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Biobleaching of pulp with dioxygen in laccase-mediator system—effect of variables on the reaction kinetics

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Abstract

Comparative studies were carried out on the kinetics and mechanism of pulp biobleaching with laccase-mediator system (LMS) with two different mediators, 1-hydroxybenzotriazole (HOBT) and *N*-hydroxyacetanilide (NHAA). The optimal NHAA and laccase charge was found to be 0.1 mmol and 10 U per gram of pulp with pulp consistency of 10%, at the reaction temperature of 40 °C for 8 h under atmospheric pressure, respectively. The kinetic studies on Kappa number reduction and dioxygen uptake suggest that a very fast rate of delignification with NHAA at the beginning of the process is the result of fast formation of the oxidized mediator species. However, a very slow delignification rate after the initial phase (0.5–1 h) could be caused by low stability of the mediator species. After the reaction time of 2 h, the degree of delignification is higher when HOBT is used as mediator. In contrast to the delignification with NHAA, the formation of the oxidized mediator species is the rate-determining step of the pulp biobleaching with dioxygen in the LMS using HOBT as mediator. Increase in temperature increases the rate of chemical reactions, but decreases the laccase stability. The optimal temperature for pulp biobleaching with HOBT and laccase from *Coriolus versicolor* is 40 °C. Increasing oxygen pressure improves the efficiency of delignification due to better penetration of the reagents, but does not affect the rate of chemical reactions. The reaction mechanism is discussed based on the kinetic data.

Keywords: *Coriolus* laccase; Laccase-mediator system (LMS); Biobleaching of pulp; Totally chlorine free bleaching; Kinetics; Delignification; Oxidation; Dioxygen uptake

1. Introduction

Recently, the mechanisms of pulp delignification with the laccase-mediator system (LMS) have been intensively investigated. Currently, the most accepted reaction mechanism can be presented in a simplified manner as a consequence of redox cycles (Scheme 1) [1]. Accordingly, dioxygen oxidizes the reduced form of laccase to the native laccase species ($Laccase_{ox}$), which in turn oxidizes a mediator to produce oxidized mediator species ($Mediator_{ox}$) and the original laccase. Lignin moieties in the residual lignin in pulp then undergo oxidation by the $Mediator_{ox}$ species, resulting in degradation of the lignin ($Lignin_{ox}$) and reduction of mediator. This completes the redox cycle. However, studies on the kinetics of veratryl alcohol oxidation with LMS have showed a rather complex role of mediator in the reaction [2,3]. These

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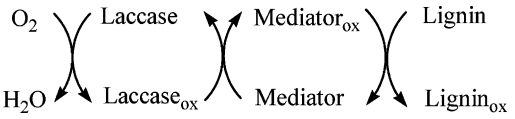

Scheme 1. Redox cycles of laccase-catalyzed oxidation of lignin moieties in the presence of mediator.

results also suggest that at certain reaction conditions, oxidation of the non-phenolic substrate directly by the enzyme activated by the mediator could occur. In addition to reactions involving mediator, oxidation reactions of phenolic lignin moieties catalyzed directly by laccase without participation of mediator also take place [4]. These reactions can result in both degradation and dehydrogenetive polymerization of lignin. Moreover, hydroxyl radicals have been showed to play an important role in wood degradation with white rote fungi [5]. The role of so-called "activated oxygen" in the oxidation of lignin with dioxygen in LMS is also discussed [6]. In addition, most of mediators suggested are decomposed during the reaction causing deactivation of laccase [7–10]. Thus, the laccase-mediator oxidation of residual lignin is a complex network of reactions.

Although an appreciable progress in understanding of the reaction mechanism has been made in recent years, important questions such as the rate-determining step(s) in lignin biodegradation, and the effect of different mediators on the reaction mechanism are not elucidated completely. Among different experimental approaches used to explore the reaction mechanism, the contribution of kinetic studies is rather few. Only a few comprehensive investigations on the effects of the reaction variables on the pulp delignification with 2,2'azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) [7] and 1-hydroxybenzotriazole (HOBT) [11–13] were conducted to optimize the biobleaching process but they did not provide sufficient information on the reaction mechanism. In addition, very little is known on the reaction kinetics of the delignification of pulp with *N*-hydroxyacetanilide (NHAA), which was suggested as a promising mediator for the LMS [9]. In the present work, comparative studies on the kinetics of pulp biobleaching with LMS using HOBT and NHAA as mediators were carried out to elucidate the effect of the process variables on the reaction kinetics and mechanism.

2. Results and discussion

2.1. Comparative studies on the kinetics of pulp delignification with NHAA and HOBT

Recently, NHAA has been suggested as a promising mediator for the LMS [9,13]. However, kinetic data on the delignification of pulp with this mediator are very limited and the optimum conditions for the biodelignification have not well established. Therefore, the effects of the NHAA and laccase charges on the reaction kinetics were elucidated.

Increase in the NHAA charge up to 0.1 mmol/g pulp increases the degree of delignification for the pine-Kraft pulp in the LMS almost linearly (Fig. 1). Nevertheless, further increase in the mediator charge does not affect the degree of delignification. In contrast, the rate of dioxygen uptake noticeably increases with increasing mediator charge up to 0.2 mmol/g pulp (Fig. 2). There is a linear correlation between the NHAA concentration and oxygen uptake up to the reaction time of 8 h (Fig. 3). This indicates that the rate of dioxygen uptake is directly proportional to the mediator concentration under the reaction conditions investigated.

Studies on the effect of laccase charge on the degree of delignification show that the higher the NHAA and laccase charges, the lower the effect (Table 1). When the laccase charge increases from 5 to 10 units/g pulp, the degree of delignification appreciably increases at the mediator charge of 0.1 mmol/g pulp. However, the effect is very small at the NHAA charge of 0.2 mmol/g.

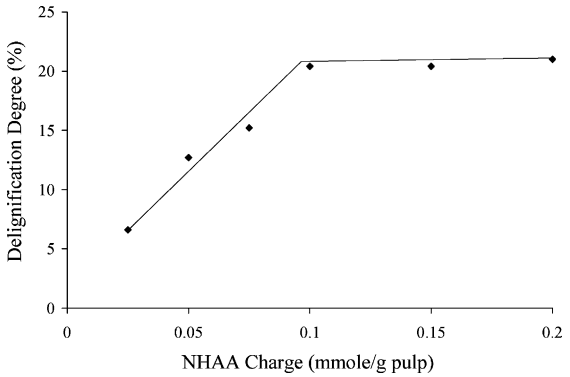

Fig. 1. Effect of NHAA charge on the degree of delignification. Reaction time of 8 h, laccase charge of 10 U/g pulp, reaction temperature 40 °C, atmospheric pressure.

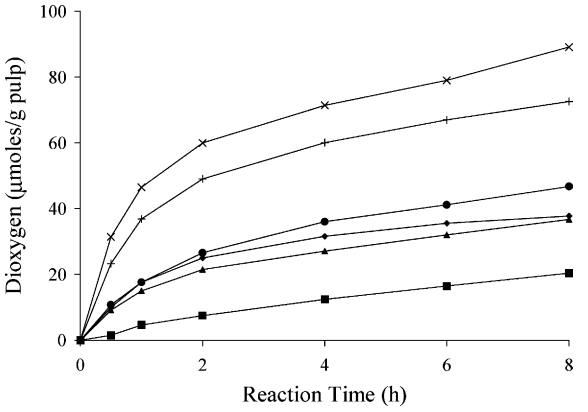

Fig. 2. Effect of NHAA charge on the rate of dioxygen uptake. NHAA charge (mmol/g pulp): (■) 0.25; (▲) 0.05; (◆) 0.075; (●) 0.1; (+), 0.15; (×), 0.2. Laccase charge 10 U/g pulp, reaction temperature 40 °C, atmospheric pressure.

Table 1
Effect of NHAA and laccase charge on the pulp delignification[a]

NHAA charge (mmol/g pulp)	Laccase charge (U/g pulp)	Kappa number[b]
0.05	5	15.2
0.05	10	15.8
0.05	20	15.4
0.10	5	15.5
0.10	10	14.4
0.10	20	14.4
0.20	5	14.6
0.20	10	14.3
0.20	20	14.3

[a] Conditions: pine-kraft-AQ pulp (Kappa number 21.8); 10% consistency; 40 °C, atmospheric pressure, 8 h; pH 4.5.

[b] After E-stage (E-stage: 10% consistency; 20 kg NaOH/t pulp, 60 °C, 1 h).

Further increase in the laccase charge from 10 to 20 U/g pulp slightly increases the degree of delignification at the mediator charge of 0.05 mmol/g pulp but does not show any effect at the higher NHAA charges. Thus, for the delignification of pine-Kraft pulp in the LMS, the optimum NHAA and laccase charges are of 0.1 mmol/g pulp and 10 U/g pulp, respectively. Moreover, in contrast to the effect of NHAA concentration on the rate of dioxygen uptake, the laccase concentration does not affect the rate of dioxygen uptake within accuracy of the experiment (Fig. 4). This implies that laccase does not participate in the rate-determining step of the reaction under the conditions employed.

Comparative studies on the kinetics of delignification of the pine-Kraft pulp (Fig. 5) were conducted at the optimum NHAA charge of 0.1 mmol/g pulp, which also corresponds to the optimum HOBT charge [14]. In the acetate buffer (pH 4.5), the rate of delignification, i.e. Kappa number reduction, is very fast in the initial phase of the process (30–60 min) using NHAA as mediator and faster than that when HOBT was used as mediator. After the initial phase, however, the rate of delignification with the laccase-NHAA

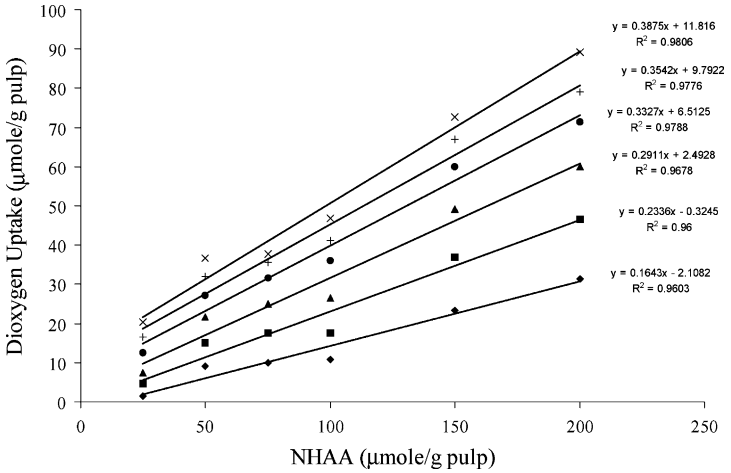

Fig. 3. Dioxygen uptake vs. NHAA charge. Reaction time (h): (◆) 0.5; (■) 1; (▲) 2; (●) 4; (+), 6; (×), 8. Laccase charge 10 U/g pulp, reaction temperature 40 °C, atmospheric pressure.

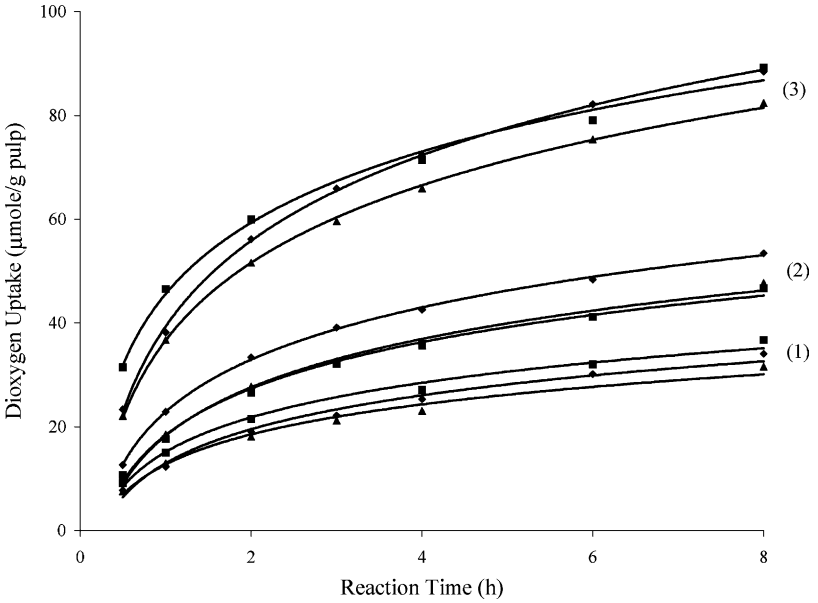

Fig. 4. Effect of laccase concentration on the rate of dioxygen uptake in the laccase-NHAA system. Laccase charge (U/g pulp): (▲) 20; (■-) 10; (◆) 5. NHAA charge (mmol/g pulp): (1) 0.05, (2) 0.10, (3) 0.20.

system sharply slows down and the degree of delignification in the process using HOBT is then higher after the reaction time of 2 h. The rate of dioxygen uptake is the same in the first 30 min of reaction using HOBT and NHAA as mediator (Fig. 6). The rate of dioxygen uptake in the biobleaching with HOBT then becomes much faster than that with NHAA.

The difference in the kinetics of the biobleaching with NHAA and HOBT shows different chemical behavior of the mediators in the reaction system. The rate of dioxygen uptake in a blank experiment with HOBT, i.e. the reaction in the absence of pulp, levels off after the reaction time of 8 h with consumption of approximately 0.25 mol of dioxygen per mol of

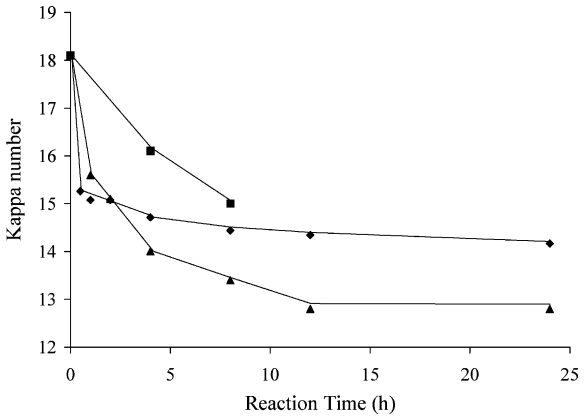

Fig. 5. Effect of mediator on the kinetics of delignification. (▲) HOBT in buffer; (◆) NHAA in buffer, (■-) NHAA in distilled water. Laccase charge 10 U/g pulp, mediator charge 0.1 mmol/g pulp, reaction temperature 40 °C, atmospheric pressure.

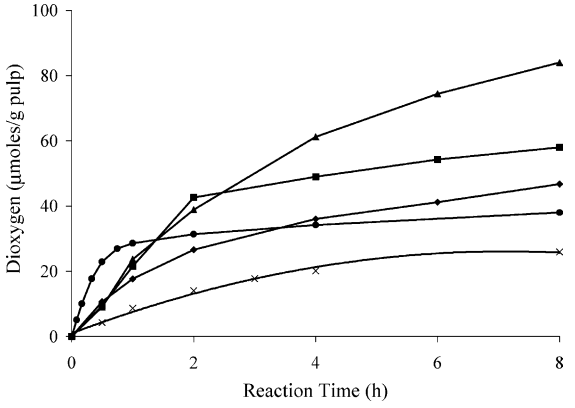

Fig. 6. Effect of mediator on the kinetics of dioxygen uptake. (▲) HOBT in buffer; (◆) NHAA in buffer; (■-) NHAA in distilled water; (×) HOBT in buffer (blank test); (●) NHAA in buffer (blank test). Laccase charge 10 U/g pulp, mediator charge 0.1 mmol/g pulp, reaction temperature 40 °C, atmospheric. pressure.

HOBT (Fig. 6). Accordingly, this amount of dioxygen is required to oxidize HOBT to the corresponding radical species by a single-electron-transferring oxidation [15]. The low rate of dioxygen consumption suggests the reaction of laccase with HOBT (Scheme 1) is the rate-determining step or one of the rate-determining steps in the oxidation of lignin in the LMS. In contrast, the rate of dioxygen uptake in the blank experiment with NHAA is rather fast (Fig. 6). The oxygen uptake of 0.25 mol per mol of NHAA is reached in less than 45 min of the reaction. It is then slowed down. The dioxygen uptake is 0.38 mol per mol of NHAA at the reaction time of 8 h. The fast dioxygen uptake in the initial phase is in good agreement with the easy formation of the corresponding radical species from NHAA reported by Freudenreich et al. [16] as well as with the redox potential of 1.04 and 0.83 V for laccase-HOBT and laccase-NHAA systems in the first single-electron-transferring oxidation stage [17]. The fast formation of the NHAA radicals leads in turn to the fast rate of delignification in the initial phase. However, the resulting NHAA radicals undergo decomposition by a second single-electron-transferring oxidation [16] resulting in a low concentration of the active $Mediator_{ox}$ species after the initial phase. The laccase-NHAA system has a second redox potential of 1.01 V [17]. Because of the decomposition of NHAA, the rate of delignification slows down sharply after the reaction time of 0.5–1 h.

The rate of dioxygen uptake in the biobleaching of pulp with the laccase-HOBT system is appreciably higher than the dioxygen uptake without pulp, even at the initial phase (Fig. 6). This is consistent with the observed synergistic effect of laccase and HOBT on the oxygen uptake in experiments with low pulp consistency and short reaction time [18]. The difference in the oxygen uptake (Fig. 6) could not result only from recycling of the mediator by the reaction with lignin because in the initial phase, the concentration of HOBT both in the blank experiment and in the biobleaching of pulp with the LMS is rather close. The higher reaction rate could be caused by reactions of laccase directly with lignin moieties. The synergism could be a result of the formation of active intermediates in the oxidation of lignin with the LMS. This would increase the concentration of lignin moieties, which can react directly with laccase. Another reason for the lower dioxygen uptake in the blank experiment could be rather intensive deactivation of laccase by mediator species in the absence of pulp. In contrast, the rate of dioxygen uptake in the blank experiment in the laccase-NHAA system is appreciably higher than that in the presence of pulp, up to the reaction time of 1 h (Fig. 6). The similar phenomenon was observed in the biobleaching of pulp in the LMS with ABTS as mediator [14,18].

Pfaller et al. [19] has reported that one of the advantages of NHAA as mediator is the possibility to conduct the biobleaching in neutral media. Nevertheless, our result shows that the rate of Kappa number reduction in the initial phase of delignification in distilled water is considerably lower than that in the acetate buffer with the NHAA charge of 0.1 mmol/g pulp (Fig. 5). However, the rate of delignification does not slow down as quickly as that in the buffer solution, and after the reaction time of 8 h the degree of delignification is quite close to that in the buffer solution. In contrast to the kinetics of Kappa number reduction, the rate of dioxygen consumption is the same in the first 30 min of reaction for using NHAA as mediator in buffer solution and in water (Fig. 6). The rate of dioxygen uptake then is much faster when the delignification in the laccase-NHAA system is carried out in the neutral solution than that in the buffer solution. Conceivably, the higher pH of the reaction mixture could increase the rate of side reactions involving dioxygen because of easier dissociation of phenolic hydroxyl groups to give the corresponding phenoxide anions and also results in higher frequency of dehydrogenative polymerization processes.

The results from the studies on the use of NHAA as a mediator did not show appreciable advantages for using this mediator as compared to HOBT. In addition, Potthast et al. [20] have reported that in the delignification process, NHAA is decomposed to produce toxic compounds such as aniline and nitrobenzene. It is evident, therefore, that there are more disadvantages over advantages in using NHAA as the mediator in the biobleaching of pulp with the LMS. For this reason, further experiments were conducted only with HOBT.

2.2. Effect of oxygen pressure and the reaction temperature on the kinetics of delignification

The kinetic data on the delignification of the pine-Kraft pulp with the LMS using HOBT as mediator

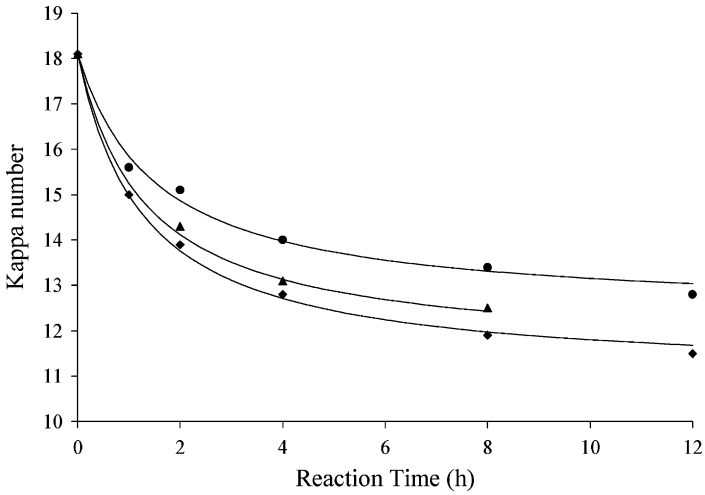

Fig. 7. Effect of dioxygen pressure on the rate of delignification (●) atmospheric pressure; (▲) 3 bar; (◆) 10 bar. Points present experimental results, lines were calculated using kinetic parameters of Table 2. Mediator: HOBT, 0.1 mmol/g pulp.

indicate that only a part of residual lignin can be removed in one stage process (Fig. 7). As showed before [14,21], the most important reasons for the delignification limit are likely accumulation of oxidatively degraded lignin fragments with high reactivity and problems with diffusion of chemical reagents into fibers. The amount of the "available lignin" (L_a) was calculated as follows:

$$L_a = (\text{Kappa}_0 - \text{Kappa}_\infty) \quad (1)$$

where Kappa_0 and Kappa_∞ are Kappa number of the original pulp and that of pulp at a delignification limit, respectively.

The kinetics of the pulp biobleaching with the LMS also shows that increase in the dioxygen pressure from 0 to 3 bar increases appreciably the rate of Kappa number reduction (Fig. 7). However, further increase in the oxygen pressure to 10 bar is not very effective. The kinetics of delignification follows experimentally a pseudo-second order law with respect to "available lignin" (L_a):

$$v_D = \frac{d(\text{Kappa})}{dt} = \kappa_D (L_a)^2 \quad (2)$$

where κ_D is the rate constant of delignification.

The kinetic parameters determined (Table 2) show that an increase in the dioxygen pressure at 40 °C does not affect the delignification rate constant appreciably, but increases the final degree of delignification. Since the rate constant for delignification is a near constant with respect to the increase in dioxygen pressure, the increase in the delignification rate is caused by the increase in L_a according to Eq. (2). The reaction rate is proportional to the maximum amount of lignin removable from the pulp in the second degree. When the dioxygen pressure increases from 0 to 3 bar at 40 °C, the rate of delignification increases by 35%. Further increase in the dioxygen pressure from 3 to 10 bar increases the rate of delignification by only 11%.

The very close magnitude of the second order rate constants for the delignification indicates that the dioxygen pressure does not affect the rate of chemical reaction in the same amount of the residual lignin in pulp. This is consistent with the role of dioxygen in laccase-catalyzed oxidation postulated by Solomon et al. [22]. Accordingly, dioxygen is required to convert the reduced form of laccase to the oxidized one, and the rate of this reaction does not limit the overall rate of the oxidation. The dioxygen concentration did not affect the rate of the laccase-ABTS-catalyzed oxidation of veratryl alcohol in the LMS either [3], indicating that dioxygen does not participate in the rate-determining step in the laccase-mediator system. Thus, the kinetics of delignification shows that the increase in the delignification rate with increasing dioxygen pressure is due to physical factors rather

Table 2
Effect of the dioxygen pressure and reaction temperature on the kinetics of pulp in the laccase-HOBT system. HOBT charge, 0.1 mmol/g pulp

Temperature (°C)	Pressure (bar)	Delignification					Oxygen uptake $\kappa_O \times 10^5$ (s^{-1})
		$Kappa_\infty$	$\kappa_D \times 10^{5a}$	Reaction order	$v \times 10^4$ (Kappa/s)	Maximal degree of delignification	
30	0	13.2	5.37	1	2.66	27.1	5.78
40	0	12.4	3.19	2	10.4	31.5%	7.54
60	0	14.5	12.5	1	4.86	19.9	6.33
40	3	11.5	3.20	2	14.0	36.5%	–
40	10	11.0	3.08	2	15.5	39.2%	–

[a] s^{-1} for the reactions of pseudo-first order and $s^{-1}Kappa^{-1}$ for the reactions of pseudo-second order.

than chemical factors. Conceivably, increase in the amount of the available lignin with increasing dioxygen pressure could result from a better diffusion of reactive chemical species into pulp fibers.

The rate of Kappa number reduction increases when the reaction temperature increases from 30 to 40 °C (Fig. 8). This effect is particularly appreciable at the beginning of the delignification. With increase in the reaction time, this difference in the reaction rates is getting smaller, and the final values of Kappa number at the reaction time of 24 h are rather close. By contrast, a further increase in the temperature to 60 °C resulted in a decrease in the reaction rate. Moreover, after the reaction time of about 4 h at 60 °C, the delignification process actually leveled off. A qualitative test on the laccase activity with ABTS did not show any enzyme activity after 4 h of the reaction at 60 °C, since the reaction mixture did not change color to blue after addition of ABTS.

The effect of the reaction temperature on the rate of delignification could be caused by the effect of temperature on the laccase stability. The laccase activity does not decrease noticeably with the reaction time when the enzyme was incubated at 30 °C without mediator and pulp (Fig. 9). At 40 °C, approximately a half of the original laccase activity has been detected at the incubation time of 48 h. However, the laccase loses almost all activity after the incubation time of 2 h at 60 °C.

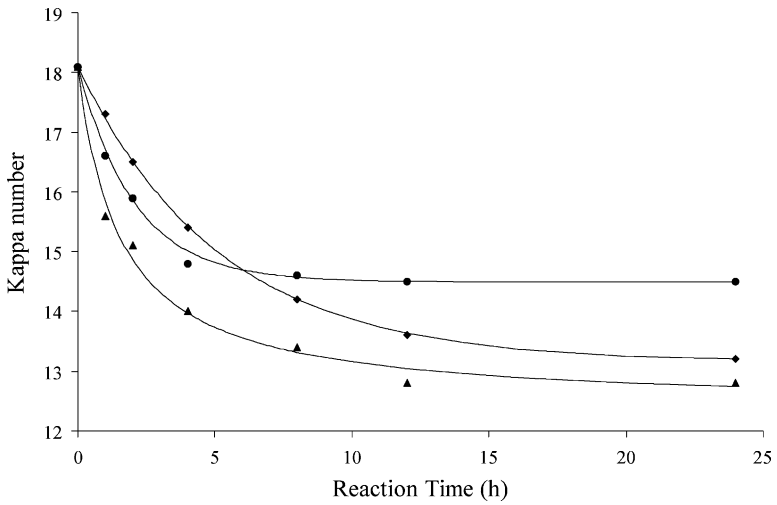

Fig. 8. Effect of temperature on the rate of delignification. (●) 60 °C; (▲) 40 °C; (◆) 30 °C. Points present experimental results, lines were calculated using kinetic parameters of Table 2. Mediator: HOBT, 0.1 mmol/g pulp.

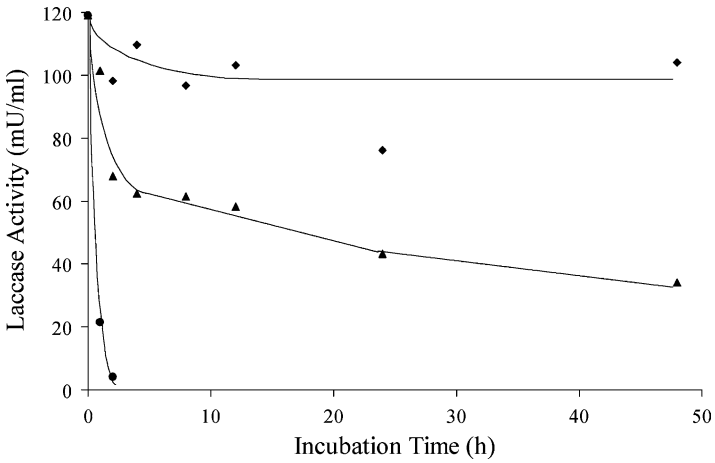

Fig. 9. Effect of temperature on the laccase stability. (●) 60 °C; (▲) 40 °C; (◆) 30 °C. Mediator: HOBT, 0.1 mmol/g pulp.

The reaction temperature affects not only the rate of delignification, but the reaction order as well. At the reaction temperature of 40 °C, the kinetics of Kappa number reduction follows a pseudo-second order reaction law. However, the reaction kinetics follows a pseudo-first order reaction law at 30 and 60 °C (Table 2, Fig. 8). In general, the rate of delignification depends on the concentration of the substrate (residual lignin) and the activity of the catalytic system, i.e. the laccase and HOBT activity:

$$v_D = f(L_a, [\text{Lac}][\text{HOBT}])$$

At 40 °C, the concentrations of all the components are changed with the reaction time. At 30 °C, the enzyme is rather stable to thermal decompsition (Fig. 9). It is possible that at that temperature the rate of laccase deactivation could be relatively low even in the presence of HOBT although no data are available so far, and the rate of delignification mainly depends on the residual lignin concentration. In contrast, the laccase activity at 60 °C decreases much faster than the lignin concentration and the rate of delignification is controlled mostly by the enzyme activity. Consequently, the lowest Kappa number (Kappa_∞) at 60 °C does not represent the amount of the reactive lignin, because the enzyme loses its activity before all the available lignin is degraded in the reaction.

Because of rather complicated reaction kinetics, such as different reaction order and different Kappa_∞, the effect of the reaction temperature on the reaction rate was estimated using the initial rate of delignification. It was calculated according to the kinetic equations for the pseudo-first and second order reaction laws at 30 and 60 °C and at 40 °C, respectively:

$$v = \kappa_D(L_a),$$

and

$$v = \kappa_D(L_a)^2$$

These calculations show (Table 2) that the increase in the reaction temperature from 30 to 40 °C increases the rate of delignification approximately 4 times. Further increase in the reaction temperature to 60 °C results in decreasing the initial reaction rate more than twice.

Call and Mücke [12] have reported that the optimal reaction temperature is 45 °C for the LMS-delignification with laccase from *Coriolus versicolor*. In contrast, the results from experiments with *Trametes versicolor* laccase [7] showed increase in the degree of delignification up to the reaction temperature of 80 °C. This suggests that the optimal temperature could depend on the origin of the enzyme.

The temperature coefficient i.e., the increase in the reaction rate per increase in the reaction temperature by 10 °C, at the reaction temperatures of 30–40 °C is rather high, which is caused by the increase in the rate of chemical reactions, but not an increase in the efficiency of diffusion. This indicates that in spite of an important role of the reagent diffusion as discussed above, the rate of delignification appreciably depends

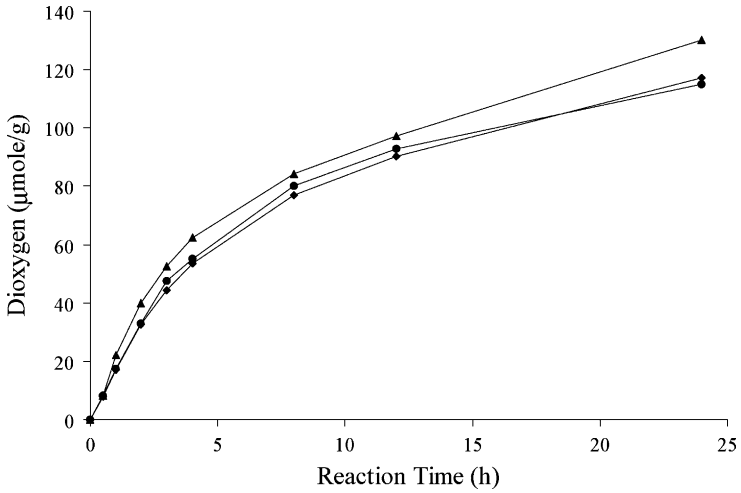

Fig. 10. Effect of temperature on the rate of dioxygen uptake. (●) 60 °C; (▲) 40 °C; (◆) 30 °C. Mediator: HOBT, 0.1 mmol/g pulp.

on the rate of chemical reactions. Conceivably, the lignin oxidation on the surface of fibers occurs in the kinetic region.

2.3. Effect of reaction temperature on the kinetics of dioxygen uptake

The effect of the reaction temperature on the rate of dioxygen uptake (Fig. 10) has similar character as the effect of the temperature on the rate of delignification, but the effect is much smaller. It is noteworthy to mention that at 60 °C the rate of dioxygen uptake is appreciably high even after the reaction time of 4 h, that is when the laccase lost its activity.

The kinetics of dioxygen uptake follows a pseudo-first order reaction law up to the reaction time of 8–12 h. Mathematical evaluation of the kinetics data showed that the maximum dioxygen uptake in the first phase is about 95 mmol/g pulp at all reaction temperatures. The estimation by the first order reaction rate

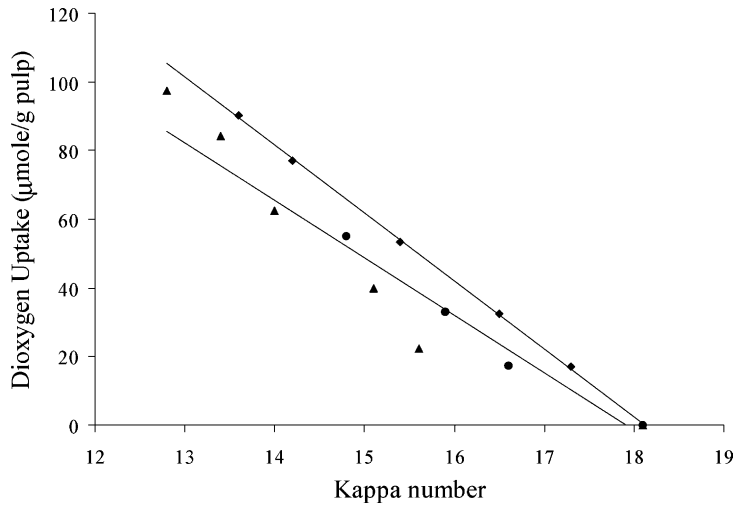

Fig. 11. Dioxygen uptake vs. Kappa number. (●) 60 °C; (▲) 40 °C; (◆) 30 °C. Mediator: HOBT, 0.1 mmol/g pulp.

constants showed that the increase in the reaction temperature from 30 to 40 °C resulted in increase in the rate of dioxygen uptake by 1.3 times (Table 2). In contrast, the further increase in the reaction temperature to 60 °C resulted in the decrease in the rate of dioxygen uptake by approximately 20%.

The plot of dioxygen uptake versus Kappa number reduction (Fig. 11) shows linear correlation at 30 and 60 °C. However, this relationship is not linear at 40 °C because of different reaction order for the kinetics of delignification and the kinetics of dioxygen uptake. The slope of the lines shows that removal of each C_9-unit of the residual lignin requires dioxygen uptake of about 2.4 and 2.1 mol at 30 and 60 °C, respectively. Again, as in the pulp biobleaching with NHAA, the delignification kinetics and the kinetics of dioxygen uptake do not correlate directly. This indicates that the part of dioxygen involved in the reactions responsible for the delignification is relatively low. Most of dioxygen is probably consumed in side reactions. This postulation is consistent with a rather high dioxygen uptake per one C_9-unit removed (Fig. 11). It seems that the reaction temperature does not appreciably affect the rate of these reactions. Experiments at 60 °C indicate that these reactions can occur even when the enzyme has already lost its activity, i.e. after the reaction time of 4 h.

3. Experimental

3.1. Materials

A pine-Kraft-AQ pulp with extended alkaline cooking (Kappa number 21.8) were obtained from Westvaco Corporation Covington Mill, VA. The pulp was washed exhaustively with deionized water and finally with distilled water. 1-Hydroxybenzotriazole hydrate (HOBT) was purchased from Aldrich Co., Milwaukee, WI. *N*-hydroxyacetanilide (NHAA) was synthesized according to the published procedure [23]. Laccase from *C. versicolor* was obtained from Mercian Corp., Fujisawa, 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 mmol of 4-hydroxybenzaldehyde per min in 50 mM sodium acetate buffer (pH 4.5) at 30 °C with 4-hydroxymandelic acid as substrate. The laccase preparation had a laccase activity of 302 U/ml.

3.2. Delignification of pulps

3.2.1. Experiments under atmospheric pressure

Moist pulp (6.45 g; 2 g o.d.), an appropriate amount of a mediator and 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 [3], and placed on a water bath that was kept at an appropriate bath temperature. The flask was flushed with dioxygen for 2 min, and dioxygen uptake was measured during the reaction.

3.2.2. Experiments under dioxygen pressure

The pulp mixed with the solution of 30.6 mg HOBT and 20 U of laccase in 13.5 ml of acetate buffer was placed in a 300 ml reactor. The reactor was then closed tightly and an appropriate amount of dioxygen was introduced into the reactor. The delignification was carried out at 40 °C. After the reaction, the pulp was washed with warm H_2O, then extracted with a NaOH solution (2% per pulp) at 60 °C for 1 h. The Kappa number was determined after the alkaline extraction according to Tappi method.

All experiments were run at least twice. The relative error in the parallel experiments was lower than 1% in the magnitude of Kappa number and about 5% in the dioxygen uptake measurements.

4. Conclusions

The kinetic results do not contradict to the general reaction mechanism of pulp delignification with the LMS presented in Scheme 1 as a consequence of redox cycles. Accordingly, the chemical reaction of dioxygen with laccase is fast and does not limit the rate of the overall process. The rate of pulp delignification is determined by the oxidation of a mediator with laccase or/and by the reaction of the oxidized mediator with the residual lignin in pulp. The kinetic results suggest that formation of the oxidative form of HOBT is the rate-determining step in the pulp delignification. In contrast, the oxidation of NHAA with laccase is very fast resulting in the fast rate of Kappa

number reduction in the initial period. One of the limiting factors in the pulp delignification with NHAA as mediator could be low stability of the activated species derived from the mediator resulting in termination of the delignification after the short initial period. The reaction temperature increases the rate of chemical reactions but decreases the laccase stability. The optimal reaction temperature has been found to be about 40 °C.

The rate of dioxygen uptake does not show direct correlation with the rate of the delignification. This indicates that most of dioxygen is involved in side reactions which do not affect the delignification. The mechanism of these reactions is more complex than the mechanism of the oxidation of the residual lignin in pulp and could include a wide variety of the reaction pathways.

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References

[1] M.G. Paice, R. Bourbonnais, I.Reid, F.S. Archibald, in: Proceedings of 9th International Symposium on Wood and Pulping Chemistry, Montreal, Canada, Vol. 1, 1997, p. PL-1–PL-4.
[2] M.Yu. Balakshin, C.-L. Chen, J.S. Gratzl, A.G. Kirkman, H. Jakob, Holzforschung 54 (2000) 165–170.
[3] M.Yu. Balakshin, C.-L. Chen, J.S. Gratzl, A.G. Kirkman, H. Jakob, Holzforschung 54 (2000) 171–175.
[4] T. Higuchi, Wood Sci. Technol. 24 (1990) 23.
[5] S. Baska, J. Gierer, T. Reitberger, T. Nilsson, Holzforschung 47 (1993) 181–187.
[6] K. Li, R.F. Helm, K.L. Eriksson, Biotechnol. Appl. Biochem. 27 (1998) 239–243.
[7] R. Bourbonnais, M.G. Paice, Tappi 76 (6) (1996) 199–204.
[8] C.-L. Chen, J.S. Gratzl, A.G. Kirkman, T. Miyakoshi, in: Proceedings of 5th Brazilian Symposium on the Chemistry of Lignins and Other Wood Components, Curitiba, PR, Brazil, 1997, pp. 547–556.
[9] M. Amann, in: Proceedings of 9th International Symposium on Wood and Pulping Chemistry, Montreal, Canada, Vol. 1, 1997, pp. F4-1–F4-5.
[10] R. Bourbonnais, M.G. Paice, B. Freiermuth, E. Bodie, S. Borneman, Appl. Environ. Microbiol. 63 (12) (1997) 4627–4632.
[11] H.P. Call, I. Mücke, in: Proceedings of Non-chlorine Bleaching Conference, Amelia Isl., FL, 1995.
[12] H.P. Call, I. Mücke, J. Biotechnol. 53 (1997) 163–202.
[13] K. Poppius-Levlin, T. Tamminen, K. Rajanen, A.-S. Jaaskelainen, L. Viikari, in: Proceedings of 10th International Symposium on Wood and Pulping Chemistry, Jokohama, Japan, Vol. 1, 1999, pp. 556–560.
[14] M.Yu. Balakshin, C.-L. Chen, J.S. Gratzl, A.G. Kirkman, H. Jakob, Holzforschung 54 (2000) 390–394.
[15] A. Potthast, H. Koch, K. Fisher, in: Proceedings of 9th International Symposium on Wood and Pulping Chemistry, Montreal, Canada, Vol. 1, 1997, pp. F2-1–F2-4.
[16] J. Freudenreich, M. Amann, E. Fritz-Langhals, J. Stohrer, in: Proceedings of IPBC, Helsinki, Finland. 1998, p. 71–76.
[17] R. Bourbonnais, D. Rochefort, M.G. Paice, D. Leech, Development of stable redox complexes to mediate delignification of Kraft pulp by lacccase, in: D.S. Argyropoulos (Ed.), Oxidative Delignification Chemistry, ACS Symposium Series 785, Washington, DC, 2001, pp. 391–399.
[18] M.-L. Niku-Paavola, W. Wang, K. Poppius-Levlin, H. Anke, L. Viikari, in: Proceedings of 9th International Symposium on Wood and Pulping Chemistry, Montreal, Canada, Vol. 2, 1997, pp. 80-1–80-3.
[19] R. Pfaller, M. Amann, J. Freudenreich, in: Proceedings of 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, BC, Canada, Vol. A, 1998, p. A99–102.
[20] A. Potthast, H. Jakob, A. Kuver, A. Treimanis, H. Koch, K. Fisher, in: Poster Presentation, ACS Meeting, San Francisco, 1997.
[21] M.Yu. Balakshin, C.-L. Chen, J.S. Gratzl, A.G. Kirkman, H. Jakob, in: Proceedings of 5th European Workshop on Lignocellulosics and Pulp, Aveiro, Portugal, 1998, pp. 585–588.
[22] E.I. Solomon, U.M. Sundaram, T.E. Machonkin, Chem. Rev. 96 (1996) 2563–2605.
[23] P.W. Oxley, B.M. Adger, M.J. Sasse, M.A. Forth, Org. Synth. 67 (1989) 187–192.