Final Report: Mill-Designed BioBleaching Technologies

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Overview of Biotechnology in the Pulp and Paper Industry

The production and usage of paper products is a cornerstone of modern societies and is interwoven into most major societal activities including education, government, business, and leisure. Indeed, it is well established that the consumption of paper on a national basis can be correlated to a nation's GDP.[1] Although many pulping technologies have been developed over the past century, the dominant global pulping process is the kraft process, producing more than 65% of 1997 virgin pulp production.[2] The German chemist C. F. Dahl invented this process in 1879 and the first kraft mill was built in 1890 in Sweden.[3] Interestingly, the telegraph system was patented 30 years before Dahl's discovery. The discoveries of the telegraph and the kraft process were, for their time, quantum leaps in technology that contributed to the industrial revolution. These and other technological advances of the day significantly altered the development of Western civilization. Now, at the beginning of a new millennium, we are experiencing another dramatic change in modern society. The advent of inexpensive, powerful personal computers, broadband telecommunications and other information technologies has begun to dramatically redefine our concepts of business, life-styles, education, and government.

Recent events now necessitate the development of new breakthrough manufacturing technologies for the pulp and paper industry. These breakthrough technologies need to be

revolutionary in design and operation and must positively impact: raw material costs; manufacturing costs; energy costs; environmental performance; and the production of high-quality products demanded by the consumer.

In 1986, Foster[4] analysed the life-cycle of technologies and proposed that most technologies follow an S-curve relationship between productivity and investment of resources. The basic premise was that the older, more established technologies have upper performance limits that are determined by a combination of physical, chemical and/or regulatory rules. As mature technologies approach the top part of their S-curves, major investments are required for only marginal gains in performance. The key to improving the return on investment is to identify and develop new technologies that develop along a new S-curve.

This challenge presents a unique opportunity for our scientific research community to discover a new S-curve of pulp and paper that will provide a new set of "winning" biomanufacturing technologies for the pulp and paper industry. Certainly, biotechnology research in pulp and paper has already demonstrated that new products can be developed that provide distinct operational benefits. For example, xylanase pretreatments for kraft bleaching have developed from laboratory experiments to commercial products.[5] Many North American and Scandinavian mills have performed mill trials with xylanase and some have incorporated their use into routine production operations. Mill use of xylanase usually can reduce chemical bleaching costs up to 20%. For chlorine-based bleaching technologies, xylanase pretreatments of kraft pulps have also been shown to reduce AOX discharges by 5-20% depending on the furnish and the type of pulping system employed. The use of an X-stage has also been successfully incorporated into commercial TCF bleaching operations. A xylanase pretreatment stage has also been shown to reduce chemical bleaching costs, and higher brightness ceilings have been achieved with an X-stage for a variety of bleach sequences.

Cellulases have been studied for drainage,[6] deinking,[7] and fiber modification. Lab work and mill use have demonstrated the ability of cellulase to enhance drainage properties of

recycled furnish. Several commercial ventures have installed deinking facilities in North America, Europe, and Australia over the last decade and have examined the application of enzymatic systems for improved operations.

Fungal and enzyme pitch degradation products have found applications in some TMP and sulfite mills.[8] Lipase has been used in mill operations to control pitch buildup and also found a niche market for deinking applications in cases where the inks contain vegetable oil formulations.[9] Ascomycete albino fungi have been used as chip pretreatment to reduce pitch and save up to 36% of bleach costs.[10]

These early successes certainly provide a high level of optimism that future research activities in biotechnology will lead to new manufacturing systems that will dramatically improve pulp and paper operations. One of the promising fields of future biotechnology research in the next decade is to improve the performance of traditional paper and board manufacturing technologies. Research goals of value to the industry that could be achieved employing a biotechnology approach include:

- Biological pretreatments to improve kraft pulping specificity to lignin.
- Novel biological pretreatments that reduce the loss of cellulose/hemicellulose during kraft pulping and bleaching operations.
- Development of simplified kraft bleaching technologies.

The continued reduction of fresh-water usage for the production of paper is anticipated to impact almost all aspects of pulp and paper production. Low effluent discharge practices for papermaking operations will present several unique difficulties for papermakers that could be resolved by employing new biotechnology approaches.

These few examples provide only a glimpse of what could be accomplished in the future.[11] Certainly, the biotechnology revolution that is occurring in textiles,[12] detergents,[13] food,[14] and other mature industries suggests that we do not fully appreciate the potential of biotechnology in the pulp and paper industry. Nonetheless, it is well appreciated that enzymatic systems are catalytic, highly selective, and operable under

mild temperature and pressures. These features alone indicate that the development of new bio-manufacturing technologies for pulp and papermaking will substantially reduce capital and operating cost requirements while yielding products with improved performance. In addition, enzymatic treatments offer the potential to selectively modify pulp fibre surfaces to yield new products that cannot be manufactured via chemical and/or mechanical methods. The ability to tailor the surface of pulp fibres will provide pulp manufacturers with new opportunities to develop differentiated, intellectually protected, high-value-added products for the consumer.

Overview of Laccase

Laccase (*p*-diphenol: dioxygen oxidoreductase, EC 1.10.3.2) belongs to the multicopper enzyme family, which includes ascorbate oxidase and ceruloplasmin.[15, 16] Laccases, both fungal and plant, are relatively large proteins with molecular masses ranging from 64-160 kDa.[15,17] The variation in molecular weight has been attributed in part to the differing glycosylation levels in these proteins. Indeed, the carbohydrate content in laccases varies and can constitute 7-45 (% by wt) of the protein molecule, depending on the source of the enzyme.[15, 17] In general, the carbohydrate content in plant laccases is higher than in fungal laccases.[17,18] Typically, laccases comprise of approximately 500 amino acids.[19] Table I.1 summarizes some properties of several well-characterized laccases.

Table I.1. Properties of some Fungal and Plant Laccases.

Source	MW[a]	%Carb	Redox Potential[b]	References
Plant Laccase				
Rhus vernicifera	110	45	394-434	[20, 21]
Rhus succedanea	130	ND	ND	[21, 22]
Acer pseudoplatanus	97	40-45	ND	[23]
Fungal Laccase				
Polyporus versicolor	64.65	10.12	775-785	[24, 25, 26]
Neurospora crassa	64-64.8	11-12	ND	[27, 28]
Pleurotus ostreatus	59-64	12.5	ND	[29, 30]
Polyporus pinsitus	126	7	760-790	[31, 32]
Myceliophthora thermophilia	160	14	450-480	[31]

[a] expressed in kDa; [b] expressed in mV vs. NHE.

Functionally, all the multicopper enzymes catalyze a four-electron reduction of O_2 to water.[17] Optical and magnetic spectroscopy as well as crystallography has elucidated several features of the active site of the multicopper enzymes. It is now well accepted that laccase, the simplest of the multicopper enzymes, contains four coppers at the active site.[15-17,33, 34] These cupric ions have been classified in accordance with their differing spectroscopic signatures. Accordingly, they have been divided into three different classes known as type 1 copper (T1), type 2 copper (T2), and type 3 copper (T3). Laccase contains one copper each of T1 and T2, and two of T3. The T2 and the two T3 copper centers have been shown to be in close proximity and together form what is referred to as the "trinuclear copper cluster."[17,35,36] The binding and reduction of dioxygen occurs at this site.

It has been shown that overall, the copper ligation of the trinuclear copper cluster is very similar in the multicopper oxidases[37]; the peptide ligands of the T2/T3 trinuclear cluster involve eight histidines, where six histidines coordinate the T3 copper and the other two coordinate the T2 copper.[17] However, differences in the coordination of the T1 copper exist between fungal and tree laccases.[17, 38] The T1 copper coordination environment in ascorbate oxidase and tree laccases involves a Cu-S from cysteine, a Cu-S from methionine, and two Cu-N from histidine. On the other hand, coordination studies on fungal laccases suggest that the T1 copper ligation involves a leucine (Leu) or phenylalanine (Phe) rather than a methionine (Met) at the axial position.[38] Leu and Phe are amino acids that do not contain functional groups that can ligate to the copper. This coordination environment has been recently corroborated by the crystal structure of *Coprinus cinereus* laccase. The T1 copper ligation for that laccase consisted of two Cu-N from histidine, and one Cu-S from cysteine.

In general, redox potentials of the T1 copper sites (see Table 1) in plant laccases (approx. 394-434 mV) are lower than in fungal laccases (480-790 mV).[17, 38] Similarly, significant differences exist in redox potentials amongst fungal laccases. The reason (s) for these

differences in redox potentials remains unclear.[17] Nonetheless, it has been suggested that the variation in redox potentials may be due in part to the differing T1 copper coordination environment.[38]

The T1 copper site in laccase accepts electrons from the substrate and then shuttles them to the trinuclear T2/T3, 13 Å away along a cysteine-histidine pathway, where dioxygen is reduced to water.[17] The importance of the T2/T3 trinuclear copper cluster in the reduction of dioxygen to water has been established[36] and it is generally accepted that the T2/T3 trinuclear copper cluster is the minimal structural unit required for the reduction of dioxygen to water.[36]

Recently, Solomon[17,39] proposed a mechanism that involves an initial reduction by two electrons from the T3 copper center yields a peroxide intermediate. This step occurs upon the addition of oxygen. The new formed peroxide bridges between the T2 copper and T3 copper. The water molecule present between the two type-3 coppers is believed to produce the hydroxide bridge at the oxidized T3 site. Upon further reduction of the peroxide, a water molecule is expelled. The hydroxide bridge between T3 copper and T2 copper, in the native intermediate, is then cleaved, and the resting enzyme state is regained.[17, 39]

Overview of Laccase Substrate Specificity

One of the unique features of laccases is their broad specificity for substrates. Accordingly, laccases have been shown to oxidize an array of compounds such as *p*-diphenols, *o*-diphenols, aryldiamines, aminophenols[15] benzenethiols,[40] and hydroxyindoles.[41] The reactivity of laccases isolated from different sources, towards diphenols and diamines may vary. For example, while laccase isolated from *Trametes versicolor* can oxidize 1,2,4,5 tetramethoxybenzene,42 laccase from sycamore maple cannot.

Laccase Oxidation of Lignin

The reactions of laccase with substructure lignin model compounds have been extensively studied. It is widely believed that laccase catalyzes solely the oxidation of phenolic lignin moieties via a one-electron abstraction. The phenoxy radicals can then undergo depolymerization reactions via several degradation pathways or polymerization via radical coupling. Laccase has been shown to degrade phenolic β-O-4 model compounds[43] whereas nonphenolic lignin compounds are not oxidized by laccase.[44, 45]

For example, Kawai et al.[44, 45] (see Figure I.1) found that substrate (**1**) (syringylglycerol-β-guaiacyl ether) was converted mainly to the α-carbonyl dimer (**2**), 2,6-dimethoxyhydroquinone (**3**), glyceraldelhyde-2-guaiacyl ether (**4**) via alkyl-phenyl cleavage, and to guaiacol by O-Cβ cleavage (**5**).

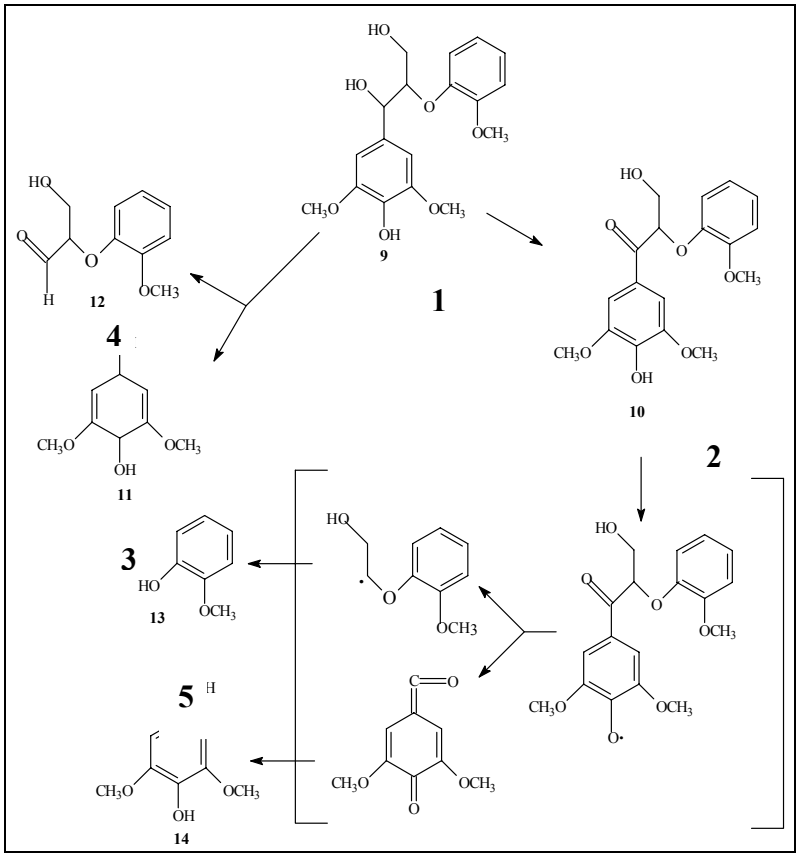


Figure I.1. Possible mechanism for side chain cleavage of a phenolic β-O-4 lignin substructure model by laccase from *Coriolus versicolor*.[45]

Laccase has also been shown to degrade β-1 lignin substructure phenolic lignin model compounds.[45, 46]

Laccase Catalyzed Polymerization of Lignin

Although fungal laccase has been shown to degrade and to depolymerize lignin and lignin model compounds, other reports have indicated that low molecular weight lignin compounds treated with this enzyme can be polymerized. Leonowicz et al.[47] have observed that low molecular weight lignosulphonate fractions incubated with laccase from *Trametes versicolor* led to poylmerization. Similarly, Potthast et al.[48] have detected that creosol was polymerized upon treatment with laccase. Ikeda et al. have also shown that 2,6-dimethylphenol incubated with laccase led to polymerization.[49] Solution conditions, in particular, the pH, has been shown to influence the transformation of substrates by fungal laccases.[50, 51] Indeed, the products obtained upon the incubation (see Figure I.2) of vanillin and syringic acid with laccase from *Trametes versicolor* and *Rhizoctonia praticola* varied depending on the pH range.

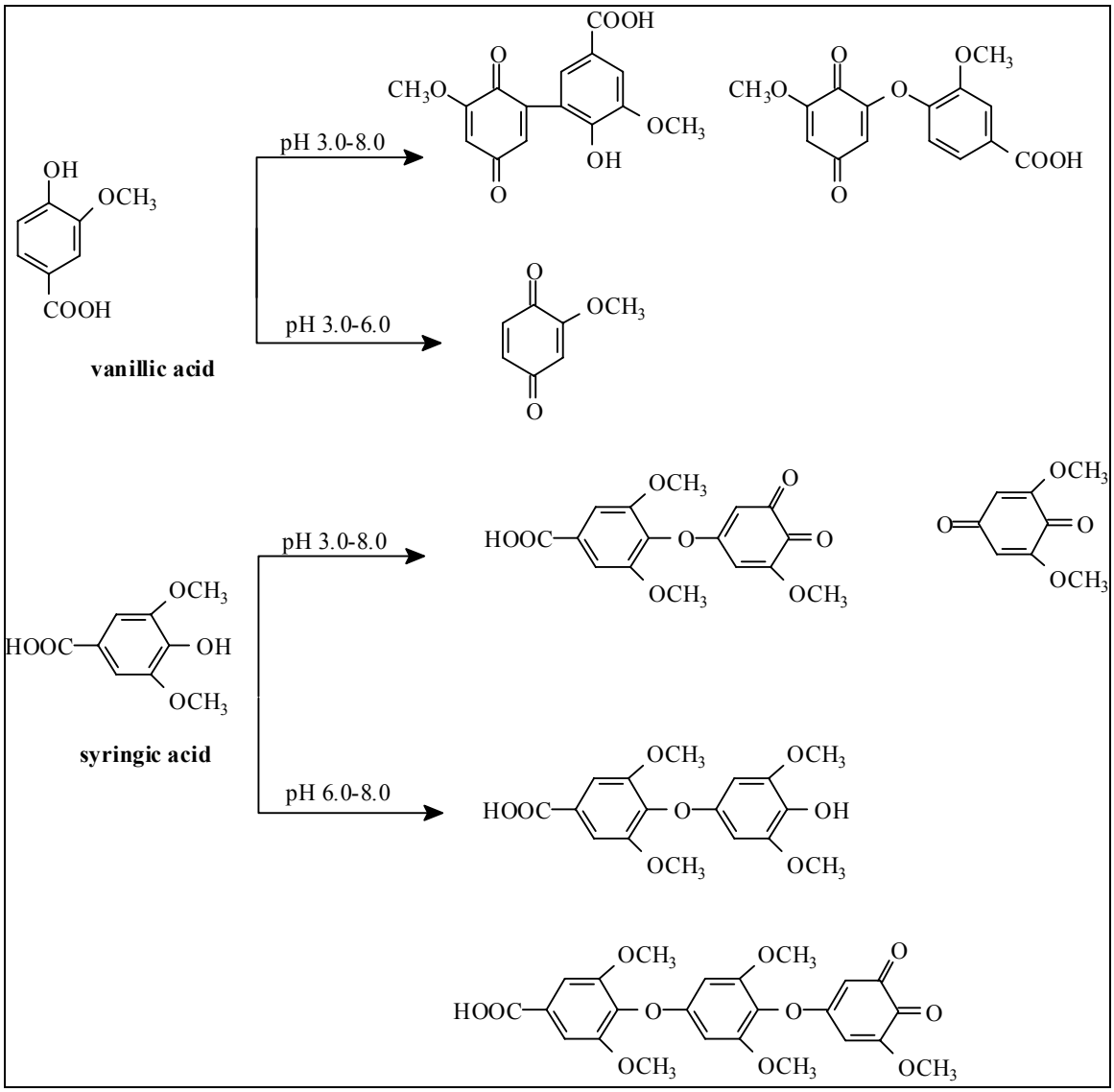


Figure I.2. Products of syringic acid and vanillin upon incubation with laccase from *T.versicolor* and *R. praticola* at different pH[51]

Nonetheless, it has been noted that the polymerization activity of laccase can be circumvented by introducing chemical mediators or other enzymes. [52, 53, 48]

Laccase Mediator Systems (LMS) and Biodelignification of Kraft Pulps

Fungal laccase has been shown to oxidize substructure lignin model compounds; however, the oxidative bleaching of chemical pulps is very poor. The generally accepted

explanation for this phenomenon has been attributed[54, 55] to the large size of the enzyme, preventing diffuse of laccase into the secondary wall of a fiber to contact the lignin. Recently, however, it was shown that it is possible to circumvent this problem by using small organic molecules, referred to as mediators, thus enhancing the delignification response in actual pulps. It is accepted that the so-called mediator is the active agent in delignification. In addition, studies have demonstrated that the specificity of laccase, which is limited to phenolic moieties, can be extended to nonphenolic substrates with the aid of mediators.

Laccase – 2-2'azinobis(3-ethylbenzthiazoline-6-sulfonate (LMS_{ABTS}) Biobleaching

Bourbonnais and Paice56 reported that laccase from *Coriolus versicolor*, in the presence of ABTS, oxidized nonphenolic lignin model compounds (see Figure I.3). It has been suggested that such reactions are initiated via abstraction of a hydrogen atom from the C-α position to yield hydroxy substituted benzyl radicals.[57] These radicals are subsequently oxidized to aldehyde or ketone moieties and are further degraded subsequent to an alkaline extraction stage.[58] In the absence of ABTS, no oxidation via laccase took place with any model compounds. Similarly, in the presence of $ABTS^{\cdot+}$ alone, no oxidation was observed; the dark green and stable $ABTS^{\cdot+}$ is produced upon the oxidation of ABTS.[59] This indicated that both the enzyme and the mediator must be present during the reaction.

The laccase-ABTS concept was also carried out on chemical pulps. In one study, Bourbonnais and Paice,[60] reported 24% delignification when a hardwood pulp with an initial kappa number of 12.1 was reacted with laccase from *Trametes versicolor*. The study was carried out at 2% consistency in an air atmosphere at 25°C. The reduction in kappa number was gradual and reached the 24% mark after five days of treatment. The decrease in pulp viscosity was minimal (i.e., initial= 24.2 mPa.s, final=21.9 mPa.s). The researchers also monitored the amount of methanol that was generated during this experiment. They noted that the methanol released during the five-day period reached a

plateau after the first day. The detection of methanol is suggestive of demethylation reactions.[60] Delignification with the laccase-ABTS system in this study was also carried out at a pH ranging from 3.5-7.0. The optimal pH for this system was found to be 4.5.

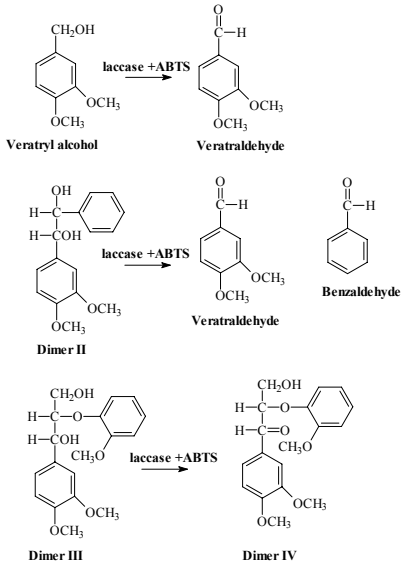


Figure I.3. Reaction between dimeric lignin model compounds and laccase/ABTS.[56-58]

In a more recent study,[61] Bourbonnais and Paice reported about a 32% delignification (after extraction stage) when a softwood kraft pulp was reacted with laccase (from *Trametes versicolor*) and ABTS under 100-400 KPa of oxygen for two hours. The experiment was carried out at a pH 5, 60^{o}C, and at 10% consistency. In the absence of the enzyme and ABTS, the extent of delignification was negligible.[61, 62]

The mechanism involved in delignification using a laccase-ABTS system is as follows: laccase is oxidized in the presence of oxygen, and in turn the enzyme oxidizes the mediator.[55, 63, 64] Subsequent diffusion of the oxidized mediator in the pulp oxidizes the lignin. It is believed that two intermediary active species are involved in delignification, where the $ABTS^{\cdot+}$ reacts with phenolic substrates and the $ABTS^{2+}$ oxidizes nonphenolic lignin moieties.[58,65]

Laccase – 1-hydroxybenzotriazole (LMS_{HBT}) Biobleaching

Recently, Call[66, 67] introduced a new class of mediators that are based on N-oxide or N-hydroxyl structures.[68] Primarily studies suggested that 1-hydroxybenzotriazole (HBT, see Figure I.4) was shown to be the most promising mediator for both hardwood and softwood kraft pulps, as well as for oxygen delignified softwood and hardwood kraft pulps. Call reported LMS_{HBT} reductions in kappa as high as 60% for low-lignin content kraft pulps after an alkaline extraction stage, without any significant losses in viscosity. These rates of delignification can be obtained in a single LMS_{HBT} treatment and in one to four hours. The system can operate at a wide range of temperatures (40 to 65°C), pH (4-6.5), consistencies (1-20%), and oxygen pressures (1-10 bars). However, it seems that the optimal conditions are approximately 45°C, 10% consistency, pH 4.5, and 10 bars oxygen pressure. These positive results have prompted great interest in the research community for further understanding of the chemistry of a laccase-HBT system, both from a fundamental and an applied point of view.

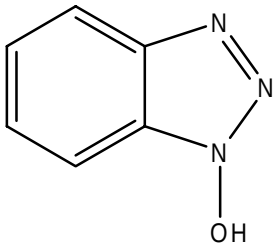


Figure I.4. 1-Hydroxybenzotriazole (HBT)

Several studies have examined the reactivity of a laccase/HBT system with nonphenolic dimeric lignin model compounds. It has been demonstrated that β-1 model compounds are oxidized with laccase/HBT via abstraction of an H atom from the C-α position.69 On this basis, the initial oxidation pathways using either ABTS or HBT are similar. However, differences in the degradation products have been observed. While the oxidation of dimer **I** with ABTS led to syringaldehyde and veratraldehyde, the oxidation

of the same dimer with HBT led to syringaldehyde, veratraldehyde and dimer **II** (see Figure **I.**5).

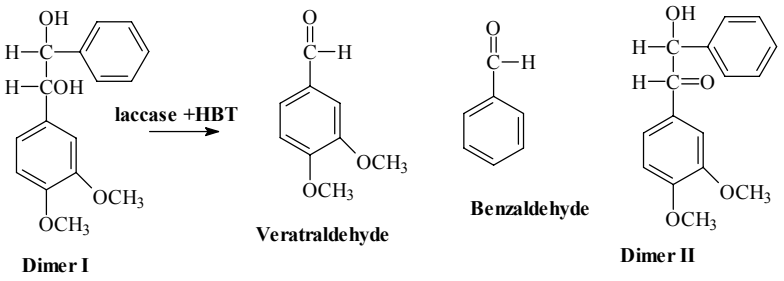


Figure I.5. Reaction of an etherified β-1 lignin model compound with laccase/HBT.[69]

Nonphenolic β-O-4 lignin models have also been examined. Dimer **III** was oxidized to dimer **IV** in a manner similar to the laccase/ABTS system (see Figure I.6). Cleavage of dimer **IV** was only observed after an alkaline extraction stage; however, the degradation products were not characterized.[69]

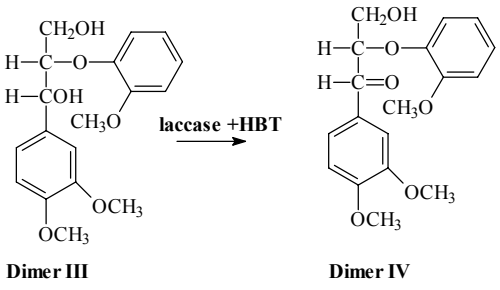


Figure I.6. Reaction of a non-phenolic β-O-4 lignin model compound with laccase/HBT[69]

Kawai et al.[70] also examined the reactivity of nonphenolic β-O-4 model compounds using laccase from *Trametes versicolor* and HBT. Dimers **IX** and **X** were converted to various compounds (dimers **XI- XV**; see Figure I.7) suggesting that the degradation can proceed via several pathways, such as β-ether cleavage, Cα-Cβ cleavage, and Cα oxidation.

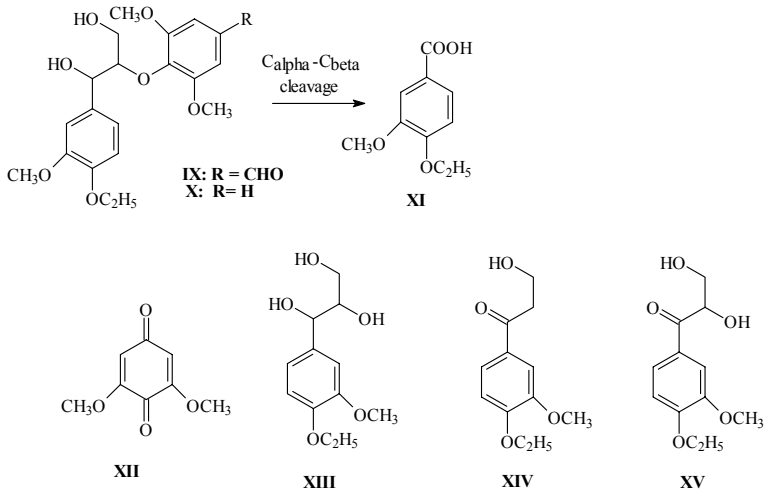


Figure I.7. Reaction of an etherified model compound with laccase/HBT according to Kawai.[70]

Another interesting result from the Kawai et al. study is that the reaction of dimer **X** (see Figure I.8) with laccase/HBT led also to compounds **XVI** and **XVII** via aromatic ring cleavage.

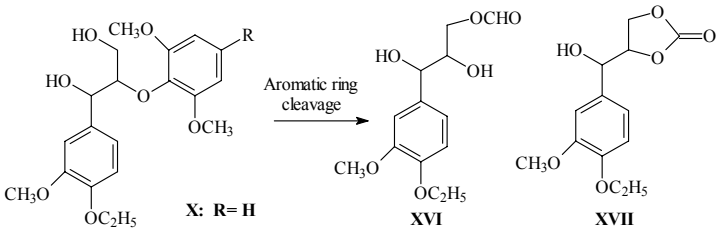


Figure I.8. Aromatic ring cleavage from the reaction of dimer X with laccase and HBT.[70]

The general mechanism involved in delignification with a laccase-HBT system is similar to a laccase-ABTS system. The oxygen present in the solution oxidizes the enzyme,

which in turn oxidizes HBT.[67] The oxidized HBT then diffuses into the pulp and oxidizes the lignin. The next cycle begins when oxygen is reduced to water. This generally accepted mechanism is shown in Figure I.9.[67] The active delignification mechanism for a laccase-HBT system has been shown to be radical based. Using EPR, Potthast proposed that the active functionality in delignification is the $NO^{\cdot}$ (radical), generated in situ from the action of laccase and HBT.[71]

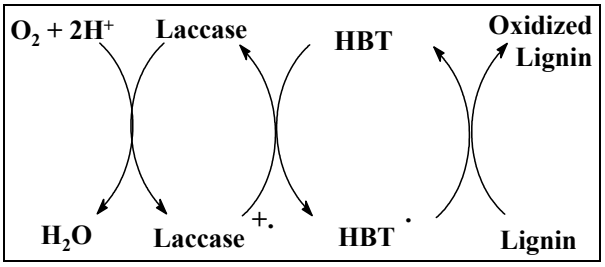


Figure 9. Proposed mechanism of laccase$_{HBT}$ in biobleaching.[67]

Sealey et al.[71] have also demonstrated that the mechanism of LMS$_{HBT}$ delignification is radical based employing by superoxide dismutase as a radical scavenger. Sealey and Ragauskas[72,73] have also characterized the nature of the residual lignin before and after a laccase-HBT treatment. A softwood kraft pulp with an initial kappa number of 25.5 was reacted with laccase (from *Polyporus versicolor*) in the presence of HBT for 24 hrs at 45°C, and at 10 bars of oxygen pressure. Subsequent alkaline extraction yielded a pulp with a final kappa of 17.3. The residual lignin from the starting pulp and the enzyme-treated pulp were extracted via acid hydrolysis, and the ^{13}C NMR spectra were recorded. The spectra revealed that the laccase treated-pulp had fewer methoxyl group when compared to the starting pulp. In addition, an enrichment in β-O-4 lignin structures was observed, suggesting that these lignin moieties are unreactive to towards LMS. This observation contradicts the results noted on model compound studies. On this basis, it is possible that the affinity of LMS in a polymeric system to β-O-4 structures is redirected towards other lignin functional groups. The results of lignin phosphorylation and ^{31}P NMR spectra revealed that the residual lignin from the enzyme treated pulp contained substantially fewer phenolic hydroxyl groups than the residual lignin isolated from the

starting pulp. These results suggested that the mediator preferentially attacks phenolic substrates. Further analysis of C-5 noncondensed and condensed at C-5 lignin groups showed that a laccase-HBT system oxidizes both types of phenolic groups.[72] The reactivity of condensed structures in a laccase-mediator system demonstrates a unique feature of this system since such structures are typically unreactive towards conventional oxidative bleaching technologies such as oxygen delignification.

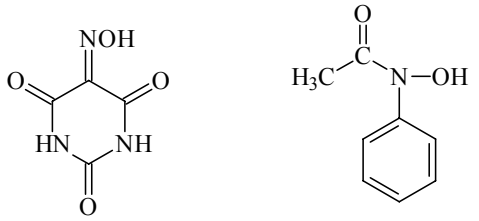
The reactivity of laccase and laccase/HBT towards phenolic condensed lignin structures has been demonstrated by Tamminen et al.[74] A pine kraft pulp (kappa no. 24.7) and a two-stage oxygen delignification pine kraft pulp (kappa no. 8.6) were treated with laccase and laccase/HBT. The residual lignins isolated after the treatments were subjected to oxidative degradation. Their results indicated decreases in phenolic 5-5 condensed lignin structures and hence are consistent with Sealey and Ragauskas' NMR data. Another essential observation was the enrichment in *p*-hydroxyphenyl-type compounds, suggesting that such structures are not reactive towards laccase/HBT treatments.

The substantial reduction in phenolic lignin moieties has also been reported by Poppius-Levlin et al.[75,76] A pine kraft pulp (kappa no. 24.7) was reacted with laccase (from *Trametes hirsuta*) in the presence of HBT at 45°C for 2 hours under five bars of oxygen pressure at a pH of 4.5. Subsequent alkaline extraction yielded a pulp with a final kappa of 15.4. The residual lignins were extracted and analyzed by FTIR. These results supported the findings of Sealey and Ragauskas that HBT preferentially attacks free phenolic sites. Accordingly, Poppius et al. reported a 42% drop in free phenolic groups for the residual lignin isolated from the laccase-HBT treated pulp.[75]

Laccase –N-acetyl-N-phenylhydroxylamine (LMS_{NHA}) and Violuric Acid (LMS_{VA}) Biobleaching

At the beginning of this project two additional mediators were reported in the literature by Amann, *N*-acetyl-*N*-phenylhydroxylamine (NHA) and violuric acid (VIO) (see Figure I.10). Preliminary results recently published by Amann suggest that NHA and VIO may

be potential candidates as mediators.[77] A softwood kraft pulp (initial kappa=15.7) was treated with laccase/NHAA, laccase/HBT, and laccase/VA in a TCF sequence (LEQP). In addition, a control sequence was performed by exchanging the LE with an oxygen delignification stage (O). The results shown in Table I.2 illustrate that the presence of NHA and VA were effective mediators, and that the loss in viscosity was not very significant.



VIO **NHA**

Figure I.10. Chemical structures of VIO and NHA.

Table I.2. Bleaching response of VIO, HBT, and NHA in a LEQP sequence.[78]

	Kappa	**Viscosity (dm^3/Kg)**
Control (OLQP)	5.1	845
VIO	5.5	890
NHA	5.7	920
HBT	5.8	880

In an effort to explain the reactivity of various mediators, Amann measured the redox potential of ABTS, HBT, NHA, and VA (see Table I.3). All values fell within a narrow range. This suggests that no conclusive relationship can be established between redox potential and mediator reactivity, although the redox potential must be an important variable.

Table I.3. Redox potentials of mediators.[67]

Mediator	**Redox potential (V); vs. NHE**
ABTS	0.74/1.15
HBT	1.13
VIO	0.97
NHA	1.00/1.21

In summary, at the initiation of this project it had been known for several decades that white-rot fungi utilize lignin peroxidase, manganese-peroxidase, and laccase in the course of extracellular lignin degradation of wood.[79] Hence, the reactions of laccase with lignin and lignin model compounds have been extensively studied. Despite the efficient degradation of lignin by laccase, the utilization of this enzyme during pulp bleaching is not feasible. This has been attributed to the inability of the enzyme to diffuse into a pulp fiber, where most of the residual lignin is located. This limitation was overcome Bourbonnais and Paice demonstrated that the addition of ABTS could lead to delignification of kraft pulps after an alkaline extraction stage.[56] This discovery initiated a flurry of fundamental research studies into the mechanism of ABTS/laccase bleaching. The results of these studies led to the suggestion that the mechanism of delignification for laccase/ABTS is based on a series of connective oxidative reactions in which laccase is oxidized by oxygen, laccase then oxidizes ABTS, and in turn the oxidized ABTS diffuses into the fiber and oxidizes the lignin. The reduced mediator is then re-oxidized by laccase and the system becomes catalytic. Unfortunately, the mediator ABTS is not cost-effective. Recently, Call identified a new mediator, N-hydroxybenzotriazole that exhibited improved bleaching performance under mild operating conditions.[66] Since this initial discovery, the Ragauskas/IPST research team has extensively investigated this biobleaching system and has made many important discoveries including:

- The active delignification agent for laccase/NHB is a radical species that is quenched by superoxide dismutase;
- Laccase/NHB delignification is relatively insensitive to metal cations;
- Greater than 60% delignification can be accomplished with a single laccase/NHB-stage;
- High brightness pulps (ISO brightness >85) can be achieved with ClO_2 after laccase/NHB stage;
- NHB is converted to benzotriazole under the biobleaching conditions and benzotriazole is not a mediator.[71-73, 80]

This research project was directed at examining the fundamental LMS biobleaching process and utilizing this information to develop improved LMS delignification technologies. This report summarizes our research activities over the duration of this program.

Summary of Modeling Studies.

At the beginning of this project research efforts were directed at developing computational modeling capabilities to predict and guide our efforts at evaluating laccase mediator chemistry. A significant effort was directed at understanding the improved biodelignification of N-hydroxyl mediators. The active species involved in lignin degradation employing laccase and an N-hydroxy mediator is the RR´NO• radical. Computational results from PM3 indicate that the bond dissociation energy and electronic factors of the radical may contribute to the efficiency of the mediator for LMS delignification.

This was explored initially by using structural analogues of 1-hydroxybenzotriazole. The mediators studied in this report are shown in Figure II.1.

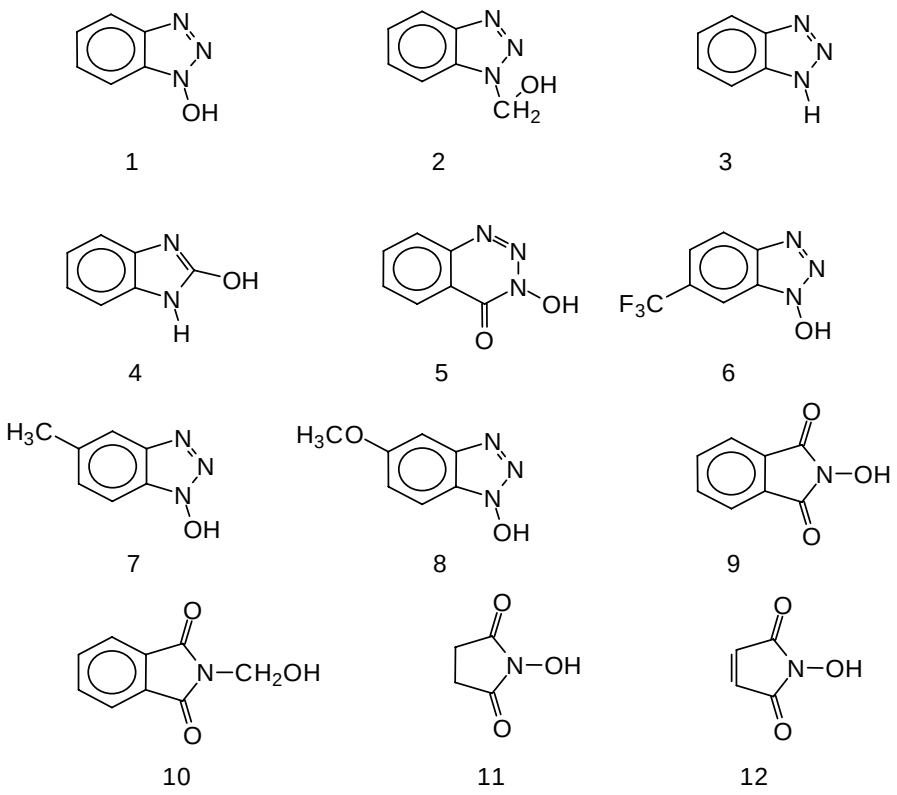


Figure II.1. Mediators employed for LMS-stage.

For the most part, the mediators that were examined for a LMS stage were chosen so as to evaluate the importance of the N-hydroxy group and proposed stability of the nitroxyl radical as it affects the delignification properties of the LMS process. The efficiency of the laccase-mediator delignification process was explored by treating a southern softwood kraft pulp with standardized LMS biobleaching conditions for 24 h and these results are summarized in Table II.1.

Table II.1. Biobleaching results employing a laccase mediator system for delignifying a 26.8 kappa number softwood kraft pulp.[a]

Mediator	% Delignification	Mediator	% Delignification
No Mediator	14	6	10
1 + no Laccase	9	7	23
1	41	8	-15
2	12	9	28
3	8	10	11
4	12	11	16
5	17	12	10

[a]% delignification values were determined by measuring the initial kappa number and the value after LMS.

Interestingly, the use of compounds 2 or 3 as a mediator for laccase failed to yield any significant delignification of the kraft pulp. Clearly, the failure of 2 to act as an effective mediator for delignification supports the proposed mechanism of delignification involving a benzotriazolyl oxide radical. The necessity of the N-hydroxy group is further validated by the observation that 1-H benzotriazole (compound 3) is also inactive as a mediator for laccase. Studies by Sealey and Ragauskas[81] and others[82] have reported that 1-hydroxy-benzotriazole is converted into 1-H benzotriazole during a LMS-stage and clearly this conversion is detrimental towards the overall delignification effect.

Biobleaching experiments also demonstrated that 3-hydroxy-1,2,3-benzotriazin-4(3H)-one or 2-hydroxybenzimidazole were ineffective as mediators for delignification of kraft pulps with laccase. Although several factors may be contributing to the lack of delignification with these two mediator structures it appears that the laccase/1-hydroxybenzotriazole biobleaching system has rather high structural specificities for delignification to occur.

To explore the influence of the aromatic ring on the delignification properties of 1-hydroxybenzotriazole we examined the use of triazole derivatives 6 to 8. The introduction of a strong electron withdrawing group on the aromatic ring such as, trifluoromethyl was detrimental with respect to delignification process. Compound 7 with a 5-methyl group was an active mediator for the delignification of kraft pulps but not as effective as 1-hydroxybenzotriazole. Interestingly, the use of additive 8, with a 5-methoxy group raised the apparent kappa number of the pulp after the LMS-stage. This was tentatively attributed to the generation of a reactive intermediate that coupled to the kraft pulp thereby affording a higher kappa number than the initial value.

Given the rather stringent limitations placed on the 1-hydroxy benzotriazole structure for effective delignification to occur during a LMS stage we explored several alternative N-hydroxy mediator structures. One of our first candidates to be examined was N-hydroxyphthalimide. Literature results by Ishii et *al.*[83] have shown that this reagent can be employed as a co-oxidant in the presence of $Co(acac)_2$ and oxygen. A variety of structures have been oxidized by this system including benzylic alcohols, primary and secondary alcohols. The active oxidizing agent in this believed to be the nitroxyl radical of 1-hydroxy-phthalimide. Given the clear similarities with the proposed mechanism for delignification by laccase and 1-hydroxybenzotriazole it was of interest to determine if 1-hydroxyphthalimide could act as a mediator for laccase. Employing the same pulp and experimental conditions as described above the kraft pulp was treated with 1-hydroxyphthalimide and laccase for 24 h. Following the usual alkaline extraction procedure, we determined that the pulp had a experienced a 28% decrease in kappa number. Although this is not as substantial a decrease in kappa number as observed with 1-hydroxybenzotriazole it is certainly much more than was observed with any other benzotriazole derivative. Interestingly, insertion of a methylene group between the nitrogen and the hydroxy group of 1-hydroxyphthalimide halted the delignification capabilities of this mediator (see Table II.1 compound 10). These results support the suggestion that the N-hydroxy functionality is an important structural feature to the effectiveness of 1-hydroxyphthalimide and 1-hydroxybenzotriazole.

To determine if a simpler pyrrolidine or maleimide structure could act as an effective

mediator for laccase we examine the biobleaching chemistry of additives 11 and 12 (see Table II.1). In each case, we were unable to measure any significant delignification in the laccase mediator bleaching treatment. These latter results re-emphasis the unique structural requirements needed for an effective mediator for LMS delignification.

Computational modeling

The calculated bond dissociation energies for the dehydrogenation of mediator compounds (Figure II.1) are as shown in Table II.2. This is, of course, a thermodynamic term describing the energy required for the generation of the free radicals. The additional values reported in Table I. the energy of the singly occupied molecular orbital (SOMO), charge, spin density and SOMO density, all refer to the mediator radicals and are measures of reactivity toward the substrate.

Given the intrinsically soft nature of radicals, it is proposed that their reactions are under frontier orbital control, in this case relying on the interaction of the SOMO of the radical with the HOMO (highest occupied molecular orbital) of the substrate. If creosol is considered as a lignin model, with a HOMO energy of –8.783 eV, it can be seen that all of the SOMO energies of the mediators are lower, such that the higher values of the latter will minimize the HOMO-SOMO gap, promoting the reaction in question. Furthermore, in frontier molecular orbital theory, the magnitude of the orbital coefficients for the reactive center, as measured by the SOMO density, is reported as an important factor in determining relative reactivity. More familiarly, total charge may be taken as a measure of nucleophilicity and the spin density is an assessment of amount of unpaired spin character at the site of reaction. For these radical species, charge, SOMO density and spin density were measured at the atom from which the hydrogen was removed (i.e. the nitrogen in compound 3 and oxygen in all other compounds).

Table II.2. Computational Modeling of N-hydroxyl Radicals for Compounds 1 – 12 (see Fig. II.1).

Compound	Bond dissociation energy (kcal/mole)	SOMO energy (eV)	Charge	Spin density	SOMO density
1	79.066	-9.2831	-0.405	0.455	0.234
2	104.966	-9.3252	-0.182	0.876	0.007
3	97.112	-10.2602	-0.020	0.719	0.195
4	80.842	-9.3055	-0.269	0.601	0.057
5	85.962	-9.7048	-0.347	0.803	0.040
6	80.224	-9.6665	-0.391	0.447	0.238
7	79.219	-9.1807	-0.409	0.430	0.120
8	78.825	-9.0829	-0.413	0.430	0.180
9	80.315	-9.7392	-0.365	0.533	0.341
10	97.625	-10.3673	-0.190	0.878	0.018
11	81.011	-9.9004	-0.357	0.541	0.339
12	80.514	-9.9453	-0.358	0.536	0.348

Among compounds 1-5, it was found experimentally that only 1-hydroxybenzotriazole was an effective delignification agent. In accord with this result, the bond dissociation energy for (1) is the lowest among these compounds, the SOMO-HOMO energy gap is the smallest, the SOMO density is largest and it has the largest partial negative charge. Interestingly, the spin density of compound 1 is the lowest among compounds 1-5. This is indicative of increased delocalization of the single electron that is less likely among the other compounds.

The SOMO energies of compounds 6-8, relative to 1-hydroxy-benzotriazole are in concert with the predictions of frontier molecular orbital theory, in which electron withdrawing groups and electron donating groups will lower and raise this energy term, respectively. Compound 7 was found to be an active compound and indeed its bond dissociation energy is only slightly below (1), while both the SOMO energy and partial negative charge are both slightly larger than for compound 1. The observed behavior of

compound 8 is somewhat puzzling, since based on the theoretical results it is comparable, and in some cases better than, 1-hydroxy-benzotriazole. These terms include the lower bond dissociation energy, indicative of a lower energy required for the removal of the hydrogen atom; the higher SOMO energy, resulting in a smaller SOMO-HOMO gap; and a larger partial negative charge at the oxygen, possibly making this radical more nucleophilic than the corresponding structure for 1-hydroxybenzotriazole. Perhaps these data are consistent with the proposed formation of reactive intermediates that might couple with the lignin to give an elevated kappa number.

Within the phthalimide compounds, the most efficacious with respect to delignification is compound 9, which among its analogues has the lowest bond dissociation energy, largest partial negative charge. Indeed the values reported for compound 9 are not markedly different from 1-hydroxybenzotriazole itself. Compound 9 also has the lowest spin density that again may be interpreted in terms of increased delocalization as proposed earlier.

Given the undoubted complexity of the reactions involved in the LMS system it is to be expected that that several factors control the overall magnitude of delignification. Nonetheless, the computational approaches employed in this study demonstrate that this approach can be used to predict relative effectiveness of LMS mediators provided that their structures are comparable.

To further examine the fundamental principals governing the reactivity of the RR'NO• radical, we modeled its reactivity with a β-O-aryl ether structure, as shown in Figure II.2.

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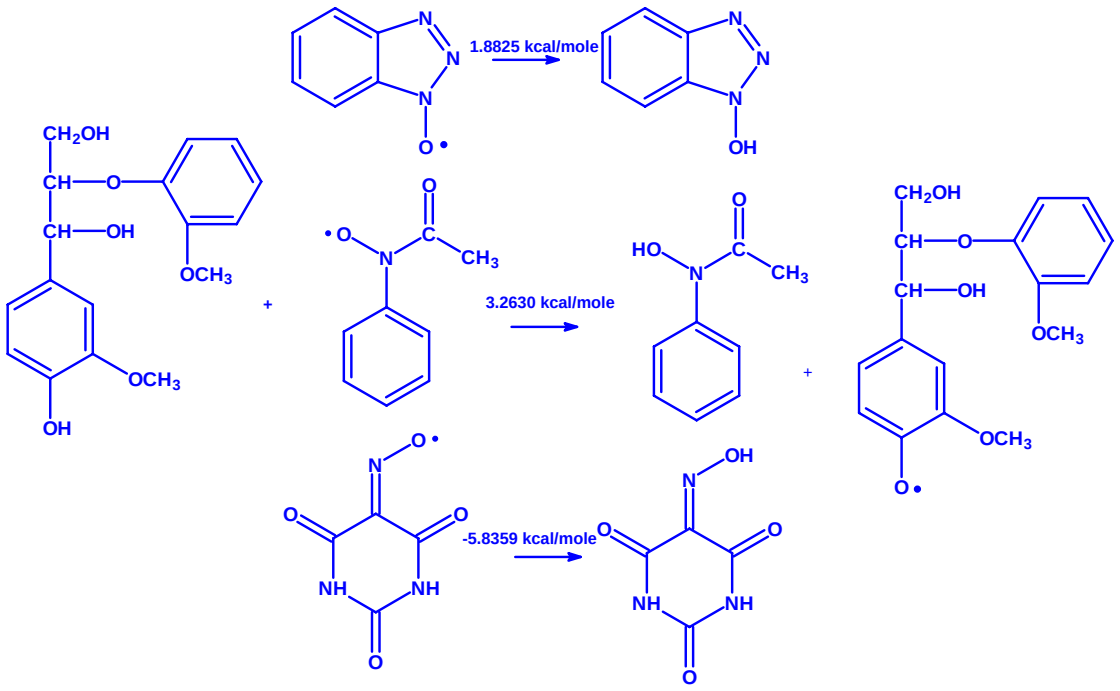


Figure II.2. Computational results from modeling the oxidation of a model dilignol by the action of the RR'NO• radical from VA, HBT, and NHA.

Exploratory LMS biobleaching results had shown that laccase mediator systems employing violuric acid are superior to other first and second-generation mediators. The underlying factors controlling the behavior of laccase mediators has been the subject of some degree of speculation, since an understanding at this level would provide considerable insight into the development of mediators with improved efficacy. The current work addresses these questions by applying computational chemical methods in an examination of the energetics and electronics associated with the oxidation of a lignin model compound

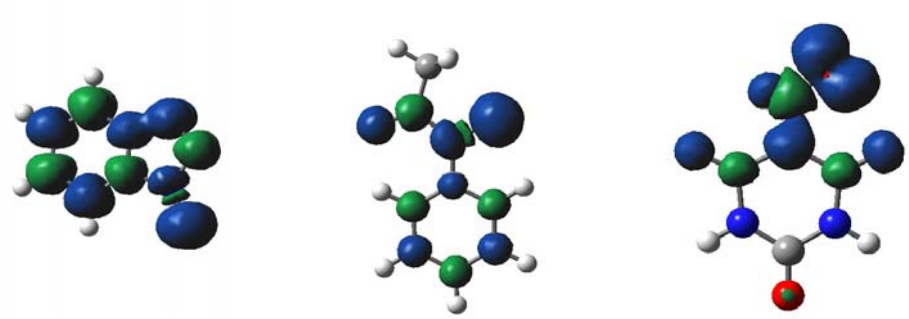
From an energetic standpoint, it can be seen (see Figure II.2) that the oxidation of the model dilignol by the action of the VA is an exothermic reaction, while HBT and NHA are both endothermic reactions. With respect to energetics, therefore, the reaction of VA should be preferred over the other mediators. Electronically, it was determined that the spin density (i.e., the degree of unpaired electron density in the free radicals) at the

reactive oxygen atom is the largest in nitroxyl radical derived from VA, followed by NHA and HBT, which are quite similar. These data summarized in Table II.3 are consistent with the literature indicating the superior performance of violuric acid and a slight advantage of NHA over HBT.

Table II.3. Calculated unpaired electron density and Mulliken charges for nitroxyl mediator.

Mediator	**Unpaired electron density**	**Mulliken charge**
VA	0.98	-0.097
NHA	0.92	-0.146
HBT	0.91	-0.158

It is also interesting to note the degree of delocalization of the unpaired electron as shown by its density among the other atoms in the various structures (Figure II.3). The unpaired nitroxyl electron seems to be tightly constrained in VA, while HBT exhibits considerable delocalization, with NHA being somewhat intermediate in this regard. Finally, the Mulliken atomic charge at the reactive oxygen, although consistently negative, is less negative for violuric acid, such that its reduction may be favored over the other two mediators.



HBT NHA VA

Figure II.3. Spin density plots of the nitroxyl radical from VA, HBT, and NHA.

Computational Experimental Methods

Computational work on the mediator compounds was performed at the semi-empirical level, using the PM3 Hamiltonian operator and full geometry optimizations (Stewart 1990). Open-shell structures were optimized using the Unrestricted Hartree-Fock method, followed by a single-point calculation with the half-electron approximation. The former method optimizes more rapidly, while the latter provides energies that can be compared with closed-shell structures. All calculations were done using Sybyl under license from Tripos Associates, running on a Silicon Graphics Indigo workstation in the School of Forestry at Auburn University

Ab initio calculations have been performed on the closed-shell and free radical forms of the HBT, NHA, VA, and a β-O-4 dilignol using Gaussian 98W. These calculations were done with full geometry optimization at the HF/3-21G* and UHF/3-21G* levels for the closed-shell and free radical structures respectively.

III: Initial Evaluation of LMS Biobleaching SW and HW Kraft Pulps

As pulp producers continue to address environmental concerns, new research opportunities are rapidly developing. In particular, wood utilization practices in North America are becoming consequential as the availability of and accessibility to inexpensive fibers is diminishing. These issues will be vital if U.S.A. pulp producers are to remain competitive in this global market. Research efforts have begun to focus on developing novel manufacturing technologies that address these issues. One of the most promising approaches to improving the economics of kraft pulp production consists of increasing overall pulp yields. This can be achieved by halting the kraft cook at a relatively high kappa (> 45) prior to reaching the terminal phase. The pulp is then subjected to a single or double oxygen stage before it is bleached. Jameel *et al.*[84] and others[85,86] have shown that this approach can improve the overall yield of bleached kraft pulps by 2-4 %. For example, Magnotta, et al. [87] reported that halting a SW kraft cook at kappa 44 and then performing a OO-stage followed by D(E+O+P)D provides a 3.8% yield increase over OD(E+O+P)D for a 30-kappa SW kraft pulp. By extension, an LMS-stage could potentially replace an O or OO-stage for high kappa kraft pulps providing improved delignification properties over a typical O-stage.

As an initial goal in this program we examined the biodelignification of an LMS stage with three of the most effective N-hydroxyl mediators (HBT, NHAA, VA see Figure III.1) identified in preliminary studies of this program for high-kappa kraft pulp.

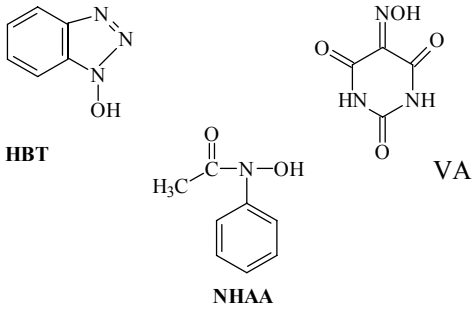


Figure III.1. N-hydroxyl-based Laccase Mediators.

This phase of our studies was directed at improving the LMS biobleaching conditions using NHAA as the mediator. This task was accomplished by carrying out a two-phase

central composite design. In Phase I, 20 enzymatic treatments were performed at various times, doses of NHAA and laccase. In Phase II, 13 treatments were carried out at various doses of NHAA and laccase and at a constant time of 2 hours. Phases I and II, along with the kappa results, are summarized in Tables II.1 and II.2, respectively. In order to simplify the regression analysis, the original variables (i.e. time, % NHAA , and dose of laccase) were non-dimensionalized and coded. Tables III.3 and III.4 define the relationships for the coded variables for Phases I and II respectively.

Table III.1. LMS(E)[a] Experimental Conditions and Data for Phase I Studies.

Experiment #	Time,	% NHAA	Laccase solution	Kappa
Starting pulp	-	-	-	73.4
1	2.0	1.0	1.0	67.2
2	2.0	1.0	9.0	68.1
3	2.0	4.0	9.0	67.2
4	2.0	4.0	1.0	63.7
5	6.0	4.0	9.0	67.5
6	6.0	1.0	1.0	67.1
7	6.0	4.0	1.0	63.0
8	6.0	1.0	9.0	68.4
9	4.0	2.0	3.0	66.7
10	4.0	2.0	3.0	66.0
11	4.0	2.0	3.0	66.4
12	4.0	2.0	3.0	66.9
13	4.0	2.0	3.0	66.6
14	4.0	2.0	3.0	66.0
15	7.4	2.0	3.0	66.0
16	4.0	6.4	3.0	63.8
17	4.0	2.0	19.0	69.2
18	0.6	2.0	3.0	67.1
19	4.0	0.6	3.0	68.5
20	4.0	2.0	0.5	65.3

[a]E stages were performed with 2.5% NaOH, for 1 h, at 80^{o} C and 10% csc.

Table III.2. Experimental Conditions and Data for Phase II Studies.

Experiment #	Time,	% NHAA	Enzyme Solution	Kappa
Starting pulp	-	-	-	73.4
1	2.0	2.00	0.50	65.3
2	2.0	2.00	1.50	66.5
3	2.0	6.82	1.00	62.2
4	2.0	4.00	1.00	63.8
5	2.0	4.00	1.0	63.7
6	2.0	1.17	1.00	67.8
7	2.0	4.00	1.70	64.9
8	2.0	4.00	0.29	62.5
9	2.0	6.00	0.50	61.1
10	2.0	4.00	1.00	63.6
11	2.0	6.00	1.50	63.8
12	2.0	4.00	1.00	63.4
13	2.0	4.00	1.00	63.0

Table III.3. Definition of the Relationships for the Coded Variables for Phase I Studies.

Original Variable	Coded Variable	Coding Relationship	Sample Values
Time, h	T	T= (time-4)/2	-1 when Time = 2.00; 0 when Time= 4.00; +1 when Time = 6.00; -1.682 when Time = 0.64; +1.682 when Time = 7.40
NHAA , %	M	LogM= 0.301NHAA +0.301	-1 when NHAA = 1.00, 0 when NHAA = 2.00; +1 when NHAA = 4.00; -1.682 when NHAA = 0.62 +1.682 when NHAA = 6.40
Laccase, ml	L	Log L= 0.477Laccase+0.477	-1 when Laccase = 1.00; 0 when Laccase = 3.00 +1 when Laccase = 9.00; -1.682 when Laccase = 0.47; +1.682 when Laccase =19.00

Table III.4. Definition of the Relationships for the Coded Variables for Phase II Studies.

Original Variable	Coded Variable	Coding Relationship	Sample Values
NHAA , %	M	M= (NHAA -4)/2	-1 when NHAA = 2.00, 0 when NHAA = 4.00; +1 when NHAA = 6.00; -1.414 when NHAA = 1.17 +1.414 when NHAA = 6.83
Laccase, ml	L	L= (Laccase-1)/0.5	-1 when Laccase = 0.50; 0 when Laccase = 1.00 +1 when Laccase = 1.50; -1.414 when Laccase = 0.29; +1.414 when Laccase = 1.71

The final regression model obtained the data in Table II.1 was as follows:

$$\text{Kappa} = 66.33 - 1.26\ M + 1.23\ L + 0.74\ M*L + 0.28\ L^2$$

The R^2 value obtained is 0.960 and the standard deviation is 0.37. The variable time was not included in the regression model since this term was not statistically significant (at the 95% confidence level). In turn, this highlights the efficiency of an LMS in that most of the delignification takes place in a relatively short time. Figure III.2 demonstrates that there is good agreement between the observed and predicted values.

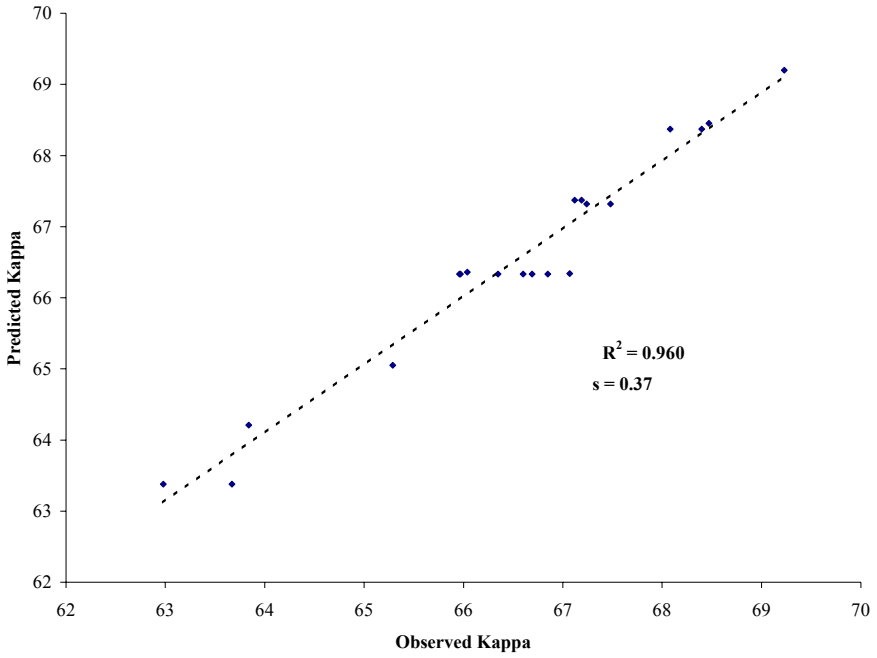


Figure III.2. Predicted kappa Number by the regression Mode vs. the Observed Kappa for Phase I Studies.

The best delignification response was observed at a 4% dose of NHAA and at 1 ml of laccase/10g of o.d. pulp, resulting in 9.8 kappa units with respect to the brownstock kappa. Although the results were promising, we believed that we could further improve the delignification, and thus we proceeded to carry out a second set of LMS studies and these results are summarized in Table III.2. The final regression model from these studies in summarized below:

$$\text{Kappa} = 63.55 - 1.86\ M + 0.91\ L + 0.37\ M*L + 0.69\ M^2$$

The R^2 value obtained is 0.984 and the standard deviation is 0.28. As in the initial study, there was a good agreement between the predicted and observed kappa numbers (see Figure III.3).

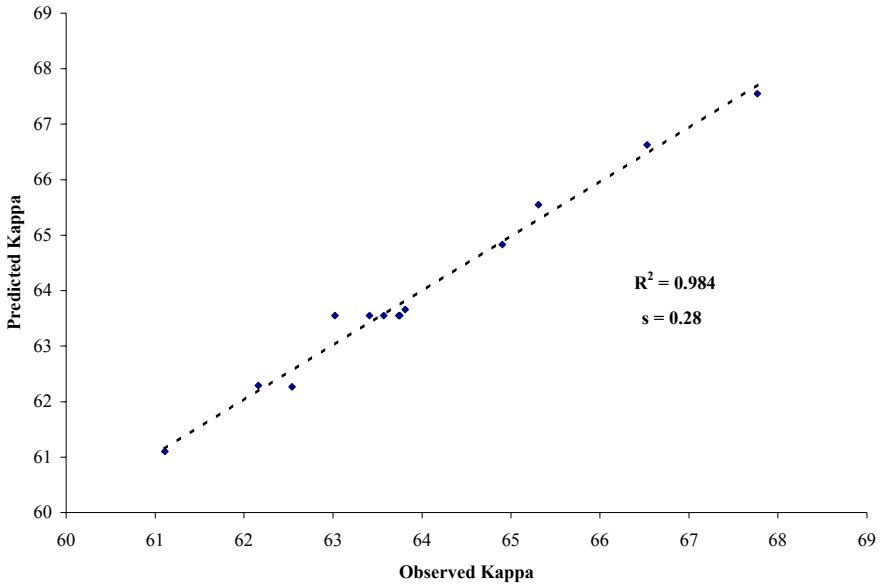


Figure III.3. Predicted Kappa Number by the Model vs. Observed Kappa for Phase II Studies.

Based on our experimental studies, the best delignification response was observed at a dose of 6% of NHAA and 0.50 ml of enzyme solution. These conditions enabled us to reduce the kappa number by additional 2.50 units using 50% less of enzyme solution. The dose of mediator had to be increased from 4% to 6%. In turn, this suggests that an LMS_{NHAA} treatment is not truly catalytic. Freudenreich et *al.* [88] have recently shown that NHAA can be reduced to a nondelignifying agent, and that may explain in part, the need for higher doses of NHAA.

Although we anticipated that the delignification properties of LMS_{NHAA} could be further improved, preliminary concurrent investigative studies employing violuric acid (VA) as a LMS mediator indicated that this agent exhibited superior biodelignification properties. Employing identical LMS conditions and the same pulp, an LMS_{VA}(E) treatment was found to substantially outperform the LMS_{NHAA} system. Indeed, we were able to reduce the pulp kappa number by 34.0 kappa units. In addition, we found this trend holds true regardless of the LMS conditions and the starting kappa number. For example, an LMS_{VA}(E) treatment (4% molar equivalence of NHAA on o.d pulp; 5% consistency; 3ml

of laccase solution/10 g of o.d. pulp; 45°C; 2 hr. Extraction: 2.5% NaOH on o.d. pulp; 80°C; 2 hr.) on a 50-kappa SW kraft pulp led to a drop of 17 kappa units. When the identical treatment was carried out using NHAA, we observed a total drop of 7.2 kappa units.

A notable observation of these studies is the dramatic differences in delignification efficiency despite the fact that both VA and NHAA are N-hydroxyl-based mediators. Following these investigations, we further explored the response of the LMS_{VA}(E) system to laccase charge. These results are shown in Figure III.4. In general, the behavior of an LMS_{VA} treatment was consistent with the trends observed with the LMS_{NHAA} system.

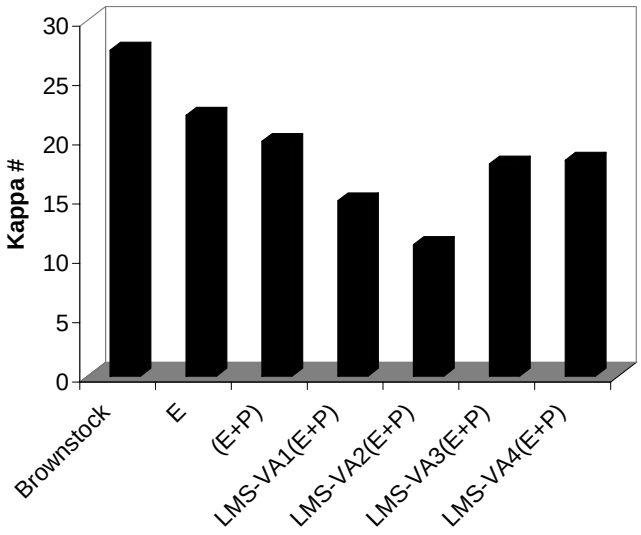


Figure III.4. Delignification of 27.5 Kappa SW Kraft Pulp with LMS_{VA}(E*).[a]

[a:] VA1 used 1.87 E+04 U of laccase, VA2 used 5.61 E+06 U laccase, VA3 used 11.22 E+06 U of laccase, VA4 used 22.4 E+06 U of laccase. Charge of VA was 4%, E-stage was at 80°C, 10% csc, 2.5% NaOH, for 1.5 h. (E+P) stages were performed in a comparable manner except 0.5% H_2O_2 was added.

Our interest in LMS treatments is based not solely on its delignification response but also on its low reactivity with cellulose. Indeed, treatment of an LMS system with a fully

bleached pulp was shown by Haynes[89] to have no impact on pulp viscosity, suggesting that the LMS system is highly specific for lignin. This high bleaching specificity should provide very high pulp bleaching yields. To test this hypothesis, a series of HW and SW kraft pulps were treated to an LMS_{VA}(E+P) sequence and gravimetric yields were determined. The results shown in Table III.5 demonstrate the improved selectivity and yield benefits that can be achieved from an LMS_{VA}(E+P) stage for both high- and low-kappa kraft pulps.

Hexenuronic acid analysis of the two HW kraft pulps was performed in accordance with a modified literature.[90] The analysis indicated that the contribution of these groups to the initial kappa number was 18.2% (initial kappa no. 26.9) and 24.0% (initial kappa no. 10.8), respectively. It is known that an LMS treatment does not oxidatively remove significant amounts of hexenuronic acid groups.[91,92] Hence, further substantial delignification could be accomplished after an LMS_{VA}(E+P) treatment if the two HW kraft pulps had been treated with a mild acid stage.

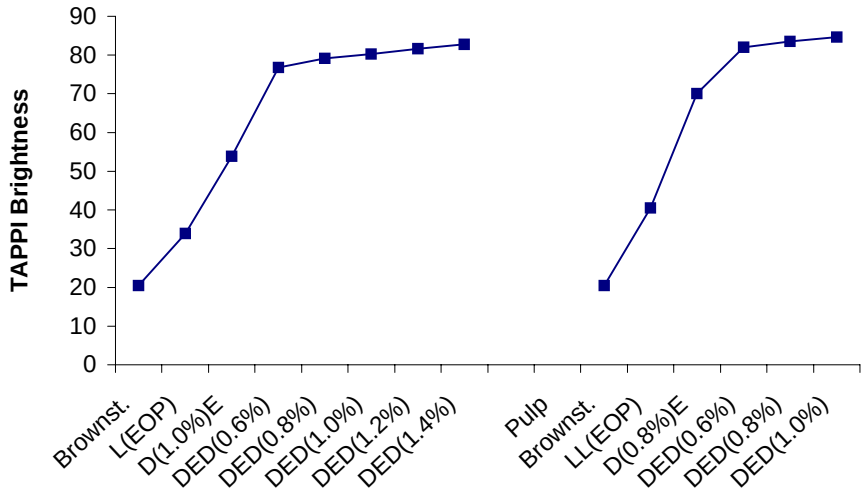
Table III.5. Yield and delignification Response after Treating High- and Low-Kappa SW and HW Kraft pulps with LMS_{VA}(E+P).[a]

Pulp	SW 50.0 kappa 45.0 cP	SW 27.5 kappa 24.2 cP	HW 26.9 kappa 67.6 cP	HW 10.8 kappa 21.6 cP
% Delign.	42.6	61.1	65.4	70.4
% Viscosity Loss	25.8	20.4	45.9	22.6
Yield[2]	99.9	100.0	99.4	99.1

[a](E+P) stage was for 1.5 h, 80°C, 10% csc, 0.5% H_2O_2, 2.5% NaOH; [2]gravemetric yield corrected for loss of lignin (i.e., mass lignin lost = 0.15% (initial – final kappa #).

The pulp bleachability of the LMS_{VA}-treated pulps was examined using a DED sequence. These results are summarized in Figure III.5 and Table III.6. These results indicated that

the LMS-treated pulps can achieve +80 TAPPI brightness values employing ECF



bleaching technologies.

Figure III.5. Changes in TAPPI Brightness Bleaching SW Kraft Pulp (kappa 30) with LMS_{VA}(E+P+O)DED and $LMS_{VA}LMS_{VA}$(E+P+O)DED.

Table III.6. Bleaching Conditions Employed for LMS_{VA}(E+P+O)DED and $LMS_{VA}LMS_{VA}$(E+P+O)DED.

LMS_{VA}	(E+P+O)	D
2% charge VA. See experimental section for remaining details.	70°C, 10% csc, 1 h, 0.05% $MgSO_4$, 2.5% NaOH, 15 min 60 psi, 12 psi vented every 5 min.	D_0, 10% csc, 50°C, 45 min. D_1 70°C, 3 h, 10% csc See Fig III.5 for ClO_2 charges in brackets

Section III Conclusions

The results of this study yield a series of important discoveries. First, high-kappa pulps respond favorably to both LMS_{NHAA} and LMS_{VA}, although the latter mediator exhibits a superior biodelignification response. For the LMS_{NHAA} system studied, the reaction is complete within 40 minutes, and high doses of laccase do not provide improved delignification. In contrast, higher doses of mediator provide improved lignin removal. These experimental trends appear to also hold true with the LMS_{VA} system. The high selectivity of the LMS_{VA} system provides exceptionally high pulp yields after alkaline extraction. Finally, the LMS-treated pulps are amendable to ECF bleaching conditions providing +80 brightness pulps. Despite the significant delignification that is achievable with NHAA and VA on high- and low-kappa pulps, these mediators cannot be considered ideal. Hence, this creates once again an opportunity for finding other mediators compatible with laccase.

Experimental Details for Section III

Materials. All materials employed in this study were purchased from Aldrich Chemicals, Milwaukee, WI, and used as received except for *N*-acetyl-*N*-phenylhydroxylamine (NHAA) and laccase. NHAA was synthesized in accordance with Oxley's method and laccase from *Trametes villosa* was donated by Novo Nordisk Biochem.

Furnish. Table III.7 provides a brief description of the pulps employed in this study. In brief, the LMS_{NHAA} and LMS_{VA} treatments were performed on a series of high-lignin-content laboratory-prepared conventional batch southern softwood and hardwood kraft pulps. In addition, two commercial hardwood and softwood kraft pulps were employed with the LMS_{VA} system.

Table III.7. Description of Kraft Pulps Employed.

Pulp	Kappa #	Viscosity/cP
SW-lab prepared	73.4	-
SW-lab prepared	50.0	45.0
SW-commercial	27.5	24.2
HW-lab prepared	26.9	67.6
HW-commercial	10.8	21.6

Laccase Assay. Laccase activity was measured by monitoring the rate of oxidation of syringaldazine. One unit of activity (U) was defined as the change in absorbance at 530 nm of 0.001 per minute, per ml of enzyme solution, in a 100 mM phosphate buffer (2.2ml) and 0.216 mM syringaldazine in methanol (0.3ml). The procedure was carried out at 23°C. The activity of the enzyme was 1.87 E+06 (U/ml of enzyme solution).

Laccase-mediator treatment procedure. All LMS experiments were carried out using a 1000-ml capacity Parr reactor equipped with a pressure gauge, a stirrer, and a temperature controller. The pulp (20g o.d. basis) was placed in the reactor and the consistency was adjusted to 5% using distilled water. The slurry was then heated to 45^{o}C while mixing and was maintained at that temperature throughout the incubation time. The appropriate dose of mediator was added and further mixing (approx. 5 min) was allowed. The pH was then adjusted to 4.5 and the appropriate dose of laccase was added. The reactor was sealed and pressurized with oxygen (10 bar). Subsequent to the treatment, the pulp was removed from the reactor and thoroughly washed with distilled water. The treated pulp was subjected to an alkaline extraction stage and then characterized.

Pulp Characterization. The delignification response of the LMS_{NHAA} (E) treatments was expressed as kappa number. Kappa numbers were determined in accordance with TAPPI Standard Method T-236. Each reported kappa number represents the average of two individual measurements. Typically, the kappa number of duplicates varied by +/- 0.3 kappa units.

Experimental Design. A two-phase central composite design (CCD) was carried out in this study. A total of 33 LMS_{NHAA} (E) treatments were performed at various times, doses of NHAA , and laccase. The data obtained from Phases I and II were then subjected to conventional multiple linear regression analysis. Regression models from each phase were constructed from a set of variables consisting of the original variables, together with their squares and pair wise cross products. The regression models contained only those terms that were justified by a significance test at a 95% confidence level (t-test) and that yielded the highest value of the multiple correlation coefficient (R^2). In addition, the lack of fit (LOF) for the two regression models was calculated and was determined to be insignificant at the 95% confidence level.

IV. Comparative Analysis of Laccase-Mediator Systems and Oxygen Delignification

Based on our previous studies, we demonstrated that an LMS_{NHAA} , an LMS_{HBT}, and an LMS_{VA} treatment can yield substantial delignification when applied on high-kappa pulps. However, under the experimental conditions used in our studies, we noted that VA significantly outperformed both NHAA and HBT as a mediator for biodelignification. This study summarizes our continued research efforts in this field. A conventional SW kraft pulp (kappa # 73.4) was subjected to LMS_{VA}, oxygen, double oxygen, and a combination of LMS and oxygen treatments. The pulps were then characterized for viscosity, kappa, and brightness.

LMS Delignification Results.

The delignification results shown in Figure IV.1 clearly demonstrate than an LMS system can yield substantial delignification on a high-kappa pulp. As expected, the delignification effect was further enhanced after an E stage, as the NaOH solubilizes the oxidized lignin.

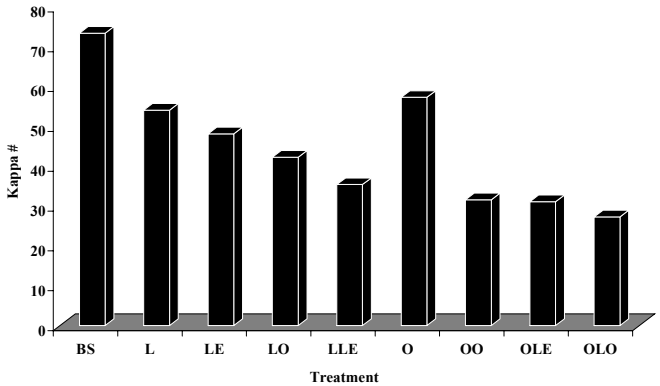


Figure IV.1. Kappa numbers of Brownstock (BS) and Pulps Treated with O, LMS_{VA}, and O/LMS_{VA} (Note: L=LMS_{VA}).

The data also suggest that under the experimental conditions employed in this study, an oxygen stage subsequent to an LMS treatment did not yield substantial delignification. This inefficiency could be linked to the oxidized nature of the residual lignin after an LMS. Presumably, the alkalinity of the oxygen system is partially directed towards the ionization of lignin oxidized by the prior LMS stage acid groups and this reduces the efficiency of the oxygen-stage. The same rationale could also be applied to the observed delignification response of the O stage subsequent to the OLMS treatment (i.e., $O(LMS_{VA})O$).

Based on the experimental protocol used in this study, the delignification response of the $LMS_{VA}LMS_{VA}E$ and $OLMS_{VA}E$ sequence was comparable to that of an OO stage and better than that of a single O stage. In addition, the $LMS_{VA}E$ treatment responded more favorably than a single O. Overall, the LMS_{VA} delignification system exhibits an additive effect that is reflected in the percent delignification of the $LMS_{VA}E$ and $LMS_{VA}LMS_{VA}E$ treatments. Clearly, these results highlight the efficiency of the laccase-mediator system.

LMS Viscosity Results.

Accompanying the substantial delignification response of a laccase-mediator system was a high retention in pulp viscosity. The viscosity results shown in Figure IV.2 indicate that LMS, LMS(E), and LMS(LMS)E treatments are much more selective than an O or an OO stage. An LMS treatment applied after an oxygen delignification stage was more beneficial to the viscosity than when applied before, which re-emphasizes the uniqueness of the enzymatic system.

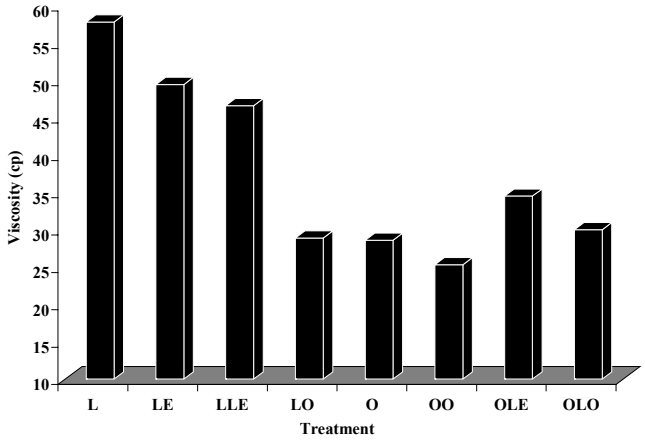


Figure IV.2. Terminal Viscosity of Pulps Treated with O, LMS_{VA}, and O/LMS_{VA} (Note: L=LMS_{VA}).

The incorporation of an LMS treatment between a double oxygen stage was also beneficial, since the terminal viscosity was higher than that of a double oxygen treatment. Furthermore, the viscosity of an O(LMS_{VA})O treatment was comparable to a single O stage. In turn, this may prove to be an attractive approach in the future for obtaining the typical enhanced delignification benefits from a double oxygen stage without further loss in pulp viscosity. Nonetheless, further experimental work will be needed to support this claim.

LMS Brightness Results

The brightness data shown in Figure IV.3 demonstrate that an LMS treatment leads to pulp darkening. This effect is attributed to the formation of quinone structures during an L treatment. The alkaline extraction stage subsequent to an LMS_{VA} treatment was beneficial in regaining some of the brightness loss, obviously because of the well-known ability of NaOH to destroy quinone type structures. The combination of LMS_{VA} with oxygen had less of a detrimental effect on brightness than LMS_{VA} alone.

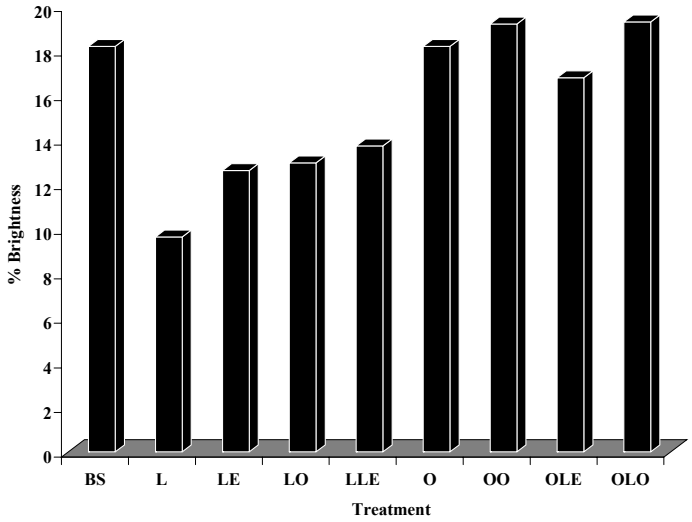


Figure IV.3. Brightness of Brownstock (BS) and Pulps Treated with O, LMS_{VA}, and O/LMS_{VA} (Note: $L=LMS_{VA}$).

Section IV Conclusions

In summary, the results from this study suggest that substantial delignification with high retention in pulp viscosity can be achieved *via* a laccase-violuric acid system. This study examined the delignification capabilities of an LMS system operating under extreme lignin content conditions. The observed selectivity of the LMS system should provide a technology in the future to delignify kraft pulps with high yields. It is now well established that the yield benefits of stopping a kraft cook prior to reaching the residual phase can be achieved with SW kraft pulps of kappa # 40-50. Based on our studies using a high kappa SW kraft pulp, we believe that a 50% delignification level using the lower kappa pulps (i.e. kappa # 40-50) is readily achievable with retention of pulp viscosity.

Section IV Experimental Details

Materials. All materials were purchased from Aldrich Chemical Co., Milwaukee, WI, and used as received. Laccase from *Trametes villosa*, was donated by Novo Nordisk Biochem, Franklinton, NC.

Furnish. A conventional southern USA softwood kraft pulp (kappa # 73.8) was prepared from *Pinus taeda* chips at Potlatch Corp. facilities in Cloquet, MN. The chips were cooked to an H-factor of 573 using 18.5% active alkali. The pulp was thoroughly washed, screened, centrifuged, fluffed, and stored at 4°C prior to carrying out experiments.

Methods

Enzyme assay. Laccase activity was measured by monitoring the rate of oxidation of syringaldazine. One unit of activity (U) was defined as the change in absorbance at 530nm 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, pH 6.7). The procedure was carried out at 23°C. The activity of the laccase was 1.87 E + 06 U/ ml of enzyme solution.

Laccase-mediator delignification procedure. A 300-ml capacity Parr reactor equipped with a stirrer, a pressure gauge, a heating mantle, and connected to a Parr 4842 temperature controller was charged with 10 g of o.d. fibers. The pulp consistency was adjusted to 10% with distilled water. The slurry was then heated to 45°C and was maintained at this temperature throughout the incubation period. VA (4.4 mmol/ 10 g of o.d. pulp) was then added to the heated slurry. Subsequent to mixing the slurry (approx. 5 minutes), the pH was adjusted to 4.5 with glacial acetic acid or saturated sodium bicarbonate solution. Laccase (93,500 U, or 0.05 ml of enzyme solution/ g of o.d. fiber) was added, and the reactor was sealed and pressurized with oxygen to 145 psig. After a mixing period of 1 hour, the pulp was removed from the reactor and thoroughly washed

with distilled water (12L per 10 g of o.d. pulp). The treated and washed pulp was either followed by subsequent treatments (oxygen delignification or LMS) or simply subjected to an alkaline extraction stage (E).

Alkaline extraction stage. Alkaline extractions (E) were carried out for 1 hour at 80°C, 10% consistency in 4mm thick heat-sealable Kapak pouches. All E treatments employed 2.5% charge (o.d. basis) of NaOH.

Oxygen delignification. Oxygen delignification was carried out in a 300-ml capacity Parr reactor. All treatments were conducted at 95 °C, 10 % consistency, for 1 hour. A charge of 2.5% NaOH (o.d. basis) was employed. Subsequent to a treatment, the pulp was thoroughly washed with distilled water.

Hexenuronic acid content in brownstock. The content of hexenuronic acids in the brownstock was indirectly measured in accordance with a modified procedure reported by Vuorinen et *al.* [93] In brief, a 1000-ml round bottom flask was charged with 25 g of pulp (o.d. basis). The pulp consistency was adjusted to 3% by adding distilled water. The pH was then lowered to 3 using concentrated sulfuric acid. The slurry was refluxed for three hours at 100°C. The change in kappa number before and after the treatment was then determined and served as indirect measurement of hexenuronic acids (see Table IV.1). Clearly, the hexenuronic acid content of this pulp was negligible.

Table IV.1. Changes in Kappa # after Acid Treatment of Kraft Brownstock Pulp.

Replicate #	Initial	Final kappa	% change
1	73.4	71.5	2.6
2	73.4	71.9	2.0

Pulp characterization. The kappa, brightness, and viscosity before and after treatments were measured in accordance with TAPPI Test Methods T236 and UM246, T452, and T230, respectively.(94) Only the terminal viscosity of the treated pulps was measured. The initial viscosity of the starting material could not be determined using TAPPI Test Method T230 due to the high-lignin content in the pulp. Kappa and viscosity

measurements were duplicated. Each brightness value was based on the average of five readings.

V. Evaluating the Interactions between LMS and Modified E-stages for High -Kappa Kraft Pulps

The purpose of this study was to examine the delignification chemistry of LMS systems and the subsequent alkaline extraction stage on a high-lignin content kraft pulp. The LMS_{HBT} system served as a reference to the understudied LMS_{NHAA} system. The conditions of the alkaline extraction stage were varied so that the effects of peroxide (E+P), oxygen (E+O), and peroxide and oxygen (E+P+O) on the LMS treated pulp could be established. The outcome of the LMS(E*) treatments was determined by measuring the changes in lignin content and brightness of the treated pulps. In addition, the structural changes in residual lignins isolated after LMS_{NHAA}(E), LMS_{HBT}(E) and LMS_{NHAA}(E+P+O), LMS_{HBT}(E+P+O) treatments were ascertained by NMR. All LMS_{NHAA} (E*) and LMS_{HBT} (E*) treatments were performed under identical conditions. This enabled us to relate the changes in biobleaching to the mediator. These changes were assessed by determining structural differences in the residual lignins as well as by measuring the kappa and brightness of the treated pulps.

Extent of LMS Delignification and Brightness Results

Delignification results from control experiments. Our previous biobleaching studies had shown that substantial delignification could be accomplished with an LMS treatment. After an annual industrial panel review of research progress, we were requested to perform a series of control experiments in the absence of laccase. Hence, the pulps were first treated in the presence of HBT and/or NHAA (MS_{HBT} and MS_{NHAA}) and then subjected to the E* stages. In addition, an alkaline extraction (E) was carried out on the untreated brownstock (BS(E)). The kappa numbers measured subsequent to the MS_{HBT}(E), MS_{NHAA}(E), and BS(E) treatments were the comparable (see Figure V.1).

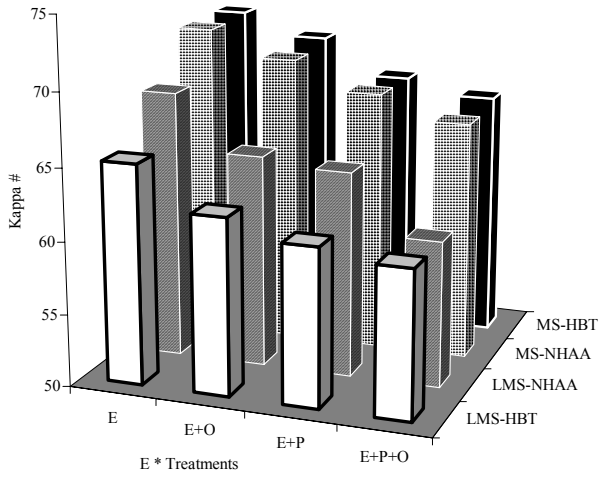


Figure V.1. Kappa results of Control Treatments in the Absence of Laccase (MS-NHAA and MS-HBT) and LMS Treated Pulps using NHAA and HBT(LMS-NHAA and LMS-HBT) Followed by the Alkaline Extraction Stages E, E+O, E+P, and E+P+O.

Clearly, this indicates that the presence of the mediator alone does not delignify the pulp. Furthermore, we can conclude that the decrease in kappa number of pulps treated with either mediator and followed by an E+O, E+P, and E+P+O stage is attributed to the oxidative reinforcement and not to the mediator.

Delignification results from LMS treated pulps. The LMS (E*) delignification kappa data are depicted in Figure V.1. Clearly, these results suggest that both an LMS_{HBT} and an LMS_{NHAA} delignified the high-kappa pulp. However, based on the experimental conditions employed in this study, the use of HBT yielded a higher degree of delignification than NHAA. The use of oxidatively reinforced alkali extractions after both the LMS_{HBT} and LMS_{NHAA} treatments further enhanced this effect. The use of an (E+P+O) alkaline extraction stage seems to narrow the difference of the kappa number after an LMS_{NHAA} and an LMS_{HBT} treatment. The addition of peroxide in the alkaline extraction stage leads to both brightening and delignification. This differs from the typical response of D_0 pulps to E+P treatments, where the peroxide essentially brightens the pulp and does not significantly delignify it. In our case, the alkaline peroxide

response could be due to several factors, including the presence of transitional metals in the pulp.

Brightness results from LMS treated pulps. Figure V.2 illustrates the brightness values for the LMS (E) and LMS (E*) treated pulps. Pulp darkening was observed subsequent to the LMS_{HBT} (E) and LMS_{NHAA} (E) treatments. However, the loss in brightness was more pronounced when NHAA was used. Pulp darkening after LMS_{HBT} and LMS_{ABTS} stages on low-lignin content pulps has been reported by several researchers.[95]

We speculate that this may be attributed to a greater content of quinone type structures generated during an LMS_{NHAA} than during an LMS_{HBT} treatment. The oxidatively reinforced alkali extraction stages were effective at countering this loss in brightness, especially when peroxide was used (i.e., E+P and E+P+O). Evidently, the oxidative reinforcement of an alkaline extraction stage leads to the destruction of chromophores.

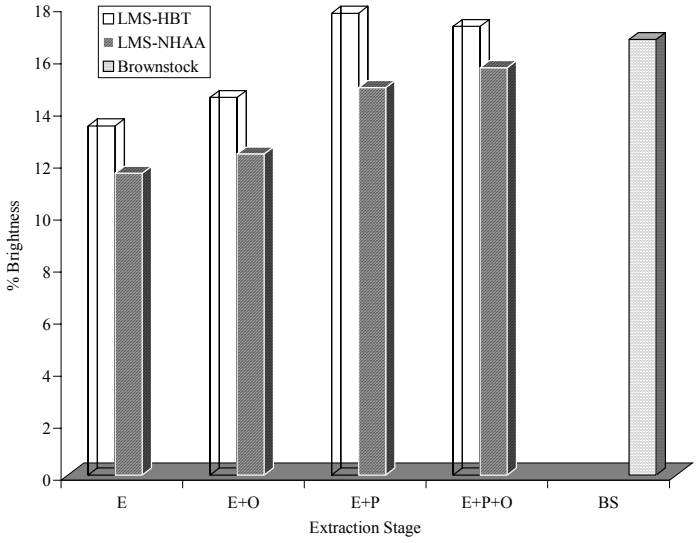


Figure V.2. TAPPI Brightness Results of Brownstock as well as of LMS Treated Pulps using NHAA and HBT (LMS_{NHAA} and LMS_{HBT}) Followed by the Alkaline Extraction Stages E, E+O, E+P, and E+P+O. N.B: Each data point represents the average of 5 brightness readings

Analysis of phosphitylated residual lignins. Having characterized the LMS (E*) treated pulps for lignin content and brightness, we proceeded further with our study by examining the structural changes in the residual lignins. The residual lignins from the brownstock and from LMS_{HBT} (E), LMS_{HBT} (E+P+O), LMS_{NHAA} (E), and LMS_{NHAA} (E+P+O) treated pulps were isolated, phosphitylated, and characterized *via* ^{31}P NMR. This facile and effective technique enabled us to canvass several important lignin functional groups, including carboxylic acid groups, aliphatic hydroxyl groups, and phenolic hydroxyl groups in non-condensed and condensed at C-5 lignin moieties. Figure V.3 illustrates phenolic lignin moieties that were quantified using this procedure.

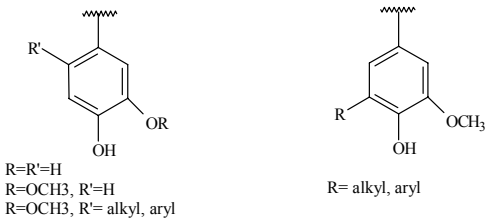


Figure V.3. Phenolic Hydroxyl Groups in C-5 Condensed and Non-condensed at C-5 Lignin Structures.

Carboxylic acid groups. The results shown in Figure V.4 clearly indicate that relative to the brownstock residual lignin, the carboxylic acid groups content increased after an LMS_{HBT} (E) and LMS_{NHAA} (E) treatment on the high-kappa pulp.

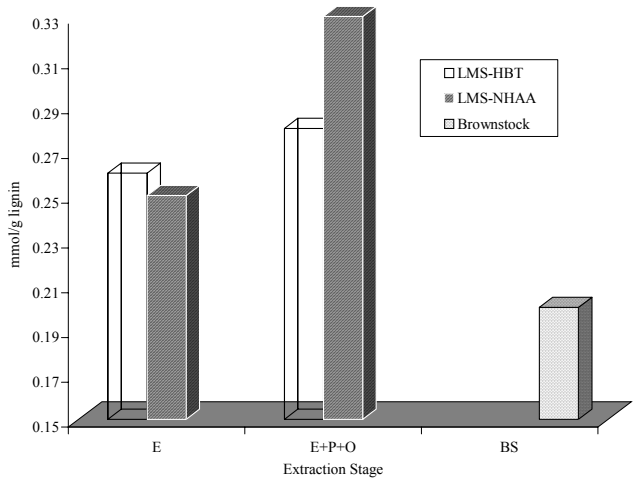


Figure V.4. Carboxylic Acid Hydroxyl groups in Residual Lignins Isolated from the Brownstock as well as from LMS Treated Pulps using NHAA and HBT (LMS_{NHAA} and LMS_{HBT}) Followed by the Alkaline Extraction Stages E and E+P+O.

An analogous increase in carboxylic acid groups has been reported in previous LMS_{HBT} work using low-lignin content kraft pulps which will be reviewed in the subsequent chapter. Our results also showed that the reinforcement of the alkaline extraction stage with peroxide and oxygen further increased the content of carboxylic acid groups. The content of carboxyl groups after an LMS_{NHAA} (E+P+O) was greater than after an LMS_{HBT} (E+P+O). This difference must be due to the different delignification chemistry of the two mediators.

Phenolic hydroxyl groups in lignin structures non-condensed at C-5. The data shown in Figure V.5 indicate that the residual lignins isolated after an LMS_{NHAA} (E) and LMS_{HBT} (E) treatment were depleted of non-condensed at C-5 lignin structures with respect to the brownstock lignin. Nonetheless, the decrease in this moiety was more pronounced with NHAA than with HBT.

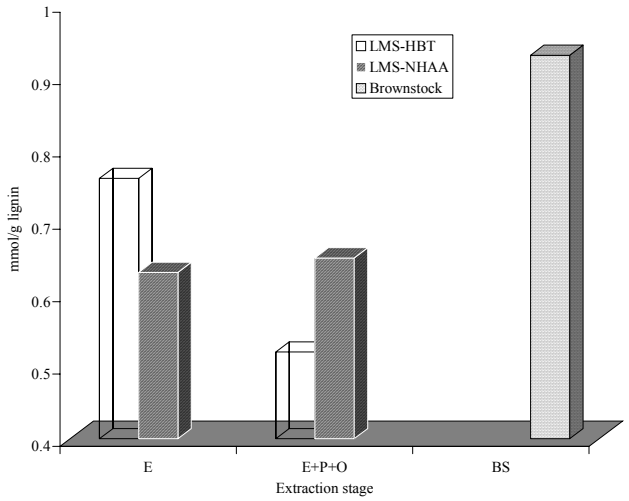


Figure V.5. Phenolic Hydroxyl Groups in Non-condensed Lignin Structures at C-5 in Residual Lignins isolated from the Brownstock as well as from LMS Treated Pulps using NHAA and HBT (LMS_{NHAA} and LMS_{HBT}) Followed by the Alkaline Extraction Stages E and E+P+O.

Phenolic hydroxyl groups in C-5 condensed lignin structures. Inspection of Figure V.6 reveals that relative to the brownstock residual lignin, the concentration of phenolic hydroxyl groups in C-5 condensed lignin structures after an LMS_{HBT} (E) and an LMS_{NHAA} (E) treatment was comparable. The reinforcement of the alkaline extraction stage after an LMS_{HBT} treatment substantially decreased the content of phenolic hydroxyl groups in these condensed lignin structures. A decrease of this magnitude was not observed after an LMS_{NHAA} treatment.

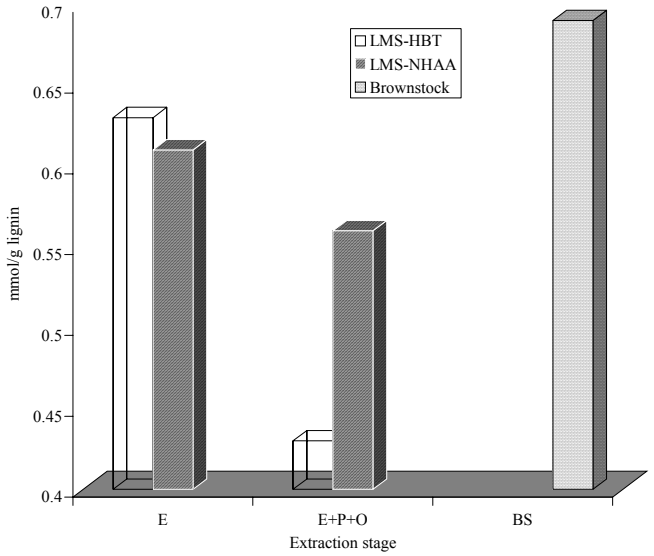
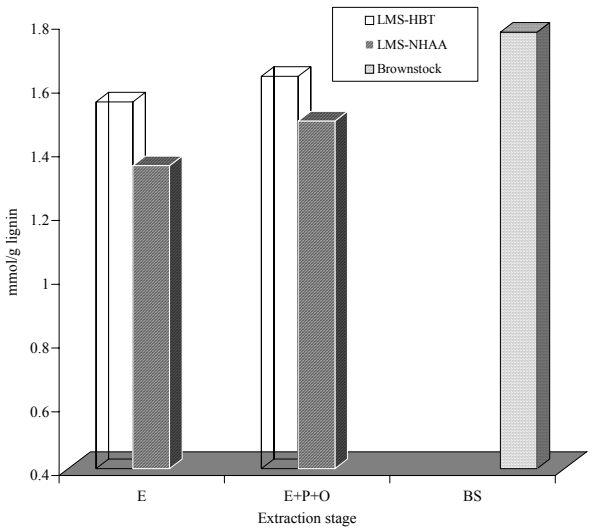


Figure V.6. Phenolic Hydroxyl groups in C-5 condensed Lignin Structures in Residual Lignins Isolated from the Brownstock as well as from LMS Treated Pulps using NHAA and HBT (LMS_{NHAA} and LMS_{HBT}) Followed by the Alkaline Extraction Stages E and E+P+O.

Overall, the phenolic hydroxyl data in non-condensed and condensed lignin structures seem to suggest that the LMS delignification chemistry on high-kappa pulps exhibits a higher selectivity towards non-condensed at C-5 lignin structures than towards condensed C-5 lignin moieties. These results are in general agreement with recent studies on LMS_{HBT} (E) treatments on low-lignin content pulps, which highlight the oxidative selectivity of an LMS towards phenolic hydroxyl groups.[96,97]

Aliphatic hydroxyl groups. The results shown in Figure V.7 illustrate a decrease in the content of aliphatic hydroxyl groups after an LMS_{NHAA} (E) and an LMS_{HBT} (E) treatment relative to the brownstock residual lignin. However, the decrease was greater with NHAA than with HBT.



FigureV.7. Aliphatic Hydroxyl Groups in Residual Lignins isolated from the Brownstock as well as from LMS Treated Pulps using NHAA and HBT (LMS_{NHAA} and LMS_{HBT}) Followed by the Alkaline Extraction Stages E and E+P+O.

This observation is indicative of side chain oxidation, and is consistent with Freudenreich *et al*. [98] and Li *et al*. [108] recent observation of side chain oxidation and fragmentation of model compounds during LMS treatments.

Hexenuronic acids in the brownstock. It is well known that the presence of hexenuronic acids (HexA) in kraft pulps, especially in hardwood kraft fibers, has an impact on the kappa number. Indeed, Vuorinen et *al.* [101] and Chakar et *al.* [99] have reported that hexenuronic acids can contribute as much as 50% to the kappa number of hardwood kraft pulps. Clearly, in such cases, a kappa number would not be a good reflection of the lignin content. In this study, we proceeded to indirectly measure the HexA content in the brownstock in accordance with the literature.[101] The change in kappa number before and after the acid hydrolysis treatments averaged 2.15% (see Table **V.1**). Consequently, this indicates that the kappa number is a good reflection of the lignin content in the pulp used in this study.

Table V.1. Changes in Kappa Number after Acid Treatment.

Initial kappa number of	Kappa number after	%
75.4	73.6	2.4
75.4	74.0	1.9

Section V Conclusions

In summary, the delignification response of the pulps clearly indicates that an LMS treatment can be effectively employed on high-lignin content kraft pulps. Oxidative reinforcement of the alkali extraction stages is beneficial in delignifying and countering the darkening phenomenon observed after LMS treatments. Based on the spectral analysis of residual lignins, an LMS_{NHAA} (E) treatment led to a greater decrease in the content of phenolic hydroxyl groups in non-condensed at C-5 lignin structures than an LMS_{HBT} (E) stage. Nonetheless, the results seem to indicate that both an LMS_{NHAA} and an LMS_{HBT} principally favors the oxidation of free phenolic moieties. Oxidation of side chain aliphatic hydroxyl groups was more pronounced after an LMS_{NHAA} than after an LMS_{HBT} treatment. In conclusion, our LMS studies on high-kappa kraft pulps suggest that differences exist in the delignification chemistry of NHAA and HBT, despite the fact that both mediators operate *via* nitroxyl radicals.(100) As previously discussed, the formation of quinones could be occurring during the LMS treatment. This hypothesis is supported by the brightness response of the pulps to E vs. (E+P+O). In addition, the structural changes in the phenolic hydroxyl content of the LMS (E*) residual lignin are consistent with our hypothesis. Further studies are ongoing to confirm this important issue.

Section V Experimental Details

Materials and Methods. All materials were purchased from Aldrich Chemical Co., Milwaukee, WI, and used as received, except for *p*-dioxane, NHAA and laccase. A conventional southern USA softwood kraft pulp was prepared from *Pinus taeda* chips at Potlatch Corp facilities in Cloquet, MN. The chips were cooked to an H-factor of 573 using 18.5 % active alkali. The pulp was thoroughly washed, screened, centrifuged,

fluffed, and stored at 4°C prior to LMS bleaching treatments. Laccase, from *Trametes villosa*, was donated by Novo Nordisk Biochem, Franklinton, NC.

Enzyme assay. Laccase activity was measured by monitoring the rate of oxidation of syringaldazine. One unit of activity (U) was defined as the change in absorbance at 530 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, pH 6.7). The procedure was carried out at 23°C. The activity of the laccase was 1.87E+06 U/ml of enzyme solution.

Laccase-mediator delignification procedure. A 1000-mL capacity Parr reactor equipped with a stirrer, a pressure gauge, a heating mantle, and connected to a Parr 4842 temperature controller was charged with 15 g of o.d. fibers. The pulp consistency was adjusted to 9 % by adding distilled water. The slurry was then heated to a temperature of 45°C and was maintained at this temperature throughout the incubation period. HBT (2.2 x 10^{-3} moles) was then added (or 2.2 x 10^{-3} moles of NHAA) to the heated slurry. Subsequent to mixing the slurry (ca. 5 minutes), the pH was adjusted to 4.5 with glacial acetic acid. Laccase was then added (372,000 U per gram of o.d. pulp) and the reactor was sealed and pressurized with oxygen to 145 psi. After the four- hour treatment, the pulp was thoroughly washed and subjected to various oxidatively reinforced alkali extraction stages (E*). All E* stages were performed for one hour at 80°C in 4mm thick heat-sealable Kapak pouches. The E* conditions are summarized in Table V.2. Kappa and brightness measurements were performed on the extracted pulps in accordance with TAPPI methods T236 and T452, respectively (TAPPI Test Methods 1999).

Table V.2. Summary of Extraction Stage Conditions.

Extraction Stage	%NaOH (o.d. basis)	%H_2O_2 (o.d. basis)	O_2 (psi)
E	2.5	-	-
E+O	2.5	-	60
E+P	2.5	0.5	-
E+P+O	2.5	0.5	60

Hexenuronic acid content in brownstock. The content of hexenuronic acids in the brownstock was indirectly measured in accordance with a modified procedure reported by Vuorinen *et al.*[101] In brief, a 1000-ml round bottom flask was charged with 25 g of pulp (o.d. basis). The pulp consistency was adjusted to 3 % by adding distilled water. The pH was then lowered to 3 using a 4.0 N solution of sulfuric acid. The slurry was refluxed for three hours at 100°C. The change in kappa number before and after the treatment was then determined and served as an indirect measurement of hexenuronic acids (see Table V.1).

Control experiments. Control experiments (see Table V.3) were performed on the brownstock in accordance with the LMS experimental protocol, except no laccase was employed. The treated pulps were then subjected to the E* stages under the conditions outlined in Table V.3.

Table V.3. Summary of Control Experiments.

Experiment [a]
Brownstock followed by E stage
Brownstock treated with HBT and followed by E stage
Brownstock treated with NHAA only and followed by E
Brownstock treated with HBT only and followed by E+O
Brownstock treated with NHAA only and followed by E+O
Brownstock treated with HBT only and followed by E+P
Brownstock treated with NHAA only and followed by E+P
Brownstock treated with HBT only and followed by E+P+O
Brownstock treated with NHAA only and followed by

[a]: Mediator treatments were performed without the laccase (see LMS and extraction procedures for experimental details).

Laccase-mediator procedure for lignin isolation purposes. In order to isolate the residual lignin from the LMS treated pulps, larger batches were needed. A 2000-ml capacity Parr reactor was employed and was charged with 60 g of never-dried fibers (solid basis). The experimental protocol for the larger batches was identical to the one

described above, except 8.9 x 10^{-3} moles of HBT and NHAA were added instead of 2.2 x 10^{-3} moles.

Isolation of residual lignins. The isolation of residual lignins was carried out following standard literature methods.(102) In brief, a 5000-ml three-necked round bottom flask was charged with 50 g of o.d. pulp and the consistency was adjusted to 4 % by adding a 0.10 N HCl 9:1 *p*-dioxane:water solution. The slurry was then refluxed for two hours under an argon atmosphere. The pulp was filtered and the filtrate was filtered through celite, neutralized, and concentrated under reduced pressure to approximately 10 % of the original volume. Water (ca. 400 ml) was added and the mixture was concentrated again under reduced pressure to remove the last traces of *p*-dioxane. The solution's pH was then adjusted to 2.5 with 1.00 N HCl. The precipitated lignin was collected, washed several times, and freeze-dried. Lignin yields ranged from 45.4 to 48.3 %. Lignin yields were calculated as follows:
% lignin yield = {mass of lignin isolated/ (initial kappa of brownstock) x 0.15)}x100.

Characterization of residual lignins. The residual lignins isolated from the brownstock (kappa # 75.4) and from LMS_{HBT} (E), LMS_{HBT} (E+P+O), LMS_{NHAA} (E) and LMS_{NHAA} (E+P+O) treated pulps were phosphitylated and characterized by ^{31}P NMR in accordance with established literature methods.(103) NMR data were acquired with a DMX400 MHz Bruker spectrometer.

NMR error analysis. The NMR error analysis was conducted by isolating the residual lignin from the brownstock three separate times under identical conditions and comparing the results. The isolated lignin samples were then phosphitylated and analyzed by ^{31}P NMR. A least significant difference (LSD) value at a 95 % confidence interval was obtained by using the standard deviation along with the Student-t value. The calculated LSD values for the functional groups acquired by ^{31}P NMR are illustrated in Table V.4.

Table V.4. ^{31}P NMR Least Significant Difference Values.

Functional group	Average (mmol/g	SD.	LSD
Carboxyl OH	0.19	0.006	0.037
Non-condensed at C-5	0.91	0.013	0.078
Condensed at C-5 phenolic	0.69	0.022	0.133
Aliphatic OH	1.73	0.007	0.042

V. The Effects of Oxidative Alkaline Extraction Stages after Laccase$_{HBT}$ and Laccase$_{NHAA}$ Treatments on a Conventional SW Kraft Pulp

The purpose of this study was to compare the delignification efficiency of an LMS$_{HBT}$ and LMS$_{NHAA}$ treatment employing conventional a SW kraft pulp. This was accomplished by using the same molar equivalence of mediators for all LMS$_{HBT}$ and LMS$_{NHAA}$ treatments with all other experimental conditions held constant. These experimental conditions were selected so that the difference in biobleaching and subsequent alkaline extraction stage could be assessed. The effects of reinforcing the subsequent alkaline extraction stages to the laccase treatments with oxygen and peroxide were examined employing an E, E+P, E+O, and E+O+P stage. The changes in biobleaching were studied by measuring the physical and optical properties of the pulps, and the structural changes of the residual lignins. HBT was chosen as the reference mediator since it is probably one of the most studied mediators currently available for kraft pulps.

All LMS treatments were performed on a laboratory-prepared southern softwood conventional kraft pulp with an initial kappa of 33.8. The LMS$_{HBT}$ and LMS$_{NHAA}$ pulps were then subjected to either an E, E+O, E+O, E+O+P stage and the extent of delignification, brightness, and viscosity were measured after LMS (E*) treatments. The residual lignins were then isolated from the brownstock and from LMS$_{HBT}$ (E), LMS$_{HBT}$ (E+P+O), LMS$_{NHAA}$ (E) and LMS$_{NHAA}$ (E+P+O) treated pulps and characterized via ^{31}P NMR.

Physical and Optical Properties of LMS Treated Pulps

The changes in TAPPI brightness and viscosity results are depicted in Figures VI.1 and VI.2, respectively. Oxidative reinforcement of the alkaline extraction stage was beneficial after either an LMS$_{HBT}$ or an LMS$_{NHAA}$ treatment. These results are in agreement with the trends seen in the literature when a softwood kraft pulp is treated with

LMS_{ABTS} and subsequently treated with a QP stage. (104) The largest increases in brightness were observed after an (E+P) and an (E+P+O) stage. Interestingly, relative to the brownstock (BS), an LMS_{NHAA} (E) treatment suffered a greater loss in brightness than an LMS_{HBT} (E) treatment. This greater loss in brightness may be attributed to a higher content of quinone-type structures.

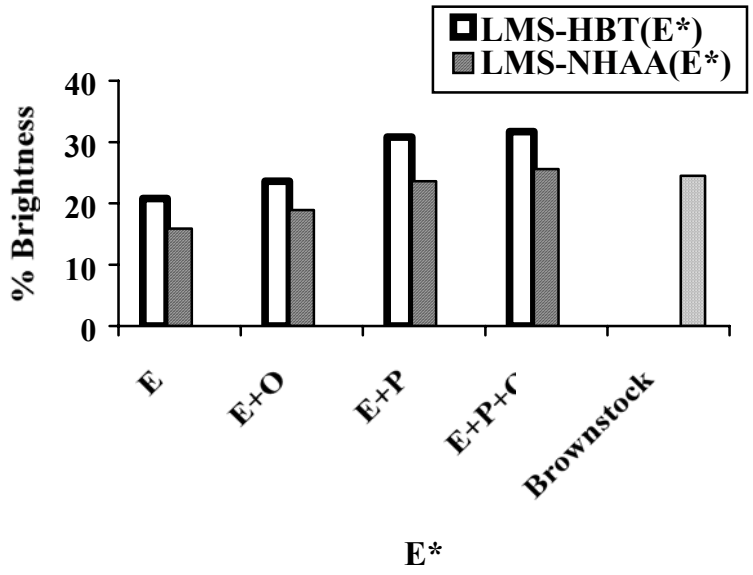


Figure VI.1. TAPPI Brightness after LMS_{HBT}(E*) and LMS_{NHAA}(E*) Treatments on a Softwood Kraft Pulp with a Starting Kappa Number of 33.8.

Viscosity measurements are in agreement with previously reported data [105, 106] and once again confirm the selectivity of a laccase-mediator system. Although both treatments were selective, the LMS_{NHAA} system exhibited a higher degree of selectivity than the LMS_{HBT} system. This trend was evident despite the type of reinforcement used in the extraction stage.

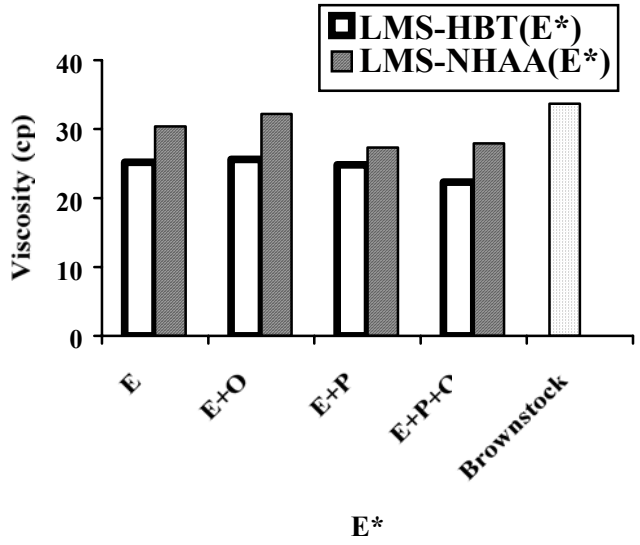


Figure VI.2. Viscosity after LMS_{HBT}(E*) and LMS_{NHAA}(E*) Treatments on a Softwood Kraft Pulp with a Starting Kappa Number of 33.8.

The delignification results shown in Figure VI.3 suggest that both an LMS_{HBT} and LMS_{NHAA} treatment yielded substantial levels of delignification. However, under the conditions used for this study, HBT was more effective than NHAA. The reinforcement of the alkaline stage further extended the level of delignification. Interestingly, reinforcement of the alkaline extraction stage with both peroxide and oxygen seemed to have narrowed the lignin content difference between the two LMS systems.

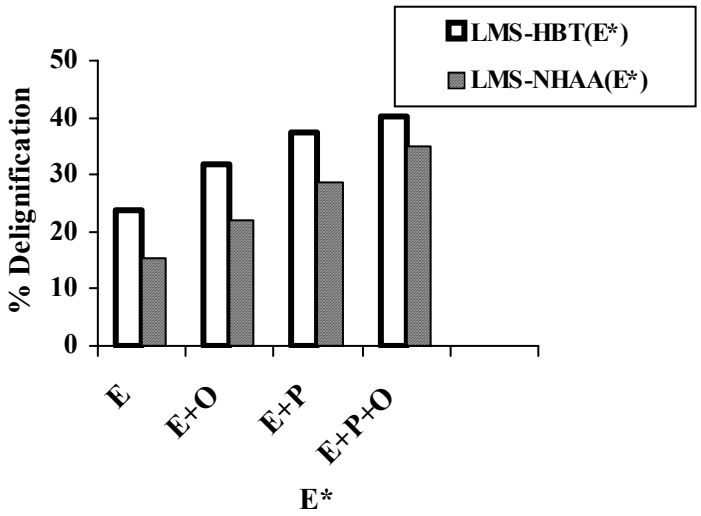


Figure VI.3. % Delignification after LMS_{HBT}(E*) and LMS_{NHAA}(E*) Treatments on a Softwood Kraft Pulp with a Starting Kappa Number of 33.8.

Control Experiments. Past literature results indicate that in order for an LMS system to yield significant levels of delignification, both the mediator and the laccase must be present.[107] In this study, we carried out a series of control experiments, which enabled us to discriminate between the effects of LMS and the oxidatively reinforced alkaline extraction stages on the brownstock. The alkaline extraction conditions for these control experiments are summarized in Table VI.1 and these results demonstrate that in all cases, an LMS stage before an E* improves delignification. The improved delignification effects were unfortunately accompanied by a decrease in pulp brightness and a slight decrease in viscosity.

Table VI.1. Kappa, Tappi Brightness and Viscosity of Control Experiments, LMS_{NHAA}(E*) and LMS_{HBT}(E*) Treatments.

Pulp	Kappa[1]	Brightness[2] (%)	Viscosity[3](cp)
Brownstock (BS)	33.8	24.5	33.7
BS(E)	31.7	25.6	33.5
BS(E+O)	28.9	26.4	28.8
BS(E+P)	27.4	30.2	24.6
BS(E+P+O)	25.1	31.9	22.5
LMS_{HBT}(E)	25.9	20.8	25.2
LMS_{HBT}(E+O)	23.1	23.6	25.6
LMS_{HBT}(E+P)	21.2	30.8	24.8
LMS_{HBT}(E+O+P)	19.9	31.7	22.3
LMS_{NHAA}(E)	28.6	15.9	30.4
LMS_{NHAA}(E+O)	26.3	18.9	32.2
LMS_{NHAA}(E+P)	24.1	23.6	27.3
LMS_{NHAA}(E+P+O)	22.0	25.6	27.9

[1] The pooled standard deviation of all kappa measurements was 0.1.

[2] The pooled standard deviation of all brightness measurements was 0.41.

[3] The pooled standard deviation of all viscosity measurements was 0.58.

^{31}P NMR Analysis. Based on the physical and optical properties, an LMS_{NHAA} and an LMS_{HBT} treatment followed by an (E+P+O) extraction stage yielded the highest brightness and level of delignification, whereas enzymatic treatments followed by a simple E stage yielded the opposite. To understand this biobleaching effect the residual lignins from the kraft pulps treated LMS_{NHAA}E*and LMS_{HBT}E* were isolated and characterized employing NMR techniques.

^{31}P NMR was used to evaluate the structural changes in phosphitylated residual lignins isolated from the brownstock and after an LMS_{HBT} (E), LMS_{HBT} (E+P+O), LMS_{NHAA} (E) and LMS_{NHAA} (E+P+O) treatment. ^{31}P NMR is a facile and effective method for evaluating various types of hydroxyl groups such as those present in carboxyl, free phenolic, condensed phenolic and aliphatic lignin moieties.

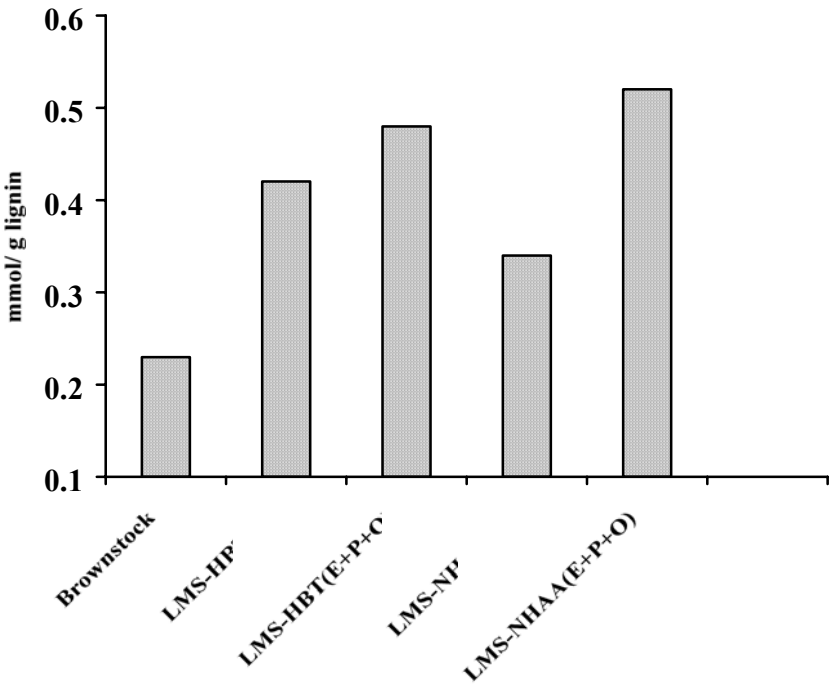


Figure VI.4. Carboxyl Acid Groups in Residual Lignins Isolated after LMS_{HBT}(E), (E+P+O), and LMS_{NHAA}(E), (E+O+P) Treatments.

It is clearly evident from the data shown in Figure VI.4 that relative to the brownstock lignin, the LMS_{HBT} (E) and LMS_{NHAA} (E) lignins were both enriched in carboxyl groups. However, this enrichment was more pronounced with HBT than with NHAA. As expected, reinforcement with peroxide and oxygen further increased the content of carboxylic acid of both LMS_{NHAA} and LMS_{HBT} residual lignins. However, this increase was more substantial when NHAA was used. We had earlier suggested that the greater loss in brightness after an LMS_{NHAA} treatment than after an LMS_{HBT} could be attributed to a greater content in quinone- type structures. If our speculation in regard to this matter is correct, then the greater increase in carboxylic acid content after the LMS_{NHAA} (E+P+O) treatment stage could be attributed to the well-known ring opening reactions of quinones to generate muconic acid-type structures.

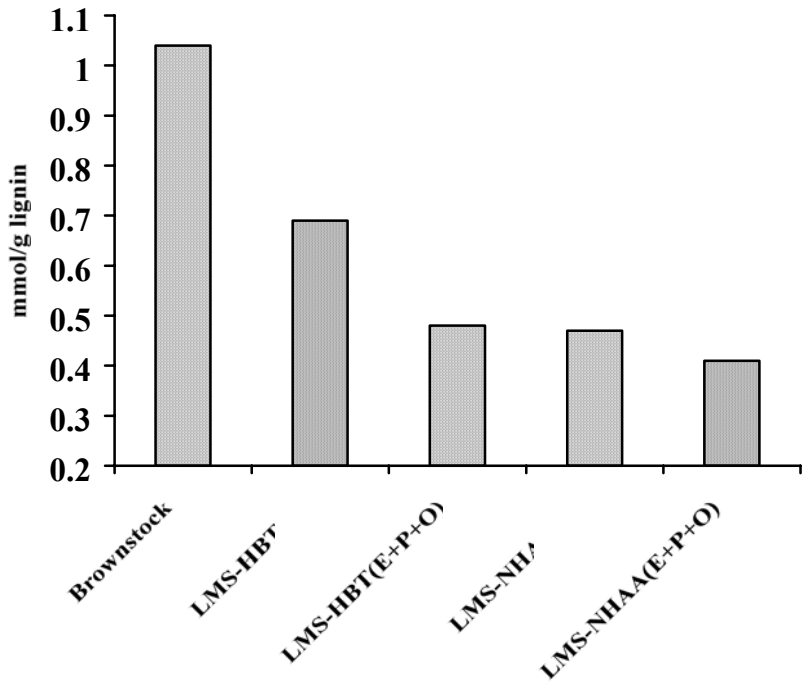


Figure VI.5. Guaiacyl Phenoxy Groups in Residual Lignins Isolated after LMS_{HBT}(E), (E+P+O), and LMS_{NHAA}(E), (E+O+P) Treatments.

Inspection of Figures VI.5 and VI.6 suggests a depletion of guaiacyl and condensed phenolic hydroxyl groups, and is consistent with trends seen by Sealey and Ragauskas when using $LMS_{HBT.}$[96] This decrease was greater with NHAA than with HBT. In turn, this may suggest that the oxidative selectivity of LMS_{NHAA} toward phenolic lignin structures may be different than that of LMS_{HBT}. Reinforcement of the alkaline extraction stages seems to narrow the gap between the two systems.

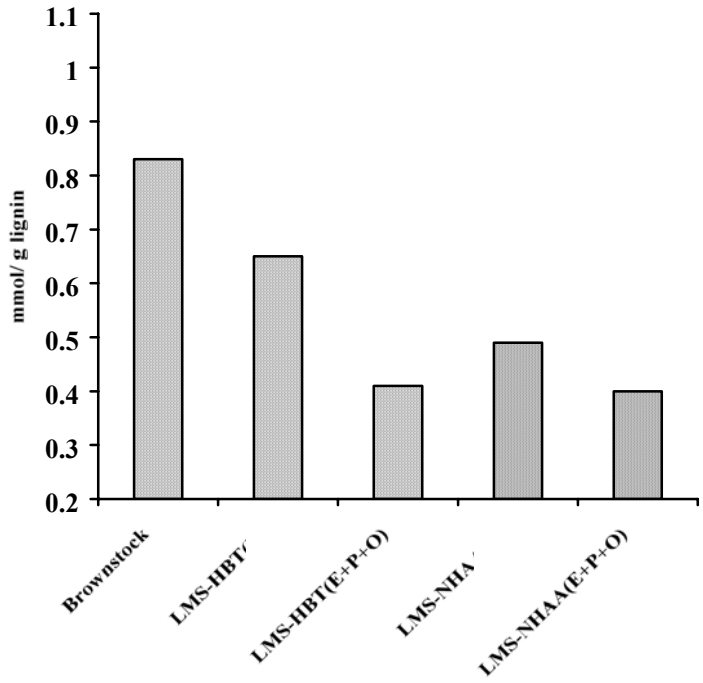


Figure VI.6. Condensed Phenolic Groups in Residual Lignins Isolated after LMS_{HBT}(E), (E+P+O), and LMS_{NHAA}(E), (E+O+P) Treatments.

The aliphatic lignin hydroxyl groups content, shown in Figure VI.7, also decreased relative to the brownstock. This decrease is consistent with recent observations of side chain oxidation and fragmentation of model compounds during LMS (E) treatments reported by Freudenreich *et al*. [88] and Li *et al*. [108] Reinforcement of the alkaline extraction stages with peroxide and oxygen did not further deplete these types of groups, as expected.

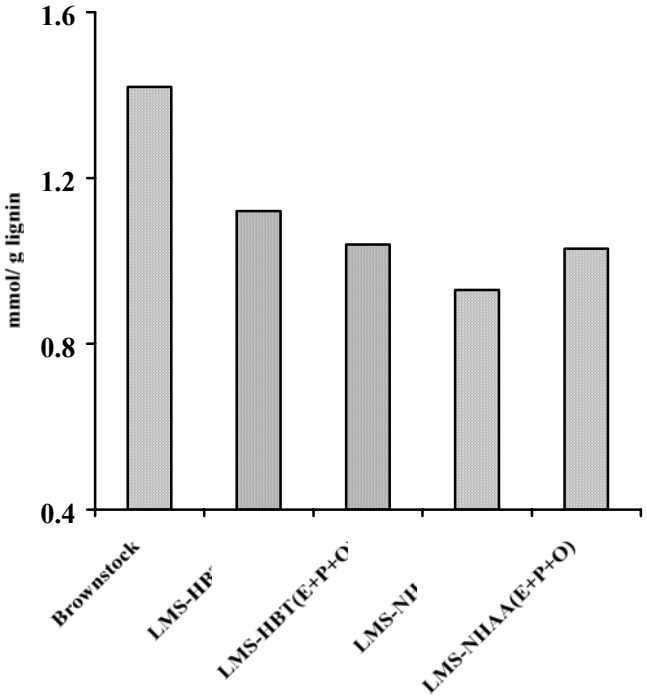


Figure VI.7. Aliphatic OH groups in Residual Lignins Isolated after LMS_{HBT}(E), (E+P+O), and LMS_{NHAA}(E), (E+O+P) Treatments.

Overall, the structural analysis of the residual lignins suggests that the oxidative chemistry of an LMS_{NHAA} and LMS_{HBT} system is different. This is supported by the observed differences in the structure of the isolated residual lignins after the alkaline extractions. If the LMS bio-delignification chemistry was proceeding via the same pathway, when either mediator was used, then the residual lignins after an LMS_{NHAA}(E) and an LMS_{HBT}(E) would have had identical ^{31}PNMR spectra, and this was not the case. Obviously, the same rationale applies to the residual lignins isolated after an LMS_{NHAA}(E+P+O) and LMS_{HBT}(E+P+O) treatments.

Section VI Conclusions

In summary, these results confirmed the reported effectiveness of HBT and NHAA as mediators in LMS systems. Based on the conditions used in this study, we observed that HBT yielded higher levels of delignification than NHAA. The oxidatively reinforced alkaline extraction stages were shown to be very beneficial and seem to narrow the gaps between the two LMS systems. Overall, the structural analysis of the residual lignins

was consistent with the delignification properties of the pulp. The improvements in the LMS_{HBT} vs. LMS_{NHAA} systems were reflected in the NMR analysis of the lignin samples. For example, the residual lignin isolated after an LMS_{HBT}(E) treatment was enriched in lignin carboxylic acid moieties more so than after an LMS_{NHAA} (E) treatment. The increased delignification observed when using an E+P+O stage after the LMS treatment was accompanied with increased amounts of carboxylic acid groups in the residual lignin.

The spectral analysis of the residual lignin samples after LMS_{NHAA}(E*) and LMS_{HBT}(E*) treatments indicated that NHAA, as a mediator, has different selectivity than HBT. Studies into the effect of quinone-type structures on LMS systems are currently underway.

Section VI Experimental Details

Materials. All materials were purchased from Aldrich Chemical Co., Milwaukee, WI, and used as received, except for *p*-dioxane, NHAA, and laccase. *p*-Dioxane was freshly distilled over $NaBH_4$ prior to using it for the lignin isolation experiments. NHAA was synthesized in accordance with Oxley's method.[109] Laccase, from *Trametes villosa*, was donated by Novo Nordisk Biochem. The conventional southern softwood kraft pulp was prepared at Potlatch Corp. facilities in Cloquet, MN. The wood source originated from *Pinus taeda* and was acquired from Union Camp. The wood was approximately 25 years of age, void of visual disease and of compression wood. The chips were cooked to an H-factor of 1390 using 19.5 % active alkali and a 4:1 liquor:wood ratio. The pulp was thoroughly washed, screened, centrifuged, fluffed, and stored at 4°C prior to LMS bleaching treatments.

Enzyme Assay. Laccase activity was measured by monitoring the rate of oxidation of syringaldazine. One unit of activity (U) was defined as the change in absorbance at 530 nm of 0.001 per min per mL of enzyme solution, in a 100 mM phosphate buffer (2.2 mL) and 0.216 mM syringaldazine in methanol (0.3 mL). The procedure was carried out at 23°C. The activity of the laccase used in this study was 1.87E+06 (U/mL of enzyme solution).

Laccase-Mediator Delignification Procedure. A 1000-mL capacity Parr reactor equipped with a stirrer, a pressure gauge, a heating mantle, and connected to a temperature controller was charged with 15 g of never-dried fibers (solid basis). The pulp consistency was adjusted to 9% by adding distilled water. The slurry was then heated to a temperature of 45°C and was maintained at this temperature throughout the incubation period. HBT (2×10^{-3} moles) was then added (or 2.2×10^{-3} moles of NHAA when NHAA was used) to the heated slurry. Subsequent to mixing the slurry (ca. 5 min), the pH was adjusted to 4.5 with glacial acetic acid. Laccase was then added (372,000 U per gram of o.d. pulp) and the reactor was sealed and pressurized with oxygen to 145 psig. Subsequent to the four-hour treatment, the pulp was thoroughly washed and

subjected to various reinforced alkaline stages (E*). All E* stages were performed for one hour at 80°C. These stages are summarized in Table 1. Kappa, brightness, and viscosity measurements were performed on the extracted pulps in accordance with TAPPI methods T236, T452, and T230, respectively.[94]

Control experiments were also conducted on the brownstock in the absence of laccase and mediator to evaluate the effect of E* treatments. The conditions for the E, E+O, E+P, and E+P+O stages are summarized in Table VI.2.

Table VI.2. Summary of Extraction Stage Conditions.[a, b]

Extraction Stage (E*)	%NaOH (o.d. basis)	$\%H_2O_2$ (o.d. basis)	O_2 (psig)
E	2.5	-	-
E+O	2.5	-	60
E+P	2.5	0.5	-
E+P+O	2.5	0.5	60

[a] All E* were applied to LMS_{HBT} and LMS_{NHAA} treated pulps.

[b] Similarly, All E* conditions were applied on the kraft softwood brownstock (initial kappa # = 33.8). These experiments served as controls.

Isolation of Residual Lignins. The isolation of residual lignins was carried out in accordance with standard literature methods. (102) A 5000-mL three-necked round bottom flask equipped with a Friedrichs condenser was charged with 50 g of o.d. pulp (air-dried). The consistency of the pulp was adjusted to 4% by adding a 0.10N (HCl) 9:1 *p*-dioxane:water solution. The slurry was then refluxed for 2 hr under an argon atmosphere. Subsequent to the treatment, the pulp was filtered and the filtrate was passed through celite to remove any fines. The filtrate was then neutralized and concentrated under reduced pressure to approximately 10% of the original volume. Water (ca. 400 mL) was added and the mixture was concentrated again under reduced pressure to

remove the last traces of *p*-dioxane. The solution's pH was then adjusted to 2.5 with 1.00 N HCl. The precipitate (i.e., the lignin) was collected, washed several times, and freeze-dried. Lignin yields ranged from 45.4 to 48.3%.

Characterization of Residual Lignins. The residual lignins isolated from the brownstock (kappa # 33.8) and from LMS_{HBT} (E), LMS_{HBT} (E+P+O), LMS_{NHAA} (E), and LMS_{NHAA} (E+P+O) treated pulps were phosphitylated and characterized by ^{31}P NMR in accordance with established literature methods.[103] NMR data was acquired with a DMX400 MHz Bruker spectrometer.

NMR Error Analysis. The NMR error analysis was performed by repeating the isolation of the brownstock residual lignin three times under identical conditions and comparing the results. The isolated lignin samples were then phosphitylated and analyzed by ^{31}P NMR, as described above. A least significant difference (LSD) value at a 95% confidence interval was calculated by using the standard deviations along with the Student-t value. The LSD values for the functional groups acquired by ^{31}P NMR are illustrated in Table VI.3.

Table VI.3. ^{31}P NMR Least Significant Difference Values.

Functional group	Average (mmol/g lignin)	St. dev.	LSD
Carboxyl OH	0.23	0.003	0.013
Guaiacyl OH	1.02	0.025	0.095
Condensed OH	0.82	0.016	0.060
Aliphatic OH	1.40	0.019	0.069

VII Biobleaching Chemistry of Laccase-Mediator Systems on High Lignin Content Kraft Pulps

The biodelignification chemistry of LMS employing violuric acid vis-a-vis lignin structural changes remains largely unknown. To provide a fundamental basis from which new LMS bleaching technologies can be further advanced, this study examines the changes in lignin structure before and after an LMS treatment using violuric acid and compared these results against NHAA and HBT. This report examines the delignification chemistry of the newest generation of N-hydroxyl mediators VA and NHAA and compares these results against HBT employing a high lignin content softwood kraft pulp.

The delignification response of LMS treatments employing NHAA , HBT, and VA as mediators was established softwood kraft pulp with an initial lignin content of 10.7% by mass. Furthermore, control experiments (MS) were carried out in accordance with the LMS procedure, except laccase was not used. The results shown in Figure VII.1 clearly indicate that a treatment in the presence of a mediator and no laccase led to no delignification. This lack of activity has been reported on pulps treated solely with laccase and no mediator was observed in prior chapters.

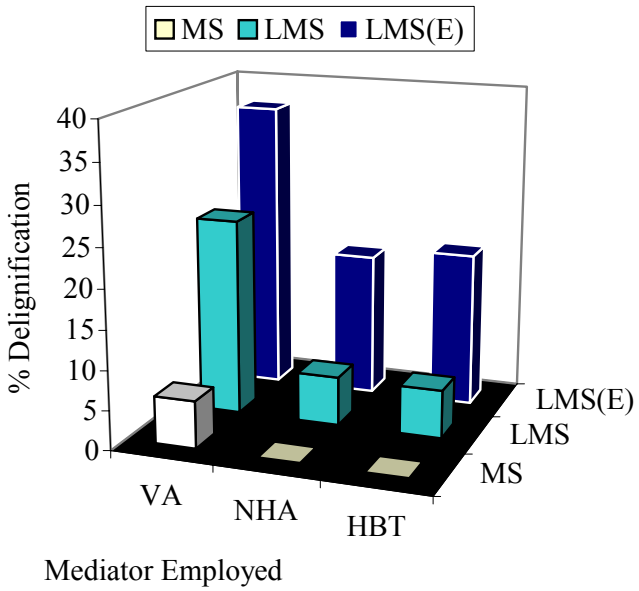


Figure VII.1. Extent of Pulp Delignification for MS, LMS, and LMS(E) Treated Kraft Pulps using VA, NHAA , and HBT as Mediators Employing a SW Kraft Pulp with an Initial Kappa Number 71.4.

Based on the results summarized in Figure VII.1 the delignification response of the LMS_{NHAA} and LMS_{HBT} system was similar. The effectiveness of LMS_{VA} vis-à-vis delignification is evident as the LMS_{VA} system outperformed both the LMS_{HBT} and LMS_{NHAA} treatments. Alkaline extraction of the LMS treated pulps resulted in a further reduction in the lignin content of the pulp, to be expected since the alkaline conditions enhance the dissolution of oxidized lignin fragments. Overall the LMS treatment followed by an alkaline extraction removed 20, 19, and 37% lignin from the starting pulp employing HBT, NHAA , and VA as mediators, respectively. The improved efficiency of VA as a mediator for LMS is not limited to high lignin content pulps as comparable results have been reported for low lignin content pulps.[110] The biodelignification properties of LMS_{VA} have been shown to be accompanied by a darkening of the pulp due to the formation quinoidal lignin structures[111] that can be oxidatively removed in subsequent bleaching treatments. To further define the fundamentals of LMS

delignification on the kraft pulp used in this study, the structure of residual lignins before and after the biobleaching treatments was examined.

NMR analysis

Residual lignins were isolated from the starting pulp as well as the MS, LMS, and LMS(E) treated pulps following literature methods.[102] Figure VII.2 summarizes the lignin functional groups examined in this study. Quantification of these functional groups in lignin was determined using ^{13}C NMR and ^{31}P NMR after lignin phosphitylation.

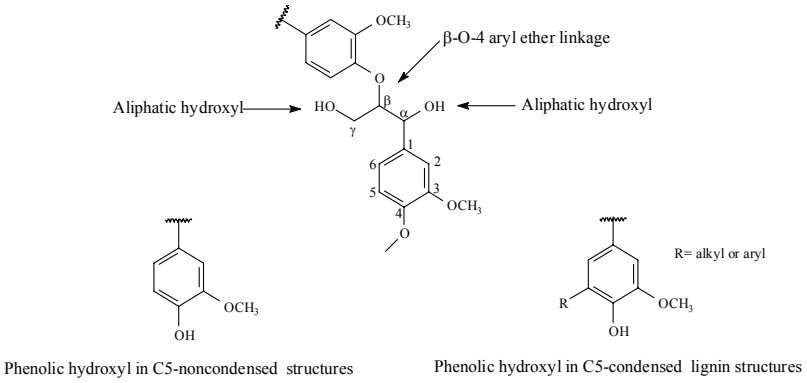


Figure VII.2. Structural Components of Lignin.

NMR analysis of the residual lignin isolated from the MS_{VA}, MS_{HBT}, and MS_{NHAA} treated pulps (i.e., pulps treated with mediator and no laccase) indicated no changes in the content of lignin functional groups with regards to the lignin isolated from starting pulp. This result was consistent with the results of the lignin content determinations of the MS treated pulps and supports the conclusion that laccase is a critical component in the overall LMS delignification process. Figures VII. 3 and 4 summarize the ^{13}C and ^{31}P NMR spectral data acquired for the residual lignin isolated from starting softwood kraft pulp.

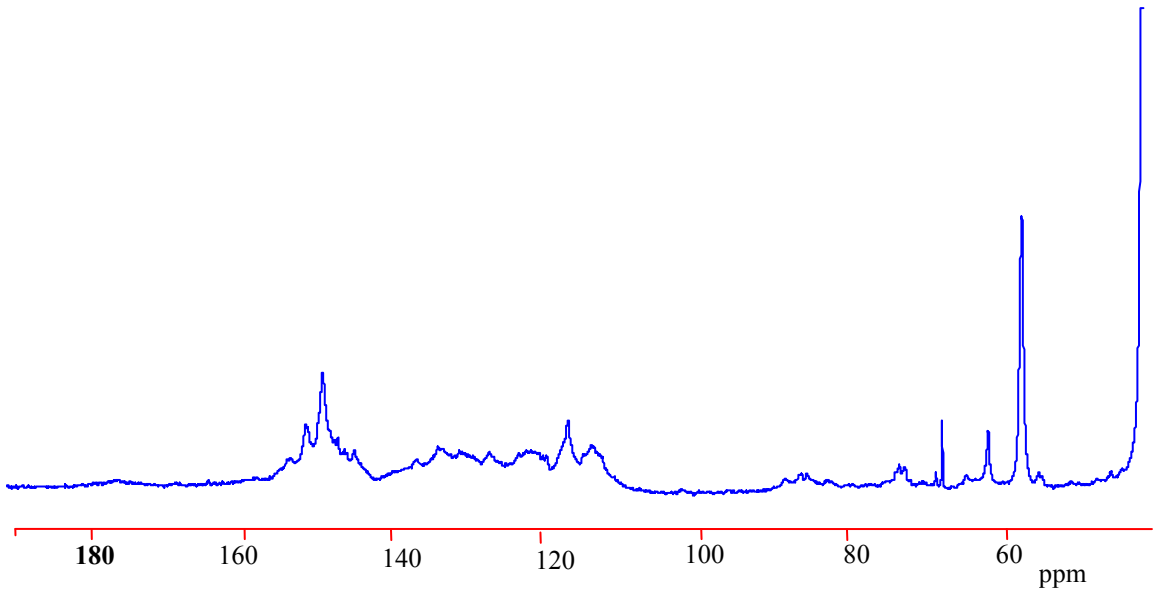


Figure VII.3. ^{13}C NMR Spectrum of Residual Lignin Isolated from Starting Pulp.

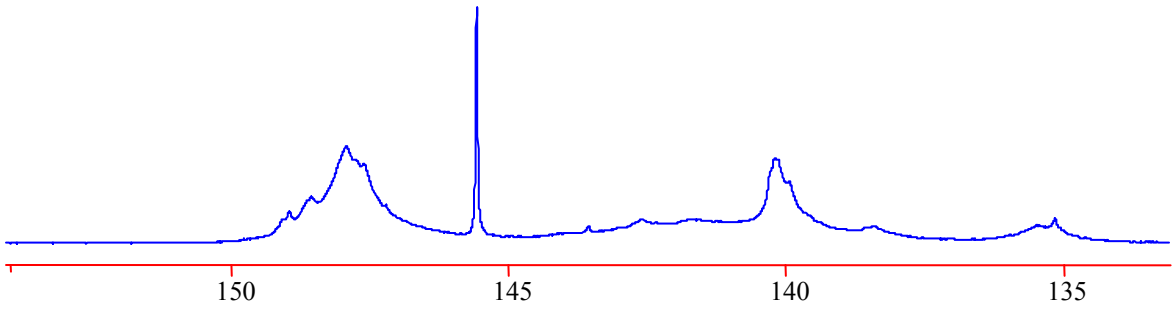


Figure VII.4. ^{31}P NMR Spectrum of Residual Lignin Isolated from Starting Pulp. ppm

Of the various structural features of lignin, changes in carboxylic acid group content are often a strong indicative sign of oxidative degradation of lignin during pulp bleaching. The acid groups content of the residual lignin from the starting pulp, LMS, and LMS(E) treated pulps is summarized in Figure VIII.5.

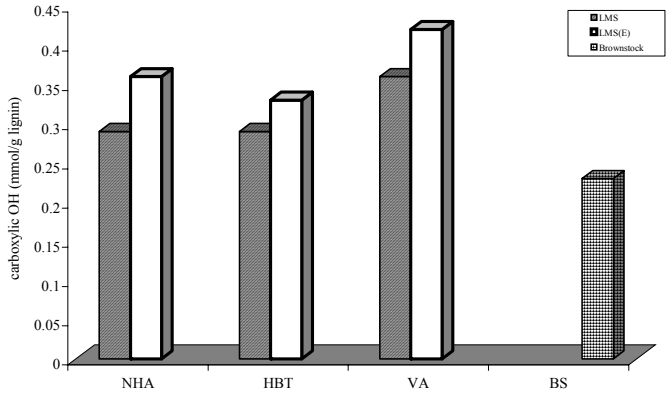
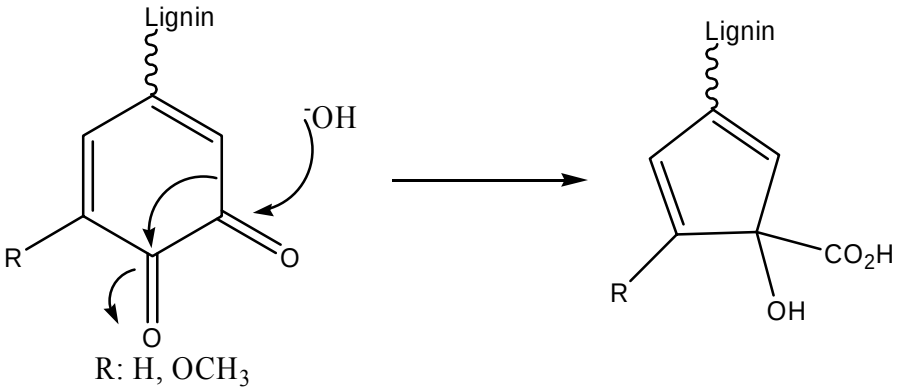


Figure VII.5. ^{31}P NMR Determination of Carboxylic Acid Groups in Residual Lignins isolated from Starting Pulp, LMS, and LMS(E) Treated Pulps Employing NHAA , HBT, and VA as Mediators.

These results indicate the residual lignin after an LMS stage is enriched with acid groups. The increase in acid group content of the LMS_{HBT} and LMS_{NHAA} treated pulps were comparable (26%), whereas the LMS_{VA} treated pulp had 57% greater increase in acid groups. These results mirror the observed changes in delignification of the pulps after the LMS treatments. Interestingly, the acid group content for all LMS treated pulps increased slightly after the alkaline extraction stage. Since the extraction process removes oxidized lignin fractions it seems unlikely that this effect is due solely to an enrichment process. Previous studies by our group have shown that LMS treated pulps are enriched with quinones, and these units are known to undergo a benzylic acid rearrangement upon exposure to base as shown in Scheme VII.1.



Scheme VII.1. Hydroxide Addition and Rearrangement of Ortho-quinones.

These and other reactions presumably contribute to the increase in acid groups after an LMS(E) treatment. The oxidative formation of carboxyl groups in lignin during pulp bleaching is most frequently accompanied with oxidative degradation of phenoxy groups. The phenoxy content of the starting, LMS, and LMS(E) treated pulps is presented in Figures VII. 6 and 7. The results in Figure VII.6 clearly demonstrate that all the residual lignins isolated after LMS treatments were depleted of C5 noncondensed phenolic hydroxyls.

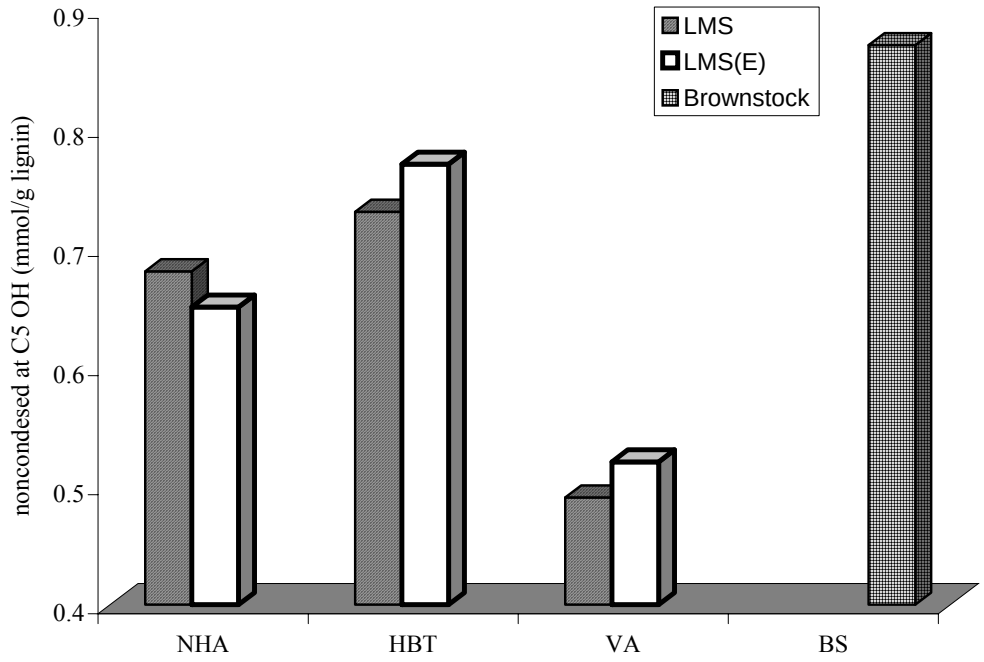


Figure VII.6. ^{31}P NMR Determination of C5 Noncondensed Phenolic Hydroxyls Groups in Residual Lignin isolated from Brownstock and LMS, and LMS(E) Treated Pulps employing NHAA , HBT, and VA as Mediators.

The LMS_{NHAA} treatment removed 22% of noncondensed phenoxy, LMS_{HBT} removed 16%, and LMS_{VA} removed 44% with respect to the starting pulp. The improved delignification properties of LMS_{VA} were reflected in the increased degradation of C5 noncondensed phenolics. The minor increase in phenolics after the LMS(E) stage suggests that the alkaline extraction is not causing further oxidative damage to phenolic lignin and is perhaps enriching the remaining lignin with phenolic units.

Figure VII.7 summarizes the changes in C5 condensed phenoxy groups after LMS and LMS(E) treatments. In general, bleaching chemistry of kraft pulps has shown that C5 noncondensed phenolics are resistant to assorted oxidative bleaching agents including chlorine dioxide[112] and alkaline-oxygen conditions. [113]

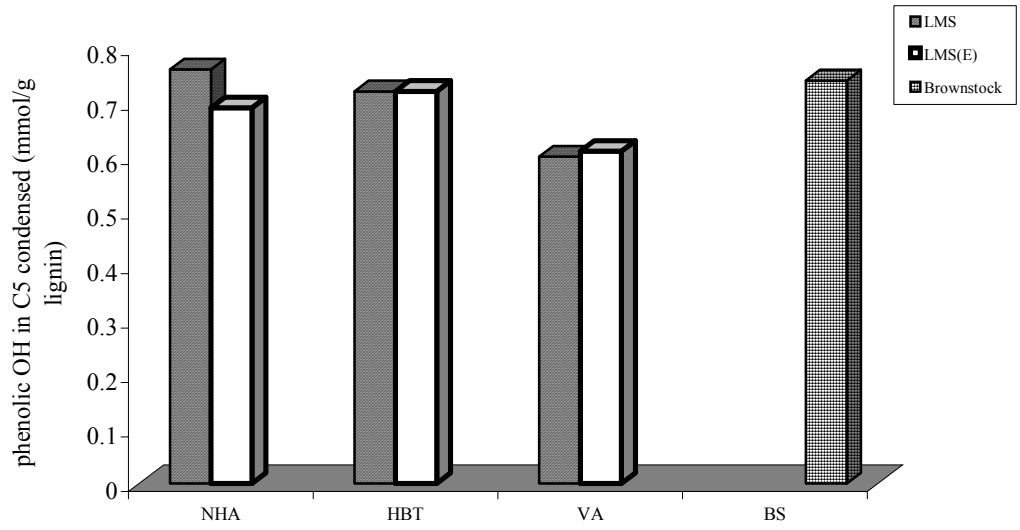


Figure VII.7. ^{31}P NMR Determination of C5 Condensed Phenolic Hydroxyl Groups in Residual Lignin isolated from Brownstock and LMS, and LMS(E) Treated Pulps Employing NHAA , HBT, and VA as Mediators.

The biobleaching chemistry of an LMS_{VA} treatment was the only LMS process capable of significantly degrading C5 condensed phenolics in the pulp. These results suggest that this mediator exhibits an enhanced oxidative capability towards phenolic units in lignin. These data also clearly demonstrate that the LMS_{HBT} or LMS_{NHAA} treatments do not oxidatively degrades C5 condensed phenolics in this high kappa kraft pulp. This result differs from our previous studies employing low lignin content kraft pulps (i.e., pulps having < 5% lignin content) in which we observed that a $LMS_{NHAA\ or\ HBT}$ treatment would degrade both C5 condensed and noncondensed phenolic units with only a slight preference for C5 noncondensed lignin phenolic units. On this basis, the ^{31}P NMR results suggest that the oxidative selectivity of the LMS_{VA} system is different than that of LMS_{HBT} and LMS_{NHAA}. Nonetheless, the overall trend of these LMS systems indicates that the preferred site of phenolic oxidation is directed toward C5 noncondensed units in lignin.

Along with the oxidative degradation of phenolic units in lignin, side chain oxidation of lignin has been proposed to occur to lignin during an LMS treatment. Figure VII.8 reveals that the content of aliphatic hydroxyl groups in residual lignins isolated after treatment with LMS_{NHAA} , LMS_{HBT}, and LMS_{VA} is decreased by 11-28% with respect to the brownstock lignin, with the greatest change occurring when VA and NHAA were employed as mediators.

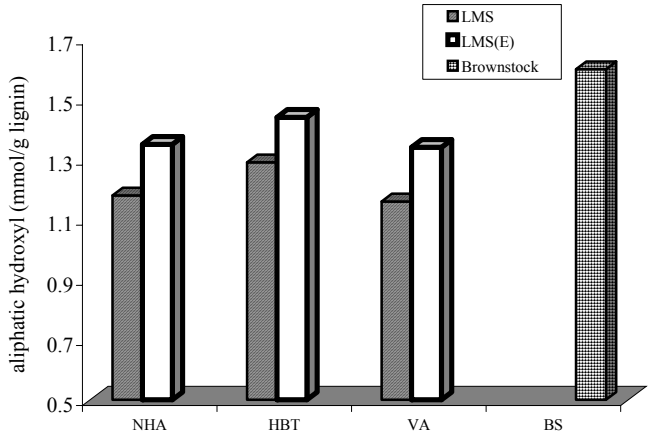


Figure VII.8. ^{31}P NMR Determination of Aliphatic Hydroxyl Groups in Residual Lignin Isolated from Brownstock and LMS, and LMS(E) Treated Pulps Employing NHAA , HBT, and VA as Mediators.

The lack of reactivity of C5 condensed phenolic structures suggests that the residual lignin after the LMS treatment on the high lignin content kraft pulps is of a condensed character. This claim was evaluated by measuring the ratio of substituted to unsubstituted aromatic carbon groups in the ^{13}C NMR spectra of the starting, LMS and LMS(E) treated pulps. Table VII.1 summarizes the results of this investigation and the data clearly support the hypothesis that after an LMS(E) treatment of a high kappa kraft pulp the lignin is further enriched in condensed lignin structures.

Table VII.1. Ratio of Substituted to Unsubstituted Aromatic Carbons[a] in Residual Lignin Samples as Determined by Quantitative ^{13}C NMR.

Residual lignin isolated from:	Ratio of substituted to unsubstituted aromatic carbons
Brownstock	1.67:1.00
LMS_{NHAA}	2.07:1.00
LMS_{HBT}	1.92:1.00
LMS_{VA}	1.97:1.00
LMS_{NHAA} (E)	1.96:1.00
LMS_{HBT}(E)	1.85:1.00
LMS_{VA}(E)	1.87:1.00

[a]frequency distribution for substituted aromatic lignin is δ154-123 and for unsubstituted aromatic carbons the frequency range is δ 123-106 ppm.

Figure VII.9 summarizes the changes in β-O-4 aryl ether content of the lignin structures before and after the LMS treatment as determined by ^{13}C NMR. The results indicate that such structures are resistant to LMS treatments. Similar trends have been reported in previous LMS studies using HBT as the mediator on low lignin content kraft pulps. In contrast, reports on lignin model compound studies have indicated that such structures are reactive towards LMS. [114] The factors contributing to this inactivity in kraft pulps remain unknown.

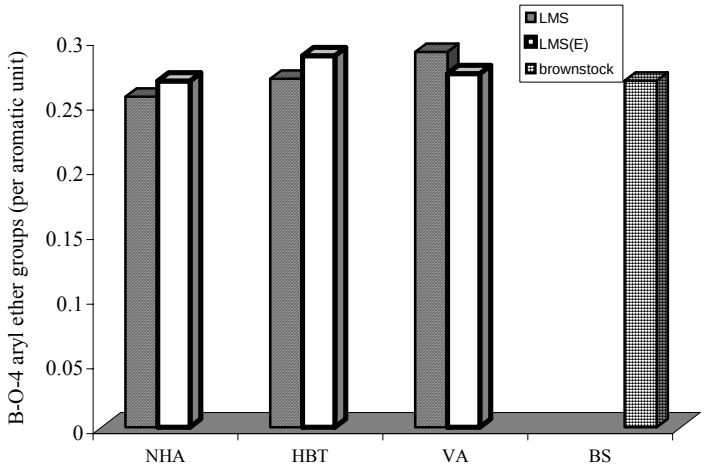


Figure VII.9. ^{13}C NMR Determination of the Content of β-O-aryl Ether Groups in Residual Lignins Isolated from Brownstock and LMS, and LMS(E) Treated Pulps Employing NHAA , HBT, and VA as Mediators.

However, it may be possible that this behavior is related to a selectivity phenomenon. Although a dimeric β-O-aryl ether lignin model compound may respond to an LMS treatment, a different scenario may be envisioned when dealing with the actual lignin macropolymer that contains an array of functional groups. On this basis, an LMS treatment may have a higher affinity towards other functional groups than β-O-aryl ether, and in turn this may explain the resistance observed in this study and in the literature.

The results shown in Figure VII.10 reveal a decrease in the content of methoxy groups. These results are consistent with previous LMS studies on low lignin content kraft pulps employing ABTS and HBT as mediators.[115]

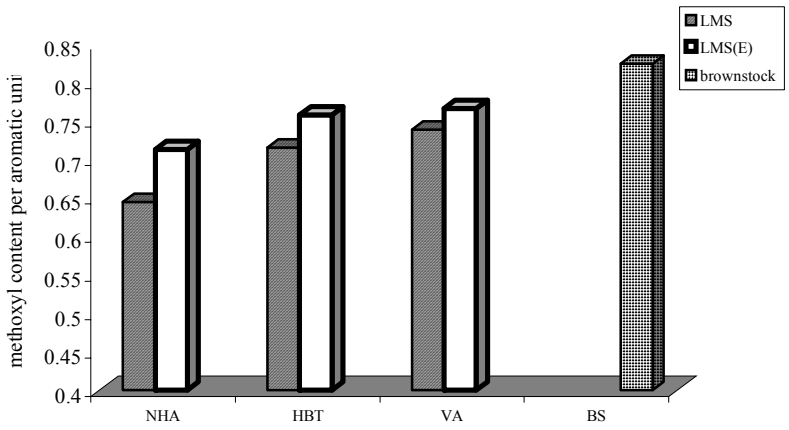


Figure VII.10. Content of Methoxy Groups in Residual Lignins Isolated from Brownstock and LMS, and LMS(E) Treated Pulps Employing NHAA , HBT, and VA as Mediators.

Section VII Conclusions

In summary, these results established that LMS_{VA} outperforms LMS_{HBT} and LMS_{NHAA} with respect to delignification of high lignin content kraft pulps. The mediator effectiveness of VA was further supported by the magnitude of lignin structural changes with respect to NHAA and HBT used as mediators for an LMS treatment. Spectral analyses of residual lignins isolated before and after LMS treatments, employing VA, NHAA , and HBT as mediators, revealed that these mediators preferentially react with C5 noncondensed phenolic lignin structures, whereas C5 condensed phenolic lignin structures were to a large extent inactive. Nonetheless, in the presence of laccase and VA, our results indicate a decrease in the latter type of lignin functional groups, suggesting that the selectivity between these mediators may be different.

t is now well established that HBT, VA, and NHAA undergo degradation reactions during an LMS reaction.[116] The results in this report further emphasis the sensitivity of an LMS stage to the structure of the mediator. The improved delignification properties of LMS_{VA} are likely to be due, in part, to the unique properties of nitroxyl radical derived

from the oxime as opposed to a dialkyl N-hydroxyl derivative. Further studies directed at exploring these issues are ongoing.

Section VII Experimental Details

Materials and methods. 1-Hydroxybenzotriazole, violuric acid, and all other reagents and solvents were purchased from Aldrich, Milwaukee, WI, and used as received except for *p*-dioxane and N-acetyl-N-phenylhydroxylamine. *p*-Dioxane was freshly distilled over $NaBH_4$ before use. N-acetyl-N-phenylhydroxylamine was synthesized in accordance with the Oxley et al. procedure.[109] Laccase from *Trametes villosa* was donated by Novo Nordisk Biochem. A high lignin content southern softwood kraft pulp was prepared from *Pinus taeda* chips at Potlatch Corporation facilities in Cloquet, MN. The pulp was thoroughly washed, screened, centrifuged, and stored at 4°C. Prior to an LMS treatment the pulps were Soxhlet extracted with acetone and air dried.

Pulp characterization. The lignin content of the kraft pulps in this study was determined by a $KMnO_4$ titration of the pulp in accordance with TAPPI Standard Methods T236-cm85.[117] This procedure yields a value known as the kappa number which is an indirect measurement of the lignin content in pulp; % mass lignin = 0.15 x kappa number. The kappa number of the Soxhlet-extracted starting kraft pulp was 71.4.

Enzyme assay. Laccase activity was measured by monitoring the rate of oxidation of syringaldazine as described in the previous section

Laccase-mediator system (LMS) general procedure. The LMS treatments were performed with a 2000-mL-capacity 316 Parr reactor equipped with a stirrer, a pressure gauge, and a heating mantle and connected to a Parr 4842 temperature controller was charged with 60.00 g of pulp and distilled water (1137 mL), as described in prior sections.

Alkaline extraction. Pulp fibers (60 g) were extracted with an aqueous 0.5 N NaOH (75.00 mL) solution for one hour at 80.0ºC with mixing. The fibers were then filtered and extensively washed until the filtrate was colorless and pH neutral. Prior studies with this pulp have shown that only 4% of the lignin present in the fiber is alkaline extractable without an LMS pretreatment.

Control bleaching experiments (MS). The softwood kraft pulp was treated in accordance with the laccase-mediator delignification procedure described above, except no laccase was employed. Kappa number analysis of the pulp after a mediator system (MS) employing VA, HBT, or NHAA indicated no change in lignin content of the pulp.

Isolation of residual lignins. The isolation of residual lignins was carried out as described in previous sections.

NMR characterization of residual lignins. The residual lignins isolated from the brownstock, MS, LMS, and LMS(E) were analyzed using a 400 MHz Bruker DMX spectrometer. Quantitative ^{13}C NMR spectra were acquired and analyzed in accordance with established literature methods (118). In brief, lignin (160-168 mg) was dissolved in 450 μL of DMSO-d_6 and the spectral data was acquired using an inverse gated decoupling sequence, 90º pulse angle, 14-s pulse delay, 23,000-Hz sweep, 10-12,000 transients, at 50.0ºC. The Fourier transformed spectra were integrated in accordance with reported chemical shifts for lignin functional groups. The integrals were normalized to the aromatic signals, which were assigned a value of six.

Lignin samples were also derivatized with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane and analyzed by ^{31}P NMR following literature methods (119). ^{31}P NMR spectra were recorded using an inverse gated decoupling sequence, 90º pulse angle, 25-s pulse delay, 13,000-Hz sweep, and 150 transients, at room temperature. NMR spectral acquisition and analysis were accomplished using Bruker's XWINNMR 2.1 software. The lignin functional groups characterized by ^{13}C and ^{31}P NMR had standard

errors of 2.0% and 1.2%, respectively, this was experimentally determined by three separate NMR measurements on three separately isolated lignin samples.

VIII. Detection and Quantification of Quinoid Structures in LMS Treated Pulps.

The research studies in sections III – VII have documented that an LMS treatment, using VA, HBT or NHAA as mediators, can remove significant amounts of lignin from high-kappa kraft pulps. NMR analysis of the residual lignin in the pulp after an LMS treatment indicated that the bio-delignification treatment extensively oxidizes C-5 noncondensed phenolic lignin structures, whereas C-5 condensed phenolic lignin structures were overall resistant to oxidation. In addition, side chain oxidation did occur on the propane-linking unit of lignin. The primary product detected from these oxidative treatments has been the formation of carboxylic acid groups. The presence of quinone groups in an LMS treated pulp has been frequently proposed.[120, 121] Lignin model compounds studies with laccase indicate that this can occur. [122] Recently, Poppius-Levlin et al.[123] presented FT-IR data suggesting that the residual lignin from LMS treated pulp has an enriched level of quinonoid structures. The formation of quinones in LMS treated pulps could readily explain the substantial increases in brightness when a kraft pulp is first subjected to LMS and then treated with alkaline hydrogen peroxide.[104] It is well established that alkaline hydrogen peroxide readily reacts with para and ortho-quinones. [124, 125] The removal of these intensively colored bodies from kraft pulp with alkaline peroxide would significantly improve the brightness of the pulp. The purpose of this study was to determine the relative amounts of quinones in residual lignins isolated from a softwood high-kappa kraft pulp before and after LMS treatments, using HBT, NHAA , and VA as mediators.

Extent of Biodelignification and Brightness.

The delignification and brightness responses of laccase-mediator systems employing HBT, NHAA , and VA on a softwood kraft pulp (kappa # 71.4) were evaluated before and after an alkaline extraction stage (E). In addition, a series of control experiments in the absence of the laccase were carried out. The kappa and brightness measurements relative to the initial brownstock are summarized in Table VIII.1.

Table VIII.1. **Kappa and TAPPI Brightness for a Softwood Kraft Pulp Treated with MS[a], LMS[b] and LMS(E)[c] using HBT, NHAA , and VA as Mediators[d].**

Pulp/Treatment	Kappa #	St.dev	TAPPI Brightness	St.dev
Brownstock	71.4	0.19	18.4	0.11
MS_{NHAA}	71.3	0.11	18.5	0.20
MS_{HBT}	71.0	0.18	18.7	0.33
MS_{VA}	71.2	0.08	18.5	0.15
LMS_{NHAA}	67.0	0.29	7.8	0.43
LMS_{HBT}	65.3	0.11	11.6	0.45
LMS_{VA}	53.6	0.09	9.8	0.39
LMS_{NHAA} (E)	58.4	0.37	10.7	0.21
LMS_{HBT}(E)	57.4	0.01	15.5	0.35
LMS_{VA}(E)	45.1	-	13.7	0.37

[a]MS treatment in the absence of laccase but in the presence of mediator. [b]LMS treatment in the presence of both laccase and mediator. [c]LMS(E) treatment in the presence of both laccase and mediator and followed by an alkaline extraction stage (E). [d]see experimental section for details.

The results clearly indicate that in the absence of laccase and in the presence of the mediator only, delignification did not occur. In addition, our previous LMS studies have demonstrated that the delignification response of a laccase treatment in the absence of a mediator is insignificant. Hence, both the mediator and the laccase must be present in order to achieve delignification. Based on the experimental conditions employed in this study and prior sections, VA was a superior mediator with respect to HBT and NHAA on this high-kappa kraft pulp. The extent of delignification of both NHAA and HBT was comparable It is well known that a high content of hexenuronic acids (HexA) has an adverse impact on the kappa number since HexA consume potassium permanganate.[93] As summarized in Table VIII.2, the change in kappa number after the acid stage was approx. 2%, implying that the HexA content is insignificant and that the kappa numbers in this study were a good reflection of the lignin content.

Accompanying the LMS delignification, the treated pulps suffered a loss in brightness. The brightness data shown in Table VIII.1 indicate that the LMS treatment always darkens the pulp with respect to the brownstock. This effect was most significant with

NHAA and VA. The extraction stage with sodium hydroxide improved the final brightness of the LMS treated pulps relative to the brownstock; however, it never exceeded the initial brightness. Based on our prior studies, we have shown that this darkening effect can be further alleviated with the reinforcement of the extraction stage with peroxide, and with peroxide and oxygen. This effect is consistent with the proposed quinone chemistry of an LMS stage.

LMS Pulp Quinone Content.

The role of quinones in the observed LMS delignification chemistry was explored by isolating the residual lignin from the SW kraft brownstock, and after the MS, LMS, and LMS(E) treatments, as described in the experimental section. The combined content of ortho- and para-quinones was examined using a trimethylphosphite derivatization procedure and ^{31}P NMR. Studies by Zawadzki and Ragauskas[126, 127] have shown that trimethylphosphite can readily be used to tag ortho- and para-quinones and after hydrolysis yield a stable phosphate ester adduct. This adduct is detected via ^{31}P NMR experiments and is a means to establish a semi-quantitative relationship of the quinone content. The combined ortho- and para-quinone data are presented in Figure VIII.1.

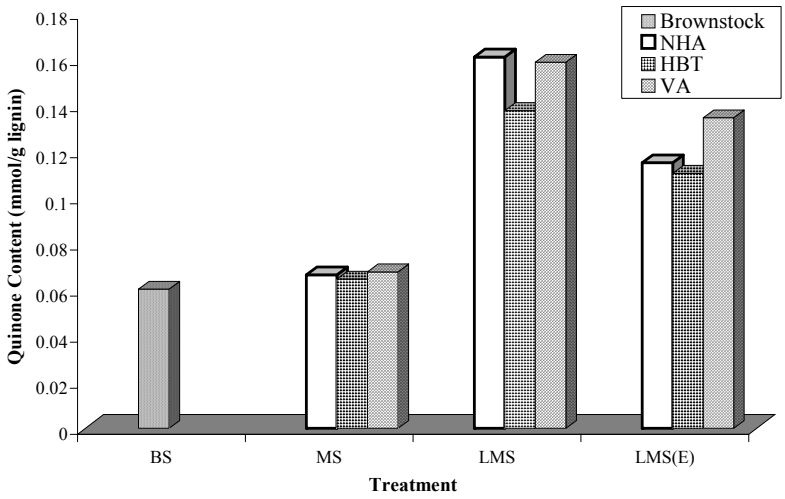


Figure VIII.1. Semiquantitative Quinone Content of Residual Lignins Isolated from the Brownstock (BS) and after MS, LMS, and LMS(E) Treatments using NHAA , HBT, and VA as Mediators.

The experimental results indicate that the content of quinone structures in the brownstock is minute. This value is comparable to that reported by Zawadzki.[126] Treatment of the pulps in the presence of mediators and oxygen failed to introduce any further quinones into the residual lignins. Repeating these experiments in the presence of both laccase and mediator led to an approximate 2.7-fold increase in detectable quinone structures when NHAA and VA were used. The relative trend also suggests that the content of quinones was lower when HBT was employed.

The subsequent alkaline extraction stage reduced the quinone content, on average, by approximately 21%. The loss of quinones during the alkaline extraction stage can be attributed to the reactivity of NaOH with such structures. The nucleophilic addition of OH^- to quinonoid structures can result in increased solubility. This type of chemistry can lead to the formation of hydroxyl substituted catechols *via* a Michael addition of hydroxide anions and also to alpha-hydroxyl-carboxylic acid cyclopentadiene structures

(see Figure VIII.2). The latter structures are postulated to stem as a result of a nucleophilic addition of OH^- followed by a benzylic acid rearrangement.[128]

Despite the loss in quinone structures subsequent to the extraction stage, the data suggest that the residual lignin still contained approximately 50% more quinone structures than the brownstock.

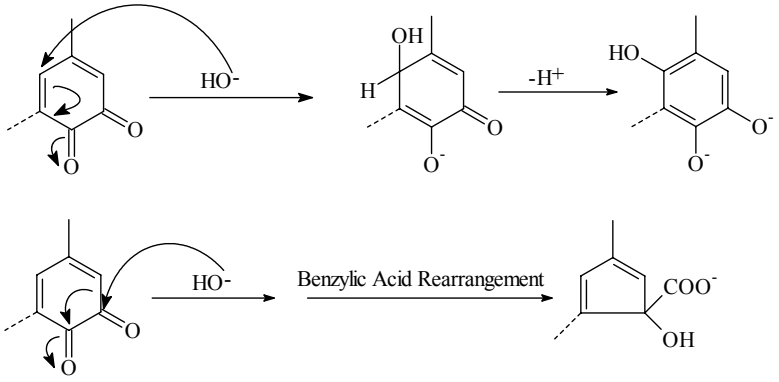


Figure VIII.2. Proposed Sites of Addition of Hydroxide Anions to Quinone Structures. [128]

One possible explanation for this observation could be attributed to the proposed propensity of *o*- and *p*-benzoquinones to undergo condensation reactions leading to the formation of bi-phenyl linkages.[129] As a result, the solubility of such structures may be adversely affected. Another possible explanation may be linked to the ability of catechols to readily be oxidized back to quinone structures.

Section VIII Conclusions

In summary, this study provides some of the first spectroscopic data that establishes conclusively the formation of quinones in LMS and LMS(E) treated softwood kraft

pulps. The data provide an explanation, in part, for the darkening of kraft pulps after an LMS stage and its subsequent partial brightening after an LMS(E) stage. The observed formation of quinones after an LMS stage is also consistent with the reported brightness benefits of alkaline peroxide bleaching of LMS treated pulps. The formation of quinones and the darkening effect of pulps are important aspects of the chemistry of LMS delignification. This issue will need to be addressed and further understood if LMS technology is to be implemented commercially.

Methods and Materials

Chemicals. All chemicals were purchased from Aldrich Co., Milwaukee, WI, and used as received, except for *p*-dioxane, NHAA , and laccase. *p*-Dioxane was freshly distilled over $NaBH_4$ prior to using it for lignin isolation experiments. NHAA was synthesized in accordance with Oxley's method.[109] Laccase from *Trametes villosa* was donated by Novo Nordisk Biochem, Franklinton, NC.

Furnish. The softwood kraft pulp employed in this study originated from a 25-year-old *Pinus taeda* tree that was donated by Union Camp (now International Paper), Savannah, GA. The chips were cooked at Potlatch Corp. facilities in Cloquet, MN, to an H-factor of 573 using 18.5% active alkali. The pulp was thoroughly washed, screened, centrifuged, fluffed, and stored at 4°C. Prior to executing the experimental design called for in this study, the pulp was Soxhlet extracted with acetone for 24 hours and then thoroughly washed with distilled water to remove the residual acetone.

Hexenuronic acid in pre-acetone extracted brownstock. The content of hexenuronic acids in the brownstock was indirectly measured in accordance with a modified procedure reported by Vuorinen et al. (93) In brief, a 1000-mL round bottom flask was charged with 25 g of pulp (o.d. basis). The pulp consistency was adjusted to 3% by adding distilled water. The pH was then lowered to 3 using 4.0 N sulfuric acid. The slurry was refluxed for three hours at 100°C. The change in kappa number before and after the treatment was then determined and served as an indirect measurement of hexenuronic acids (see Table VIII.2).

Table VIII.2. Changes in Kappa # After Acid Treatment of Brownstock.

Replicate #	Initial Kappa	Final Kappa	%Change
1	73.4	71.5	2.6
2	73.4	71.9	2.0

Laccase assay. Laccase activity was measured as described in prior chapters.

Laccase-mediator delignification procedure (LMS). The biodelignifications were performed employing a 2000-mL capacity Parr reactor as described in previous sections

Alkaline extraction stage (E). Alkaline extractions were performed in 4 mm-thick Kapak heat sealable pouches for 1 hour, at 80°C, and 10% consistency. All E treatments employed 2.5% charge of NaOH.

Pulp characterization. The brownstock, MS, LMS, and LMS(E) pulps were characterized for kappa number and brightness in accordance with standard TAPPI Standard Methods T236-cm85 and T452-om98, respectively.[94]

Isolation of residual lignins. The isolation of residual lignins was carried out following methods described in prior sections

Derivatization of residual lignins with trimethylphosphite (TMP). Derivatization of residual lignins with trimethylphosphite was performed in accordance with Zawadzki's method.[126] In brief, a 30 mg sample of lignin previously dried at 40°C under vacuum for 24 hours was treated with 500 μL of 50% TMP/DMF (v/v) under an argon atmosphere for 7 days. Subsequent to the incubation period, excess trimethylphosphite was removed by first adding 250 μL of DMSO and then placing the lignin solution under vacuum at 45°C until the sample was nearly dry (approx. 6 hours). The treated lignin samples were then dissolved in 500 μL 60% of DMSO-d_6/pyridine (v/v) containing tri-meta-tolylphosphate (0.7 mg/mL) and chromium–acetylacetonate (0.9 mg/mL). Deionized water (5 μL) was then added and the lignin solution was allowed to mix for 12 hours prior to acquiring the ^{31}P NMR spectrum.

^{31}P NMR of derivatized residual lignins. ^{31}P NMR spectra of derivatized lignins were acquired using a 90° pulse, a 5-second pulse delay, inverse-gated broad-band proton

decoupling and 1000 scans per spectrum (approx. 1 hr 36 min total acquisition time). All ^{31}P NMR spectra were recorded on a DMX 400 MHz Bruker spectrometer.

Section IX: Application of LMS Biobleaching Technology for the Production of High-Kappa Kraft Pulps

Our interest in LMS biobleaching resides, in-part, on the well-established phenomena that the selectivity of kraft pulping decreases as the pulping process completes the bulk phase and begins the residual phase. Several researchers[130] have noted that it is possible to improve pulp yields by halting the kraft cook prior to the residual phase and removing the additional lignin via oxygen delignification. In general, for SW kraft pulps, this requires employing an aggressive O or OO stage on a brownstock pulp having a kappa number greater than 40. The overall wood savings following this approach have been reported to be in the range of 2–6% for a modern pulp mill.

The application of LMS delignification as an alternative to an extended O or OO stage is an attractive technology to delignifying high-kappa kraft pulps. Indeed, Haynes and Ragauskas[89] had reported that LMS treatment of cellulose led to no change in pulp viscosity, suggesting that laccase mediator delignification of kraft pulps is highly selective for lignin. Earlier studies in this program have demonstrated the high selectivity of an LMS system with a kappa 70 SW kraft pulp yielding minimal losses in pulp viscosity. Based on these observation, it appeared that an LMS stage could be used to process high-kappa kraft pulps with nominal loss in pulp carbohydrates providing improved pulp yields. LMS bleaching studies with a 70-kappa SW kraft pulp indicated that violuric acid was a superior mediator for the delignification of high-kappa kraft pulps compared with respect to either HBT or NHA. The results of biobleaching a high and low lignin content SW kraft pulp with LMS(E+P), employing violuric acid as a mediator, are summarized in Table IX.1.

Table IX.1. LMS(E+P) Delignification Properties of SW Kraft Pulps.

Pulp	SW-50.0 kappa	SW-27.5 kappa
% Delignification	42.6	61.1
% Viscosity Retained	74.2	79.6
Yield	99.9	100.0

This research study examines the integration of this biodelignification technology into modern ECF bleaching systems for the production of fully bleached kraft pulps.

Full-Sequence Bleaching

Past studies by Poppius-Levlin et al.,[92] Sealey et al.,[96] and others have all demonstrated that low-kappa pulps can be readily delignified with an LMS stage and then bleached to full brightness using either an ECF or TCF bleaching sequence. The nature of the physical pulp properties has not been as well established. This study examines the bleachability of high and low-kappa SW kraft pulps and defines their physical properties. The bleaching sequences examined are summarized in Table IX.2.

Table IX.2. Bleaching Sequences Applied to Kappa 27.5 and 50.0 SW Kraft Pulps.

Kappa 27.5 SW kraft pulp	**Kappa 50.0 SW kraft pulp**
D(E+P+O)DED	OOD(E+P+O)D
LMS_{VA}(E+P+O)DED	$LMS_{VA}LMS_{VA}$(E+P+O)DED
OOD(E+P+O)D	LMS_{VA}OD(E+P+O)D
LMS(O)D(E+P+O)D	$OLMS_{VAD}$(E+P+O)D

The results of the bleaching sequence studies are summarized in Figures IX.1 to 5. In each case, it was possible to readily achieve a final brightness of ≈ 85.

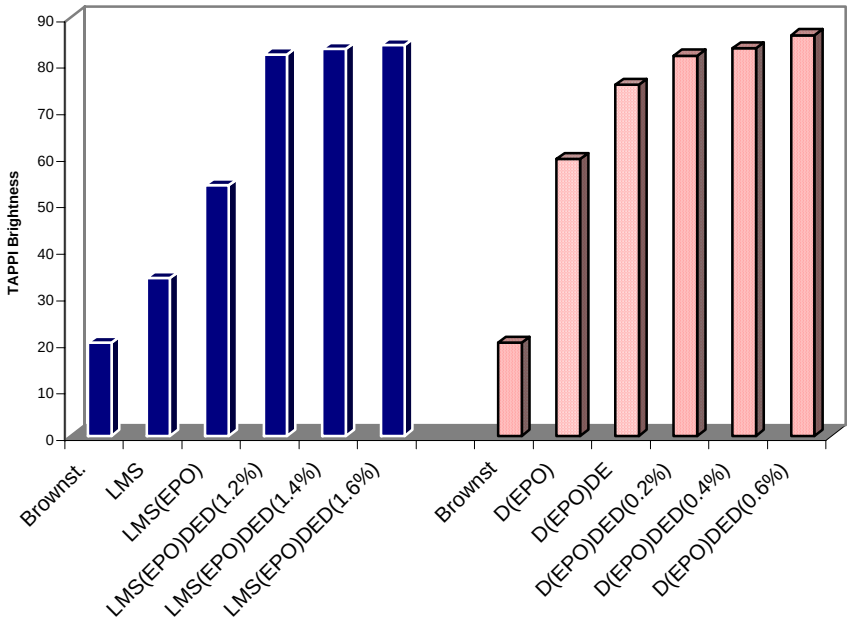


Figure IX.1. Brightness Development for Bleaching Kappa 27.5 SW Kraft Pulp via LMS_{VA}(E+P+O)DED and D(E+P+O)DED.

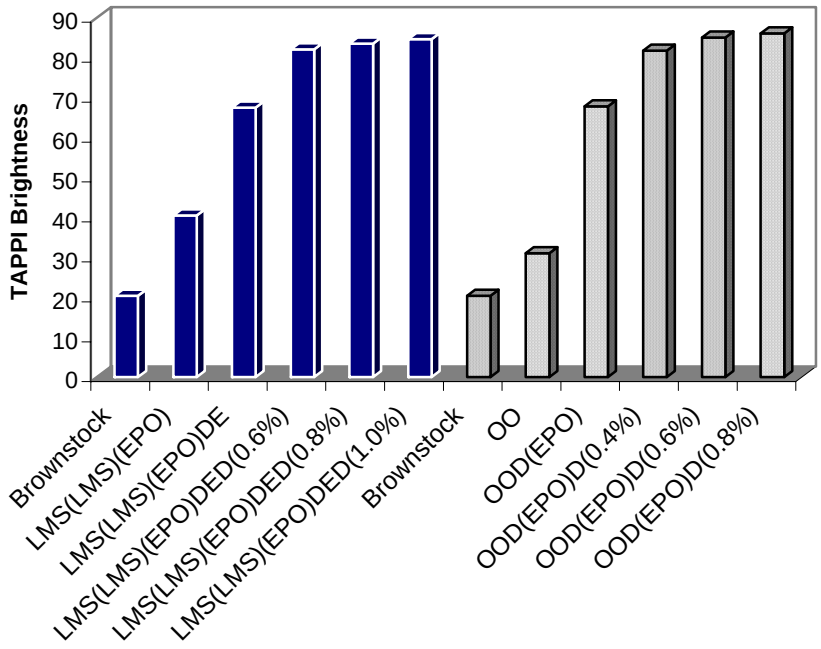


Figure IX.2. Brightness Development for Bleaching Kappa 27.5 SW Kraft Pulp via $LMS_{VA}LMS_{VA}$(E+P+O)DED and OOD(E+P+O)D.

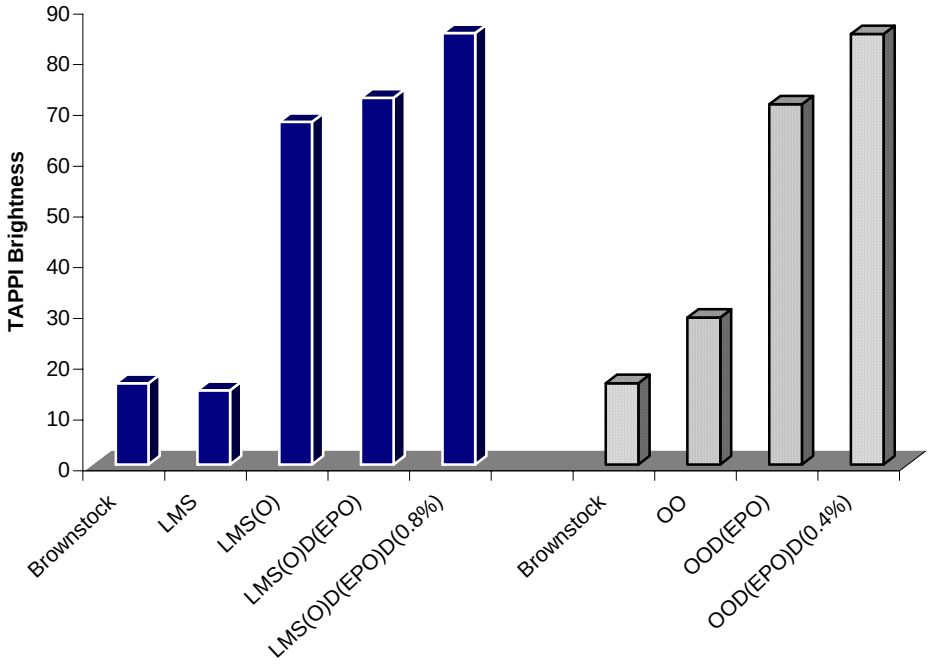


Figure IX.3. Brightness Development for Bleaching kappa 27.5 SW Kraft Pulp via $LMS_{VA}OD(E+P+O)D$ and OOD(E+P+O)D.

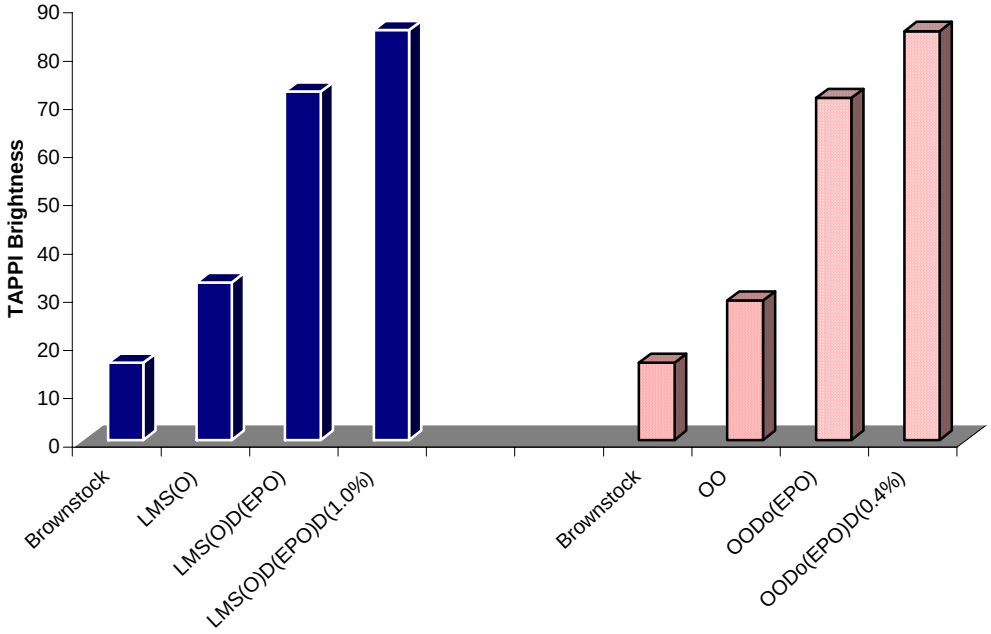


Figure IX.4. Brightness Development for Bleaching Kappa 50.0 SW Kraft Pulp via $LMS_{VA}OD(E+P+O)D$ and OOD(E+P+O)D.

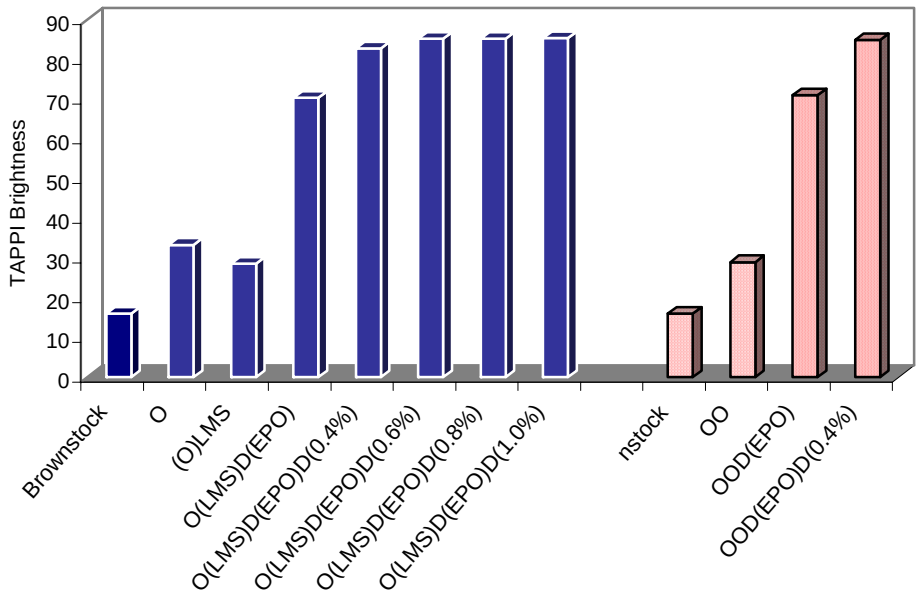


Figure IX.5. Brightness Development for Bleaching Kappa 50.0 SW Kraft Pulp via O(LMS$_{VA}$)D(E+P+O)D and OOD(E+P+O)D.

Samples of the fully bleached pulp samples (TAPPI Brightness ≅85) were PFI refined to 2000 and 4000 revolutions and then analyzed for tensile, tear, and burst index values. These physical testing results are summarized in Table IX.3.

Table IX.3. Tensile, Tear, and Burst Index for Fully Bleached SW Kraft Pulp with a Final TAPPI brightness of 84.5 ±0.50.

SW Kraft Pulp	Physical Properties Following 0–2000–4000 Revolutions		
	Tensile Index Nm/g	**Tear Index mNm^2/g**	**Burst Index $kPam^2/g$**
Kappa 27.5			
D(E+P+O)DED	15.7 – 69.4 – 75.4	12.4 – 18.4 – 15.8	0.8 – 6.0 – 6.7
LMS_{VA}(E+P+O)DED	22.8 – 77.6 – 87.6	13.9 – 14.5 – 11.5	1.4 – 6.2 – 6.8
OOD(E+P+O)D	23.9 – 80.3 – 87.9	15.2 - 12.2 – 11.0	1.6 – 6.3 – 6.8
LMS(O)D(E+P+O)D	24.4 – 80.4 – 87.2	16.6 – 12.8 – 10.5	1.5 – 6.2 – 6.8
Kappa 50.0			
OOD(E+P+O)D	41.1 – 98.5 – 104.4	19.1 – 13.4 – 11.4	3.3 – 8.5 – 9.4
LMS_{VA}OD(E+P+O)D	45.2 – 98.8 – 103.5	20.0 – 12.7 – 11.5	3.2 – 8.0 – 9.0
OLMS_{VA}D(E+P+O)D	40.8 – 101.6 – 102.5	22.7 – 13.0 – 11.4	2.8 – 8.5 – 9.6

The physical testing of the fully bleached pulps indicates that the LMS-delignified pulps have physical strength properties comparable to conventional ECF bleached pulps. This results is significant given the significant improvements in pulp yield achieved with an LMS stage and the resulting bleaching processes employed in Table IX.3

LMS Degradation of Lignin

As we have previously discussed, the biobleaching chemistry of an LMS stage is influenced by the nature of the mediator and furnish employed. For low-kappa pulps, it appears that the LMS_{HBT} system vigorously oxidizes C-5 condensed and noncondensed phenolics. For high-kappa pulps, this same delignification system removes primarily C-5 noncondensed phenolic structures. Furthermore, in comparing the reactivity of a LMS_{HBT} vs. LMS_{NHA} vs. LMS_{VA} system for high-kappa SW kraft pulps we observed that VA was approximately two fold more effective at removing lignin than HBT or NHA.

To further explore the fundamental chemical basis by which the LMS_{VA} system delignified the SW kraft pulps presented in Table IX.1, the residual lignin from the kraft brownstocks and after an LMS_{VA}(E+P) were isolated and characterized by NMR. Residual lignin was recovered from the kraft pulps employing a standard dioxane-water acidic hydrolysis procedure, which provided on average 47% lignin recovery. These lignin samples were then analyzed by quantitative ^{13}C and ^{31}P NMR techniques and these results are summarized in Figures IX 6–8. The ^{31}P NMR analysis technique is well suited for characterizing the hydroxyl functionality of lignin, whereas the ^{13}C NMR method provides a complete structural analysis of lignin.

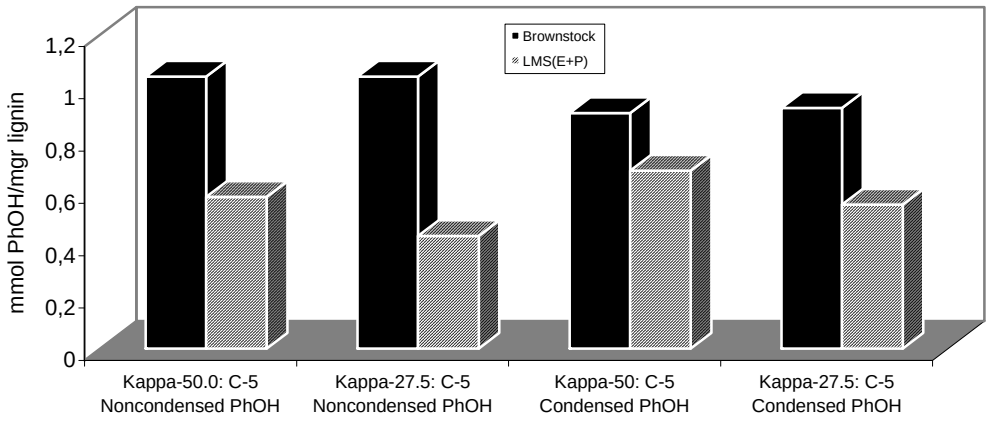


Figure IX.6. Changes in C-5 Condensed and Noncondensed Phenolic Guaiacyl Content of Residual Lignin isolated from Kappa 50.0 and Kappa 27.5 Brownstocks and After LMS_{VA}(E+P) Treatments, as Determined by Phosphitylation and ^{31}P NMR Analysis.

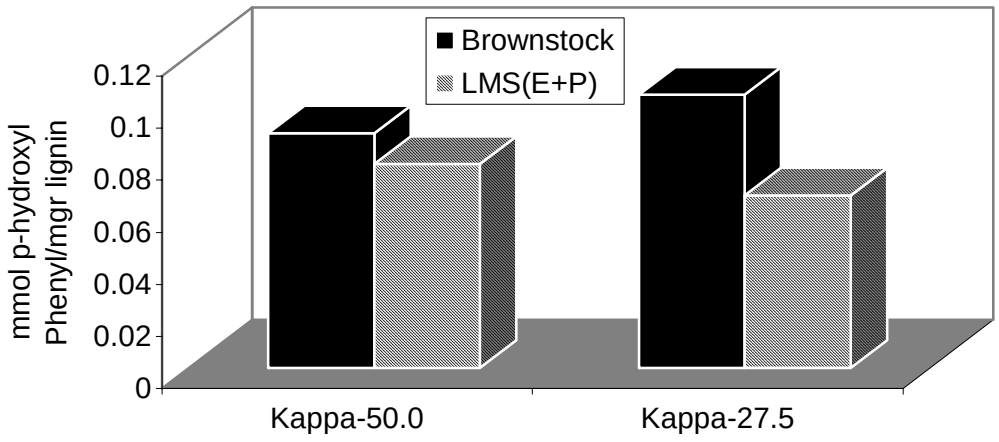


Figure IX.7. Changes in p-Hydroxyl Phenyl Content of Residual Lignin Isolated from Kappa 50.0 and Kappa 27.5 Brownstocks and after LMS_{VA}(E+P) Treatments, as Determined by Phosphitylation and ^{31}P NMR.

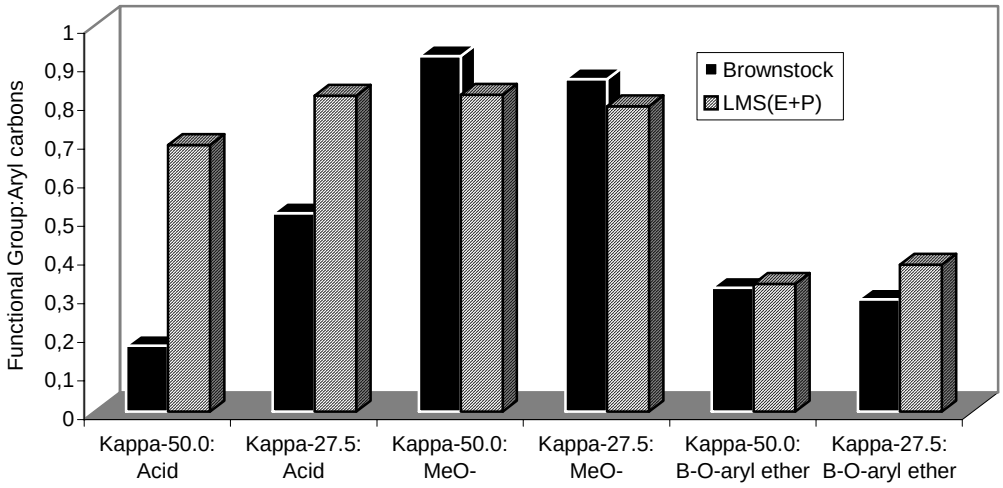


Figure IX.8. Changes in Acid Group, Methoxyl, and β-O-aryl Ether Content of Residual Lignin isolated from Kappa 50.0 and Kappa 27.5 Brownstocks and After LMS_{VA}(E+P) treatments, as Determined by ^{13}C NMR.

The results of this analysis clearly indicated that the primary pathway of lignin degradation during an LMS_{VA}(E+P) treatment is the degradation of phenoxy groups with

a preference for C-5 noncondensed phenoxy groups. This latter effect is attenuated for the low-kappa SW kraft pulp. It is interesting to note the slight decrease in p-hydroxyl phenyl groups. The loss in phenoxy groups is accompanied by a large increase in acid groups. It is also apparent that the β-O-aryl ether group is resistant to oxidation, and only slight demethoxylation was observed to occur under the biobleaching conditions employed.

The results of these studies are consistent overall with lignin model compounds studies with LMS but some differences do exist, such as the lack of reactivity with non phenolic and β-O-aryl ether structures observed in this study versus LMS lignin model compound studies. This effect has been tentatively attributed to differences in LMS selectivity when reacting with one or two functional groups (i.e., lignin model compound) versus the lignin macropolymer containing an array of functional groups with which the oxidized mediator can react.

Section IX Conclusions

The use of LMS-delignification technologies can be applied to high-kappa kraft pulps that can be subsequently processed to yield high-quality fully bleached pulps. The key parameter involved in this biotechnology is the mediator used in conjunction with laccase. To date, we have observed that violuric acid is a superior agent for laccase. Nonetheless, the propensity of VA to degrade during the LMS stage significantly limits its practical applications to remove lignin. The bleaching of high-kappa kraft pulps with a laccase mediator system (LMS) was found to provide 43-61% delignification following an (E+P) stage when violuric acid was used as the mediator. Pulp yields after the LMS(E+P) treatment were +99.9%. Structural analysis of the residual lignin after LMS_{VA} treatments indicated that the LMS stage oxidizes primarily the phenolic structures of lignin. Full sequence ECF bleaching of high and low-kappa SW kraft pulps after a LMS_{VA}(E+P+O) or (LMS_{VA})(LMS_{VA})(E+P+O) indicated the pulps could be readily bleached to +85 TAPPI brightness. The physical strength properties of the fully bleached LMS-treated pulps exhibited tensile, tear, and burst indexes comparable to

control ECF-bleached pulps. The challenge for commercial LMS systems is the development of catalytic mediators that can selectively remove > 60% of the lignin in pulp fibers in a cost-effective manner.

Experimental Details for Section IX

Materials. Violuric acid and all other chemical reagents and solvents were purchased from Aldrich, Milwaukee, WI, and used as received except for *p*-dioxane, which was freshly distilled over $NaBH_4$ before use. Laccase from *Trametes villosa* was donated by Novo Nordisk Biochem. The kappa 27.5 SW kraft pulp was acquired from a southern USA pulp mill, whereas the kappa 50.0 SW pulp was laboratory-prepared from a conventional batch cook. The pulps were thoroughly washed, screened, centrifuged, fluffed, and stored at 4°C prior to use.

Pulp Characterization. All pulp testing was done according to TAPPI Test Methods.

Laccase Assay. Laccase activity was measured by monitoring the rate of oxidation of syringaldazine as described previously.

LMS Delignification. LMS_{VA} was accomplished as described in the literature[8] employing 1.87×10^7 U of laccase/gr o.d. pulp and a 4% charge of violuric acid, as described previously

Pulp Bleaching Conditions. Table IX.4 summarizes the bleaching conditions employed for the remaining bleaching stages described in Table 2.

Table IX.5. Summary of Bleaching Conditions Employed.

Treatment	Bleaching Conditions
D_0	0.20 k.f., 10% csc, 50°C, 45 min.
E+P	2.5% NaOH, 0.05% $MgSO_4$, 10% csc, 70°C, 1 h
E+P+O	0.5% H_2O_2, 2.75% NaOH, 0.05% $MgSO_4$, 10% csc, 70°C, 1.0 h O_2 60 psi for 15 min. and then reduced 12 psi/5 min.
O	3.00% NaOH for low-kappa pulp, 4.00% for high-kappa pulp, 0.05% $MgSO_4$, 10% csc, 80 psi O_2, 95°C, 1.3 h
OO	3.00% NaOH for low-kappa pulp, 4.00% for high-kappa pulp, 0.05% $MgSO_4$, 10% csc, 80°C, 130 psi O_2 for 30 min., 95°C 60 psi for 60 min.
D_1	1% ClO_2, 10% csc, 70°C, 3 h
E	NaOH: 50% of equiv. Cl_2 charge, 10% csc, 70°C, 1 h
D_{final}	See figure for charge of ClO_2 employed, 10% csc, 70°C, 3 h

NMR Characterization of Residual Lignins. The isolated residual lignin samples were analyzed using a 400 MHz Bruker DMX spectrometer. Quantitative ^{13}C NMR spectra were acquired and analyzed in accordance with established literature methods. Lignin samples were also derivatized with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane and analyzed by ^{31}P NMR as previously described.

Section X: Influence of Process Parameters on LMS Biobleaching Technology for the Production of High-Kappa Kraft Pulps

Delignification of kraft pulps with laccase is critically dependent upon the nature of the mediator employed and the process conditions employed. In this chapter we summarize our research studies focused primarily on the role that nonprocess elements, pH, temperature have on the LMS system when violuric acid is employed as a mediator. Although many challenges remain prior to the development of a practical LMS-delignification system it is most likely these biobleaching systems will need to operate in an environment where the level of trace metals is much higher than is typically present in most LMS systems studied to date in the laboratory. To explore the sensitivity of an LMS system to non-process elements a 50.0 SW kraft pulp was treated to an $LMS_{VA}(E)$ sequence with and without additional trace metal salts added. The extent of delignification and pulp viscosity values were determined before and after this treatment and the results of these experiments are summarized in Figures X.1 and X.2.

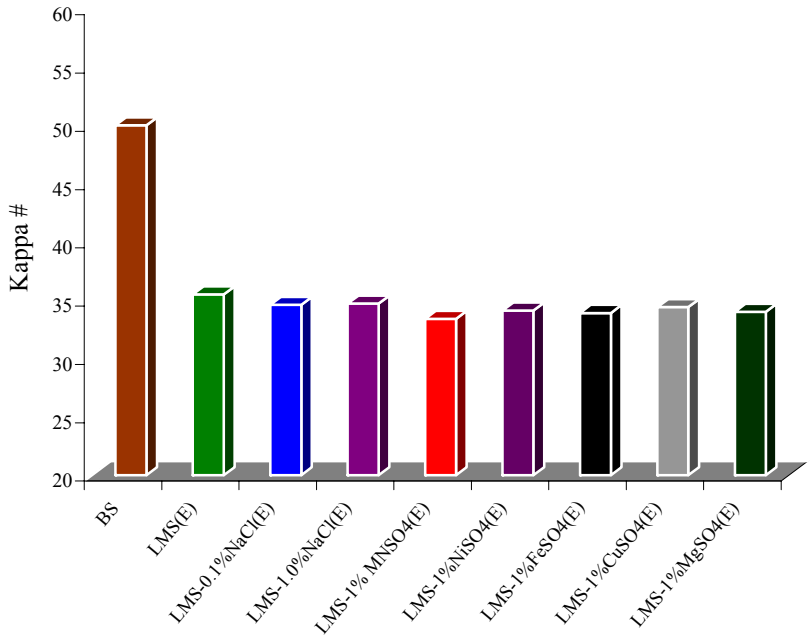


Figure X.1: Influence of nonprocess elements on the delignification performance of an LMS_{VA} treatment (containing1% black liquor carryover) evaluated after $LMS_{VA}(E)$.

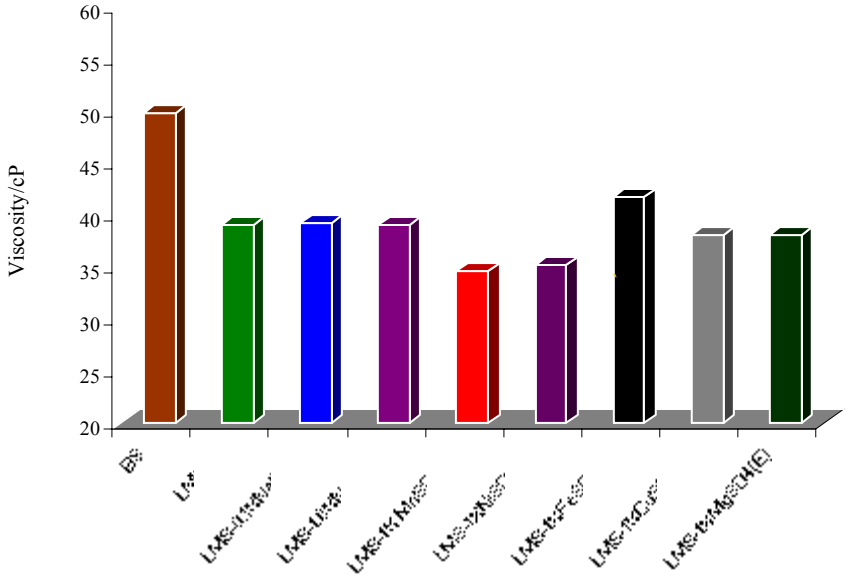


Figure X.2: Influence of nonprocess elements on the viscosity properties of an LMS_{VA} treatment (containing1% black liquor carryover) evaluated after $LMS_{VA}(E)$.

The results summarized in Figures X.1 and X.2 suggest that the LMS_{VA} system is not overtly sensitive to the presence of trace inorganic metals, with Mn^{2+} only slightly detrimentally impacting the performance the viscosity properties of the biodelignified pulp.

Although the optimal pH and temperature profile for the laccase employed in this study is approximately 4.5 and 45°C, these conditions are far from preferred mill conditions. It is anticipated that future genetic research will provide access to differing laccases with varying reaction profiles, nonetheless it was interest to profile the temperature and pH sensitivity of the enzyme employed in this study and these results are summarized in Figures X.3 and X.4.

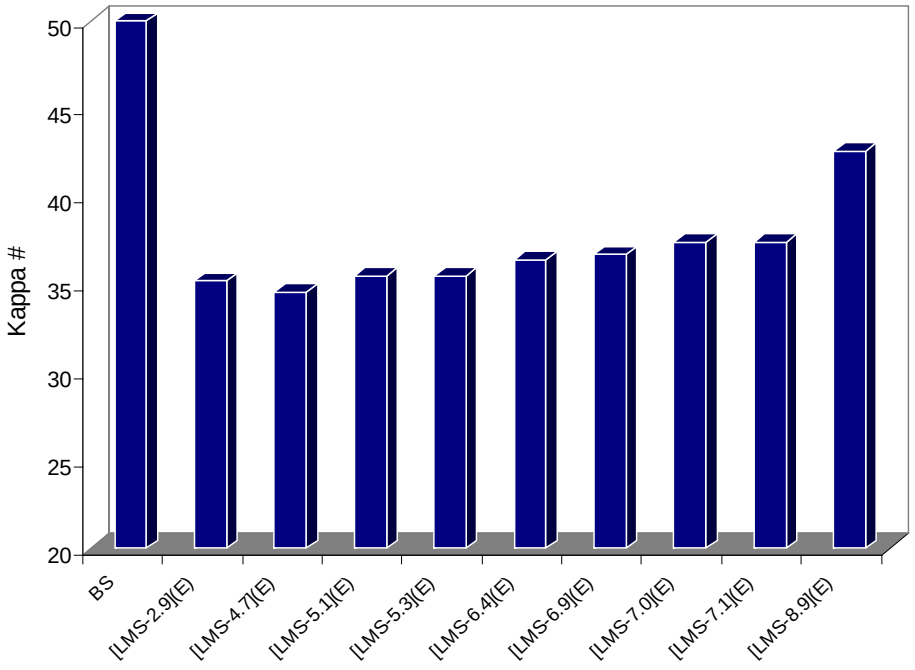


Figure X.3. Influence of LMS pH [LMS pH value is denoted in brackets] on the delignification performance of LMS_{VA} treatment evaluated after LMS_{VA}(E).

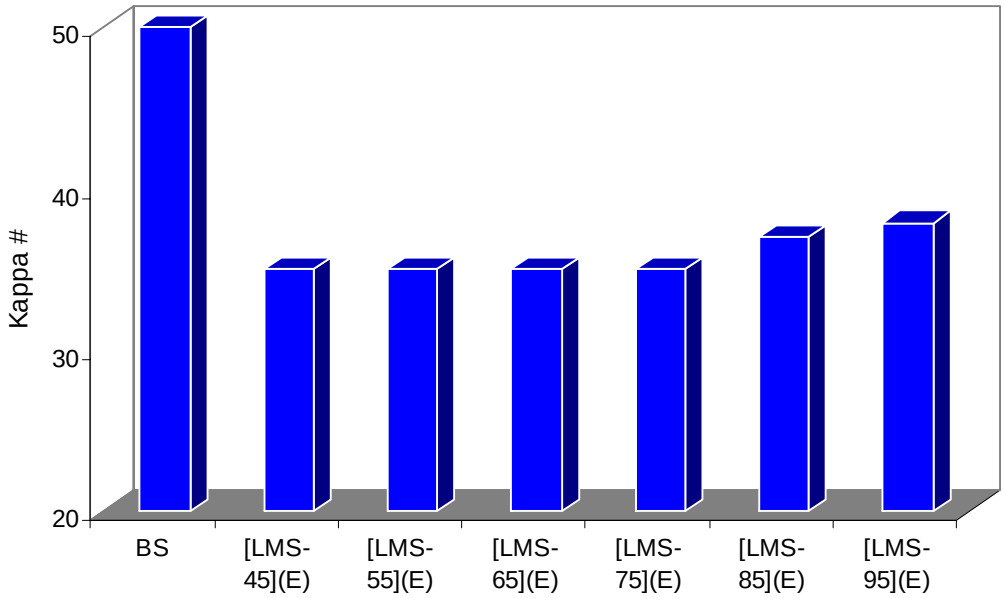


Figure X.4. Influence of LMS temperature [LMS temperature/^{o}C value is denoted in brackets] on the delignification performance of LMS_{VA} treatment evaluated after LMS_{VA}(E)

The biobleaching results summarized in Figures X.3 and X.4 clearly indicate that the laccase employed in this study has a broad range of operating parameters. Nonetheless, process benefits could be captured if laccases could be designed with higher temperature and pH operating ranges.

The final experimental parameter examined in this study was the evaluation of carryover on the LMS delignification process. Most prior LMS delignification studies have used fiber samples that have been exhaustively washed, a situation that does not replicate actual mill conditions. Hence, pulp samples were mixed with varying amounts of black liquor solids and then treated to an $LMS_{VA}(E)$ sequence and these results are summarized in Figures X.5 and X.6. This data indicates the addition of small amounts of black liquor carryover is not detrimental to the overall performance of an LMS_{VA} treatment.

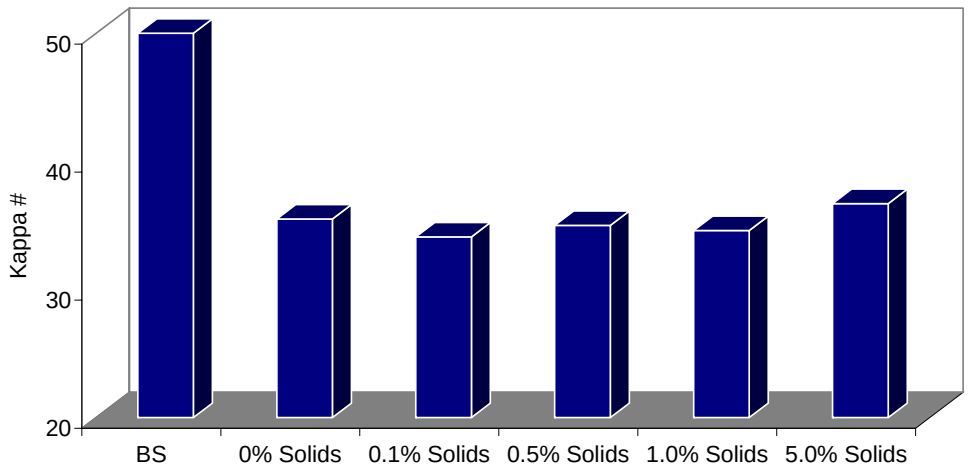


Figure X.5. Influence of carryover on the delignification performance of LMS_{VA} treatment evaluated after $LMS_{VA}(E)$

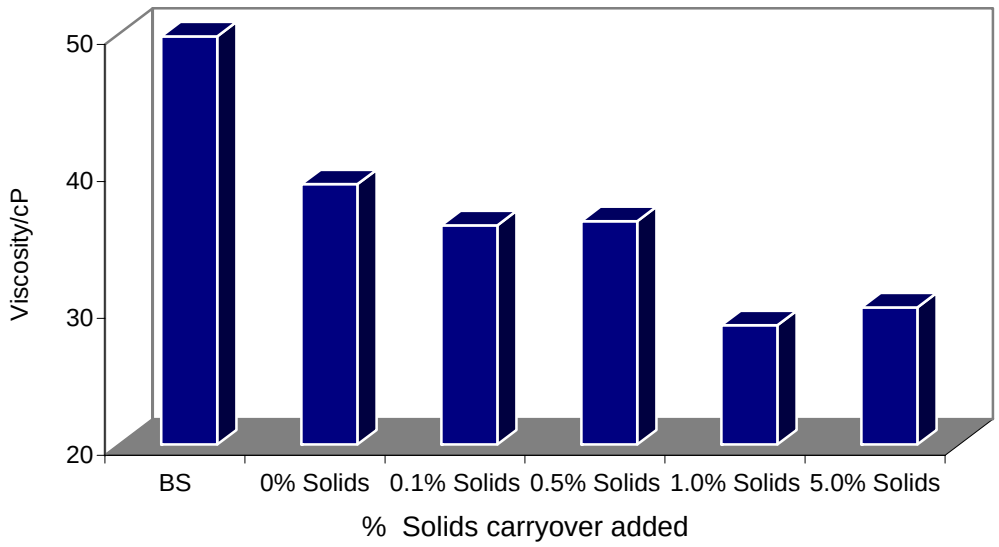


Figure X.6. Influence of carryover on the LMS_{VA} treatment after LMS_{VA}(E) as evaluated by viscosity (cP).

In addition to these key chemical process parameters another important factor that will influence the performance of laccase biobleaching technology is it's response to the high shear mixing environmental in a pulp mill. This issue has taken on extra concern after a recently report by Paice et al that the performance of an LMS stage is detrimentally influenced by mixing.[131] To examine the effects of mixing on an LMS-stage we examined several degrees of mixing on an LMSVA(EPO) sequence. The experimental biobleaching parameters examined are summarized in Table X.1 and under all the conditions employed it was apparent that the LMS stage is not sensitive to mixing speed and/or consistency effects studied. A closer examination of the studies undertaken in this program and those reported by Paice et al.[131] could be possibly attributed to the addition of a chelant in the latter study.

Two other biobleaching parameters that have not been fully examined are the effects of oxygen pressure and presence of nitrogen species in eth biobleaching waste products. To evaluate the sensitivity of the LMS stage to the applied pressure a SW kraft pulp was biobleached at a series of oxygen pressures as summarized in Table X.2. These results clearly demonstrated the potential of enhancing the performance of a LMS stage with

increased oxygen pressure but these elevated pressures are not practical for mill applications. In the future, it may be possible to attain comparable performance in biodelignification at lower oxygen pressures by genetically engineering improved lactase performance.

Table X.1 Effects of Varying Mixing Speed and RPM's on LMS(EPO)[a] Efficiency employing a SW Kraft Pulp.[b]

LMS Mixer	LMS Csc.	Mixing Speed (rpm)	Mixing Time Over 1 h Reaction Time	Kappa #	LMS(EPO) Viscosity/cP	Tappi Brightness
Quantum	10%	600	10 sec/2 min	14.6	23.6	34.2
	10%	1200	5 sec/2 min	14.4	23.2	34.2
	10%	2400	2.5 sec/2 min	14.3	23.1	33.3
	10%	600	10 sec/2 min	14.2	23.8	32.9
	10%	3600	10 sec/2 min	14.0	22.7	32.6
	20%	600	10 sec/2 min	14.2	22.9	--
	20%	2400	10 sec/2 min	14.1	23.6	--
Stir Paddle	10%	48	1 h	16.3	--	--

[a]LMS: Laccase Dose: 1.4 x 10^6 U/gr., 45 min., pH:4.5, 4% VA, 120 psi O_2, 25^{o}C (EPO): 70^{o}C, 10% csc, 1 h, 60 psig O_2, 0.5% H_2O_2, 2.5% NaOH , all EPO stages were preformed with pin mixer reactor; b Kraft SW Brownstock Kappa #:33.3; Viscosity:30.4 cP; TAPPI Brightness:25.9

Table X.2 Biobleaching Conditions and Physical Properties of Pre and Post Oxygen Delignified SW Pulps treated via $LMS_{VA}E$[a]

LMS_{VA} O_2 Pressure (psig)	Pulp Kappa #
SW Kraft Brownstock	31.3
0	22.8
65	20.9
145	14.4
Post-O SW Kraft pulp	10.0
5	6.0
25	5.5
65	5.3
105	3.6
145	3.8
185	3.0

[a]LMS stage was conducted with a 4% charge of VA, 0.5 ml laccase/gr pulp; 55oC, 45 min., 10%csc; E-stage was conducted at 10% csc, 2.5% NaOH; 0.5% H_2O_2; 60 psig O_2 pressure, 1 h, 70^oC

Research studies also determined what level will a LMS-treatment increase NO_x discharge limits if the effluents of an LMS treatment were directed towards the recovery system. This parameter was studied by performing a series of LMS stages on a high kappa kraft pulp as summarized below.

Table X.3: Experimental LMS and (E+P+O) Delignification Conditions.

LMS Stage	
Pressurized Reactor:	Parr Pressure Reactor
O_2 pressure:	120 psig
Reaction Time:	45 min
Reaction Temperature:	55^o C
Consistency:	10%
Violuric Acid (VA) Charge:	1.0 – 4.0%
Laccase Charge:	7.0 x 0^5 U/gr OD pulp
(E+P+O) Stage:	
Pressurized Reactor:	Parr Reactor
O2 pressure:	60 psig
Reaction Time:	60 min
Reaction Temperature:	70^o C
Consistency:	10%
H_2O_2 Charge:	0.50%
NaOH Charge:	2.50%

Following the LMS and (EPO) treatment pulp samples were analyzed for kappa and viscosity values. The effluents from the LMS, (EPO) and control stages were stored near 0^oC until NO_x analysis. The latter analysis was accomplished via by charring the effluents and measuring NO_x generation and these results are summarized in Table X.4.

Table X.4: Analysis of LMS_{VA}(E+P+O) effluents for NO_x generation.

Sample	Stage	Kappa #	Viscosity(cP)	NO_2(mg/L)	NO_3(mg/L)
Brownstock		33.3	30.4		
LMS with 4% VA followed	LMS			<0.05	5.00
by (EPO)	(LMS)(EPO)	15.0	23.33	11.4	6.23
LMS with 2% VA followed	LMS			<0.05	3.68
by (EPO)	(LMS)(EPO)	17.6	24.06	4.03	2.13
LMS with 1% VA followed	LMS			<0.05	2.26
by (EPO)	(LMS)(EPO)	22.0	23.45	1.20	1.21
2% VA, no laccase followed	MS			---	---
by (EPO)	(EPO)	24.6	21.58	<0.05	1.02
No VA No Laccase followed	Control			<0.05	0.40
by (EPO)	(EPO)	24.6	20.86	<0.05	0.85
Laccase No VA followed	Laccase			<0.05	1.25
by (EPO)	(EPO)	24.8	21.89	<0.05	0.36

The data in Table X.4 indicates that at most the addition of laccase in a bleaching stage could increase NO_x emissions by approximately 30%. Of greater concern is the substantially greater generation of NO_x when the required mediator is present in the LMS stage, as demonstrated when 1-4% VA is employed. This suggests that a highest priority is to examine alternative waste treatment systems or the need to discover an alternative mediator technology.

Section X Conclusions

Although the search of new mediators for LMS remains a high priority, the results reported in this paper indicate that additional issues need to be addressed in the performance and conditions under which laccase operates. A review of xylanase operating performance over the past year certainly suggests that alternative sources of laccase and genetic modification will eventually provide laccases with differing pH, temperature, and other operating performance properties. The ability to operate laccase in differing pH, and temperature profiles will facilitate the development of commercial

biobleaching technologies, although for LMS mill applications the need to identify new mediator systems remains critical.

Experimental Details for Section X

Materials. Violuric acid and all other chemical reagents and solvents were purchased from Aldrich, Milwaukee, WI, and used as received. Laccase from *Trametes villosa* was donated by Novozymes Biotech, Raleigh, NC. The kappa 50.0 SW kraft pulp was acquired from a laboratory-prepared conventional kraft cook employing chips from a commercial southern USA pulp mill. The pulps were thoroughly washed, screened, centrifuged, fluffed, and stored at 4°C prior to use.

Pulp Characterization. All pulp testing was done according to TAPPI Test Methods.

Laccase Assay. Laccase activity was measured by monitoring the rate of oxidation of syringaldazine as described in earlier chapters.

LMS Delignification. LMS_{VA} was accomplished as described in the literature[8] employing 1.87×10^7 U of laccase/gr o.d. pulp and a 4% charge of violuric acid

OVERALL RESEARCH PROGRAM CONCLUSIONS

A key finding of this research program was that LMS treatments on high-kappa kraft could be successfully accomplished providing substantial delignification (i.e., > 50%) without detrimental impact on viscosity and significantly improved yield properties. The efficiency of the LMS was evident since most of the lignin from the pulp was removed in less than one hour at 45 degrees C. Of the mediators investigated, violuric acid was the most effective vis-à-vis delignification. A comparative study between oxygen delignification and violuric acid revealed that under relatively mild conditions, a single or a double LMS_{VA} treatment is comparable to a single or a double O stage. Of great notability was the retention of end viscosity of LMS_{VA} treated pulps with respect to the end viscosity of oxygen treated pulps. These pulps could then be bleached to full brightness values employing conventional ECF bleaching technologies and the final pulp physical properties were equal and/or better than those bleached in a conventional ECF manner employing an aggressively O or OO stage initially.

Spectral analyses of residual lignins isolated after LMS treated high-kappa kraft pulps revealed that similar to HBT, VA and NHA preferentially attack phenolic lignin moieties. In addition, a substantial decrease in aliphatic hydroxyl groups was also noted, suggesting side chain oxidation. In all cases, an increase in carboxylic acid was observed. Of notable importance was the different selectivity of NHA, VA and HBT towards lignin functional groups, despite the common N-OH moiety. C-5 condensed phenolic lignin groups were overall resistant to an $LMS_{NHA,\ HBT}$ treatments but to a lesser extent to an $LMS_{VA.}$ The inactiveness of these condensed lignin moieties was not observed when low-kappa kraft pulps were biobleached, suggesting that the LMS chemistry is influenced by the extent of delignification.

We have also demonstrated that the current generation of laccase has a broad spectrum of operating parameters. Nonetheless, the development of future genetically engineered laccases with enhanced temperature, pH and redox potentials will dramatically improve the overall process. A second challenge for LMS bleaching technologies is the need to develop effective, catalytic mediators. From the literature we already know this is feasible since ABTS and some inorganic mediators are catalytic.

Unfortunately, the mediators that exhibit catalytic properties do not exhibit significant delignification properties and this is a challenge for future research studies.

Potential short-term mill application of laccase has been recently reported by Felby132 and Chandra[133] as they have demonstrated that the physical properties of linerboard can be improved when exposed to laccase without a chemical mediator. In addition, xxx has shown that the addition of laccase to the whitewater of the papermachine has several benefits for the removal of colloidal materials. Finally, this research program has presented important features on the delignification chemistry of LMS_{NHA} and LMS_{VA} that, in the opinion of the author, are momentous contributions to the overall LMS chemistry/biochemistry knowledge base which will continue to have future benefits.

Appendix 1: Industry Reporting of Mill Designed Biobleaching Technologies

The results of this research program have been presented at several DOE/AFPA industry review panels, invited company visits, TAPPI conferences and in formal publications. List below is a summary of these accomplishments.

PUBLICATIONS:

1. Biobleaching chemistry of laccase-mediator systems on high lignin content kraft pulps, Chakar, F.S.; Ragauskas, A.J., accepted for publication in Can. J. Chem.
2. Evaluating Laccase-Facilitated Coupling of Phenolic Acids to High-Yield Kraft Pulps. Chandra, R.P., Ragauskas, A.J., Enzyme Microbiol. Techn., 30(7) 855-861 (2002).
3. Delving into the Fundamental LMS Delignification of High-Kappa Pulps. Chandra, R.P., Chakar, F.S., Allison, A., Kim, D.H., Ragauskas, A.J. Elder, T.J., Biotechnology in the Pulp and Paper Industry, 151-164 (2002).
4. Formation of Quinonoid Structures in Laccase-Mediator Reactions. Chakar, F.S., Ragauskas, A.J. In ACS Series Fundamentals and Catalysis of Oxidative Delignification Processes. In Oxidative Delignification Chemistry. Fundamentals and Catalysis. Ed. Argyropoulos, D.A., ACS Symposium Series, Oxford University Press, Washington, 785, 130 (2001).
5. Laccase N-Hydroxybenzotriazole Full-Sequence Bleaching with Hydrogen Peroxide and Chlorine Dioxide. Sealey, J.E., Runge, T.M., Ragauskas, A.J., TAPPI J., 83(9), 66(2000).
6. The Kismet of Residual Lignins During LMS Delignification of High-Kappa Kraft Pulps. Chakar, F.S., Ragauskas, A.J., Holzforschung, 54, 647(2000).
7. The Effects of Oxidative Alkaline Extraction Stages after $Laccase_{HBT}$ and $Laccase_{NHAA}$ Treatments – An NMR Study of Residual Lignin. Chakar, F.S.; Ragauskas, A.J., J. Wood Chem. Technol., 20(2), 169(2000).
8. Investigations into Laccase-Mediator Delignification of Kraft Pulps. Sealey, J.; Ragauskas, A.J.; Elder, T.J., Holzforschung, 53, 498(1999).

CONFERENCE PROCEEDINGS:

1. Nano-Biotechnology Changing the Challenge in Pulp & Paper Research, Ragauskas, A.J. TAPPI Fall Technical Conference: Engineering, Pulping & PCE&I, Oct., Chicago, IL.(2003).
2. LMS Biobleaching Studies, Allison, L.; Ragauskas, A.J., TAPPI Pulping Conference, San Diego, CA(Sept. 2002).
3. Laccase: The Renegade of Fiber Modification. Chandra, R.; Ragauskas, A.J., TAPPI Pulping Conference, Seattle, WA(Nov. 2001).
4. Biotechnology in the Pulp and Paper Industry: A Challenge for Change. Ragauskas, A.J., 8th International Conference on Biotechnology in the Pulp and Paper Industry, Helsinki, Finland O/2(June 2001).
5. Delving into the Fundamental LMS Delignification of High Kappa Kraft Pulps, Chandra, R.P.; Chakar, F.S.; Allison, L.; Kim, D.H.; Ragauskas, A.J., Elder, T., 8th International Conference on Biotechnology in the Pulp and Paper Industry, Helsinki, Finland, 54(June, 2001).
6. Elucidating the Effect of Laccase on the Physical Properties of High Kappa Kraft Pulps. Chandra, R.P.; Ragauskas, A.J., 8th International Conference on Biotechnology in the Pulp and Paper Industry, Helsinki, Finland, 255 (June 2001).
7. Extending the Limits of Oxygen Delignification. Chakar, F.S.; Lucia, L.; Ragauskas, A.J., 2000 International Pulp Bleaching Conference, NS, Canada, 123(June 2000).
8. The Path Forward to Practical Nascent Laccase Biobleaching Technologies. Chakar, F.S.; Allison, L.; Kim, D.H.; Ragauskas, A.J., TAPPI Pulping Conference, Boston, MA (Nov. 2000).
9. The Challenge of Change. Ferris, J.; Ragauskas, A.J., TAPPI Pulping Conference, Boston, MA (Nov. 2000).
10. Laccase-Lignin Interactions, Chakar, F.S.; Ragauskas, A.J., 6th European Workshop on Lignocellulosics and Pulp – Advances in Lignocellulosics Chemistry Towards High Quality Processes and Products, Bordeaux, France, 53(Sept. 2000).

11. Laccase-Mediator Systems and Oxygen Delignification-A Comparative Study. Chakar, F.S.; Ragauskas, A.J.; McDonough, T.J., 2000 International Pulp Bleaching Conference, Halifax, NS, 59(June, 2000).
12. Fundamental Investigations of Laccase Mediator Delignification on High Lignin Content Kraft Pulps. Chakar, F.S.; Ragauskas, A.J., 10th International Symposium on Wood and Pulping Chemistry, Yokohama, Japan, 566(June 1999).

PRESENTATIONS:

1. Redefining The Pulp and Paper Industry with New Chemo-Enzymatic Technologies. Ragauskas, A.J., Gordon Polysaccharide Conference, Italy (May, 2003).
2. Laccase: An Ancilla to Kraft Pulping. Dyer, T.; Kim, D.; Ragauskas, A.J., 225th ACS National Meeting, New Orleans, LA (2002).
3. Invigorating High Kappa Kraft Pulps with Laccase: Chandra, R.P; Ragauskas, A.J. 225th ACS National Meeting, New Orleans, LA (2002).
4. Chemoenzymatic Fiber Modification, Asian Institute of Technology and Department of Forest Products, Kasetsart University, Thailand (2002).
5. Decade of Pulp and Paper Research, Kaunas University of Technology, Lithuania (2002).
6. Biotechnology in the Pulp and Paper Industry: A Challenge for Change. Ragauskas, A.J., 8th International Conference on Biotechnology in the Pulp and Paper Industry, Helsinki, Finland (June 2001).
7. Fundamental Delignification Chemistry of Laccase-Mediator Systems on High-Lignin Content Kraft Pulps-A Synopsis of Contributions. Chakar, F.S.; Ragauskas, A.J., 125th ACS National Meeting, San Diego, CA(2001).
8. Laccase-Lignin Oxidative Chemistry. Ragauskas, A.J.; Allison, L.; Chakar, F.S., International Chemical Congress of Pacific Basin Societies, Honolulu, HI (2000).
9. Parsing Laccase's Effect on Modifying Lignin. Chandra, R.; Ragauskas, A.J., International Chemical Congress of Pacific Basin Societies, Honolulu, HI (2000).
10. Structural Enhancement of Laccase-Lignin Reactions. Chakar, F.S., Ragauskas, A.J., 219th ACS National Meeting, San Francisco, CA (2000).

11. Insight into Laccase-Mediator Delignification of Softwood Kraft Pulps. Chakar, F.S.; Ragauskas, A.J., 1999 217th ACS National Meeting, Anaheim, CA (1999).

INVITED INDUSTRY SPEAKER

- Pulp/Bleach Mill of the Future. Innovase hosted Mini-Symposium, San Diego, CA(2001).
- Developing New Pulp Fibers. Kimberly-Clark Corporation, Neenah, WI (2000).
- Fundamentals of Laccase Mediator System Delignification. Hercules Incorporated, Wilmington, DE (2000).
- Laccase Biobleaching Technologies. International Paper, Tuxedo Park, NY (2000).
- Laccase Biobleaching. IPST Industrial Consortium Program review (2000).

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