was highly selective for lignin and did not induce significant oxidative degradation with the cellulose component of the kraft pulp fibers.

The mechanisms contributing to the laccase/N-hydroxybenzotriazole biobleaching effect were further explored by characterizing the nature of residual lignin before and after the biobleaching/alkaline extraction stage. The residual lignin of the two starting kraft pulps was

isolated employing Gellerstedt's acidic dioxane extraction procedure for kraft pulps.¹³ These conditions are believed to catalyze the hydrolysis of lignin-carbohydrate bonds facilitating the dissolution of lignin into an aqueous dioxane solution. Subsequent work-up procedures provide lignin samples free of carbohydrate containment that can be readily analyzed by high-field NMR methods. Employing the same isolation procedure for the alkaline-extracted, laccase-treated pulps yielded residual lignin samples for the pre-O₂ and post-O₂ biobleached pulps. In addition, the lignin fragments present in the effluents of alkaline extracts from each laccase-treated pulp were acid precipitated, purified, and analyzed by NMR. Inspection of these spectra indicates that the lignin fragments from the alkaline extraction contained a significant enrichment of acid groups (δ 180-165 ppm) depletion of phenoxy groups (C-3, 4 of phenyl ring, δ 154-140 ppm), and little change in amounts of β -O-aryl ether linkages (δ 85-80 ppm). Table 2 summarizes some of the more significant functional group alterations that occurred during the biobleaching process.

Table 2. Lignin functional group distribution for kraft pulps before and after biobleaching treatment as determined by quantitative ¹³C NMR.

Lignin Sample ^a	RCO ₂ H	C-3,4 of substituted guaiacyl units	C-3,4 of guaiacyl and demethylated phenolics	β -O-aryl ether C- β
Pre-O ₂ pulp	0.44	2.20	0.39	0.38
Pre-O ₂ pulp after LE	0.44	1.75	0.15	0.39
E-effluents	0.84	1.76	0.35	0.55
Post-O ₂ pulp	0.47	2.06	0.29	0.49
Post-O ₂ pulp after LE	0.56	1.79	0.24	0.53
E-effluents	1.05	1.72	0.33	0.54

^a all values are relative and were determined by integrating individual spectrum, assigning the aromatic section of the spectrum (δ 160.1-106.4 ppm) a value of 6, and then measuring all other portions of the spectrum relative to this arbitrary assignment.

Structural elucidation of the residual lignin samples was further explored by phosphilating the lignin samples with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane and then recording the ³¹P NMR spectra for the derivatized lignin samples. The phosphilating agent, is selective for hydroxy groups and provides a versatile method of characterizing free hydroxy groups of residual lignin by ³¹P NMR.¹⁴ The results of this analysis are summarized in Table 3.

As observed previously in the ¹³C NMR analysis, the carboxylic content of the residual lignin after LE treatment and the lignin fragments in the alkaline extraction are significantly enriched over the starting pulp. Presumably, the acids are generated in part by the oxidative

degradation of free phenoxy groups in the lignin since it was observed that a decrease in phenoxy content occurred with the lignin after the LE treatment. The decrease in phenoxy content of the LE-treated pulps can be further analyzed in terms of guaiacyl and condensed phenoxy groups and as summarized in Table 4. Both types of phenoxy groups appear to be oxidized by the laccase biobleaching treatment. Finally, the residual lignin remaining after a LE treatment appears to have a reduced amount of aliphatic hydroxy groups, which is most likely to be due to an enhancement of hydrophobic groups in the pulp after alkaline extraction.

Table 3. Characterization of lignin hydroxy groups by phosphorylation and ^{31}P NMR.

Functional Group^a					
Lignin Sample	RCO ₂ H	Total Phenoxy Content	Guaiacyl and demethylated phenolics	Condensed phenoxy groups	Aliphatic hydroxy groups
Pre-O ₂ pulp	0.293	2.031	1.082	0.949	1.702
Pre-O ₂ pulp after LE	0.467	1.073	0.540	0.533	1.170
E-effluents	0.711	0.747	0.331	0.416	1.384
Post-O ₂ pulp	0.408	1.477	0.759	0.718	2.201
Post-O ₂ pulp after LE	0.679	0.737	0.336	0.401	1.005
E-effluents	0.622	0.414	0.169	0.245	1.107

^a all values are expressed as mmol/gr of lignin.

Conclusions

In summary, these results demonstrate that the laccase/N-hydroxybenzotriazole bleaching system can very effectively remove lignin from kraft pulps via a series of oxidative degradation reactions. It appears the principle site of oxidative attack is the free phenoxy groups of lignin. The role of the chemical mediator, N-hydroxybenzotriazole, is key to these reactions. Further studies will be needed to fully define the mechanistic reactions that occur between laccase, N-hydroxybenzotriazole, and the lignin present in kraft pulps.

Acknowledgments

The gift of the laccase by Novo Laboratories Inc. is great fully appreciated. Portions of this work were used by J.S. as partial fulfillment of the requirements for the Ph.D. degree at the Institute of Paper Science and Technology.

References

1. Reeves, D. W. The principles of bleaching. *Tappi/ Bleach Plant Operations Short Course*, 1992 1-12.
2. Mcdonough, T. Recent advances in bleached chemical pulp manufacturing technology. *Tappi J.*, 1995, 78 (March), 55-61.
3. Call, H. Multicomponent bleaching system. WO #94/29425.
4. Kantelinen, A. Effects of fungal and enzymatic treatments on isolated lignins and on pulp bleachability. *Holzforchung*, 1993, 47 (1) 29-35.
5. Jurasek, L. Toward a three-dimensional model of lignin structure. *J. of Pulp and Paper Sci.*, 1995, 21 (August), J274-J279.
6. Bourbonnais, R. and Paice, G. M. Enzymatic delignification of kraft pulp using laccase and a mediator. *Tappi J.*, 1996, 79 (June), 199-204.
7. Call, H. P. State of the art of enzyme bleaching and disclosure of a breakthrough process. *Tappi/Emerging Pulping and Bleaching Technology Workshop*, Durham, NC, 1995 15-17.
8. Call, H. P. and Mucke, I. The laccase-mediator system (LMS) - a new concept. *6th International Conference on Biotechnology in the Pulp and Paper Industry*, Vienna, Austria, 1996 27-32
9. Santiago, D., Rodriguez, A., Hamilton, J., Senior, D.J., Szwec, J., Ragauskas, A.J. "Applications of Endo-(1,4)- β -D-Xylanase in the Pulp and Paper Industry" in *Industrial Biotechnological Polymers*, Ed. Gebelein, C.G., Carraher, C.E., Jr., Technomic Publishing Company, Inc., Lancaster, PA (1995).
10. Zwierzak, A. Cyclic organophosphorus compounds. I: synthesis and infrared spectral studies of cyclic hydrogen phosphites and thiophosphites. *Canadian Journal of Chemistry*, 1967 (45) 2501-2512.
11. *Tappi Test Methods*, Tappi Press, Atlanta, GA (1992).
12. Gellerstedt, G. and Robert, D. Quantitative ¹³C NMR analysis of kraft lignins. *Acta Chemica Scandinavica*, B41, 541-546 (1987).
13. Gellerstedt, G. and Lindfors, E. On the structure and reactivity of residual lignin in kraft pulp fibers. *International Pulp Bleaching Conference*, Stockholm, Vol. 1 (1991), 73-88.
14. Granata, A., and Argyropoulos, D. 2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, a reagent for the accurate determination of the uncondensed and condensed phenolic moieties in lignins. *J. Agric. Food Chem.* 1995,43, 1538-1544.

