Mokchae Konghak 32(3) : 52~58, 2004

Mediator-Assisted Biobleaching of Kraft Pulp by Laccase from *Botrytis cinerea*[*1]

Myungkil Kim[*2†]

ABSTRACT

The use of N-hydroxyphthalimide (NHPI) as a mediator for laccase has proven to be comparable to N-hydroxybenzotriazole (HBT) for the delignification of kraft pulp, and the transformation of a number of industrial dyes. The advantages of NHPI derivatives are the biodegradation of these compounds compared to HBT, which has been shown to be recalcitrant in the environment, and the more reasonable cost of synthetic process.

Keywords : Mediator, biobleaching, laccase, HBT, NHPI derivatives

1. INTRODUCTION

Among various lignin-degrading enzymes, over a period of several years, laccase has been studied in white-rot fungi, soft-rot and some soil fungi. In white-rot fungi, laccase is active in lignin and lignosulfonate biotransformation, it occurs in the multiple, inducible and constitutive forms and manifests a bifunctional, oxidative and demethylating character.[8,10] Especially, laccase has attracted much attention in recent years of its potential application in pulp bleaching.[1,3,9,11] But there are no significant commercial success in pulping and bleaching. One problem is that the enzyme cannot penetrate into the fiber walls because it is too large to enter the fiber pores. Another is that is known to react only with the phenolic lignin structures, leaving the nonphenolic structures that are abundant in intact lignin. However, in the presence of some low molar mass organic compounds (mediators), laccases are able to delignify pulp and to improve its final brightness. A mediator could be a small molecule that acts as a sort of 'electron shuttle': once it is oxidized by the enzyme, it diffuses away from the enzymatic pocket and in turn oxidizes any substrate that, due to its size, could not directly enter the enzymatic pocket.[6] Alternatively, the oxidized mediator could rely on an oxidation mechanism not available to the enzyme, thereby extending the range of substrates accessible to it. Among the most promising mediators is 1-hydroxybenzotriazole (HBT), although it has been found to be partly converted under delignification conditions to benzotriazole (BT), which did not

*1 Received on September 10, 2003; accepted on December 15, 2003.
*2 Research Scientist at Div. Wood Chemistry & Microbiology, Dept. of Forest Products, Korea Forest Research Institute
† Corresponding author : Myungkil Kim (mkkim@foa.go.kr)

mediate laccase delignification.[4] It has been postulated that the mediator is converted by laccase in the presence of oxygen to a co-mediator which, because of its small size, is able to diffuse into the fiber and react with lignin.[5,7,12] Delignification mechanisms are based on the formation of radicals.[2] It has been shown that the principal sites of oxidative attack of the laccase/HBT system are the free phenolic group in lignin.

Bio-bleaching of pulps has been demonstrated by a laccase-mediator-O_2 treatment followed by a chemical treatment stage to remove or further degrade the oxidized residual lignin. Bourbonnais and Paice[3] reported that the lignin was removed by a combined laccase-ABTS-O_2 and alkaline extraction treatments.

I have recently described a constitutive, extracellular, high specific-activity laccase from *Botrytis cinerea*, which is as potent as many widely-studied white-rot fungal laccases in the oxidation of wide spectra of substrates. In this paper, I report the distinct of several laccase-mediated oxidation of kraft pulp. This and continuing studies may facilitate further research toward the development of an environmentally benign biodelignification process.

2. MATERIALS and METHODS

2.1. Enzyme Preparation

Laccase was isolated from *B. cinera* 61-34 grown in a liquid culture for 7 days as described previously[12] and was essentially purified to homogeneity by HPLC on a phenyl 5-PW column. Its activity, maintained in acetate buffer at pH 3.5, was determined with ABTS 3.7 μkat/mℓ.

2.2. Substrate

An EMCC kraft pulp (kappa number 27.9) was supplied by Ahlstrom Machinery Corp., Glens Falls, New York, U. S. A.

2.3. Preparation & Synthesis of NHPI Derivatives

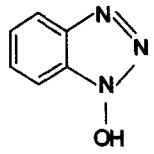
N-hydroxyphthalimide was synthesized as illustrated in Fig. 1. These compounds were prepared by following known literature procedures [(Orndorff & Pratt (1912), Fieser & Fieser (1967), Rougny & Daudon (1976), Keumi et al (1986), Morreal et al (1990), Chan et al (1987)] (3-amino-, 4-carboxylic-, 4-methyl-, 4,5-dichloro-, 3,6-dichloro-, 3-fluoro- and 4-nitro- derivatives) involving reaction of the corresponding substituted phthalic anhydrides with hydroxylamine. The purified NHPI derivatives exhibited NMR and MS spectra in keeping with their anticipated physical chemical properties.

2.4. Decolorization of the Industrial Dye as Different Mediators

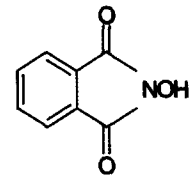
The laccase treatment of Orange II solution (2.51×10^{-5} M) with 50 nkat/mℓ was conducted in citrate phosphate buffer solution (pH: 4.0) at 2×10^{-5} M mediator. And then decolorization was observed at 483 nm wavelength.

2.5. Laccase Oxidation of Veratryl Alcohol and 3,4-dimethoxy Toluene with NHPI Mediators

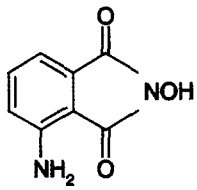
Substrate (0.1 mmol) and mediator (0.01 mmole) in 25 mℓ acetate buffer (0.05 M, pH 4.5) with 10% DMSO (V/V) as cosolvent was flushed with oxygen for 3 mins.; then the purified laccase (35 IU) was added. The reaction mixture was agitated for 24 hrs. under air. Direct analysis was carried out by HPLC using isoelectric elution (water-acetonitrile 40/60) on a Bondapak C18 column.



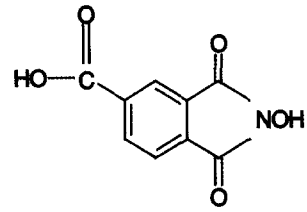
1-hydroxybenzotriazole(HBT)



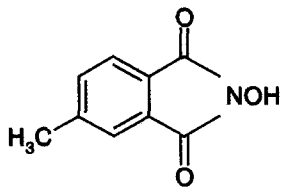
N-hydroxyphthalimide(NHPI)



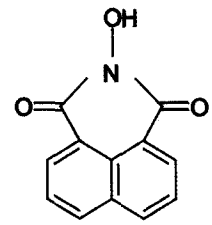
3-amino-N-hydroxyphthalimide(IMD-1B)



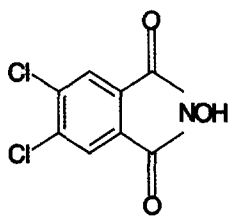
carboxylic-N-hydroxyphthalimide(IMD-2)



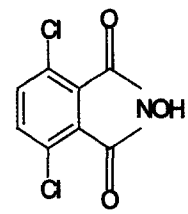
4-methyl-N-hydroxyphthalimide(IMD-3)



N-hydroxynaphthalimide(IMD-4)

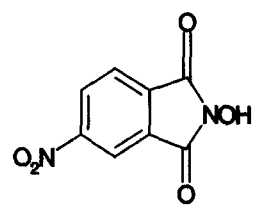


4,5-dichloro-N-hydroxyphthalimide(IMD-5)



3,6-dichloro-N-hydroxyphthalimide(IMD-7)

3-fluoro-N-hydroxyphthalimide(IMD-9)



4-nitro-N-hydroxyphthalimide(IMD-11A)

Fig. 1. Chemical structures used as mediators for enzyme oxidation.

2.6. Enzymatic Oxidation in a Bio-reactor

The initial laccase treatment of kraft pulps with different enzyme dosages (20 IU/g pulp) was conducted in a sodium acetate buffer solution (pH: 4.0) at 1% consistency containing 2% HBT and NHPI derivatives per pulp oven dry weight pulp and under atmospheric conditions with a continuous flow of O_2 through the system for 3 days. The treated pulp was then throughly washed with distilled water. A portion of the laccase-treated sample was then extracted with dilute sodium hydroxide solution using a 2.5% alkali charge and O_2 flow at 10% consistency and 80°C for 1h.

After alkaline extraction, laccase, alkali treated, O_2 flow treated pulp were analyzed for kappa number.

2.7. Enzymatic Oxidation at Pressurized Quantum Reactor

A 20 g pulp was suspended in pH 4.5 sodium acetate buffer solution. The pulp slurry was introduced into a Quantum Mark IV reactor in laccase activity 40 IU/g pulp, 2% HBT and NHPI derivatives and 1% consistency at 40°C, 4 h and at 90 psi. The reactor was set to mix every 2 min for 10 seconds. The mixing intensity was set at 10% so that less of shear force was applied to the laccase and thus decreased the damage to the laccase due to the mechanical reaction.

The kappa number of treated pulp was analyzed as TAPPI standard method.

3. RESULTS & DISCUSSION

3.1. Decolorization of the Industrial Dye as Different Mediators

Using decolorization of the industrial dye,

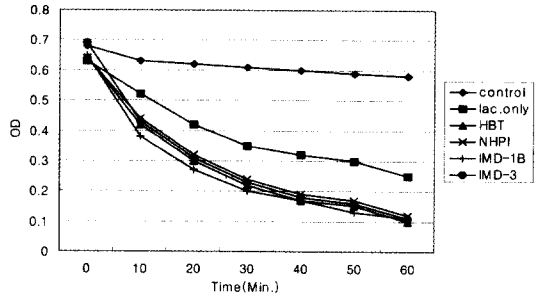


Fig. 2. Relative mediator effectiveness using Orange II oxidation

Orange II, as a model system, I have screened a number of putative mediator compounds and compared them to HBT. As shown an Fig. 2, decolorization rate of Orange II was similar HBT, NHPI, 3-amino- and 4-methyl- derivatives of NHPI compared to using control and laccase only. It was significant results to understand the laccase-mediator system according as delignification process. Above all, both the 3-amino- and the 4-methyl- derivatives of NHPI compare favorably in terms of their oxidation and possess the added advantages of low cost and biodegradability.

3.2. Laccase Oxidation of Veratryl Alcohol and 3,4-dimethoxy Toluene with NHPI Mediators

As shown Table 1 and Table 2, 3-amino- and 4-methyl- derivatives were higher than other derivatives at oxidation of veratryl alcohol and 3,4-dimethoxytoluene. As kinds of functional group of benzene ring, oxidation rate of these chemicals was different. These results were significant because they evaluated the oxidation of veratryl alcohol using laccase-mediator combinations. Again the 3-amino- and 4-methyl- derivatives were comparable and in fact, superior to HBT and may provide a degradative route for this commonly occurring lignin intermediate.

Table 1. NHPI mediator-assisted oxidation of veratryl alcohol

mediator	untreated(%)	veratryl alcohol(%)
none	100	-
HBT	12.4	72.0
NHPI	8.7	82.3
IMD-1B	1.4	87.7
IMD-2	71.6	18.6
IMD-3	8.1	82.9
IMD-4	77.8	12.8
IMD-5	72.9	16.7
IMD-7	83.6	7.4
IMD-9	87.4	2.7
IMD-11A	90.5	2.6

Similar results were obtained with 3,4-dimethoxytoluene. Interestingly, the dichloro-derivatives was comparable to HBT and the best NHPI derivatives.

3.3. Enzymatic Oxidation

As shown in Table 3 and 4, five of the derivatives (IMD-1B, IMD-3, IMD-4, IMD-5, IMD-7) indicate an effective degree of delignification as exhibited by reduction in Kappa number. In addition, more effective degree of delignification as exhibited superior results to HBT, two additional derivatives, IMD-1B and IMD-3, resulted in Kappa number reductions which were comparable. It is unclear why the dichloro derivative (IMD-7), which was the good performer under pressurized conditions, was much less effective under atmospheric pressure. I was encouraged by these data and feel that further refinement in the incubation of pulp and enzyme under pressure will lead to even effective reduction in Kappa number. I have some concern that mixing under pressure may denature the enzyme resulting in premature inactivation. I am currently investigating other reactor designs which will reduce the potential for shearing of the protein during the incubation period.

4. CONCLUSIONS

The present study clearly demonstrates that the mediator assisted laccase from *Botrytis cinerea* catalyzes enzymatic oxidation of kraft pulp. These reactions are enhanced in the presence of redox mediators, NHPI derivatives,

Table 2. NHPI mediator-assisted oxidation of 3,4-dimethoxy toluene

mediator	untreated(%)	veratryl alcohol(%)	veratryl aldehyde(%)	Total productivity(%)
none	100	-	-	
HBT	16.5	22.7	48.2	70.9
NHPI	23.2	10.1	50.9	61.0
IMD-1B	17.5	5.0	61.3	66.3
IMD-2	66.8	12.2	8.9	21.1
IMD-3	10.6	6.8	65.8	72.6
IMD-4	21.6	11.0	54.0	65.0
IMD-5	18.4	5.4	64.0	69.4
IMD-7	78.0	7.8	3.2	11.0
IMD-9	88.2	9.4	0.3	9.7
IMD-11A	21.6	11.0	54.0	65.0

Table 3. The influence of mediators in laccase-assisted delignification of kraft pulp in bioreactor

Mediator	Kappa number(Reduction rate %)		
	KL	KLE	KLEo
Blank	26.2(6.5)	24.1(13.6)	22.9(17.9)
Mediator only	26.1(6.5)	23.9(14.3)	23.0(17.6)
Enzyme only	25.0(10.4)	22.9(17.9)	22.0(21.1)
HBT	23.5(15.8)	20.6(26.1)	19.0(31.9)
NHPI	25.1(10.0)	24.0(14.0)	21.7(22.2)
IMD-1B	23.0(17.6)	20.7(25.8)	18.5(33.7)
IMD-2	23.9(14.3)	22.5(19.4)	21.8(21.9)
IMD-3	24.7(12.2)	21.8(21.9)	19.1(31.5)
IMD-4	24.3(12.9)	20.7(25.8)	20.0(28.3)
IMD-5	24.1(13.6)	20.9(25.1)	20.4(26.9)
IMD-7	24.9(10.8)	22.1(20.8)	21.5(22.9)
IMD-9	25.4(9.0)	22.6(19.0)	22.2(20.4)
IMD-11A	24.7(11.5)	23.3(16.5)	21.8(21.9)

KL: 2% mediator, purified laccase treated pulp (20IU/g pulp), pH 4.0 sodium acetate buffer, 1% consistency at room temperature, 3 days
KLE: 2.5% NaOH extraction, 10% consistency at 80°C, 1hr
KLEo: 2.5% NaOH + O_2 flow treated pulp

Table 4. The influence of mediators in laccase-assisted delignification of kraft pulp in Quantum reactor

Mediator	Kappa number(Reduction rate %)		
	KL	KLE	KLEo
Blank	-	-	-
Mediator only	-	-	-
Enzyme only	-	-	-
HBT	24.4(12.5)	21.8(21.9)	20.3(27.2)
NHPI	22.5(19.4)	21.1(24.4)	20.7(25.8)
IMD-1B	24.5(12.2)	22.0(21.1)	20.5(26.5)
IMD-2	26.0(6.8)	23.0(17.6)	22.3(20.1)
IMD-3	23.4(16.1)	20.5(26.5)	19.8(29.0)
IMD-4	22.7(18.6)	21.4(23.3)	20.7(25.8)
IMD-5	24.5(12.2)	22.5(19.4)	20.5(26.5)
IMD-7	22.8(18.3)	20.6(26.2)	20.0(28.3)
IMD-9	26.0(6.8)	21.9(21.5)	21.5(22.9)
IMD-11A	25.0(10.4)	23.5(15.8)	21.3(23.7)

KL: 2% mediator, purified laccase treated pulp (40IU/g pulp), pH 4.5 sodium acetate buffer, 1% consistency at 40°C, 4 hrs
KLE: 2.5% NaOH extraction, 10% consistency at 80°C, 1 hr
KLEo: 2.5% NaOH + O_2 flow treated pulp

being more effective than HBT.

REFERENCES

1. Bourbonnais, R., M. G. Paice. 1992. Demethylation and delignification of kraft pulp by *Trametes versicolor* laccase in the presence of 2, 2'-azinobis-(3-ethylbenzthiazoline-6 -sulphonate). Appl. microbiol. Biotechnol. 36: 823~827
2. Bourbonnais, R., D. Leech, and M. G. Paice. 1998. Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. Biochim. Biophys. Acta. 1379(3): 381~390
3. Bourbonnais, R. and M. G. Paice. 1996. Enzymatic delignification of kraft pulp using laccase and a mediator. Tappi. J. 79(6): 199~204
4. Bourbonnais, R., M. G. Paice, B. Freiermuth, E. Bodie, and S. Borneman. 1997. Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. Appl. Environ. Microbiol. 63(12): 4627~4632
5. Bourbonnais, R., M. G. Paice, I. D. Reid, P. Lanthier, and M. Yaguchi. 1995. Lignin oxidation by laccase isozymes from *Trametes versicolor* and role of the mediator 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) in kraft lignin depolymerization. Appl. Environ. Microbiol. 61: 1876~1880
6. Fabbrini, M., C. Galli, and P. Gentili. 2002. Comparing the catalytic efficiency of some mediators of laccase. J. of Molecular Cat. B: Enzymatic 16: 232~240
7. Frits-Langhals, E and B. Kunath. 1998. Synthesis of aromatic aldehydes by laccase-mediator assisted oxidation. Tetrahedron Lett. 39: 5955~5956
8. Kawai, S., T. Umezawa, and T. Higuchi. 1988. Degradation mechanism of phenolic β-1 lignin substructure model compounds by laccase of *Coriolus versicolor*. Arch. of Biochem. and Biophis. 262(1): 99~110
9. Poppius-Levlin, K., W. Wang, T. Tamminen, B. Hortling, L. Vikari, and M. L. Niku-Paavola. 1999. Effects of laccase/HBT treatment on pulp and lignin structures. J. of Pulp and Paper Science. 25(3): 90~94
10. Stone, J. E., A. M. Scallan, E. Donefer, and E. Ahlgren. 1969. Digestibility as a simple function of a molecule similar of size to an enzyme. Adv. Chem. Ser. 95: 219~241
11. Wong, K. K. Y., K. B. Anderson, and R. P. Kibblewhite. 1999. Effects of laccase-mediator system on the handsheet properties of two high kappa kraft pulps. Enzyme and Microbial Tech. 25: 125~131
12. Xu, H, Y. Z. Lai, D. Slomczymski, J. P. Nakas, and S. W. Tanenbaum. 1997. Mediator-assisted selective oxidation of lignin model compounds by laccase from *Botrytis cinerea*. Biotechnology Letters. 19(10): 957~960