

# Enzyme-Assisted Delignification of Several Pulps by Laccase from *Botrytis Cinerea*

Myung-kil Kim

## ABSTRACT

The two stage laccase-assisted delignification process led to significant lignin removal in the non-pressurized treatments. It is clearly shown that an alkaline extraction prior to the second laccase treatment significantly increased the overall delignification by ~15%. This is in line with the contention that the residual lignin has undergone structural changes during the alkaline extraction, and the resulting modified structures are susceptible to the laccase oxidation. In phenolic hydroxyl group, the pre-methylated sample was very responsive to the delignification process. The phenolic hydroxyl groups could be increased during side chain cleavage catalyzed by laccase. This finding demonstrates that the delignification of etherified structures is an important reaction in the delignification by laccase.

**Keywords:** *laccase-assisted delignification, phenolic hydroxyl group, pre-methylated pulp, kappa number*

## 1. Introduction

Lignin biodegradation occupies a central position in the earth's carbon cycle, because most renewable carbon is either in lignin or in compounds protected by lignin from enzymatic degradation. Lignin biodegradation is responsible for much of the natural destruction of wood in use, and thus is responsible for substantial economic losses. Finally, potential biotechnical applications of lignin-degrading enzymes or organisms its significance, however, lignin biodegradation is only slowly being defined chemically and biochemically<sup>1)</sup>.

Especially, the lignin removal in chemical pulping and bleaching, or lignin softening and brightening in mechanical pulping, are the

major routes to paper production. But the traditional practice of degrading most of the residual lignin by chlorination has fallen out of favor because of the chloro-organic byproducts.<sup>2)</sup> Protection of the yield and strength of the pulp requires a very selective attack on lignin without damage to the cellulose or hemicellulose polysaccharides. Because lignin is a biodegradable material, and biological, enzyme-catalyzed reactions typically have high specificity, the potential of lignin biodegradation in pulp bleaching deserves consideration.<sup>3,4,5)</sup>

The laccase as aryl alcohol oxidase is reported to cause C-oxidation, demethylation, cleavage in phenyl groups and C-C cleavage in syringyl structures. Laccase also provides the phenoxyl radicals and quinones from lignin cel-

---

• Post Doctoral Associate at State University of New York, College of Environmental Science & Forestry, U. S. A

lobiose dehydrogenase. Recently, it is found that aromatic cleavage is catalyzed by laccase.<sup>6)</sup>

A significant increase in delignification was obtained by means of HBT(1-hydroxybenzotriazole)-mediated laccase treatment compared with another treatments.<sup>7)</sup> Besides oxygen is needed as delignification by laccase treatments.<sup>8)</sup>

The goal of this experiment is evaluated to degree of delignification as lignin removal and analyzed products from degrading by laccase.

## 2. Experimentals

### 2.1 Materials

The laccase was isolated from *Botrytis cinera* 6134.<sup>9)</sup> An EMCC pulp from softwood was supplied by then Ahlstrom Machinery Corp. in Glens Falls, New York. And Aspen pulp, norway spruce pulp and TMP(thermo-mechanical pulp) were cooked at State University of New York at College of Environmental Science & Forestry.

#### 2.1.1 Oxygen bleaching of pulp

A pulp was suspended 2.5% NaOH and 0.5%  $MgSO_4 \cdot 7H_2O$  in water. The pulp slurry was introduced into a Quantum Mark IV reactor 10% consistency at 100°C, 1hr and 90psi. The reactor was set to mix every 2 minutes for 10 second. The mixing intensity was set at 10% so that less of shear force was applied to the slurry.

#### 2.1.2 Methylation of pulp

The sample, preferentially in the order of 10-100mg calculated as lignin, is dissolved or suspended in 10ml of 1,2-dimethoxyethane-methanol-water(35:35:30, v/v) in a 25ml flat-bottomed three-necked flask placed on a magnetic stirrer. Through the middle neck a pH electrode is loosely inserted so that the tip extends below

the surface of the solution. In the two other necks are placed glass tubes for the introduction of alkali solution and nitrogen gas(free from oxygen). The reaction is started by the addition of 2ml of dimethyl sulfate to the solution. Simultaneously, alkali, in the form of an approximately 15% solution of NaOH in the solvent mixture referred to above, is added to the reaction mixture via an automatic titration assembly consisting of an autoburet, titrator and pH meter. During the reaction the pH is maintained at 11.

The reaction is continued for approximately 24h with stirring at room temperature or until the consumption of alkali ceases, after which the addition of alkali is interrupted and the reaction mixture is acidified to pH 3 by the addition of 0.5M phosphoric acid. After 30min, the pH is again adjusted to 6.5 by the addition of NaOH solution and the entire sample is transferred to a 500ml round-bottomed flask. After evaporating the mixture to dryness, a small volume(20-30ml) of t-butyl alcohol-water(3:1, v/v) is added and the solution is again evaporated to dryness.

### 2.2 Pressurized oxygen-laccase treatment

A 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 40IU/g pulp, 2% HBT mediator and 1% consistency at 40°C, 4hr and 90psi. The reactor was set to mix every 2 minutes for 10 second. 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.

### 2.3 Non-pressurized oxygen-laccase treatment

A pulp was suspended in pH 4.5 sodium

acetate buffer solution. The pulp slurry was introduced into a 2l reactor in laccase activity 60IU/g pulp, 2% HBT mediator and 1% consistency under room temperature with a continuous flow of O<sub>2</sub> through the system for 3 days. The blank sample without laccase was conducted in the same conditions.

#### 2.4 Alkaline extraction

Both pressurized and non-pressurized oxygen-laccase treated pulp were then washed with distilled water. A portion of treated pulp was then extracted with dilute sodium hydroxide solution using a 2.5% alkali charge and O<sub>2</sub> flow at 10% consistency and 80°C for 1h.

#### 2.5 Pulp analysis

The treated pulp was analyzed for yield and

kappa number following the corresponding Tappi standard procedures. And phenolic hydroxyl analysis was conducted as sodium periodate oxidation method.<sup>10,11)</sup>

### 3. Results and Discussion

#### 3.1 The effect of pressurized and non-pressurized oxygen-laccase treatment premethylated pulps on lignin removal

In order to further understand the reactivity of etherified structures toward the laccase treatment, I have determined the delignification behavior of a pre-methylated softwood and hardwood pulps, in which the phenolic hydroxyl group were blocked by reacting with dimethyl sulfate.

As indicated in the Table.1, 2, the pre-methylated sample was very responsive to the deligni-

**Table 1. The effect of pressurized and non-pressurized oxygen laccase treatment of methylated aspen pulp on lignin removal in different alkaline treatments**

Pressurized oxygen-laccase treatment					
Treatment	A	MA	MLA	MLEA	MLEoA
Kappa number	31	25.6	20.5	18.7	17.6
Reduction(%)		17.4	33.9	39.7	43.2
Phenolic OH*	1.76	0.40	0.59	0.62	0.50
Non-pressurized oxygen-laccase treatment					
Treatment	A	MA	MLA	MLEA	MLEoA
Kappa number	31	25.6	20.0	18.1	17.4
Reduction(%)		17.4	35.5	41.6	43.9
Phenolic OH*	1.76	0.40	0.65	0.67	0.70
Blank non-pressurized treatment					
Treatment	A	MA	MBA	MBEA	MBEoA
Kappa number	31	25.6	23.3	22.8	22.2
Reduction(%)		17.4	24.8	26.5	28.4
Phenolic OH*	1.76	0.40	0.68	0.72	0.73

\*: mmol<sub>Cl<sub>2</sub>SO<sub>4</sub></sub>/g lignin

A: aspen pulp

MA: methylated aspen pulp

MLA: oxygen-laccase treated methylated pulp

MLEA: oxygen-laccase treated methylated + 2.5% NaOH extraction

MLEoA : oxygen-laccase treated methylated + 2.5% NaOH extraction + O<sub>2</sub> flow

**Table 2. The effect of pressurized oxygen laccase treatment of aspen pulp and norway spruce pulp on lignin removal in different alkaline treatments**

Oxygen bleached spruce pulp							
Treatment	S	OS	OES	OEOs	OLS	OLES	OLEoS
Kappa number	26	11.1	10.8	10.2	10.4	10.0	9.5
Reduction(%)		57.3	58.5	60.8	60.0	61.5	63.5
Oxygen bleached methylated spruce pulp							
Treatment	S	OMS	OMES	OMEoS	OMLS	OMLES	OMLEoS
Kappa number	26	10.9	10.6	10.2	9.5	9.0	8.6
Reduction(%)		58.1	59.2	60.8	63.5	65.4	66.9
Oxygen bleached aspen pulp							
Treatment	A	OA	OEA	OEOA	OLA	OLEA	OLEoA
Kappa number	31	15.1	13.9	13.5	12.8	12.6	12.5
Reduction(%)		51.3	55.2	56.5	58.7	59.4	59.7
Oxygen bleached methylated aspen pulp							
Treatment	A	OMA	OMEA	OMEoA	OMLA	OMLEA	OMLEoA
Kappa number	31	13.1	12.8	12.1	11.1	10.7	10.1
Reduction(%)		57.7	58.7	61.0	64.2	65.5	67.4

S: spruce pulp, A: aspen pulp

L: oxygen-laccase treated

E: 2.5% NaOH extraction

O: oxygen bleached spruce pulp

Eo: 2.5% NaOH extraction + O<sub>2</sub> flow

M: methylated

fication process. The phenolic hydroxyl groups could be increased during side chain cleavage catalyzed by laccase. This finding demonstrates that the delignification of etherified structures is an important reaction in the delignification by laccase.

The change of kappa number with different treatment was significant at both pressurized and non-pressurized laccase-oxygen treatment. Especially, the pre-methylated pulps have more significant reduction at kappa number than unmethylated pulps. It is certain that the laccase was effective a degradation of lignin.

### 3.2 The effect of two stage non-pressurized oxygen-laccase treatment on thermo-mechanical pulp and kraft pulp on lignin removal

The laccase assisted delignification of thermo-mechanical and kraft pulps summarized in

Table 3, 4 were further related to changes of the phenolic hydroxyl content in the residual lignin as determined by the periodate oxidation method. It should be noted that this analytical procedure based on the methanol formation, excludes the detection of catechol units. Accordingly, the catechol-type units possibly generated from the demethylation reaction would be accounted for as a loss of phenolic hydroxyl groups.

As indicated for the delignification of thermo-mechanical pulp (Table. 3) and kraft pulp (Table. 4), the impact of delignification on the phenolic hydroxyl content of residual lignin displays two distinct phases. The initial phase was accompanied by a significant reduction in the phenolic hydroxyl content, where subsequent alkaline extraction has little influence on this functional group. But, these data suggest that the delignification process involves not only the existing phenolic units but also the delignification of the etherified units. The subsequent alkaline extrac-

**Table 3. The effect of two stage-non-pressurized oxygen-laccase treatment on thermo-mechanical pulp on lignin removal in different alkaline treatments**

Treatment	Yield(%)	Lignin content(%)		Reduction(%)	Phenolic OH*
		Klason lignin	Acid soluble lignin		
T		28.30	0.04		1.47
LT	95.9	26.47	0.05	6.5	0.95
LET	94.9	24.25	0.05	14.3	0.89
LEoT	94.3	23.36	0.06	17.4	0.83
LELT	93.7	23.02	0.06	18.6	0.54
LELET	92.9	22.11	0.06	21.8	0.50
LLT	94.1	24.64	0.06	13.1	0.59
LLET	93.2	23.20	0.06	17.9	0.52

\*: mmol<sub>CHOH</sub>/g lignin

T: thermo-mechanical pulp(TMP)

LT: oxygen-laccase treated TMP

LET: oxygen-laccase treated + 2.5% NaOH extraction

LEoT: oxygen-laccase treated + 2.5% NaOH extraction + O<sub>2</sub> flow

LELT: oxygen-laccase treated + 2.5% NaOH extraction + oxygen-laccase treated

LELET: oxygen-laccase treated + 2.5% NaOH extraction + oxygen-laccase treated + 2.5% NaOH extraction

LLT: oxygen-laccase treated + oxygen-laccase treated

LLET: oxygen-laccase treated + oxygen-laccase treated + 2.5% NaOH extraction

**Table 4. The effect of two stage-non-pressurized oxygen-laccase treatment on kraft pulp(EMCC) on lignin removal in different alkaline treatments**

Treatment	Yield(%)	Kappa number	Reduction(%)	Phenolic OH*
K		27.9		1.86
LK	99.1	20.5	26.5	0.82
LEK	96.0	16.6	40.5	0.89
LELK	88.5	13.4	52.0	0.83
LELEK	87.9	10.7	61.6	0.87
LELEoK	86.4	9.8	64.9	0.75
LLK	85.5	17.3	38.0	0.61
LLEK	84.1	15.0	46.2	0.80
LLEoK	83.3	13.1	53.0	0.73

\*: mmol<sub>CHOH</sub>/g lignin

K: kraft pulp

tion resulted little from the aryl ether cleaved or a preferential removal of the etherified units.

As indicated Table. 4, the first laccase treatment(LK) resulted in a significant delignification(26.5%) especially after the alkaline extraction(LEK)(40.5%). Both the LK and LEK samples were responsive to a second laccase treatment resulting in additional ~8% delignification.

It is clearly shown that an alkaline extraction prior to the second laccase treatment significantly increased the overall delignification by ~15%. This is in line with the contention that the residual lignin has undergone structural changes during the alkaline extraction, and the resulting modified structures are susceptible to the laccase oxidation.

## 4. Conclusions

The present results on delignification of un- and pre-methylated and alkaline extracted pulps confirms that the laccase action is capable of mediating oxidation of etherified units of residual lignin.

## Literature Cited

1. K. T., Kent, *Recent advances in lignin biodegradation research: Lignin biodegradation; Importance and historical research perspective*, UNI PUBLISHERS CO. LTD. 1983
2. Raymond A. Young and Masood Akhtar, *Environmentally Friendly Technologies for the Pulp and Paper Industry*, John Wiley & Sons, INC, p:505, 1998
3. Reid, I. D., and M. G. Paice. *In Frontiers in industrial mycology*, G. F. Leatham, editor. Chapman & Hall, New York, p:112, 1992
4. Paice, M. G., R. Bourbonnais, I. D. Reid, F. Archbald, and L. Jurasek. *J. Pulp & paper Sci.* 21:J280, 1995
5. Archbald, F. S., R. Bourbonnais, L. Jurasek, M. G. Paice, and I. D. Reid. *J. Biotechnol.*, 53:215, 1996
6. Kawai S., T. Higuchi, K. Nabeta and H. Okuyama, *Biotechnology in pulp and paper manufacture: Degradation mechanisms of  $\alpha$ -O-4 lignin substructure model compounds by laccase of *Coriolus versicolor**, Butterworth-Heinemann, 1990
7. Poppius-Levin, K., W. Wang, T. Tamminen, B. Hortling, L. Vikari and M. L. Niku-Paavola, *J. Pulp & Paper Sci.*, 25:90, 1999
8. Xu, H., Y. Z. Lai, D. Slomczynski, J. P. Nakas, and S.W. Tanenbaum. *Biotechnol. Letts.* 19:957, 1997
9. Slomczynski, D., J. P. Nakas. and S. W. Tanenbaum. *Appl. Environ. Microbiol.* 61:907, 1995
10. Lai, Y. Z., In *Methods in Lignin Chemistry*, S. Y. Lin and C. W. Dence, editor, Springer-Verlag, p:423, 1992
11. Yang, R. and Lai, Y. Z., *J. Wood. Chem. technol.*, 17:383, 1997.