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Biobleaching of pulp with dioxygen in the laccase-mediator system — reaction mechanisms for degradation of residual lignin

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Abstract

Pine Kraft-AQ pulp was biobleached with pressurized dioxygen at 40°C in laccase-mediator system (LMS), i.e. in acetate buffer (pH 4.5) containing *Coriolus*-laccase and 1-hydroxy-benzotriazole (HOBT), the latter being as a mediator. The LMS-treatment was followed by alkaline extraction (E) under standard conditions. The structures of the residual lignins before and after the biobleaching did not differ appreciably. This indicates that only a part of the residual lignin in the pulp undergoes oxidative degradation in the LMS treatment. In contrast, the treatment resulted in strong changes in the structure of the lignin isolated from E-effluents. The 2D HMQC (^{1}H–^{13}C correlation) spectra showed the disappearance of β-*O*-4′, β-β′ and β-5′ bonds in the structure of the alkaline soluble lignin (ASL) from E-effluents, which are present in the 2D spectrum of the original residual lignin (RKL). In addition, the spectra exhibited new signals that are assigned to Ar–COOH in biphenyl (5-5′) moieties. This implies that oxidative cleavage of side chains plays an important role in the delignification of pulp. The NMR studies also indicated that intensive degradation of aromatic ring has occurred in the biobleaching. However, premethylation of neither benzyl alcohol nor phenolic hydroxyl groups of the residual lignin in pulp before the biobleaching affected the rate of delignification. The latter indicates that phenolic moieties participate not only in oxidative degradation but also dehydrogenative polymerization reactions in the biobleaching. This is consistent with an appreciable increase in the proportion of fractions with higher molecular mass in lignin isolated from E-effluents.

Keywords: Pine Kraft-AQ pulp; Biobleaching; Dioxygen; Laccase-mediator system (LMS); Residual lignin; Alkaline soluble lignins; Changes in structures of lignins; Reaction mechanisms

1. Introduction

Delignification of pulp with dioxygen in the laccase-mediator system (LMS) is under intensive investigation as a potential, environmentally benign biobleaching process. Nevertheless, the reaction mechanisms, particularly the reaction pathways of lignin oxidation in the LMS, are not well established. The most known reaction of lignin model compounds with dioxygen in the LMS is the oxidation of benzyl alcohols to the corresponding carbonyl compounds [1]. However, how reactions of this type could lead to lignin fragmentation is unclear. Freudenreich et al. [2] suggested that cleavage of β-*O*-4′ bonds in the corresponding α-carbonyl compounds occurs during alkaline extraction:

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Srebotnik et al. [3] reported that cleavage of β-*O*-4′ bonds occurs in certain lignin model compounds in the LMS treatment. This is in contradiction to earlier reports that the corresponding α-carbonyl product was the only oxidation product identified in the reactions of this type [1]. Recently, Chen et al. [4–5] reported that oxidation of 1-(3,4-dimethoxyphenyl)-1-propene with dioxygen in laccase-ABTS system results in the cleavage of C_α–C_β conjugated double bond. Moreover, Crestini and Argyropoulos [6] observed that stilbene structures undergo oxidation in the LMS, even though no degradation products were identified. In addition, oxidation of phenolic lignin moieties catalyzed directly by laccase is also possible [7].

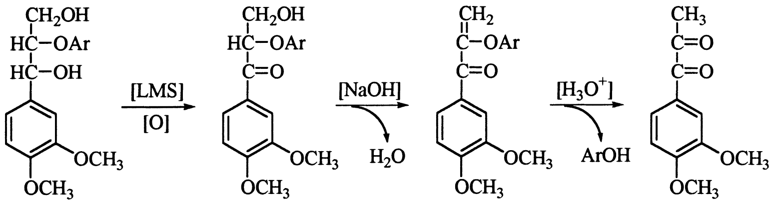
So far, studies on the reactions of lignin in pulps during delignification with dioxygen in LMS are very limited. Quantitative ^{13}C and ^{31}P NMR spectroscopic studies showed [8] that residual and soluble lignins increased carboxyl group content and decreased phenolic moieties in biobleaching of pulp with dioxygen in the laccase-HOBT system. The formation of an appreciable amount of methanol [9] and a high consumption of dioxygen [10] are consistent with possible participation of phenolic groups in the lignin oxidation. Permanganate oxidation of residual lignins suggests high reactivity of phenolic biphenyl lignin moieties in the biobleaching [11]. However, reaction mechanisms postulated by oxidation of lignin model compounds with dioxygen in the LMS was not verified by the qualitative changes in the structures of lignins in the pulp biobleaching. Thus, it is still unclear how the residual lignin reacts with dioxygen in the LMS.

Previously, we have briefly reported the main transformations of residual lignin in pulp in the biobleaching of pine Kraft-AQ pulp with dioxygen in the LMS, using HOBT as a mediator [12]. In this paper, we will report in detail changes in the structures of residual lignins in the delignification of pulp with dioxygen in the LMS.

2. Results and discussion

2.1. Isolation and chemical compositions of lignin preparation derived from residual lignin in pine Kraft-AQ pulp

Isolation of the residual lignin from pulp with minimum structural alterations is of primary importance in investigation on the chemical reactions involving in bleaching of pulp. The treatment of pulps with acidic dioxane (acidolysis) gives residual lignins free from carbohydrate contaminants, but cleavage of some aryl ether bonds occurs during the process of isolation [13] (Scheme 1). Alternatively, the treatment of pulps with cellulase gives the corresponding residual lignins with minimum structural alteration in 80–90% yield. However, the residual lignin thus isolated contains noticeable amounts of contaminates derived from the enzyme and carbohydrates, which create considerable problems in characterization of the lignin preparations with spectroscopic techniques. Application of advanced ^{1}H–^{13}C correlation 2D NMR spectroscopic techniques, such as heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond coherence (HMBC) sequences, possibly gives 2D NMR spectra with a higher resolution of signals, which is very useful in observing qualitative changes in the lignin structure. The HMQC sequence gives information about the correlation between ^{1}H and ^{13}C atoms via $^1J(C, H)$, while the HMBC sequence provides information about long range correlation



Scheme 1. Cleavage of β-aryl ether bond in β-*O*-4′ type structures by dioxygen in the laccase-mediator system (LMS), using HOBT as mediator [2].

Table 1
Elemental composition of lignin preparations from pine Kraft-AQ pulp

Lignin	C (%)	H (%)	N (%)	OCH_3 (%)
RKL	58.96	5.46	1.09	11.73
RBL	57.91	5.44	3.36	8.02
ASL	49.47	4.17	0.14	6.90

between ^{1}H and ^{13}C atoms via $^{2}J(C, H)$ and $^{3}J(C, H)$. Thus, the strategy of the present research is to isolate residual lignin from pulp with minimal structural alterations, and to characterize the resulting lignin preparations with advanced NMR spectroscopic techniques.

As expected, the data from elemental analysis (Table 1) show that the residual lignin preparations contain some amounts of proteins. In addition, the relatively low percentage of carbon in the lignin preparations indicates that they contain a rather high percentage of oxygen. This implies that the lignins also contain appreciable amounts of carbohydrates as contaminants in addition to proteins.

2.2. The advantages of HMQC 2D NMR spectra of lignin preparations derived from the residual lignin in pine Kraft-AQ pulp

To check the effect of protein impurities on the spectra of the residual lignins (Fig. 1), the HMQC spectrum of the cellulase has been recorded. It shows (Fig. 2, Table 2) that some of the cross-signals of cellulase could be overlapped with those of lignins in some regions of the lignin spectra. However, some of the very strong cross-signals in the cellulase spectrum are absent in the spectrum of the residual lignin (Figs. 1 and 2). For example, no cross-signals are observed in the spectra of lignins in the region of δ_C/δ_H 42/3.75 and 74–85/3.4–4.0 ppm, and very strong cross-signals in the region of δ_C/δ_H 90–110/4.1–5.2 and 123.5–126/7.13–7.17 ppm in the spectrum of cellulase are absent in the residual lignin spectra. This indicates that the protein contaminants do not obstruct interpretation of the HMQC spectra of the residual lignins. The signals for the carbohydrates observed in the spectra could be distinguished from the lignin signals (Fig. 1, Table 2). Thus, the protein and

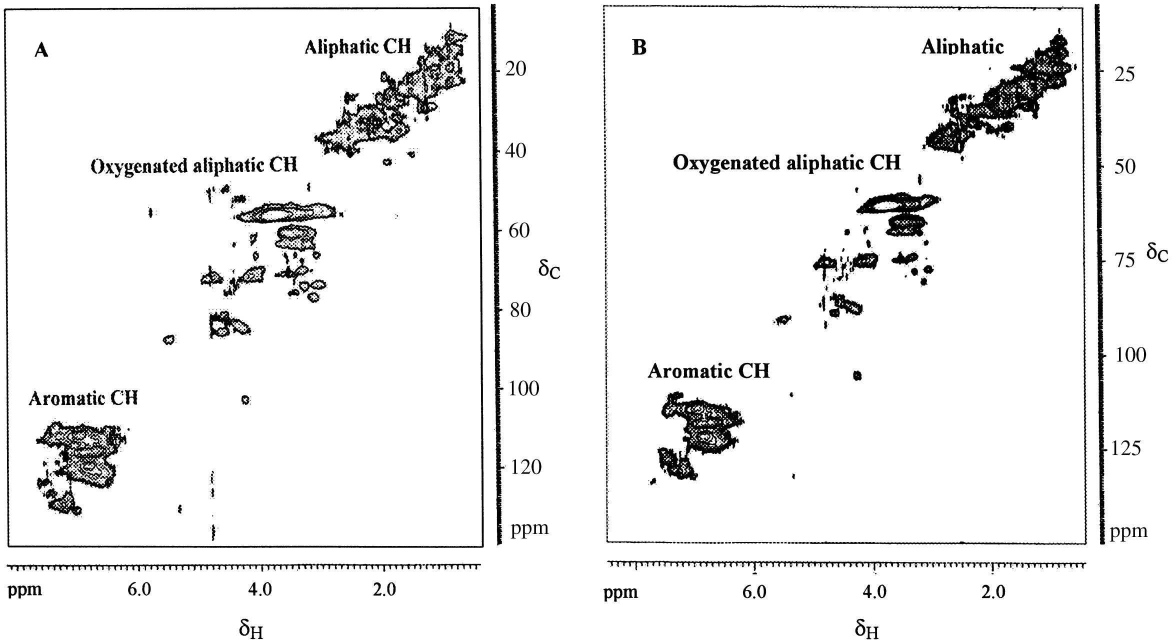


Fig. 1. (A) HMQC spectrum of residual lignin from the pine Kraft-AQ pulp (RKL); (B) HMQC spectrum of residual lignin from the LMS-E treated pine Kraft-AQ pulp (RBL). Solvent: DMSO-d_6.

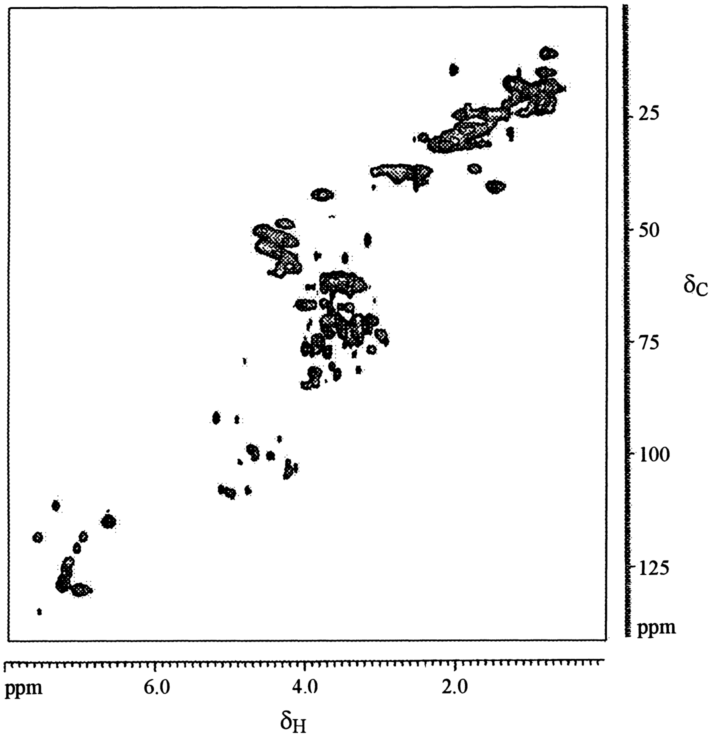


Fig. 2. HMQC spectrum of cellulase. Solvent: DMSO-d_6.

carbohydrate contamination does not hinder interpretation of the spectra for elucidation of lignin structures.

2.3. Characterization of original residual Kraft-AQ lignin (RKL)

Characterization of the original residual Kraft-AQ lignin (RKL) gives information on reactive centers, which could undergo oxidation with dioxygen in the LMS. The HMQC technique shows the correlation between ^{1}H and ^{13}C atoms bonded to each other. In general, a lignin 2D spectrum (Fig. 1) can be divided into three regions: aliphatic, oxygenated aliphatic and aromatic regions. The important cross-signals of the lignin preparations are listed in Table 2. The assignment of the cross-signals in 2D NMR spectra was basically made using the published database for ^{13}C [13–18] and ^{1}H [18–21].

The residual Kraft-AQ lignin (RKL) contains appreciable amounts of aliphatic moieties as showed in its HMQC spectrum (Fig. 3A). The spectrum exhibits groups of signals with chemical shifts of about δ_H 0.8 ppm and δ_H 1.2–1.3 ppm (Table 2). These signals correspond to saturated aliphatic moieties, which could form in the Kraft-AQ pulping. In additional, they can originate from extractives, which are likely chemically bonded to lignin.

Cross-signals in lower field (δ_H 1.5–2.5 and δ_C 20–43 ppm) could correspond to aliphatic groups neighboring to alkene and oxygen-containing groups such as alcohol, carbonyl and ethers. The FTIR spectrum shows that the lignin contains an appreciable amount of carbonyl groups. Thus, the presence of structural types such as G–CO–CH_3 (CH_3: δ_C/δ_H 25.9/2.5 ppm), and G–CO–CH_2–CH_3 (CH_2: δ_C/δ_H 31/2.5 and CH_3: δ_C/δ_H 10.5/0.8 ppm) is possible, where G is guaiacyl group. This is also supported by the signals at δ_C/δ_H 111.4/7.43 and 122.7/7.62 ppm in the HMQC spectrum of RKL (Fig. 5A), corresponding to CH at C-2 and C-6 of the aromatic ring in this type of structures, respectively.

Table 2
Chemical shift and signal assignment for lignin preparations[a,b,c]

δ_C (ppm)	δ_H (ppm)	RKL	ASL	Cell	Assignment
10.7	0.80	w	w	s	*Alk–CH$_3$, Alk–CH$_2$–Alk*
13.6	0.85	m	s	–	
15.2	1.07	–	m	–	
17.4	1.03	–	w	–	
18.3	0.80	m	m	s	
21.7	0.80	m	–	s	
20.4	1.27	w	–	–	
22.3	0.88	m	v	m	
22.0	1.21	vw	m	–	
23.7	1.35	–	v	–	
24.0	1.48	w	m	–	
20.6	1.93	w	–	–	*Ar–CH$_3$*
26.0	1.98	m	–	–	*G–CHOH–CH$_3$*
26.0	2.50	m	–	–	*G–CO–CH$_3$*
28.5	1.24	s	s	s	**Unknown**
30.5	2.08	vw	s	m	*G–CH$_2$–CH$_2$–COOH*
31.0	2.50	m	vw		*G–CO–CH$_2$–CH$_3$*
33.5	2.18	w	m	–	*G–CH$_2$–CH$_2$–COOH*
33.5	2.25	–	w	–	
34.4	1.67	w	wv	–	**Unknown**
50.0	4.55	w	–	s	*α-CH in α-condensed structures*
52.0	4.2–4.3	w	–	s	
54.0	5.75	w	w	–	
56.0	3.75	vs	vs	–	**ArOMe**
60.0	3.39	vs	s	vs	γ-**CH$_2$–OH** in β-***O*-4′**, CH-6 carbohydrates
60.0	3.55	vs	s	vs	***Cellulase ?***
62.0	4.10	vw	vw	–	γ-CH$_2$–OH in β-5′, β-*O*-4′ with α-C=O
63.0	3.62	w	–	vs	?
63.0	3.70	w	–	vs	
63.0	3.17	vw	m	–	**Carbohydrates (CH-5** in **xylane)**
63.0	3.87	vw	m	–	
65.5	3.72	–	w	s	*Ar–OCH$_2$–COOH*
65.5	4.05	w	–	m	
67.0	4.13	–	w	–	*G–CHOH–CH$_3$, R–OCH$_2$–COOH*
69.5	3.28	w	w	s	Carbohydrates
69.5	3.52	w	w	s	
	4.13	m	–	–	γ-**CH$_2$–O–** in **pinoresinol** (β-β′)
71.5	4.75	m	–	–	α-**CH–O–** in β-***O*-4′**
73.0	3.02	w	m	s	**Carbohydrates**
73.5	3.28	w	m	m	
75.5	3.41	w	–	m	
75.2	3.52	wv	m	–	
76.5	3.13	w	m	s	
77.8	3.63	–	vw	vs	
80.2	3.82	–	vw	w	
82.0	4.5–4.7	w	–		*α-CH–O–C– in LC ether bond*
84.5	4.28	m	–	–	β-**CH–O–** in β-***O*-4′**
85.5	4.62	m	–	–	α-**CH–O–** in β-β′
87.3	5.45	w	–	–	α-**CH–O–** in β-**5′**

Table 2 (*Continued*)

δ_C (ppm)	δ_H (ppm)	RKL	ASL	Cell	Assignment
102.5	4.25	w	m	–	**Carbohydrates (C-1)**
110.8	7.00	vs	s	–	**HC-2** in **G-units**
111.4	7.43	s	m	–	
115.0	6.74	vs	s	–	**HC-5** in **G-units**
115.3	6.90	vs	s	–	
118.5	6.84	vs	s	–	**HC-6** in **G-units**
120.2	6.70	s	m	–	
122.7	7.60	w	–	–	HC-6 in G–CO–R
124.5	7.25	–	w	–	HC-6 in G–CHO
124.7	7.32	–	w	–	
125.5	7.43	w	m	–	HC-6 in biphenil (5-5′) G-units with α-COOH or α-C=O group
127.5	7.10	–	w	–	*G–CH=CH–G, other olefinic moieties*
127.8	7.25	m	–	–	
127.8	7.37	w	–	–	
128.8	7.15	–	w	s	
128.8	7.23	–	w	–	
129.3	5.32	w	w	–	?
129.2	7.72	–	m	–	**Olefinic moieties**
131.9	7.68	–	m	–	
133.7	7.45	–	m	–	

[a] RKL: residual lignin isolated from pine Krafst-AQ pulp; ASL: alkaline soluble lignin isolated from alkaline effluents after biobleaching of the pulp with dioxygen in LMS; cell: cellullase.

[b] vw: very weak; w: weak; m: moderate; s: strong.

[c] vs: very strong; bold: definite assignment; italic: tentative assignment.

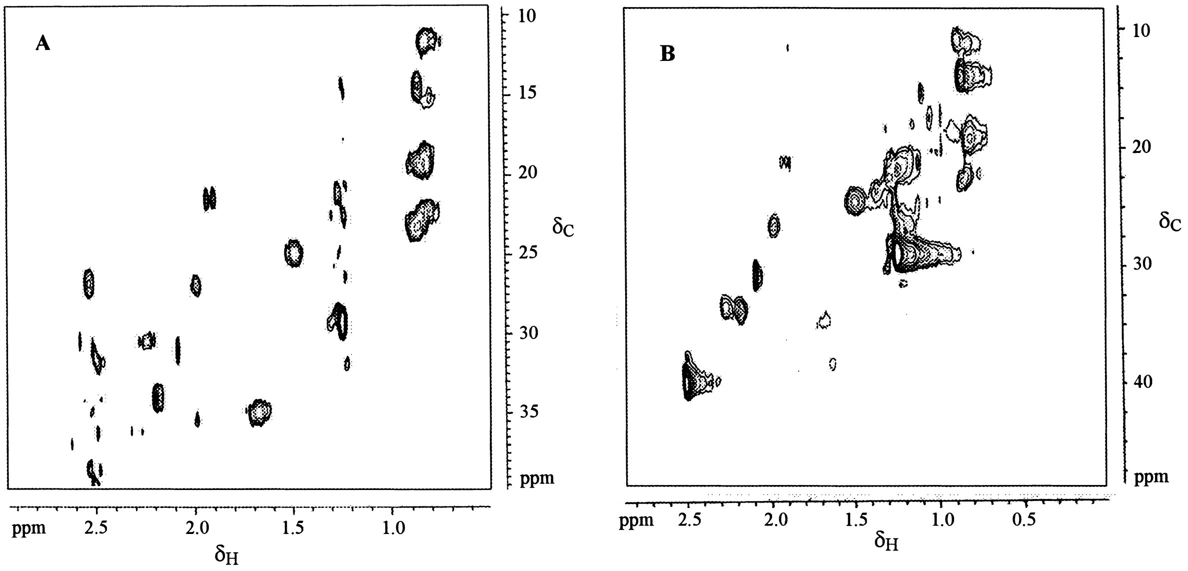
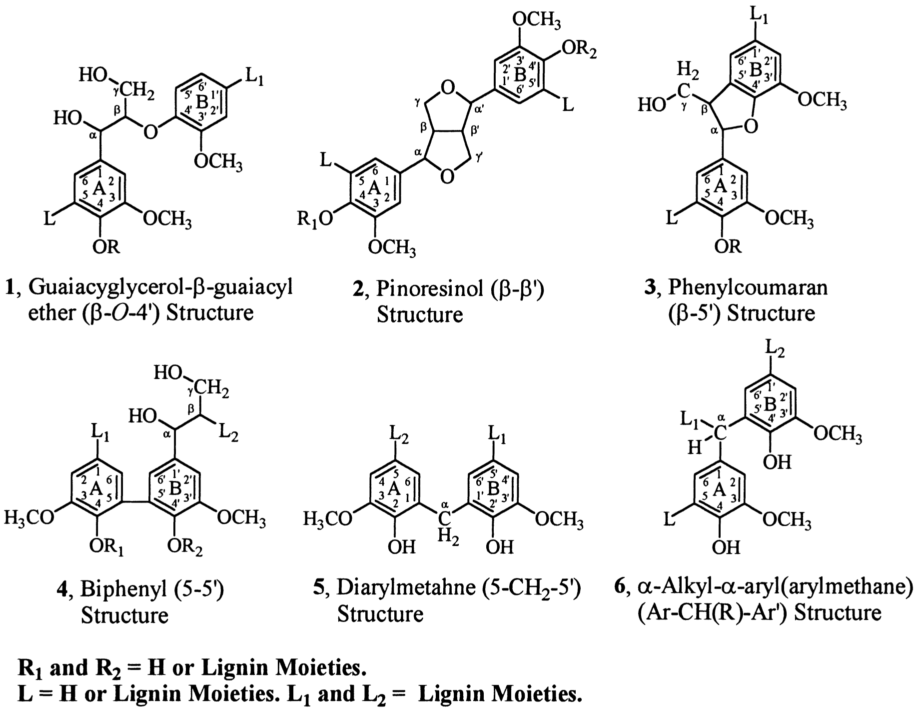


Fig. 3. (A) Expanded aliphatic region of the HMQC spectrum of residual lignin from the pine Kraft-AQ pulp (RKL); (B) expanded aliphatic region of the HMQC spectrum of lignin from the alkaline E-effluent (ASL) after the LMS-E treatment of pine Kraft-AQ pulp. Solvent: DMSO-d_6.



1, Guaiacyglycerol-β-guaiacyl ether (β-*O*-4′) Structure

2, Pinoresinol (β-β′) Structure

3, Phenylcoumaran (β-5′) Structure

4, Biphenyl (5-5′) Structure

5, Diarylmetahne ($5\text{-}CH_2\text{-}5'$) Structure

6, α-Alkyl-α-aryl(arylmethane) (Ar-CH(R)-Ar′) Structure

R_1 and R_2 = H or Lignin Moieties.
L = H or Lignin Moieties. L_1 and L_2 = Lignin Moieties.

Scheme 2. Some important structures in pine residual lignins and alkaline soluble lignins isolated from pine Kraft-AQ pulp before and after biobleaching with dioxygen in laccase-mediator system using 1-hydroxybenzotriazol as the mediator.

As we have reported previously [22], the HMQC 2D NMR technique does not show the presence of either diarylmethane ($5\text{-}CH_2\text{-}5'$; **5**, see Scheme 2 for structure) or α-alkyl-α-aryl(arylmethane) (Ar–CH(R)–Ar′; **6**) moieties in the residual Kraft-AQ lignin, condensed structures commonly believed to form in the Kraft-AQ pulping and hinder further delignification. However, weak cross-signals at δ_C/δ_H 50/4.55, 52/4.2–4.3 and 54/5.75 ppm in the HMQC spectrum of RKL (Fig. 4A) could correspond to α-CH groups in α-alklyl-α-aryl(arylmethane) condensed structures (**6**) according to data reported for the corresponding lignin model compounds [15,23].

Cross-signals in the oxygenated aliphatic region of the HMQC spectrum of the RKL (Fig. 4A, Table 2) clearly show the presence of guaiacylglicerol-β-guaiacyl ether structures (β-*O*-4′; **1**, L = H). The amount of β-*O*-4′ moieties with α-carbonyl group appears to be very small. In spite of a weak signal for $\gamma\text{-}CH_2OH$ group at δ_C/δ_H 62.5/4.10 ppm, which could also correspond to that of phenylcoumaran (β-5′; **3**, L = H) type structures [19–21], the characteristic cross-signal for $C_\beta H_\beta$ at δ_C/δ_H 81.3/5.4 ppm [18] was not observed. This implies that α-carbonyl groups discussed above mainly present in the residual lignin as terminal side chains, which do not contribute appreciably to fragmentation of lignin. The HMQC spectrum also shows the presence of pinoresinol (β-β′; **2**, L = H) and phenylcoumaran (β-5′; **3**, L = H) structures in the residual lignin (Fig. 4A, Table 2). Cross-signals at δ_C/δ_H 82/4.5–4.7 ppm could correspond to $C_\beta H_\beta$ in moieties containing β-*O*-4′ and α-*O*-4′ ether bonds simultaneously. However, the corresponding cross-signal for $C_\alpha H_\alpha$ at about δ_C/δ_H 79/5.5 ppm [18] was not observed. Alternatively, these signals can be attributable to β-*O*-4′ moieties (**1**, L = H) involving in lignin carbohydrate ether bonds at the benzylic carbon atom [24].

Cross-signals observed at δ_C/δ_H 128.5/7.2–7.4 ppm likely correspond to stilbene moieties formed during the pulping from phenylcoumaran (β-5′) type structures (**3**). A very weak signal at δ_C/δ_H 125.5/7.43 ppm

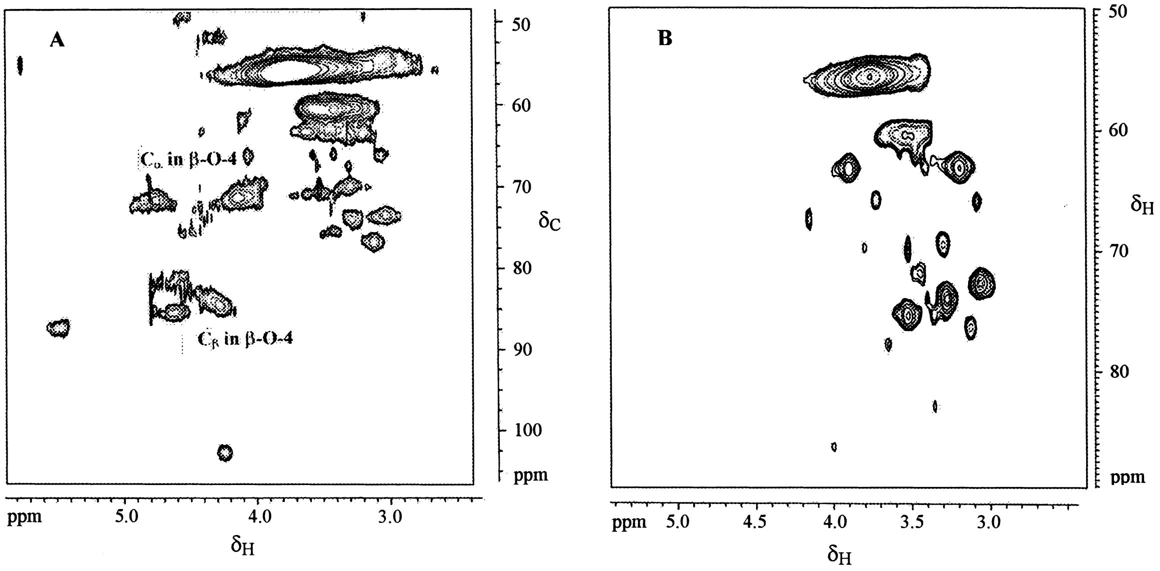


Fig. 4. (A) Expanded oxygenated aliphatic region of the HMQC Spectrum of residual lignin from the pine Kraft-AQ pulp (RKL); (B) expanded oxygenated aliphatic region of the HMQC spectrum of lignin from the alkaline E-effluent (ASL) after the LMS-E treatment of pine Kraft-AQ pulp. Solvent: DMSO-d_6.

probably corresponds to conjugated aldehyde moieties, likely benzaldehyde type since no cross-signals of coniferyl aldehyde (δ_C/δ_H 127/6.75, 153.5/7.62 and 194/9.7 ppm) was detected. The presence of a small amount of benzaldehyde moieties is confirmed by the corresponding cross-signal (δ_H 9.7–9.9 ppm and δ_C 191.5 ppm) in the aldehyde region of the HMQC spectrum. The absence of a signal at δ_C 143 ppm [15,17] indicates the absence of vinyl ether type structures in the residual lignin in the Kraft-AQ pulp, which could be potential reactive centers in the biobleaching with dioxygen in the LMS.

2.4. Structure of residual lignin after the biobleaching of pulp with dioxygen in the LMS-reaction mechanisms

The HMQC NMR (Fig. 1) and FTIR spectra and molecular mass distribution (Fig. 7) of the residual lignin after the LMS-E treatment (RBL) were very similar to those of the original residual Kraft-AQ lignin (RKL). The absence of appreciable differences in the lignin structures is in good agreement with the kinetic data obtained previously [10,12]. This indicates that residual lignin in the pulp undergoes oxidative degradation successively from the surface into the center of the fibers, which does not result in substantial quantitative changes in the structure of the residual lignin remained in pulp. The LMS then induces the reactions of lignin degradation fragments with dioxygen. This is evidenced by the structure of lignin isolated from E-effluents (ASL).

The ASL preparation contains a high percentage of oxygen (Table 1). This is in good agreement with the high dioxygen consumption in the biobleaching of pulp in the LMS reported earlier [10,12] as well as the noticeable carbohydrate content in the ASL (Table 2, Fig. 4B). In addition to the ASL fraction, the whole alkali soluble (AS) fraction was obtained from the E-effluent without acidic precipitation as in the case of ASL. The E-effluents were only neutralized and then dialyzed to remove inorganic components. The original objective of isolation of the AS fraction was to analyze all degradation products, which are not precipitated by acidification of the E-effluents. However, the ^{1}H, ^{13}C and 2D NMR studies showed that the AS fraction is very rich in carbohydrates. The presence of an appreciable amount of carbohydrates in the E-effluent contradicts with the statement of Poppius-Levlin et al. [25]. about very high stability of polysaccharides in biobleaching of pulp with dioxygen in the LMS.

The HMQC spectrum of the ASL shows clearly a drastic decrease in the amount of β-*O*-4′ (**1**), β-β′ (**2**) and β-5′ (**3**) moieties in the structures of the lignin from E-effluents (Fig. 4B). The absence of the signal at δ_C/δ_H 82/5.5 ppm indicates that the disappearance of guaiacylglicerol-β-guaiacyl ether structures (**1**) does not result in accumulation of the corresponding α-carbonyl moieties either in the residual lignin (RBL) from the biobleached pulp or in the soluble (ASL) lignins. However, new signals at δ_C/δ_H 124.5–125.5/7.25–7.43 ppm have appeared. These signals could not belong to benzaldehyde moieties since no aldehyde signal can be observed in the region of δ_C/δ_H 180–200/9–10 ppm. The new cross-signals were assigned as CH-6 in biphenyl (5–5′; **4**) type structures substituted with α-ketone or carboxyl group [18,26]. In addition, new signals in the region of δ_C 65–67 ppm, assigned by Robert and Chen [16] as carbon in the CH_2 group in Ar–OCH_2–COOH and R–OCH_2–COOH, can be observed in the HMQC spectra of ASL (Fig. 4B, Table 2). This implies that oxidative cleavage of the side chain, probably C_α–C_β bonds, plays an important role in the delignification of residual lignin in pulp with dioxygen in the LMS. This postulation is further supported by qualitatively and quantitatively discernible changes observed in the aliphatic and oxygenated aliphatic regions of the spectra (Figs. 3, 4, Table 2). The presence of stilbene structures in the residual Kraft-AQ lignin (RKL) and in the lignin from E-effluents (ASL) does not allow drawing any conclusions about their reactivity in the biobleaching of pulp.

The HMQC spectrum of ASL shows that new cross-signals appear in the region of δ_C/δ_H 127.5–134/7.1–7.7 ppm. In addition to original stilbene moieties, these signals could correspond to olefinic moieties formed in oxidative cleavage of aromatic ring. Particularly, three sharp cross-signals of moderate intensity appeared at δ_C/δ_H 129.2/7.72, 131.9/7.68 and 133.7/7.45 ppm in the spectrum of the ASL (Fig. 5B). The intensity of these cross-signals increased in the AS preparation indicating that these moieties are not readily precipitated from the alkaline E-effluents by acidification. They could correspond to olefinic moieties conjugated with carboxyl groups formed in aromatic ring cleavage. Furthermore, the ^{1}H NMR

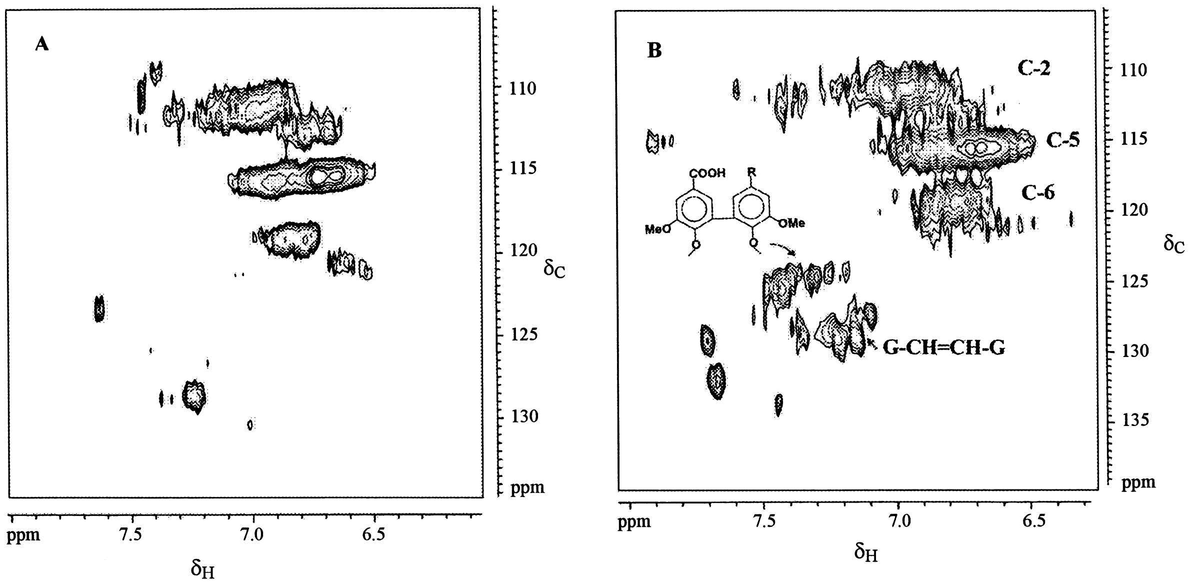


Fig. 5. (A) Expanded aromatic region of the HMQC spectrum of residual lignin from the pine Kraft-AQ pulp (RKL); (B) expanded aromatic region of the HMQC spectrum of lignin from the alkaline E-effluent (ASL) after the LMS-E treatment of pine Kraft-AQ pulp. Solvent: DMSO-d_6.

spectrum of the ASL showed that the intensity of signals in the aromatic region decreased appreciably with concomitant increase in the intensity of signals in the aliphatic region as compared to that of the RKL (Fig. 6). Similar result has been observed in different processes of lignin oxidation [27,28]. These aliphatic moieties can be produced by disproportionation reactions of side chain and ring cleavage products derived from oxidative cleavage of the aromatic rings followed by hydration. Thus, the NMR studies together

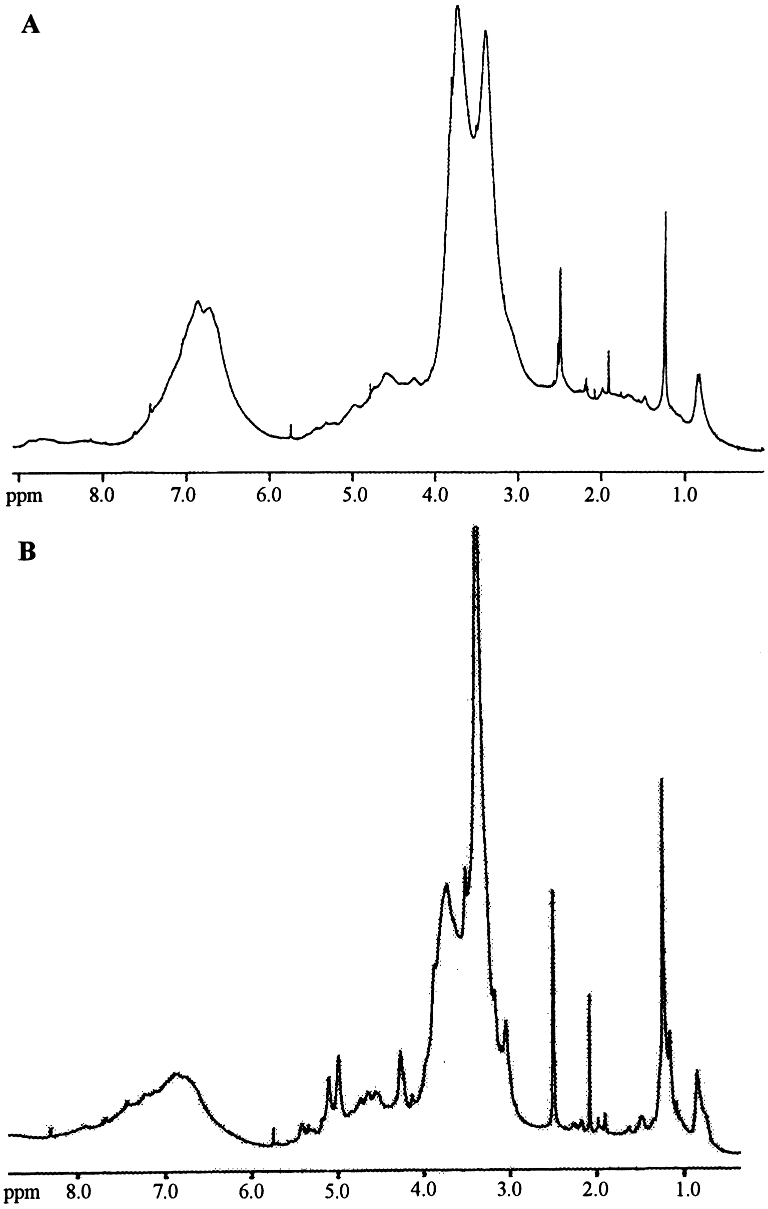


Fig. 6. (A) ^{1}H NMR spectrum of residual lignin from the pine Kraft-AQ pulp (RKL); (B) ^{1}H NMR spectrum of lignin from the alkaline E-effluent (ASL) after the LMS-E treatment of pine Kraft-AQ pulp. Solvent: DMSO-d_6.

with the decrease in methoxyl group content (Table 1) indicates that the oxidative cleavage of aromatic ring in lignin occurs in the biobleaching of pulp with dioxygen in the LMS.

2.5. Oxidative degradation of the residual lignin and dehydrogenative polymerization of its degradation products in the biobleaching with dioxygen in laccase-mediator system

Gel permeation chromatogram (GPC) of the lignin isolated from E-effluents (ASL) clearly showed (Fig. 7) that, besides a major fraction with a molecular mass of approximately 3500, the ASL has a higher molecular mass fraction as compared to the residual lignin (RKL). This fraction can be produced by dehydrogenative polymerization involving phenolic moieties leading mostly the formation of biphenyl (5–5′) type structures (**4**) in the biobleaching of pulp with dioxygen in the LMS. The dehydrogenative polymerization of phenol was also observed by Potthast et al. [29] in studies on the oxidation of phenolic model compounds with dioxygen catalyzed by laccase and in the laccase-ABTS system. Tamminen et al. [11] reported that on permanganate oxidation, the relative content of oxidation products derived from phenolic biphenyl structures is lower after treatment of pulps and residual lignin preparation with LMS. Based on this finding, they postulated that biphenyl type structures decreased in the biobleaching. Even though dioxygen in the LMS might have high reactivity towards phenolic biphenyl structures, it is worthwhile that on permanganate oxidation, the relative abundance of isohemipinic acid determined in the form of methyl ester appreciably increases in the LMS treatment [11]. This product is derived from β-5′ (**3**) and stilbene structures as well as from biphenyl structures with one etherified phenolic hydroxyl group [30]. As has been shown above, the amount of β-5′ structures in the ASL preparation is negligible. Thus, dehydrogenative polymerization of phenolic moieties to form 5–5′ type structures (**4**) could occur in the bleaching of pulp with dioxygen in the LMS. However, additional experiments are needed to further clarify this postulation.

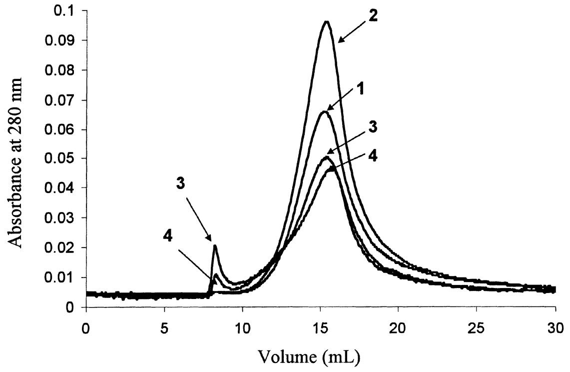


Fig. 7. Molecular mass distribution of lignin preparations. 1: residual lignin from the pine Kraft-AQ pulp (RKL); 2: residual lignin from the LMS-E treated pine Kraft-AQ pulp (RBL); 3: lignin from the alkaline E-effluent (ASL) after the LMS-E treatment of pine Kraft-AQ pulp; 4: degradation products from the alkali soluble (AS) fraction, which are not precipitated by acidification of the E-effluents. Solvent: 0.1 N NaOH solution with 0.5 M LiCl.

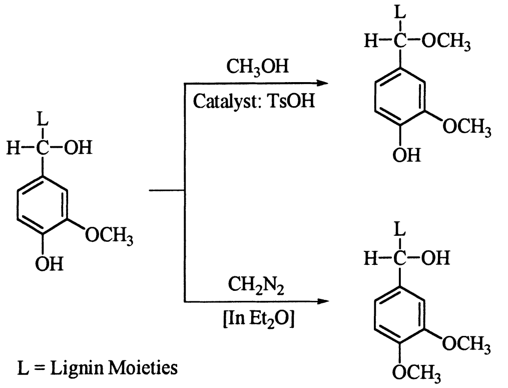
2.6. Effects of O-methylation on the biobleaching of pulp

Knowledge on the role of different functional groups of residual lignin in the delignification with dioxygen in the LMS is of primary importance to a better understanding of the reaction mechanisms. Based on the literature discussed in Section 1 and the spectroscopic studies presented above, phenolic and benzyl alcohol groups are expected to play the most important role in the degradation of residual lignin in the biobleaching of pulp. Therefore, the pulp was treated with (1) methanol using toluene-*p*-sulfonic acid (TsOH) as catalyst to selectively *O*-methylate benzyl alcohol hydroxyl groups in lignin and (2) ethereal diazomethane to *O*-methylate phenolic hydroxyl groups (Schemes 3 and 4) [31].

Results of *O*-methylation are shown in Table 3. After the *O*-methylation of benzyl alcohol hydroxyl groups with methanol, the methoxyl content of pulp did not increase, but kappa number of the pulp was reduced by approximately 30%. This implies that the

Table 3
Results of the *O*-methylation of pine Kraft-AQ pulp

Reagent	Pulp	Yield (%)	OCH_3 (%)	Kappa #
NO	N	100	1.27	21.8
CH_2N_2	D	100	3.06	16.1
CH_3OH	M	98.9	1.26	15.0



Scheme 3. *O*-Methylation of benzyl alcohol and phenolic hydroxyl groups in the residual lignin in pine Kraft-AQ pulp.

Table 4
Bleaching of the *O*-methylated pine Kraft-AQ pulps[a]

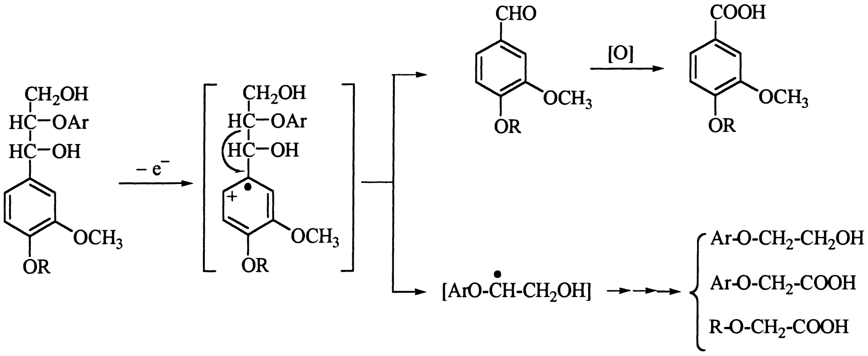
Pulp	Reaction time 4 h		Reaction time 8 h	
	Kappa #	Delignification (%)	Kappa #	Delignification (%)
N	14.8	32.0	14.0	35.8
D	11.0	32.7	10.5	34.8
M	10.6	29.3	10.3	31.3

[a] Reaction conditions: reaction temperature 40°C, pH 4.5 (acetate buffer), pulp consistency 10%, mediator charge 0.1 mmole HOBT/g pulp, laccase charge 10 U laccase/g pulp.

methoxyl group content per lignin in the pulp increases proportionally by 30%. The decrease in kappa number of the pulp after treatment with methanol could be caused by acid-catalyzed hydrolysis under the reaction conditions of *O*-methylation, particularly lignin–carbohydrate bonds [32]. In contrast, the *O*-methylation of the pulp with a great excess of ethereal diazomethane (approximately 100 times per amount of phenolic groups in residual lignin) resulted in the increase of methoxyl content by about 2.5 times and the kappa number was reduced by approximately 26% (Table 3). In contrast to the *O*-methylation of benzyl alcohol hydroxyl groups with methanol, the reduction of the kappa number value in the neutral organic solvent mixture at low temperature is rather surprising. It could be caused by reactions of diazomethane with double bonds in hexeneuronic acids, which contribute in kappa number of Kraft pulps.

Numerous model compound studies showed that aromatic methoxyl groups in etherified lignin substructures are stable under the reaction conditions [1–6,33,34]. However, α-methyl ethers may undergo acid-catalyzed hydrolysis under the acidic conditions of delignification. Therefore, the stability of α-methyl ethers was investigated using the methyl ether of veratryl alcohol as a model compound. Less than 10% of starting material were converted into veratryl aldehyde, probably by way of veratryl alcohol, with reaction time of 4 days at 40°C. Thus, non-phenolic α-methyl ethers are rather stable under the reaction conditions employed. The stability of phenolic α-methyl ethers has not been examined.

Bleaching experiments with the dioxygen in the laccase-HOBT system showed that both the pretreatment of pulp with diazomethane and TsOH in



Scheme 4. Postulated reaction mechanisms for degradation of residual lignin in the bleaching of pine Kraft-AQ pulp with dioxygen in the LMS, using HOBT as mediator.

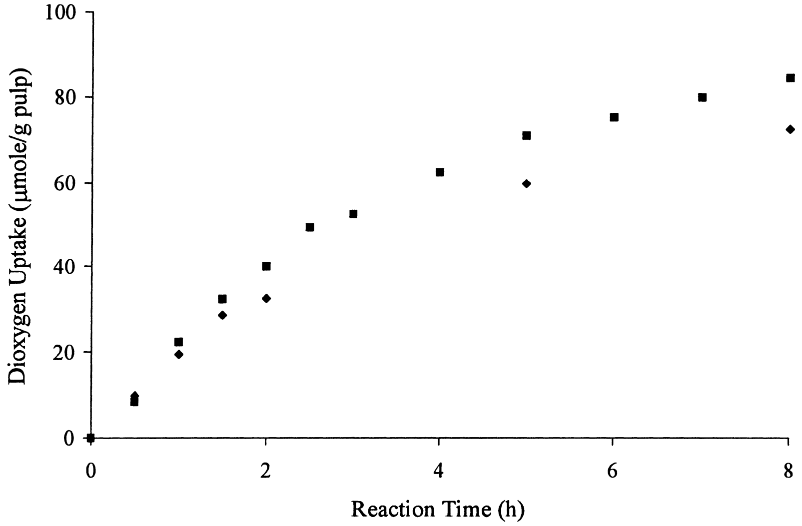


Fig. 8. Effect of *O*-methylation on the dioxygen uptake in biobleaching of pine Kraft-AQ pulp with dioxygen in laccase-mediator system (LSL). (■): non-*O*-methylated pulp; (◆): pulp *O*-methylated with ethereal diazomethane (CH_2N_2).

methanol did not affect the delignification rate of pulp significantly (Table 4). In addition, the effect of *O*-methylation of the pulp with diazomethane on the rate of dioxygen uptake during the biobleaching is rather small (Fig. 8).

The results of the investigations on the lignin structures and kinetics of delignification of the methylated pulps indicate that although degradation of the side chain plays an important role in degradation of residual lignin in the biobleaching, the reaction mechanisms should be different from that presented in Scheme 1. The cleavage of β-*O*-4′ structures (**1**) by Freudenreich et al. [2] implies primarily the oxidation of benzyl alcohol group to the corresponding α-carbonyl group. However, *O*-methylation of benzyl alcohol group of the residual lignin does not affect the rate of delignification implying that the reaction mechanism presented in Scheme 1 is not the major one. This is in agreement with the degradation of cyclic ether structures in the residual lignin (RKL) such as pinoresinol and phenylcoumarane structures showed by the HMQC spectra.

The presence of benzyl alcohol groups is not required in the mechanism suggested for cleavage of β-*O*-4′ bonds by *Phanerochaete chrysosporium* [16]. This mechanism has been proposed for degradation of softwood lignin catalyzed by lignin peroxidase in the white-rot fungi. In our earlier publication [12], we have suggested the similar mechanism for degradation of residual lignin in the biobleaching of pulp with dioxygen in the LMS. This mechanism has been verified later by Kawai et al. [35,36] in lignin model compound experiments.

The same rates for the delignification and dioxygen uptake of non- and CH_2N_2-treated pulps were unexpected in light of the discussion above. Our results are rather different from those of Potthast [34]. However, the differences could not be caused by insufficient *O*-methylation since 100 times excess of diazomethane was applied for the *O*-methylation resulting in the large increase in the methoxyl group content in the resulting pulp (2.5 times). On the other hand, our results are in agreement with the observation [37] that the reactivity of residual lignin towards dioxygen in the LMS does not depend on the amount of phenolic groups in the residual lignin in pulps. Similarly, it is also consistent with our previous kinetic results showing that the reactivity of dioxygen in the LMS towards the residual lignin does not decreased in a multistage pulp treatment [10] in spite of known fact [8,11,37] that the phenolic hydroxyl group content in the residual lignin decreases in the biobleaching of pulp. However, one should not eliminate the possible participation of phenolic moieties in the oxidative degradation of residual lignin. Conceivably, phenolic moieties participate in the degradation as well as polymerization, and the effect of these competitive processes on delignification is similar in magnitude. Nevertheless, further investigation is required to clarify the role of phenolic lignin moieties in the biobleaching of pulp with dioxygen in the LMS.

3. Conclusions

The structure of the residual lignin in pine Kraft-AQ pulp is not appreciably changed after biobleaching of pulp with dioxygen in the LMS, using 1-hydroxybenzotriazole (HOBT) as mediator. The LMS mainly induces the reactions of lignin degradation fragments with dioxygen resulting in important changes in the structure of the lignin isolated from the alkaline E-effluents. Oxidative cleavage of side chains, such as cleavage of C_α–C_β bonds, plays an important role in the delignification of pulp. Phenolic moieties undergo both oxidative degradation and dehydrogenative polymerization reactions.

4. Experimental

4.1. Materials

Pine Kraft-AQ pulp prepared by extended alkaline cooking (kappa number 21.8) was obtained from Westvaco Corporation Covington Mill, VA. Laccase from *Coriolus versicolor* was obtained from Mercian Corp., Fujizawa, Japan, as a solution in 0.1 M sodium phosphate buffer at pH 6. One unit of laccase was defined as the amount of enzyme producing 1 μmole of 4-hydroxy-benzaldehyde per minute in 50 mM sodium acetate buffer (pH 4.5) at 30°C with 4-hydroxymandelic acid as a substrate.

4.2. O-Methylation of Kraft pulp

Air-dried pine Kraft-AQ pulp was dried over P_2O_5 under vacuum to constant weight. *O*-Methylation of pulp was conducted according to known methods [31] modified for *O*-methylation of spruce sawdust [33].

4.2.1. O-Methylation with methanol

The dried pulp (10 g) was homogenized in 100 ml of MeOH-dioxan solution (2:1, v/v). To 80 ml of the above mixture in a flask was added 5 g of TsOH. The resulting mixture was kept at room temperature for 4 days. The resulting pulp was then filtered, washed with MeOH and H_2O to neutral pH.

4.2.2. O-Methylation with diazomethane

The dried pulp (10 g) was homogenized in 180 ml of MeOH-dioxan solution (5:1, v/v). To the resulting mixture was added ethereal CH_2N_2 (170 ml, obtained from 16.5 g of diazard) in several portions over 1 h. The reaction mixture was then kept for 1 week at 0°C. Acetic acid was then added to the mixture to destroy excess CH_2N_2. The resulting pulp was filtered, washed with MeOH and H_2O to neutral pH.

4.3. Delignification of pulps

4.3.1. Delignification of non-methylated and O-methylated pulps under atmospheric pressure

Moist pulp (6.45 g; 2 g o.d.), 27 mg of HOBT and 20 U of laccase in 13.5 ml of acetate buffer (pH 4.5) were placed in a 50 ml Erlenmeyer flask. The mixture was thoroughly mixed. The flask was connected to a modified Warburg's barometer [38], and placed on a water bath (bath temperature 40°C). The flask was flushed with dioxygen for 2 min, and dioxygen uptake was measured during the reaction. After the reaction, the pulp was washed with warm H_2O, and then extracted with a NaOH solution (2% on pulp) at 60°C for 1 h.

4.3.2. Delignification under oxygen pressure

A mixture of pulp (60 g, 15 g o.d.) in 90 ml of acetate buffer acetate buffer solution (pH 4.5) containing HOBT (0.2025 g) and laccase (150 U) was placed in a 300 ml reactor. The reactor was then closed tightly and dioxygen (3 bar) was introduced into the reactor. The biobleaching was carried out at 40°C for 4 h. After the washing and the alkaline extraction, the pulp was used for isolation of the residual lignin. The alkaline solution (E-effluents) was used to isolate the corresponding soluble lignin preparation.

4.4. Isolation of lignins

Residual lignins were isolated from the original Kraft-AQ pulp (RKL) and from the biobleached pulp (RBL) by cellulase treatment followed by purification with a NaOH solution [39]. Lignin in the alkaline E-effluent (ASL) was isolated by acidifying the alkaline solution with a HCl solution. The resulting precipitate was centrifuged, washed and subjected to dialysis (1000 cutoff) to obtain alkaline soluble oxidation products from the original residual lignin.

4.5. ^{1}H–^{13}C *correlation 2D NMR spectra*

The NMR spectra were recorded in a Bruker AVANCE 500 MHz spectrometer (1996 model) with the Oxford narrow bore magnet (1989 model) after dissolving approximately 60 mg of each lignin preparation in 0.75 ml DMSO-d_6 containing 0.01% of TMS as internal standard. The system was controlled by the SGI INDY host workstation and the data was processed using the XWINNMR software. The instrument was equipped with three frequency channels with waveform memory and amplitude shaping, three channel gradient control units (GRASP III) and one variable temperature unit, as well as one unit for pre-cooling and temperature stabilization. All measurements have been carried out with a 5 mm ID ^{1}H/BB (^{109}Ag–^{31}P) triple-axis gradient probe (ID500-5EB, Nalorac Cryogenic Corp.). The operational frequency for ^{1}H nucleus was 500.128 MHz and conditions for analysis included a 90° pulse width of 10 μs and a 1.5 s pulse delay (d_1).

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