FUNDAMENTAL INVESTIGATION INTO OXIDOREDUCTASE ENZYMATIC BLEACHING SYSTEMS

A Dissertation Submitted by

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in partial fulfillment of the requirements of the
Institute of Paper Science and Technology
for the degree of Doctor of Philosophy
Atlanta, Georgia

March 12, 1998

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LIST OF SYMBOLS

Mediator Symbols

ABTS	2,2' azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
BT	1-H benzotriazole
HBT	N-hydroxybenzotriazole
HTI	N-hydroxypthalimide
NHAA	N-acetyl-N-phenylhydroxylamine
VIO	violuric acid

Bleaching Stages

D	chlorine dioxide
E	alkaline extraction
E+O	alkaline extraction with oxygen reinforcement
E+O+P	alkaline extraction with oxygen and hydrogen peroxide reinforcement
L	laccase with a mediator biobleaching stage
LMS	laccase mediator biobleaching stage with an alkaline extraction
P	hydrogen peroxide

Kraft Pulp Types

WSBK25	western softwood brownstock pulp with a kappa number of 25
WSOK17	western softwood oxygen-delignified pulp with a kappa number of 17
SSBK26	southern softwood brownstock pulp with a kappa number of 26
SSOK13	southern softwood oxygen-delignified pulp with a kappa number of 13

SHBK15	southern hardwood brownstock pulp with a kappa number of 15
SHOK10	southern softwood oxygen-delignified pulp with a kappa number of 10
o.d.	oven dry

Equipment

EPR	electron spin resonance
NMR	nuclear magnetic resonance
GC	gas chromatography
GC/MS	gas chromatography with a mass spectrometer
FTIR	fourier transform infrared spectrometry
LC	liquid chromatography
TLC	thin layer chromatography
UV	ultraviolet spectrometry

Common Chemicals

DMSO	dimethyl sulphoxide
THF	tetrahydrofuran

EXECUTIVE SUMMARY

Biobleaching has been studied for many years with little success. Enzymatic bleaching systems that incorporate a mediator or a small molecular weight compound have shown promise. Of these, laccase and manganese peroxidase-based biobleaching systems have been the two most promising. The mediator's role in delignification has been proposed to be radical based with the mediator being activated by the enzyme. The purpose of this study is to examine the mechanism of laccase mediator biobleaching.

Residual lignins isolated from kraft pulps bleached with laccase hydroxybenzotriazole (HBT) were shown to be structurally altered. Oxidation appeared to be the main delignification mechanism, as indicated by measured increases in carboxylic acid groups and decreases in free phenolic groups. Demethylation of the residual lignins was also detected.

Reactions between laccase and HBT revealed a new species which was determined to be benzotriazole (BT). Since laccase BT biobleaching trials revealed no delignification, the generation of BT was concluded to be a negative by-product of the reaction. Further investigations revealed that HBT was converted to BT rapidly during laccase biobleaching, and delignification appeared to be greatly limited by the conversion of HBT to BT.

Derivatives of HBT and closely related compounds were tested as possible mediators, and the RR'N-OH functional group was needed to facilitate delignification for the class of compounds tested. Bond dissociation energies and the polar character of the mediators appear to influence reactivity of the laccase mediator biobleaching system.

Full-sequence bleaching with laccase HBT was performed, and an ISO brightness of 85 was obtained with an OL(E+O+P)LED sequence. Recently introduced mediators, violuric acid (VIO) and N-acetyl-N-phenylhydroxylamine (NHAA), were also tested and shown to delignify kraft pulps with a much lower dose of enzyme than HBT. The selectivity of all the biobleaching systems remained high.

The radical mechanism proposed for laccase mediator delignification was tested with a selective radical quenching catalyst in the presence of pulp. Results were positive, as delignification was almost completely inhibited. Almost no laccase activity remained after a 4-hour biobleaching trial regardless of the mediator used. Further investigation of laccase mediator delignification revealed that lignin was modified differently, depending on the mediator used.

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INTRODUCTION

TRENDS

The pulp and paper industry has gone through a dramatic period of change in recent years; and the last 20 years can even be considered revolutionary. Historically, the pulp and paper industry has been slow to change because of large capital costs associated with even minor changes because of the industry's large-scale operations. Environmental pressures have been the leading cause for this period of change, spawning increased interest in research. A large portion of this research has been dedicated to exploring alternative bleaching technologies that eliminate the use of chlorine. Many of the bleaching technologies that are used today would have attracted very little interest if environmental concerns were not considered (1). In the future, the pulp and paper industry may face even greater changes because environmental pressures have continued to increase dispite the numerous advances that the pulp and paper industry has already achieved.

One strong example of recent changes is the elimination of chlorine bleaching in Sweden, where Cl_2 use went from 150,000 metric tons per year in 1987 to zero in 1993 (1). Other examples include: the rapid growth in demand for elemental chlorine-free (ECF) and totally chlorine-free (TCF) products, the sudden appearance of seven commercial ozone bleach plants in 1993, and the virtual elimination of dioxins in North American mill effluents (1). Pulping operations have also changed with the introduction of Rapid Displacement Heating (RDH) and Extended Modified Continuous Cooking (EMCC®). The pulp and bleach plants are now considered integrated because changes in both areas are used to achieve the same main goal:

"reduced mill discharges." All aspects of effluents are being reduced, which include chlorinated organics, organics, and the total volume of water.

While the driving forces behind technological changes are government regulations, recent market demands have also increased. The new "Cluster Rules," which are recently proposed guidelines for regulating pulp mill emissions, have greatly increased interest in research because numerous mills throughout the United States do not meet the proposed standards and will have to shut down if the Cluster Rules are implemented in 1998 (2,3). The Cluster Rules are a function of political pressures and scientific data. The big difference between the Cluster Rules and previous legislature is the systematic limitation on air, water, and solid waste for each group of industrial operations. The combined restrictions make it difficult for many of the older mills to comply because they are based on best industrial practices which are achieved by new capital-intensive technology (1).

General guidelines on bleach plant effluents for paper grade kraft mills have been published (1). These apply to specific compounds, and several compounds are limited to detectable limits. These limits continue to change and create an increasingly difficult compliance issue. The proposed EPA regulations for the entire mill's effluent are also published (1). These govern absorbable organic halide (AOX), biochemical oxygen demand (BOD), total suspended solids (TSS), chemical oxygen demand (COD), and color. Compliance with these regulations may require extended delignification pulping, oxygen delignification, and perhaps an efficient secondary biological treatment system (1). The bleach plant would also change with the elimination of elemental chlorine stages, while ClO_2 use may be greatly limited in the long term.

All of these changes may increase production cost or may only be implemented with a high capital investment.

Extended kraft delignification is an attractive means to reduce bleach plant discharges because it can lower the residual lignin content while producing pulp with higher strength properties than conventional continuous or batch systems. If the EMCC principles are followed closely, softwood pulp with kappa numbers in the 20's can be obtained with high pulp yields and strength properties compared to conventional cooks.

Oxygen delignification is used to remove a substantial percentage of residual lignin in unbleached pulp. This lignin can then be burned in the recovery furnace and the pulp that enters the bleach plant has a reduced residual lignin content which requires less bleaching chemical to obtain a target brightness level (1). Oxygen delignification removes 40-60% of the residual lignin, which lessens the bleach plant's environmental impact while reducing chemical cost in the bleach plant. Current technology has moved to high consistencies (20-30%) or a two-stage medium-consistency (10-15%) system that can remove up to 70% of the residual lignin for softwood pulps. Oxygen delignification has become increasingly important in recent years, and any greenfield mill built today would incorporate oxygen delignification (4).

ENZYME APPLICATIONS

Enzyme bleaching studies have been performed for many years (5). Most studies were performed with hemicellulases or enzymes produced by white rot fungi. Hemicellulases were introduced because several researchers believed that if the hemicellulose bond could be selectively broken, the lignin-hemicellulose fragment could be removed from the pulp by

alkaline extraction (5,6). Hemicellulase enzymes were the first enzymes used in mill-scale trials, and a large amount of research has been dedicated to optimizing their use and understanding their reaction mechanisms.

Xylanases are the principle hemicellulase enzymes used to treat kraft pulps. While they have no real capacity to delignify kraft pulp, xylanases can significantly lower the amount of bleaching chemicals needed in succeeding bleaching stages (5-10). The mechanisms of this reaction are complex and involve the structure of the hemicellulose-lignin-cellulose composite formed during kraft pulping. During the heating stage in a kraft cook when the alkali concentration is high, some of the xylan is dissolved in the pulping liquor. When the pH of the pulping liquor falls, some of degraded xylan precipitates on the fiber. Apparently, the degraded xylan can form a crystalline-like structure as a result of the removal of glucuronic acid and arabinose units (8). The degraded xylan also forms a tightly bonded structure with the cellulose fiber, and co-crystalline structures may form. During pulping, the lignin also undergoes numerous condensation reactions which may introduce cellulose-lignin, new hemicellulose-lignin, and new lignin-lignin chemical bonds (11-13). The xylanase may hydrolyze the reprecipitated and relocated xylans on the surface of the pulp fiber and make the fiber more permeable. This increased permeability increases the amount of lignin and lignin-carbohydrate molecules that can be removed with subsequent chemical extractions. This is concluded because only 20% of the xylan in birch and pine kraft pulps is hydrolyzed with xylanase treatments regardless of xylanase dose (8).

A number of reasons are cited for the use of xylanase treatments on kraft pulps (10,14-17). If xylanase is used, the amount of AOX in bleach plant effluents may be reduced. This can

be obtained because up to 25% reduction in the consumption of active chlorine can be achieved (with a softwood DEDED sequence) while maintaining the same brightness level (8). The kappa factor (ratio of active chlorine charge to kappa number) is also decreased from 0.165 to 0.125 for a softwood D(E+O)D(E+P)D sequence (1). This reduction in bleaching chemicals can lower costs, especially in ECF and TCF bleaching operations. Higher brightness ceilings or better strength properties at a given brightness can also be achieved with xylanase treatments (for TCF pulps). Flexibility is also cited as a reason for the use of xylanase pretreatments. This refers to the ability of some mills to meet environmental regulations without introducing capital-intensive technologies such as ozone, oxygen, and extended pulping.

The advantages of xylanase treatment of kraft pulps mentioned above and the simplicity of adding an enzyme treatment into the bleaching sequence has led to mill trials and continued use in several mills (14-16). The xylanases used in these large-scale trials are produced by generations of selection or genetically engineered organisms which lack the capability of producing cellulases (5). This has led to the introduction of several suppliers of xylanases using trade names such as Ecopulp X-100, Albazyme-10, and several others (5,14,15). One example of xylanase treatment of kraft pulps is performed by acidifying the pulp with sulfuric acid to a pH 6, and enzyme treatment of 0.25 kg/ton of dry pulp for a reaction time of 30 minutes. The acid and enzyme is added directly into the brownstock decker.

Recent studies have examined the use of mannanase and compared it to xylanase treatments and mixed mannanase and xylanase treatments on softwood kraft pulps (18). It was found that mannanase acted differently than xylanase, and performed significantly better with a succeeding hydrogen peroxide delignification stage than a chlorine dioxide stage while xylanase

performed well with both. Performance was rated by the increase in delignification obtained when using the enzyme treatment. This selectivity of mannanase-treated pulp for peroxide was explained by the decreased ability of peroxide to degrade lignin as compared to chlorine dioxide; in addition, the degradation of mannan chains, which may be attached to lignin by a galactose side group, could produce a lignin carbohydrate compound that is soluble in an alkaline solution. It was also reported that mannan and xylan are not located similarly on the surface of the fiber (18). No strong synergistic effects with a combined mannanase and xylanase treatment were detected, but additive effects were seen.

The introduction of xylanase into TCF bleaching sequences has become increasingly reported in the literature (1,5,19,20). The use of xylanase with ozone bleaching has allowed a decrease in the ozone charge while increasing the brightness ceiling and pulp viscosity (19,20). For many years, low pulp viscosity with TCF bleaching sequences that use ozone have caused concern, but pulp viscosity can not always be closely compared to paper properties (20).

Hemicellulases, which may be effective in pulp activation, have severe limitations in their ability to delignify because they lack the ability to degrade lignin. Enzymes that are isolated from white rot fungi have been studied for many years, and they belong to a special class of oxidative enzymes that have the ability to degrade lignin (1,21). Difficulties in isolating active enzymes, low reaction rates, and low activities of these isolated enzymes have posed challenges for researchers in this field. A dispute over the enzyme's ability to degrade lignin has led to more obstacles that have hindered the development of an enzymatic delignification system. Recent studies with a laccase-mediator system have shown delignification of kraft pulp as high as 67%, and these studies have led to increased interest in oxidative enzymes (1).

LITERATURE REVIEW

ENZYMES

OVERVIEW

Enzymes are specialized molecules, usually proteins, that are produced by living organisms to catalyze metabolic reactions (22). Typically, enzymes cause reactions between organic molecules that have very low to no reactivity outside of the living cell. A good example of this is the reaction between glucose and oxygen (22). Glucose and oxygen react in the presence of enzymes to form carbon dioxide, water, and energy. The enzyme lowers the activation energy of the reaction, and this allows the reaction to occur at a much higher rate.

Enzyme nomenclature is often confusing, with some enzymes named after the substrate upon which they act, this is done by adding *-ase* as a suffix, but some enzymes are named without regard to their activity. Because of this confusion, an International Commission on Enzymes was established in 1956, and this commission created a systematic basis for enzyme nomenclature. While common names remain in use today, each enzyme can be classified with this system. Six classes of reactions are recognized (Table 1). Each class is broken into subclasses and each subclass has sub-subclasses in which each individual enzyme has been placed. A numbering system is used to represent classes, subclasses, sub-subclasses, and individual enzymes. This produces a four-digit number system that is used to specify a particular enzyme.

Table 1 Systematic classification (class and subclass) of enzymes according to the enzyme commission (22).

E.C. Number	Systematic Name and Subclasses
1	Oxidoreductases (oxidation-reduction reactions)
1.1	Acting on CH-OH group of donors
1.1.1	with NAD or NADP as acceptor
1.1.3	with O_2 as acceptor
1.2	Acting on the $R_2C=O$ group of donors
1.2.3	with O_2 as acceptor
1.3	Acting on the CH-CH group of donors
1.3.1	with NAD or NADP as acceptor
2	Transferases (transfer of functional groups)
2.1	transferring C-1 groups
2.1.1	Methyltransferases
2.1.2	Hydroxymethyltransferases and Formyltransferases
2.1.3	Carboxyltransferases and Carbamoyltransferases
2.2	Transferring aldehydic or ketonic residues
2.3	Acyltransferases
2.4	Glycosyltransferases
2.6	Transferring N-containing groups
2.6.1	Aminotransferases
2.7	Transferring P-containing groups
2.7.1	With an alcohol group as acceptor
3	Hydrolases (hydrolysis reactions)
3.1	Cleaving ester linkage
3.1.1	Carboxylic ester hydrolases
3.1.3	Phosphoric monoester hydrolases
3.1.4	Phosphoric diester hydrolases
4	Lyases (addition to double bonds)
4.1	C=C lyases
4.1.1	Carboxy lyases
4.1.2	Aldehyde lyases
4.2	C=O lyases
4.2.1	Hydrolases
4.3	C=N lyases
4.3.1	Ammonia-lyases
5	Isomerases (isomerization reactions)
5.1	Racemases and epimerases
5.1.3	Acting on carbohydrates
5.2	Cis-trans isomerases
6	Ligases (formation of bonds with ATP cleavage)
6.1	Forming C-O bonds
6.1.1	Amino acid-RNA ligases
6.2	Forming C-S bonds
6.3	Forming C-N bonds
6.4	Forming C-C bonds
6.4.1	Carboxylases

One of the most important roles enzymes play in living organisms is regulation. The presence or absence of enzymes can stimulate or inhibit metabolic reactions. This enables the cell to perform numerous reactions depending on the conditions. The ability to regulate is also obtained by the specificity of the enzyme which can also affect catalytic power.

FUNDAMENTAL CONCEPTS

Specificity is the ability of the enzyme to catalyze reactions with a limited group of substrates. This specificity is believed to be due to its (3-D) structure (22). An enzyme molecule is typically several orders of magnitude larger than its substrate. Its active site is only a small portion of the overall enzyme structure. The active site of the enzyme is arranged to create a special pocket, or cleft, whose three-dimensional structure is complementary to the structure of the substrate. This allows the enzyme and the substrate to "recognize" each other, which produces the selectivity of the system (22). The substrates bond to the active site with weak forces: H-bonding, ionic bonds, and van der Waals interactions. The strength of these interactions can greatly affect the kinetics of the reactions catalyzed by the enzyme (22).

This interaction between the substrate and the active site led to the "Lock and Key" hypothesis presented by organic chemist Emil Fischer. Other hypotheses such as "Induced Fit," were introduced later (22). "Induced fit" describes the ability of the enzyme to change its shape. The shape of the active site changes when the proper substrate interacts and bonds to it. This allows the substrate and the protein to "fit" each other more precisely (22).

The specificity controls the activity of the enzyme. Both hypotheses can be used to explain how reactions with smaller substrate molecules with the same reactive sites as larger molecules are not catalyzed by enzymes that catalyze reactions with the larger molecules. The active sites on an enzyme can become poisoned if molecules bond tightly to them, preventing substrate molecules to come in contact with the enzyme's active site (22).

Since enzymes are molecules that are produced by living organisms, the conditions of the surrounding environment can greatly affect their activity (22). Solution conditions

(concentration of substrates and ionic strength) pH, and temperature are factors that can greatly effect the activity of enzymes. While optimal solution conditions are specific to the particular enzyme, some enzymes remain active over a wide range of solution conditions, and some enzymes even remain active in organic solvents (22).

In many situations, the actual molar amount of the enzyme is not known, so the amount of enzymes is expressed in terms of activity. One generally accepted unit that defines the activity of the enzyme is the katal. A katal is the amount of enzyme catalyzing the conversion of one mole of substrate to product in one second (22). The turnover number, or k_{cat}, is defined as the number of substrate molecules converted into products per enzyme molecule per unit time when the enzyme is saturated with substrate. The turnover number is also referred to as the molecular activity. A full review of these terms is beyond the scope of this discussion, but it is important to note that several solution factors can greatly affect the kinetics of the enzyme-catalyzed reactions.

Coenzymes are nonprotein components that contribute to the catalytic function of the enzymes. They are made up of organic molecules or metal ions, and not all enzymes use them. The coenzymes are structurally less complex than proteins, and they tend to be stable to heat. Usually the coenzymes are actively involved in the catalytic reactions with the enzymes, often serving as intermediate carriers of functional groups in the conversion of substrates to products (22). In many cases the coenzyme is closely associated or even covalently bonded to the enzyme, and it is difficult to separate the two.

Enzyme technology is rapidly increasing with many products on the market today that were not available a few years ago. Generally, commercial enzymes that are sold for specific

applications are produced by generations of selection or by genetically engineered organisms that can be manipulated to produce the particular enzyme of interest. Enzyme isolation can be very difficult, because most isolation procedures recover enzyme solutions that are often contaminated with other molecules. Specific enzymes can also vary in the number of amino acid groups used to make up the protein molecule. The number of atoms in the active site can also vary which make-up difference types of specific enzymes. This can make it extremely difficult to determine exactly which molecules are present.

WHITE ROT FUNGI

BLEACHING

White rot fungi are comprised of a group of basidiomycetes species that have the ability to use hemicelluloses as a food source while degrading lignin (23-25). In this process, the dark color of the lignin is reduced, and the biomass becomes lighter in color which gives these fungi the name white rot fungi. While some bacteria have the ability to degrade lignin, most researchers believe they play a role secondary to that of fungi (24). Numerous white rot fungi have been studied for their ability to degrade native, synthetic, and industrial lignins (26). Some of the fungi species are *Phanerochaete chrysosporium*, *Polyporus versicolor, Polyporus zonatus, Phellinus igniarius,* and many others.

The lignin degradation mechanisms of white rot fungi are still unclear after many years of research (6). Most studies concluded that the extracellular enzymes play an important role, but

after isolation of these enzymes, the ability to polymerize and depolymerize lignin is detected with polymerization being favored in numerous conditions (6,23-26). These enzymes will be discussed in more detail later, but it is important to note that many enzymatic bleaching studies use enzymes that are isolated from white rot fungi or are produced by white rot fungi.

White rot fungi are believed to have three modes of degradative reaction on lignin:

1. Oxidative cleavage of propyl side chains between α- and β-carbons leading to the formation of benzoic acids.
2. Cleavage of β-aryl ether bonds and modification of the side chain structures.
3. Degradation of aromatic nuclei through oxidative ring opening.

Cheng and Chang also suggest that degradation of the lignin proceeds partly through a combination of reductive and oxidative reactions (23). While these studies reveal possible depolymerization mechanisms, recent studies reveal a mixture of reactions including polymerization (26).

Kantelinen et al. performed a study on how the white rot fungus *Phlebia radiata* reacts with native and industrial lignins (26). When black liquor lignin was treated with this fungus, polymerization occurred after a two-week cultivation time, and then some degradation occurred over the next three weeks, producing a more uniform lignin molecular weight distribution while slightly increasing the amount of insoluble lignin (Figure 1). No significant changes in the water soluble lignin was detected. If the same fungi was used to treat wood powdered lignin, which

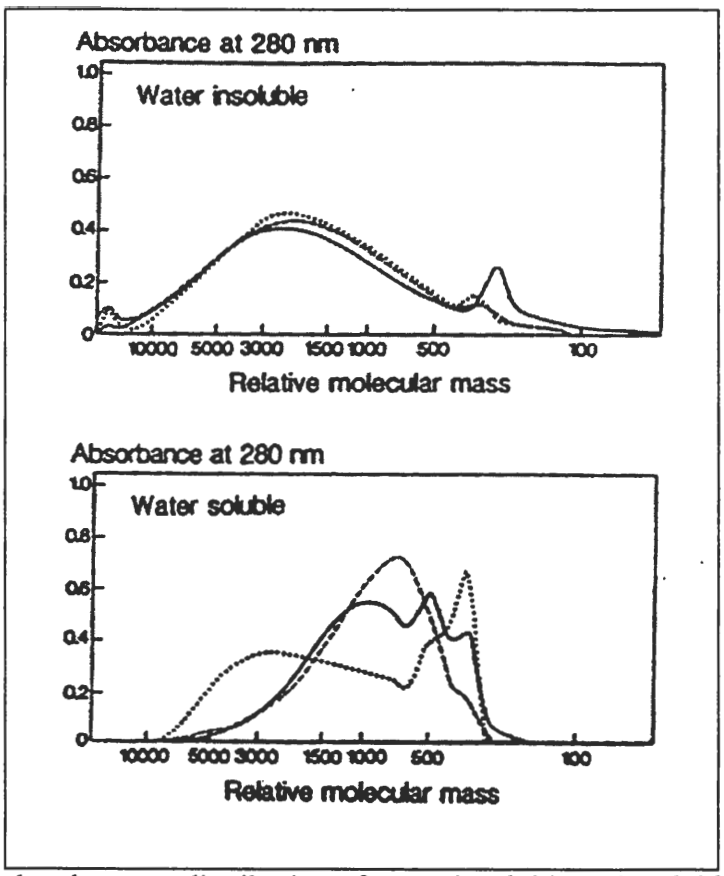


Figure 1 Relative molecular mass distribution of water-insoluble and -soluble fractions of black liquor lignin reference; ····· 2 weeks cultivation; ------- 5 weeks cultivation (26).

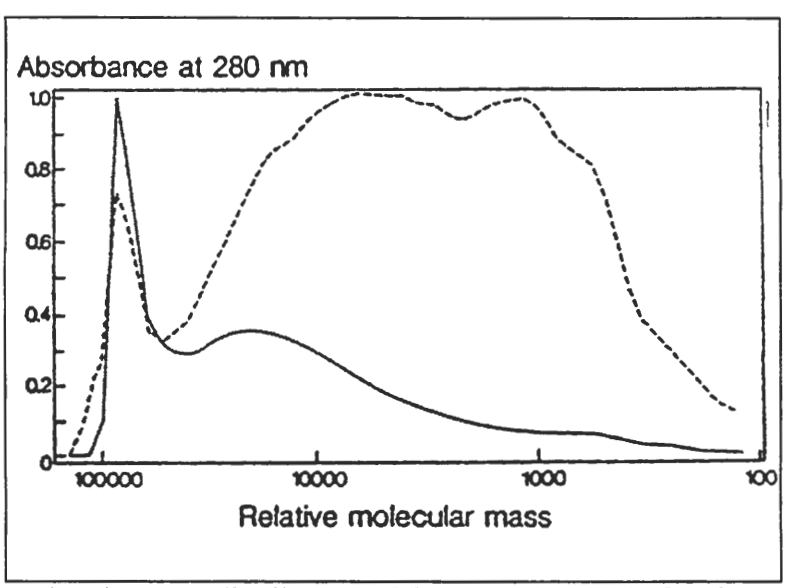


Figure 2 Relative molecular mass distribution of the water-insoluble fraction of wood powder lignin: reference, ------ 2 weeks cultivation (26).

was used to represent the native lignin, a two-week cultivation period greatly reduced the average molecular weight of the lignin (note: almost all of this lignin was water insoluble) (Figure 2). Prolonged treatment reduced the average molecular weight even further (26).

Two main directions are pursued in white rot bleaching studies: treatment of the chips before pulping or treatment of the pulped fiber (27,28). White rot fungi are studied with chips before mechanical pulping where they significantly reduce the energy required to pulp chips with only a few days of treatment. Control of the process (selectivity) and scale-up problems inhibit its use on an industrial scale. Biopulping has been performed with highly selective strains of white rot fungi *Phanerochaete chrysosporium* (selectivity for lignin with little degradation of cellulose polymer), but poor results were observed for woody species while better results were reported for grass biomass. These results have led to increased research with kraft or other pulped fiber since direct biopulping of the fibers or chips appears to face several technological barriers other than reaction rates (28).

Direct treatment of kraft pulps with white rot fungi has different degrees of success depending on the species of the white rot fungi and the wood species (29,30). After testing several white rot species, Paice et al. concluded that *Coriolus versicolor* (strain #052) produced the brightest pulp in a five-day treatment of hardwood pulp (29). The pulp brightness increases 15 points while the kappa number decreased from 11.6 to 7.9. Handsheet strength properties increased while pulp viscosity dropped significantly. Most of the brightness gains occurred during the second day of the reaction, after the characteristic lag time during the first day. A bleaching sequence with a fungi stage (F) with a DED sequence produced fiber with an 82% ISO

brightness while a CEDED sequence produced fiber with an 88% ISO brightness for the same hardwood pulp (29).

Softwood kraft pulps performed differently than hardwood pulp with less gains in brightness and kappa number. *Trametes versicolor* was found to perform better than other fungi species (29,30). The softwood pulps seem to require a longer lag time than the hardwood pulps. A recent study reported the use of an IZU-154 white rot fungi strain, which out-performed other fungi (Figure 3)(31). This study was directed to develop TCF bleaching sequences. A five-day fungus treatment (F) with a 2% NaOH extraction (E) stage and then a 4% H_2O_2 stage (P) produced an 87.3% ISO brightness of an oxygen delignified hardwood pulp (31). This OFEP bleaching sequence is comparable to conventional OCED bleaching sequences (if the five day reaction time is overlooked).

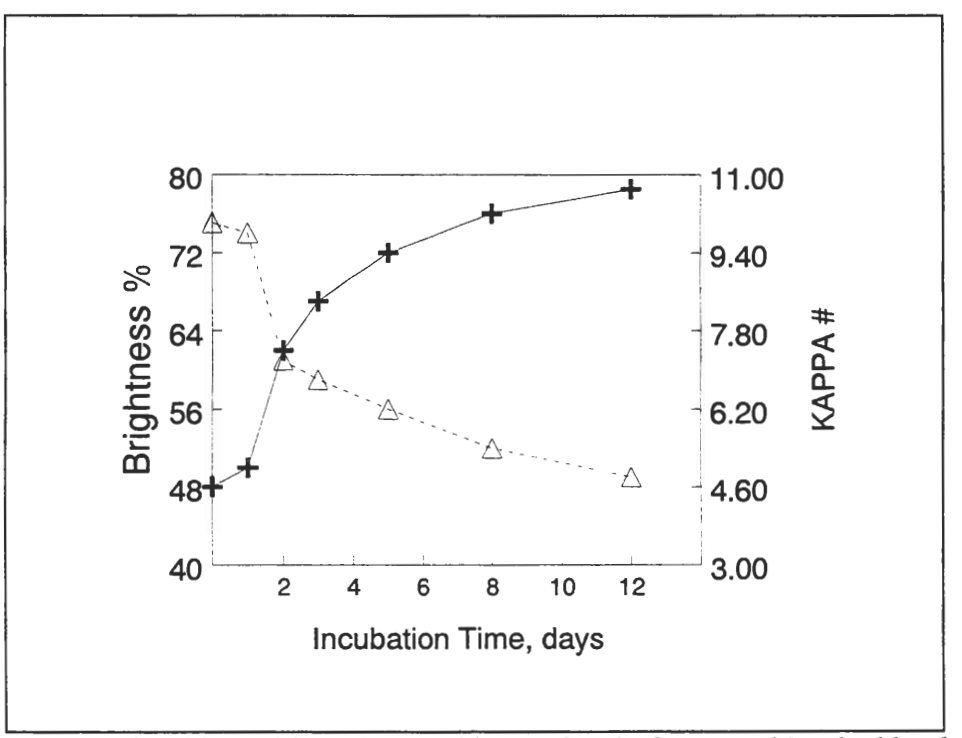


Figure 3 Biobleaching with IZU-154 white rot fungi of oxygen-bleached hardwood kraft pulp (Δ) kappa number and (+) brightness (31).

Immobilized fungi have also been shown to be effective in fungi bleaching of kraft pulps (30). Immobilized T. *versicolor* in polyurethane foam performed similarly to free fungi. This shows that intimate contact between the fungi and the pulp fiber is not needed for lignin degradation. This technique also allows the fungus to be removed from the pulp after treatment, and it can be reused with another batch (30). This data implies that extracellular enzymes may be the active agents in lignin degradation.

OXIDOREDUCTASES

Enzyme isolation and characterization studies have been performed in search of the active agents in lignin degradation of the white rot fungi (24). The enzymes that seemed to be involved in the lignin degradation were the aromatic alcohol dehydrogenases, phenol oxidases, and mono- and di-oxygenases. Most of the recent studies has been dedicated to the phenol oxidases: lignin peroxidase, manganese peroxidase, and laccase (6,24-26). These enzymes belong to the oxidoreductase class of enzymes.

Oxidoreductases are active in biological oxidation and reduction, and function in respiration and fermentation processes (Table 1)(32). This class of enzymes includes dehydrogenases, oxidases, and peroxidases, which use H_2O_2 as an oxidant; dehydroxylases, which introduce hydroxyl groups; and oxygenases, which add molecular O_2 to a double bond in the substrate (33).

Since phenoloxidases were first found in 1928 by Bevendamm, numerous studies have been performed to isolate and determine the function of each, but confusion still exist over their

function (6,33). It is generally accepted that they remove one electron from phenolic hydroxyl groups. The lignin peroxidases and manganese peroxidases are considered relatively "new" enzymes since they were not isolated until the early 1980's.

PEROXIDASE BLEACHING

Numerous lignin peroxidase and manganese peroxidase bleaching studies have been performed over the last 10 years. Two general approaches have been used for studying the abilities of these enzymes to degrade lignin: experiments with the enzyme and ligno-cellulosic fiber in the presence of H_2O_2 and experiments with the enzyme and lignin model compounds or isolated lignin fragments in the presence of H_2O_2 (33). Most work has been concentrated on the latter because of the lack of information on the enzymes and general ineffectiveness in bleaching of pulps in early tests.

Isolated lignin peroxidases have molecular weight of 41,000-42,000 and are composed of 15% carbohydrates. The native enzyme has an absorption at 407 nm (33). Soon after the isolation of lignin peroxidase, a manganese-dependent lignin peroxidase was isolated with an average molecular weight of 46,000. Both peroxidases contain iron atoms in their active site, and the oxidation and reduction of these sites are believed to cause the degradation of lignin by numerous reactions (Figures 4 and 5). Both systems are dependent on the presence of H_2O_2, but excess peroxide can render the enzyme inactive.

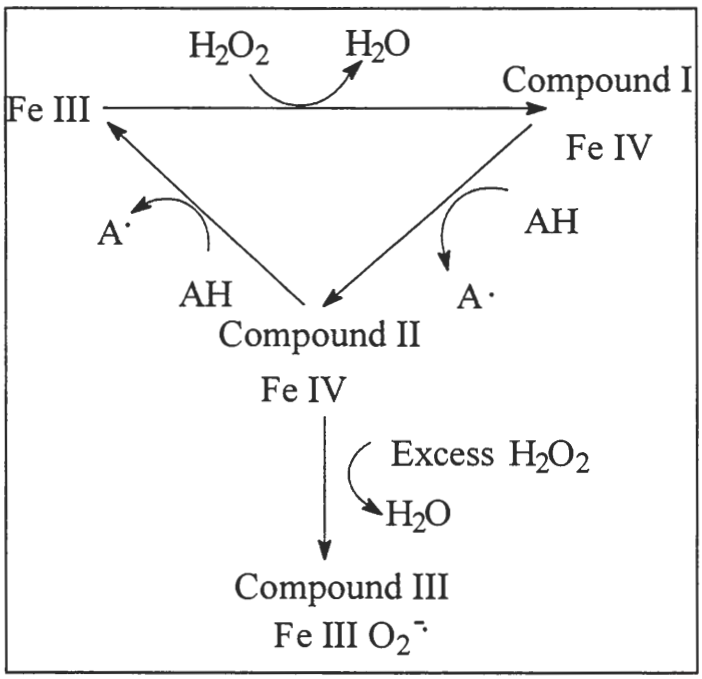


Figure 4 Catalytic cycle of lignin peroxidase in the presence of H_2O_2. (A) represents a substrate (34).

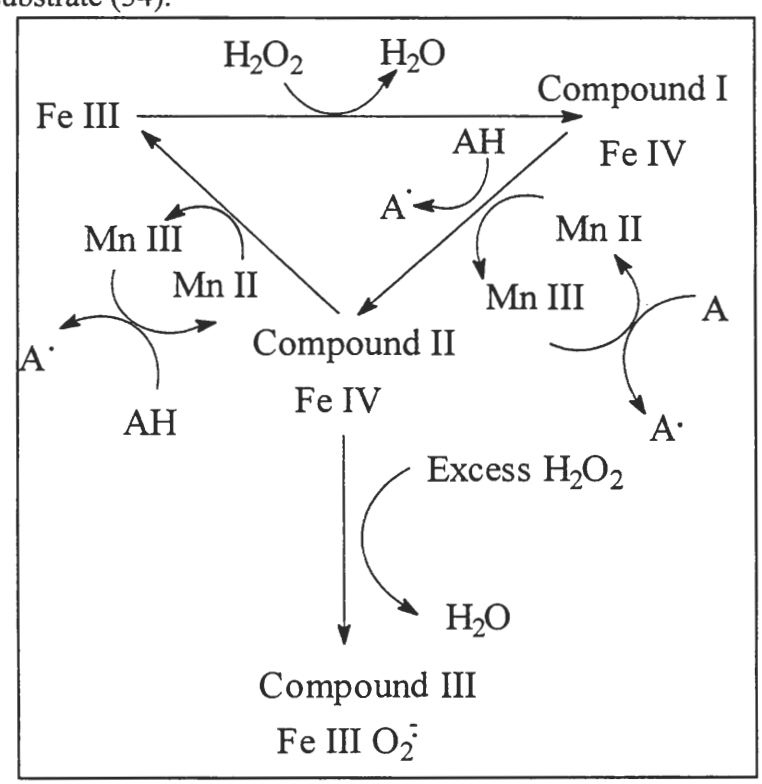


Figure 5 Catalytic cycle of manganese peroxidase in the presence of H_2O_2. (A) represents a substrate (34).

Results from model compound studies have shown that both peroxidases can degrade phenol-containing compounds and non-phenol containing compounds (23,36). Degradation of β-O-4, β-1, β-5, Cα-Cβ , β-β , and several other model compounds has been reported along with mechanisms for side chain and aromatic ring cleavages (23,37-40). Several studies are conflicting, and the ability of these enzymes to degrade some model compounds is still in dispute (23,33,37).

Early studies reported that the enzymes diffuse into the ligno-cellulosic material. Later reviews stated that this is impractical because of the physical size of the enzymes (6). This has led to the introduction of "mediator" systems where the enzyme interacts with a relatively small molecule, which can then diffuse into the pulp or wood chips. This may be the actual mechanism in white rot fungi, but these mediators may be lost during isolation of the enzyme, which could explain their absence in isolated systems (6,33).

Since lignin peroxidase catalyzes the oxidation of veratryl alcohol, a mediator system was proposed by Harvey et. al. (23,33). In this system, the H_2O_2 oxidized the active site in the ligninase which oxidized the veratryl alcohol, producing a cation radical which oxidized the lignin (Figure 6). The cation radical was not isolated, but this was attributed to the short lifetime of this species (33). Several studies reported that lignin peroxidase was not produced during the degradation of lignin by several white rot fungi species, while manganese peroxidase and laccase were detected (35,36). This has led to decreased interest in lignin peroxidases and more interest in manganese peroxidases and laccase in recent years (21,35,36).

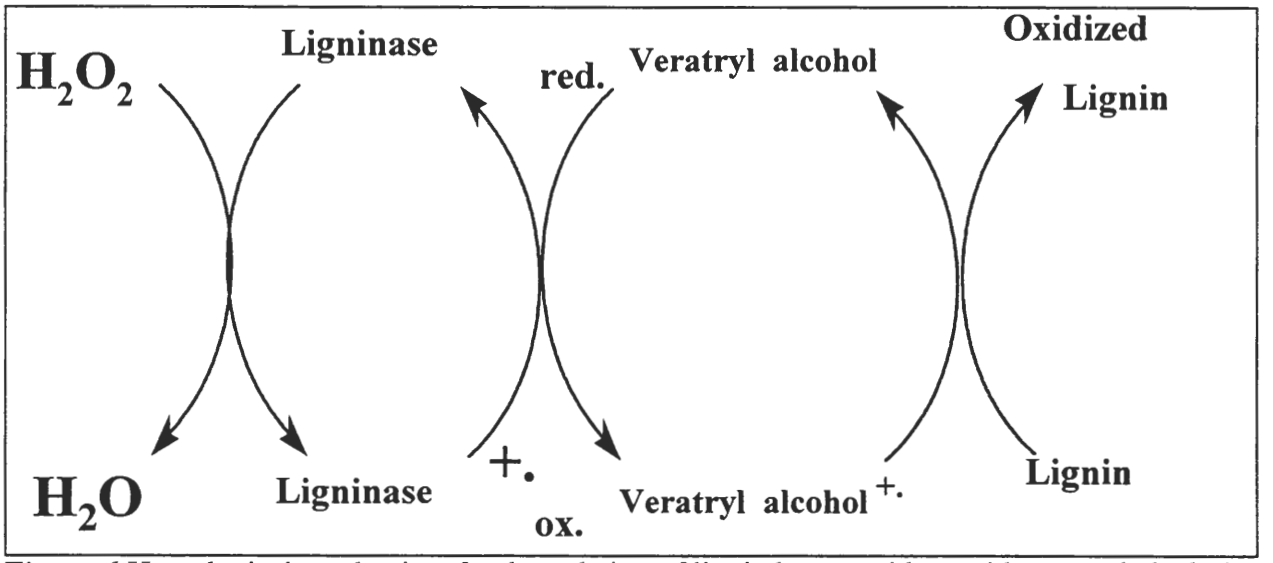


Figure 6 Hypothetical mechanism for degradation of lignin by peroxidase with veratryl alcohol as a mediator (33).

Since manganese peroxidase is secreted by many delignifying fungi during pulp bleaching, and its production peaks when peak delignification of pulp occurs with these fungi, manganese peroxidase appears to play a strong role in delignification (21). The manganese peroxidase system oxidizes Mn(II) to Mn(III) in the presence of a chelant and H_2O_2, and the chelated Mn(III) diffuses into the pulp and oxidizes the lignin. Evidence supporting this mediator system include: the isolation of naturally occurring oxalate chelants; the enzyme is inactive without the chelant or H_2O_2; and a system with active enzyme, H_2O_2, and chelant with Mn produces partially delignified pulp (21). This process is highly selective for lignin, with very low reduction in pulp viscosity and unchanged zero-span breaking lengths (21). While the overall delignification is rather small and a long reaction time is needed, the ability of manganese peroxidase to demethylate the phenolic methoxyl groups appears to be high. This is represented by the release of methanol. Manganese peroxidase remains active only with a very narrow range of H_2O_2 concentration. If H_2O_2 is present with 0.1 mM excess, manganese peroxidase becomes

inactive. This requirement for manganese peroxidase bleaching could greatly limit its ability in industrial applications (21).

LACCASE

INTRODUCTION

Laccase was first obtained from the juice of the Japanese tree *Rhus venicifera.* Laccase is also produced by many fungi of which the most studied are *Agaricus bisporus, Podospora anserina, Rhizoctonia practicola, Trametes* or *Polporus versicolor, Pholiota aegerita, Pleurotus ostreatus, Coriolus hirsitus,* and *Neurospora crassa.* Most fungi produce both intra- and extracellular enzymes, and the presence of phenols, amines, and benzoic acid can induce fungi to produce laccase (41).

Laccase is a blue oxidase under the oxidoreductase class of enzymes. The presence of copper accounts for the "blue" classification. Laccase usually contains multiple copper atoms in its active site which enables laccase to catalyze the reduction of molecular oxygen to water without the presence of H_2O_2. Laccase is broadly categorized by the Enzyme Nomenclature International Union. It is classified as EC 1.10.3.2 (benzenediol: O_2 oxidoreductase), but it also has some properties of EC 1.10.3.1 (1,2-benzenediol: O_2 oxidoreductase) or EC 1.14.18.1 (Monophenol, L-dopa: O_2 oxidoreductase) (33,91). Laccase is also broken into subclasses depending on the organism that produces it, the molecular weight, and the number of central copper atoms (Table 2).

Table 2 Properties of laccases isolated from different sources (91)

Source	Molecular Weight	Carbohydrate Content %	Copper Atoms per active site
Rhus vernicifera	110,000-141,000	45	4-6
Rhus succedanea	130,000	not reported	5-6
Polyporus versicolor			
A	64,400	10-14	4
B	64,700	10	4
Podospora anserina			
I	390,000	24	16
II	70,000	25	4
III	80,000	23	4
Neurospora crassa	64,800	11	3,4
Agaricus bisporus	100,000	15	2
Lactarius piperatus	67,000	not reported	not reported
Aspergillus nidulans	87,000	not reported	not reported
Prunus persica	70,000-90,000	not reported	not reported

Carbohydrate content of laccase can vary from 10-45% by weight (41). The molecular weight of laccase can vary widely, possibly due to the carbohydrate moiety. The amino acid chain contains about 500 amino acids, but this can also vary.

Laccase usually contains four copper atoms per active site that are classified into three types depending on their difference in EPR signal, in their surrounding features, and in their accessibility to solvents (41). Copper 1 is able to interact with various solvents including water, and it can be removed from the enzyme molecule by various chelating agents. Copper 1 can also be displaced by mercury or cobalt which causes a great loss in activity. Copper 2 in aerobic conditions is very difficult to remove from the enzyme. Copper 2 will interact with fluorine and the surrounding protein undergoes conformational changes. Copper 3 is partially classified by the capability of being re-embedded into the enzyme after cyanide treatment while Coppers 1 and 2 can not be re-embedded (41).

Laccase spectra have a λ_{max} at 280 and 605 nm. Copper 1 has a strong absorbance at 605 nm which gives the enzyme a deep blue color. Copper 2 and 3 may also be distinguished using other analytical methods.

The structure of laccase is relatively simple compared to other enzymes in its class. One of the ligands of Copper 1 seems to be cysteine or methionine, while Copper 2 is bound to three nitrogen atoms, and Coppers 2 and 3 may be bonded via a nitrogen atom. Apparently, the electron is transferred from Copper 1 to 2 to 3 because, if Copper 2 is removed, Copper 1 can not transfer the electron to Copper 3. Coppers 2 and 3 are believe to be responsible for the reduction of oxygen (41). The primary acceptors of the electron in this oxidase are the amino acids cysteine and histidine which transfer the electron to the copper 1 atom. If the literature on laccase is compiled, three main conclusions can be made about the mechanism of laccase's catalytic reduction of oxygen to water:

1. All four copper atoms are involved in the catalysis;
2. Electrons can enter the protein molecule by various ways in various sequences; and
3. The interaction with molecular oxygen can occur at all reduction steps of the reaction.

Several other trends can also be stated:

1. Oxygen molecules can interact with Copper 2 and 3 in a rapid reaction only after Copper 1 has gained one electron;
2. The limiting step seems to be the intramolecular electron transport or some related conformational change;
3. Fluoride ion can hinder laccase reoxidation; and
4. A lower (compared to peroxidases) sensitivity for metal ions of various valences in the medium (41).

Laccase has been studied extensively for application in many areas. The forest products industry is interested in its ability to degrade and polymerize lignin while other industries are

interested in biosensors and organic synthesis (laccase is detected in vascular plant tissue during lignin synthesis) (42,58). Laccase has the ability to oxidize a wide variety of molecules and is considered relatively nonspecific compared to other enzymes in its subclass (41).

REACTION WITH LIGNIN MODEL COMPOUNDS

Numerous studies with laccase and lignin model compounds have been performed over the last 15 years (23). Most of the studies involved the interaction between water soluble lignin model compounds and laccase with optimal solution conditions. Optimal reaction conditions depended on the type of isolated laccase and the organism that produced the laccase. The reaction times were usually limited to a few hours. The lignin model compounds contained bonding patterns that exist in native or industrial lignins (23). A review of these experiments follows; the discussion will concentrate on the type of lignin bonds that laccase can degrade.

Several studies show that laccase can degrade phenolic β-O-4 model compounds, but the products produced and the mechanism of the reaction are in dispute (23). One study presented by Kawai et al. showed that the syringylglycerol-β-guaiacyl ether is cleaved between the Cα and Cβ to give syringaldehyde and guaiacoxyethanol; however, this was not seen in another study performed by Higuchi (Figure 7) (23). Higuchi showed that syringylglycerol-β-guaiacyl ether is mainly converted into an α-carbonyl dimer (compound 10), 2,6-dimethoxyhydroquinone (compound 11), glyceraldehyde-2-guaiacyl ether (compound 12), and to guaiacol (compound 13). While laccase appeared to degrade phenolic β-O-4 compounds, the study showed that nonphenolic compounds were unreactive.

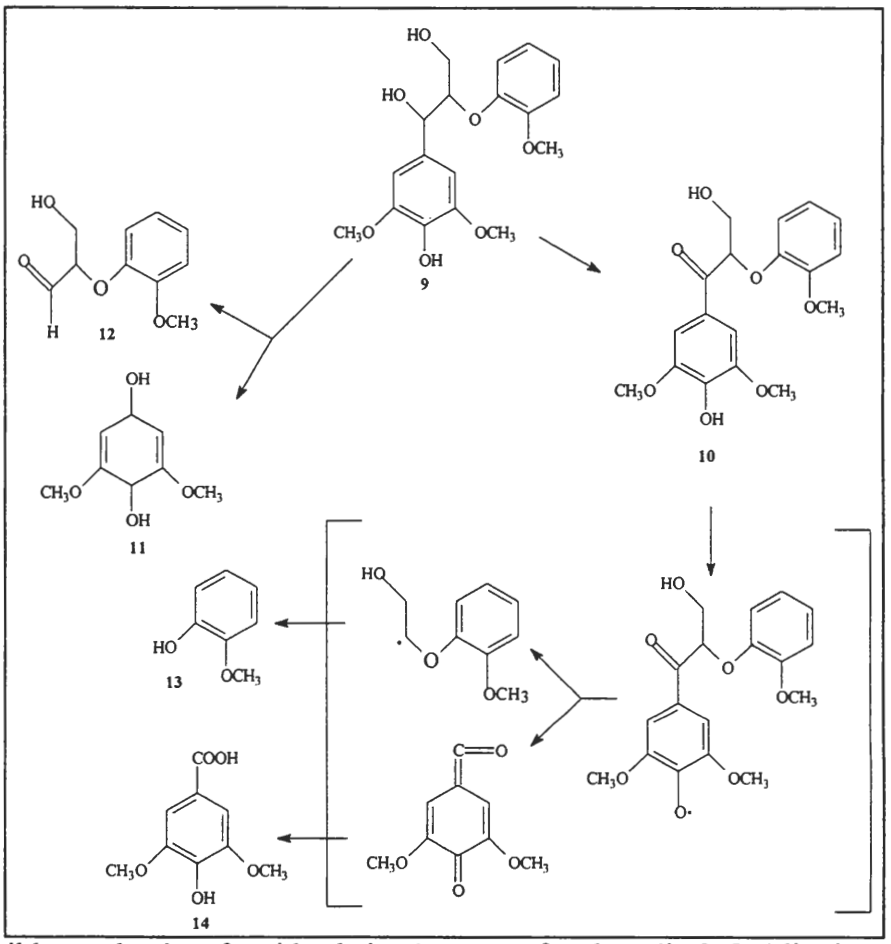


Figure 7 Possible mechanism for side chain cleavage of a phenolic β-O-4 lignin substructure model by laccase (as presented by Higuchi) (23).

Apparently, laccase degradation of phenolic lignin model compounds occurs through phenoxy radical reaction. These phenoxy radicals are either attacked by O_2 to give various degradation products or repolymerization occurs by radical-radical couplings (23,33).

Lignin model compounds that contain β-1 linkages can also be degraded by laccase (23). This degradation occurred through Cα-Cβ cleavage or by Cα oxidation (Figures 8 and 9). The degradation products of the lignin peroxidase treatment of the same model compound were very similar to the laccase study. After testing several β-1 lignin model compounds and

monophenolic model compounds, three reactions via phenoxy radicals were detected: Cα-Cβ cleavage; alkyl-phenyl cleavage between C_α and C_1; and Cα oxidation.

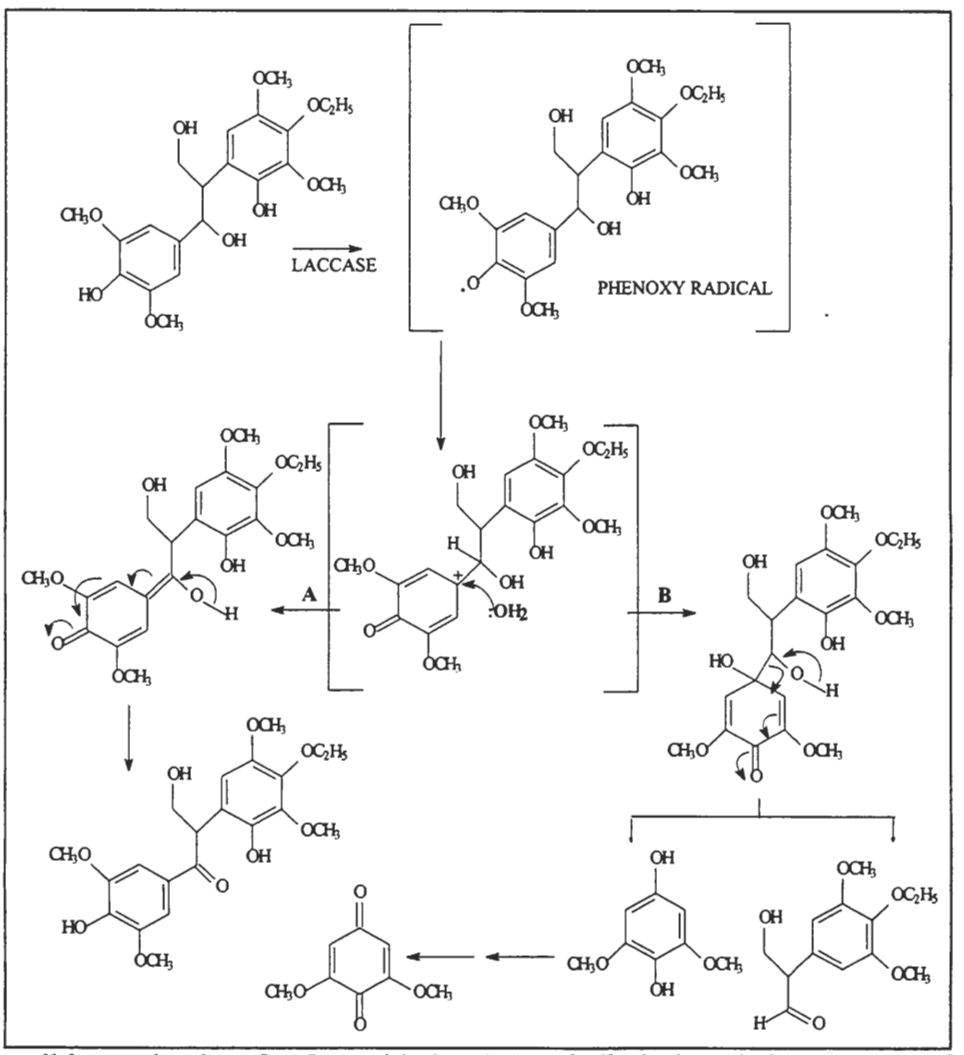


Figure 8 Possible mechanism for Cα oxidation (A) and alkyl-phenyl cleavage (B) of phenolic β-1 model compounds by laccase (as presented by Higuchi) (23).

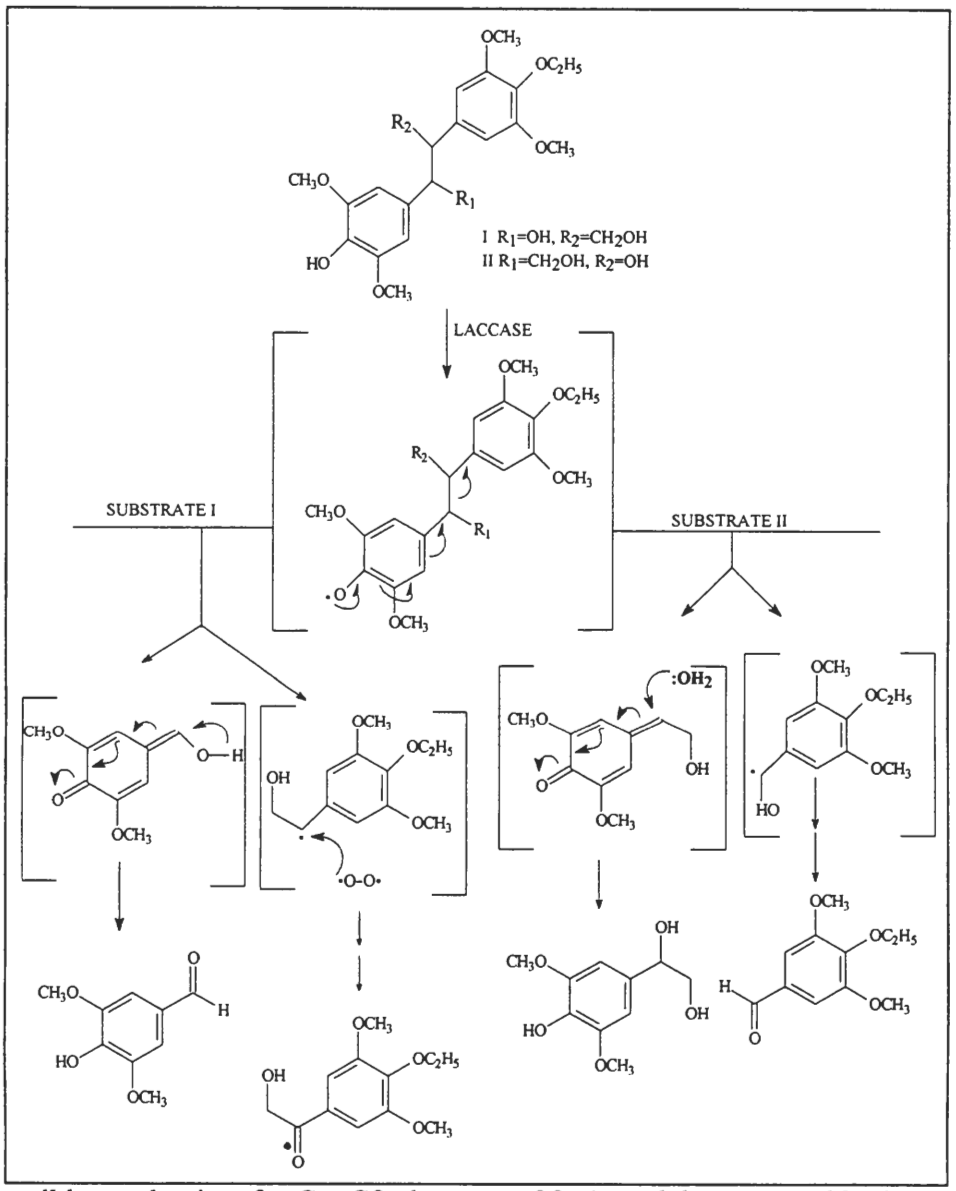


Figure 9 Possible mechanism for Cα-Cβ cleavage of β -1 model compound by laccase (as presented by Higuchi) (23).

Other studies with 4,6-di-t-butylguaiacol and laccase suggested a ring cleavage product (Figure 10)(23). It was also shown, through labeling studies, that the oxygen that incorporates into this product originated from the O_2 in the solution and not the oxygen in the water (23). Laccase has also been shown to be ineffective in demethoxylation of model compounds, which again suggests that it can not degrade nonphenol-containing lignins (48). Recent literature

suggests that when laccase and manganese peroxidase are used together, they act synergistically (43). The interaction between these and other enzymes could inhibit the repolymerization reaction of the lignin fragments when bleaching with the whiterot fungi.

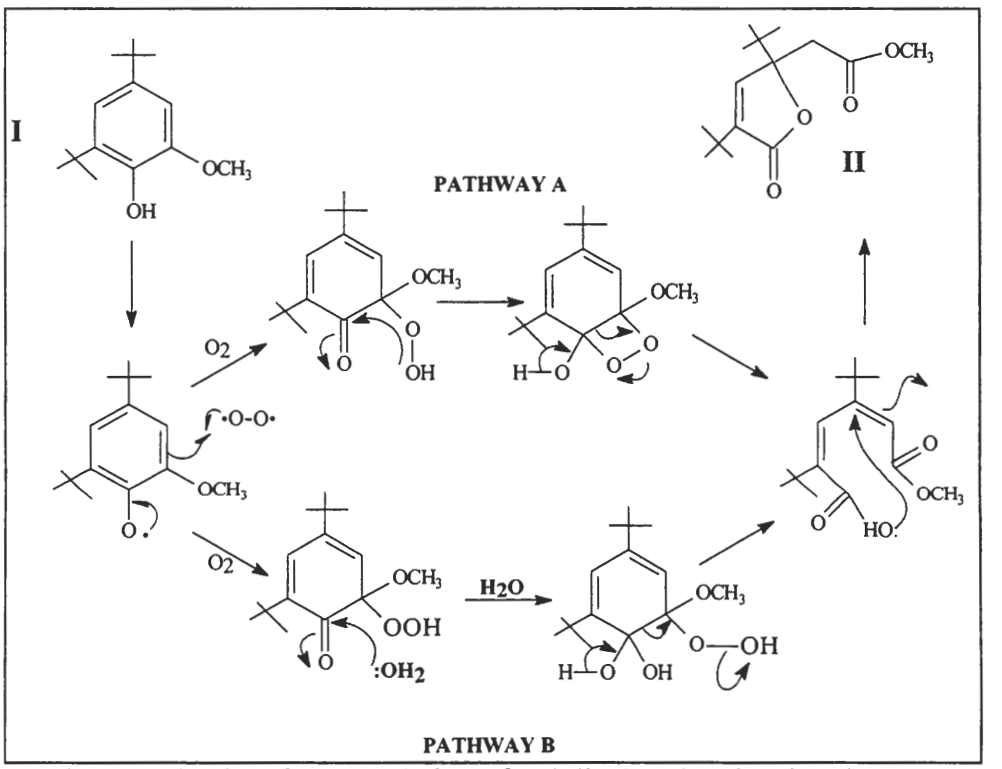


Figure 10 Possible mechanism for degradation of 4,6-di-t-butylguaiacol by laccase (as presented by Higuchi) (23).

Numerous studies have shown that laccase can also catalyze the polymerization of lignin (23,33,44-46). The source of the lignin, native or industrial, can have a large influence in creating favorable conditions for polymerization or depolymerization. Laccase has been used to dechlorinate bleach plant effluents and other compounds, PCB's (polychlorinated biphenols) and PCP's (pentachlorophenol), by polymerizing the materials (47). This work also showed how un-selective laccase can be compared to some other enzyme systems.

While these studies suggest that laccase could be an effective delignifying and brightening agent, it performs poorly with pulp fiber. Even mixtures of lignin peroxidases (which can degrade nonphenolic lignin model compounds) and laccase have relatively poor delignification ability compared to conventional chemical methods. This supports the view that direct interaction between the enzyme and the lignin molecule is not the main mechanism of lignin degradation used by white rot fungi. A mediator system seems to be involved, and recent studies have examined this possibility.

MEDIATOR SYSTEM

The mediator system of laccase was first introduced in 1990 with ABTS (2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate) as the mediator (49). ABTS has been used in standard procedures to determine the activity of isolated laccase by measuring the rate at which it is oxidized per unit of enzyme. In this experiment, lignin model compounds veratryl alcohol and 1-(3,3-dimethoxyphenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol were oxidized by the laccase mediator system. This system is the first reported laccase system that can degrade nonphenolic lignin model compounds. The Cα -Cβ linkage of the nonphenolic β-1 lignin model was oxidatively cleaved (49).

Bleaching with the laccase ABTS mediator system was performed on hardwood kraft pulps (50). This system was capable of substantial delignification, lowering the kappa number from 12.1 to 4 in five days, while only slight decreases in pulp viscosity were detected (24.2 to 21.9 mPa· s). The bleaching reactions were also monitored for methanol production, and about half of the methoxy groups present in the pulp's lignin were converted to methanol (50). The

methanol production peaked early during the reaction while kappa number decreased steadily until the later stages of the reaction. The oxidation of the phenolic OH appears to be the rate limiting step. An optimal enzyme concentration was also detected, with decreasing performance of the system if more or less enzyme was present. The optimal solution pH appears to be a trade off between the enzyme's solubility and its activity; the enzyme is more active at lower pH, but its solubility decreases as the pH is lowered. It is also important to note that the enzyme activity is higher at lower pH, but it remains active for only a short period of time (50).

Several other studies have used the laccase ABTS mediator system with similar results (51,52). Other phenolic and nonphenolic lignin model compounds have been shown to be degraded by this system. This system has been used to increase the ability of laccase to oxidize PCPs, which increased dechlorination rates and increased the ability of laccase to polymerize the organic compounds (47). The ability of oxidized ABTS to delignify kraft pulps has also been tested (50,53). While some delignification was detected, it was much lower than the laccase mediator system.

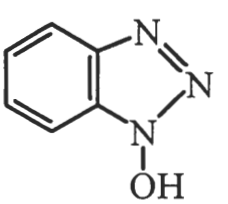
A study reported in a recent paper used the ABTS-laccase bleaching system under 100-400 kPa of oxygen pressure. After alkaline extraction, 32% delignification of softwood kraft pulp was achieved with a 2-hour bleaching stage (57). All previous ABTS-laccase systems required a reaction time of several days for effective delignification. A control sample with no enzyme or mediator revealed that only 1.7% of the delignification was a result of the pressurized oxygen treatment. Evidence suggesting loss of ABTS during the reaction also occurred. The ABTS may react with the lignin in the pulps and form chemical bonds reducing its effectiveness

as a bleaching agent, but no ABTS-lignin bond was isolated (57). The laccase was recovered after the reaction (through filtration alone) and 60% of the total activity was retained.

ABTS is not produced by the white rot fungi, and the exact function of laccase in lignin degradation is still unclear. Bourbonnais has suggested other enzymes produced by the white rot fungi (glucose oxidases and cellobiose quinone oxidoreductases) may produce effective mediators that can be used by laccase (50). However, these compounds have not been detected in extracellular fluid.

Recent studies by Call et al. have revealed a new laccase mediator system that has delignification properties comparable to conventional oxygen delignification methods with reaction time of hours instead of days (54-56). This system has proven effective with hardwood and softwood pulp before and after oxygen delignification. Removal of more than 60% of the residual lignin in oxygen delignified softwood kraft pulps can be achieved over a range of reaction conditions with little to no cellulose degradation. This enzymatic system was the first enzymatic system to display large levels of delignification in only 1-4 hours, and this breakthrough may enable enzymatic bleaching to become commercially viable (54).

The mediator revealed by Call et al. was 1-hydroxybenzotriazole (56). The oxidative site



1-Hydroxybenzotriazole

is the RR'N-OH which is believed to be oxidized to RR'NO· by the enzyme. This species diffuses into the pulp and oxidizes the residual lignin. Call's patent claims any mediator with a

RR'N-OH site, and many different compounds may be effective (56). The mechanism proposed by Call et al. shows an oxygen-oxidized mediator, via laccase, that selectively oxidizes the residual lignin (Figure 11).

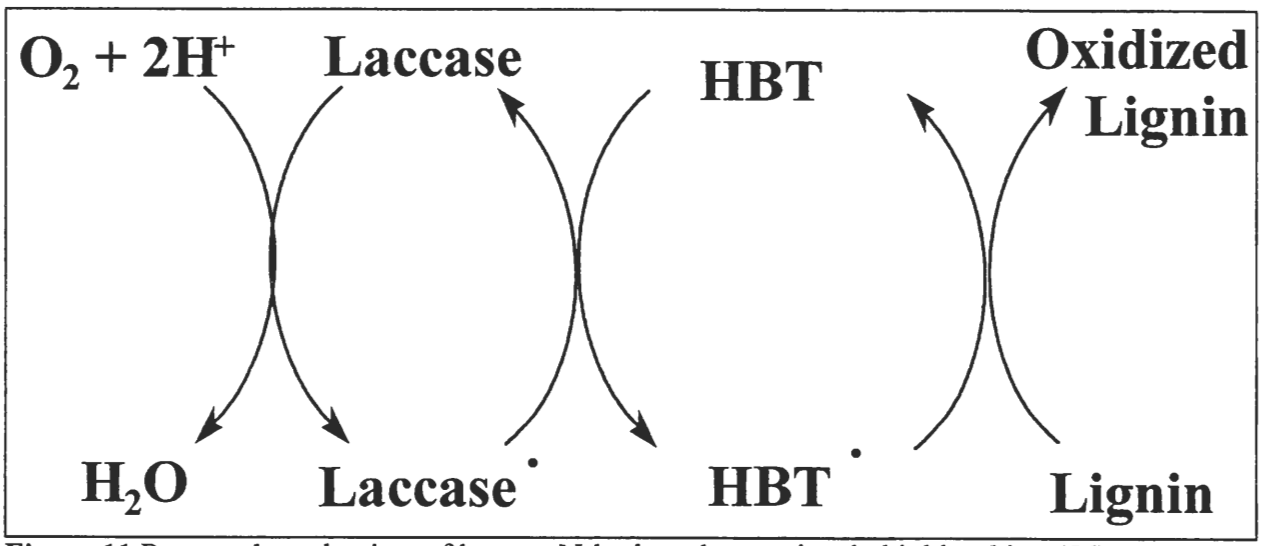
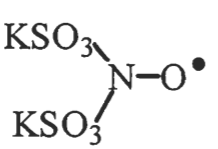


Figure 11 Proposed mechanism of laccase N-hydroxybenzotriazole biobleaching (56).

Several studies have been performed on RR'NO• bleaching agents. Fremy's salt was one of the first RR'NO• molecules studied for its delignification ability (59,60). Fremy's salt is



Fremy's salt

potassium nitrosodisulfonate, and it can react with phenols to produce benzoquinones. Fremy's salt can also oxidize many different compounds, but it has several drawbacks. One of the main disadvantages of using this bleaching compound is its tendency to oxidize explosively under certain conditions. The explosive property and high cost has limited its use as a bleaching agent.

Another RR'NO• compound that was studied is N-hydroxysuccinimide; it can be oxidized to form a relatively stable RR'NO• radical that can also react with substituted phenols to produce benzoquinones (61). A regeneration system of the RR'NO• radical after oxidation of lignin compound was proposed when NO_2 was present; however, it was found that NO_2 was an effective oxidizing agent without N-hydroxysuccinimide (60).

These studies suggest that a molecule with a RR'NO• functional group may be an effective delignification agent. If a regeneration process is performed during the reaction, only a small concentration of the bleaching agent is needed to perform significant delignification.

DISSERTATION OBJECTIVES

The objective of this research was to examine the fundamental chemical reactions of lignin in pulp with the recently proposed N-OH mediator-laccase system. An analysis of the resulting chemical changes in residual lignin was used to study the mechanism and evaluate the effectiveness of the laccase mediator system. The mechanism of this enzymatic bleaching system was examined to determine which component or combination of components acts as the active delignification agent. This information was used to obtain an understanding of how to optimize this and other delignification systems. The three objectives of this research are listed below.

1. To determine if 1-hydroxybenzotriazole is an effective mediator in the laccase mediator bleaching system for kraft softwood pulp, and determine the structural changes that occur in residual lignin.

2. To determine the chemical species that form when hydroxybenzotriazole reacts with laccase.

3. To examine the reaction between isolated residual lignin and laccase in the presence or absence of a mediator.

EXPERIMENTAL PROCEDURES

MATERIALS AND SUPPLIES

Several industrial pulps were used in this study (Table 3). The list of abbreviations in Table 3 will be used in the following chapters. Each O_2-delignified pulp was sampled from the same pulp line that produced the brownstock pulp. The Western source pulps used for earlier studies were not washed immediately, but the Southern source pulps were washed immediately. Most of the research was performed on the Southern source pulps.

Table 3 List of industrial pulps used in this study with kappa numbers and pulp viscosity.

Pulp Type	Symbol	Kappa #	Pulp Viscosity (cP)
Western Softwood kraft brownstock	WSBK25	25.5	30.7
Western Softwood kraft O_2 delignified	WSOK17	17.2	26.8
Southern Softwood kraft brownstock	SSBK26	26.8	31.9
Southern Softwood kraft O_2 delignified	SSOK13	13.6	18.0
Southern Hardwood kraft brownstock	SHBK15	15.7	54.5
Southern Hardwood kraft O_2 delignified	SHOK10	10.5	28.3

Bulk chemicals used were purchased from VWR and were used as received. acetone, pentane, sodium borohydride, 1.00 N NaOH solution, glacial acetic acid, 1.00 N HCl solution, methylene chloride, methanol, petroleum ether, magnesium sulfate, tetrahydrofuran, sodium bicarbonate (which was used to make a saturated solution in distilled water), and p-dioxane (distilled over sodium borohydride before used).

Specialty chemicals were purchased from Aldrich chemical company and were used as received. They include N-hydroxybenzotriazole, benzotriazole, 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonate), violuric acid, N-hydroxypthalimide, hydrazine hydrate (98%), ethanol (100%), 4-chloro-3-nitroanisole, 4-chloro-3-nitrotoluene, 5% rhodium on carbon, nitrobenzene, acetyl chloride, 6-trifluoro-methyl-hydroxybenzotriazole, benzotriazole-1-methanol, N-hydroxysuccinimide, N-hydroxymethylpthalimide, N-hydroxymaleimide, 1-hydroxy-7-azobenzotriazole, and 3-hydroxyanthranilic acid.

NMR solvents were purchased from aldrich in sealed glass ampoules and were dried over 4A-molecular sieves before use. Solvents used (including purity): dimethyl sulfoxide-d_6 (99.9% D), acetone-d_6 (99.8% D), chloroform-d (100.0% D), deuterium oxide (100.0% D), and tetramethylsilane-d_{12} (99.9 + % D). DMSO-d_6 used for ^{13}C NMR experiments was not dried before use. The solvent was purchase in a sure-seal bottle and flushed with argon after each use.

Superoxide dismutase (Bovine Erythrocytes) was purchased from Sigma (Product # S-2515 with an activity of 5800 U per mg of solid) and used as received.

EQUIPMENT

All NMR spectra were performed with a Bruker DMX 400 with a 5 mm QMP probe. The operating system was IRIX 5.2 run under UNIX 5.2. Mass spectra were obtained from a VG 70 - SE mass spectrometer equipped with a double sector magnetic analyzer. The columns were comprised of fused silica DB-5 30 m x 0.250 mm ID with a 25-micron film thickness, and they were purchased from J&W Scientific. The gas chromatograph was a Hewlett Packard 5890 equipped with an HP-17 capillary column and flame ionization detector. Fourier transform

infrared (FTIR) spectrometry was performed on a Nicolet 550 spectrometer with OMNIC 3.1 software. Each spectrum was comprised of 128 scans with a 4.0 resolution setting. Memory required for each spectrum was 37 K and was collected over 2 minutes and 38 seconds. The ultraviolet spectrophotometer was a Shimadzu UV-160A. All UV analyses were performed at room temperature, 23°C, with a 1 cm quartz cuvette. Pulp brightness measurements were performed on a Technibright™ Micro TB-1C instrument, and the brightness readings were recorded at 457 nm wavelength of light.

LACCASE ACTIVITY MEASUREMENTS

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

NOVO activity measurements were performed with syringaldazine 19 μM in a MES 23 mM buffer with a pH of 5.5. The temperature was 30°C with a reaction time of 60 seconds. The activity was measured at an A_{530nm}, and a laccase unit (LACU) was defined as the amount of enzyme which, under these conditions catalyzes, the conversion of 1 μmol of syringaldazine per minute. The MES buffer was prepared by adding 2.66 g of 2-(N-morpholine) ethane sulphonic acid into a 500 mL volumetric flask. Distilled water and 1 mL of 2 M NaOH was added to a volume of 500 mL. The syringaldazine solution was prepared by adding 10.0 mg of syringaldazine in 50 mL of ethanol (96%). An amount of 25.0 mL of this solution was diluted with 25 mL of distilled water to make a 0.28 mM solution of syringaldazine which 300 μl of this

MES solution and 100 µl of the enzyme solution was added together for the activity meaurement (136).

Several different laccases were used in this study. The activities and concentration of protein in the enzyme solution are listed in Table 4. Most of the laccase experiments used SP 504, which was recently called NS51002 when this laccase was introduced as a commercial product. Experiments in Chapter 1 were done with PPQ 4211, which was also a gift from NOVO, but this laccase batch was not made commercial. SP 850, which was recently called NS51003 when this laccase was introduced as a commercial product, was used in Chapter 2. NS51003 was used in NMR HBT conversion experiments (described in detail below) in Chapter 2, but the pulp biobleaching experiments were carried out with NS51002.

Table 4 Laccase batches used in this study. NOVO activity measurements were performed by NOVO, and IPST activity was performed by the author. The techniques varied by reaction mixture and reaction temperature, but both used the same substrate, syringaldazine.

Laccase Type	**NOVO Activity**	**mg protein/ mL of solution**	**IPST Activity**	**Organism Used**[a]
SP 504 NS51002 (LacNS51002)	320 LACU/ mL	3.2	584,000 U/ mL	*Trametes aspergillus*
SP 850 NS51003 (LacNS51003)	1520 LAMU/ mL	23.4	102,000,000 U/ mL	*Polyporus aspergillus*
PPQ 4211 (LacPPQ-4211)	NA	2.0	60,000 U/ mL	*Polyporus aspergillus*
Merican Laccase (LacMer)	NA	NA	258,000 U / mL	*Trametes versicolor*

[a]Laccase isolated from *Pyricularia oryzae* and purchased from Sigma was found to be inactive in biobleached kraft pulps with HBT as the mediator.

FIBER BIOBLEACHING STUDIES

Industrial kraft pulp samples were obtained from a mill site and washed with distilled water until the filtrate was clear. Two large batches were used in this study. The first batch of pulp was WSBK 28 and WSOK17. These pulps were used in the study described in Chapter 1. All the other studies were performed on the second pulp batch which was SSBK26, SSOK13, SHBK15, and SHOK10. These pulps were washed thoroughly and refrigerated until used.

Prior to bleaching, consistency, kappa number, and pulp viscosity measurements were determined for a well-mixed sample. Most studies were performed on a small scale (10 g o.d.) or on a large scale (60 g o.d.). Large-scale biobleaching trials were carried out for residual lignin isolation studies. Almost all biobleaching experiments were performed in the same vessel. The reaction vessel was a 316 stainless steel Parr bomb with a 1000 mL capacity. The Parr bomb was fixed with a sealed center port blade mixer.

Each biobleaching treatment was performed with the following procedure:
The bomb, pulp, and water was heated to 45° C. Distilled water was added to set the consistency to 10%. The mediator was added, and mixed for 3 minutes. The pH was then adjusted to 4.5 with glacial acetic acid or sodium bicarbonate. The proper dose of enzyme was added and mixed for 1 minute. The bomb was closed, and the oxygen pressure was regulated to 145 psi while mixing (reaction time varied). After biobleaching, the pulp mixture was filtered and washed with distilled water (1000 mL of water per 10 g o.d. pulp scale). Depending on the study, the filtrate was collected or discarded.

Each biobleaching experiment was followed with an alkaline extraction stage. Each alkaline extraction stage was performed with a 2% charge of NaOH at 10% consistency on o.d.

pulp. The alkaline extraction stage was carried out for 1 hr at 70°C. The pulp was then washed with distilled water (1500 mL water per 10 g o.d. pulp sample) and air dried.

The first biobleaching studies (Chapter 1) used laccase LacPPQ-4211 (defined earlier) and pulps WSBK25 and WSOK17 (0.8 mL or 24,000 U of LacPPQ-4211 was used on 1 g o.d. pulp). All the later experiments which include the conversion of HBT with time and pulp type as well as the LMS treatments on isolated lignins were carried out with laccase NS51002. The pulps used in these experiments were SSBK26, SSOK13, SHBK15, and SHOK10. The laccase dose was 3 mL of NS51002 per 10 g o.d. pulp at 3.2 mg of protein per mL and an activity of 320 LACU and 1,752,000 U.

PULP PROPERTY MEASUREMENTS

The main pulp properties tested were kappa number, Klason lignin, pulp viscosity, and brightness measurements. Kappa number was to estimate the amount of lignin or oxidizable material in the pulp. Kappa numbers were performed on air-dried pulp samples. These pulp samples were dried on a heated balance. The kappa number was performed in accordance with TAPPI Useful Method UM-246 "Micro Kappa Number" (79). This procedure was used because only ~1 to 3 g of pulp was needed (standard deviations ± 0.3).

Pulp viscosity values were determined in accordance with TAPPI Standard T-230 om-89 "Viscosity of Pulp (capillary viscometer method)"(80). The pulp was air dried and the consistency was determined (the sample was not processed unless the consistency was over 92%). The pulp was then weighed out to ± 0.0005 g under dry conditions. All pulp solutions

were monitored for insoluble material, and no data were reported if any insoluble material was detected (standard deviations ± 0.6).

The Klason lignin technique that was used was developed from the TAPPI standard by Kaar (81). This method was developed to accommodate small samples. The amount of pulp needed ranged from 0.7-3 g o.d. and was dependent upon the amount of residual lignin in the pulp. Brownstock pulps were tested with 0.7 g of sample whereas bleached pulp samples required 3 g of pulp in order to provide a significant amount of material to weigh accurately. The procedure was as follows: 1 g of o.d. pulp was measured into an autoclave bottle. 24 mL of H_2SO_4 (72%) was added to the bottle, and the solution was mixed with a glass rod for approximately 5 minutes. The vessel was placed in a water-bath at 30°C for 1 hour, and mixed with a glass rod (occasionally). The mixture was diluted to 4% acid with distilled water and autoclaved at 120°C for 1 hour. After cooling, the mixture was filtered through a weighed, clean, (medium) frit glass crucible, and the solid material was washed with distilled water. The crucible was heated at 105°C for 24 hr and then weighed (standard deviation ± 0.02 % lignin content).

Pulp brightness measurements were reported as ISO brightness and were performed in accordance with TAPPI Standard T-525 om-92 "Diffuse Brightness of Pulp (d/O°)." The handsheets were made using TAPPI Standard T-231 cm-85 "Forming Handsheets for Physical Test of Pulp." Thermal reversion was performed by heating the handsheets at 90°C for 10 hours at 50% relative humidity.

RESIDUAL LIGNIN ISOLATION

The residual lignin was isolated by acid hydrolysis. Gellerstedt's procedure was followed with some minor changes (70,71). The following procedure was used:

1. The pulps were acetone extracted in a soxhlet for 24 hr, air dried, washed with water, and air dried again.
2. Dioxane/acid extraction was performed at 9:1 ratio of dioxane to 0.1 N HCl solution at 4% consistency. The mixture was refluxed for 2 hr under argon atmosphere.
3. The mixture was filtered through a (coarse) glass frit to remove extracted pulp, and then refiltered through celite in a (medium) glass frit to remove residual carbohydrates.
4. The pH was adjusted to 6.0 with sodium bicarbonate solution (saturated) and concentrated to 1/4 the original volume under reduced pressure, and 200 mL of distilled water was added and concentrated to remove the last traces of dioxane. More water was added, if needed.
5. The solution was then acid precipitated to pH 2.5 with 1.00 N HCl at a 1.5 liter volume for 100 g o.d. pulp batch. The dilution water was added to insure good washing.
6. The mixture was transferred to centrifuge bottles and frozen. This allowed the lignin to coalesce and improved yield. The samples were then thawed, centrifuged, and decanted. This step was repeated twice.
7. The lignin sample was then freeze-dried.

The yield of residual lignin is calculated by kappa number and Klason lignin analysis. Yields calculated by kappa number ranged from 30-50%, and yields calculated by Klason lignin analysis ranged from 40-60%. Lignin yield was calculated by the following: lignin yield = [mass lignin isolated / {(kappa # of pulp before treatment - kappa number of pulp after treatment) x 0.15}] %.

EFFLUENT LIGNIN ISOLATION

This procedure was used to isolate the lignin removed in the alkaline extraction stage after a bleaching stage. Since the alkaline extraction effluent contains a substantial amount of

carbohydrates, extra steps were used to purify the lignin. The isolation procedure was based on a 60 g o.d. pulp batch and was as followed:

1. The pH of the solution was adjusted with 1.00 N HCl (~5 mL) to pH 6.5, then concentrated to 50 mL.
2. The mixture was transferred to a centrifuge bottle and diluted to 150 mL. The pH was lowered with a 1.00 N HCl (~4 mL)solution to 2.5. This precipitated the lignin fragments.
3. The mixture was frozen in centrifuge bottles. After thawing, the samples were centrifuged and decanted. This step was repeated 2 times with acidified wash water.
4. The precipitate was freeze dried and pentane extracted in a soxhlet for 24 hr. The solid was removed and air dried overnight.
5. The solid material was washed with 9:1 dioxane:water until the supernatant was clear (usually 3 times with 100 mL of solvent mixture).
6. The lignin solutions were combined, filtered through celite, and concentrated. Distilled water was added at the end of concentration to remove the last traces of dioxane.
7. The lignin was freeze dried.

NMR ANALYSIS

The three main NMR techniques used to characterize lignin included quantitative ^{13}C, ^{31}P, and ^{1}H NMR. These proposed techniques were well established in the literature and were followed closely. All NMR spectra were recorded with a DMX 400 MHz Bruker spectrometer.

^{13}C NMR ANALYSIS

The quantitative ^{13}C experiments used a 90^{o} pulse with an inverse gated decoupling (IGD) pulse sequence, which is needed to remove the nuclear overhauser effect (NOE). A pulse delay of 11 seconds was used to allow relaxation of the carbon nuclei (73). More than 10,000-30,000 scans were acquired for every sample (TD = 32,768, SI = 32,768, LB = 10 Hz). The sample was prepared by adding 100 mg of dry lignin to 0.45 mL of DMSO-d_6. The integration regions used for the analysis of the spectra are shown in Table 5. Three-point manual base line

correction was performed and the DMSO signal (39.5 ppm) was used as the reference. Each spectrum was recorded at 50°C.

Table 5 Integration regions for ^{13}C NMR spectrum.

Functional Group	Integral Region
-COOH	180.1-165.1
C3/C3' C5-C5	160.1-154.1
C3, C4 (C-Ar-O)	154.1-139.8
C1, (-C-Ar-C)	139.8-126.8
C5, (-C-Ar-C)	126.8-122.5
C6, (-C-Ar-H)	122.5-116.6
C5, (-C-Ar-H)	116.6-113.6
C2, (-C-Ar-H)	113.6-106.4
Aliphatic C-O (Cβ in β-O-4)	89.5-78.4
Aliphatic C-O	78.4-67.4
Cγ (β-O-4)	67.4-61.3
OCH_3	57.4-53.9
Cβ in β-β and Cβ in β-5	53.9-51.2

^{31}P NMR ANALYSIS

^{31}P NMR spectra were generated by reacting the lignin sample with a phosphorus-containing compound that replaces labile protons on mainly primary hydroxyl groups. This procedure has provided quantitative information on several lignin functional groups (74-78). The procedure was performed as described by Argyropoulos (74-78). A solvent mixture composed of pyridine and deuterated chloroform in a 1.6:1 ratio was prepared. A solution was prepared by adding 4.0 mg of cyclohexanol (internal standard) per mL of solvent mixture and 3.6 mg of chromium acetylacetonate (relaxation agent) per mL of solvent mixture. A 150 mL aliquot of this solution was added to approximately 25 mg of dry lignin in 400 mL of solvent mixture. This mixture was stirred for several minutes, and 0.2 mL of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (reactive agent) was added. The sample was mixed for 20

seconds and then immediately transferred into a 5-mm NMR tube, and the NMR spectrum was recorded immediately.

Each ^{31}P NMR spectrum was performed with a 25-second delay between 30° pulses. The inverse gated decoupling pulse sequence was used to obtain quantitative spectra. Each spectrum was acquired by compiling 200 acquisitions performed at room temperature, and a 61.9 ppm sweep width with a 4 Hz line broadening was used (TD = 32,768, SI = 32,768). All the chemical shifts were referenced to the product of the derivatizing agent with water which gives a sharp ^{31}P signal at 121.1 ppm. The integration regions used are listed in Table 6.

Table 6 ^{31}P integration region used to quantify lignin functional groups.

Functional Group	**Integration Region (ppm)**
Cyclohexanol (internal standard)	145.4-144.0
Aliphatic OH	150.0-145.3
Condensed phenolic OH	144.0-141.0
Guaiacyl phenolic OH	141.0-137.0
COOH	136.2-134.0

^{1}H NMR ANALYSIS

The ^{1}H NMR procedure was performed by following Lundquist's technique with a slight modification (82-88). The modification involved the internal standard. Two internal standards were used in this study, pentafluorobenzaldehyde (PFB) and trimethylsilyl propionate-d_4 (TSP). NMR spectra performed with PFB had to be prepared under anhydrous conditions because PFB is unstable in the presence of water in the lignin mixture. To avoid this problem TSP was used for the later experiments, but anhydrous conditions were still maintained. The procedure was as follows: an anhydrous, internal standard solution was prepared first by adding 30 mL of DMSO-

d_6 to molecular sieves for 24 hr. Then TSP was weighed out into a 25 mL volumetric flask (~20 mg) (when PFB was used ~9 mg was weighed out). The flask was filled with dry DMSO-d_6 by transferring under argon pressure. The TSP was allowed to dissolve completely and the solution was transferred and stored under argon. The NMR sample was prepared by adding ~25 mg of dry lignin into 0.45 mL of internal standard solution. Several molecular sieves were added to each lignin solution, and the molecular sieves were used as stir bars (stirring was performed by vibration). The lignin solution was transferred to a 5 mm NMR tube with a dry pipette.

The ^{1}H NMR spectrum was recorded under quantitative conditions using a 90° pulse and a 25-second delay at 50°C. Each spectrum was required by compiling 750 acquisitions and a 15 ppm sweep width (TD = 32,768, SI = 16,386 LB = 0.30 Hz). The integration regions that were used for the analysis of each spectrum are listed in Table 7.

Table 7 Integration regions for ^{1}H NMR lignin analysis.

Functional groups	**Integration Region (ppm)**
Carboxylic acid H	13.5-11.7
Formyl H	10.0-9.4
Total Phenolic H	9.4-8.0
Aromatic H	7.7-6.3
Aliphatic H	6.3-4.1
Methoxyl H	4.1-3.4
Internal standard TSP H_9	0.03-(-)0.10

FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

FTIR studies were accomplished with a Nicolet 5 SXC spectrometer in the transmittance mode. Each sample was analyzed by mixing 2 mg of lignin in KBr powder (0.4 g) and the pellet

was formed under pressure. All spectra were recorded at 24°C. Typically, this procedure yielded spectra data with a high degree of reproducibility (<2% deviations in relative peak ratios).

LACCASE MEDIATOR REACTIONS

Laccase HBT experiments were performed to examine product or products formed during biobleaching. LacNS51003 was used for NMR-monitored reactions. These experiments were done by adding HBT into a NMR tube with D_2O. About 2 mg of HBT was added to 0.5 mL of D_2O and 5.2 U of LacNS51003 was added. The preliminary experiments were performed at room temperature, but conversion-over-time experiments were performed at 45°C. The NMR tube was not removed from the magnet during the conversion-over-time experiment.

Several large-scale (200 mg HBT) reactions with LacNS51002 (2 mL) were performed under 145 psi O_2 pressure at pH 4.5 (the pH was adjusted with acetic acid) and 45°C with 100 mL of distilled water. The reactions were carried out for 24 hr. The mixture was then concentrated (under reduced pressure) to dryness. A small sample (~3 mg) was dissolved in D_2O and ^1H NMR analysis was performed, and the remaining material was separated by preparative silica gel plates (1000 μm thick) with CH_2Cl_2 as the mobile phase. Two more large-scale experiments were performed to isolate more benzotriazole. Benzotriazole was isolated by dry-packed silica gel columns. Silica gel 60 with 0.040-0.063 mm size particles was used with CH_2Cl_2 as the mobile phase. Benzotriazole was also isolated from large-scale laccase HBT pulp treatments. The pulp used was SSOK13 with LacNS51002. The water wash after biobleaching was collected, concentrated to dryness under reduced pressure, and isolated as described above. 1-H benzotriazole was characterized by ^1H NMR, ^{13}C NMR, EI/MS, and HRMS..

^{1}H NMR (acetone-d_6): δ 7.58 (2H, dd, J = 6.0, 3.3 Hz), 8.04 (2H, dd, J = 6.0, 3.3 Hz)
^{13}C NMR (acetone-d_6): δ 115.0, 125.5, 139.0
EI/MS m/z (rel. intensity): 119(100), 91(90), 64(60), 52(18), 40(10)
HRMS calculated for $C_6H_5N_3$: 119.0483, found 119.0488

MEDIATOR RECOVERY EXPERIMENTS

The mediator recovery experiments were performed to determine the mass balance of the mediator during biobleaching and isolate the mediator structure after biobleaching to determine its chemical nature. These experiments preceded model experiments where laccase and HBT were reacted. Since these model experiments detected a new chemical species being formed, analysis of the mediator after biobleaching was performed.

Mediator recovery experiments were first performed on acetone extracted pulp. This pulp was WSBK25. The biobleaching experiments with laccase were performed as described earlier. However, the first experiment was carried out on 5 g o.d. pulp basis, but the mediator dose (2% HBT on pulp) remained the same. The laccase dose and type of laccase was varied. The first experiment was performed with laccase Novo sample LacPPQ 4211, and 120,000 U (4 mL of enzyme solution) measured by syringaldazine per 5 g of o.d. pulp was used. LacPPQ 4211 had a lower oxidation potential and lower activity than LacNS51002 (101). All other mediator conversion experiments were carried out with SSBK26 and SSOK13 with LacNS51002. The pulp was not acetone-extracted before use.

The mediator structures were isolated by the following procedure: The biobleaching wash solution (2000 mL) was concentrated and freeze dried. The solid material was acetone extracted for 24 hr, and the extract was concentrated to ~5 mL volume. The solution was quantitatively transferred into a 25 mL round bottom flask, and concentrated to dryness. The oily solid was

placed under high vacuum overnight and then weighed. This fraction accounted for about 60% of the total weight of the original mediator added.

The biobleached pulp was air dried and acetone extracted in a soxhlet for 24 hours. The extract was concentrated as described above. This fraction accounted for about 30% of the total weight of the original mediator added.

Table 8 Mass yield of HBT during laccase (LacNS51002) biobleaching. The yield was for the water wash fraction (isolation procedure describe above). Brownstock and Oxygen delignified pulps were tested. Three SSOK13 pulp samples were reproduced and were listed.

Reaction Time SSBK26	**Percent Yield in the water wash Fraction**	**Reaction Time SSOK13 pulp**	**Percent Yield in the water wash Fraction**
15 min.	75	15 min.	73
1 hour	77	30 min.	74/ 75
4 hour	64	1 hour	63
8 hour	61	2 hour	60/ 64
24 hour	53	4 hour	59
44 hour	55	8 hour	56
		16 hour	59/ 55
		32 hour	55

MEDIATOR SYNTHESIS

Several chemical compounds were synthesized to test their effectiveness as mediators for laccase biobleaching. The compounds will be listed in the order that they will appear in later chapters.

1-HYDROXY-4-METHOXY-BENZOTRIAZOLE

1-Hydroxy-4-methoxy-benzotriazole was prepared first by adding 15 mL of hydrazine hydrate (98%) into 100 mL of ethanol (100%) and then by adding 4.64 g of 4-chloro-3-

nitroanisole (89). The mixture was refluxed for 4 hours and then cooled to room temperature. The solution was concentrated to an oil and solvated with methylene chloride (with a trace of methanol to enhance solubility). The product was isolated by column chromatography with silica gel as the stationary phase and methylene chloride as the mobile phase. The yield was 20%, and the product was characterized by ^{1}H and ^{13}C. The melting point was 216-217°C (literature 215°C) (89).

^{1}H NMR (acetone-d_6): δ 7.09 (1H, d, J = 8.7 Hz), 6.23 (1H, dd, J = 6, 1.4 Hz), 6.46 (1H, d, J = 2.9 Hz), 4.95 broad, 3.75 (1H singlet)
^{13}C NMR (acetone-d_6): δ 159.7, 145.2, 129.4, 110.2, 103.7, 100.7, 54.6

1-HYDROXY-4-METHYL-BENZOTRIAZOLE

1-Hydroxy-4-methyl-benzotriazole was prepared by adding 15 mL of hydrazine hydrate (98%) into 50 mL of ethanol (100%) and then by adding 17.16 mL of 4-chloro-3-nitrotoluene (89). The mixture was mechanically stirred under pressure (300 psi) at 150°C for 5 hours. The yield was 28%, and the melting point was 153°C (literature 151.5-152.5°C) (89).

N-ACETYL-N-PHENYLHYDROXYLAMINE

N-acetyl-N-phenylhydroxylamine was prepared from nitrobenzene by a two-step reaction with a unstable intermediate (hydroxylamine) which was acylated immediately (90). N-phenylhydroxylamine was prepared by adding 1.1 g of 5% rhodium on carbon to 200 mL of THF in a 500 mL round bottom flask with magnetic stirring (note: added 1 mL of distilled water to the carbon before adding to THF to decrease the possibility of fire). The nitrobenzene (34.3 mL) was added to the round bottom flask while stirring and the temperature of the mixture was

lowered to 15°C with an ice bath. Once the mixture was cooled, 16.5 mL of hydrazine hydrate (98%) was added slowly over 30 min. The temperature was maintained below 30°C during the addition. After addition, the reaction was stirred for 2 hours at 25-30°C. The mixture was filtered and the carbon was washed with 30 mL of THF. The solution was added immediately to a 1000 mL round bottom flask with magnetic stirring.

The acylation reaction was performed on the solution from the first reaction by adding a slurry of sodium bicarbonate (42 g in 40 mL of distilled water). The mixture was cooled to -4°C in an ice-salt bath before 23.6 mL of acetyl chloride was added slowly over 1 hour while the temperature was maintained below 0°C. After addition of the acetyl chloride, the reaction was allowed to continue for 30 minutes. Sodium hydroxide solution (20 g in 200 mL distilled water) was added while keeping the solution temperature below 20°C. The aqueous phase was separated, the THF phase was diluted with an equal volume of petroleum ether, and the aqueous phase was removed again. The organic phase was extracted with a 10% sodium hydroxide solution (2 x 50 mL). The combined aqueous phases were washed with methylene chloride (200 mL), and then the aqueous phase was neutralized with concentrated hydrochloric acid while keeping the solution cool. The mixture was extracted with methylene chloride (3 x 100 mL). The methylene chloride extracts were combined and dried with magnesium sulphate, filtered, and concentrated to ~30 mL volume. The product was crystallized by adding petroleum ether (~100 mL) until the solution became cloudy. The solution was cooled in the freezer overnight and the crystals were filtered and washed with petroleum ether. The crystals were dried under vacuum for 24 hours and characterized by ^{1}H, ^{13}C, COSY ([CO] correlation [SY] spectroscopy), and XHCORR ([XH] heteronuclear [CORR] correlation spectroscopy) NMR analysis. The melting

point was 67-68°C (literature 66-67°C) (90). The COSY (Figure 12) and the XHCORR (Figure 13) spectrum support the assignment of N-acetyl-N-phenylhydroxylamine.

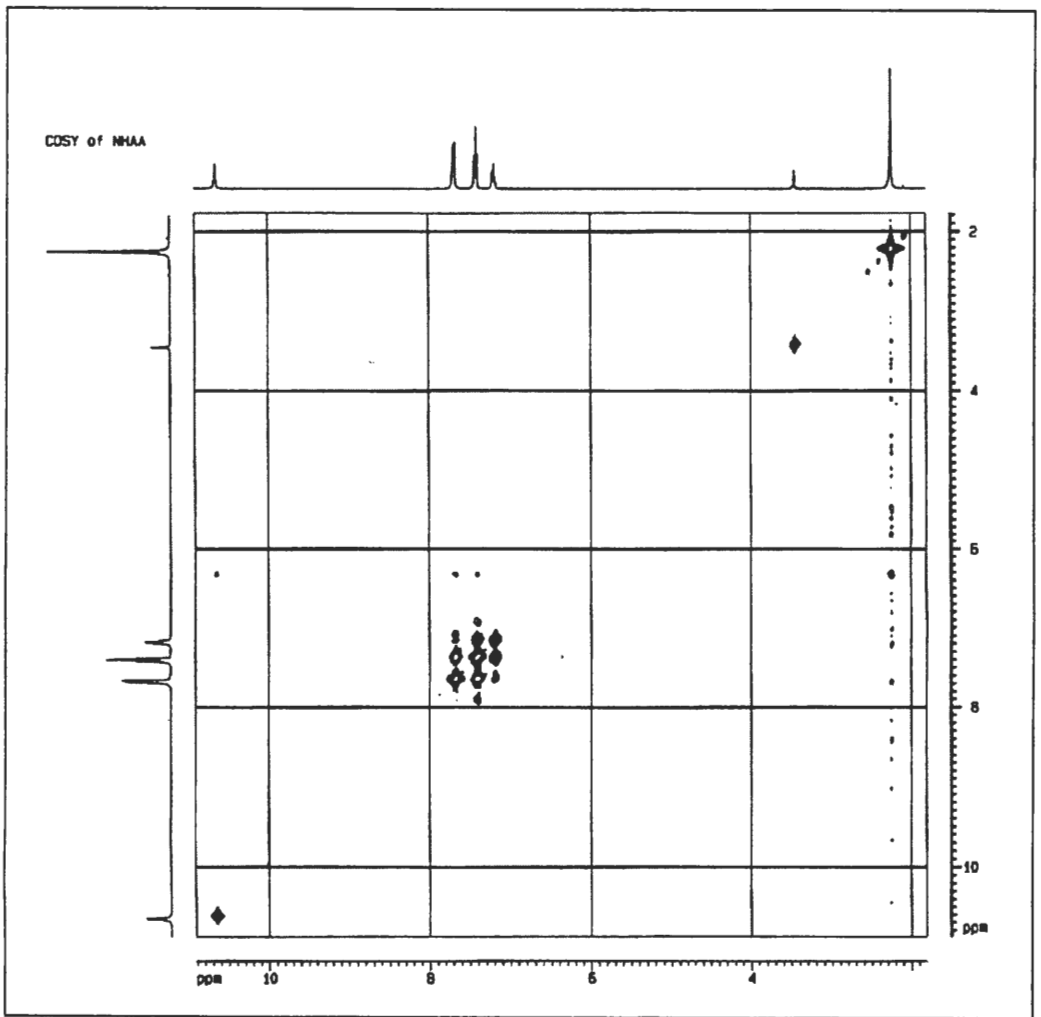


Figure 12 COSY NMR spectrum of NHAA in DMSO-d_6.

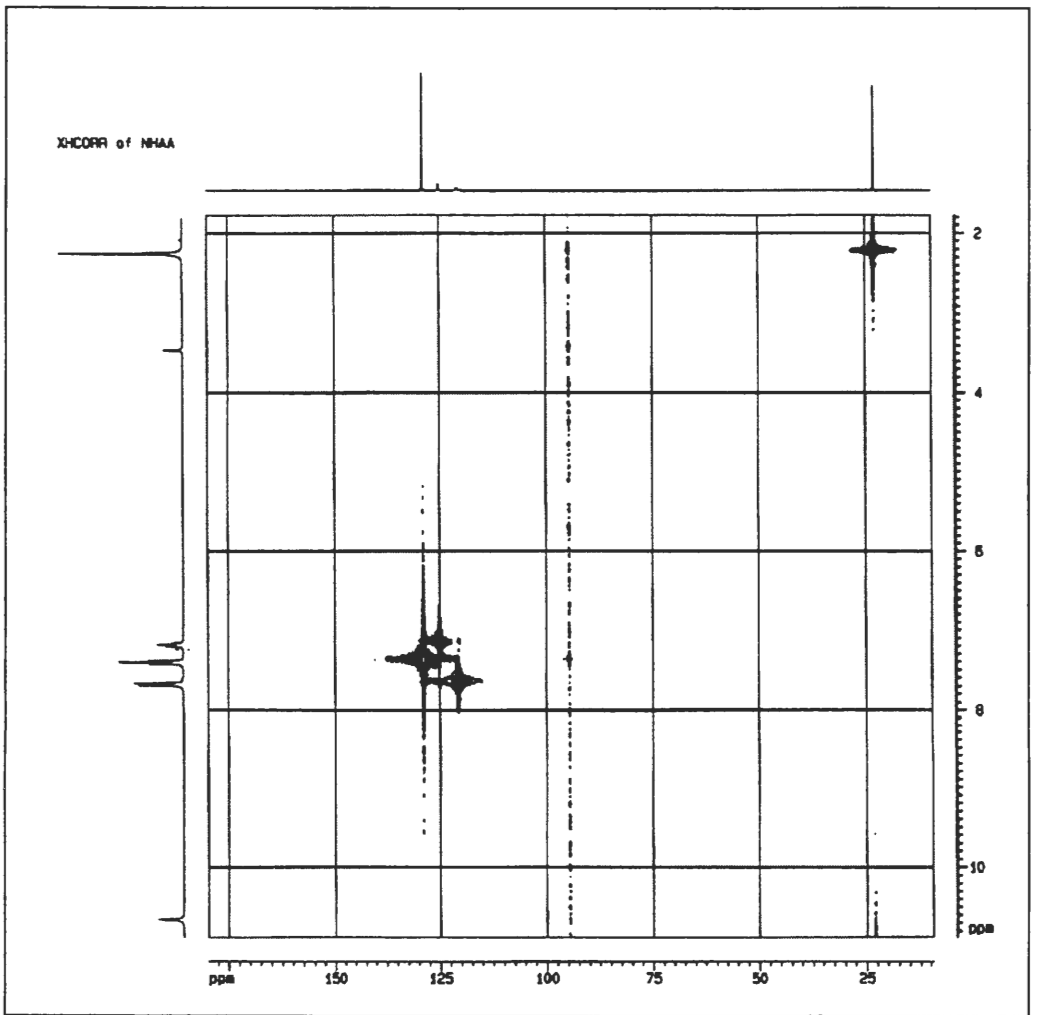


Figure 13 XHCORR NMR spectrum of NHAA in DMSO-d_6. ^{13}C spectrum is on top and ^{1}H NMR spectrum is on the left.

^{1}H NMR (DMSO-d_6): δ 10.66 (1H singlet), 7.67 (2H, brd dd), 7.40 (2H, brd dd), 7.18 (1H, brd dd), 2.25 (3H, singlet)
^{13}C NMR (DMSO-d_6): δ 128.87, 125.06, 120.69, 22.97

MEDIATOR STRUCTURE BIOBLEACHING EXPERIMENTS

Mediator structure experiments were performed on pulp SSBK26 with laccase LacNS51002 (3 mL for a 10 g o.d. pulp). All mediator doses were based on a molar equivalent of a 2% charge of HBT. The procedure was identical to the biobleaching procedure described

above, and all reaction times were carried out for 24 hours. Alkaline extraction stages were performed with a 2% NaOH charge at 70°C for 1 hour in a sealed bag.

FULL SEQUENCE BLEACHING EXPERIMENTS

The laccase stages in the full-sequence bleaching experiments were performed with HBT (2% charge) as the mediator and NS51002 laccase. The enzyme dose was 3 mL of enzyme per 10 g o.d. pulp (9.6 mg/10 g o.d. pulp), and the pulp that was used was SSOK13. All other conditions were identical to the fiber biobleaching experiments described earlier.

The alkaline extraction stage was performed in a sealed bag at 70°C and 10% consistency with a 2% alkali charge on o.d. pulp for 1 hour. The (E+O) and (E+O+P) extraction stages were also performed with a 2% alkali charge at 70°C and 10% consistency for 1 hour in the Parr reactor. The oxygen addition was performed by adding 60 psi O_2 for the first 15 minutes. The pressure was then decreased by 20 psi every 5 minutes until atmospheric pressure was reached. The peroxide charge was 0.5% H_2O_2 for the (E+O+P) stage. The pulp samples were washed with 2000 mL of distilled water per 60 g batch after each treatment.

The chlorine dioxide stages were performed at 10% consistency for 3 hours in a sealed bag at 70°C, after initial pH was adjusted to 10. The hydrogen peroxide stages were performed at 10% consistency at 90°C in a sealed bag for 4 hours. Pulp samples were washed with distilled water (2000 mL per 60 g batch) after treatment.

SUPEROXIDE DISMUTASE (SOD) EXPERIMENTS

SOD experiments were performed with the same procedure described in the fiber biobleaching section except SOD powder was added before the laccase solution. Laccase LacNS51002 was used in each experiment at a 3 mL per 10 g o.d. pulp dose. The pulp used was SSOK13. The pulp slurry was mixed for one minute after the SOD was added, and the laccase solution was added and mixed as described earlier. The thermally degraded SOD experiments were performed by adding 30 mL of distilled water to the SOD powder and heating it at 90°C for one hour. The mixture was then transferred into the pulp slurry before the laccase addition.

ISOLATED LIGNIN CONTROLS

Three lignin isolation samples were collected and analyzed by ^{1}H NMR and ^{13}C NMR analysis. This study was used to calculate "least significant difference" (LSD) analysis. LSD was used to determine if a difference in quantitative analysis was statistically significant. The control experiments were performed to allow for as many possible sources of error to be incorporated in the study. Three pulp samples from WSBK25 were treated with a control biobleaching experiment which was a biobleaching stage without laccase or a mediator. The samples were then treated with a typical alkaline extraction (described in Fiber Biobleaching section). After washing and air-drying, residual lignin was isolated from each sample separately. Chemical analysis was performed on each sample (Tables 9-10). The LSD was used in Chapters 6 and 7 when comparing differences in residual and reacted lignin structure. LSD was calculated using the following equation: $LSD = 2 * t * s$ (with $t = 2.92$ and s standard deviation, which was calculated for each functional group separately).

Table 9 ^{13}C NMR analysis of residual lignins in the control study. Least Significant Difference (LSD) analysis was performed on each functional group separately.

^{13}C NMR Analysis					
Functional Group	**Integral region (ppm)**	**Control 1**	**Control 2**	**Control 3**	**LSD**
COOH	180.1-165.1	0.394	0.392	0.413	0.067
C3/C3' C5-C5	160.1-154.1	0.125	0.118	0.093	0.098
C3,C4 (C-Ar-O)	154.1-139.8	1.738	1.776	1.776	0.129
C1 (C-Ar-C)	139.8-126.8	1.427	1.426	1.415	0.039
C5 (C-Ar-C)	126.8-122.5	0.583	0.586	0.588	0.015
C6 (C-Ar-H)	122.5-116.6	0.812	0.811	0.815	0.014
C5 (C-Ar-H)	116.6-113.6	0.456	0.438	0.463	0.075
C2 (C-Ar-H)	113.6-106.4	0.865	0.861	0.855	0.028
Aliphatic C-O (Cβ in β-0-4)	89.5-78.4	0.691	0.561	0.625	0.379
Aliphatic C-O (Cα in β-O-4)	78.4-67.4	0.838	0.693	0.814	0.454
Aliphatic C-O	67.4-61.3	0.377	0.282	0.373	0.314
Cγ (β-O-4)	61.3-57.4	0.272	0.235	0.286	0.153
OCH3	57.4-53.9	0.819	0.778	0.894	0.343
Cβ in ββ and Cβ in β5	53.9-51.2	0.227	0.191	0.247	0.168

Table 10 ^{1}H NMR analysis of residual lignins in the control study. Least Significant Difference (LSD) analysis was performed on each functional group separately.

^{1}H NMR Analysis					
Functional groups	**Integral region (ppm)**	**Control 1**	**Control 2**	**Control 3**	**LSD**
COOH	13.5-11.7	0.974	0.956	1.017	0.18
Formyl H	10.0-9.4	0.541	0.568	0.501	0.20
Total Phenolic OH	9.4-8.0	1.780	1.754	1.736	0.13
Aromatic H	7.7-6.3	10.571	10.732	10.221	1.53
Aliphatic H	6.3-4.1	9.483	9.512	8.768	2.46
Methoxy H	4.1-3.4	14.879	15.011	14.460	1.68

ISOLATED LIGNIN REACTIONS

Residual lignin was isolated from pulp SSBK26. Two lignin isolation batches (125 g o.d. pulp each) were combined and well mixed to obtain 4.6 g of isolated lignin (yield 48%). All lignin reactions were performed with this lignin sample.

Lignin reactions were performed on a 250-mg scale with the mediator and laccase (LacNS51002) charges remaining constant. The mediator and enzyme dose was based on the amount of reagents used for laccase HBT biobleaching of SSBK26. The 2% charge of HBT on o.d. weight of pulp was used with respect to the amount of lignin present in a brownstock pulp with 26 kappa number. All mediators were dosed at a molar equivalent of HBT. The procedure was as follow: 250 mg of lignin was weighed out into a 50 mL Erlenmeyer flask and magnetically stirred with 5 mL of dioxane: water in a 9:1 ratio. The flask was placed in the Parr reactor and heated to 45°C with the stopper on the Erlenmeyer flask (1 inch of water was added to the Parr reactor to allow for even heating). After heating for 1-2 hours, 0.125 g of HBT was added ($9.3x10^{-4}$ moles or other mediator with the proper weight). The mixture was mixed for 2 minutes and the pH was adjusted to 4.5 with acetic acid or a satuated solution of sodium bicarbonate, depending on the mediator used. Laccase LacNS51002 was then added (1.8 mL). The mixture was then pressurized with O_2 to 145 psi and reaction timing was started. The mediator study was performed with 4-hour reaction times, but the laccase-only study and laccase- HBT study were performed with 1,4, and 8 hour reaction times. The laccase-only experiments were performed with the same procedure described above except no mediator was added, and the pH was adjusted to 4.5 with acetic acid.

The reacted lignins were isolated by first transferring the reaction mixture to a round bottom flask, using 100 mL of distilled water. The mixture's pH was adjusted to ~6.2 and concentrated under reduced pressure to ~25 mL. Approximately 200-300 mL of distilled water was added, and the sample was concentrated under reduced pressure again to help remove all the dioxane. The mixture was then diluted to 1000 mL with distilled water. The pH of the mixture was adjusted to 2.0, and the mixture was transferred to centrifuge bottles, and then frozen. The samples were thawed, centrifuged and decanted. This step was repeated twice more, and the lignin samples were then freeze-dried. The lignin samples were then acetone-extracted in a soxhlet for 8 hours. The extracted lignin samples were air-dried and then dried under vacuum.

RESULTS AND DISCUSSION

The results and discussion section has been divided into seven chapters with each chapter describing the experimental data that related to the objectives of this thesis as described earlier. Chapter 1 concerns conditions needed for active delignification by laccase and HBT. Also presented in Chapter 1 is the structure changes that occur to the residual lignin during biobleaching with laccase and HBT. This information was used to test the proposed mechanism for delignfication by the laccase mediator biobleaching system (LMS).

The study in Chapter 2 investigates the reaction between laccase and HBT. This information was used to understand the efficiency and the mechanism of laccase mediator reaction. The chemistry that occurred during these model experiments was also examined in the presence of pulp to determine if the same reactions occurred. The study in Chapter 3 examines HBT type structures as possible mediators. These chemical structures were also examined by model programs to predict the components needed by an effective mediator.

The study in Chapter 4 concerns an applied study that determines how other bleaching chemicals react with laccase HBT-treated pulp. This study used hydrogen peroxide, chlorine dioxide, and oxygen as other bleaching chemicals in full-sequence bleaching trials. The study in Chapter 5 examines the radical mechanism that was proposed as the active delignification agent, and also examines the activity of laccase after biobleaching with several active mediators. The study in Chapter 6 examines the residual lignin of hardwood and softwood pulps treated with laccase HBT. This chapter also examined the effect of reaction time of the laccase HBT treatment on residual lignin structure.

Chapter 7 examines the changes in functional groups of isolated residual lignin that occurred when reacting laccase in the presence or absence of a mediator. This study was used to determine if each mediator reacted differently with lignin.

CHAPTER 1
RESIDUAL LIGNIN ANALYSIS OF LACCASE HBT REACTED PULPS

INTRODUCTION

The role of laccase in biological systems to catalyze the polymerization and depolymerization of lignin has been extensively investigated (26). Historically, the use of laccase to delignify kraft pulps has been limited because the enzyme can not diffuse into pulp fibers due to size limitations (92). Attempts to circumvent these limitations have focused on the use of chemical mediators, which are believed to be oxidized by the enzyme and then believed to undergo oxidative reactions with lignin in the pulp fiber. 2,2'-Azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid was one of the first reported mediators for laccase, and its application has been extensively studied (50,93,94). N-hydroxybenzotriazole has been reported to be a more effective mediator for laccase-assisted delignification of chemical pulps, (54,95) and, in recent years, several other mediators (96,97) have also been discovered. Despite these advances in biobleaching, the amounts of mediator required and the associated process costs have hindered commercial applications.

Future improvements in laccase-assisted delignification of kraft pulps would be enhanced if the fundamental chemical reactions contributing to this process were understood. This chapter examines the effect that laccase and N-hydroxybenzotriazole have on the structure of lignin during biobleaching. Several reaction conditions will also be examined to better understand the conditions needed for delignification by the laccase HBT biobleaching system.

RESULTS AND DISCUSSION

The effects of the laccase/N-hydroxybenzotriazole biobleaching system were explored with industrial softwood kraft pulps (WSBK25 and WSOK17) isolated before and after oxygen delignification. In each case, the pulp was treated with laccase HBT for 24 hours at 45° C and 10 bar of O_2 pressure and then alkaline extracted (LMS-sequence). Optimal enzyme dosage was determined by increasing the enzyme dose while holding all other reaction conditions constant (see Figure 1.1). A sharp decrease in kappa number was detected at 400,000 U for a 10g batch of oven dry pulp, and increasing the enzyme dosage did not increase lignin removal. Variations in the mediator dosage from 200 mg to 400 mg per 10g of oven dry pulp per 400,000 U laccase produced no increase in lignin removal for either the Pre- and Post-oxygen delignified kraft pulps.

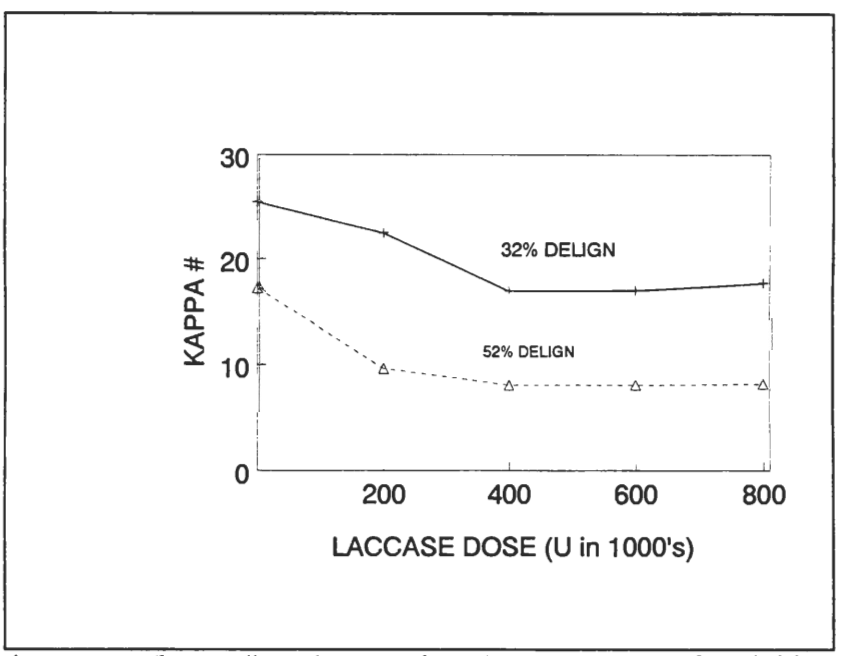


Figure 1.1 Lignin content (kappa #) vs laccase dose (LacPPQ-4211) for biobleaching of WSBK25 (+) and WSOK17 (Δ) with hydroxybenzotriazole as the mediator. The mediator dose remained constant at 200 mg/10 g of oven dry pulp. The "Delign" values were taken at a laccase dose of 400,000 U.

As shown in Figure 1.1, optimal delignification results were achieved with the post-oxygen delignified kraft pulp (WSOK17) (discussed in more detail in Chapter 4). Similar results have been noted by Call (98). The mediator dose was not changed in these experiments, but further trials, where the dose of mediator was 2 to 4 times the 2 % charge, revealed no further delignification (the enzyme dose was set at optimal or 2 times the optimal dosage shown above). A mediator dose of 1 %, which was one-half the dose used in Figure 1.1, decreased delignification. These studies revealed a large range of conditions where the laccase HBT biobleaching systems remained active, and excess dosages of bleaching chemical (laccase and HBT) had no detrimental effect on pulp viscosity.

Table 1.1 summarizes the physical properties of the pulps after maximizing the biobleaching effect (400,000 U LacPPQ-4211/ 10 g o.d. pulp) of the LMS. Although the post LMS pulps suffered viscosity losses, the values are higher than many chemical bleaching agents at comparable levels of delignification (99).

Table 1.1. Laccase/N-hydroxybenzotriazole delignification of kraft pulps.

Kraft Pulp	**Prior to Biobleaching**		**Post-LMS**	
	Lignin Content[a] (kappa #)	**Viscosity (cP)**	**% Delignification[a]**	**% Viscosity Loss**
Pre-O_2	25.5	30.7	32	20
Post-O_2	17.2	26.8	53	31

[a]% Delignification values and pulp viscosity measurements represent an average of two experiments, and these values varied by less than 1%.

The relative delignification efficiency of N-hydroxybenzotriazole versus 2,2'-azino-

bis(3-ethylbenzthiazoline-6-sulfonic acid) with laccase was examined with a Post-O_2 softwood kraft pulp. Employing molar equivalent amounts of either mediator (1.50×10^{-3} moles) and 400×10^3 units of laccase for 24 hours at 45° C, the laccase/N-hydroxybenzotriazole system achieved 52% delignification, whereas laccase ABTS provided 35% delignification (Note: Employing lower dosages of ABTS, 30% of the above charge, provided the same extent of delignification). These studies suggest that the laccase/N-hydroxybenzotriazole system was more efficient at removing lignin than laccase/ABTS. This was consistent with recent findings by Paice; although, our results indicate a larger difference in biobleaching efficiency for these two mediators (100). The source of this difference could be the type of laccase used.

The mechanisms contributing to the laccase/N-hydroxybenzotriazole biobleaching effect were explored by characterizing residual lignin before and after the biobleaching/alkaline extraction stage. The residual lignins from the two starting kraft pulps were isolated by employing an acidic dioxane extraction procedure described in the Experimental section. These conditions were believed to be catalyzed by the hydrolysis of lignin-carbohydrate bonds, facilitating the dissolution of lignin in an aqueous dioxane solution. Subsequent work-up procedures provided lignin samples that were readily analyzed by FT-IR and high-field NMR. The same isolation procedures were then employed for the LMS-treated kraft pulps. In addition to the residual lignin samples extracted from the pulp, the lignin fragments present in the effluents of alkaline extracts from each laccase-treated pulp were acid-precipitated, purified, and analyzed.

Qualitative analysis of the residual lignin samples by FT-IR suggested that the lignin isolated from the starting pulp, post-LMS/alkaline extraction treated pulps, and the alkaline

effluents were structurally similar (Figure 1.2) (73). The most notable change in functional groups was the increase in the relative signal intensity of the absorption centered at 1719 cm^{-1}. This signal has been assigned to carboxylic acid and unconjugated carbonyl groups in ketones and aldehydes, suggesting that residual lignin after an LMS-stage is enriched in these types of functional groups (73).

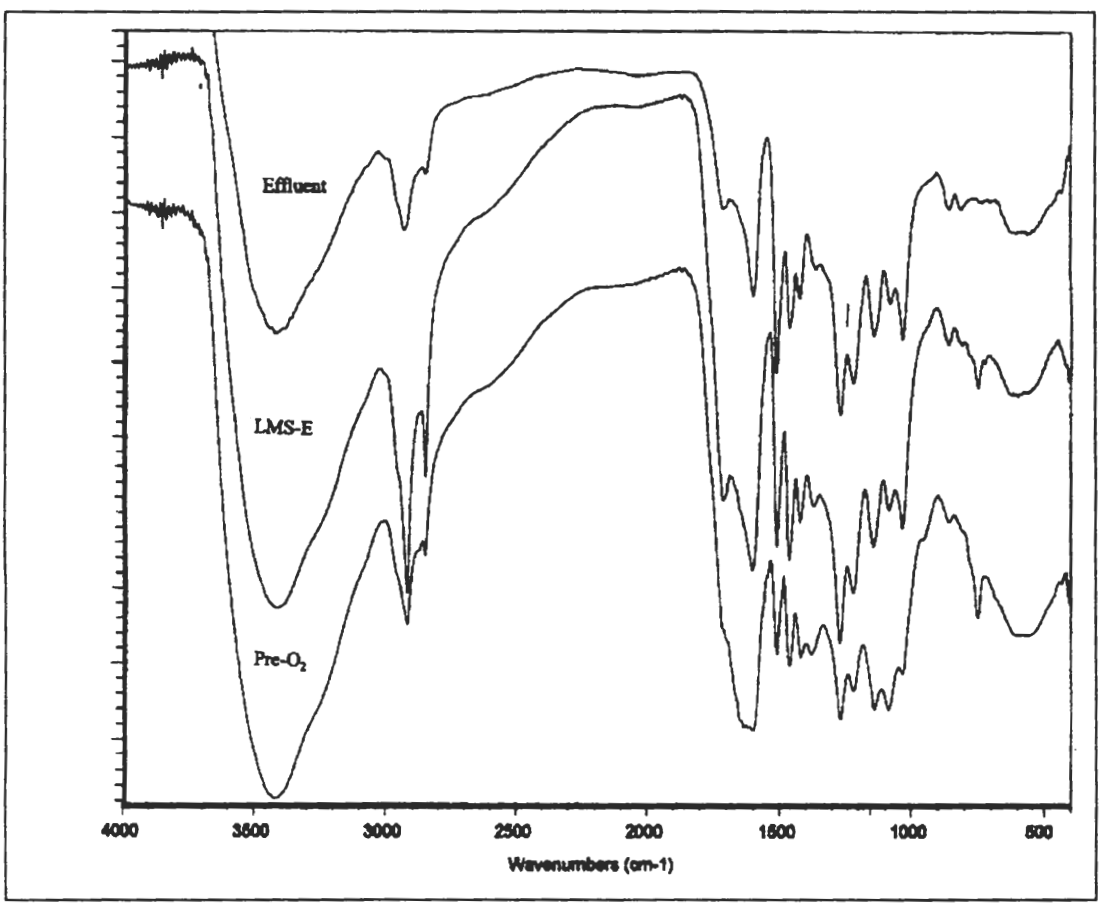


Figure 1.2 FT-IR of pre-O_2 residual lignin, LMS residual lignin, and effluent lignin in the alkali extract from the LMS treated pre-O_2. The spectrum are presented in transmittance mode.

^{13}C NMR analysis of the residual lignin samples and alkaline effluents provided additional information on the nature of the structural changes occurring to lignin during the

LMS. Figure 1.3 provides an example of the spectral data acquired for the lignin samples isolated. Some of the key lignin functional group assignments are highlighted on the spectra, and the assignments were based upon well-established literature values (73). All spectral data were acquired under quantitative conditions, and simple integration provided a facile means of monitoring changes in functional group distribution. The results were summarized in Table 1.2. The yield of residual lignin from each pulp sample ranged from 45-57 % based on kappa number analysis (Table 1.3).

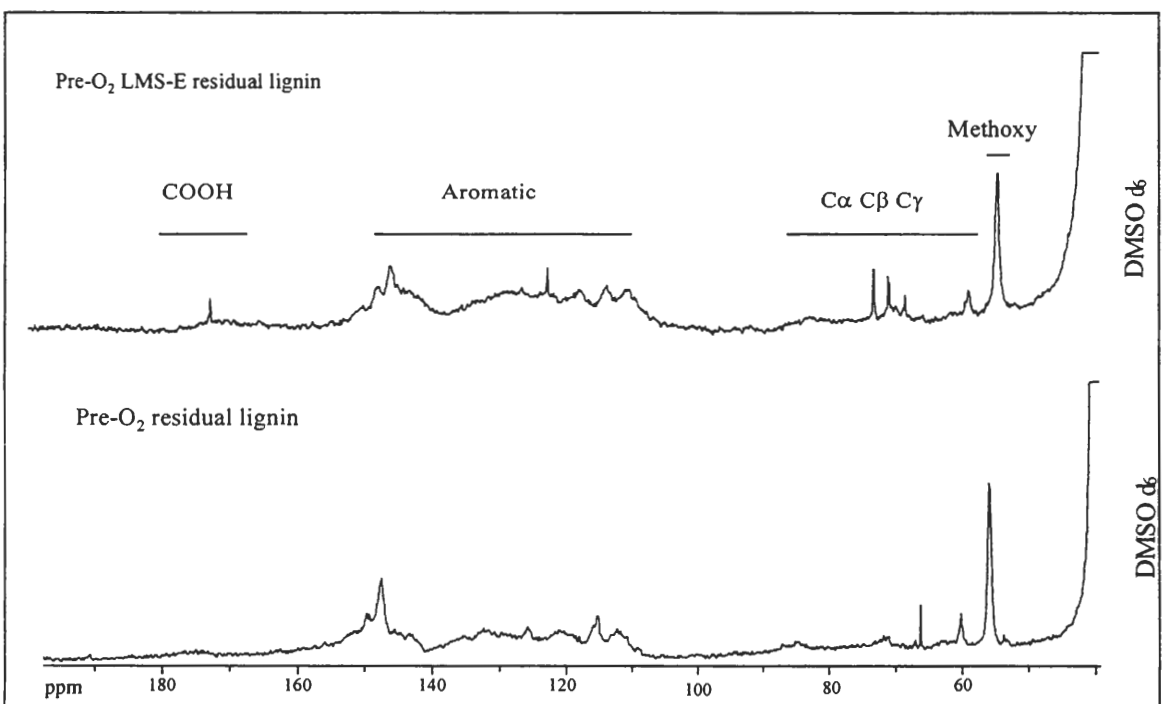


Figure 1.3 Quantitative ^{13}C NMR analysis of residual lignin from Pre-O_2 delignified kraft pulp (WSBK25) and WSBK25 LMS residual lignin.

Inspection of the data in Table 1.2 suggests that the lignin remaining in the kraft pulps after an LMS stage was enriched in acid groups and exhibited a small decrease in substituted guaiacyl groups and a slight enrichment of β-O-4 aryl ether linkages. Both the Pre- and Post-

oxygen delignified kraft pulps also appeared to undergo a loss in methoxy content and a significant decrease in phenoxy content. The removal of methoxy groups from lignin was comparable to experiments reported by Bourbonnais and Paice with laccase ABTS (93).

These results suggested that the laccase/N-hydroxybenzotriazole treatment selectively oxidizes free phenoxy groups in lignin, which were then removed during the alkaline extraction stage. Equally significant was the apparent unreactivity of the β-O-4 aryl ether linkage of lignin toward the LMS bleaching protocol.

Table 1.2. Lignin functional-group distribution for kraft pulps before and after biobleaching treatment as determined by quantitative ^{13}C NMR.

Lignin Sample[b]	**RCO_2H (δ 180.1-165.1 ppm)**	**C-3,4 of substituted guaiacyl units (δ 154.1-139.8ppm)**	**C-3,4 of guaiacyl and demethylated guaiacyl units (δ 160.1-154.1ppm)**	**β-O-aryl ether Cβ (δ 89.5-78.4ppm)**	**CH_3O- (δ 57.4-53.9ppm)**
Pre-O_2 pulp	0.44	2.20	0.39	0.38	0.84
Pre-O_2 pulp after LMS	0.50	1.75	0.15	0.39	0.77
Pre-O_2 LMS Alkaline-effluent Lignin	0.84	1.76	0.35	0.55	0.62
Post-O_2 pulp	0.47	2.06	0.29	0.49	0.81
Post-O_2 pulp after LMS	0.56	1.79	0.24	0.53	0.57
Post-O_2 Alkaline-effluents Lignin	1.05	1.72	0.33	0.54	0.66

[a]All assignments based on literature values for lignin functional groups[14]; [b] all values are relative and were determined by integrating individual spectrum, assigning the aromatic section of the spectrum (δ 160.0-106.5 ppm) a value of 6, and then measuring all other portions of the spectrum relative to this arbitrary assignment.

Table 1.3. Yield of residual lignin isolated from the kraft pulps and alkaline extractions.

Pulp Yield%[a]	Pre-O2 (WSBK25)	Post-O2 (WSOK17)	WSBK25 LMS treated	WSOK17 LMS Treated
Residual Lignin	55	41	57	45
E Effluent Lignin	------	------	15	13

[a]The yield of lignin was calculated following literature methods (73), lignin yield = [mass lignin isolated/{(kappa # of pulp before treatment-kappa # of pulp after treatment) x 0.15}]%.

The analysis of the effluents was complicated by the fact that the multi-step acid-precipitation procedure provided only 10-15% (theoretically recoverable amount calculated by kappa number analysis) of the suspended lignin released into solution. For the effluents recovered after a LMS (Pre and Post-O2), the spectra exhibited a significant increase in acid content but otherwise as comparable in structure to the residual lignin structures.

^{31}P NMR analysis of the residual and effluent lignins agrees with the ^{13}C NMR analysis, namely the concentration of carboxylic acid groups increased with a LMS stage. The concentration of phenolic functional groups decreased with an LMS stage. The effluent lignins had lower phenolic and higher carboxylic acid group concentrations than the residual lignins. The residual lignins after a LMS stage were not enriched in condensed phenolic structures.

Table 1.4 ^{31}P NMR analysis of residual and effluent lignin. All function group values are expressed as mmol/g of lignin.

Lignin Sample	COOH	Total Phenoxy content	Guaiacyl and Demethylated Phenolics	Condensed Phenoxy groups	Aliphatic Hydroxy Groups
Pre-O_2 Pulp (WSBK25) Residual Lignin	0.29	2.03	1.08	0.95	1.70
WSBK25 LMS Residual Lignin	0.47	1.07	0.54	0.53	1.17
WSOK17 LMS E Effluent Lignin	0.71	0.75	0.33	0.42	1.38
Post-O_2 Pulp (WSOK17) Residual Lignin	0.41	1.48	0.76	0.72	2.20
WSOK17 LMS Residual Lignin	0.68	0.74	0.34	0.40	1.01
WSOK17 LMS E Effluent Lignin	0.62	0.41	0.17	0.25	1.11

CONCLUSIONS

In summary, these results demonstrate that the laccase/N-hydroxybenzotriazole bleaching system can very effectively remove lignin from kraft pulps via a series of oxidative degradation reactions. It appears, from the NMR analysis, the principal site of oxidative attack is the free phenoxy groups of lignin, but condensed free phenolic groups were not enriched with the LMS treatment. The role of the chemical mediator, N-hydroxybenzotriazole, is key to these reactions. Literature presented after this work confirms these conclusions (144).

CHAPTER 2
LACCASE HYDROXYBENZOTRIAZOLE REACTIONS

INTRODUCTION

Laccase-mediator biobleaching involves many reactions between several substrates. The mediator, lignin fragments, and oxygen are proposed to react in an oxidative and reductive environment, but the intermediates or exact mechanism is unknown. Before the laccase-mediator biobleaching system can be optimized, a better understanding of the mechanism is needed. The mechanisms of oxidation and reduction begin with the reaction between the active site of laccase and oxygen.

The active site of laccase has been shown to contain four copper ions constituting three different oxidation-reduction sites, all of which are essential for the catalytic activity of this enzyme (91). Xu has reported that the redox potential for the three types of copper (Note: frequently referred to as type 1, 2, & 3) atoms in laccase varies from +0.35 to +0.79 V (101). The active site of laccase has been studied in detail, and it has been shown that the type 2 and 3 coppers function as a trinuclear copper complex with respect to oxygen and other ligands (102-106). Solomon has extensively studied the redox chemistry of laccase and has suggested that the type 2 and 3 copper sites form a peroxide-copper complex after binding oxygen at the active site (106). The proposed peroxide structure is shown in Figure 2.1.

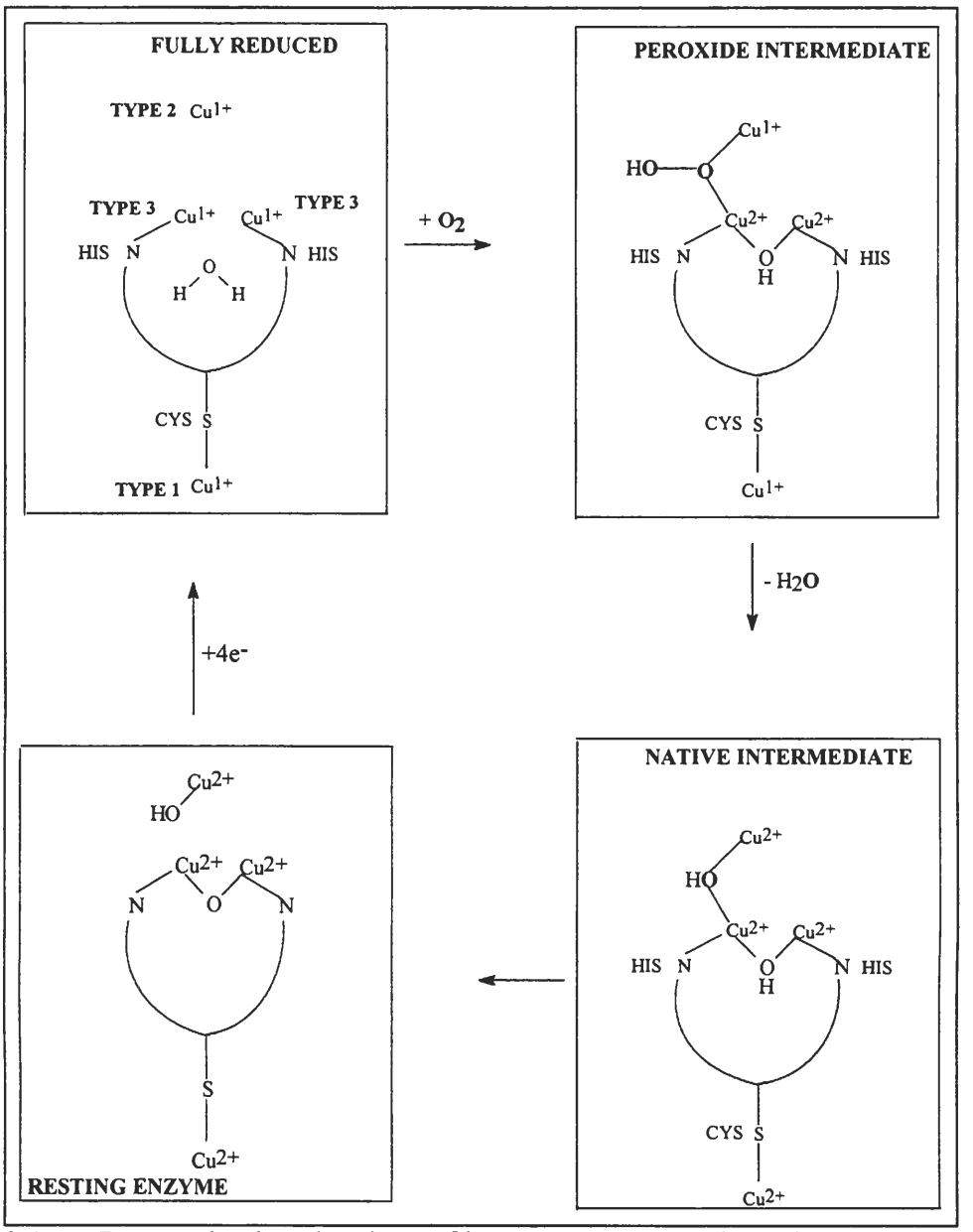


Figure 2.1 Proposed redox chemistry of laccase as presented by Solomon (106).

The peroxy-copper complex is then further reduced by type 1 and type 2 copper, yielding a molecule of water. The oxidized copper species can then be reduced by an exogenous substrate. The oxidative properties of these copper atoms become the key functional unit involved in oxidizing lignin and future biobleaching technologies.

An overall picture of the active site has been modeled by Xu (101). This model provides an overview of the oxidation and reduction reactions, and it also shows the point of inhibition of fluorine ions and hydroxide ions (Figure 2.2). In this model the phenolic substrate, which was used to picture the reducing substrate, was replaced with HBT, which is a mediator structure. Very little has been published on the chemistry between mediators and laccase (94,95).

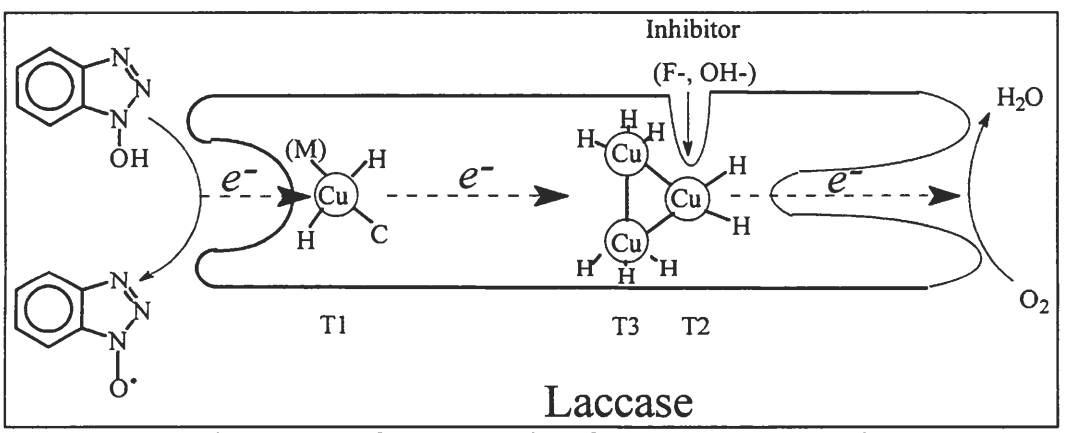


Figure 2.2 Proposed overview of the active site of laccase as presented by Xu.. The T1-T3 represent the copper atom type in laccase (101).

The reaction between the activated copper atoms in laccase and a substrate has been examined mainly with lignin model compounds. It has 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 (6,24,25). Hence, the reactions of laccase with lignin and lignin model compounds have been studied extensively. Figure 2.3 illustrates typical laccase-catalyzed degradation reactions of lignin (23). Most researchers believe that a phenolic group must be present before laccase can react with a lignin model compound (23-25).

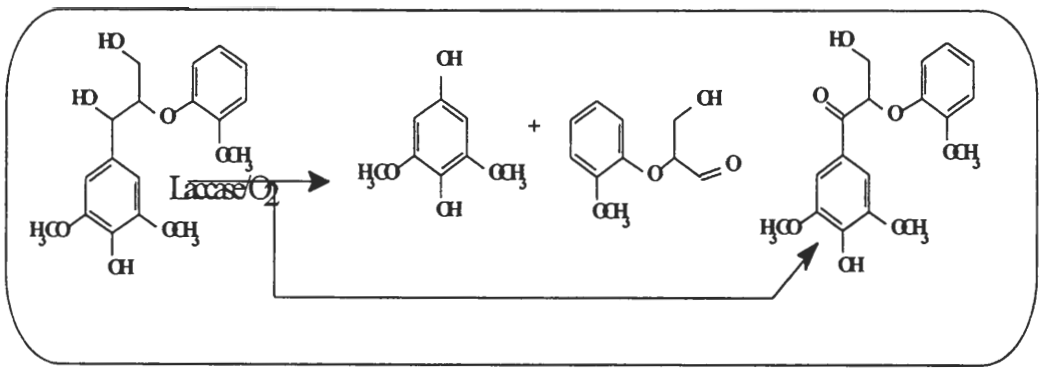


Figure 2.3 Proposed laccase degradation of lignin model compound (23).

Despite the rather efficient degradation of lignin by laccase, the utilization of laccase during pulp bleaching has been not feasible because the enzyme can not diffuse into a lignocellulosic fiber where most of the residual lignin is located. The first true breakthrough in this field came about when Bourbonnais and Paice demonstrated that the addition of ABTS (2,2'azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) could lead to substantial delignification of kraft pulps (50). Although the cost and extent of delignification achieved with an ABTS-laccase system prevented commercialization, this advancement initiated a flurry of fundamental research studies into laccase-assisted bleaching technologies (94-95).

RESULTS AND DISCUSSION

LACCASE HBT REACTIONS

As a preliminary investigation into the nature of the active delignification agent present during biobleaching with laccase and N-hydroxybenzotriazole, we elected to examine the chemical reactions that occur between these two species in the absence of

kraft pulp. Purified laccase (5.2 units of activity) was added to 2 mg of N-hydroxybenzotriazole in 0.8 mL of deuterium oxide (see Experimental section). This mixture was periodically analyzed by ^{1}H NMR and it gradually became apparent that the enzymatic treatment converted N-hydroxybenzotriazole into a new material (see Figure 2.4).

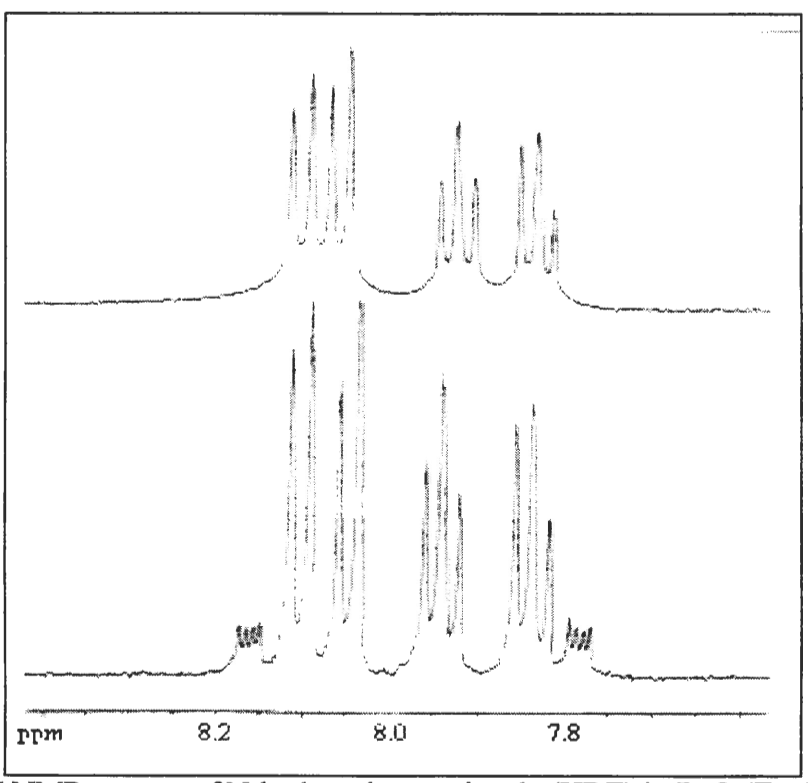


Figure 2.4 ^{1}H NMR spectra of N-hydroxybenzotriazole (HBT) in D_2O (Top) and ^{1}H NMR spectrum of the reaction between laccase (5.2 U LacNS51003) and HBT for 4 hr. at 23° C (Bottom).

Preparative TLC chromatography allowed for isolation of the converted material from two separate large-scale reactions between laccase and HBT. The new compound was characterized by NMR (^{1}H and ^{13}C) and mass spectroscopy (EI and exact mass) analysis, and all analyses indicated that this new component was benzotriazole (data shown in the

Experimental section). Only two products were detected by TLC. The other compound that was isolated and characterized by ^{1}H and ^{13}C NMR was HBT. This previously unreported reaction suggested that N-hydroxybenzotriazole was converted into benzotriazole through a reductive pathway in the presence of laccase. The rate of this conversion process was readily determined by monitoring the reaction by ^{1}H NMR; the results are summarized in Figure 2.5.

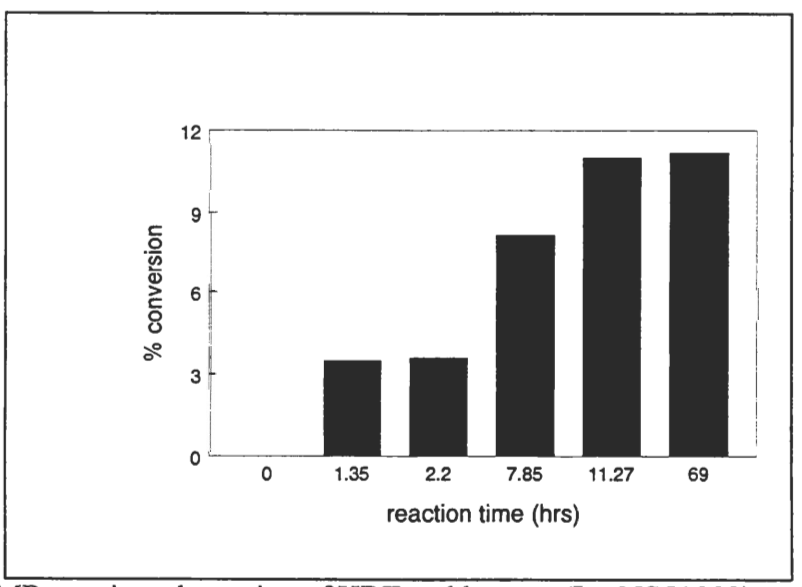


Figure 2.5 ^{1}H NMR monitored reaction of HBT and laccase (LacNS51003) at 45° C. Percent conversion was calculated from the ratio of the integration of HBT and the new species.

The time studies demonstrate that laccase was capable of converting 11% N-hydroxybenzotriazole into benzotriazole. Several control experiments were performed. HBT was heated at 45° C under 145 psi O_2 pressure for 24 hours, and no converted product was detected. HBT was also reacted with denatured laccase, and no converted product was detected. On this basis, it became evident that active laccase was needed to convert HBT into benzotriazole (BT).

MEDIATOR, LACCASE, AND PULP EXPERIMENTS

To determine if the conversion of HBT into BT was relevant to the laccase/N-hydroxybenzotriazole biobleaching system, we examined the fate of the mediator at the conclusion of a laccase-mediator stage (LMS). An acetone-extracted brownstock kraft pulp (SSBK26) was treated with the LMS-stage (LacPPQ-4211) for 24 hours. After the LMS stage, the pulp mixture was washed with 1200 mL of distilled water. The pulp was air-dried and acetone-extracted. Analysis of the soxhlet extracts by ^{1}H NMR indicated a 16:84 mixture of N-hydroxybenzotriazole to benzotriazole.

The aqueous biobleaching effluents were freeze-dried, soxhlet-extracted with acetone and then analyzed by ^{1}H NMR. Analysis of the acetone extracts by ^{1}H NMR indicated the presence of only N-hydroxybenzotriazole and benzotriazole in a 16:84 mixture. The combined yield from the pulp (65 %) and effluents (34 %) was 99 %. This suggested that the conversion of the mediator into benzotriazole was a dominant reaction during an LMS.

To explore the relevancy of this mediator-conversion pathway to the biobleaching process, we repeated the LMS-bleaching stage with a brownstock kraft pulp (SSBK26), replacing N-hydroxybenzotriazole with benzotriazole. After 24 hours with the same oxygen pressure, solution pH, and enzyme dose used for the earlier active biobleaching conditions, no delignification was detected when compared to a control taken after an alkaline extraction. Hence, the conversion of HBT into benzotriazole must be viewed as a deleterious pathway that reduces the efficiency of an LMS-stage.

CONVERSION OVER TIME EXPERIMENTS

The conversion of HBT into benzotriazole was examined further by monitoring the conversion over time with brownstock (SSBK26) and Post-O_2 delignified pulp (SSOK13) (these pulps were not acetone extracted). The degree of delignification and pulp viscosity were also monitored to determine if a relationship existed between HBT conversion and delignification or pulp viscosity. The brownstock pulp treated with laccase HBT showed that significant delignification continued for up to 8 hours, and no real increase in delignification occurred between 8 to 44 hours of treatment. The enzyme and mediator dose was held constant for these experiments (Figure 2.6).

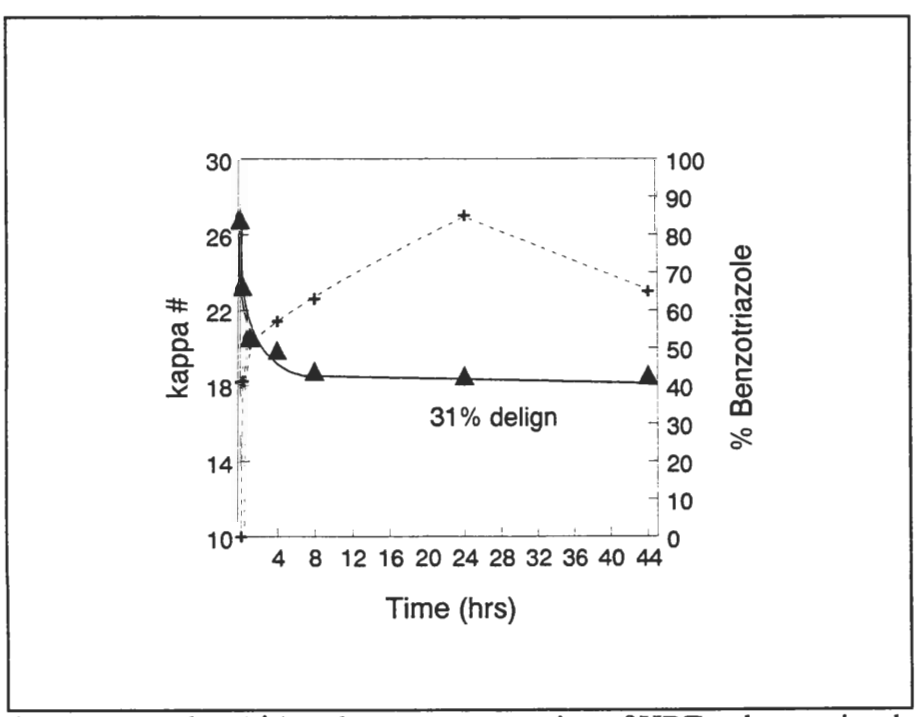


Figure 2.6 Kappa number (▲) and percent conversion of HBT to benzotriazole (+) vs. reaction time for a brownstock kraft pulp (SSBK26). All reactions were performed under 145 psi O_2 with a 2% HBT charge and 9.6 mg of laccase protein (LacNS51002) per 10 g of o.d. pulp.

The conversion of HBT into benzotriazole occurred rapidly, with over 40 % conversion of the mediator after 15 minutes of treatment. The percent BT peaked after 24 hours, and a strong trend existed between the conversion of HBT into an inactive mediator and pulp delignification. The analysis of the mediator was performed on the water wash fraction, which represented 53-75 % of the total mass of the mediator (see Experimental section for details). The analysis of the acetone extract of the biobleached pulp, which represented about +20 % of the total mass of the mediator, was complicated with extractives and derivatives of benzotriazole. This was determined by GC MS (Note: these pulps were not acetone-extracted before biobleaching and a new batch of laccase was used for these experiments; see experimental section). The amount of mediator recovered in the water wash decreased for the pulp experiments that were treated for a longer time. It appears that more benzotriazole or benzotriazole derivatives remained on the pulp after water washing (see Experimental section). This data may explain why the concentration of benzotriazole in the water extract fraction decreased after 24 hours.

Two biobleaching control experiments were performed with benzotriazole only and 25:75 mixtures of HBT and benzotriazole to determine if laccase could convert benzotriazole into HBT. No HBT was detected in the water wash or pulp acetone extract for either experiment, which strongly suggests that laccase can not convert benzotriazole to HBT.

The pulp viscosity measurements of the above experiments showed a slight decrease in the pulp viscosity for the first few hours of reaction, then no decrease in pulp viscosity was seen after delignification remained constant (Figure 2.7). The constant

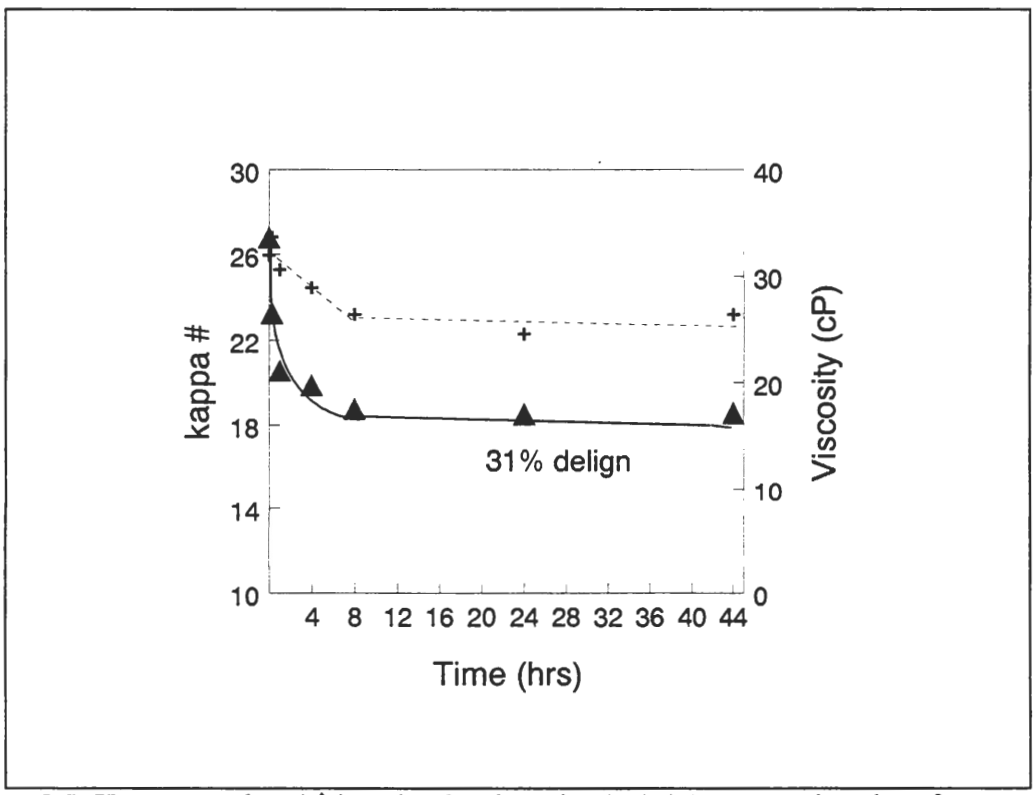


Figure 2.7 Kappa number (▲) and pulp viscosity (cP) (+) vs. reaction time for a brownstock kraft pulp (SSB26). All reactions were performed under 145 psi O_2 with a 2% HBT charge and 9.6 mg of laccase protein (NS51002) per 10 g of o.d. pulp.

pulp viscosity and constant kappa numbers at longer treatment times suggested that this bleaching system becomes inactive as the reaction time increases. These results also agreed with earlier studies where high dosages of laccase or mediator had no effect on pulp viscosity or kappa number (Chapter 1).

The conversion of HBT into benzotriazole and the optimal delignification time occurred faster for the Post-O_2 delignified pulp than for brownstock pulp (Figure 2.8).

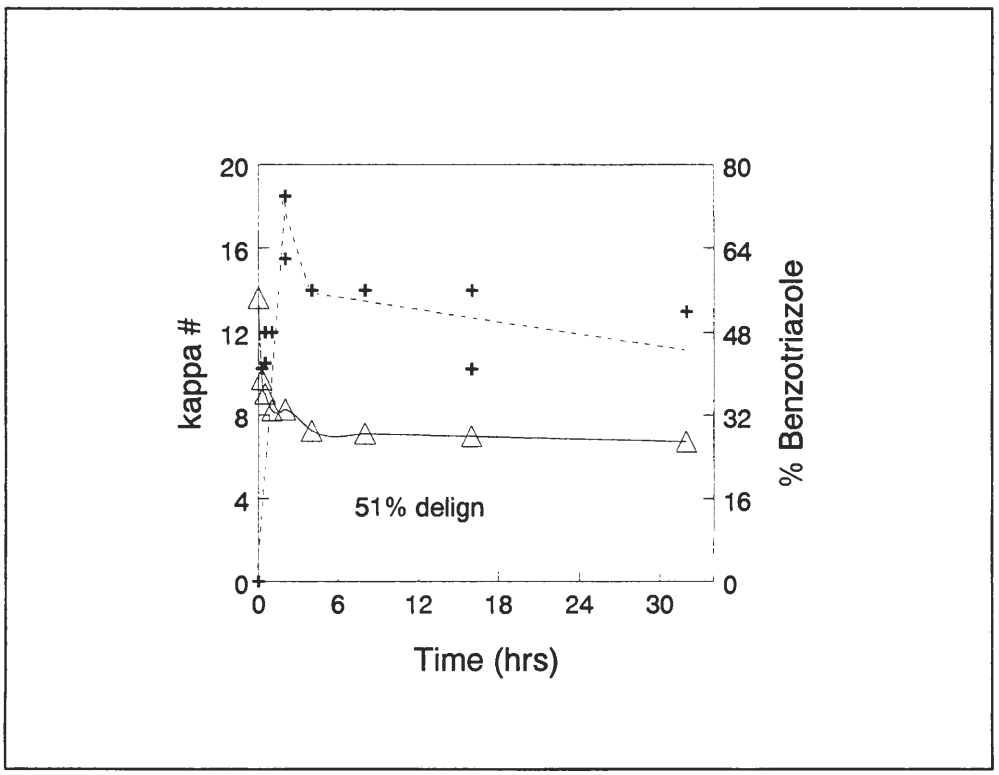


Figure 2.8 Kappa number (Δ) and percent conversion of HBT to benzotriazole (+) vs. reaction time for a Post-O_2 delignified kraft pulp (SSOK13). All reactions were performed under 145 psi O_2 with a 2% HBT charge and 9.6 mg of laccase protein (NS51002) per 10 g of o.d. pulp.

The optimal delignification time was 4 hours for the Post-O_2 delignified sample as compared to 8 hours for the brownstock pulp. The amount of lignin present appears to affect the rate of reaction (Note: the amount of lignin removed from the brownstock and Post-O_2 delignified pulp was nearly identical; 0.124 g for the brownstock pulp and 0.104 g for the Post-O_2 delignified pulp on a 10 g o.d. pulp bases). The conversion of HBT into benzotriazole for the Post-O_2 pulp also closely followed delignification, which agreed with the brownstock pulp experiments. Experiments at 0.5, 2, and 16 hours were repeated

to determine if the conversion trend was reproducible. These experiments agreed with earlier experiments within 10 % conversion.

The pulp viscosity measurement for the Post-O_2 delignified pulp samples showed the same trend as observed for the brownstock pulps (Figure 2.9). No significant drop in pulp viscosity was seen after optimal delignification time was reached.

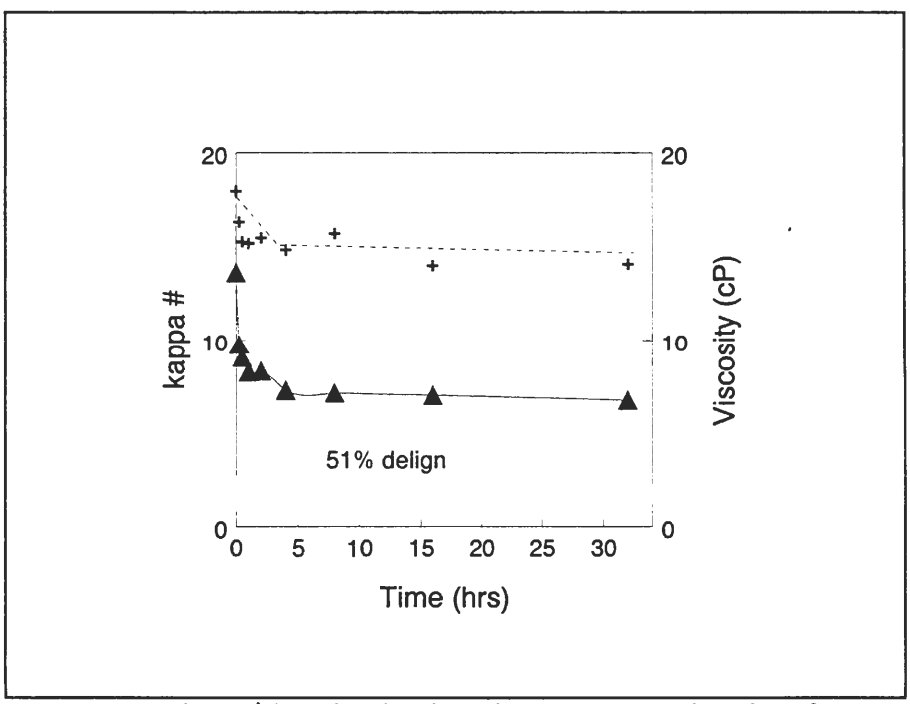


Figure 2.9 Kappa number (▲) and pulp viscosity (+) vs. reaction time for a Post-O_2 delignified kraft pulp (SSOK13). All reactions were performed under 145 psi O_2 with a 2% HBT charge and 9.6 mg of laccase protein (NS51002) per 10 g of o.d. pulp.

CONCLUSIONS

These studies provided the first report on the fate of N-hydroxybenzotriazole during an LMS-stage. Clearly, the mediator was not stable under these conditions and was partially converted into benzotriazole. The mechanism of conversion has not yet

been determined, but control studies indicate that active laccase must be present for this reaction to occur, and laccase can not convert benzotriazole to HBT. The conversion of HBT into an inactive mediator, benzotriazole, appears to limit the amount of delignification for the laccase HBT biobleaching system.

After our study was presented, the conversion of HBT into benzotriazole has also been reported by Paice and Potthast (100,113). Studies by Potthast with model compounds lead to the suggestion that the conversion of HBT to benzotriazole occurs via a radical coupling process between a phenolic radical and the radical intermediate of HBT (Figure 2.10). The coupled product, involving lignin, rearranges to from benzotriazole, but this does not explain why HBT was converted to benzotriazole in the presence of laccase only. One possible explanation involves the interaction of HBT and the amino acid tyrosine, which contains a phenolic functional group, in laccase.

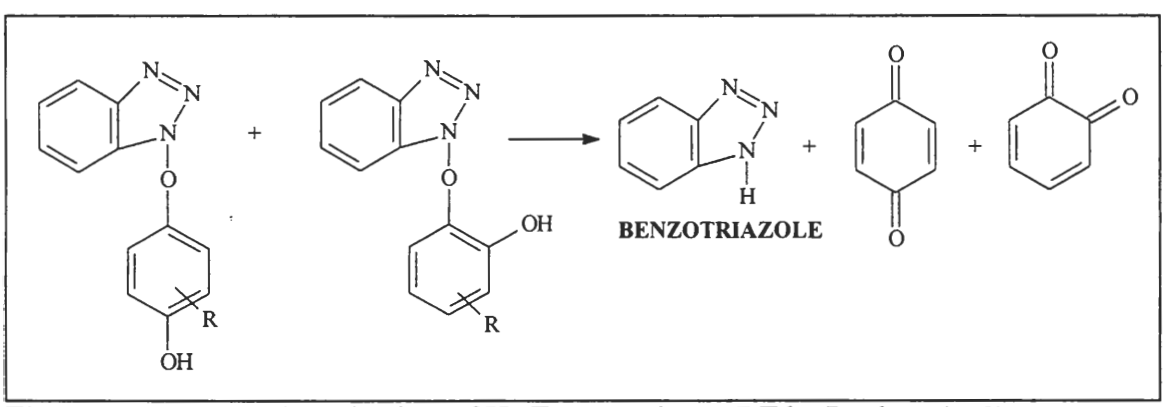


Figure 2.10 Proposed mechanism of HBT conversion to BT by Potthast (113).

CHAPTER 3
THE EFFECT OF MEDIATOR STRUCTURE ON DELIGNIFICATION WITH LACCASE BIOBLEACHING

INTRODUCTION

Since the laccase-mediator biobleaching system was introduced, most of the breakthroughs were due to mediator development. Researchers have agreed that, before the laccase mediator system can be implemented into a mill-scale process, the mediator must be low in cost and have no adverse effect on the environment. Almost all reported mediators for laccase contain nitrogen. This could create a problem in mill effluents where nitrogen levels are already too high, and the toxicity of the nitrogen compounds must also be considered.

The cost of the mediator also appears to be a barrier for all reported mediators, but this may be overcome if the mediator can be reused. The stability of the mediator, which has been shown to be a problem during biobleaching, becomes an important issue. Theoretically, a very low dose of mediator would be required for laccase biobleaching if the mediator reacted rapidly with the enzyme and had a high selectivity to lignin oxidation while remaining structurally stable during the entire redox cycle. Today, most mediator dosages have been reported to be around 1 to 2 % on weight of o.d. pulp which would suggest that very little of the mediator was reoxidized in this process.

The rate of reaction between the mediator and laccase or lignin also is critical for industrial success of laccase mediator biobleaching. Reaction time between 1 to 4 hours would be the longest allowed with most current industrial bleaching processes. Several laccase biobleaching studies have reported active biobleaching condition with these reaction times, but the reactivity of laccase mediator biobleaching stage (LMS) appears to be limited to 50 % or less.

The reactivity or ability to remove lignin with the LMS may be the biggest limitation of all. Several factors may limit delignification with LMS: stability of the mediator, stability of the enzyme, and selectivity with lignin. To understand the limitations and improve the LMS, fundamental understanding of what makes up an active mediator is needed. Most reported active delignification mediators contain an RR'N-OH functional group. The proposed radical mechanism has been presented with no real experimental proof (Figure 3.1). The radical mechanism may exist, but this mechanism does not explain why a heterocyclic compound, HBT, was needed.

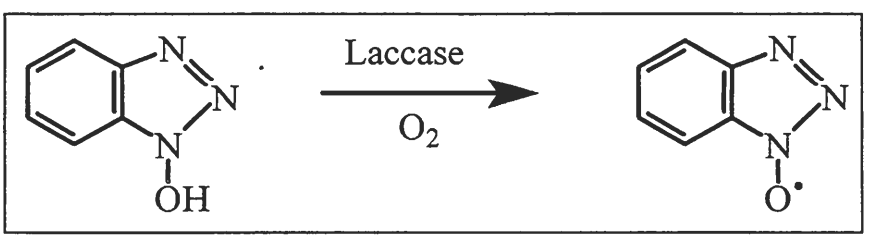


Figure 3.1 Proposed laccase mediator active delignification reagent (95).

The purpose of this chapter was to examine the structural components of an active mediator. This was performed by studying the ability of HBT derivatives to delignify kraft pulp. A small screening of possible active mediators containing RR'N-OH functional group was also tested. For this study, we assumed that the mediator's active delignification mechanism was due to a radical mechanism. The radical mechanism will be studied in more depth in a later chapter (Chapter 5).

RESULTS AND DISCUSSION

Preliminary experiments, summarized in Figure 3.2 and Table 3.1, indicated that if either the mediator or the laccase was omitted from the LMS, only minor amounts of lignin were removed from the pulp presumably due to alkaline leaching (see Experimental section for details). Nonetheless, examination of the extent of delignification when both laccase and N-hydroxybenzotriazole were present confirmed previous reports on this biobleaching system (Chapter 1). Interestingly, the use of compound **2** or **3** as a mediator for laccase failed to yield any significant delignification of the kraft pulp. These observations may be related to the relative energies required for the formation of radicals from each of the proposed mediator compounds. Indeed, computational results from PM3 (107) molecular orbital calculations indicate that the bond dissociation energies for the removal of a hydrogen atom from compounds **1**, **2** and **3** are 79.239, 104.959 and 97.095 kcal/mole, respectively. These values are similar to those reported from laboratory measurements on N-phenylhydroxylamines which range from 74.8 to 84.2 kcal/mole (108). The insertion of a methylene group between the nitrogen and the hydroxy group and the absence of the hydroxyl group both raised the predicted heat of reaction for radical formation over that calculated for N-hydroxybenzotriazole. Furthermore, we and others have reported that N-hydroxybenzotriazole was converted into 1-H benzotriazole during a LMS-stage, and clearly this conversion was detrimental towards the overall delignification effect (Chapter 2).

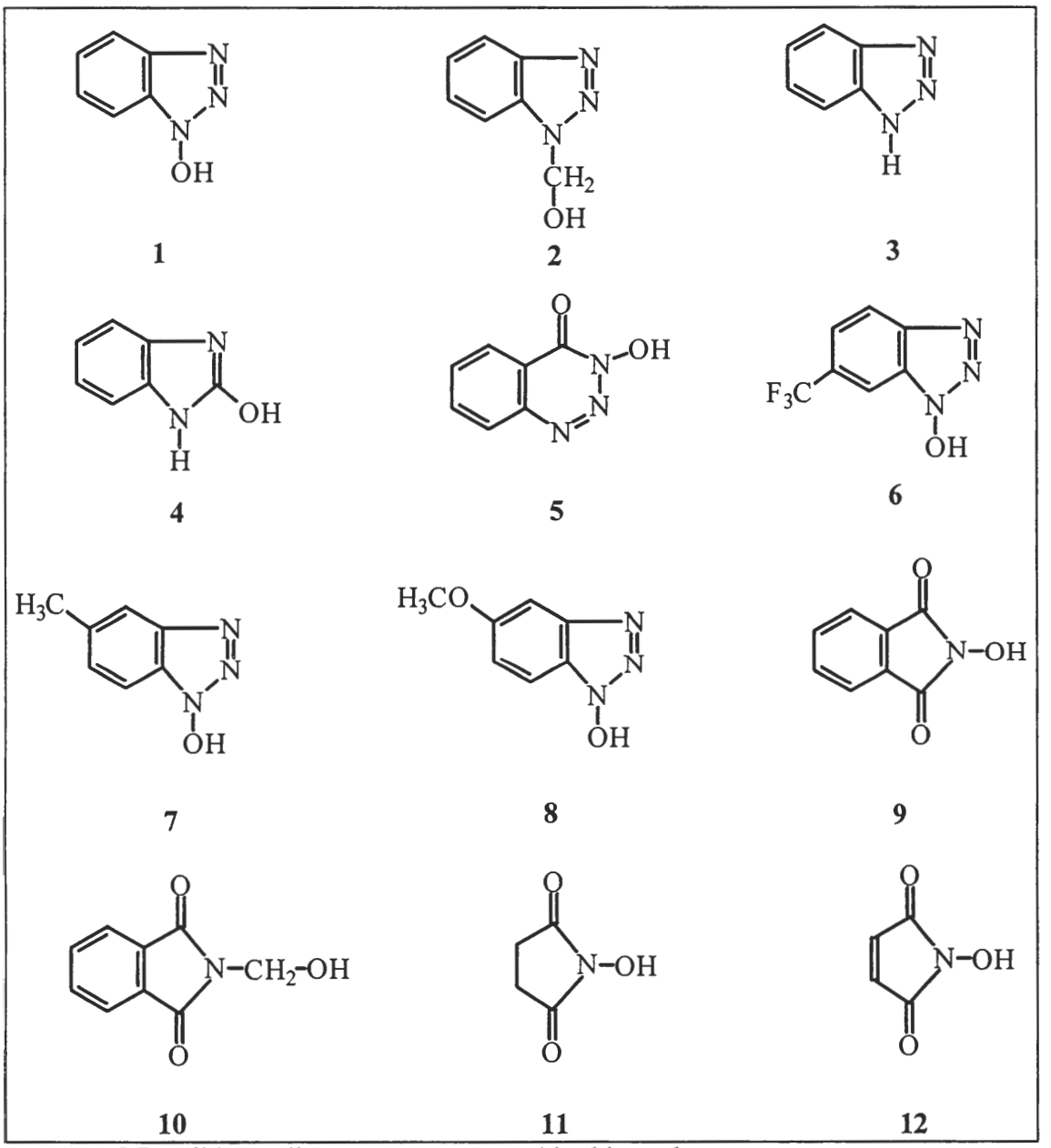


Figure 3.2 Possible mediator structures tested in this study.

Table 3.1 Biobleaching experiments with laccase and a mediator. The experiments were performed with the same molar concentrations of mediator, based on a 2 % charge by weight on o.d. pulp. The laccase dose (LacNS51002 3 mL/10 g o.d. pulp, SSBK26) remained constant, and percent delignification was calculated by kappa number differences ([kappa $\#_{initial}$ - kappa $\#_{LE}$]/ kappa $\#_{initial}$]).

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

Biobleaching experiments also demonstrated **5** and **4** 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 mediator system has rather stringent structural specificities for delignification to occur.

To explore the influence of the aromatic ring on the delignification properties of N-hydroxybenzotriazole, we examined the use of triazole derivatives **6** to **8**. The introduction of a strong electron withdrawing group, such as trifluoromethyl, on the aromatic ring 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 N-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 strict limitations placed on the N-hydroxybenzotriazole structure for effective delignification to occur during a LMS stage, we explored several alternative N-hydroxy mediator structures. The first candidate to be examined was N-hydroxyphthalimide (compound **9**). Literature results by Ishii have shown that this reagent can be employed as a co-oxidant in the presence of $Co(acac)_2$ (109). A variety of structures have been oxidized in the presence of N-hydroxyphthalimide and $Co(acac)_2$, including benzylic alcohols, and primary and secondary alcohols. The active agent believed to oxidize these organic substrate is the N-hydroxy radical of N-hydroxyphthalimide. Given the clear similarities with the proposed mechanism for delignification by laccase and N-hydroxybenzotriazole, it was of interest to determine if N-hydroxyphthalimide could act as a mediator for laccase. Employing the same pulp and experimental conditions as the earlier experiments, pulp (SSBK26) was treated with N-hydroxyphthalimide and laccase for 24 hours. Following the usual alkaline extraction, we determined that a 28% decrease in kappa number of the pulp had occurred. Although this is not as substantial a decrease in kappa number as observed with N-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 N-hydroxyphthalimide halted the delignification capabilities of this mediator (see Table 3.1 compound **10**).

To determine if a simpler pyrrolidine or maleimide structure could act as an effective mediator for laccase, we examined the biobleaching chemistry of additives **11** and **12**

(see Table 1). In each case, there appeared to be no significant delignification in the laccase mediator bleaching treatment. Presumably the N-hydroxy radical generated in this process needs the additional delocalization capabilities available in N-hydroxyphthalimide to become an effective delignification agent or other structural limitations exist with these last two chemical structures.

One possible mechanism, that influences the ability of a chemical structure to be an active mediator, could be represented by solubility in water or hydrophilic: hydrophobic character. The literature suggests that the micro-environment around the active site of copper containing proteins was slightly hydrophobic (91,110,111). To test this theory, the octanol: water partition coefficients were calculated (Table 3.2). Compounds **11** and **12** had the lowest ratios with the active mediator ranging from 1.37 to 1.63. This suggests that the polar character of the mediator may be an important factor influencing reactivity between the enzyme and lignin in the pulp.

Table 3.2 Octanol : Water partition coefficient ratios for tested chemical structures. The chemical compound with the lowest ratio was the most water soluble.

Mediator	Octanol : Water Partition Coefficients
1	1.37
2	1.37
3	1.59
4	0.56
5	1.59
6	2.69
9	1.63
10	1.49
11	0.31
12	0.30

Another important property of the laccase mediator biobleaching system was selectivity. Viscosity of the pulps after treatment was used to rate selectivity (Table 3.3).

Table 3.3 Pulp viscosity values for pulp (SSBK26) treated with different mediator compounds. The laccase dose (LacNS51002 3 mL/10 g o.d. pulp) remained constant.

Mediator	Pulp Viscosity (cP)	Mediator	Pulp Viscosity (cP)
Starting Pulp	31.9	7	28.5
No Mediator	27.7	8	27.5
1 + no laccase	29.7	9	28.0
1	25.8	10	27.5
2	28.5	11	26.7
3	29.5	12	28.2
4	28.6		
5	30.8		
6	28.6		

Very little loss of pulp viscosity was detected for all the treated pulps. This suggests that the active delignification systems were selective for lignin and the non-delignification mediator systems remained unreactive with cellulose.

CONCLUSIONS

This study revealed the rather stringent structural requirements for delignification of kraft pulps with laccase and N-hydroxybenzotriazole like structures. The results of these investigations support the proposed importance of N-hydroxy radicals as the active delignification agent for kraft pulps. The observation that both **7** and **9** were mediators for

laccase (while less effective than N-hydroxybenzotriazole) suggested that the steric requirements about the active site of laccase were not overly specific, which was also consistent with the ability of the enzyme to oxidize ABTS. The lack of substantial drop in pulp viscosity also showed the good selectivity for the laccase mediator system.

While many substrates can react with laccase, the laccase mediator delignification system appears to require very narrow conditions. Mediators with chemical structures close to N-hydroxybenzotriazole appear to be limited to N-OH functional groups with a finite ability to delocalize an electron. The polar nature of the compound may also be important.

CHAPTER 4
LACCASE N-HYDROXYBENZOTRIAZOLE FULL-SEQUENCE BLEACHING WITH HYDROGEN PEROXIDE AND CHLORINE DIOXIDE

INTRODUCTION

Biobleaching systems that incorporate a mediator compound with isolated enzymes have been used to delignify kraft pulp. Recently, laccase, which is an oxidoreductase enzyme isolated from white-rot fungi, mediator biobleaching systems have shown promise. While the currently proposed laccase-mediator biobleaching systems are far from benign, the perceived environmental advantages have been the impetus behind most of the recent biobleaching research (94,95). However, other properties of the laccase-mediator system may be more valuable than its environmental advantages. Selectivity and a potentially recoverable bleaching agent may prove to be the most intriguing aspects of this novel bleaching system. Bleachability of the laccase-mediator treated pulps will also be an important aspect of the commercial application of the process.

A review of mediator structures reveals that the RR'N-OH mediators are the most effective for bleaching kraft pulps. While several obstacles exist with HBT for commercial-scale bleaching, it has been shown to be as effective, or more effective than any other reported mediator in the literature. Two new mediators have been shown to be closely comparable to HBT (Figure 4.1). N-Hydroxy-acetanilide (NHAA) has been introduced as a biodegradable mediator that could be cost effective in kraft pulp bleaching. Unfortunately, very little has been reported about this mediator or the recently reported mediator violuric acid (VIO) (97). Both of these new mediators may have an advantage over HBT. It has been reported that the residual

activity of the enzyme was much higher for NHAA and VIO than for HBT (97). This will be examined in more detail in Chapter 5.

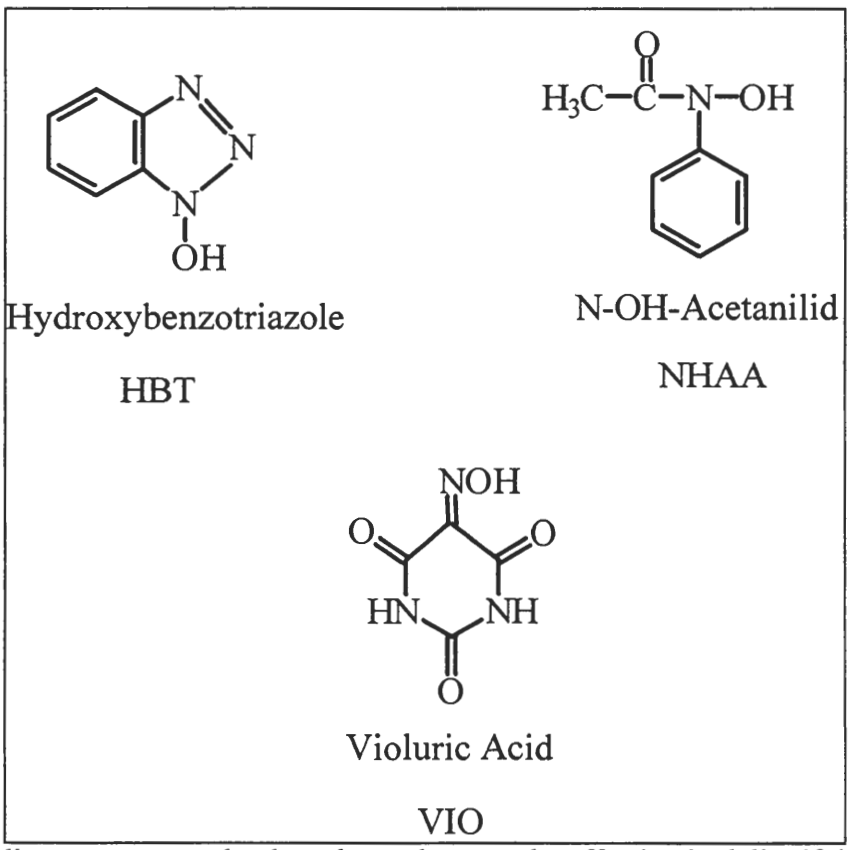


Figure 4.1 Mediator structures that have been shown to be effective in delignifying kraft pulps (97).

The structural changes in the residual lignin in pulps treated with the laccase HBT system were reported in Chapter 1. Overall, the residual lignin was mildly altered with some demethylation and a decrease in hydroxyl group concentrations. This suggested that the residual lignin of laccase-HBT treated pulps remained relatively reactive. To test this theory, we performed full-sequence bleaching trials. Several studies have been reported with full sequence bleaching with LMS, but all of them used TCF sequences (116). Most of these reports also used

low kappa brownstock pulps (15.3-11.0), which are not representative of typical industrial conditions today (54).

The purpose of this study was to determine the response of laccase-HBT treated pulps to hydrogen peroxide and chlorine dioxide. Oxidant-reinforced alkaline extractions of laccase-HBT treated pulps and mediators NHAA and VIO were also examined.

RESULTS AND DISCUSSION

BLEACHING SEQUENCE STUDY

The initial experiments were performed to determine the effect of reinforced alkaline extraction on laccase-HBT treated pulps. For a standard laccase-HBT treatment (L), about 50 % delignification can be achieved with a typical industrial softwood kraft Post-O_2 pulp (SSOK13) with an initial kappa number of 13. If oxygen reinforced alkaline extraction (E+O) was used after an L stage, close to 60 % delignification was achieved. If an oxygen and hydrogen peroxide reinforced alkaline extraction (E+O+P) was used, over 70 % delignification was obtained with a single laccase-HBT treatment (Figure 4.2). When the L(E+O+P) pulp was treated with another laccase-HBT stage, the kappa number decreased significantly again. This observation suggested that a laccase-HBT biobleaching system was capable of reacting with the last vestiges of residual lignin, which are typically very unreactive. The selectivity of this biobleaching system also appeared to be equal to, if not significantly better than, most TCF sequences (112). Surprisingly, a significant drop in pulp viscosity was observed when oxidant-reinforced alkaline extractions were used. This could be due to increased metal ion concentrations in the pulp, which originated from the enzyme solution.

The L(E+O+P)LE treated pulp was subjected to hydrogen peroxide and chlorine dioxide brightening stages to examine how laccase-HBT treated pulps would respond to brightening stages (Figure 4.3 and 4.4). The hydrogen peroxide-treated pulp approached 80 ISO brightness when the highest peroxide charge of 3.5 % was used. The brightness loss of 3 to 5 ISO brightness points after thermal reversion was low compared to other TCF processes (112).

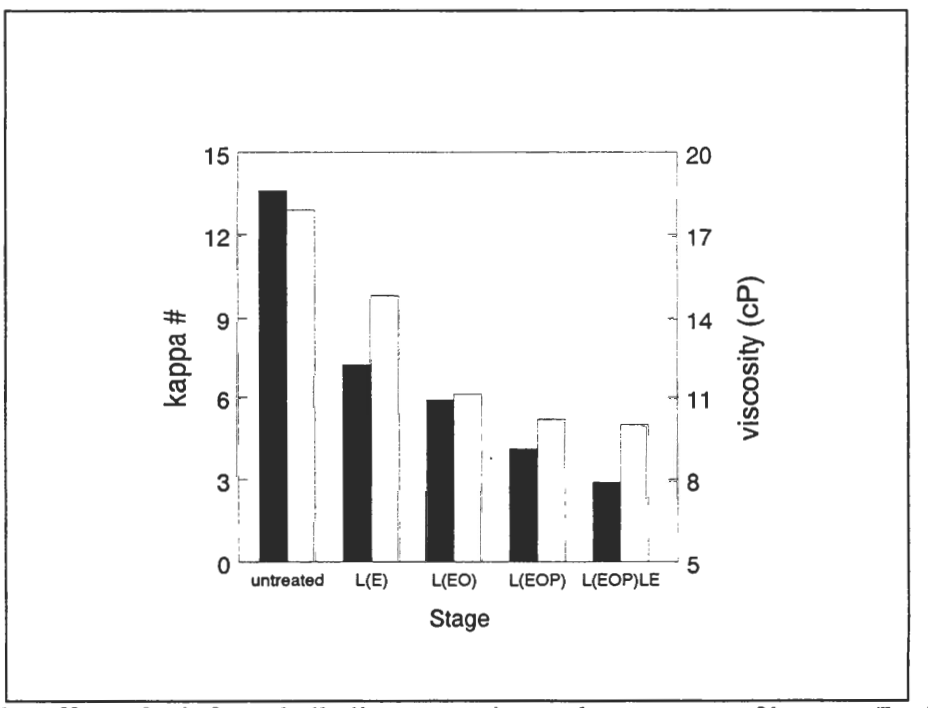


Figure 4.2 The effect of reinforced alkaline extraction and two stages of laccase (LacNS51002) HBT treated softwood kraft Post-O_2 industrial pulp (SSOK13). Solid bar is kappa number, and open bar is viscosity. The (E+O+P) stages were performed with a 0.5 % P and an O_2 charge of 60 psi for the first 15 minutes. The pressure was decreased by 20 psi every 5 minutes until atmospheric pressure was reached.

The L(E+O+P)LE pulp responded better to chlorine dioxide than to hydrogen peroxide. Indeed, an 85 ISO brightness pulp was obtained when the highest chlorine dioxide charge of 1.0 % was employed. Even a low dosage of chlorine dioxide, 0.2 %, increased the ISO brightness over 78 (Figure 4.4). The brightness lost due to thermal reversion was lower than

with hydrogen peroxide brightened pulp. A series of experiments were performed with OL(E+O+P)DED bleached pulp to further examine the reactivity of laccase-HBT treated pulps with chlorine dioxide. The OL(E+O+P) pulp (the O stage was performed on an industrial scale) was identical to the pulp treated in the experiments described above. The D_1 stage was performed at two different charges, 0.75 and 1.25 %, and the temperature was raised to

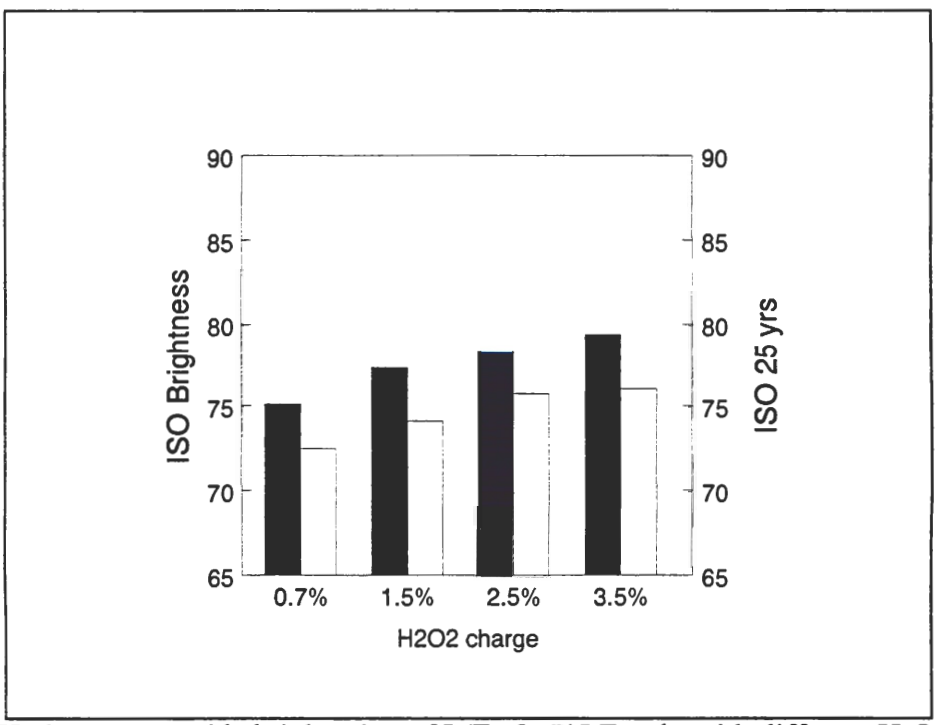


Figure 4.3 Hydrogen peroxide brightening of L(E+O+P)LE pulp with different H_2O_2 charges. Solid bar is ISO brightness, and open bar is ISO brightness after thermal reversion equal to 25 years. The P stages were performed at 10 % consistency at 90° C in a sealed bag for 4 hours.

70° C for 3 hours. The second alkaline extraction was performed with a 2 % NaOH charge at 70° C for 1 hour at 10 % consistency. The final D stage was performed with a 0.50 and 0.75 % charge, and the reaction was continued at 70° C for 3 hours. The brightness of the pulp, after the higher charge D_1, was surprisingly high at 83 ISO; surprisingly because the kappa number entering the stage was over 4 (Table 4.1). The lower charge D_1 also reacted well; the final ISO

brightness over 78. The final brightnesses achieved after the D_2 stage were less impressive, with a range of 84 to 87.

These experiments were only used to obtain an understanding of how laccase-mediator delignified pulp would respond to a full bleaching sequence. Numerous other studies should be performed before any overall conclusions can be made about the advantages or disadvantages of bleaching LMS pulp. However, the data in this study proved interesting when comparing it to other enzymatic delignification studies. Earlier reports of laccase HBT biobleaching studies only achieved an 82.7 ISO brightness with a LELEQP sequence on softwood sulfite pulps (54).

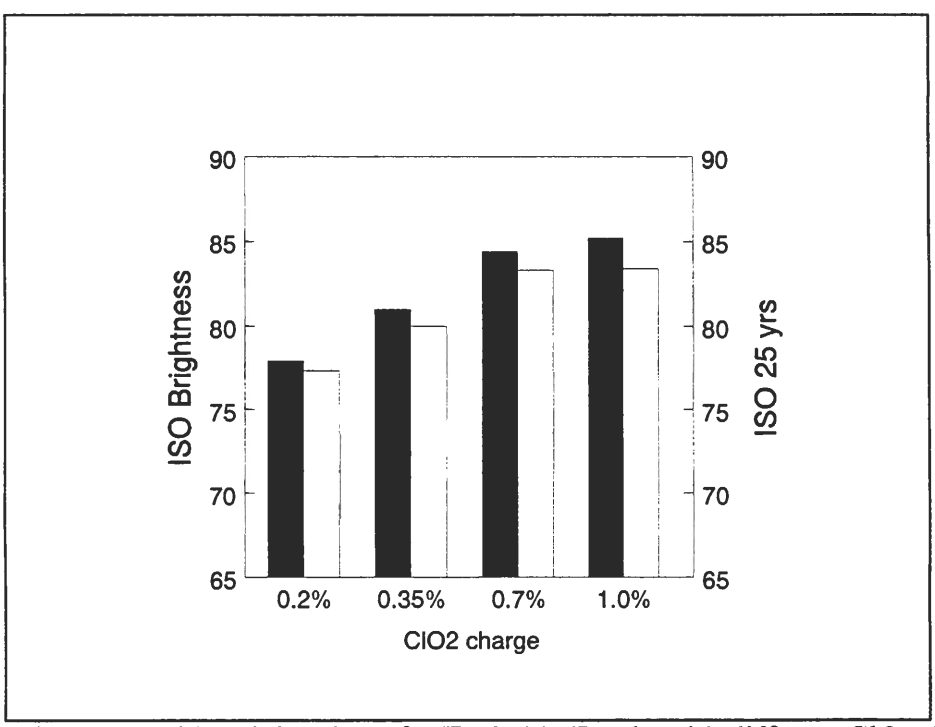


Figure 4.4 Chlorine dioxide brightening of L(E+O+P)LE pulp with different ClO_2 charges. Solid bar is ISO brightness, and open bar is ISO brightness after thermal reversion equal to 25 years. The D stages were performed at 10 % consistency for 3 hours.

MEDIATOR STUDY

Another series of experiments were performed to understand how the laccase biobleaching system would perform with two new mediators, NHAA and VIO. One of the greatest disadvantages of HBT was the amount of laccase needed to create an active delignification environment. Several other researchers have also detected this limitation with HBT. Their experimental data suggested that the HBT radical attacks the amino acids in laccase, causing the protein to unfold and denature, which destroys the enzyme's activity (97,100,113,114). Biobleaching effluents from pulp samples treated with NHAA and VIO mediators were reported to have much higher residual enzyme activities.

Table 4.1 Brightness and pH values for OL(E+O+P)D_1ED_2 sequence pulp. Starting pulp was SSOK13, and laccase LacNS51002 was used.

Bleaching stage	**ISO Brightness**	**Initial (D) stage pH**	**Final (D) stage pH**
OL(E+O+P)D (0.75%) D_1	78.4	5.4	2.5
OL(E+O+P)D (1.25%) D_1	83.0	5.3	2.3
OL(E+O+P)$D_{0.75}$ ED (0.5%) D_2	84.6	8.0	3.9
OL(E+O+P)$D_{0.75}$ ED (0.75%) D_2	86.0	8.0	4.3
OL(E+O+P)$D_{1.25}$ED (0.5%) D_2	85.1	8.0	4.6
OL(E+O+P)$D_{1.25}$ED (0.75%) D_2	87.4	8.0	4.4

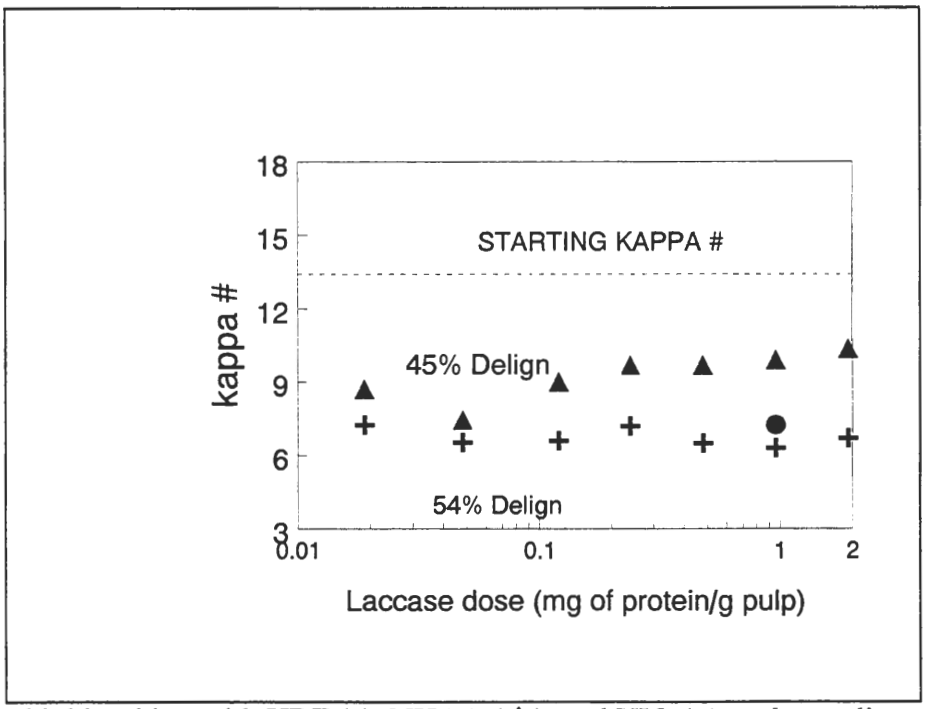


Figure 4.5 Laccase biobleaching with HBT (•), NHAA (▲), and VIO (+) as the mediator. The pulp used was a southern kraft softwood O_2 delignified (SSOK13). Mediator doses were held constant at a molar equivalent of a 2% charge based on HBT , and LacNS51002 was used for all experiments.

HBT, NHAA, and VIO mediators were tested with the same pulp (SSOK13) and laccase batch (LacNS51002) to examine their effectiveness in delignifying kraft pulp. The mediator dose, which was based on a 2 % charge of HBT, was held constant at a molar equivalent for each mediator. The only variable that changed was the enzyme dose (Figure 4.5).

After the first few experiments, the large difference between HBT and VIO or NHAA became apparent (Note: The data point for HBT was the optimal dose. Any dose below this level drastically lowered its effectiveness). At 1/50 of the laccase dose needed for HBT, VIO remained active. While VIO had a higher overall delignification for all experiments when compared to HBT or NHAA, the difference between NHAA and VIO decreased at lower enzyme dosage trials. The trends for NHAA and VIO were also different with higher doses of enzyme,

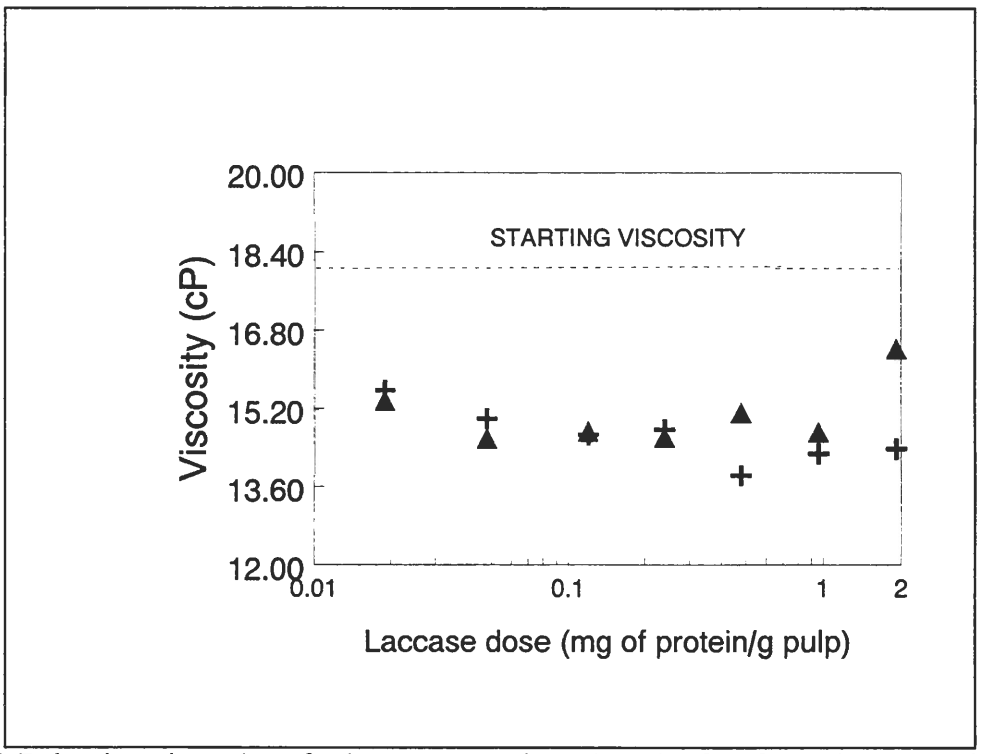


Figure 4.6 Pulp viscosity values for laccase treated southern kraft softwood Post-O_2 delignified samples with NHAA (▲) and VIO (+) as the mediators.

hindering delignification for NHAA but having very little effect on VIO performance. Very little difference was detected with pulp viscosity values between NHAA and VIO with both systems remaining highly selective (Figure 4.6).

A biobleaching experiment with laccase VIO was performed with an (E+O+P) extraction to determine pulp yields. A pulp yield of 97 % was recorded for an L(E+O+P) treated pulp. The pre-alkaline extraction kappa number was 9.5 (starting kappa number of 13.6), and the post-alkaline extraction kappa number was 5.1, which represents 62 % delignification. The L(E+O+P) sequence with VIO as the mediator was slightly less effective than when HBT was used as a mediator. However, it should be noted that 1/20 of the enzyme dose was used for the VIO experiment. Both experiments were performed on the same pulp sample, SSOK13.

All reports of laccase HBT biobleaching trials have shown that low kappa brownstock pulp or pulps after O_2 delignification, ranging between 12-15, have the highest delignification percentages. More typical brownstock pulps with higher kappa numbers, ranging between 24-30, seemed to be less reactive when comparing delignification percentages. This can be misleading because the amount of lignin removed was greater for the higher kappa pulp (see chapter 1), but the delignification percentage was due less to the higher starting lignin content. The lignin in higher kappa pulps generally has a much higher reactivity with oxidizing bleaching chemicals. Laccase-HBT delignification appears to be more limited by the amount of material it can oxidize, rather than by the reactivity or structure of the residual lignin. Since VIO seems to be a more efficient mediator than HBT when delignifying kraft pulps, biobleaching trials were performed with laccase VIO on a brownstock kraft pulp (SSOK26) with a kappa number of 26 (Figure 4.7).

The enzyme dose was first applied at the original optimal dose for HBT, and the enzyme dose was decreased sequentially by 50 % for four separate bleaching trials. The response of VIO was drastically different with the brownstock pulp than the Post-O_2 delignified pulp. Even at one-half the original enzyme dose, a significantly lower delignification percentage was observed. At each lower enzyme dosage, a stronger decrease in delignification was detected. This suggested that the delignification was closely related to the enzyme dose. The factors that limit delignification appeared to be different between the two pulp samples since both the brownstock and Post-O_2 delignified pulp samples were treated with the same batch of laccase and mediator dose. The selectivity of the laccase VIO trials remained high, with little to no difference in pulp viscosity between all pulp samples.

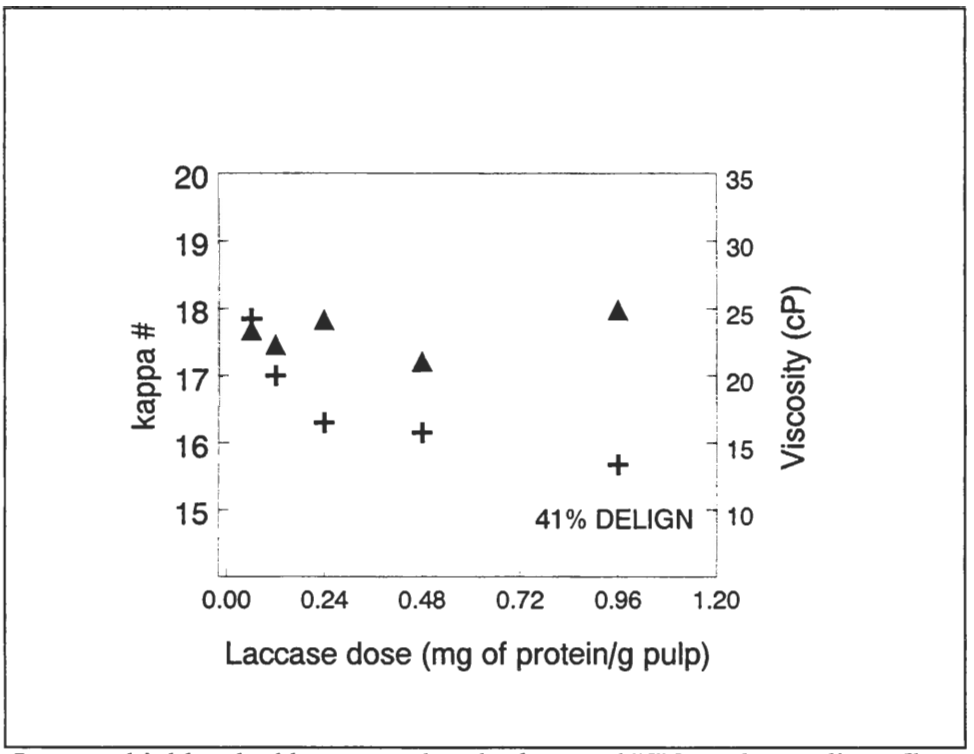


Figure 4.7 Laccase biobleached brownstock pulp that used VIO as the mediator [kappa number (+) and pulp viscosity (▲)]. The starting kappa number was 26.8, and all experiments were performed with the same mediator dose of a mole equivalent of 2 % HBT. Reaction conditions were 4 hours at 45° C and a pH of 4.5.

CONCLUSIONS

Pulp bleached with the laccase HBT-biobleaching system appeared to respond well to oxidant-reinforced alkaline extractions with over 70 % delignification achieved with an L(E+O+P) sequence. A bleaching sequence with two laccase-HBT stages continued to decrease the pulp's kappa number. An L(E+O+P)LED bleaching sequence can produce a pulp with 85 ISO brightness, while L(E+O+P)LEP bleached pulps appear to be limited to 79-80 ISO brightness. Further investigation into the bleachability of laccase-HBT bleached pulps revealed a >83 ISO brightness achieved with a OL(E+O+P)D sequence.

Laccase-mediator bleaching trials with HBT, NHAA, and VIO showed a vast improvement in the requirements of enzyme dose with NHAA and VIO. NHAA and VIO were able to produce a significant delignification percentage at 1/50 the enzyme dose required by HBT. While the response to enzyme dose was different for NHAA and VIO, NHAA and VIO appeared to be highly selective for lignin oxidation. VIO was the best delignification mediator tested for all pulp samples used in this study and remained active with brownstock pulps. However, a higher enzyme dose was needed to reach optimal delignification conditions with a brownstock pulp. The factors that limit delignification appeared to vary depending on the amount of lignin in the pulp and the mediator used.

CHAPTER 5
ANALYSIS OF RADICAL MECHANISM AND ACTIVITY DURING BIOBLEACHING

INTRODUCTION

Since the laccase mediator biobleaching system contains many components, several factors, or combinations of factors, could limit delignification of kraft pulps. While researchers have generally accepted a radical mechanism as the active delignfication mechanism, very little experimental proof has been presented in the literature. Paice has shown that reactions between ABTS and laccase generated a radical cation species and a dication species (100,115). This study used cyclic voltammetry to determine the oxidation potential of the $ABTS^{\bullet+}$ and the dication $ABTS^{++}$. These oxidation potentials did not change for several cycles which suggested that ABTS radicals could be formed and quenched several times during the experiment. HBT was also tested and only one oxidation peak was detected. An HBT oxidation potential was not detected in the second cycle. This suggested that HBT was structurally altered during the experiment, and this could be explained by the well established conversion of HBT to BT (Chapter 2). ABTS has also been shown to be structurally unstable during biobleaching, but it appeared to be stable in this model system (113). Cyclic voltammetry experiments were also carried out in the presence of veratryl alcohol. The presence of veratryl alcohol seemed to enhance the oxidation peaks for ABTS and HBT. This suggested that veratryl alcohol enhanced the regeneration of the mediator compounds.

Another study that examined the radicals generated during model conditions was conducted using EPR spectroscopy (113). The ABTS cation radical was shown to be very stable when compared to the HBT radical because the HBT radical species was only detected for a few

minutes after generation. These experiments were performed without the presence of pulp or lignin model compounds. Cyclic voltammetry studies were also reported, and they confirmed the studies performed by Paice (113).

While the presence of radical species during the reaction between laccase and a mediator has been established, very little has been presented on the presence of radicals during an active biobleaching experiment or if the radical mechanism was responsible for delignification. The purpose of this study was to determine if radical species created during biobleaching experiments with laccase and HBT actively participate in delignification. This was performed with a selective radical quenching agent. The activity of laccase, after biobleaching with different mediators, was also monitored to determine how enzyme activity was affected by different biobleaching conditions.

RESULTS AND DISCUSSION

RADICAL STUDY

A selective radical quenching agent had to be used that would not act as an oxidizable material. This prerequisite eliminated the use of radical scavenger agents that could act as oxidizable material or resemble more lignin present in the pulp slurry. A catalytic radical quenching system, as exists with superoxide dismutase, appeared to be a possible candidate.

Superoxide dismutase (SOD), an oxidoreductase enzyme, is comprised of a dimeric protein with two identical subunits (Figure 5.1). Each subunit contains one Cu^{2+} and one Zn^{2+} ion which catalyze the dismutation of superoxide, $O_2^{\cdot-}$, to dioxygen and hydrogen peroxide (116). This oxidoreductase enzyme has also been shown to react with oxy-radicals in general (110,118-

120). The cyclic redox pattern that causes the dismutation of superoxide has been shown by the following:

$$O_2^- + \text{Protein-Cu(II)} \rightarrow O_2 + \text{Protein-Cu(I)}$$

$$O_2^- + \text{Protein-Cu(I)} \rightarrow H_2O_2 + \text{Protein-Cu(II)}$$

It appears that all of the redox cycle occurs around the chelated Cu atom. The Zn atom is used to hold the protein together. It has also been reported that the oxidation and reduction steps occur at about the same rate, suggesting that the reaction is limited by diffusion in most cases.

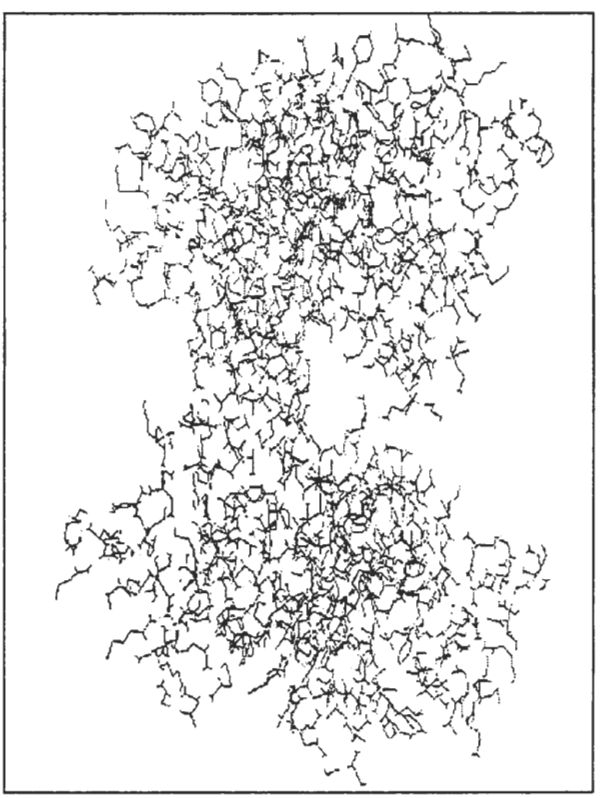


Figure 5.1 Model picture of Superoxide dismutase. This picture shows the protein bridge and dimeric character of SOD.

The solution pH requirements for bovine SOD is very broad. SOD exhibits only slight changes in activity between pH 5 to 9.5 (117). This was very important for its use during biobleaching conditions performed at pH 4.5. While pH 4.5 is not the optimal condition for

bovine SOD, it still remains active, and this allowed the use of SOD without changing active biobleaching conditions with laccase and HBT. The size of SOD, MW ~ 31,000, is also too large to fit into the active site of laccase and would only react with radical species outside of laccase.

SOD experiments with laccase have been reported in the literature (46). For example, Milstein carried out experiments between laccase and organosolv lignin (OL). He reported that laccase polymerized the OL after several hours of incubation (121). These studies were performed in dioxane-H_2O (7:3), and the generation of superoxide was monitored by the oxidation of cytochrome c (cytochrome c is a substrate used to measure the activity of superoxide dismutase protein). A sharp increase in superoxide concentration was measured within the first few minutes after laccase was added to the OL solution. The increase in the measured superoxide concentration continued until about 1 hour into the reaction, and then it remained constant until the experiment ended at 4.5 hours. No oxidation of cytochrome c was detected when either the laccase or lignin was absent from the system. When SOD was added, less polymerization of the OL was detected, and very little superoxide was measured in the reaction mixture.

Milstein stated that these experiments do not prove that superoxide was present in the reaction mixture. The oxidation of cytochrome c only indicated that radicals are present, and lignin free radicals were most likely present, which caused the oxidation of cytochrome c (121). From this experiment, we derived the test of SOD with the laccase HBT biobleaching system.

The radical quenching experiments were performed with SOD under standard biobleaching conditions for 4 hours on a Post-O_2 delignified kraft pulp (SSOK13). The

experiments were monitored by kappa number and pulp viscosity measurements. The starting kappa number of the untreated pulp was 13.6, and a laccase HBT treatment of this pulp removed 50 % of the residual lignin after an alkaline extraction (Figure 5.2). When the lowest dose of

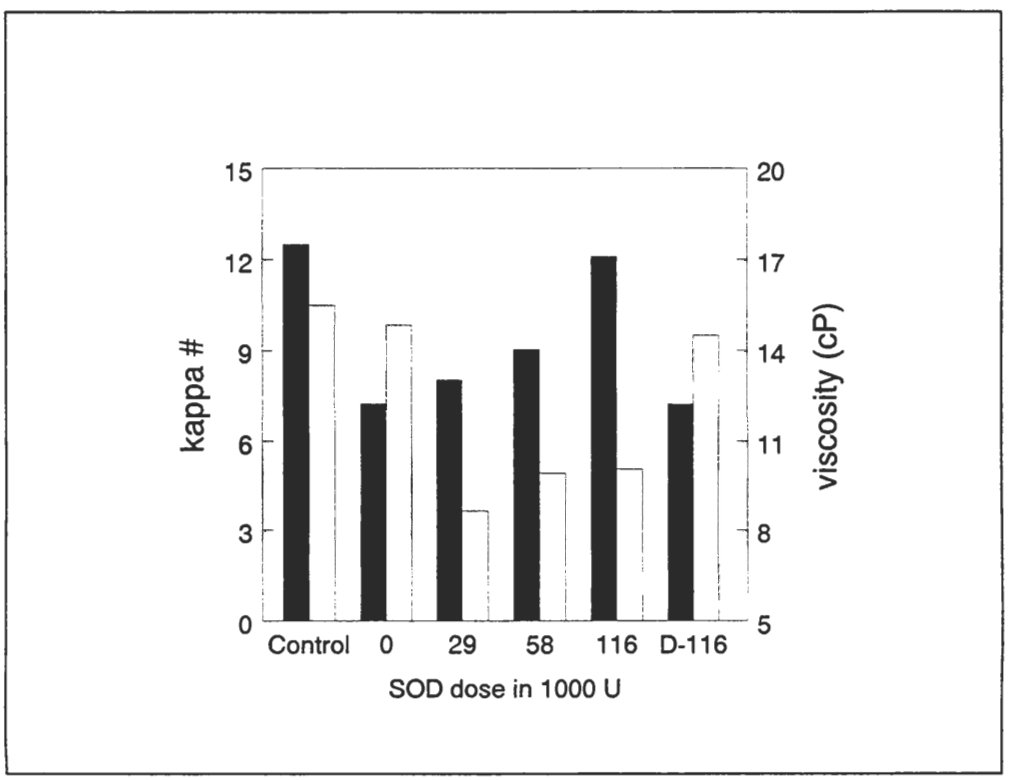


Figure 5.2 Superoxide dismutase treatments of active biobleaching conditions of laccase HBT (pulp SSOK13, laccase LacNS51002). D-116 was thermally denatured SOD with a 116,000 U starting activity. The "Control" experiment was performed under typical biobleaching conditions with no enzyme or mediator followed by an alkaline extraction stage. Solid bar is kappa number, and open bar is viscosity.

SOD (29,000 U) was added to the biobleaching system, a slight decrease in delignification was detected. However, a large decrease in pulp viscosity was observed. When the dose of SOD was doubled, the pulp delignfication was even less. When 116,000 U of SOD was added, delignification was equal to the control (the control was performed under standard biobleaching conditions without laccase or HBT, and the pulp was then treated with an alkaline extraction

stage.). Several control experiments were also carried out to determine if this observation was attributed to radical quenching of SOD. One of the most interesting controls involved denaturing the SOD before addition to the biobleaching system. A sample of 116,000 U SOD was denatured by heating it at 90° C for 1 hour. The sample was then added to a biobleaching system (represented by D-116 in Figure 5.2), and no detrimental effect on delignification was detected. This suggested that active SOD was needed to hinder delignification.

The viscosity drop of the SOD treated samples was surprising. The exact cause of this observation remains unknown. One possible explanation can be explained by the mechanism of SOD. If SOD reacted with a radical species that was selective to lignin or closely associated to lignin and generated hydrogen peroxide in the process (116), the hydrogen peroxide would be unstable in the acidic medium. The hydrogen peroxide could readily decompose into hydroxyl radicals which would be very reactive. The hydroxyl radical would most likely react with the cellulose in pulp since 98 % of the material present was carbohydrate.

ACTIVITY STUDY

A series of experiments were performed to determine which factors affect the enzyme activity during biobleaching. Several researchers have reported that the activity of laccase was drastically reduced during biobleaching treatments with HBT as the mediator. The residual activity of the laccase was higher for biobleaching trials with other mediators, NHAA and VIO (97,100). Our first activity study was performed under typical biobleaching conditions with the pH at 4.5 and the O_2 pressure at 145 psi with a reaction time of 4 hr. All mediator doses were constant at a molar equivalent of 2 % HBT, and the enzyme dose was held constant (laccase

LacNS51002 with 3 mL / 10 g o.d. pulp, pulp SSOK13) (Table 5.1). No residual laccase activity was detected after biobleaching, regardless of the mediator used. This was surprising since Amann reported that the laccase activity remained high after biobleaching with NHAA (97). One main factor was different between the studies. The reaction time was 1 hour for Amann's study while we used 4 hours.

Several control experiments were also carried out. The laccase activity decreased by 9 % when the laccase was just mixed with pulp for one minute at room temperature with a pH of 4.5 and filtered (Note: the 9 % loss in activity was the difference between the measured activity of the pulp mixing control and the dilution control). This suggested that some amount of the protein became associated with the pulp fiber, or the filtering process caused the enzyme to denature. The former was more likely than the latter. The data from the control experiment, in which laccase biobleaching was performed without the addition of a mediator, suggested that the elevated oxygen pressure during biobleaching may have contributed substantially to the loss of activity. Another set of experiments was performed to test this theory.

A series of experiments were carried out on the same pulp sample used in the previous experiments, but no O_2 pressure was applied. All other reaction conditions were identical to the earlier study. Sampling was performed before and after washing and filtering of the pulp.

Table 5.1 Laccase activity measurements of filtrates after biobleaching.[a]

Biobleaching Conditions	Lac U in	Lac U out	Percent Laccase activity recovered	Kappa # Starting kappa # 13.6
Laccase no mediator	960	207	22%	11.5
Laccase HBT	960	0	0%	7.2
Laccase NHAA	960	0	0%	9.9
Laccase VIO	960	0	0%	6.3
Control when laccase was mixed with pulp for one minute and then filtered	960	731	76%	----
Control when laccase was diluted with water	960	820	85%	----

[a]The trials were performed on a 10 g o.d. pulp (SSOK13) scale under typical biobleaching conditions (LacNS51002 was used with a dose of 3 mL/ 10 g o.d. pulp). The pH of the pulp mixture was adjusted to 4.5 with glacial acetic acid or sodium bicarbonate. The pulp was then washed after treatment with ~1000 mL of distilled water and the amount of filtrate collected was accurately measured and frozen immediately. The activity test was performed by the LACU procedure (see experimental section) and the filtrates were thawed just before testing.

Sampling of the supernatant was done by pushing a 1 mL syringe into the pulp mat immediately after biobleaching and drawing a sample into the syringe. This was done to test the activity of the supernatant before filtering and washing to eliminate these variables as possible sources of enzyme activity losses.

Table 5.2 Laccase activity measurements of filtrates after biobleaching.[a]

Biobleaching Conditions No O_2 Pressure	LacU in	LacU out	Percent Laccase Activity Recovered
Laccase HBT before washing	960	38	4.0%
Laccase HBT after washing	960	55	5.7%
Laccase NHAA before washing	960	13	1.4%
Laccase NHAA after washing	960	195	20%
Laccase VIO before washing	960	12	1.2%
Laccase VIO after washing	960	14.6	1.5%

[a]The trials were performed on a 10 g o.d. pulp (SSOK13) scale under typical biobleaching conditions except no oxygen pressure was used (LacNS51002 was used with a dose of 3 mL/ 10 g o.d. pulp). The pH of the pulp mixture was adjusted to 4.5 with glacial acetic acid or sodium bicarbonate. The pulp was then washed after treatment with ~1000 mL of distilled water and the amount of filtrate collected was accurately measured and frozen immediately. The activity test was performed by the LACU procedure (see experimental section) and the filtrates were thawed just before testing.

Residual laccase activity was detected for all the mediators tested. The sampling of the supernatant before washing and filtering appeared to be inadequate since the recovered laccase activity was higher for the washed and filtered samples. NHAA had the highest residual laccase activity, but the 20 % recovery of laccase activity was still quite low compared to the 80 % recovery rate reported by Amann (97). The HBT and VIO residual laccase activities were both lower than NHAA which agrees with Amann's data. The elevated oxygen pressure appeared to have a significant affect on residual laccase activity.

This data suggested that the recycling of laccase would have little to no advantage for the overall efficiency of the laccase mediator biobleaching system. The exact cause for the difference between our study and Amann's study was not know, but differences in reaction time may have been the main contributing factor.

CONCLUSIONS

The main delignification mechanism in laccase HBT biobleaching experiments was radical based. The SOD treatment appeared to block the laccase biobleaching process between the third and fourth cycle (Figure 5.3). Clearly, the delignification that occurred in the presence of the denatured SOD indicated that SOD acted as a selective radical quenching agent. This study did not prove or disprove if superoxide was present or involved in laccase HBT biobleaching. The decrease in delignification that occurred in SOD treatments was highly dependent on the SOD dose. An extremely high dose of SOD was needed to detrimentally affect delignification. A large pulp viscosity drop was detected in SOD treatments. The exact cause of this was unknown. However, it is possible that the pulp viscosity drop occurred because of the

generation of hydrogen peroxide by SOD, which was a by-product of radical quenching reactions.

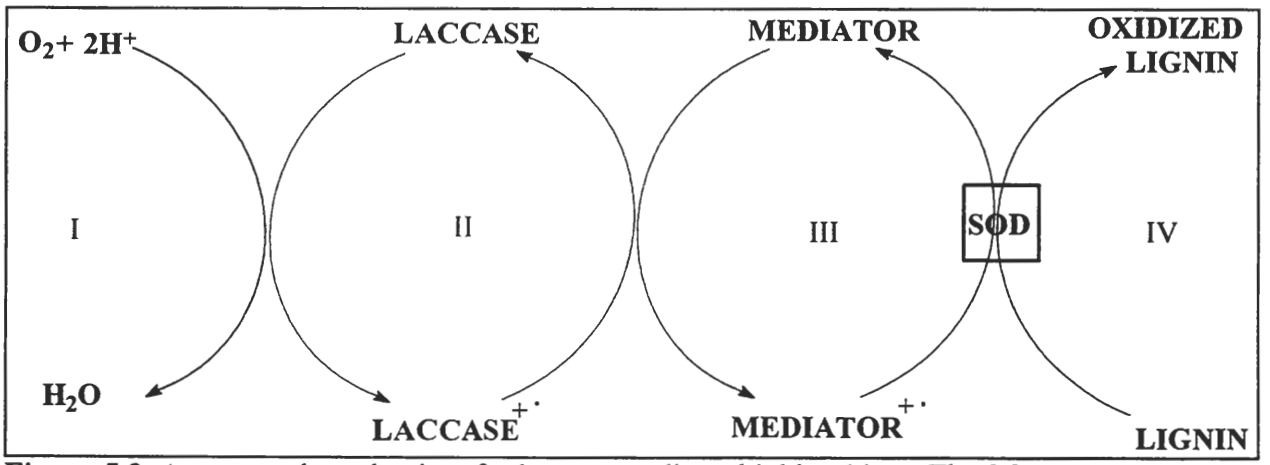


Figure 5.3 A proposed mechanism for laccase mediator biobleaching. The SOD treatment would theoretically block the interaction between the III and IV cycle.

Residual laccase activity studies revealed that very little to no laccase activity remained after a 4 hour biobleaching experiment, regardless if HBT, VIO or NHAA was used as the mediator. Almost 80 % of the laccase activity was lost during laccase biobleaching with no mediator present. This suggested that the mediator was not the main factor contributing to the laccase activity loss. Oxygen pressure also appeared to have some effect on residual laccase activity, but overall it only represented a few percentage points on recoverable laccase activity. A small portion of laccase also appeared to be trapped by the pulp during filtering, and this could cause problems for industrial applications.

CHAPTER 6
ANALYSIS OF LACCASE HBT TREATED HARDWOOD AND SOFTWOOD KRAFT PULPS

INTRODUCTION

Fundamental investigation into laccase based biobleaching has been performed for several years by using lignin model compounds as substrates. While most of this work was done without mediator compounds, several recent publications have presented data with mediator compounds (47-49). The main difference reported between laccase studies with or without the presence of a mediator was that laccase mediator systems were able to oxidize non-phenolic lignin model compounds, while laccase alone was not. Predicting how a laccase mediator system will react with pulp has been very difficult. The laccase biobleaching system involves the interactions of numerous substrates, and the detailed mechanisms have not been elucidated. An investigation into the residual lignin structure was needed to better understand the oxidation chemistry that occurred during biobleaching.

Since the evolution of laccase biobleaching technology has occurred at a rapid pace, analysis of kraft pulps treated with laccase mediator biobleaching was performed with a commercially available laccase source, LacNS51002. This laccase differs from the laccase used in the studies in Chapter 1. Besides the type of laccase, the mediator choice was also important. While a large class of compounds have been shown to be suitable substrates for laccase, only a few compounds are effective in delignifing kraft pulps. Among this subclass of compounds, HBT, NHAA, and VIO were considered. Since very little was known about NHAA and VIO, it was decided to study the effect of HBT as the mediator. HBT has also been shown to be as effective, or even more effective, than any reported mediator in removing lignin from kraft pulp.

While several properties of laccase HBT biobleaching may hinder its use on a commercial scale, this biobleaching system should provide insight into the mechanism of laccase mediator biobleaching.

The pulp type was also a consideration. Since laccase biobleaching has been shown to be more effective with low kappa number pulps, a range of lignin content in the pulp was considered (54). A typical softwood brownstock pulp with a kappa number of 26 was chosen. An oxygen delignified pulp from the same source was also used. This provided a range of lignin concentrations in the pulp and also allowed a comparison between a laccase biobleaching stage and an oxygen stage. Hardwood pulp was also tested, and this provided a comparison of lignin types. A brownstock and an oxygen delignified hardwood pulp was selected to see if the same trends observed with the softwood pulps occurred with the hardwood pulps.

RESULTS AND DISCUSSION

SOFTWOOD STUDY

The softwood kraft pulp was treated with laccase HBT, and delignification percentages were comparable to earlier experiments in Chapter 1 (Table 6.1). The laccase HBT stage followed by an alkaline extraction (LE) produced higher delignification percentages in a O_2 delignified pulp than in a brownstock (BS) pulp. However, the amount of lignin removed was about the same for each. The selectivity of the LE stage was much higher than the oxygen stage when comparing treatments on the brownstock pulp. The LE stage remained selective with the oxygen delignified kraft pulp.

Table 6.1 Pulp properties and yields of isolated residual lignin of laccase (LacNS51002) HBT biobleached softwood kraft pulps (SSBK26 and SSOK13).

Pulp Type	Yield(%) of lignin by kappa #	Yield (%) of lignin by Klason	Kappa # of pulp	% Delignification	Pulp Viscosity (cP)	% Loss of Pulp Viscosity
Brownstock	45	48	26.8		31.9	
LE treated Brownstock	38	47	15.8	41	28.0	12
O_2 delignified	31	37	13.6	49	18.0	44
LE treated O_2 delignified	37	38	6.44	53	14.1	21

[a]Percent delignification was calculated by the difference in kappa number between the treated and untreated pulp divided by the starting kappa number. Pulp viscosity loss was calculated in the same manner. The percent lignin yield was calculated by [mass of lignin isolated/{(kappa number of pulp before treatment - kappa number of pulp after treatment}x 0.15}]x 100.

The residual lignin was isolated via acid hydrolysis, a method first introduced by Gellerstedt (70-71)(see Experimental Procedures for details). Once a carbohydrate-free lignin sample was collected, lignin functional group analysis was performed by ^{1}H and ^{13}C NMR analysis. The ^{1}H NMR spectrum was integrated for the following functional groups: carboxylic acid H, formyl H, phenolic H, aromatic H, aliphatic H, and methoxy H (Figure 6.1). Each integration region was based on lignin model compound experiments, and literature assignments were followed (see Experimental Procedures for details) (82-86). Since some overlap between the integration regions does occur, the quantitative values reported do not represent absolute values. The data was used to evaluate trends.

The carboxylic acid concentration in the residual lignin was used in part to rate the amount of chemical oxidation that had occurred to the residual lignin. The brownstock lignin had significantly lower concentration of carboxylic acid groups than bleached pulps (Figure 6.2)

(122-125). Oxygen delignification has been shown to increase carboxylic acid concentrations in the residual lignin, and this study corroborated that claim (126). The LE treatment appeared to

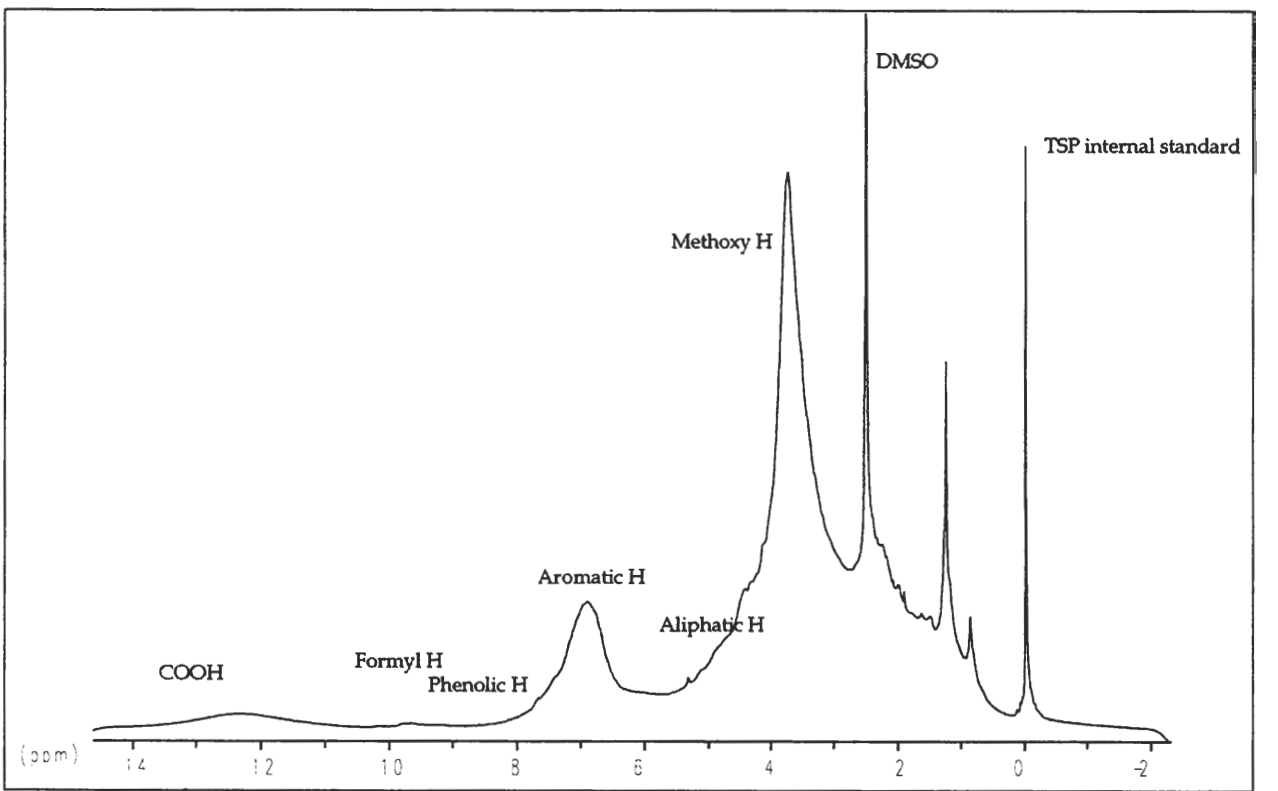


Figure 6.1 A ^{1}H NMR spectrum of a softwood brownstock residual lignin. The spectrum's intensity was reduced so all signals could be see in this graft. The solvent was DMSO-d_6.

increase the acid group content as much as the oxygen delignfication stage. However, no further increase in acid groups concentration occurred in the LE treated pulp after oxygen delignification. This was not too surprising since other studies have shown that the acid group concentration levels off after the first two stages of a four stage bleaching sequence (127). The apparent ceiling in carboxylic acid concentration may have occurred because once the acid group

concentration reaches a certain level further oxidation caused the lignin to be removed from the fiber matrix.

The phenolic concentrations in the residual lignins were used in part to understand the reactivity of the residual lignins. A correlation between phenolic concentrations in the residual lignin and bleachability has been shown. The higher the phenolic concentration the more reactive the residual lignin was with most commercial bleaching agents (124,126). The decrease

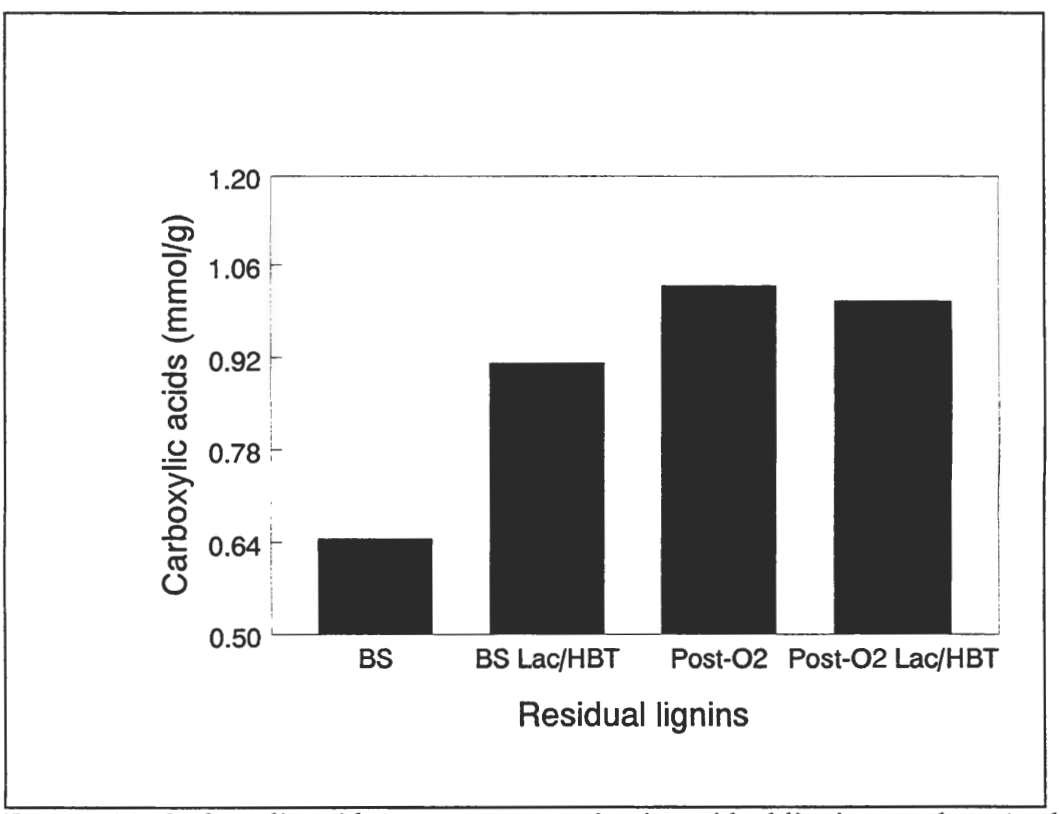


Figure 6.2 Carboxylic acid proton concentration in residual lignin samples. Analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 0.18 mmol/g is significant (with 95% confidence).

in phenolic group concentration in the residual lignins after oxygen delignification has been well established, and this was observed in this study (Figure 6.2). The LE residual lignin had a higher

phenolic content than the oxygen-delignified residual lignin when the phenolic content remained constant between the O_2 and the O_2LE residual lignins (Figure 6.3). The data suggested that free phenolic groups in lignin are quite reactive toward laccase HBT. However, laccase HBT also appears to react with non-phenolic groups or possible demethylated non-phenolic structures. This has been shown to occur in laccase ABTS experiments, and it also appeared to be true with laccase HBT treatments (50). The higher phenolic concentrations in the laccase HBT treated pulps suggested that the residual lignins remained reactive.

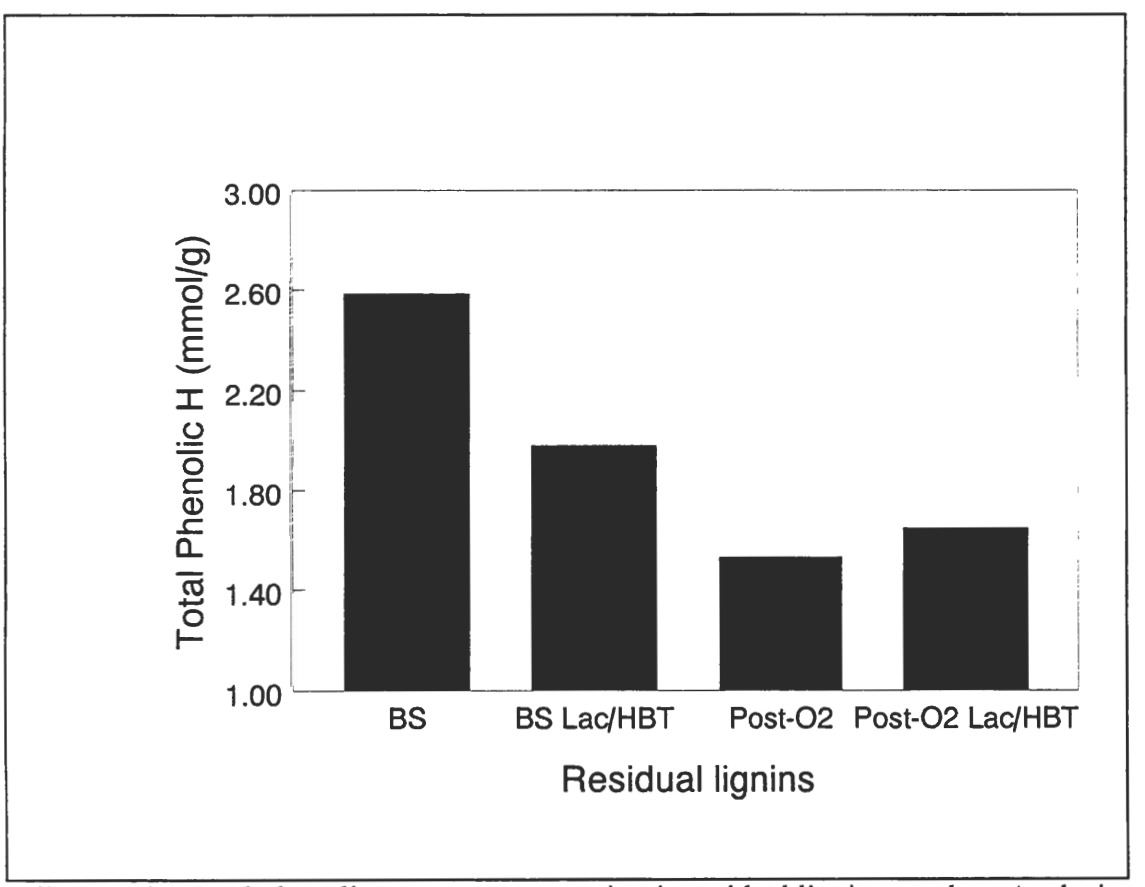


Figure 6.3 Total phenolic proton concentration in residual lignin samples. Analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 0.20 mmol/g is significant (with 95% confidence).

The methoxyl concentration for LE treated residual lignin was drastically decreased and the drop of methoxyl concentration was much higher for LE samples than oxygen delignified samples (Figure 6.4). The laccase HBT biobleaching appeared to cause demethylation in the residual lignin which agreed with the phenolic data presented above. The demethylation trend appeared to increase if the concentration of free phenolics was low. Demethylation appeared to be highly favored for the LE treated Post-O_2 delignified pulp than the LE treated brownstock pulp.

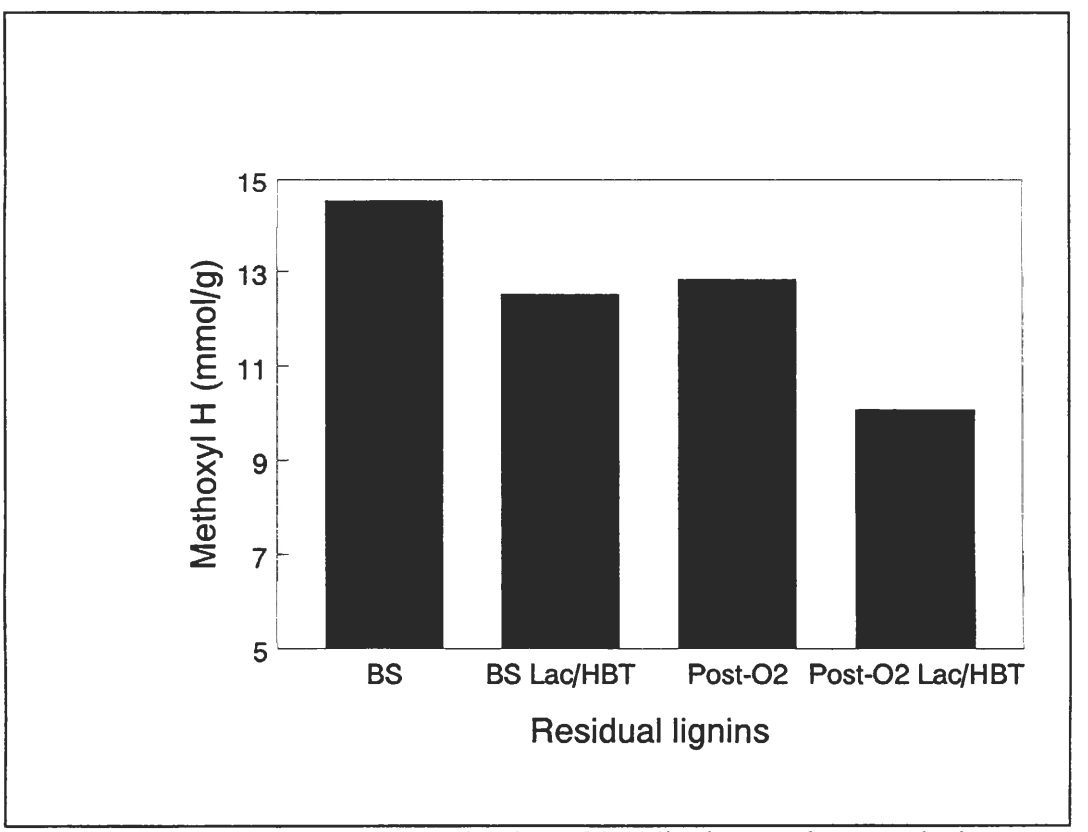


Figure 6.4 Methoxyl proton concentration in residual lignin samples. Analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 1.69 mmol/g is significant (with 95% confidence).

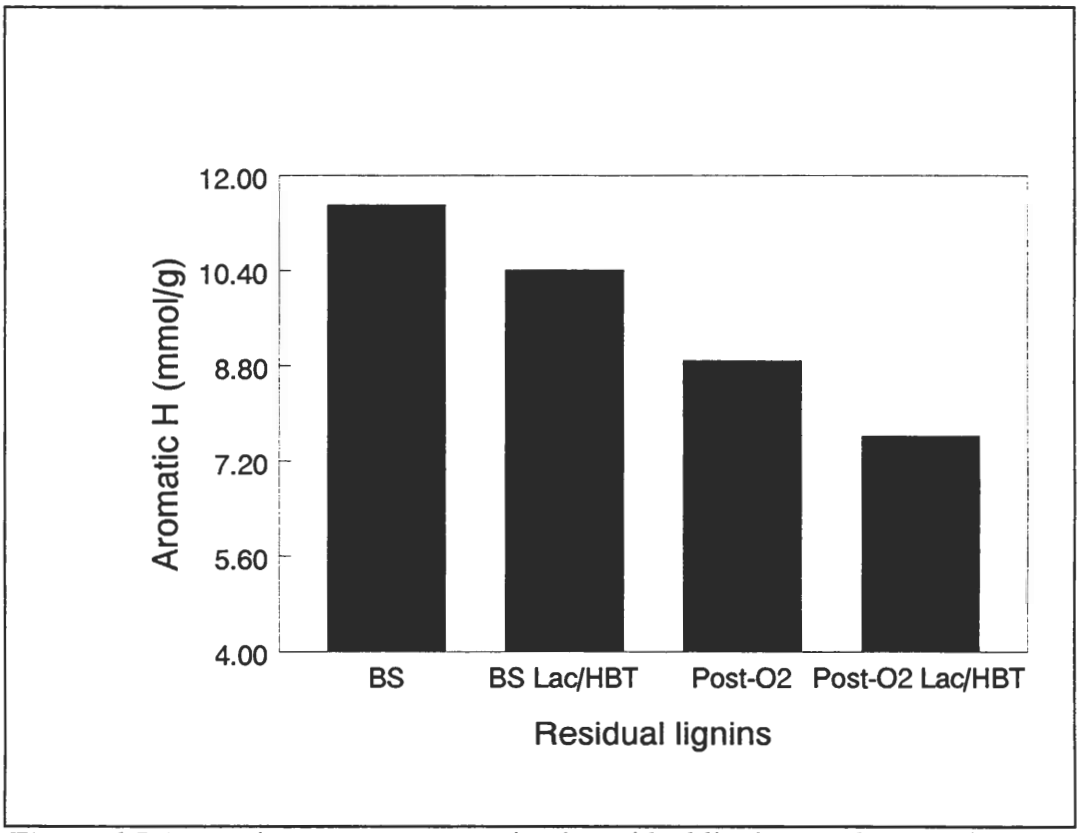


Figure 6.5 Aromatic proton concentration in residual lignin samples. Analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 1.53 mmol/g is significant (with 95% confidence).

The aromatic proton concentrations in the residual lignin was used to obtain an indirect measurement of the level of condensed structures, which is reflected in the amount of substituents on the aromatic rings of lignin. A decrease in aromatic proton concentrations in lignins can also represent ring opening. The analysis was used in part to predict the reactivity of the residual lignin. Generally, the lower the kappa number of a pulp sample, the more condensed the residual lignin becomes. This most likely occurs through enriching these higher substituted lignins, but some crosslinking may occur during bleaching with radical based systems. Aromatic proton concentrations decreased in LE and oxygen delignified samples. However, the oxygen-

delignified residual lignin appeared to be more condensed than the LE treated brownstock sample (Figure 6.5).

HARDWOOD STUDY

A study similar to the softwood residual lignin experiments described above was performed on hardwood pulp samples. Surprisingly, a lower percent of delignification was observed for O_2 delignified LE treated pulp than LE treated brownstock pulp (Table 6.2). The selectivity was also higher for the LE treatment than the oxygen stage.

Residual lignin analysis showed significant differences between hardwood and softwood laccase HBT treated pulps (Figure 6.6). The carboxylic acid concentration in the residual lignins did not change for the brownstock samples after the LE treatment. The oxygen stage increased

Table 6.2 Pulp properties and yields of isolated residual lignin from laccase (LacNS51002) HBT biobleached hardwood kraft pulps (SHBK15 and SHOK10).

Pulp Type	Yield (%)of lignin by kappa #	Yield (%) of lignin by Klason	Kappa # of pulp	% Delignification	Pulp Viscosity (cP)	% Loss of Pulp Viscosity
Brownstock	54	61	15.7		54.5	
LE treated Brownstock	34	39	9.4	40	33.0	40
O_2 delignified	33	39	10.5	33	28.3	48
LE treated O_2 delignified	34	39	7.1	33	21.1	25

[a]Percent delignification was calculated by the difference in kappa number between the treated and untreated pulp divided by the starting kappa number. Pulp viscosity loss was calculated in the same manner. The percent lignin yield was calculated by [mass of lignin isolated/{(kappa number of pulp before treatment - kappa number of pulp after treatment}x 0.15}]x 100.

the concentration of carboxylic acid group in the residual lignin; but, after an LE treatment, the residual lignin had the same concentration of acid groups as the brownstock sample. The data

suggested that laccase HBT treatments did not favor ring opening or aldehyde oxidation to a carboxylic acid. Treatment of the oxygen delignified pulp with laccase HBT seemed to cause the removal of the carboxylic acid rich fraction of the residual lignin but very little, to no, oxidation to carboxylic acid groups occurred.

A decrease in the concentration of phenolic groups in residual lignins of LE treatments was shown, but no real difference between oxygen delignification or LE treatment was seen (Figure 6.7). The overall concentration of free phenolics remained unchanged after an LE

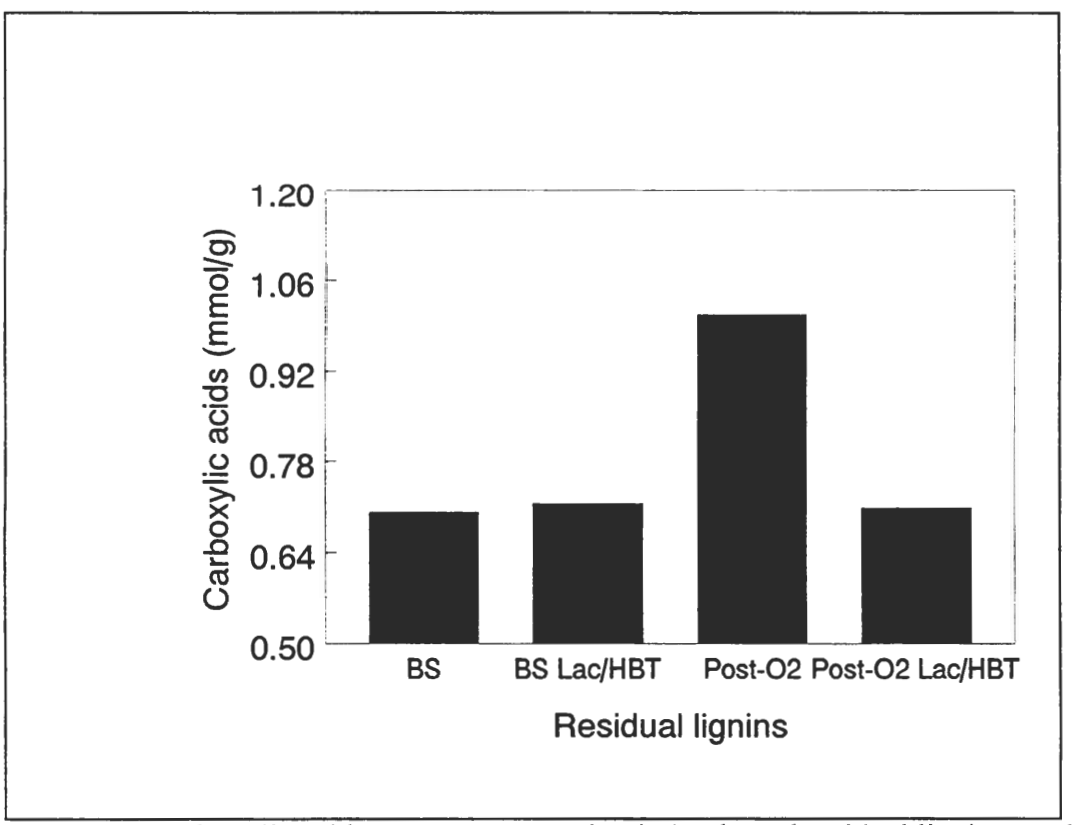


Figure 6.6 Carboxylic acid proton concentration in hardwood residual lignin samples. Analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 0.18 mmol/g is significant (with 95% confidence).

treatment of the oxygen delignified pulp. This suggested that the chemical oxidation that occurred during the LE treatment was not limited to free phenolic groups.

The methoxyl proton concentration in the residual lignin samples for both LE treatments decreased greatly (Figure 6.8). The oxygen delignification stage slightly decreased the methoxyl proton concentration in the residual lignin sample, but the decrease was only a fraction of the decrease that occurred during the LE treatment of the brownstock pulp. It appeared that the laccase HBT treatments favored demethoxylation of the hardwood pulp samples.

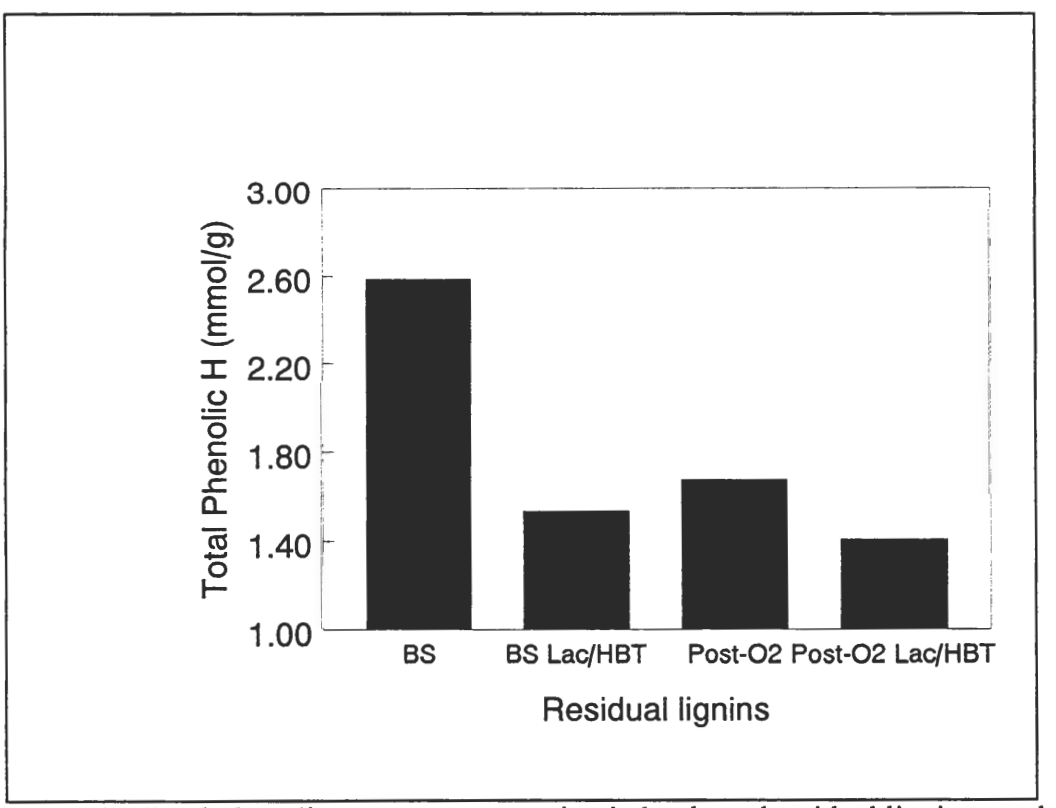


Figure 6.7 Total phenolic proton concentration in hardwood residual lignin samples. Analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 0.20 mmol/g is significant (with 95% confidence).

The aromatic proton concentration of the residual lignin samples decreased slightly with the LE treated samples. The oxygen sample was slightly less condensed than the LE residual

lignin when comparing the oxygen delignified residual lignin with the LE treated brownstock pulp sample. The concentration of aromatic protons in the residual lignin of the LE treated O_2 pulp was substantially lower than the oxygen delignified residual lignin sample. A significant difference in oxidation chemistry occurred when comparing softwood samples to hardwood samples.

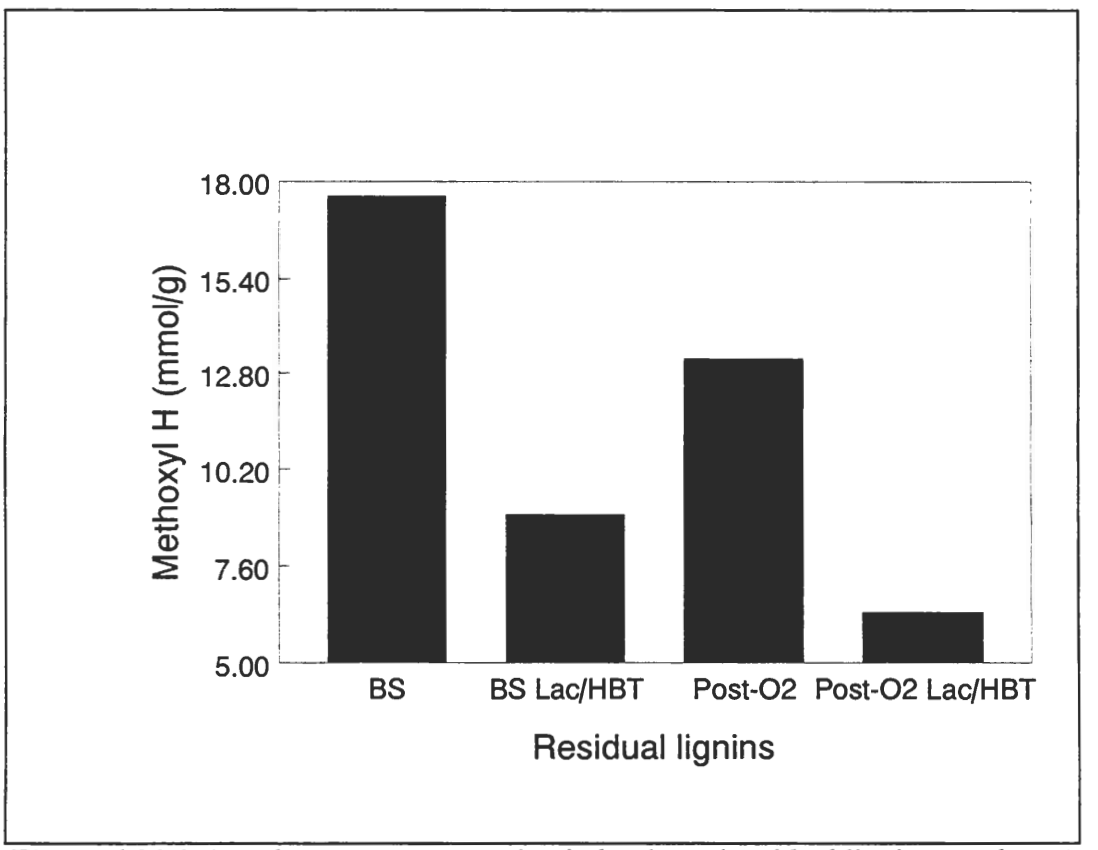


Figure 6.8 Methoxyl proton concentration in hardwood residual lignin samples. Analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 1.69 mmol/g is significant (with 95% confidence).

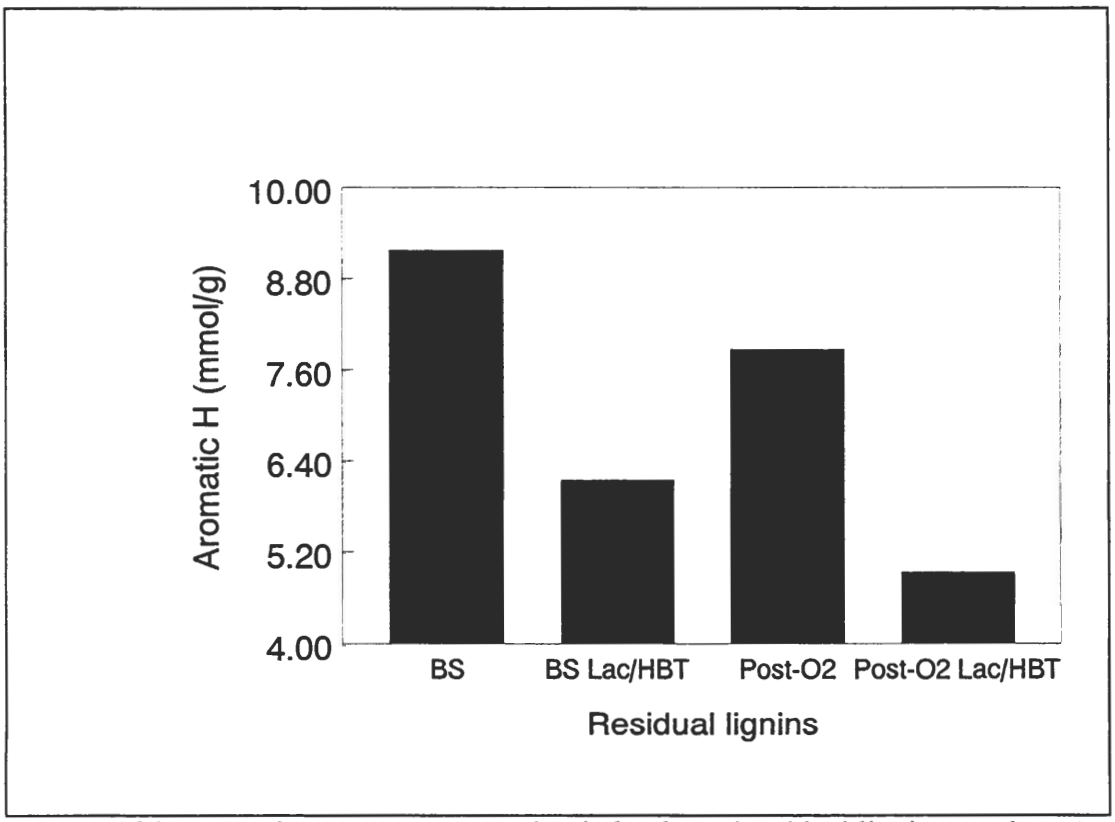


Figure 6.9 Aromatic proton concentration in hardwood residual lignin samples. Analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 1.53 mmol/g is significant (with 95% confidence).

TIME STUDY

Several large scale biobleaching trials of softwood oxygen delignified pulp were performed at different time intervals. Oxidation of the residual lignin was monitored with respect to reaction time to determine if changes to the functional groups of the residual lignin occurred over time, even if delignification remained constant.

The softwood Post-O_2 delignified pulp was treated with the same LE treatments used in the above studies for reactions times of 15 minutes, 1 hour, 4 hours, and 8 hours (SSOK13). The percent delignification ranged from 28 to 48 percent, and the pulp viscosity was nearly constant.

The 4 and 8 hour treatments produced almost the same kappa number pulp, and this was used to determine if modifications to the residual lignin occurred even if delignification remained unchanged. Very limited differences should have occurred between the 4 and 8 hour sample since the enzyme should have very limited activity after 4 hours (see Chapter 5).

Table 6.3 Pulp properties and yields of isolated residual lignin from laccase (LacNS51002) HBT biobleached softwood kraft pulps (SSOK13).

Pulp Type	Yield (%)of lignin by kappa #	Yield (%) of lignin by Klason	Kappa # of pulp	% Delignification	Pulp Viscosity (cP)	% Loss of Pulp Viscosity
1/4 hour LE	31	34	9.7	28	16.3	9
1 hour LE	31	39	8.2	39	15.2	15
4 hour LE	34	37	7.2	47	14.8	17
8 hour LE	34	37	7.1	48	15.7	13

[a]Percent delignification was calculated by the difference in kappa number between the treated and untreated pulp divided by the starting kappa number. Pulp viscosity loss was calculated in the same manner. The percent lignin yield was calculated by [mass of lignin isolated/{(kappa number of pulp before treatment - kappa number of pulp after treatment}x 0.15}]x 100.

The carboxylic acid concentrations of the residual lignin were unchanged for all four samples. The residual lignin appeared to change very little. The data agreed with the softwood study described earlier when all laccase HBT trials were performed for 24 hr. The carboxylic acid data could be misleading because a ceiling in the carboxylic acid concentrations seems to be apparent. When further oxidation occurred, the highly oxidized lignin was removed. With this explanation, the laccase HBT treatments could have generated carboxylic acid groups in the lignins of these pulps; but, the acids groups were not detected because the highly oxidized lignin fragments were removed.

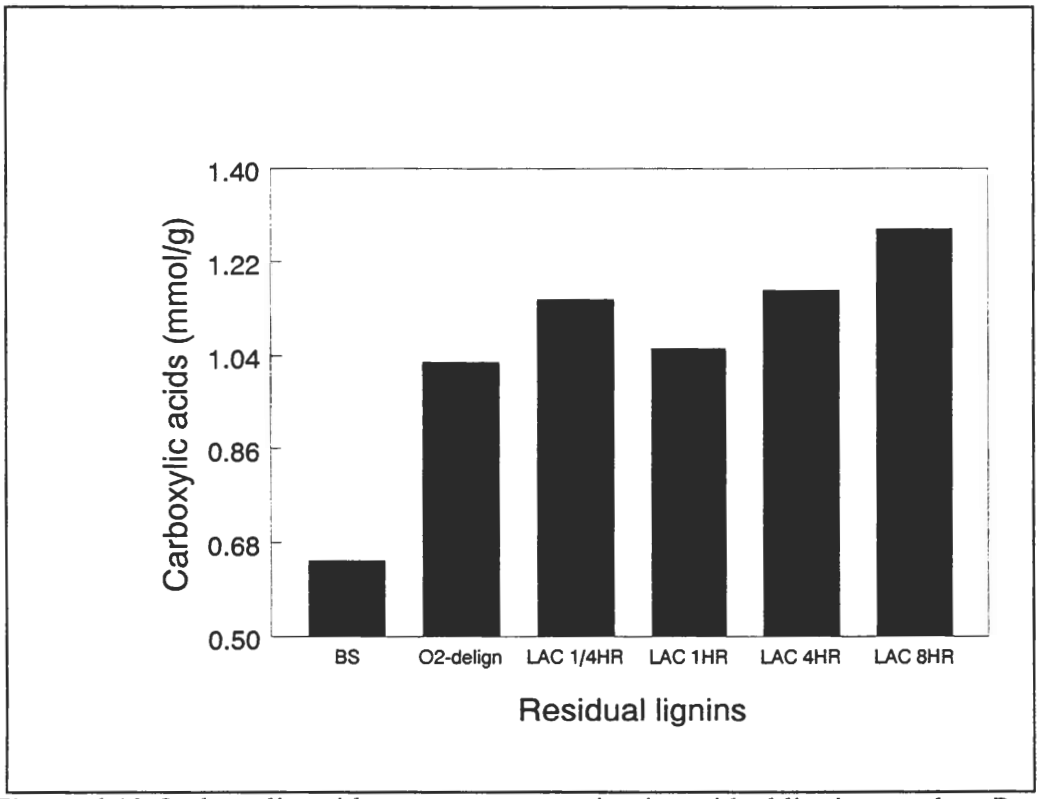


Figure 6.10 Carboxylic acid proton concentration in residual lignin samples. Post-O_2 delignified pulp (SSOK13) was treated with laccase (LacNS51002) and HBT for 0.25, 1, 4, and 8 hours reaction times. Analysis was performed by ^1H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 0.18 mmol/g is significant (with 95% confidence).

The phenolic concentrations in the residual lignins were also unchanged for all the tested samples (Figure 6.11). The phenolic concentrations was not too surprising since the laccase HBT system appeared to generate phenolic groups during delignification, but a significant selectivity for phenolic groups, at least with reaction time as a variable, was theorized with the laccase HBT biobleaching system. This was not apparent in our study.

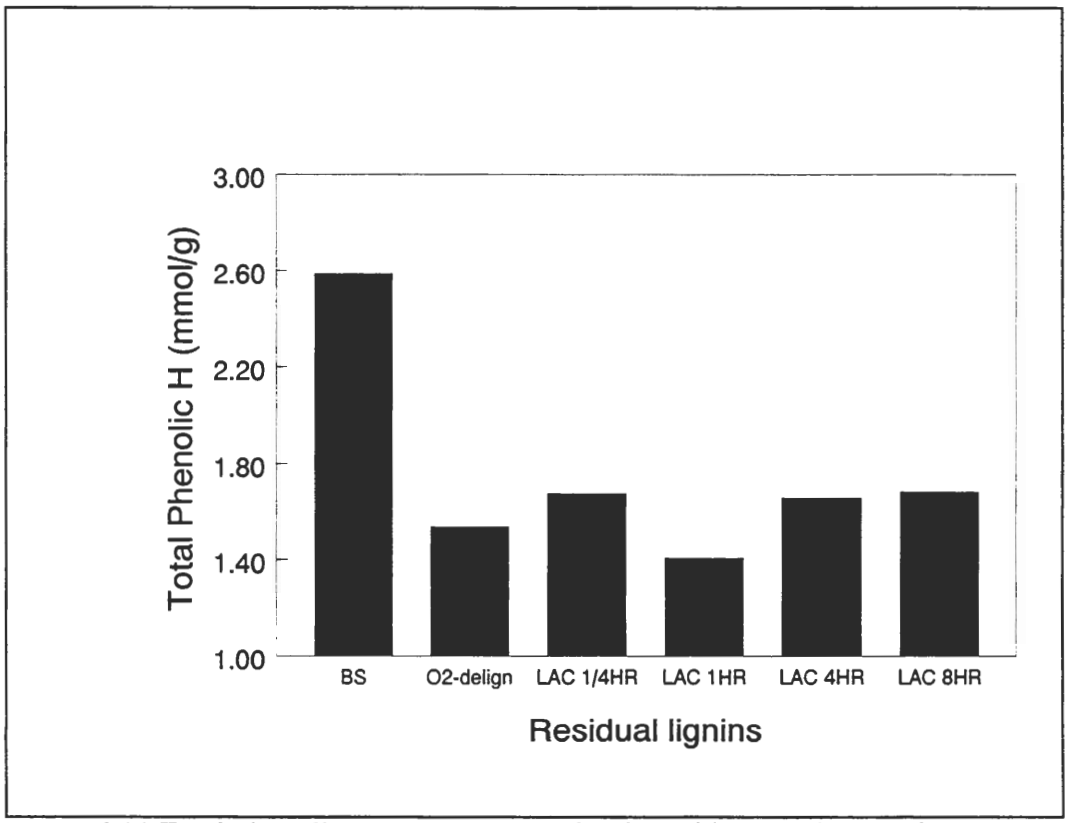


Figure 6.11 Total phenolic proton concentration in residual lignin samples. Post-O_2 delignified pulp (SSOK13) was treated with laccase (LacNS51002) and HBT for 0.25, 1, 4, and 8 hours reaction times. Analysis was performed by ^1H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 0.20 mmol/g is significant (with 95% confidence).

The methoxyl group analysis revealed a slight trend. The methoxyl group concentration for the 15 minute reaction was slightly higher than the other samples, but the difference was not statistically significant. The laccase HBT treated samples were significantly different than the oxygen delignified samples. The methoxy data suggested that a large part of the chemical oxidation of the residual lignin occurred within the first few minutes in the laccase HBT reaction. The lignin that was removed after the longer reaction times occurred through slight structural changes in the lignin.

The aromatic proton analysis showed no real change in the concentration of aromatic protons in the laccase HBT treated O_2 delignified samples (Figure 6.12). The aromatic data suggested that no further enrichment or generation of condensed structures occurred at the longer reaction times. The 24 hour laccase HBT study described above also revealed residual lignin aromatic proton concentrations equal to the time study, and the aromatic proton trend also agreed with the laccase HBT residual lignin studies presented earlier in Chapter 1.

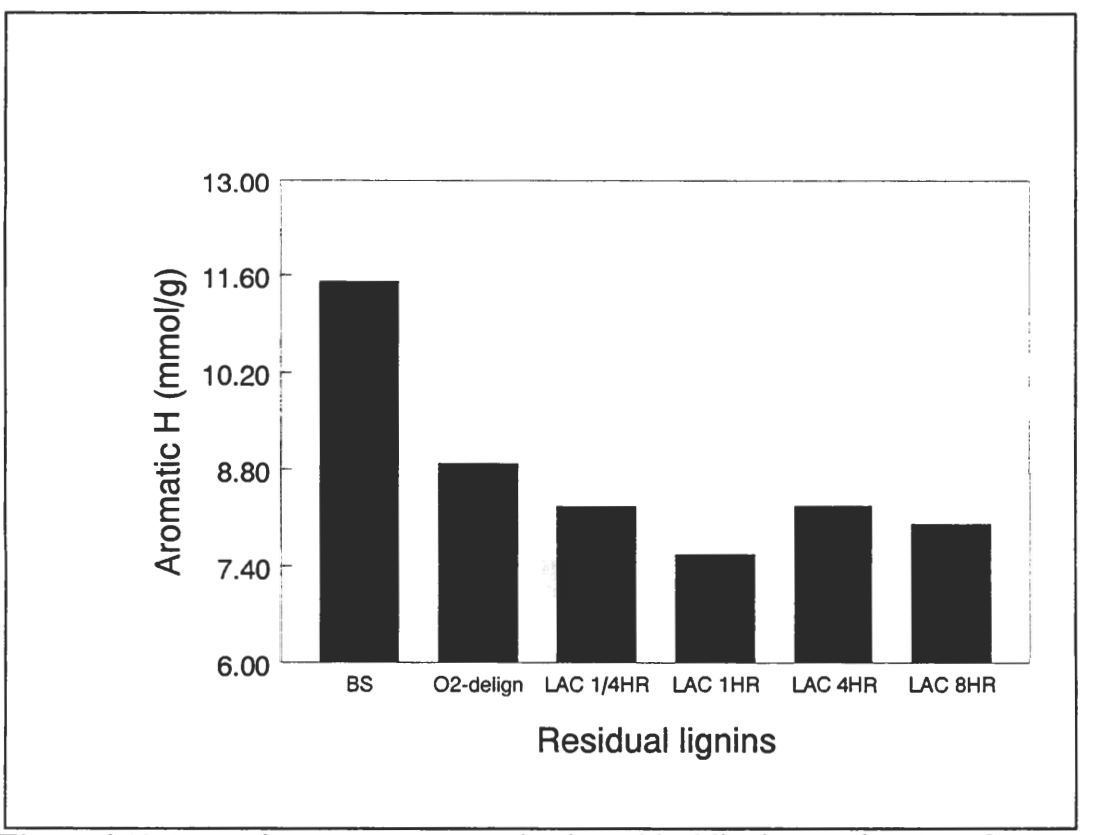


Figure 6.12 Aromatic proton concentration in residual lignin samples. Post-O_2 delignified pulp (SSOK13) was treated with laccase (LacNS51002) and HBT for 0.25, 1, 4, and 8 hours reaction times. Analysis was performed by ^1H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 1.53 mmol/g is significant (with 95% confidence).

ELEMENTAL ANALYSIS

The pulp samples from the previous studies were analyzed for carbon, hydrogen and nitrogen content (table 6.4). Elemental analysis was performed to determine if HBT was incorporated into the pulp. In theory, the oxidized mediator could covalently bond to the lignin matrix and increase the nitrogen content of the pulp. HBT contains 38.5 % nitrogen, while the brownstock pulp or starting material was void of nitrogen. Several controls were performed to determine if the laccase or mediator was incorporated into the pulp. A slight increase in nitrogen was detected in the pulp when laccase or HBT was added alone, and it appeared that more nitrogen was deposited on the pulp from the enzyme than the mediator. The highest nitrogen content pulp was detected after a laccase HBT treatment with no alkaline extraction. This was expected, and the nitrogen content decreased after an alkaline extraction. The same

Table 6.4 Elemental analysis of pulps treated with the laccase HBT. Several controls with mediator only and enzyme only were performed. The E represents an alkaline extraction.

Pulp Type	**% C**	**% H**	**% N**
Brownstock	44.33	6.28	0.00
Laccase only, E	44.35	6.19	0.04
HBT only, E	44.32	6.17	0.02
Laccase HBT, no E	44.25	6.18	0.14
Laccase HBT 4 hr., E	44.17	6.30	0.06
Laccase HBT 8 hr., E	44.27	6.24	0.04
Pulp Type	**% C**	**% H**	**% N**
Post-O_2	43.86	6.22	0.00
Laccase only, E	43.92	6.23	0.02
HBT only, E	43.82	6.34	0.02
Laccase HBT, no E	44.10	6.14	0.06
Laccase HBT 4 hr., E	43.87	6.23	0.05

study was performed with the oxygen delignified pulp, and similar results were seen. While the overall percentages of nitrogen incorporated in the pulp was low, this could be significant for industrial applications.

CONCLUSIONS

The laccase mediator biobleaching system removed lignin via an oxidative mechanism, and the laccase HBT system altered the residual lignin structure differently than oxygen delignification. While phenolic functional groups in the residual lignin after treatment with laccase HBT did decrease, the laccase HBT system did not appear to be limited to oxidizing these structures. Demethylation of the residual lignin was apparent in all laccase HBT experiments, and the ability to remove methyl groups from the residual lignins was greater for the laccase HBT system when compared to oxygen delignification.

A large difference in laccase HBT treatments of softwood and hardwood pulp was detected. Surprisingly, lower delignification percentages were seen with hardwood pulps than softwood pulps. Demethylation of the residual lignin was the main difference detected with the residual lignin analysis of the hardwood pulps.

The incorporation of nitrogen into the pulp was detected by elemental analysis. The data suggested that more nitrogen was incorporated into the pulp from the enzyme rather than from the mediator. While the overall percentages of nitrogen in the pulp was low, this could be significant for certain applications.

CHAPTER 7
ANALYSIS OF ISOLATED RESIDUAL LIGNIN REACTED WITH LACCASE IN THE PRESENCE OR ABSENCE OF A MEDIATOR

INTRODUCTION

Numerous studies have been performed on model systems that were used to predict laccase's reactivity with lignin in pulp or solid wood (23,33). These experiments used lignin model compounds with various linkages or immobilized laccase experiments with isolated suspended lignin. However, no real success was made in predicting how the enzymatic system would react with lignocellulosic materials. One limitation to the model studies may have been underestimating the size of the enzyme molecule which would limit it's ability to diffuse into a fiber matrix and react with the lignin (6). Model laccase experiments have provided some information on the increased reactivity of laccase mediator systems when compared with laccase only treatments. Paice and others have shown that laccase ABTS can react with non-phenolic lignin model compounds, while laccase alone could not. This data was used to explain the ability of the laccase mediator system to delignify kraft pulp (49,51).

Since laccase pulp bleaching model systems can produce useful information on the mechanism of laccase mediator delignification of kraft pulps, a study was performed to examine the affect of different laccase delignification mediators on lignin functional groups. An isolated residual lignin for kraft pulp was used for this study. Isolated residual lignin reactions, in theory, allowed us to compare lignins reacted with laccase and different mediators without substantial yield losses that occur with pulp lignin isolation techniques. The ability of each mediator to alter the residual lignin structure was compared to the mediator's ability to delignify kraft pulps.

The reactivity of laccase in the absence of a mediator could also be tested with isolated lignin experiments. The isolated residual lignin was suspended into an organic solvent system to facilitate a homogeneous reaction mixture. The reactivity of laccase was also considered. Several studies have been reported with laccase suspended in dioxane and water mixtures (121, 128). The activity of laccase in dioxane water mixtures was reported to be over 80 % of it's aqueous activity (128). Our preliminary activity measurement confirmed this, with laccase NS51002 retaining 90 % of it's activity in a 9:1 dioxane/ water mixture.

RESULTS AND DISCUSSION

MEDIATOR STUDY

The residual lignin was isolated from a brownstock kraft pulp (SSBK26). Several isolation batches were performed to accumulate several grams of residual lignin. The batches were well mixed, and all reactions were performed on this lignin sample. Four mediators were tested in this study: N-hydroxypthalimide (HTI), HBT, NHAA, and VIO. All of the mediators contained RR'NOH functional groups, and all dosages were based on molar equivalents. The mediator and enzyme dose used were based on the amount of reagents used in laccase HBT biobleaching treatments of brownstock kraft pulps. A 2 % charge of HBT on o.d. weight of pulp was used with respect to the amount of lignin present in a 26 kappa number brownstock pulp. For example, the amount of lignin in 10 g of brownstock pulp was ~400 mg, and 200 mg of HBT was a normal mediator dose; when 250 mg of isolated residual lignin was reacted with laccase and HBT in 9:1 dioxane/ water, 0.125 g of HBT was used, and 1.88 mL of NS51002 laccase was used. The reaction time was set to 4 hours with all the mediators, but the reaction

time was varied for laccase HBT and laccase only experiments. A reaction temperature of 45° C and an oxygen pressure of 145 psi was used, which also mimicked laccase biobleaching conditions.

The work-up of the reacted lignin was a concern since contamination from the mediator or the enzyme was possible. Preliminary trials revealed, by ^{13}C NMR, that laccase HBT lignin contained HBT and benzotriazole (a by-product produced when HBT reacts with laccase, see Chapter 2). These lignins were worked-up by the following technique: neutralizing the reaction mixture, evaporating off the dioxane, acid precipitating the lignin, and washing the solid material with distilled water several times. Another step was added to the isolation procedure, acetone extraction of the reacted lignin in a soxhlet. Acetone was chosen because all the mediators used were soluble in acetone. Yield was a concern because the highly oxidized lignins may be removed with an acetone extraction; however, all yields were above 85 %. All ^{13}C NMR spectra were also void of signals due to mediator compounds. Nitrogen analysis revealed low levels of nitrogen in the reacted lignins; most of the nitrogen contamination was most likely attributed to the enzyme and not the mediator species (Table 7.1). (If the nitrogen in the 4 hour laccase HBT lignin was only due to HBT contamination, the weight percent of HBT would be 6.4 % with HBT.) Surprisingly, the mediator compounds used in this study did not appear to be covalently bond to the lignin. This data agreed with the nitrogen analysis of laccase HBT treated pulps (Chapter 6).

Table 7.1 Percent yield and nitrogen contents for reacted lignins. Yields were calculated on a weight percent of the starting material added to the reaction vessel. Nitrogen contents were performed on 5 mg of lignin sample. All reactions were performed in 9:1 dioxane/ water with laccase NS51002.

Lignin	Percent Yield	Nitrogen content % ± 0.05
Starting material		0.00
Laccase only 1 hr reaction	95	2.11
Laccase only 4 hr reaction	95	1.94
Laccase only 8 hr reaction	98	2.07
Laccase HBT 1 hr reaction	88	2.19
Laccase HBT 4 hr reaction	92	2.47
Laccase HBT 8 hr reaction	96	2.51
Laccase HTI 4 hr reaction	98	2.76
Laccase NHAA 4 hr reaction	97	2.08
Laccase VIO 4 hr reaction	98	2.79

The reacted lignins were analyzed by ^{13}C and ^{1}H NMR. Three sets of comparisons were made: different mediators, laccase HBT reactions over time, and laccase reactions over time. Significant differences in the concentration of lignin functional groups were detected when the lignins treated with different mediators were compared. The carboxylic acid concentration, by ^{1}H NMR analysis, in the reacted lignin revealed a trend with delignification (Figure 7.1). The data in Figure 7.1 were arranged in order of delignification ability: laccase only has the least delignification of kraft pulp and violuric acid has the best. HBT and NHAA had about the same

ability to delignify kraft pulps. The response of an isolated lignin to reactions with different mediators was used to better understand the ability of each mediator system to delignify kraft pulp.

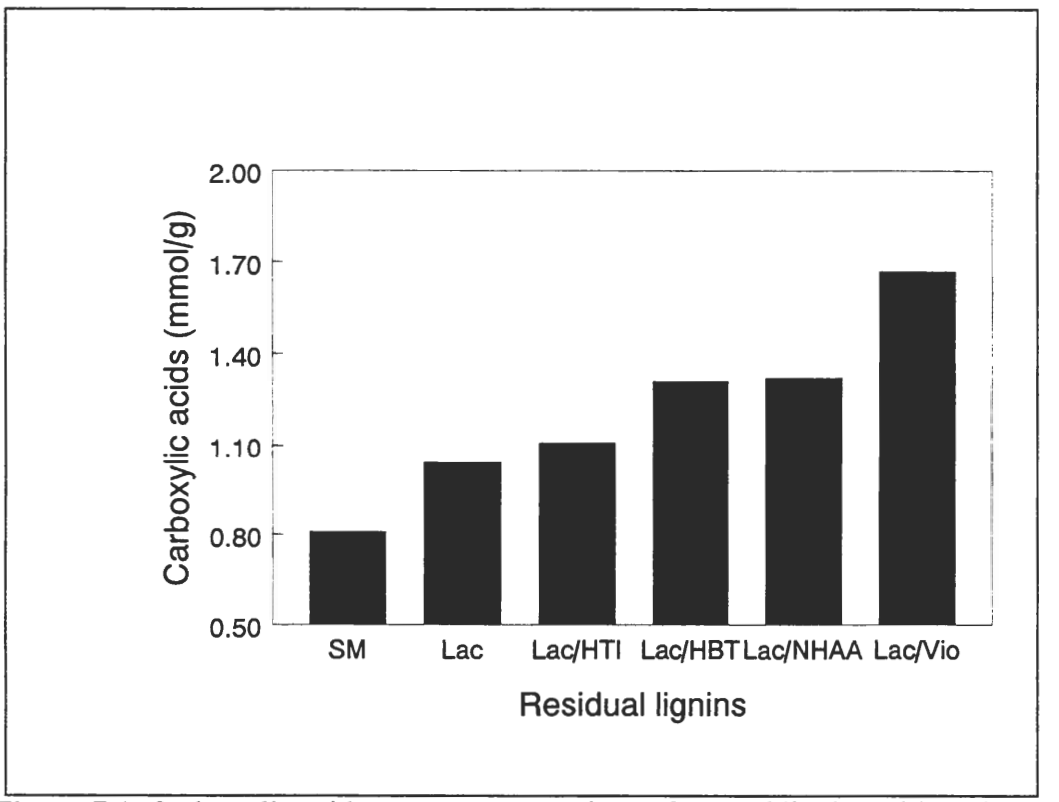


Figure 7.1 Carboxylic acid proton concentrations of reacted lignins with various mediators or laccase alone. All reactions were performed on isolated residual lignin from softwood brownstock kraft pulp. The analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggest that any difference greater than 0.18 mmol/g is significant (with 95 % confidence).

A steady increase in carboxylic acid groups was detected for mediators with higher delignification responses with kraft pulp (also see figure 4.5 and Table 3.1). The exact mechanism for generating acid groups in lignin was not known. Two possible mechanism involve the generation of muconic acids through a ring opening chemistry or the oxidation of gamma carbons to aldehydes and then to acids. However, our experiments can not determine

which mechanism or combination of mechanism occurred. The increase in acid groups in lignin appears to be an important mechanism in delignification with the laccase mediator system.

The methoxyl H concentrations revealed a different trend (Figure 7.2). Laccase alone and laccase HTI did not change the methoxyl group concentrations in the reacted lignins, but laccase HBT drastically lowered the methoxy content. Laccase NHAA also lowered the methoxyl group concentration, but much less then HBT. Laccase VIO treated lignin was only slightly lower in

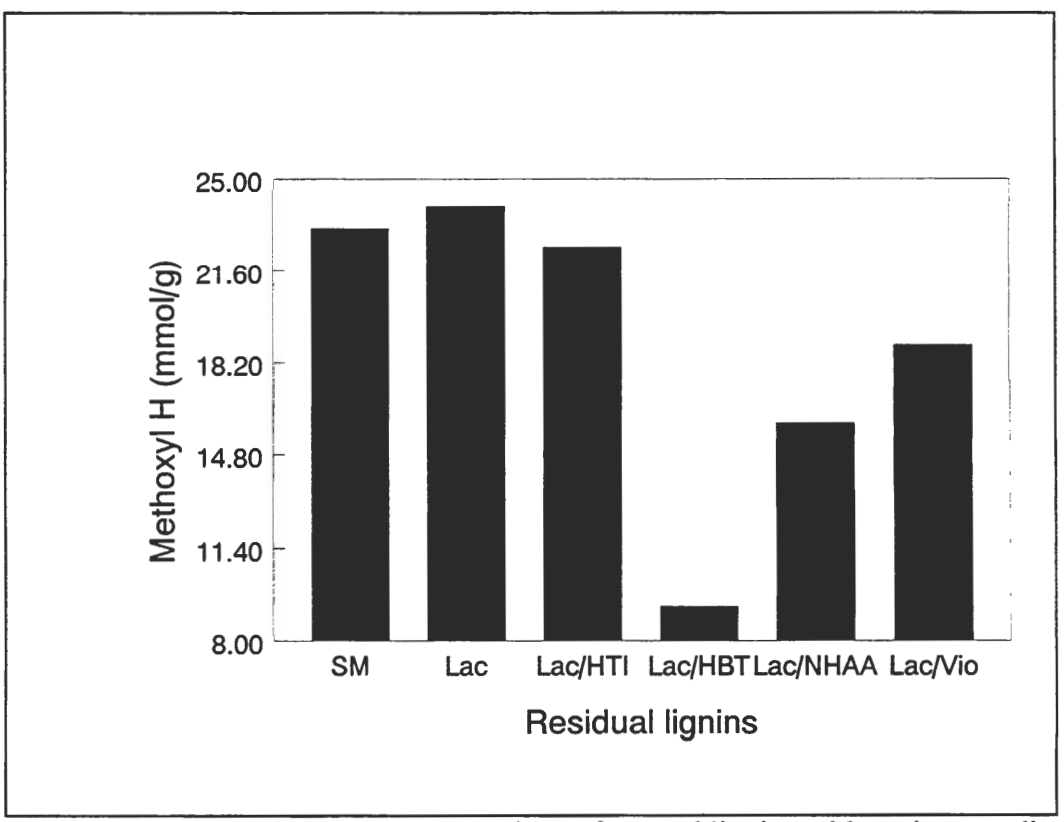


Figure 7.2 Methoxyl proton concentrations of reacted lignins with various mediators or laccase alone. All reactions were performed on isolated residual lignin from softwood brownstock kraft pulp. The analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 1.68 mmol/g is significant (with 95 % confidence).

methoxyl group concentration than the starting material. The different mediators appear to react with lignin with varying selectivity.

The aromatic proton concentrations revealed a trend similar to the methoxyl group analysis describe above (Figure 7.3). The laccase HBT reacted lignin had the lowest concentration of aromatic H which would suggest that the lignin was more condensed or crosslinked than the other lignins. NHAA and VIO treated samples were also lower in aromatic protons but to a much lower degree than HBT.

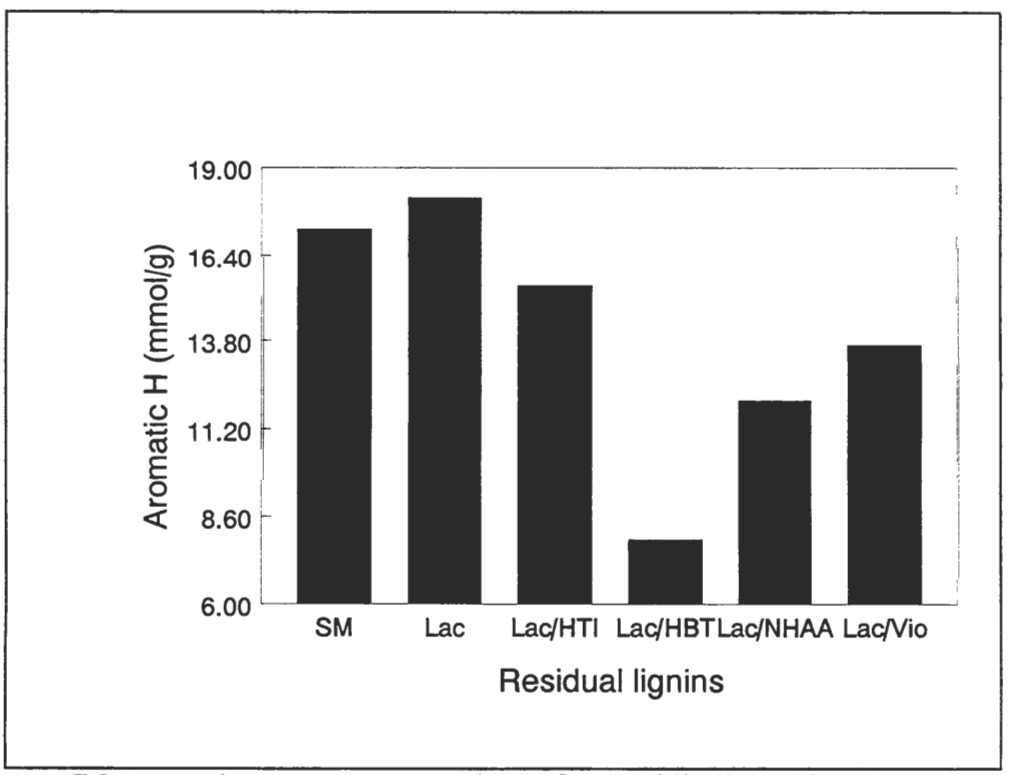


Figure 7.3 Aromatic proton concentrations of reacted lignins with various mediators or laccase alone. All reactions were performed on isolated residual lignin from softwood brownstock kraft pulp. The analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 1.53 mmol/g is significant (with 95 % confidence).

Formyl proton concentrations increased for all the mediator reactions but was unchanged for the laccase alone experiment (Figure 7.4). The formyl proton concentrations were used to

determine the concentration of aldehyde functional groups in the lignin samples. This reflects the degree of oxidation in the lignins, similar to the concentration of carboxylic acid groups. The amount of formyl protons in the lignin samples varied between mediators; the VIO treated lignin had the highest concentration.

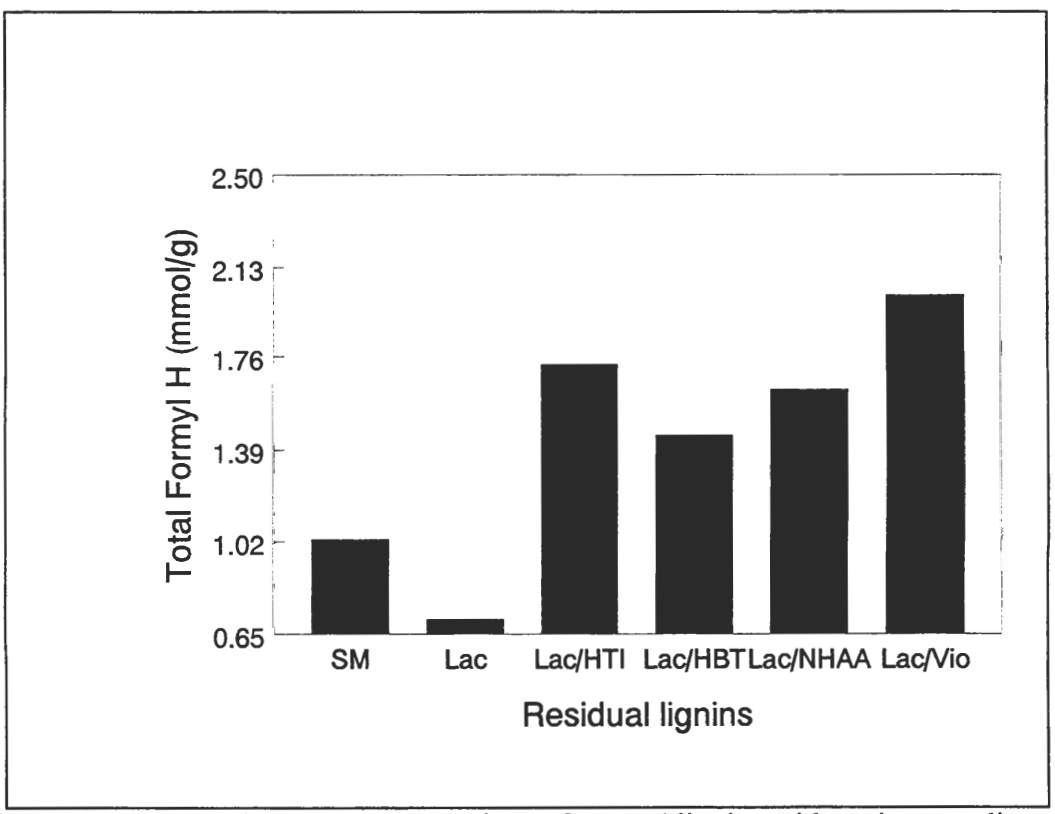


Figure 7.4 Formyl proton concentrations of reacted lignins with various mediators or laccase alone. All reactions were performed on isolated residual lignin from softwood brownstock kraft pulp. The analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 0.20 mmol/g is significant (with 95 % confidence).

The ^{13}C data showed no change in β-O-4 concentrations. This agreed with data presented in Chapter 1 and with the work presented by Levlin (114). The aromatic proton concentration decreased for the mediator treated samples, which agreed with the proton data. The spectra were

void of mediator or small MW compounds that would provide sharp peaks. While carbon analysis by ^{13}C NMR has been reported to be reproducible to ±5 %, the control study revealed higher variability for certain functional groups (Appendix 3).

LACCASE HBT REACTION TIME STUDY

Laccase HBT time experiments were performed to determine if functional groups changed with respect to reaction time. The carboxylic acid group rose substantially with laccase HBT treatments (Figure 7.5). The one hour treatment was as high as the eight hour treatment.

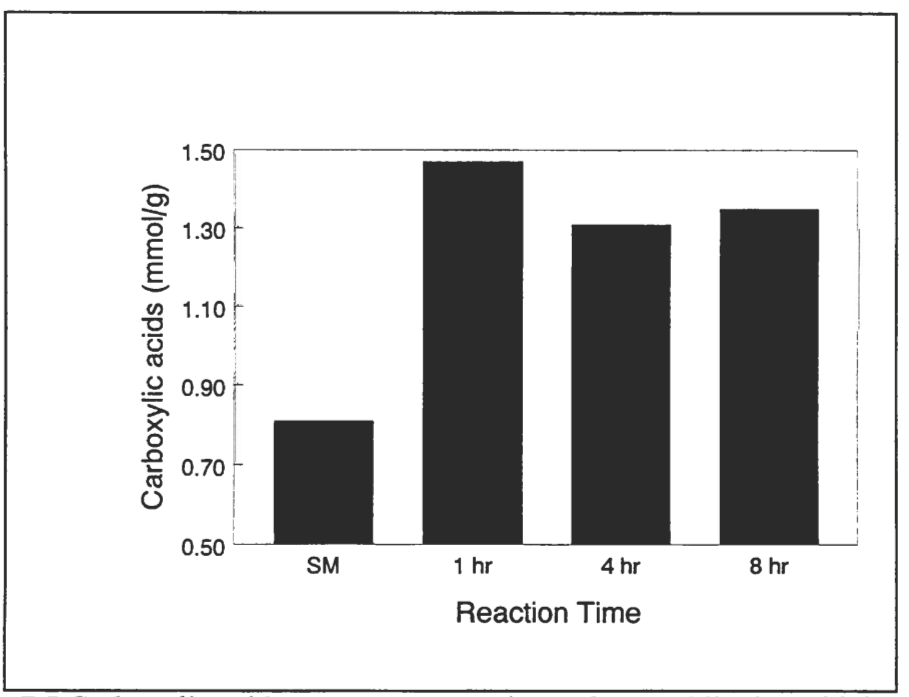


Figure 7.5 Carboxylic acid proton concentrations of reacted lignins with laccase HBT. All reactions were performed on isolated residual lignin from softwood brownstock kraft pulp. The analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 0.18 mmol/g is significant (with 95 % confidence).

The level of acid groups added to the lignin was much higher than that observed with the residual lignins isolated from pulps treated with laccase HBT. Besides no pulp being present during the

lignin reaction experiments, the reacted lignins were not exposed to alkaline extraction conditions or fractionated. The conclusions made from these experiments were only relevant to the chemistry between the laccase mediator system and isolated lignin.

The methoxyl proton concentration for the laccase HBT treated lignins dropped substantially in the first few hours of reaction (Figure 7.6). No real difference was detected between the four and eight hour experiments.

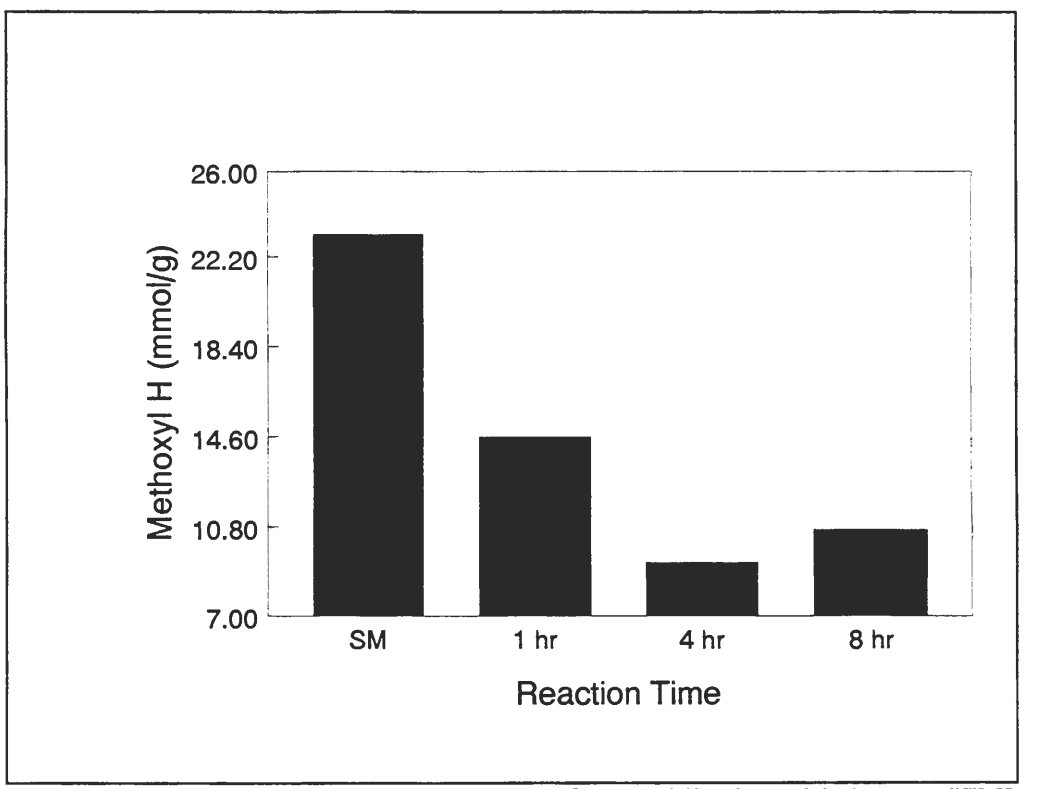


Figure 7.6 Methoxyl proton concentrations of reacted lignins with laccase HBT. All reactions were performed on isolated residual lignin from softwood brownstock kraft pulp. The analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 1.68 mmol/g is significant (with 95 % confidence).

The aromatic proton concentrations revealed the same trend as the methoxyl proton data (Figure 7.7). A drop in aromatic proton concentration was seen for the one hour experiment and the concentration of aromatic protons continued to decrease for the 4 hour experiment. This suggested that the lignin was becoming more condensed or more ring-opened structures were present as the reaction time increased.

The ^{13}C NMR data agree with the ^{1}H NMR with regard to the carboxylic acid groups increasing with the laccase HBT treatment. No real change to the β-O-4 linkages was detected, which agreed with the mediator study described above. A lower concentration of aromatic protons was also detected with the ^{13}C data.

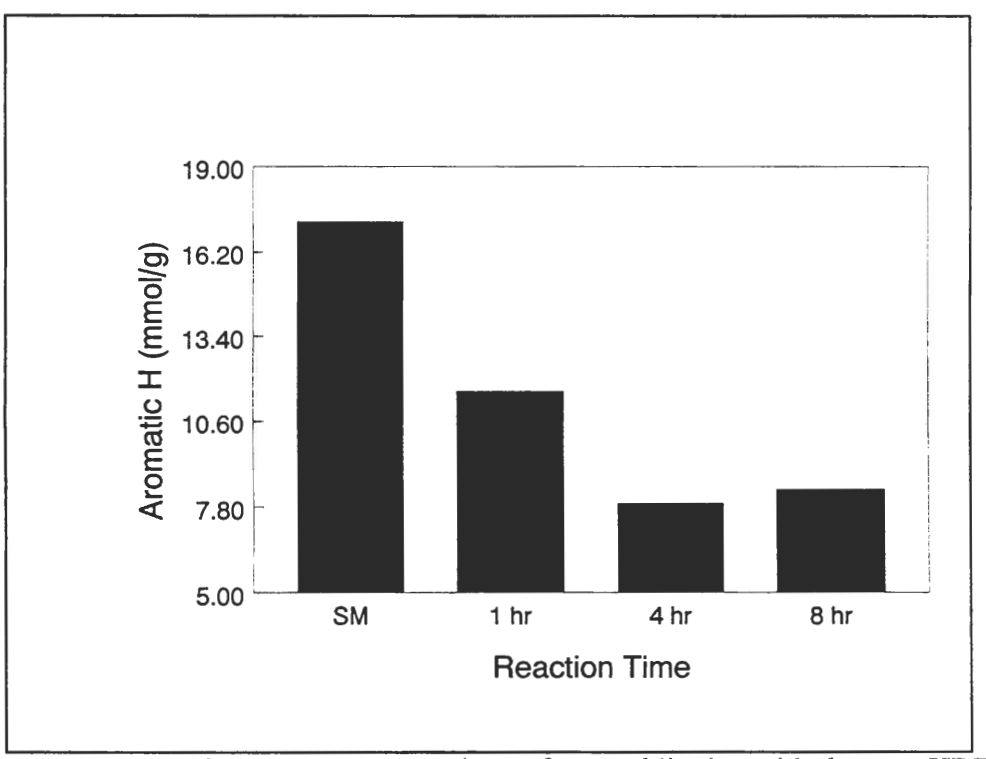


Figure 7.7 Aromatic proton concentrations of reacted lignins with laccase HBT. All reactions were performed on isolated residual lignin from softwood brownstock kraft pulp. The analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 1.53 mmol/g is significant (with 95 % confidence).

LACCASE ONLY REACTION TIME STUDY

A laccase only study was performed to determine the reactivity of laccase with an isolated residual lignin. Since the lignin was suspended in a homogeneous mixture, laccase should be able to react directly with the lignin. The concentration of carboxylic acid groups increased only slightly, with no difference between the 1, 4, or 8 hour reaction times (Figure 7.8). The increase of acid groups in the reacted lignins was only a fraction of the increase detected when HBT or VIO was present.

The methoxyl proton concentrations were not significantly changed by the laccase treatments (Figure 7.9). This was very different from laccase mediator treatments of isolated

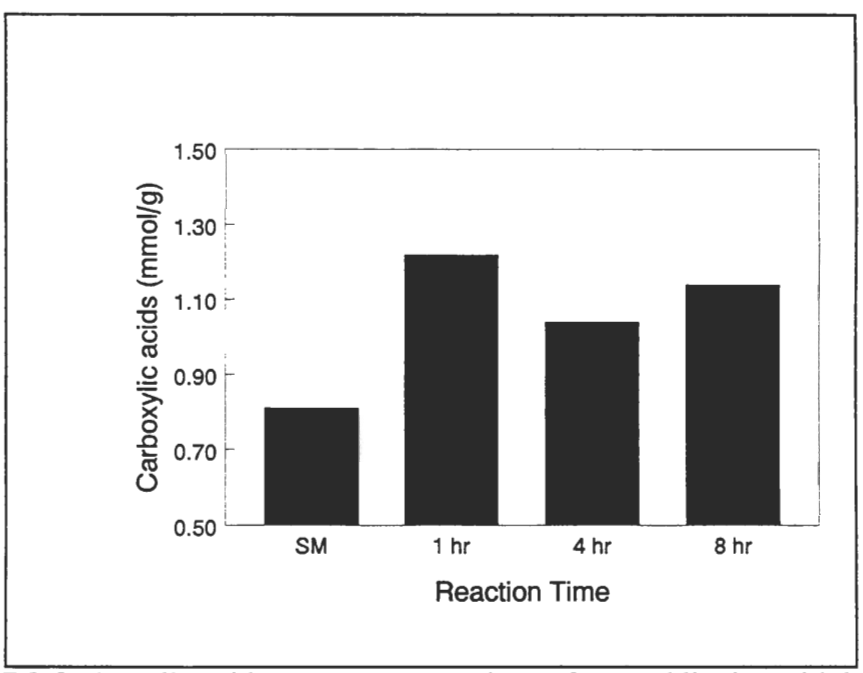


Figure 7.8 Carboxylic acid proton concentrations of reacted lignins with laccase alone. All reactions were performed on isolated residual lignin from softwood brownstock kraft pulp. The analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 0.18 mmol/g is significant (with 95 % confidence).

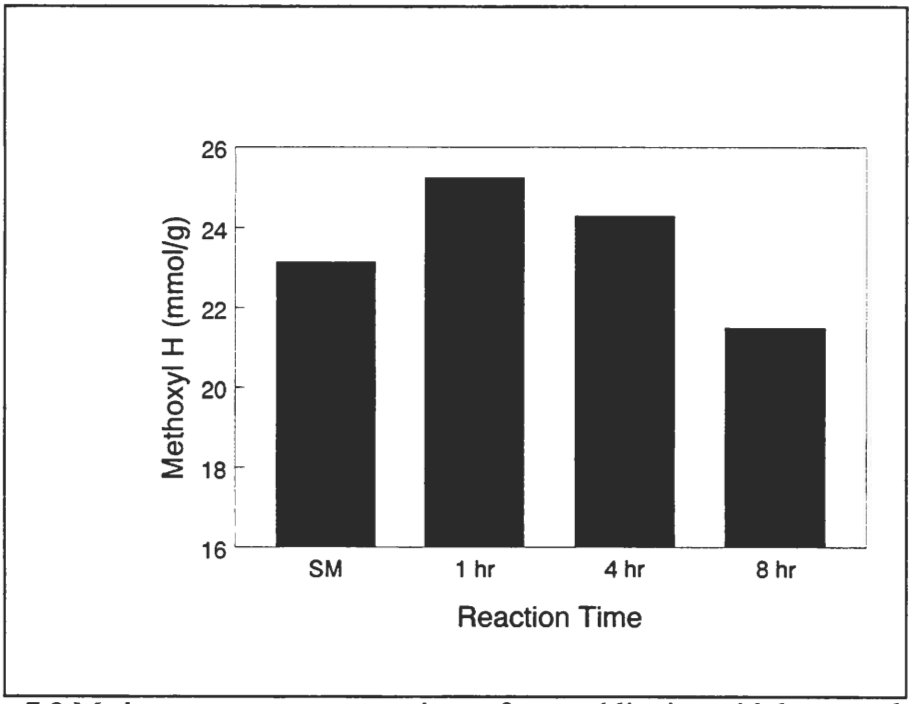


Figure 7.9 Methoxy proton concentrations of reacted lignins with laccase alone. All reactions were performed on isolated residual lignin from softwood brownstock kraft pulp. The analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 1.68 mmol/g is significant (with 95 % confidence).

lignin. Laccase alone appeared to be unable to demethylate lignin. This observation was in agreement with a study performed by Paice (50). In Paice's study, no release of methanol was detected with laccase alone, but when the mediator ABTS was used, a steady increase in methanol was observed from a pulp mixture. Paice also stated that delignfication of kraft pulps with the laccase enzymatic system was preceded by substantial demethylation. The presence of certain mediators appeared to greatly alter the reactivity of the enzymatic system with lignin.

The concentration of aromatic protons were unchanged with the laccase treatments, which again differed from mediator experiments. A visual difference between the reacted lignins treated with laccase alone or with mediator compounds was also detected. The lignin in

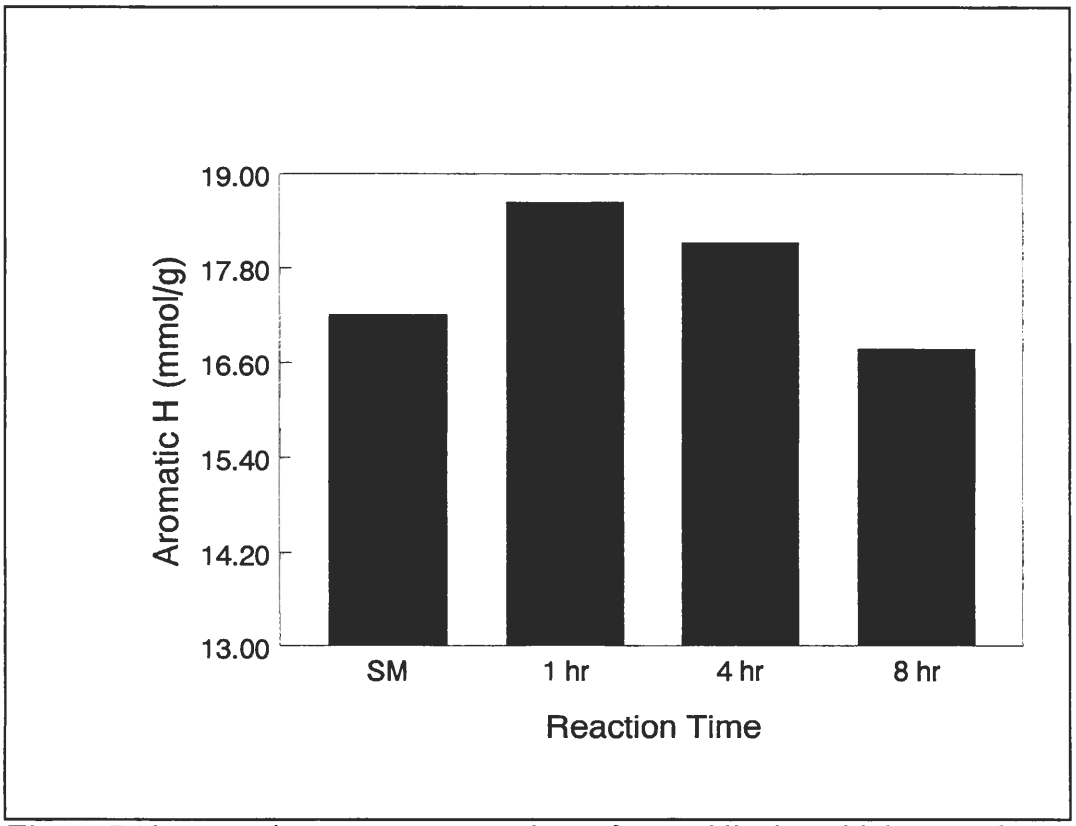


Figure 7.10 Aromatic proton concentrations of reacted lignins with laccase alone. All reactions were performed on isolated residual lignin from softwood brownstock kraft pulp. The analysis was performed by ^{1}H NMR in DMSO-d_6. The statistical analysis suggested that any difference greater than 1.53 mmol/g is significant (with 95 % confidence).

the laccase only reactions precipitated, and the amount of precipitated lignin appeared to increase with reaction time. This did not occur with most of the mediator reactions; however, HTI was the exception. The ability of laccase to polymerize lignin or phenolic compounds has been well established in the literature (26,44,42,129-134). Experiments with laccase polymerizing organosolv lignin in dioxane/ water mixtures were reported (121,128).

All of the reacted lignins were acetylated for GPC molecular weight analysis, but the acetylated reacted lignins were not soluble in THF. This could be due to the MW of the reacted lignin or problems with acetylating highly oxidized lignins. Oxidized lignins with high

concentrations of carboxylic acid groups could cause problems during acetylation. The anhydride formed would be unstable in the basic pyridine mixture and would be removed. Regardless of the cause, MW analysis was not obtained. Further investigation was beyond the scope of this study.

One possible explanation for no changes between the reacted lignin with 4 or 8 hour reaction times was the activity of the laccase. The data in Chapter 5 showed that the activity of laccase after 4 hour under elevated oxygen pressure was very low to inactive. This data was not known when the above experiments were performed.

CONCLUSIONS

Lignin functional groups significantly changed when reacted with the laccase mediator system, and each mediator used appeared to alter the isolated lignin structure differently. The isolated residual lignin was highly oxidized when laccase and a mediator was used, but very little changes to the chemical functional groups of lignin occurred when laccase was used alone. The concentration of carboxylic acid groups in reacted lignins appears to predict delignification ability of the laccase mediator system. The mediator that removed the most lignin from kraft pulps, also generated the highest concentration carboxylic acid groups in the reacted lignin. Surprisingly, the mediator compounds did not appear to covalently bond to lignin, even when reacting with isolated lignins.

THESIS CONCLUSIONS

The laccase HBT biobleaching system effectively removes residual lignin from kraft pulps via an oxidation mechanism. Phenolic functional groups were reacted selectively, but demethylation was also detected, especially with low kappa number pulps. Surprisingly, no significant decrease in β-O-4 structures was detected in the residual lignins of the LMS treated pulps.

The mechanism of delignification of the laccase HBT systems was radical-based. Selective radical quenching agents were effective in completely hindering delignification by laccase HBT. Since the radical quenching agent was too large to fit into the active site of laccase, the radical chemistry that was hindered occurred outside of the active site of laccase.

A strong limitation to delignification was the conversion of HBT to an inactive mediator species, BT. Laccase activity was lost after biobleaching with laccase mediator systems. Since the enzyme was deactivated and the mediator was converted into an inactive species, recycling of the bleaching agents in the laccase HBT biobleaching system appears to be impractical.

The LMS reacted better with O_2 delignified brownstock pulps than brownstock pulps when delignification percentages were compared. The amount of lignin removed was about the same in all cases. The delignification results suggested that the amount of material that LMS can oxidize was limited, regardless of the amount of enzyme or mediator used. However, if another LMS sequence was performed, delignification was detected.

RECOMMENDATION FOR FUTURE WORK

Future work in the area of enzymatic base biobleaching should involve a better understanding of mediator chemistry. The ability to selectively apply the oxidation power of oxygen to lignin, regardless of the catalysis, may provide a significant breakthrough into bleaching chemistry. Despite all of the recent work in laccase mediator chemistry, very little is known about what makes an activated mediator react with lignin, and what mechanism causes the mediator to be structurally unstable. If a recyclable active delignfication agent can be identified, the chemical cost associated with bleaching kraft pulp may be drastically reduced.

ACKNOWLEDGMENTS

I would like to thank the member companies of IPST for their support. I would like to thank Novo Nordisk for their laccase and access to Neal Franks and Claus Felby. It was an honor and a pleasure to work with them. I would also like to give special thanks to the Analytical, Pulping and Bleaching, and Wood Chemistry groups for their skillful assistance. I would like to single out Troy Runge and Mark Turner for their help with the bleaching sequence work.

I would like to thank my advisor Art Ragauskas for his guidance and support throughout my sentence at IPST. Art was the individual who introduced me to biobleaching, and his skillful knowledge and forethought in this area enabled me to have an excited and enjoyable project that incorporated numerous scientific disciplines. I would also like to give special thanks to the numerous faculty members who expressed negative opinions about my project which provided the drive and determination I needed.

Finally, I would like to thank my family for their support and encouragement over the years. I would have not been able to make it through the system without them. I also want to give special thanks to my wife to be, Kaaren. She has help me through some of the most difficult times, and completion of this degree would have not occurred without her.

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APPENDIX I NMR DATA FROM CHAPTER 1

Appendix 1 contains the ^{13}C and ^{31}P NMR spectrum of residual and effluent lignin samples presented in Chapter 1. ^{13}C NMR values were calculated by integrating the aromatic region 160.0-106.5 ppm to 6.

^{31}P NMR values were calculated by the following: the signal area of the cyclohexanol derivatized with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, which was due to $6.01x10^{-4}$ g of cyclohexanol, was integrated to 1.0 on the ^{31}P spectrum. Since the molecule contains one hydroxyl group and has a molecular weight of 100.16 g/ mol, $(100.16x10^{-4})/ 100.16 = 5.9994x10^{-6}$ was the number of moles of hydroxy groups present in the internal standard in the sample. Each unit of 1 in the integration of the spectrum was equal to $5.9994x10^{-6}$ moles of hydroxyl groups. The moles of functional groups in lignin was calculated by multiplying each integration region by $5.9994x10^{-6}$ and dividing by the weight of the lignin sample.

The filename listed for each spectrum is the filename used to store the spectrum in the UNIX data base.

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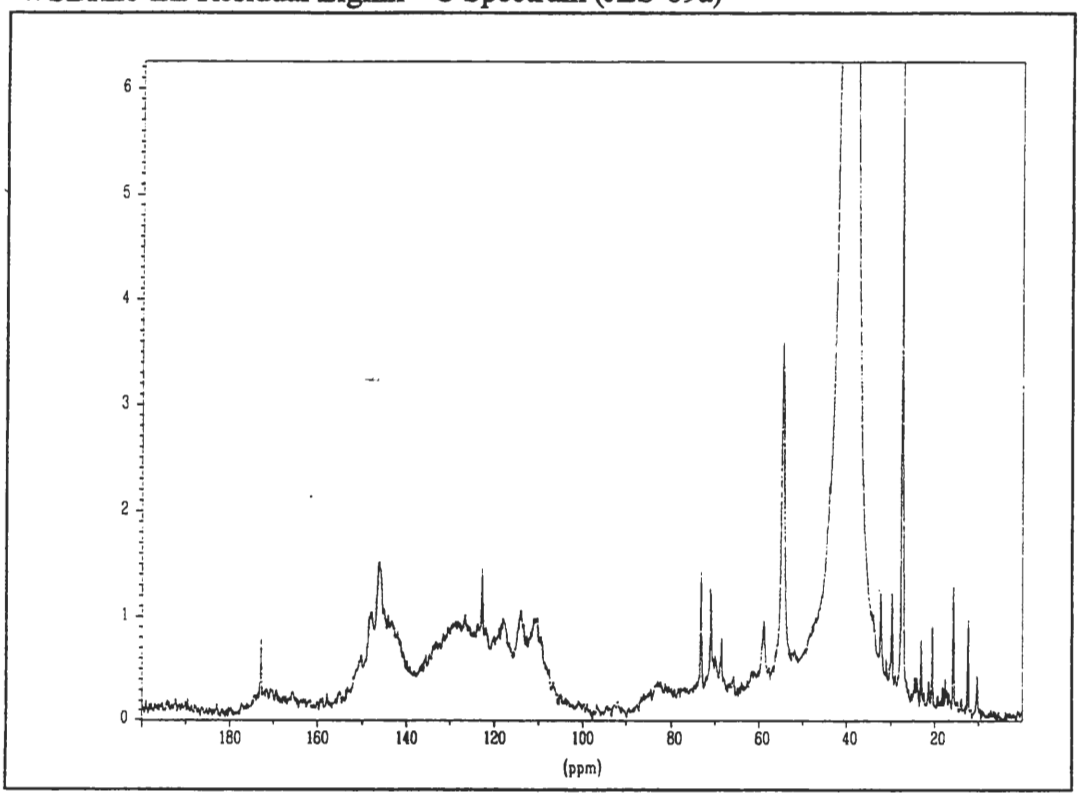

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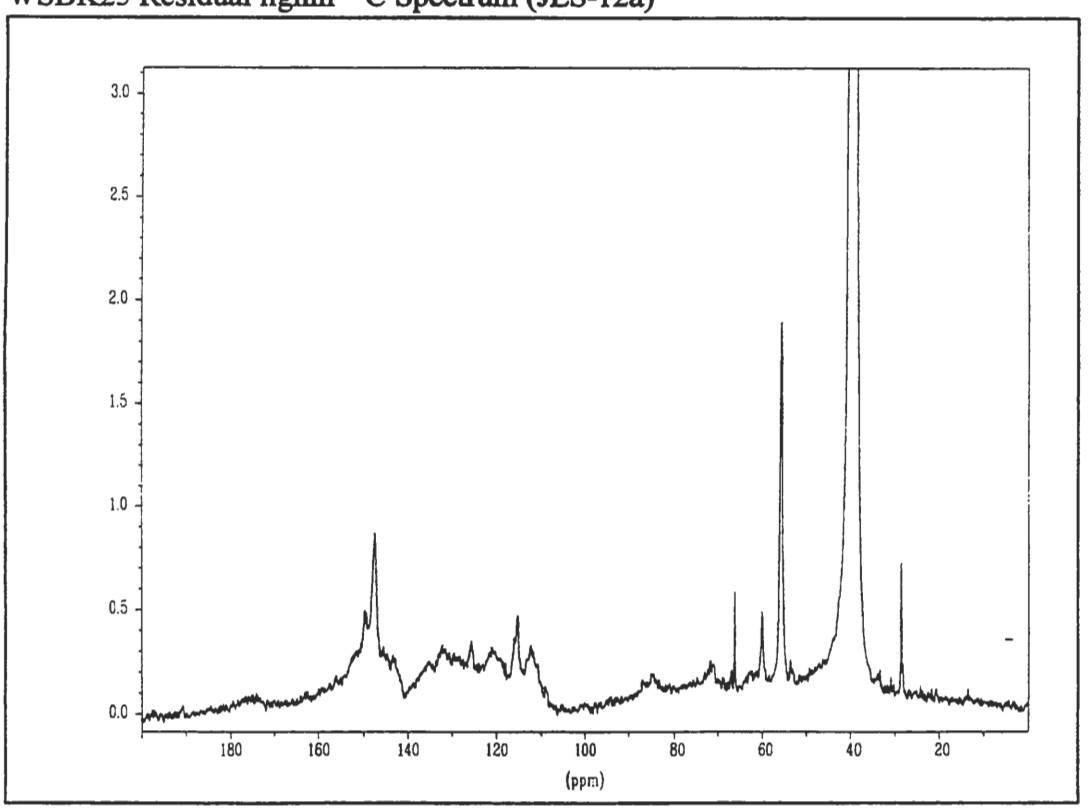

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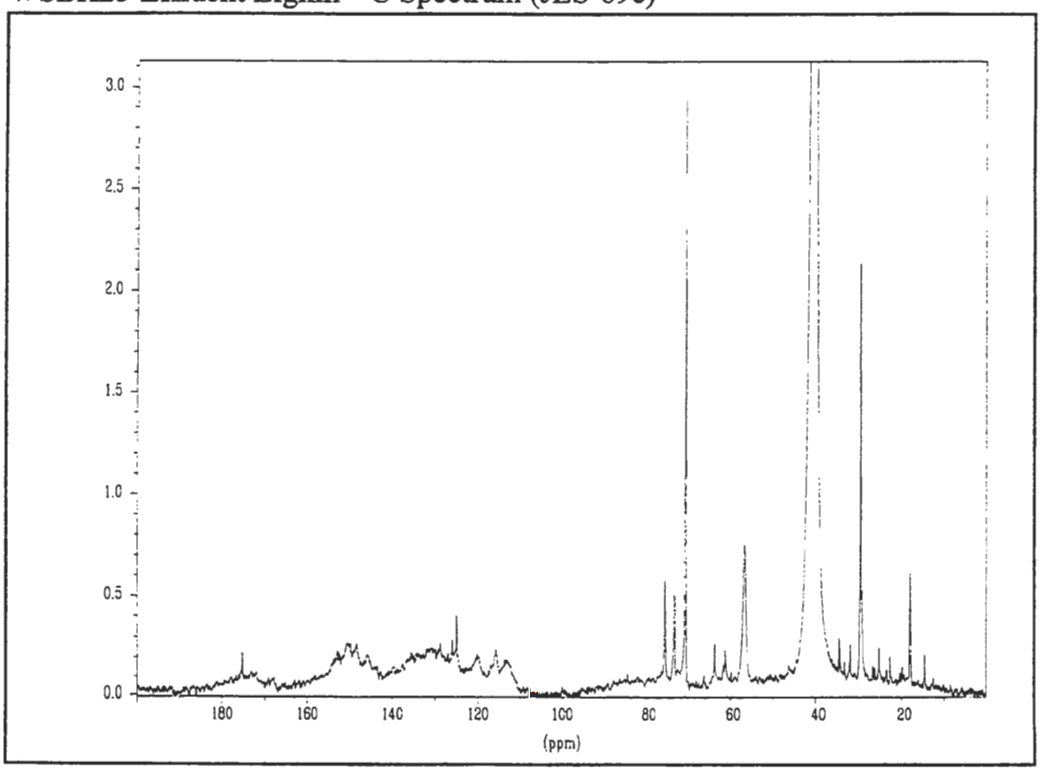

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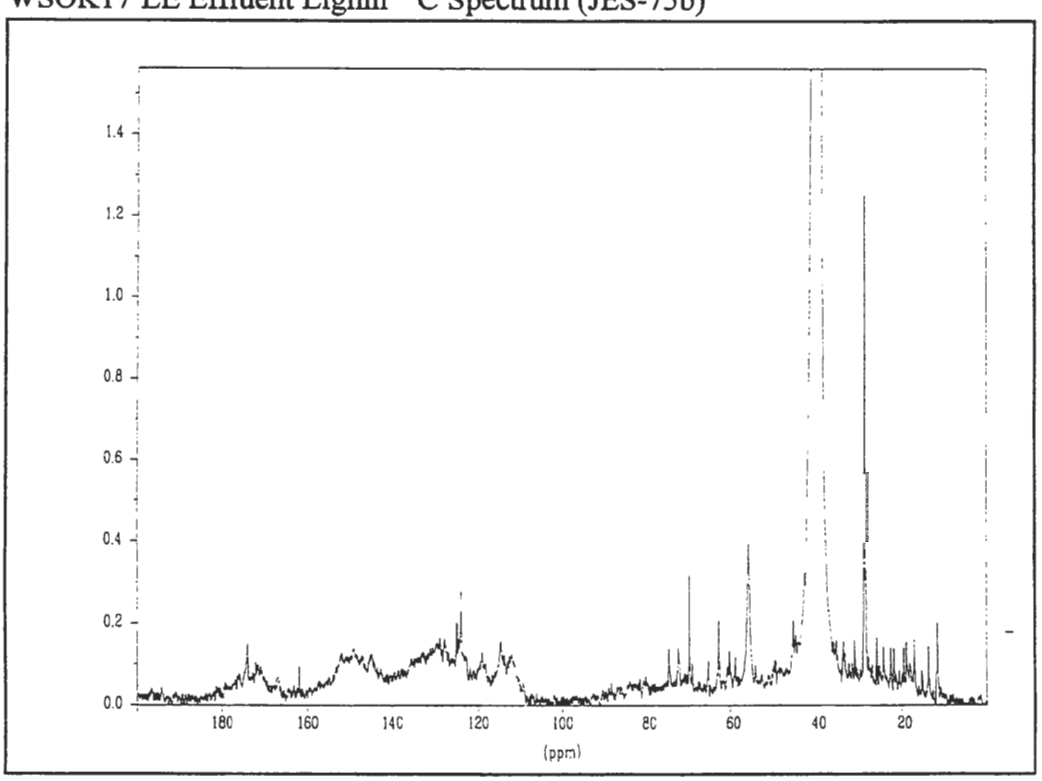

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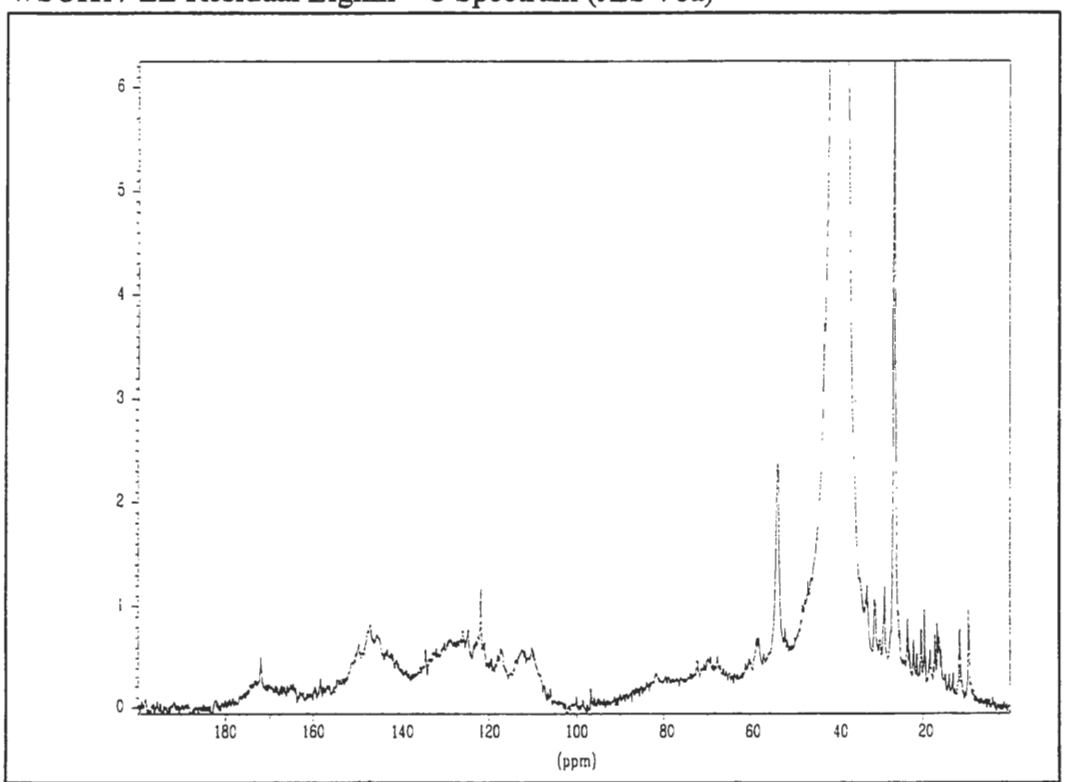

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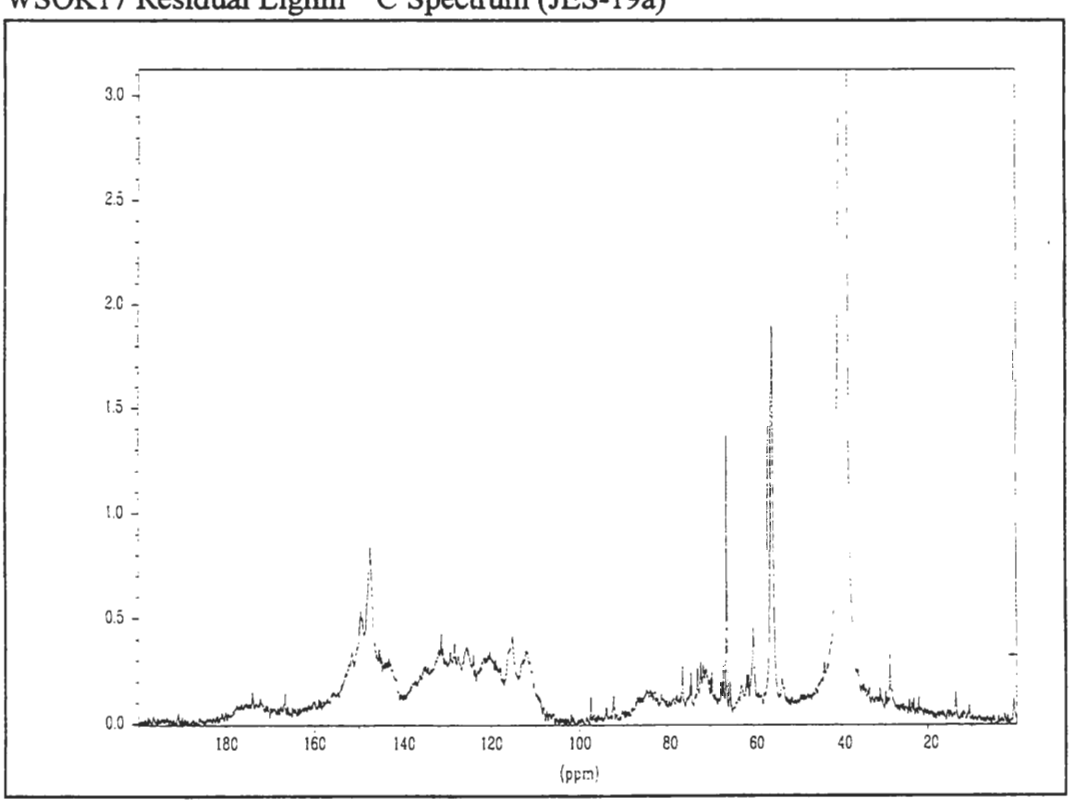
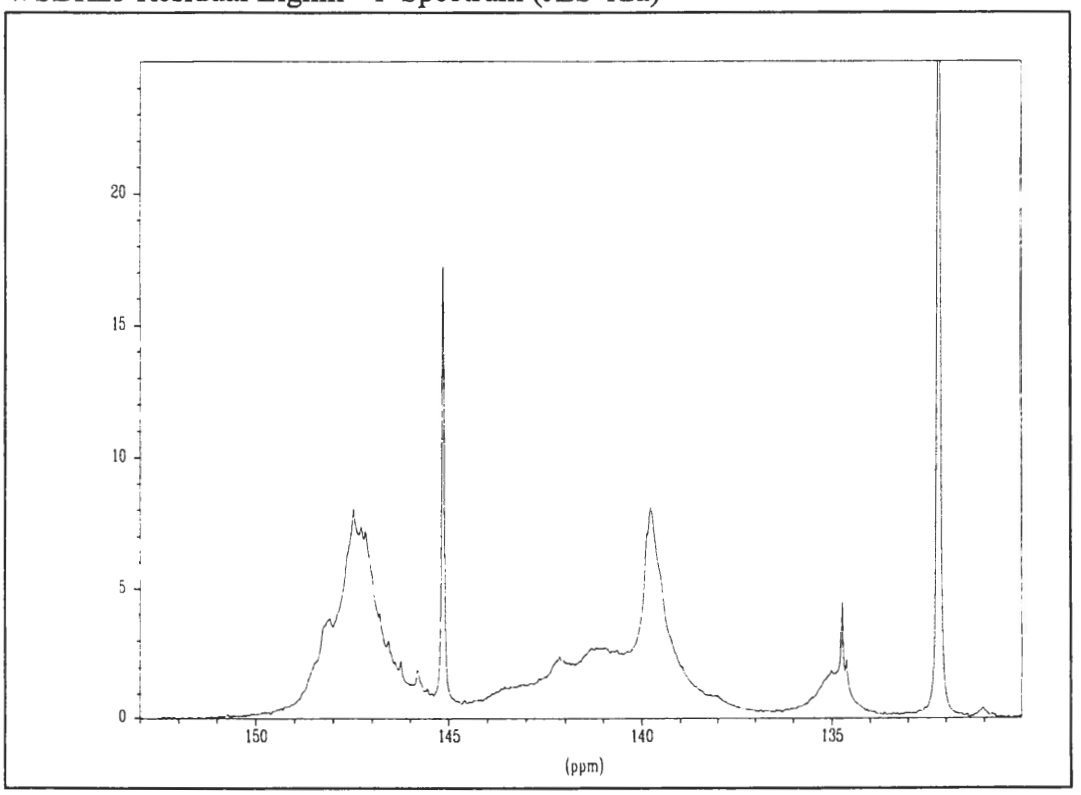


WSBK25 Residual Lignin ^{31}P Spectrum (JES-12a)



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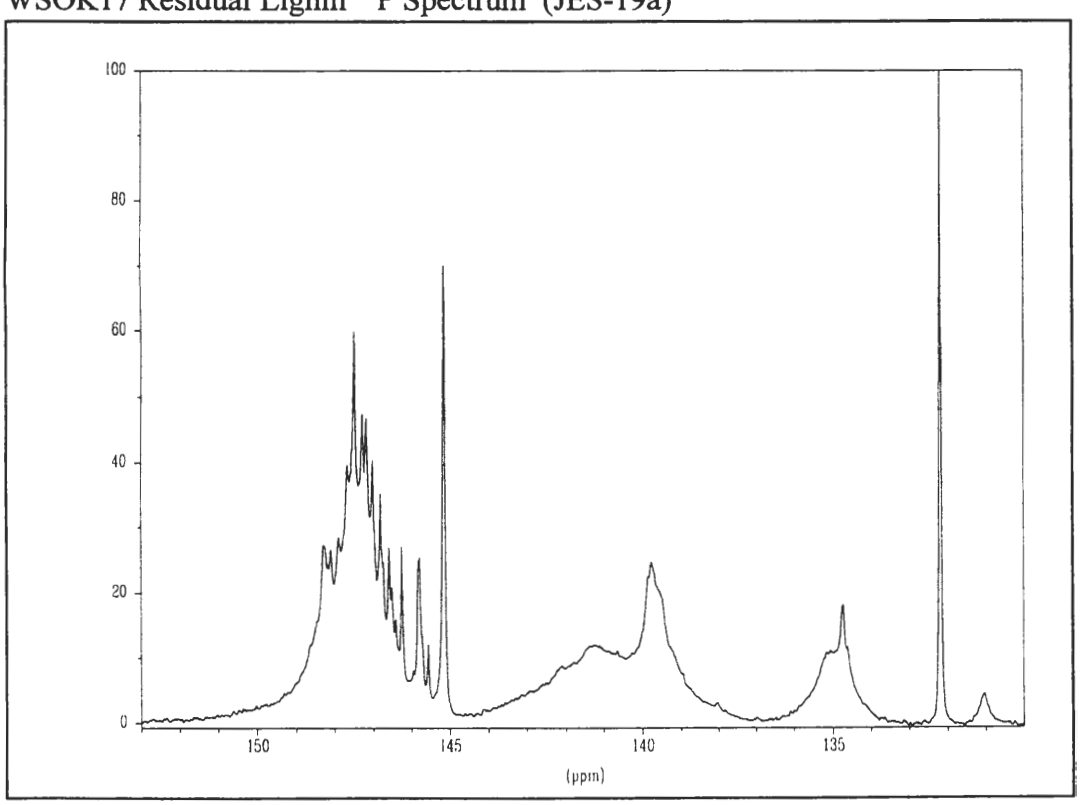

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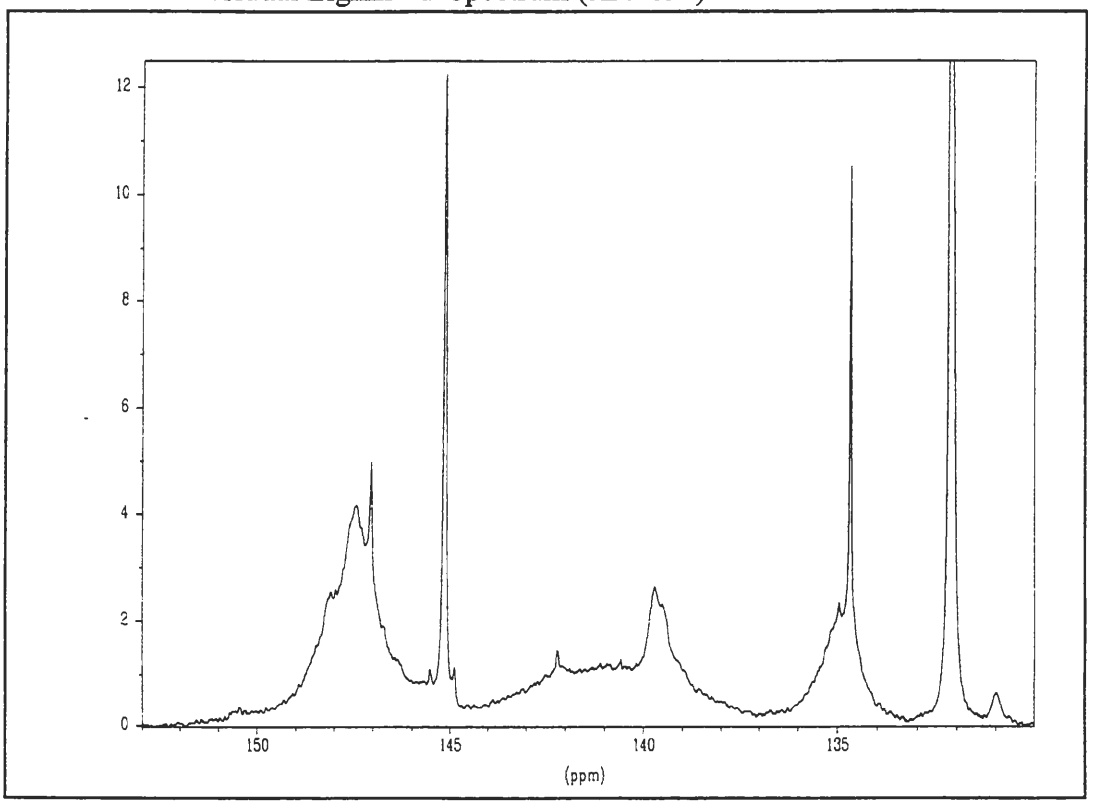
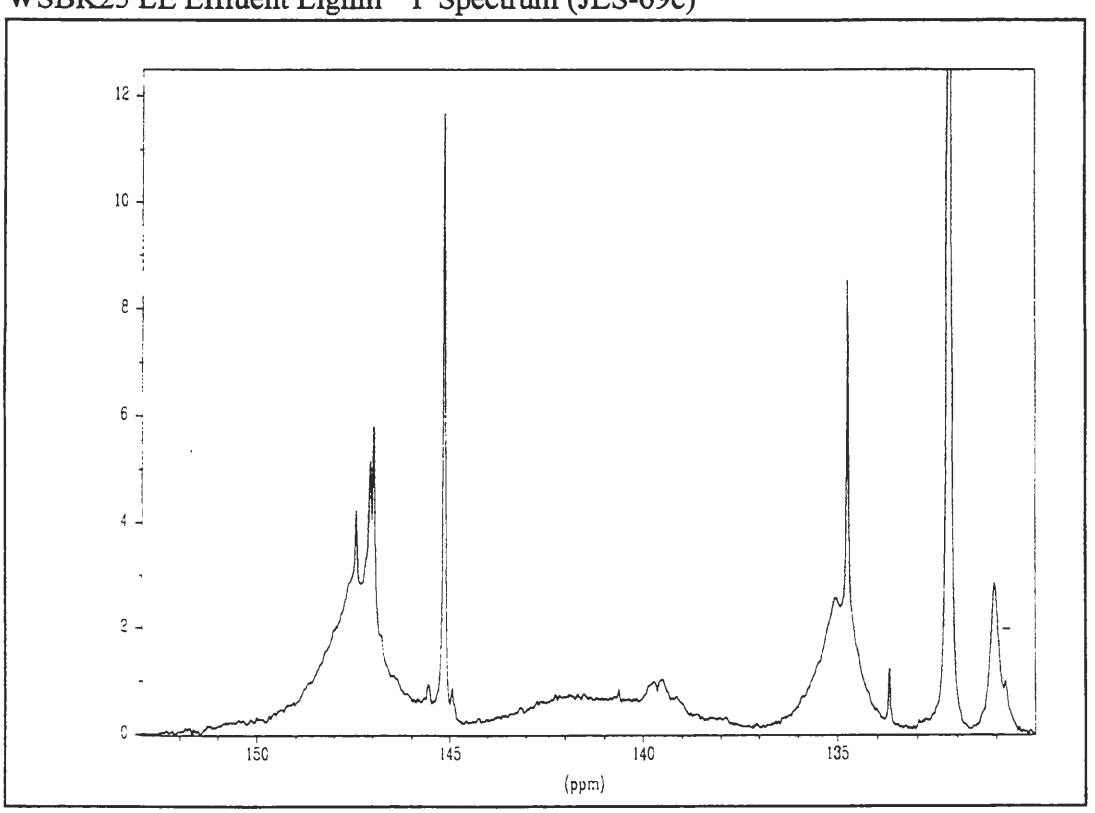
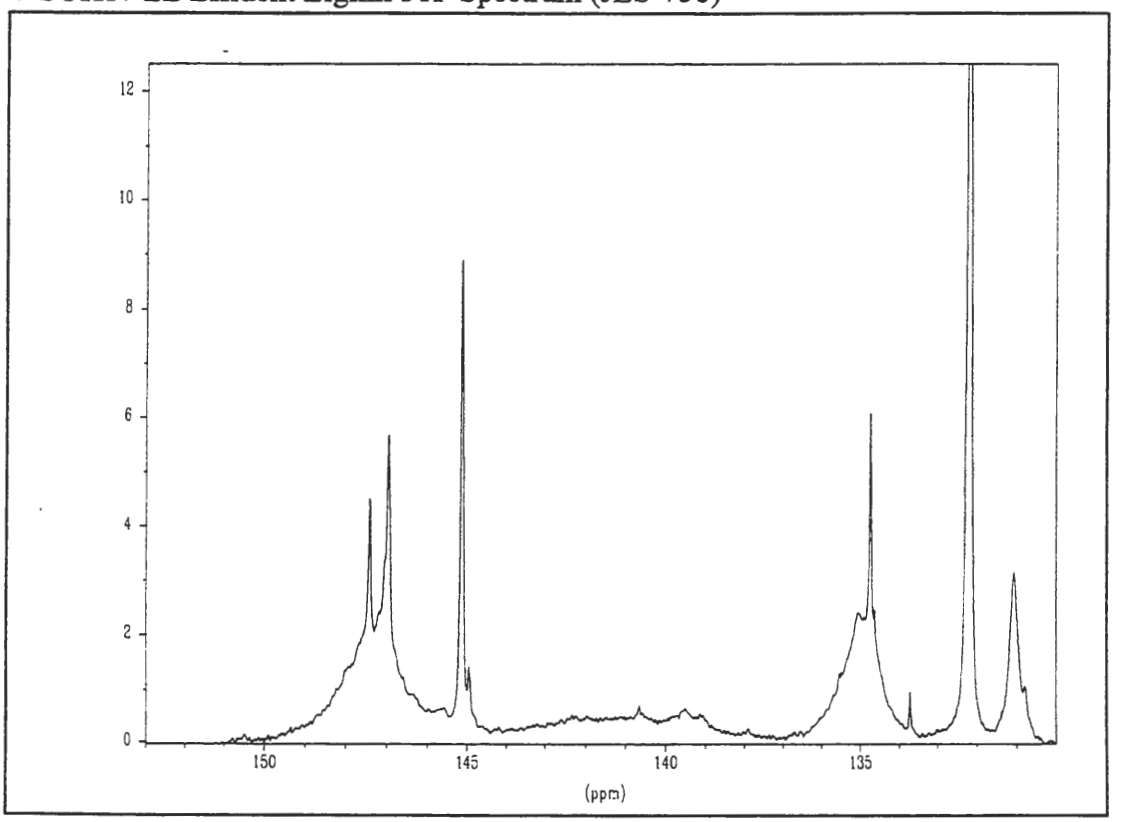


WSBK25 LE Effluent Lignin ^{31}P Spectrum (JES-69c)



WSOK17 LE Effluent Lignin 31P Spectrum (JES-75b)



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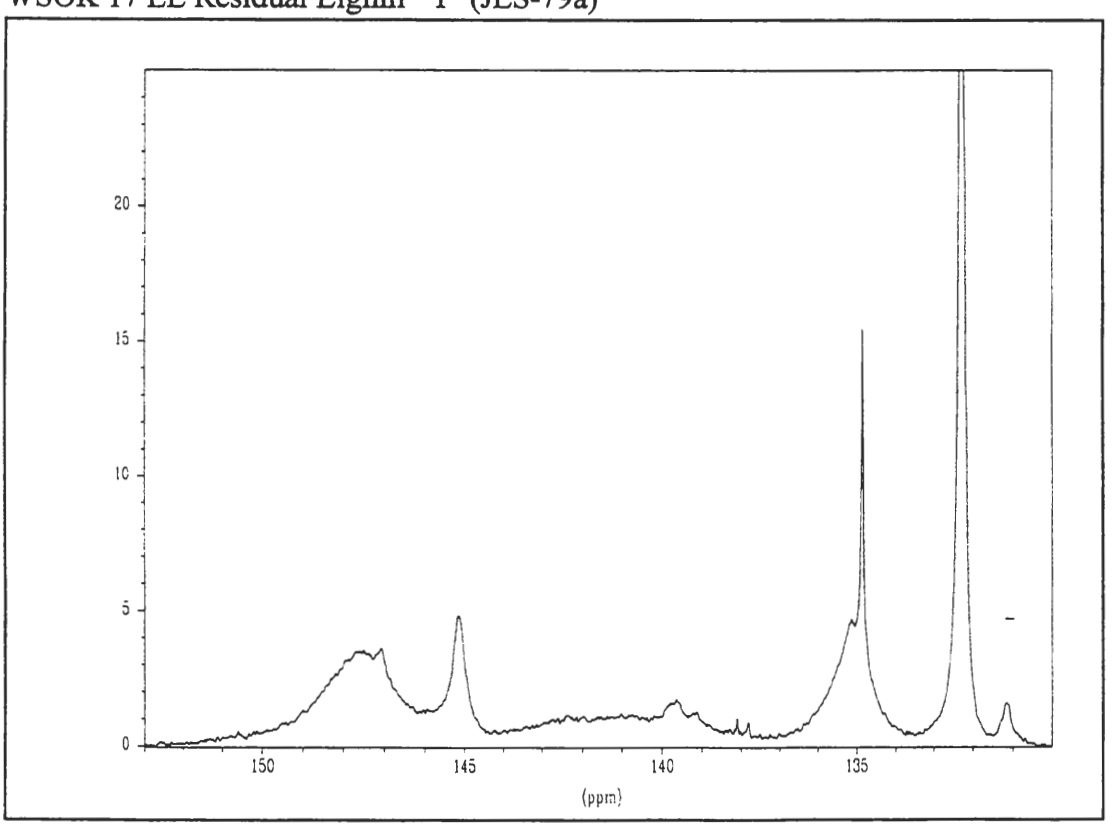


APPENDIX II NMR DATA FROM CHAPTER 6

Appendis II contains ^{13}C and ^{1}H NMR spectrum of residual lignin samples presented in Chapter 6. ^{13}C NMR values were calculated by integrating the aromatic region 160.0-106.5 ppm to 6. ^{1}H NMR calculations were performed by the following: The PFB signal was set to 1. $4.8x10^{-3}$ g of PFB was added to each lignin sample (a stock DMSO-d_6 solution was made), and $4.8x10^{-3}$ / 196.07 MW of PFB = moles of H in the internal standard (PFBM). (PFBM x integration region for the lignin functional group)/ (the weight of lignin in sample x 1000) = mmol/ g of lignin.

^{13}C NMR data on residual lignin from SSBK26 and SSOK13 pulps treated with laccase (LacNS51002) HBT.

	13C NMR softwood pulp STUDY					
Functional group	Intergal region	BS	Pre-O2 LE	Post-O2	Post-O2 LE	LSD
COOH	180.1-165.1	0.368	0.534	0.427	0.716	0.06
C3/C3' C5-C5	160.13-154.14	0.155	0.18	0.158	0.193	0.09
C3,C4 (-CAr-O)	154.14-139.79	1.742	1.631	1.675	1.685	0.129
C1	139.79-126.75	1.47	1.498	1.473	1.461	0.04
C5	126.75-122.54	0.574	0.619	0.619	0.641	0.015
C6	122.54-116.57	0.772	0.79	0.801	0.809	0.014
C5	116.57-113.58	0.452	0.433	0.425	0.36	0.08
C2	113.58-106.36	0.853	0.866	0.866	0.871	0.03
Aliphatic C-O (Cb in Bo4)	89.53-78.44	0.557	0.657	0.695	0.33	0.379
Aliphatic C-O (Ca in Bo4)	78.44-67.37	0.615	0.804	0.832	0.554	0.454
Aliphatic C-O	67.37-61.33	0.28	0.408	0.396	0.253	0.31
Cg (Bo4)	61.33-57.39	0.204	0.252	0.25	0.193	0.153
OCH3	57.39-53.88	0.74	0.718	0.759	0.661	0.343
CB in BB and CB in B5	53.88-51.23	0.18	0.238	0.22	0.281	0.168

^{13}C NMR data on residual lignins from SHBK15 and SHOK10 pulps treated with laccase (LacNS51001) HBT.

	13C NMR hardwood pulp study					
Functional group	Intergal region	bs hd	Pre-O2 LE	Post-O2	Post O2 LE	LSD
COOH	180.1-165.1	0.433	0.752	0.77	0.81	0.06
C3/C3' C5-C5	160.13-154.14	0.142	0.244	0.234	0.256	0.09
C3,C4 (-CAr-O)	154.14-139.79	1.923	1.654	1.827	1.542	0.129
C1	139.79-126.75	1.7317	1.697	1.665	1.652	0.04
C5	126.75-122.54	0.491	0.585	0.547	0.592	0.015
C6	122.54-116.57	0.616	0.684	0.659	0.683	0.014
C5	116.57-113.58	0.322	0.332	0.325	0.34	0.08
C2	113.58-106.36	0.784	0.804	0.757	0.8	0.03
Aliphatic C-O (Cb in Bo4)	89.53-78.44	0.607	0.813	0.643	0.854	0.379
Aliphatic C-O (Ca in Bo4)	78.44-67.37	0.814	1.083	0.896	0.924	0.454
Aliphatic C-O	67.37-61.33	0.364	0.574	0.423	0.498	0.31
Cg (Bo4)	61.33-57.39	0.296	0.327	0.285	0.385	0.153
OCH3	57.39-53.88	1.101	0.904	0.937	0.912	0.343
CB in BB and CB in B5	53.88-51.23	0.245	0.417	0.295	0.458	0.168

^{13}C NMR data on residual lignins from SSOK13 pulp treated with laccase (LacNS51002) and HBT.

	13C NMR softwood pulp time STUDY						
Functional group	Intergal region	Post-O2	15 min lac/HBT	1 hr lac/HBT	4 hr lac/HBT	8 hr lac/HBT	LSD
COOH	180.1-165.1	0.427	0.592	0.858	0.754	0.921	0.06
C3/C3' C5-C5	160.13-154.14	0.158	0.174	0.381	0.369	0.404	0.09
C3,C4 (-CAr-O)	154.14-139.79	1.675	1.64	1.967	1.97	1.978	0.129
C1	139.79-126.75	1.473	1.513	1.593	1.571	1.616	0.04
C5	126.75-122.54	0.619	0.604	0.55	0.56	0.549	0.015
C6	122.54-116.57	0.801	0.793	0.643	0.653	0.626	0.014
C5	116.57-113.58	0.425	0.43	0.339	0.338	0.332	0.08
C2	113.58-106.36	0.866	0.863	0.5345	0.539	0.501	0.03
Aliphatic C-O (Cb in Bo4)	89.53-78.44	0.695	0.643	0.486	0.495	0.457	0.379
Aliphatic C-O (Ca in Bo4)	78.44-67.37	0.832	0.848	0.67	0.694	0.637	0.454
Aliphatic C-O	67.37-61.33	0.396	0.394	0.308	0.328	0.31	0.31
Cg (Bo4)	61.33-57.39	0.25	0.254	0.234	0.246	0.222	0.153
OCH3	57.39-53.88	0.759	0.766	0.722	0.786	0.71	0.343
CB in BB and CB in B5	53.88-51.23	0.22	0.252	0.191	0.211	0.176	0.168

^{1}H NMR data Laccase treated SSBK26 and SSOK13 residual lignins. All reaction times were for 24 hrs.

Functional group	Brownstock	Brownstock LE	Post-O2	Post-O2 LE
COOH	0.65	0.91	1.03	1.01
Formyl H	0.63	0.59	0.48	0.54
Total Phenolic OH	2.59	1.48	1.54	1.65
Aromatic H	11.51	10.41	8.89	7.64
Aliphatic H	8.91	8.46	7.50	8.34
Methoxy H	14.52	12.52	12.83	10.08

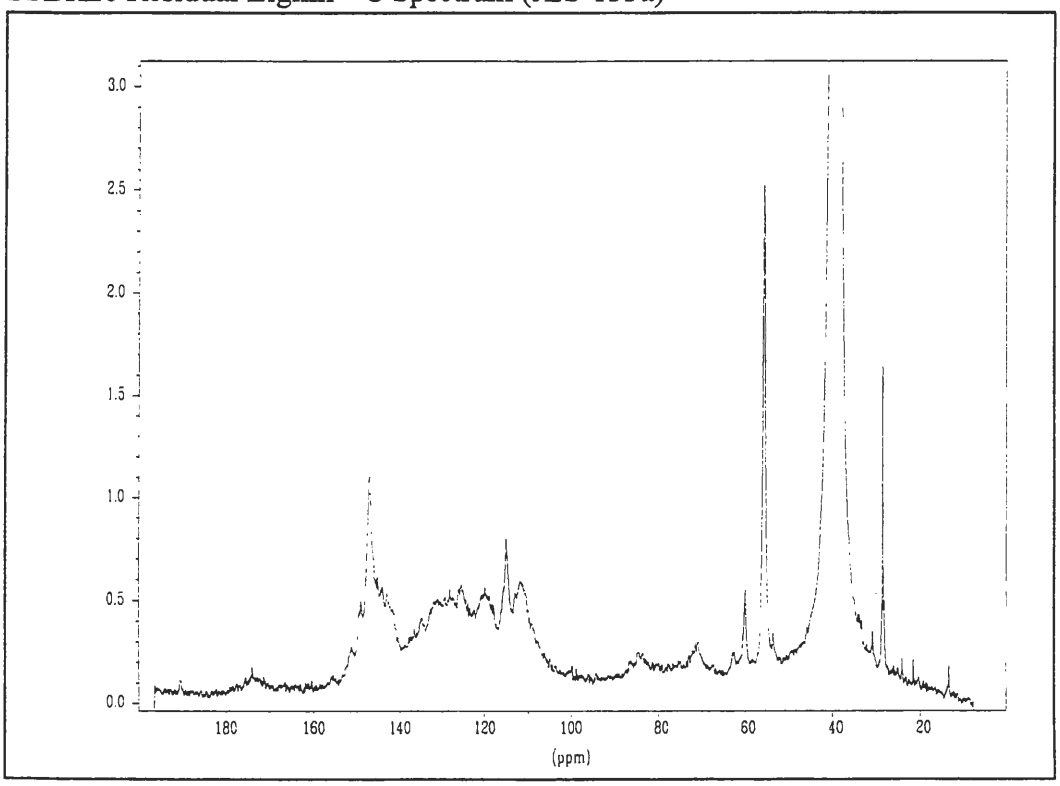
^{1}H NMR data Laccase treated SHBK15 and SHOK10 residual lignins. All reaction times were for 24 hrs

Functional group	Brownstock	Brownstock LE	Post-O2	Post-O2 LE
COOH	0.71	0.72	1.01	0.71
Formyl H	0.50	0.58	0.69	0.76
Total Phenolic OH	2.08	1.56	2.16	1.57
Aromatic H	9.17	6.14	7.87	4.93
Aliphatic H	9.29	7.70	9.33	7.22
Methoxy H	17.61	8.98	13.18	6.37

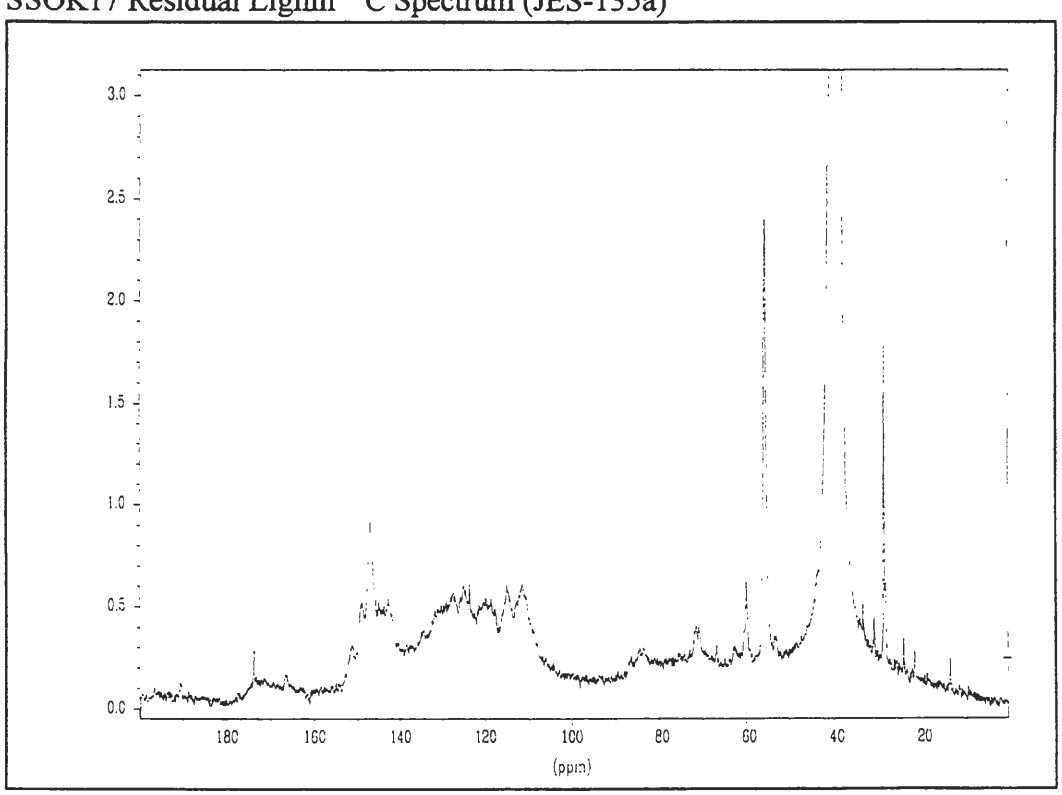
^{1}H NMR data Laccase treated SSOK13 residual lignins. Reaction time was varied.

Functional group	Post-O2	Post-O2 LE 0.25 hr	Post-O2 LE 1 hr	Post-O2 LE 4 hr	Post-O2 LE 8 hr
COOH	1.03	1.15	1.05	1.16	1.28
Formyl H	0.48	0.56	0.43	0.53	0.57
Total Phenolic OH	1.54	1.68	1.14	1.66	1.68
Aromatic H	8.89	8.27	7.58	8.28	8.01
Aliphatic H	7.54	8.59	7.57	8.36	8.28
Methoxy H	12.83	11.28	10.24	10.79	10.11

SSBK26 Residual Lignin ^{13}C Spectrum (JES-133a)



SSOK17 Residual Lignin ^{13}C Spectrum (JES-135a)



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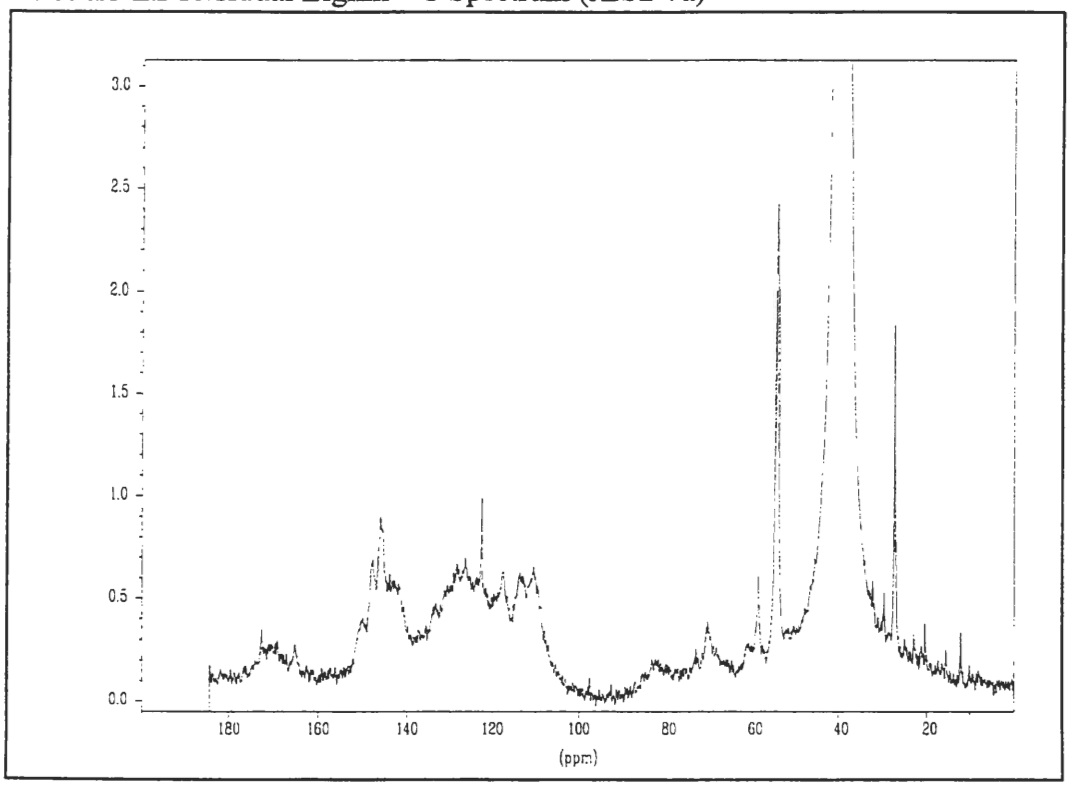
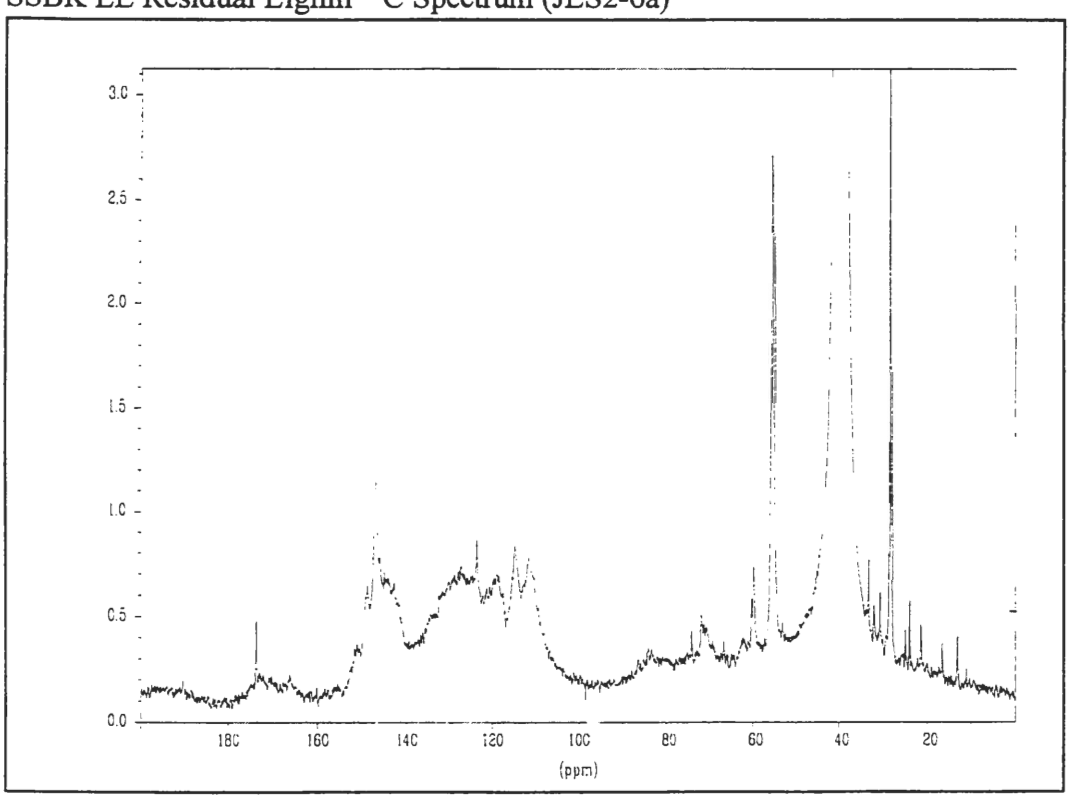
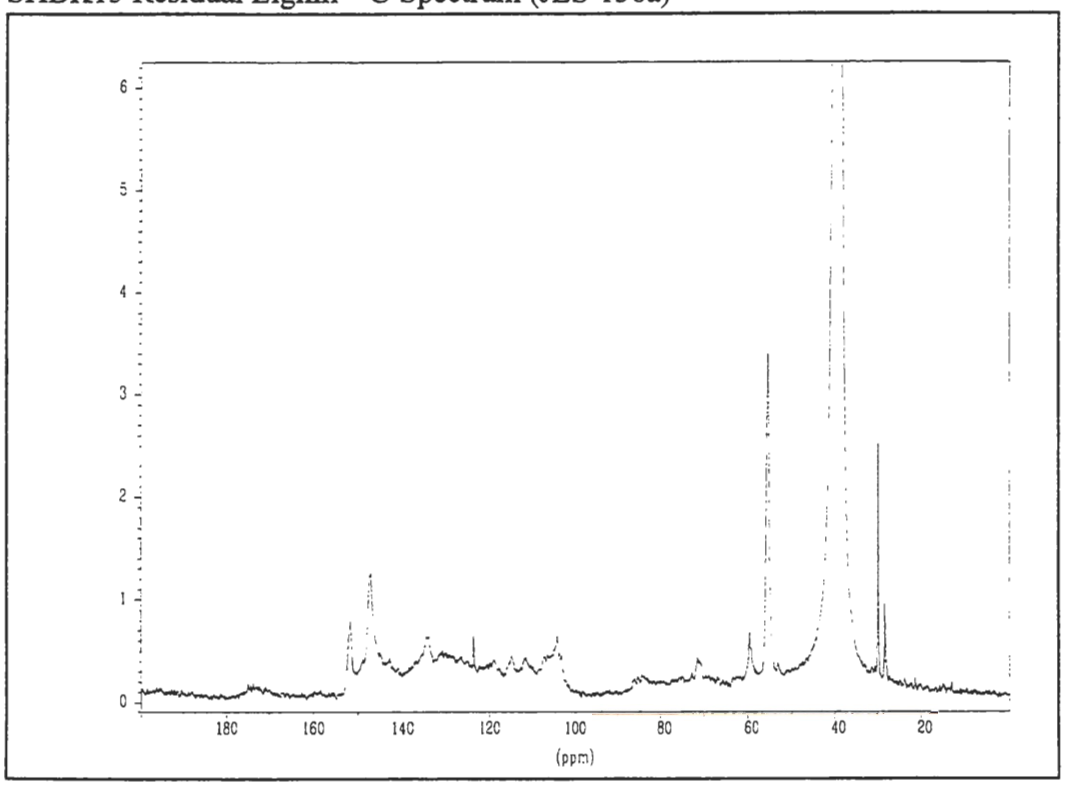


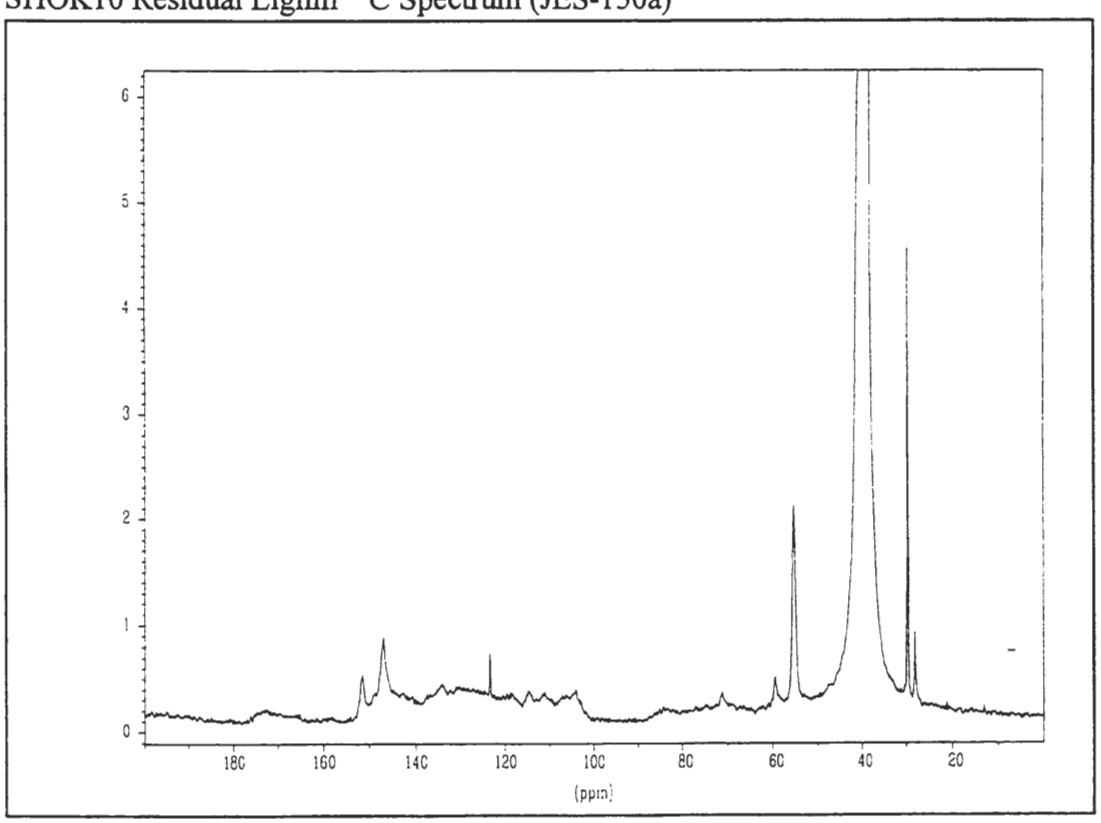
SSBK LE Residual Lignin ^{13}C Spectrum (JES2-6a)



SHBK15 Residual Lignin ^{13}C Spectrum (JES-138a)



SHOK10 Residual Lignin ^{13}C Spectrum (JES-150a)



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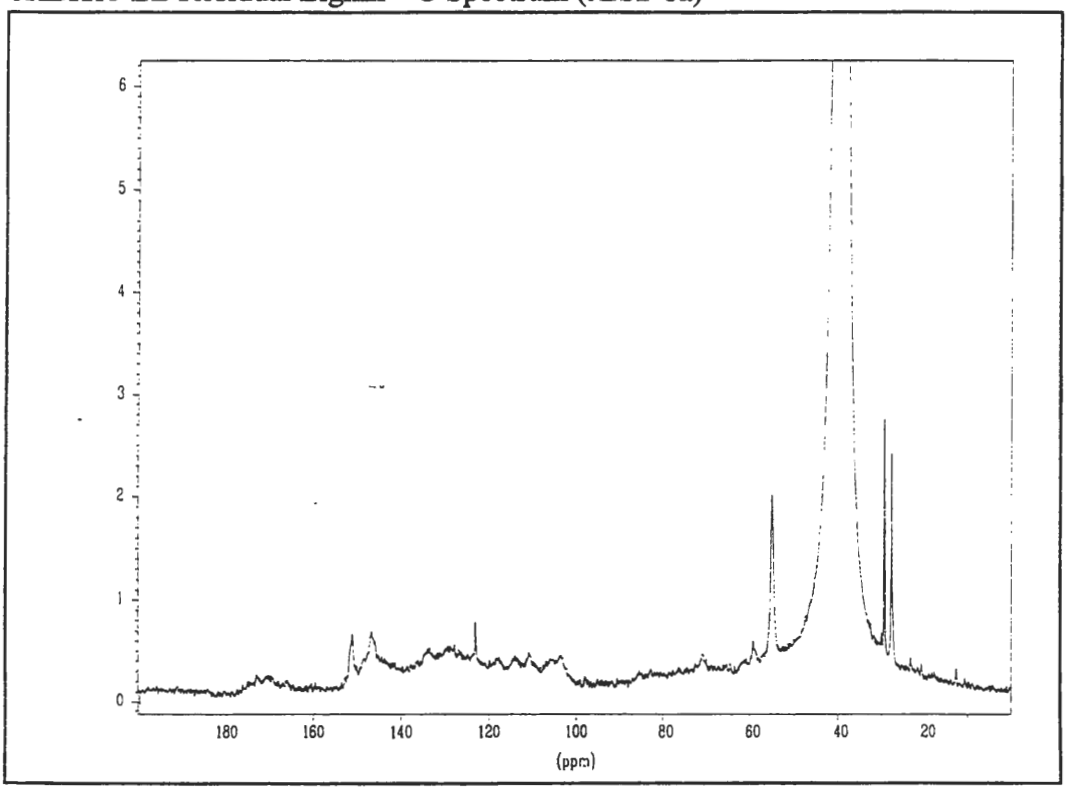
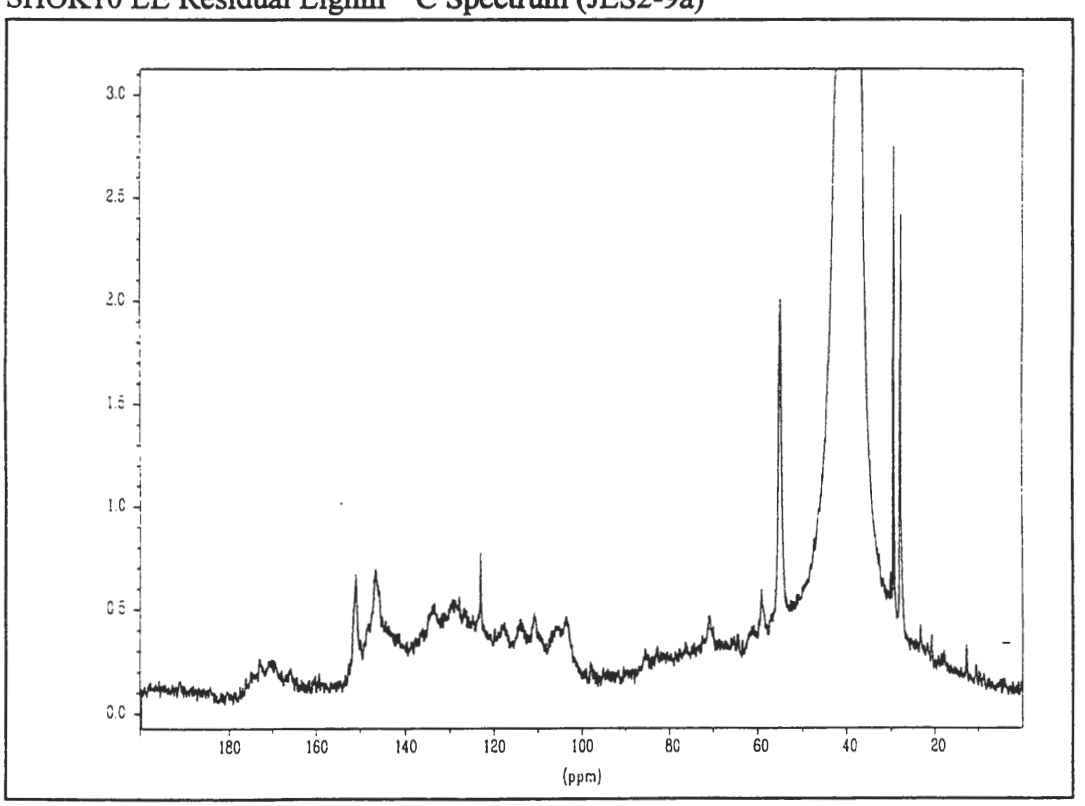
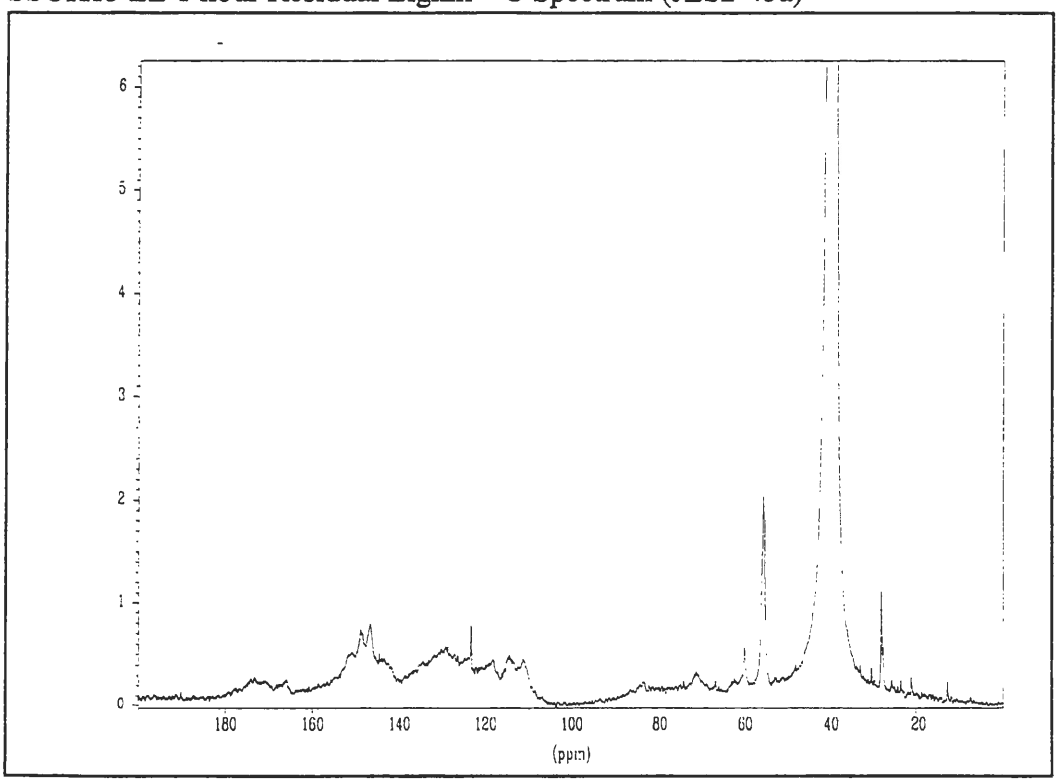


SHOK10 LE Residual Lignin ^{13}C Spectrum (JES2-9a)



SSOK13 LE 1 hour Residual Lignin ^{13}C Spectrum (JES2-45a)



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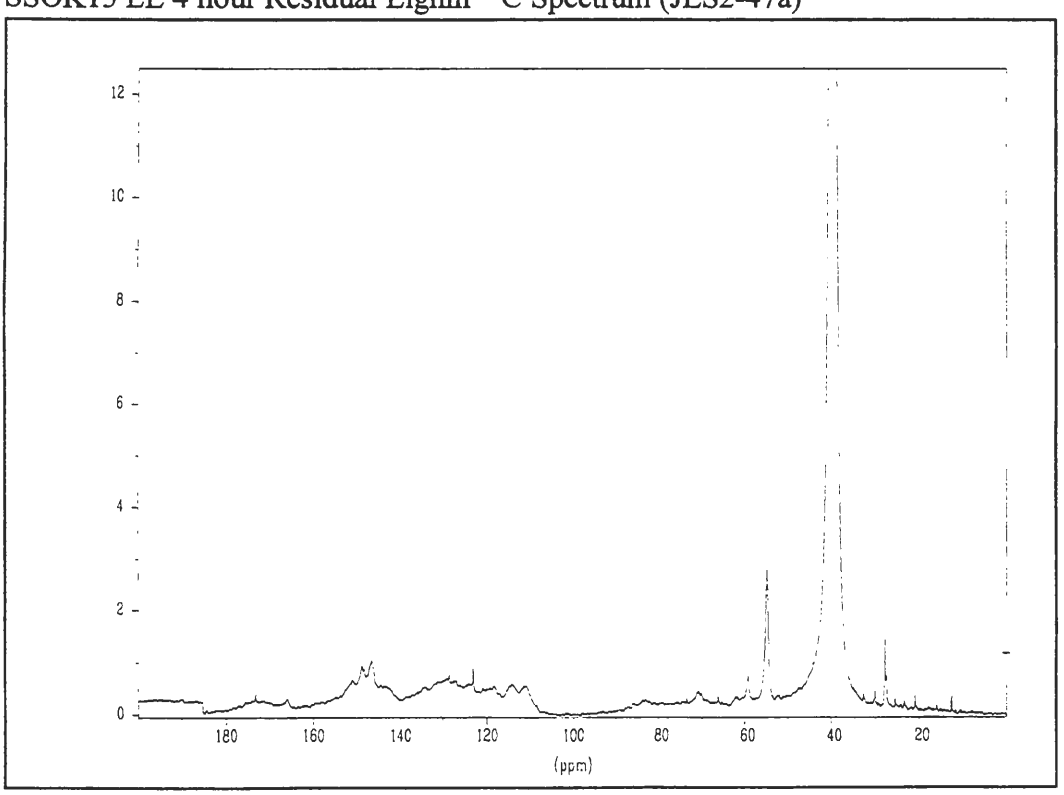

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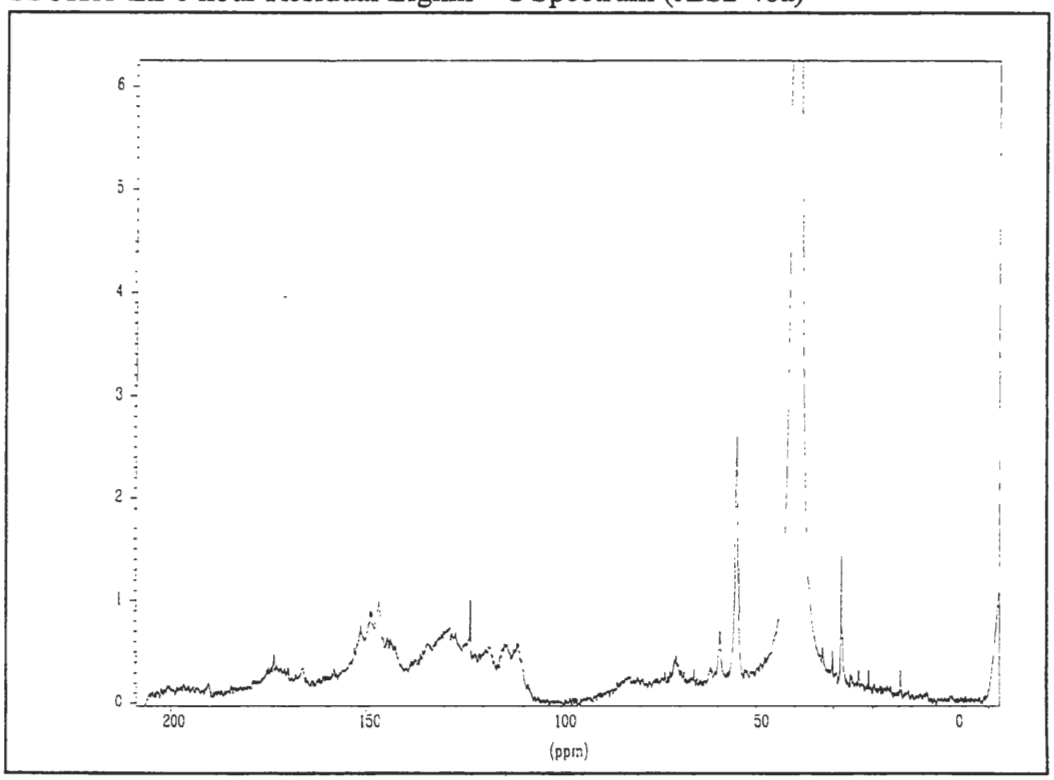
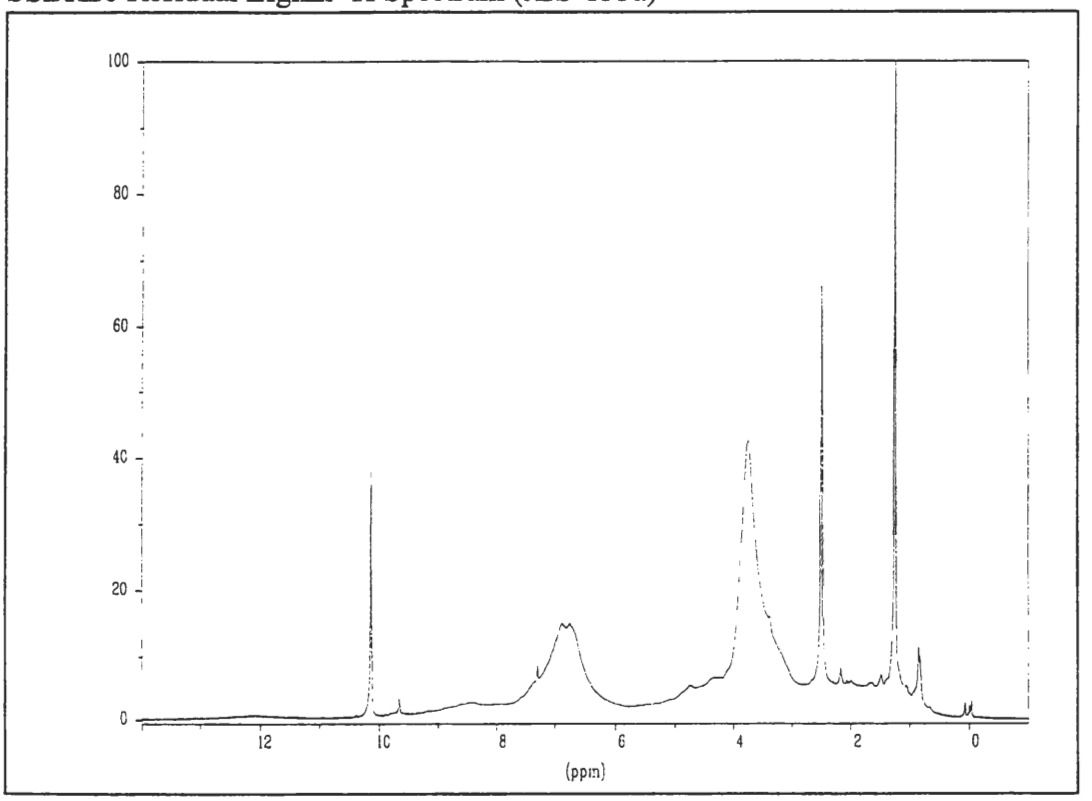
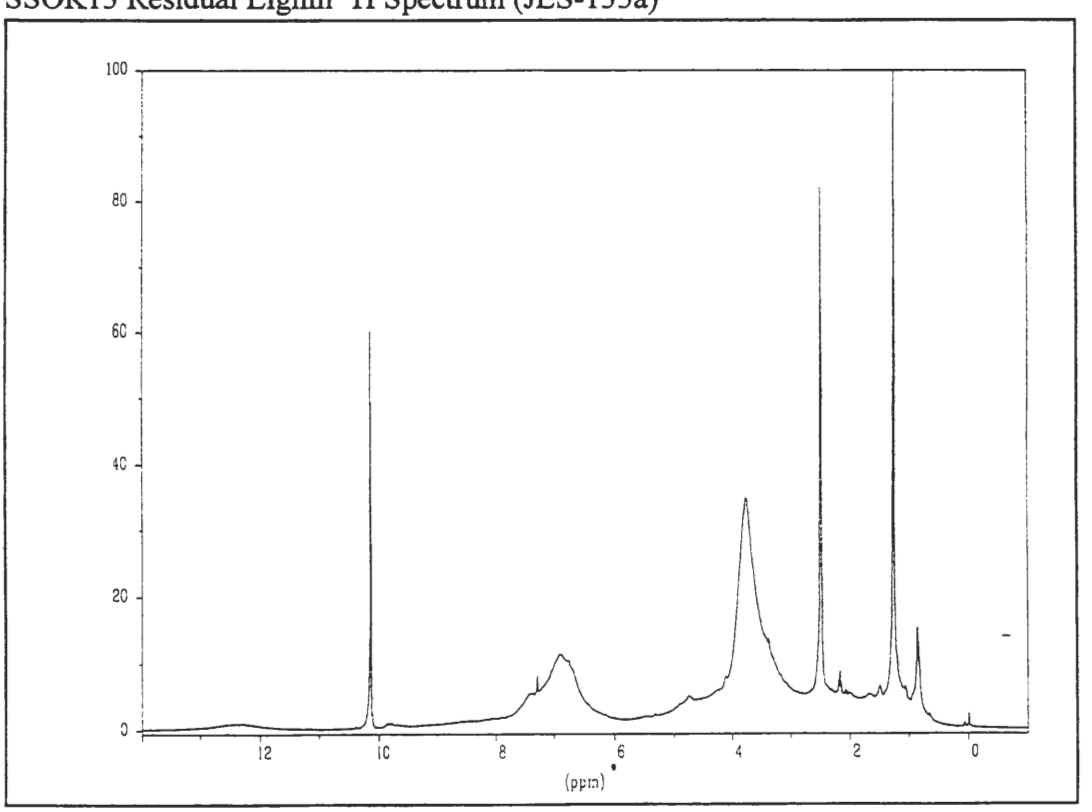


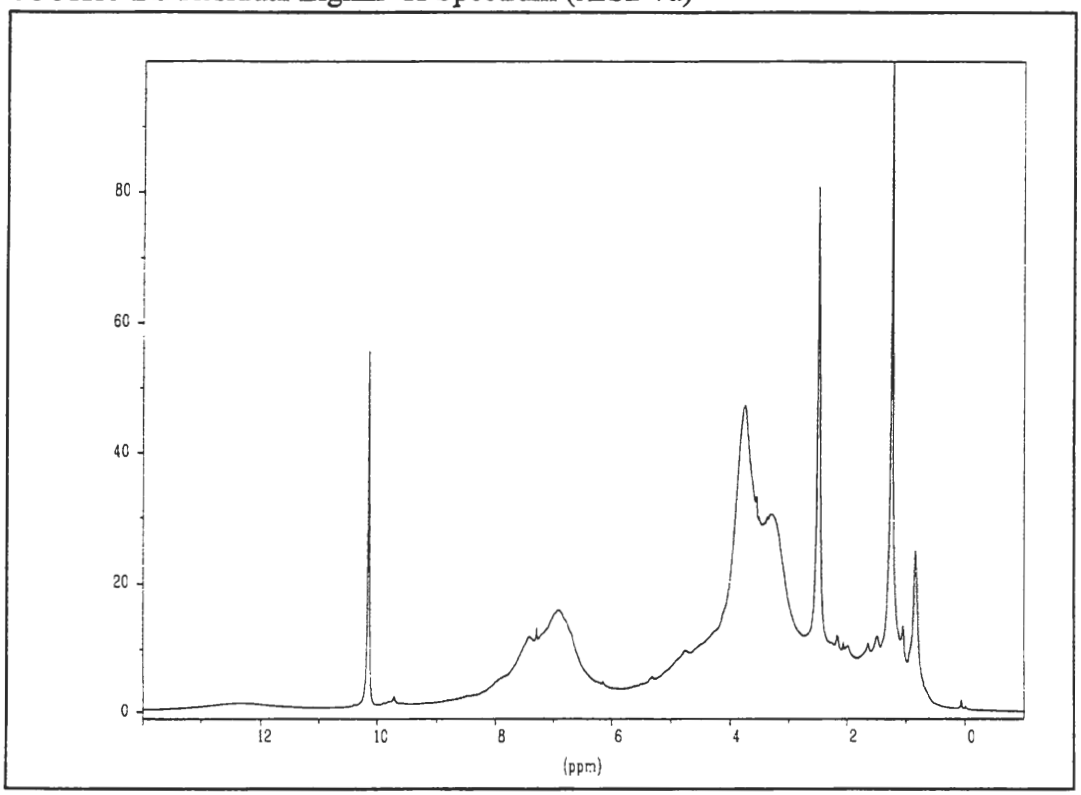
SSBK26 Residual Lignin ^{1}H Spectrum (JES-133a)



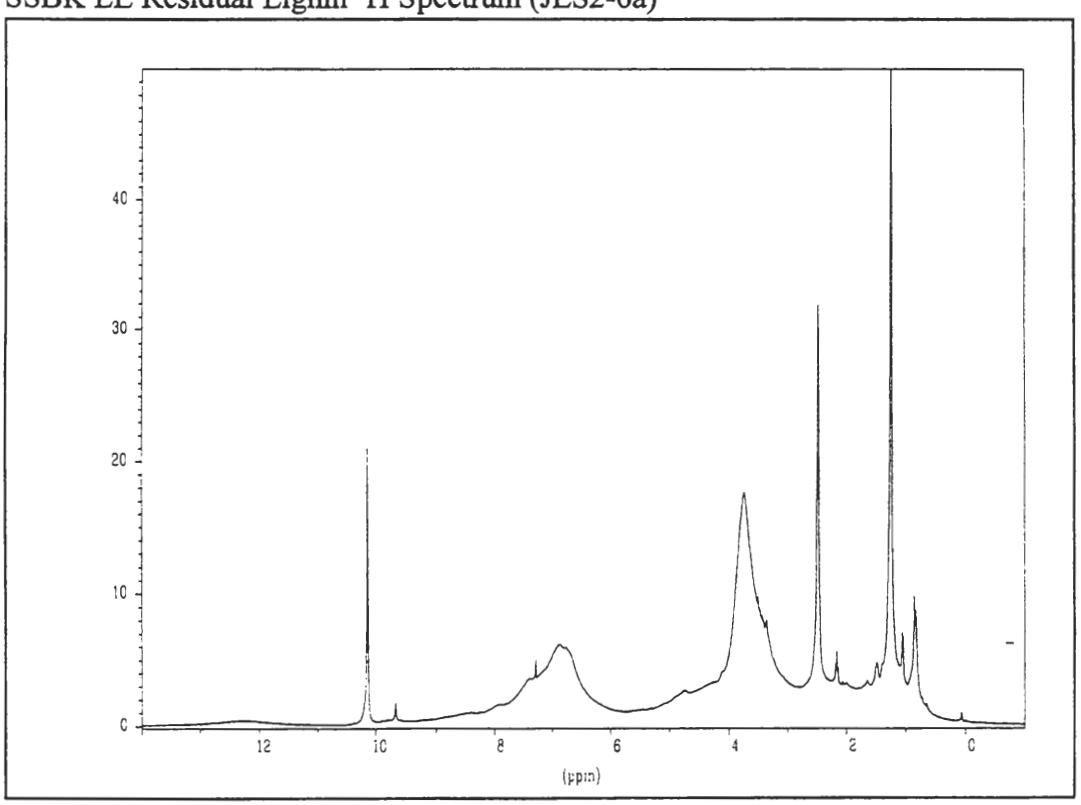
SSOK13 Residual Lignin ^{1}H Spectrum (JES-135a)



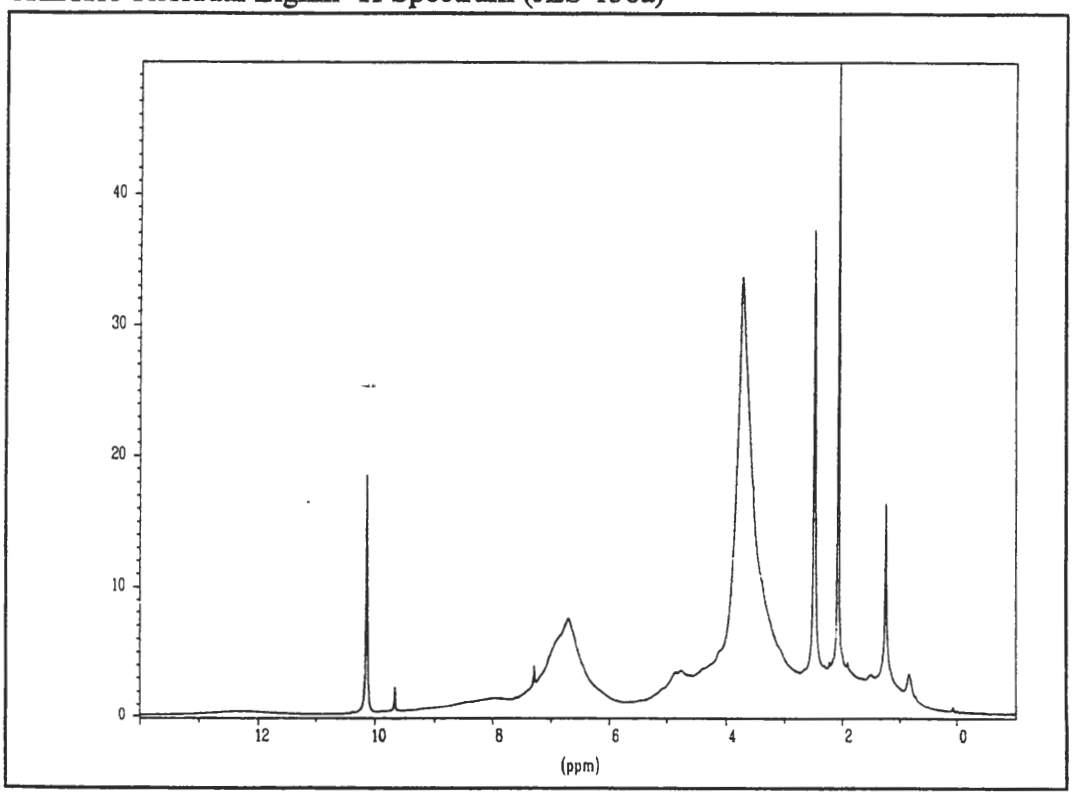
SSOK13 LE Residual Lignin ^{1}H Spectrum (JES2-7a)



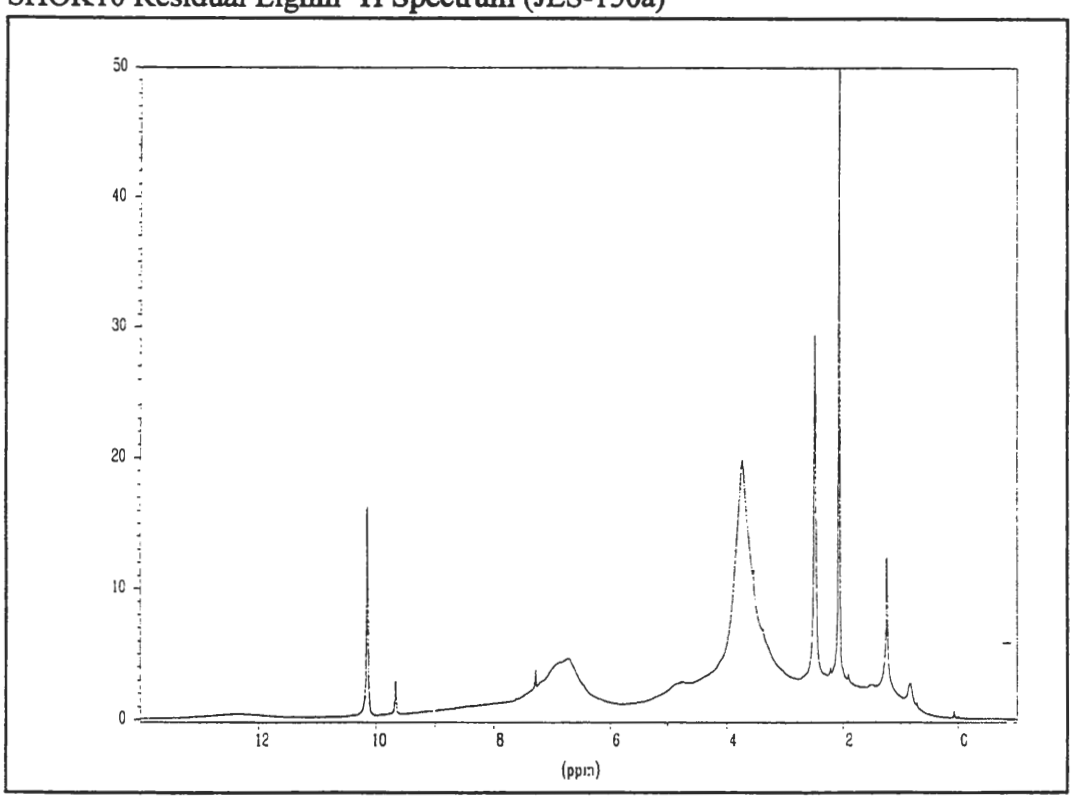
SSBK LE Residual Lignin ^{1}H Spectrum (JES2-6a)



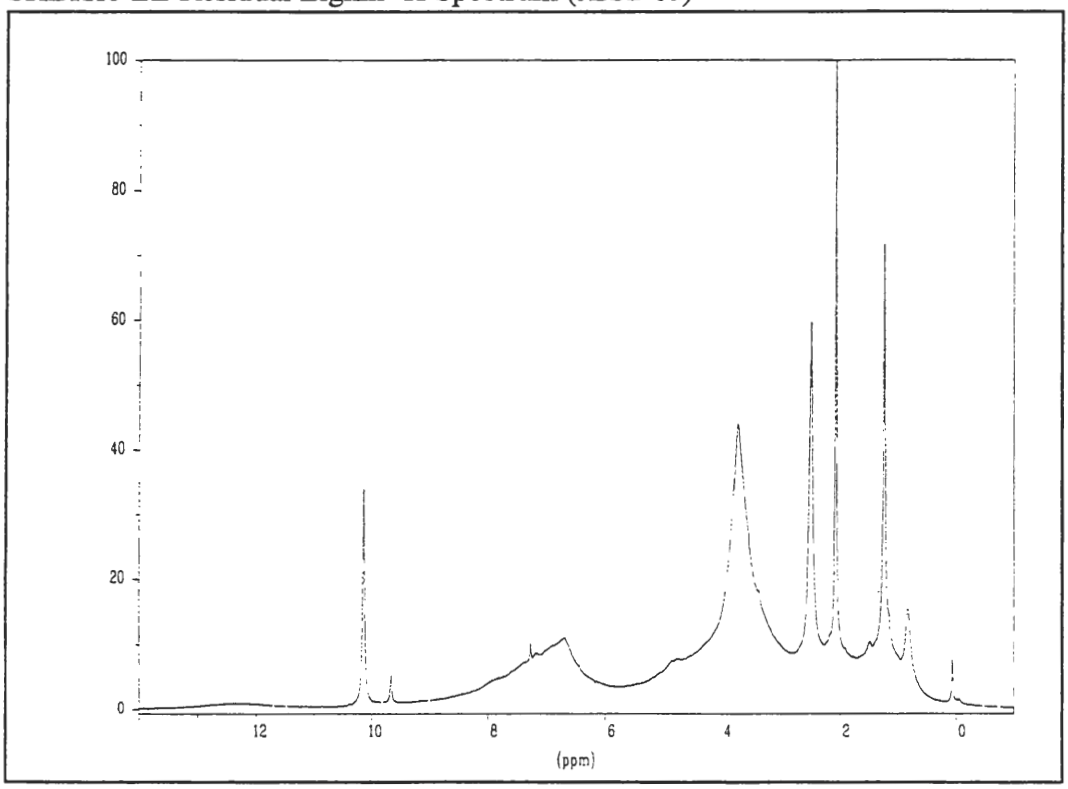
SHBK15 Residual Lignin ^{1}H Spectrum (JES-138a)



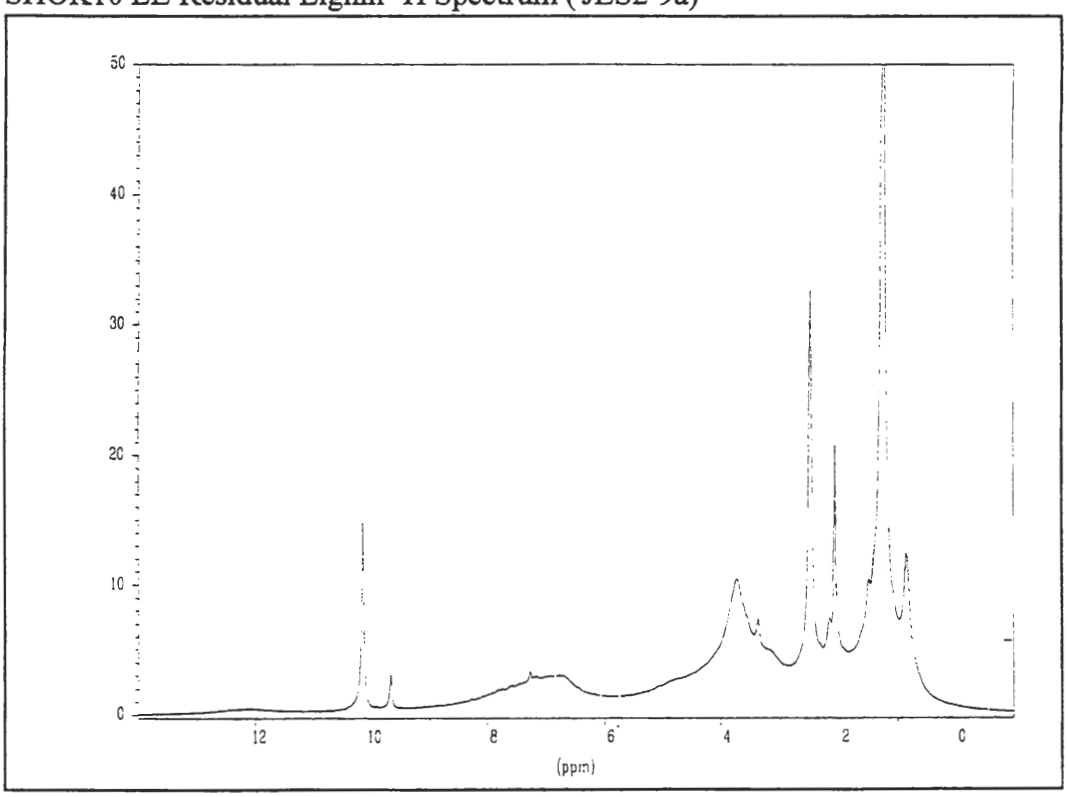
SHOK10 Residual Lignin ^{1}H Spectrum (JES-150a)



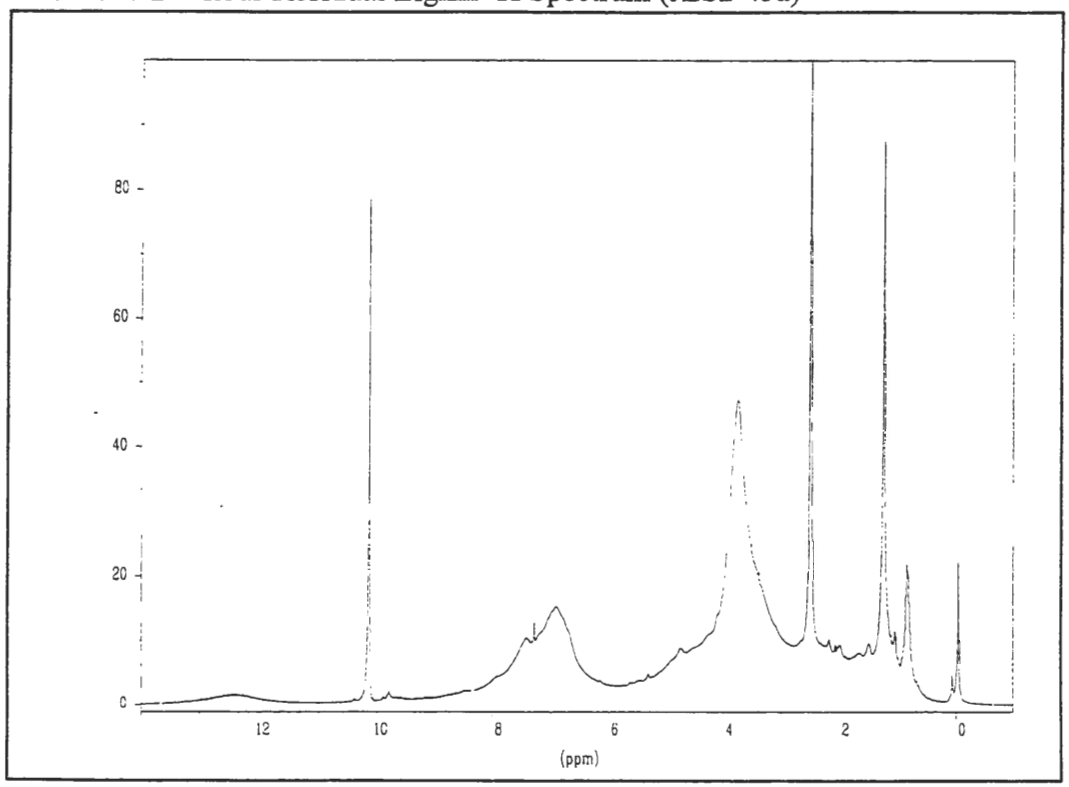
SHBK15 LE Residual Lignin ^{1}H Spectrum (JES2-8a)



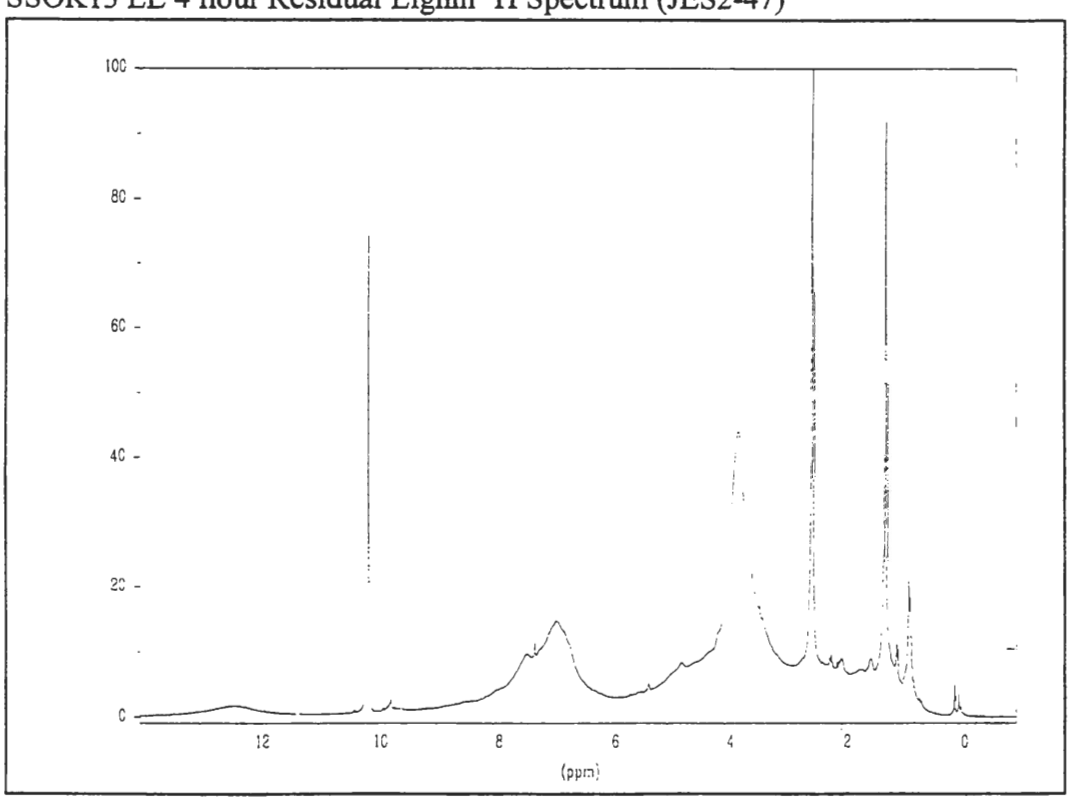
SHOK10 LE Residual Lignin ^{1}H Spectrum (JES2-9a)



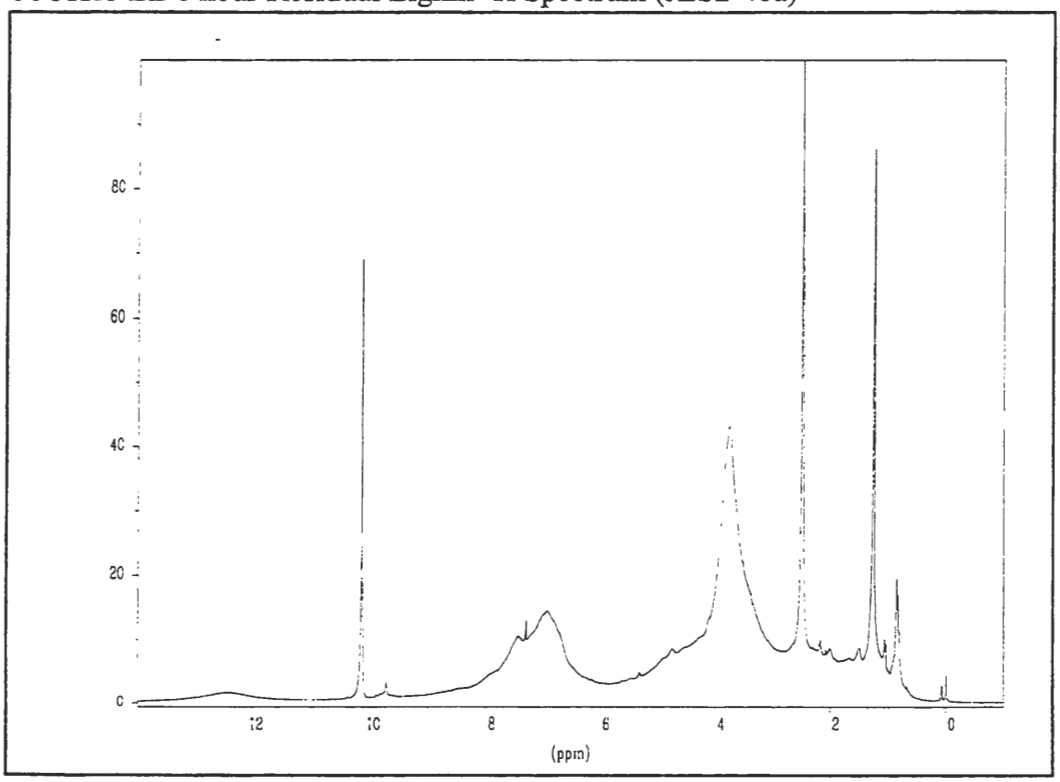
SSOK13 LE 1 hour Residual Lignin ^{1}H Spectrum (JES2-45a)



SSOK13 LE 4 hour Residual Lignin ^{1}H Spectrum (JES2-47)



SSOK13 LE 8 hour Residual Lignin ^{1}H Spectrum (JES2-48a)



APPENDIX III NMR DATA FROM CHAPTER 7

Appendix III contains ^{13}C and ^{1}H NMR Spectrum of reacted lignin samples persented in Chapter 7. Calculations were persented in Appendix II.

^{13}C NMR values for reaction lignins with different mediators used. All reaction times were 4 hrs.

	13C NMR CONTROL STUDY						
Functional group	Intergal region	sm	lac	lac/HTI	lac/HBT	lac/NHAA	lac/VIO
COOH	180.1-165.1	0.275	0.462	0.584	0.529	0.88	0.735
C3/C3' C5-C5	160.13-154.14	0.121	0.132	0.118	0.109	0.134	0.198
C3,C4 (-CAr-O)	154.14-139.79	1.809	1.805	1.711	1.676	1.746	1.778
C1	139.79-126.75	1.462	1.493	1.587	1.721	1.764	1.593
C5	126.75-122.54	0.59	0.589	0.602	0.686	0.674	0.598
C6	122.54-116.57	0.773	0.749	0.766	0.703	0.715	0.706
C5	116.57-113.58	0.45	0.447	0.435	0.389	0.325	0.381
C2	113.58-106.36	0.805	0.795	0.777	0.722	0.641	0.756
Aliphatic C-O (Cb in Bo4)	89.53-78.44	0.46	0.512	0.538	0.262	0.297	0.394
Aliphatic C-O (Ca in Bo4)	78.44-67.37	0.55	0.674	0.827	0.627	0.687	0.747
Aliphatic C-O	67.37-61.33	0.261	0.319	0.421	0.362	0.311	0.339
Cg (Bo4)	61.33-57.39	0.205	0.237	0.255	0.243	0.243	0.248
OCH3	57.39-53.88	0.784	0.823	0.853	0.784	0.846	0.849
CB in BB and CB in B5	53.88-51.23	0.167	0.204	0.284	0.258	0.248	0.271

^{13}C NMR values for reacted lignin with HBT as the mediator, and reaction time was varied.

	13C NMR CONTROL STUDY				
Functional group	Intergal region	sm	lac/HBT 1hr	lac/HBT 4 hr	lac/HBT 8 hr
COOH	180.1-165.1	0.275	0.474	0.523	0.722
C3/C3' C5-C5	160.13-154.14	0.121	0.081	0.109	0.167
C3,C4 (-CAr-O)	154.14-139.79	1.809	1.743	1.676	1.485
C1	139.79-126.75	1.462	1.619	1.721	1.665
C5	126.75-122.54	0.59	0.62	0.686	0.646
C6	122.54-116.57	0.773	0.76	0.703	0.697
C5	116.57-113.58	0.45	0.41	0.389	0.395
C2	113.58-106.36	0.805	0.779	0.722	0.743
Aliphatic C-O (Cb in Bo4)	89.53-78.44	0.46	0.397	0.262	0.564
Aliphatic C-O (Ca in Bo4)	78.44-67.37	0.55	0.604	0.627	0.879
Aliphatic C-O	67.37-61.33	0.261	0.295	0.362	0.447
Cg (Bo4)	61.33-57.39	0.205	0.21	0.243	0.248
OCH3	57.39-53.88	0.784	0.815	0.784	0.658
CB in BB and CB in B5	53.88-51.23	0.167	0.211	0.258	0.264

^{13}C NMR values for reacted lignin with laccase only used. Reaction times were varied.

	13C NMR CONTROL STUDY				
Functional group	Intergal region	sm	lac 1 hr	lac 4 hr	lac 8 hr
COOH	180.1-165.1	0.275	0.609	0.462	0.703
C3/C3' C5-C5	160.13-154.14	0.121	0.148	0.132	0.157
C3,C4 (-CAr-O)	154.14-139.79	1.809	1.737	1.805	1.759
C1	139.79-126.75	1.462	1.521	1.493	1.543
C5	126.75-122.54	0.59	0.585	0.589	0.601
C6	122.54-116.57	0.773	0.762	0.749	0.738
C5	116.57-113.58	0.45	0.436	0.447	0.433
C2	113.58-106.36	0.805	0.811	0.795	0.778
Aliphatic C-O (Cb in Bo4)	89.53-78.44	0.46	0.601	0.512	0.532
Aliphatic C-O (Ca in Bo4)	78.44-67.37	0.55	0.833	0.674	0.816
Aliphatic C-O	67.37-61.33	0.261	0.373	0.319	0.393
Cg (Bo4)	61.33-57.39	0.205	0.244	0.223	0.251
OCH3	57.39-53.88	0.784	0.8	0.823	0.891
CB in BB and CB in B5	53.88-51.23	0.167	0.24	0.204	0.257

^{1}H NMR data of reacted lignin with different mediators used. All reaction times were 4 hrs.

Lignin functional groups	SM	Laccase 4 hrs	Laccase HBT 4 hrs	Laccase HTI 4 hrs	Laccase NHAA 4 hrs	Laccase Vio 4 hrs
COOH	0.81	1.04	1.31	1.11	1.32	1.68
Formyl H	1.03	0.71	1.45	1.73	1.63	2.03
Total Phenolic OH	4.19	3.78	5.00	7.45	5.49	6.03
Aromatic H	17.21	18.12	7.92	15.45	12.04	13.67
Aliphatic H	13.64	17.90	10.59	23.33	14.86	16.85
methoxy H	23.16	24.31	9.27	23.53	15.95	18.85

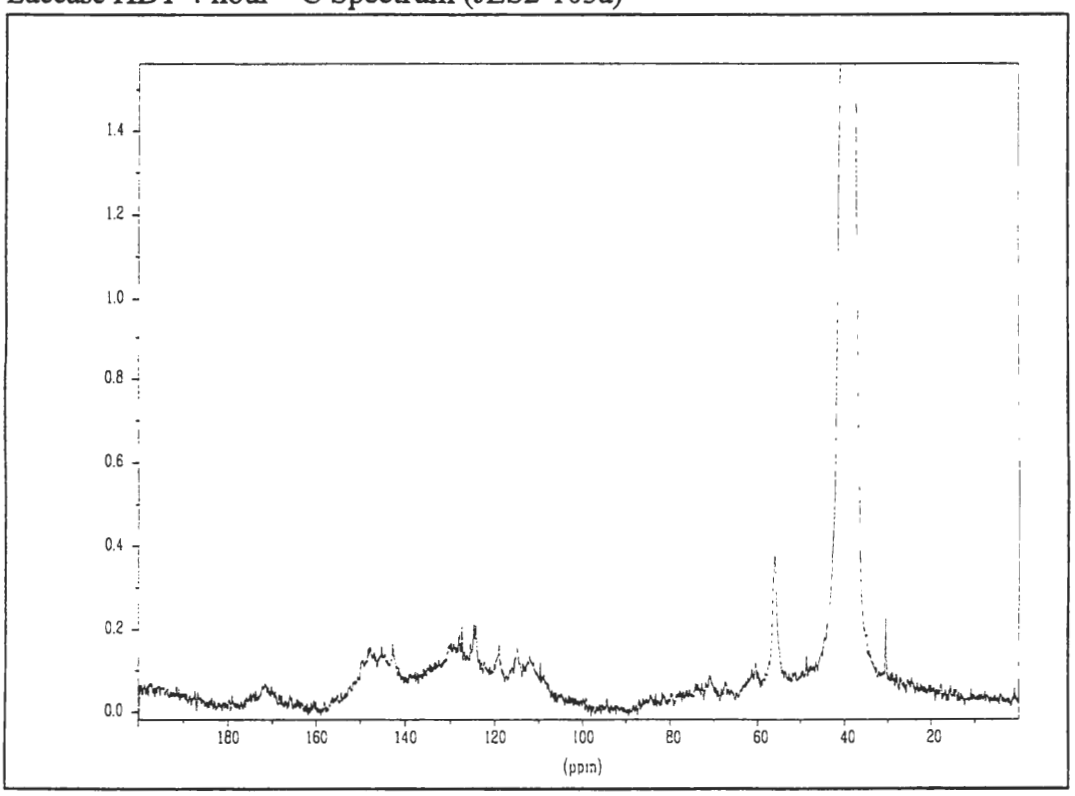
^{1}H NMR data of reacted lignin with HBT as the mediator and reaction time varied.

Lignin functional groups	SM	Laccase HBT 1 hr	Laccase HBT 4 hr	Laccase HBT 8 hr
COOH	0.81	1.47	1.31	1.34
Formyl H	1.03	1.95	1.45	1.42
Total Phenolic OH	4.19	6.13	5.00	5.37
Aromatic H	17.21	11.6	7.92	8.38
Aliphatic H	13.64	12.91	10.59	13.63
methoxy H	23.16	14.59	9.27	10.67

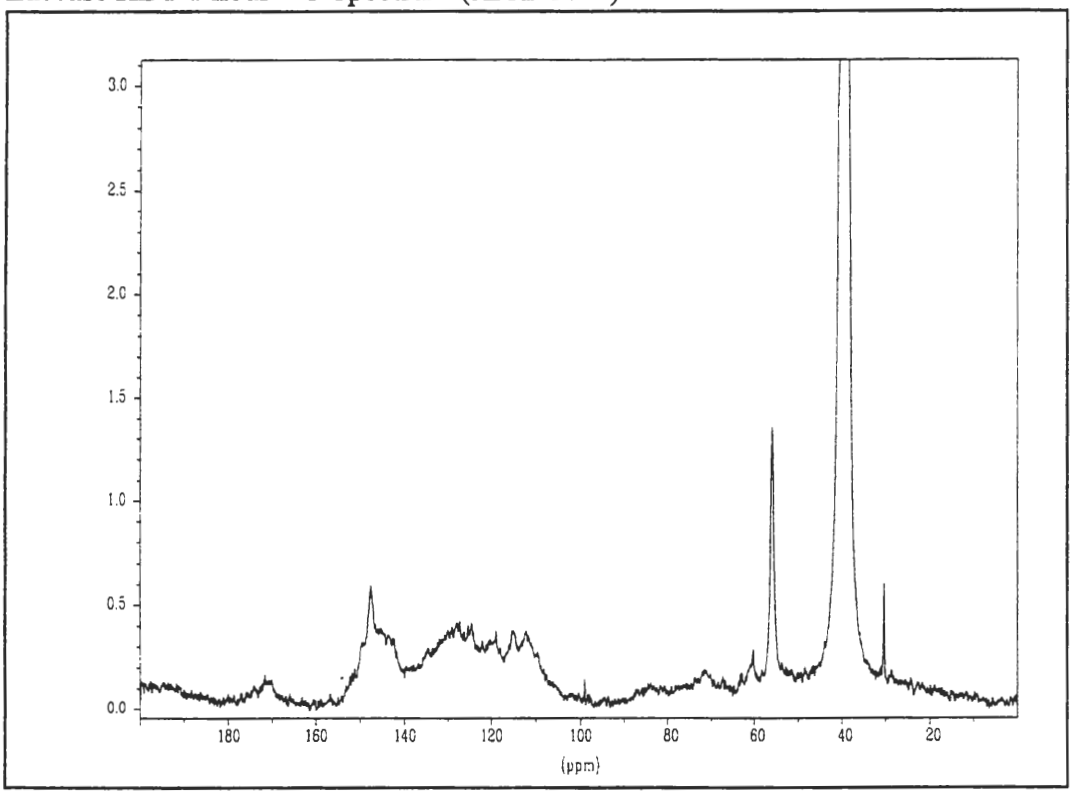
^{1}H NMR data for reacted lignin with laccase only and the reaction time varied.

Lignin functional groups	SM	Laccase 1 hr	Laccase 4 hr	Laccase 8 hr
COOH	0.81	1.23	1.04	1.14
Formyl H	1.03	1.28	0.71	1.30
Total Phenolic OH	4.19	5.22	3.78	5.31
Aromatic H	17.21	18.64	18.12	16.71
Aliphatic H	13.64	20.05	17.90	21.34
methoxy H	23.16	25.25	24.31	21.50

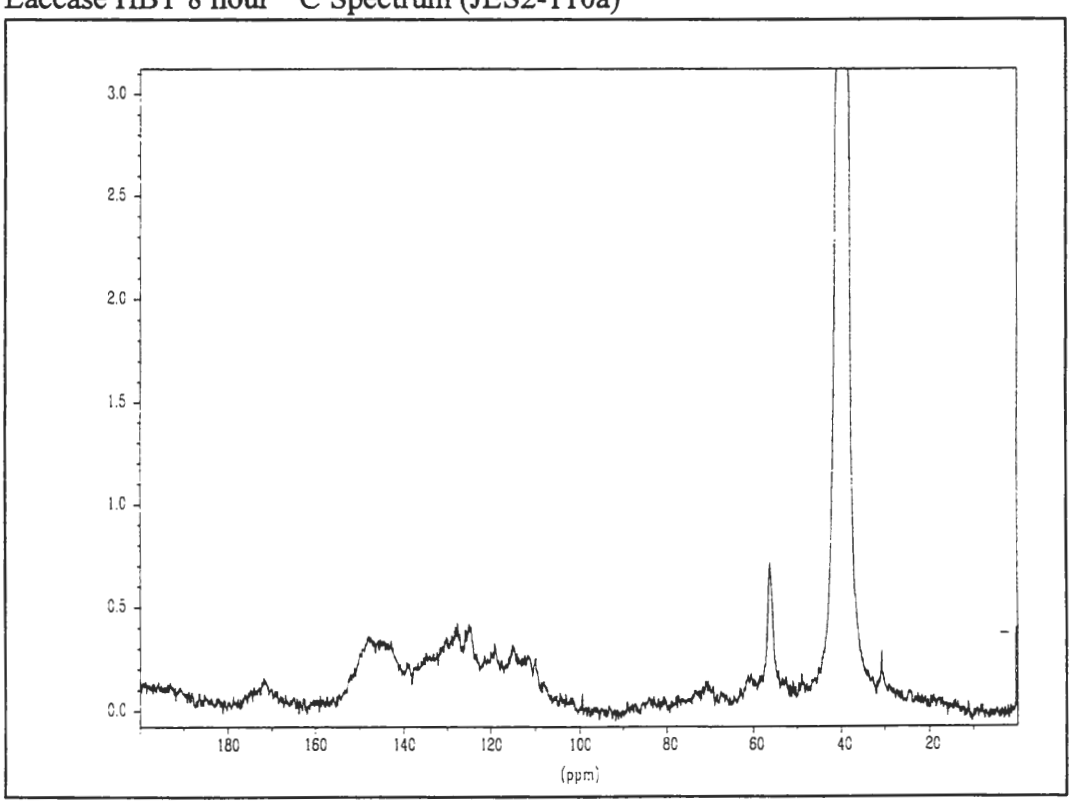
Laccase HBT 4 hour ^{13}C Spectrum (JES2-103a)



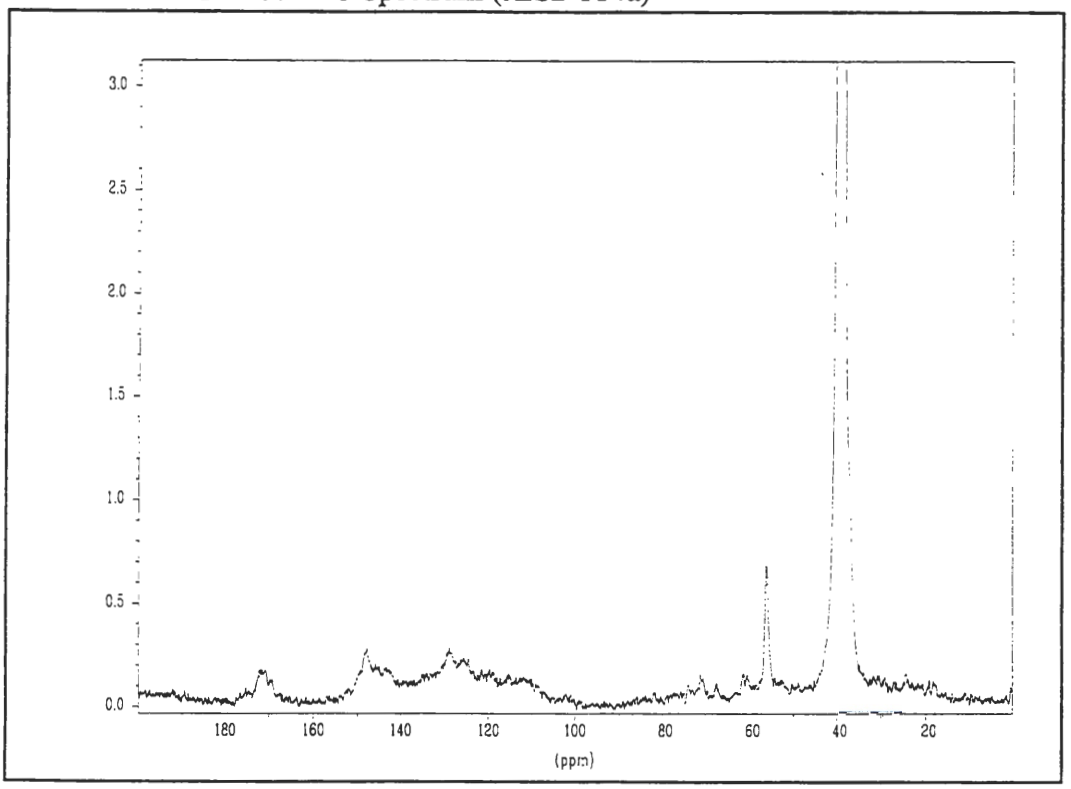
Laccase HBT 1 hour ^{13}C Spectrum (JES2-104a)



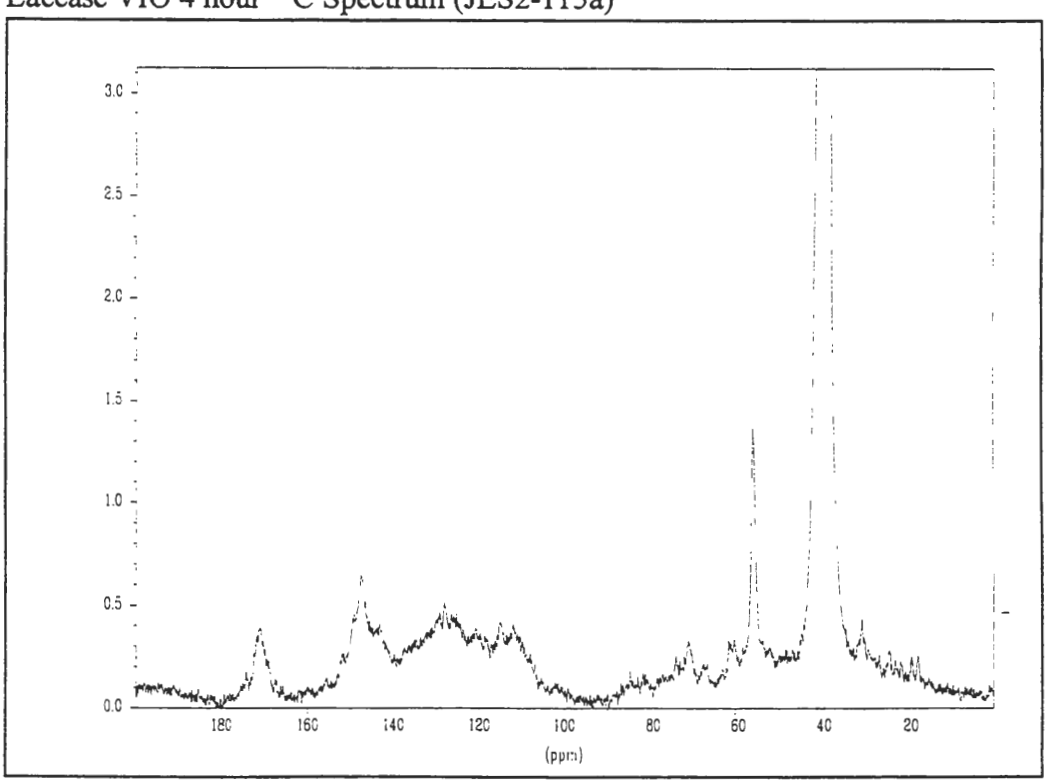
Laccase HBT 8 hour ^{13}C Spectrum (JES2-110a)



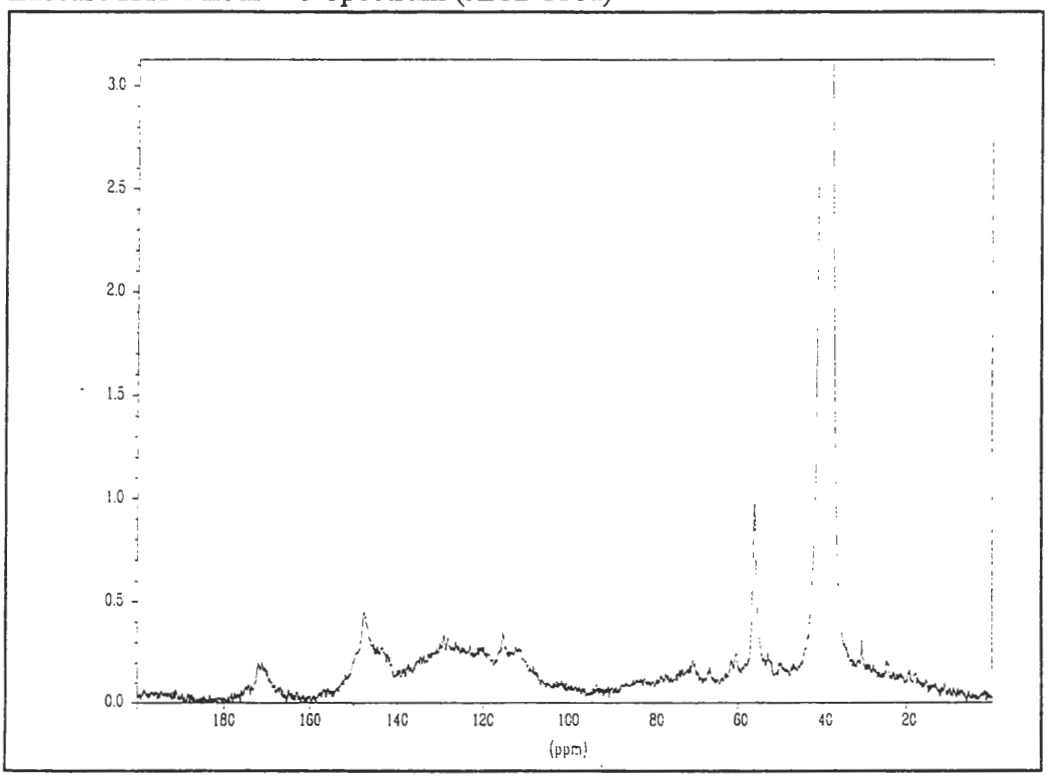
Laccase NHAA 4 hour ^{13}C Spectrum (JES2-114a)



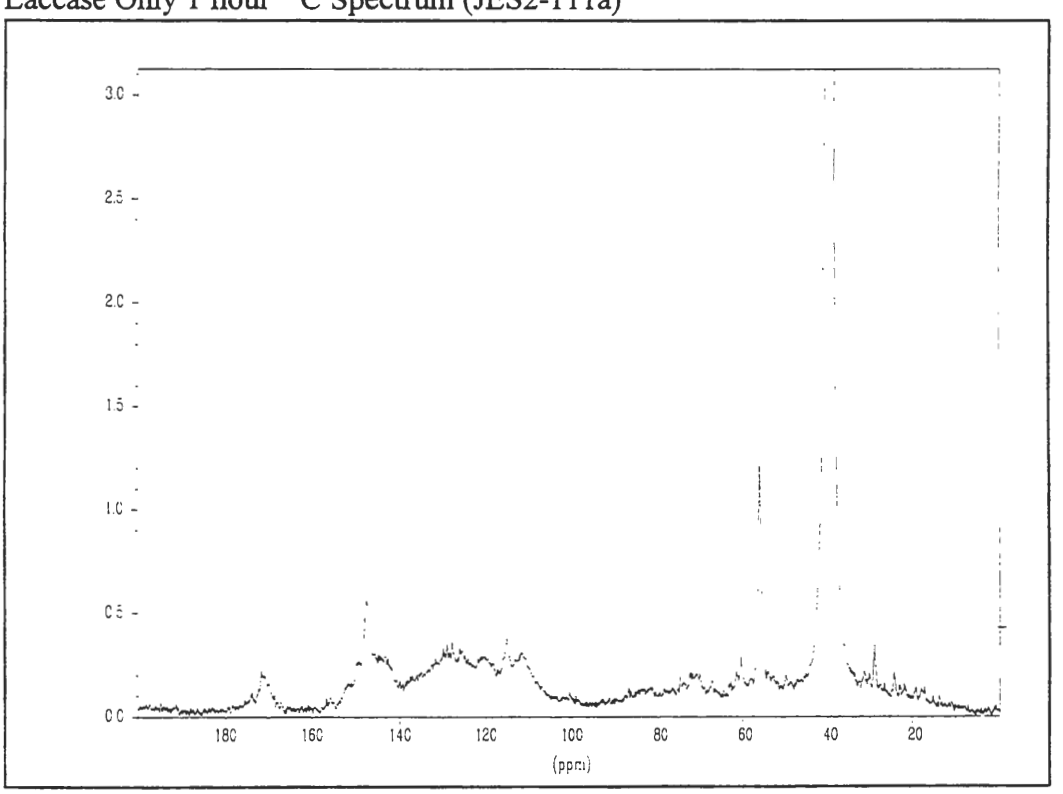
Laccase VIO 4 hour ^{13}C Spectrum (JES2-115a)



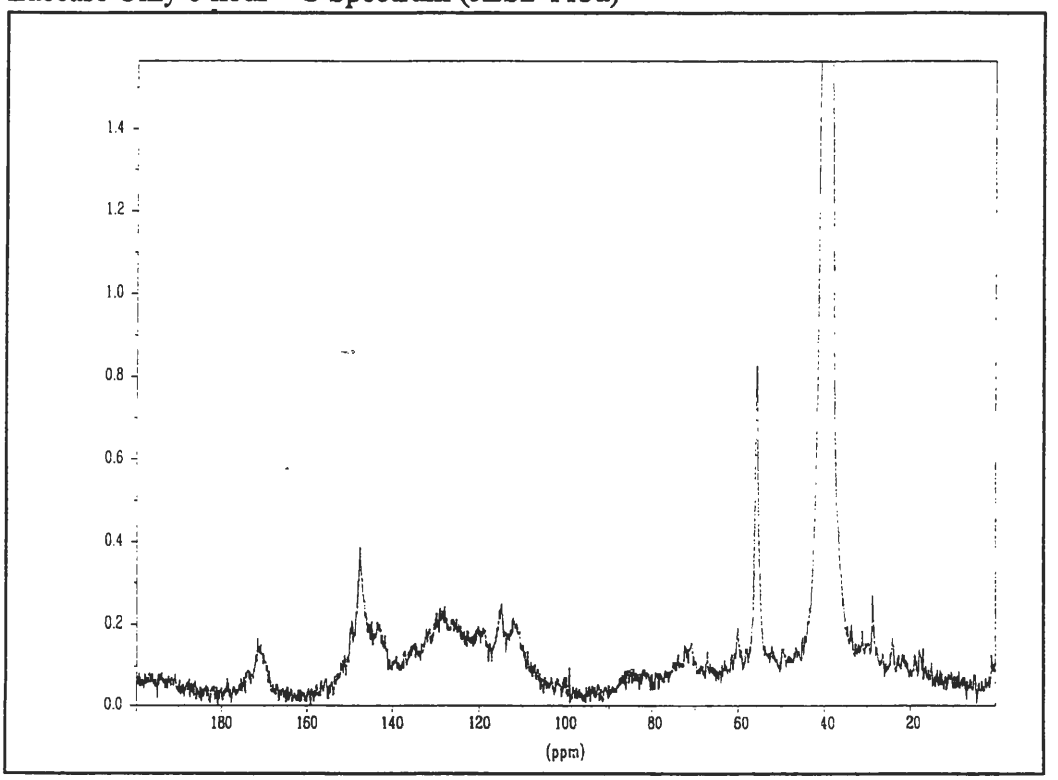
Laccase HTI 4 hour ^{13}C Spectrum (JES2-116a)



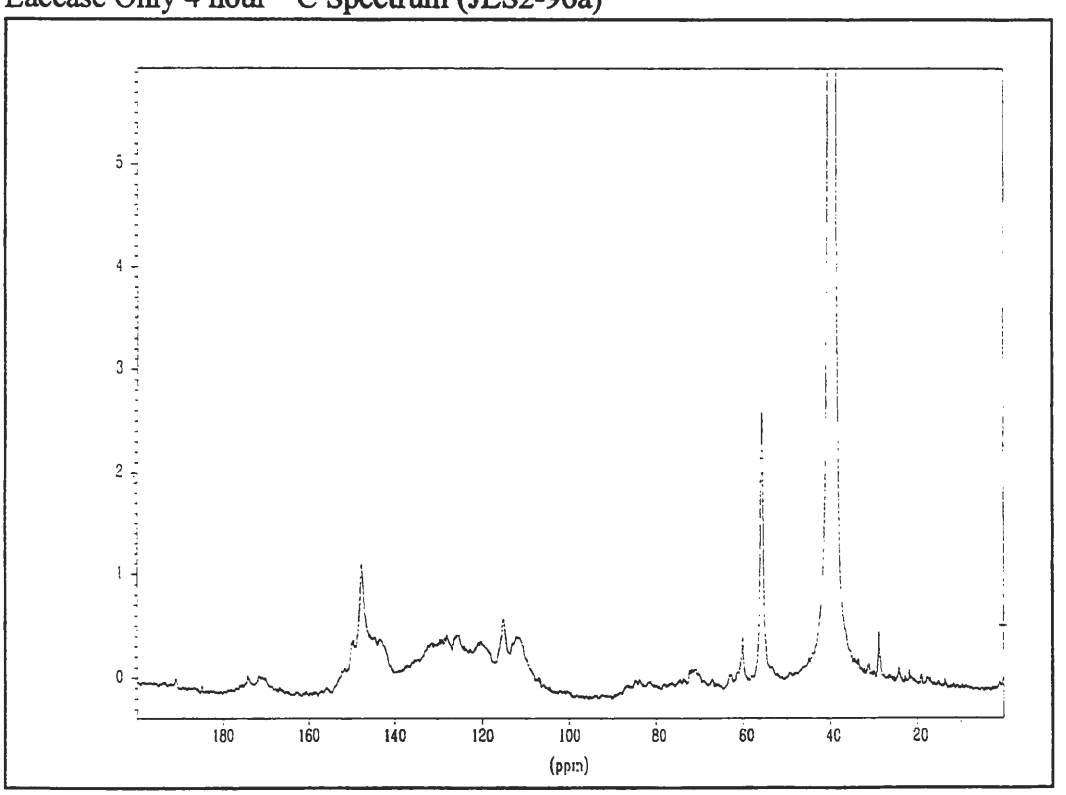
Laccase Only 1 hour ^{13}C Spectrum (JES2-111a)



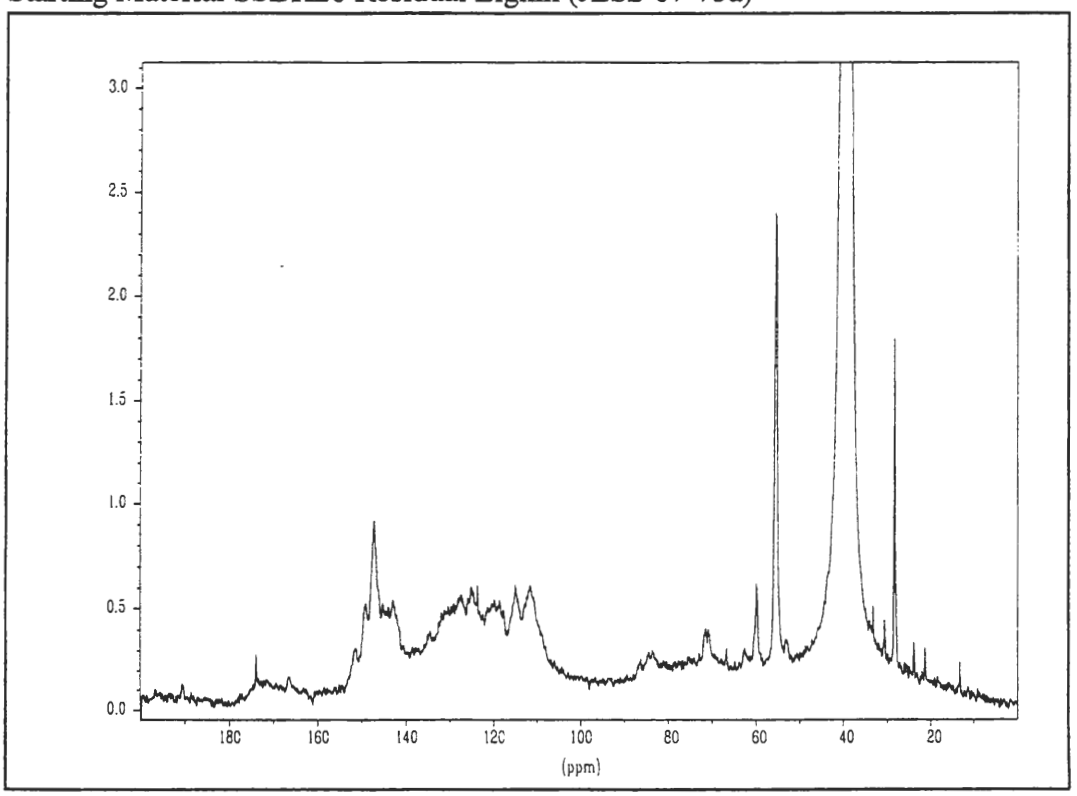
Laccase Only 8 hour ^{13}C Spectrum (JES2-113a)



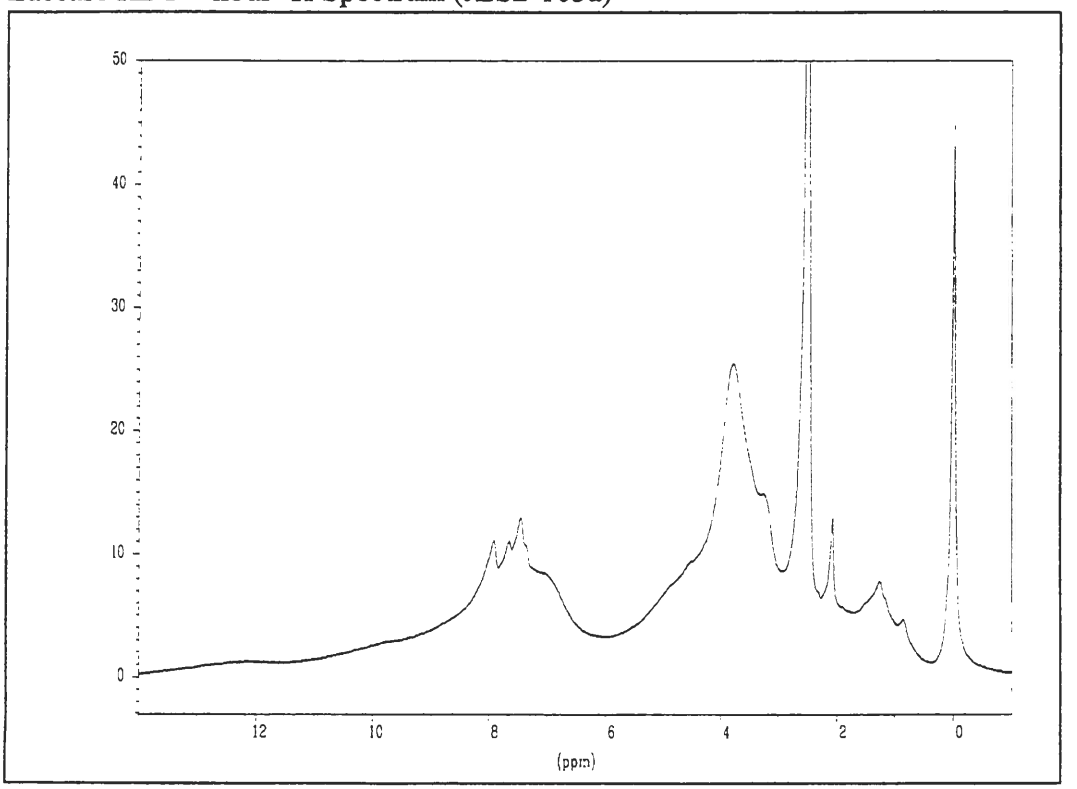
Laccase Only 4 hour ^{13}C Spectrum (JES2-96a)



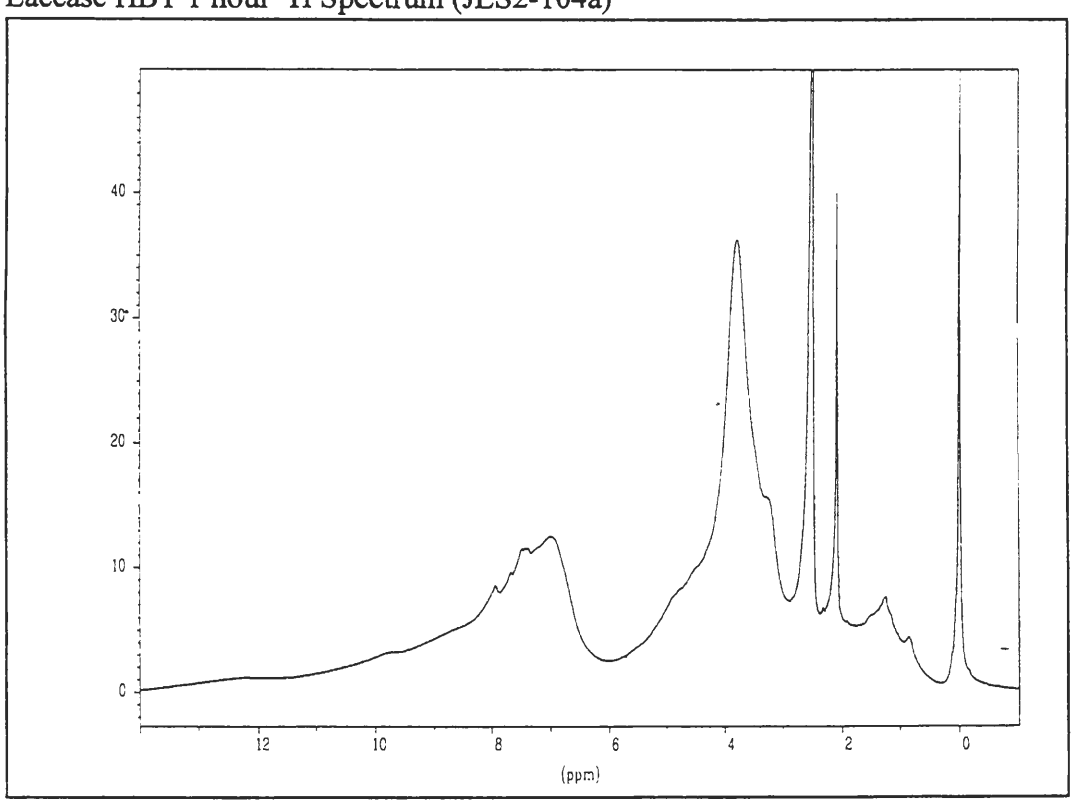
Starting Material SSBK26 Residual Lignin (JES2-67-73a)



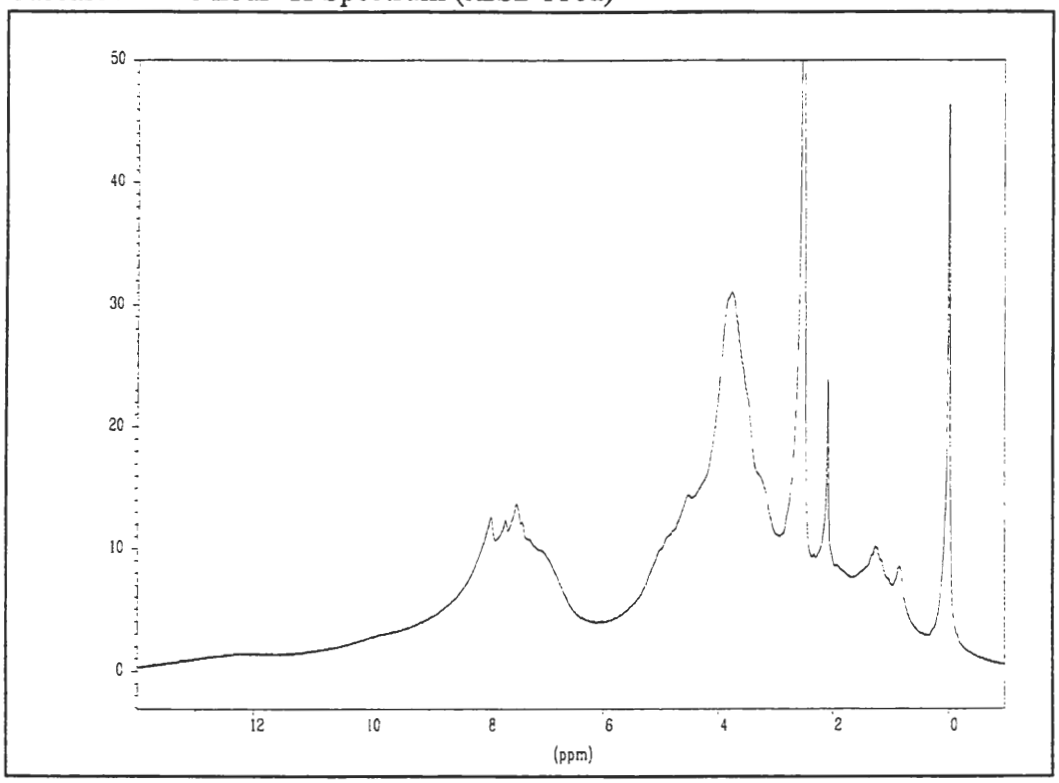
Laccase HBT 4 hour ^{1}H Spectrum (JES2-103a)



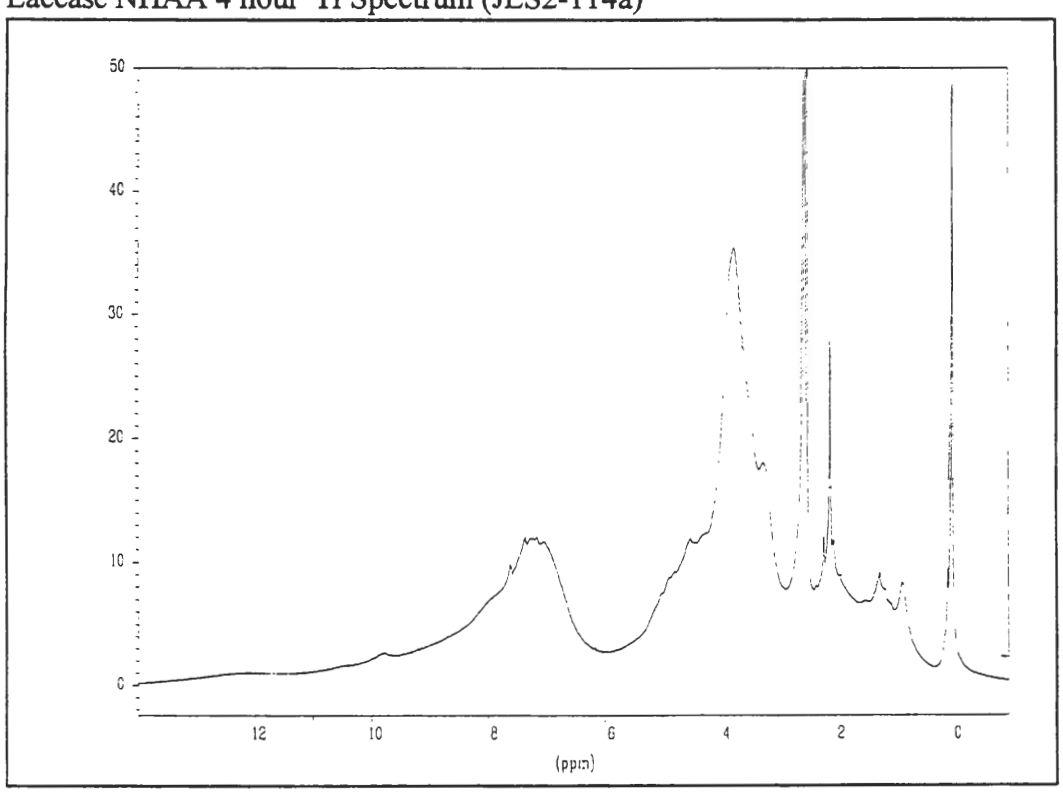
Laccase HBT 1 hour ^{1}H Spectrum (JES2-104a)



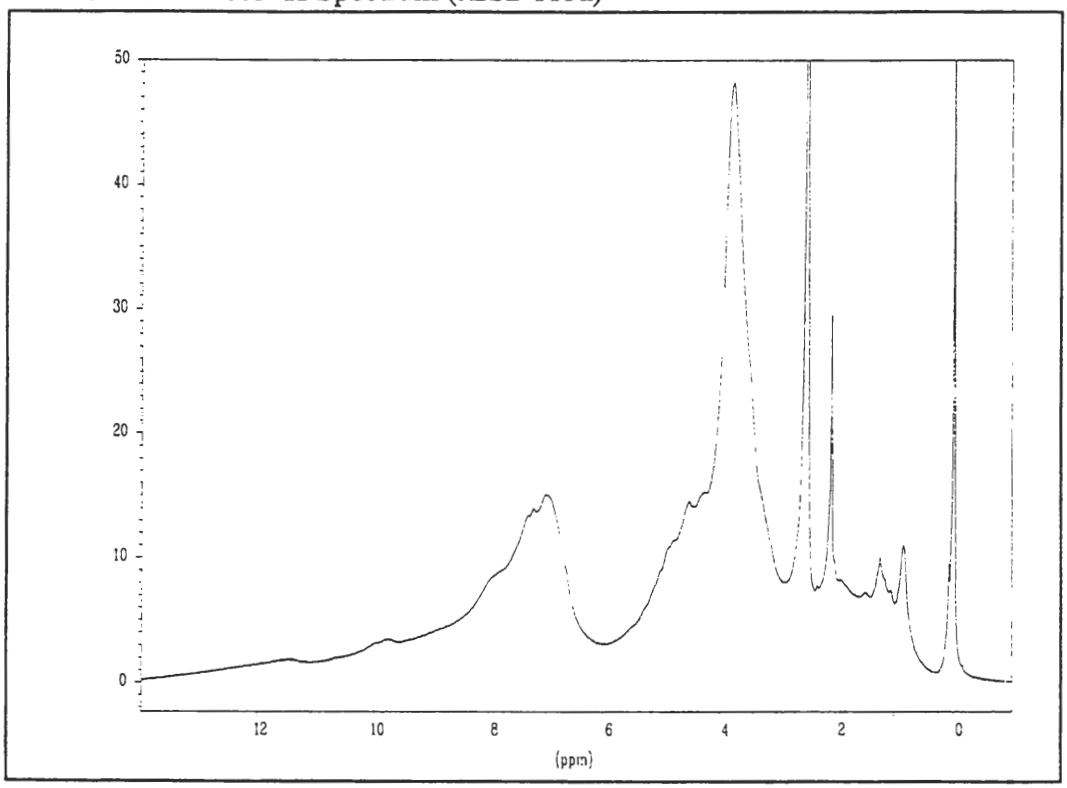
Laccase HBT 8 hour ^{1}H Spectrum (JES2-110a)



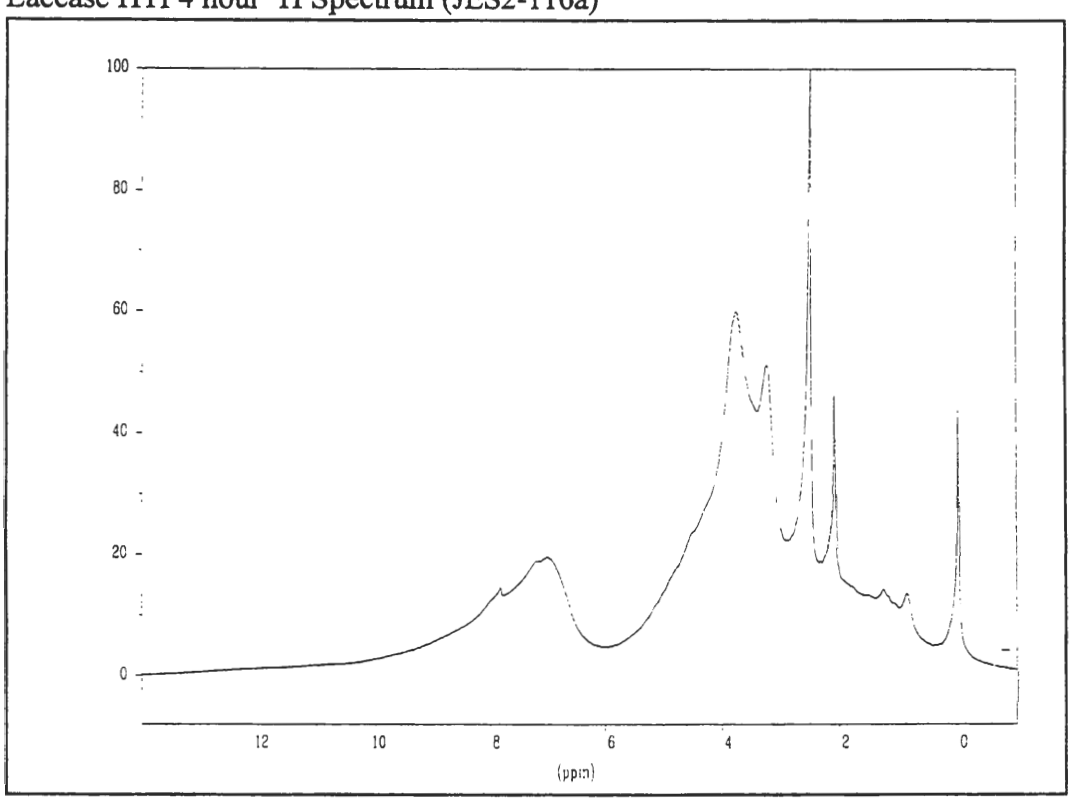
Laccase NHAA 4 hour ^{1}H Spectrum (JES2-114a)



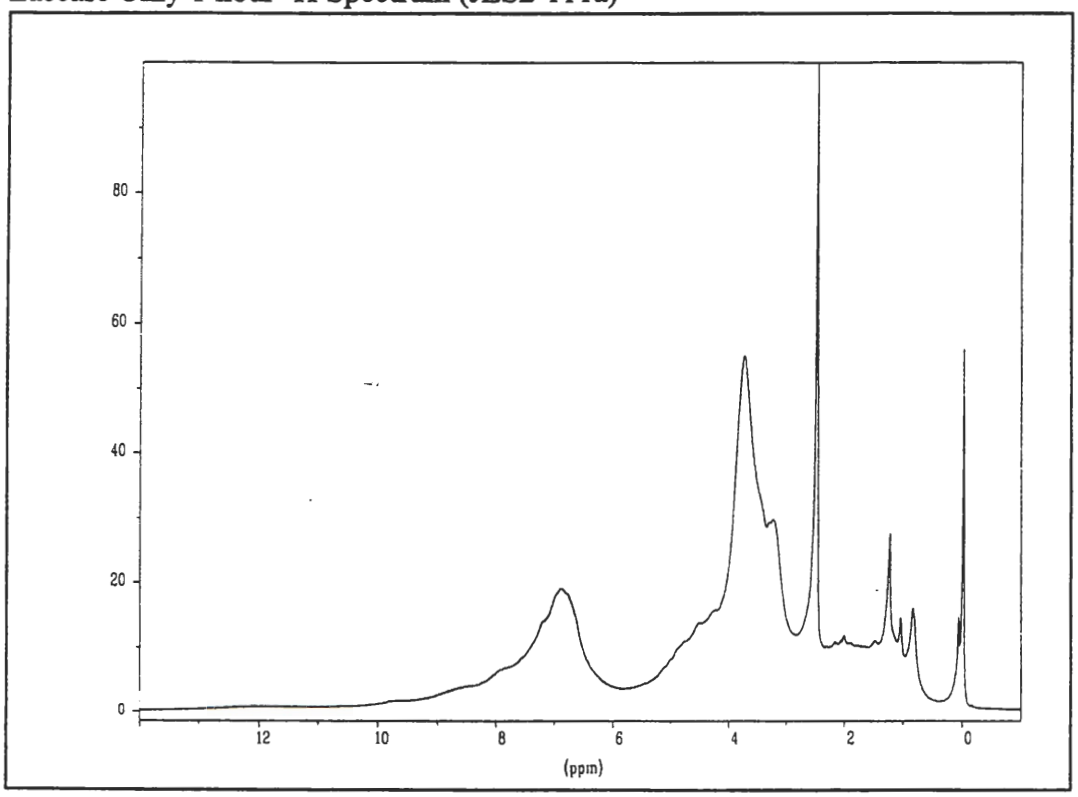
Laccase VIO 4 hour ^{1}H Spectrum (JES2-115a)



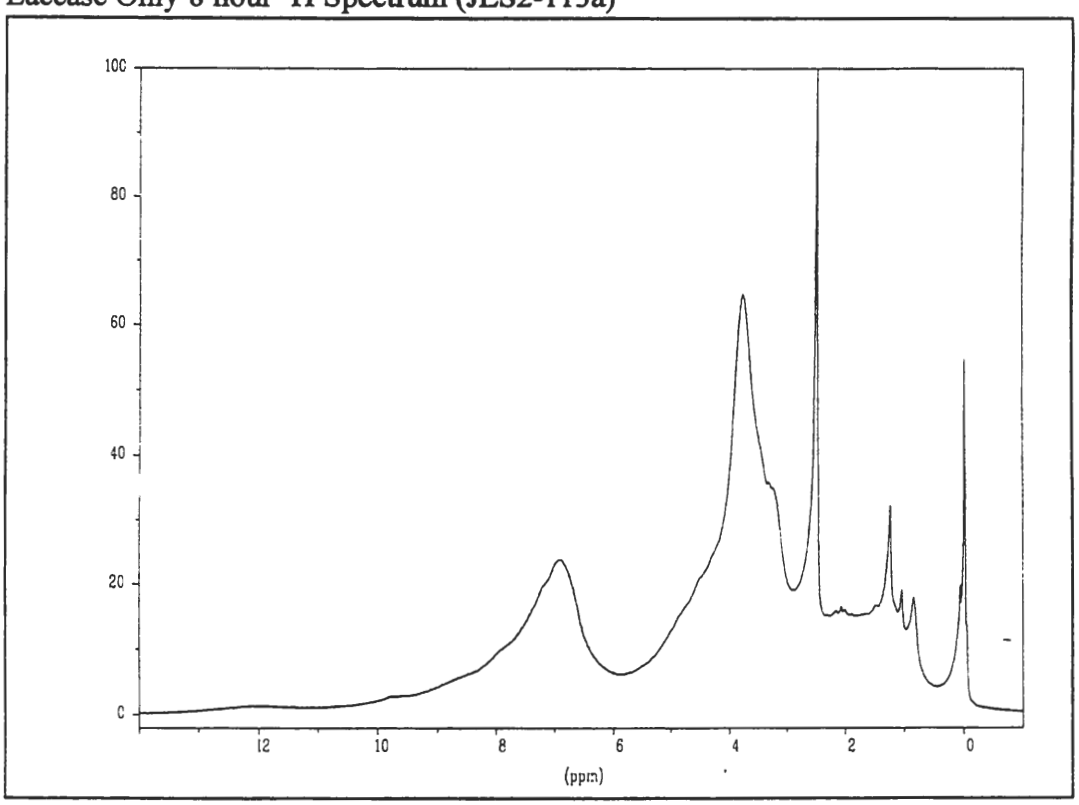
Laccase HTI 4 hour ^{1}H Spectrum (JES2-116a)



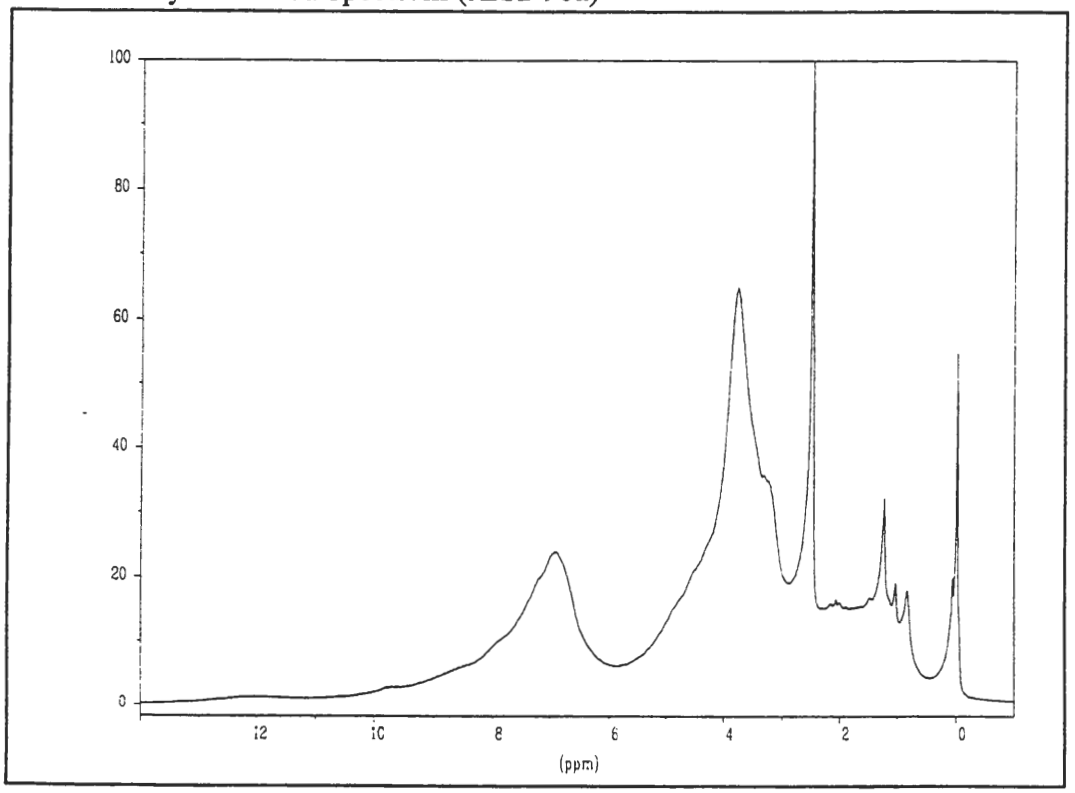
Laccase Only 1 hour ^{1}H Spectrum (JES2-111a)



Laccase Only 8 hour ^{1}H Spectrum (JES2-113a)



Laccase Only 4 hour ^{1}H Spectrum (JES2-96a)



Starting Material SSOK13 Residual Lignin ^{1}H Spectrum (JES2-67-73a)