Enhancing Antimicrobial Activity in Unbleached Kraft Pulp using Laccase and Phenolic Compounds

Jicheng Pei, Ying Zhang, Fangdong Zhang, Xiuling Yu, and Xiaoting Yan

Unbleached kraft pulp fibers were reacted with laccase in the presence of different phenolic compounds (isoeugenol, butyl *p*-hydroxybenzoate, *p*-coumaric acid, and ferulic acid) in order to confer them with new properties. After the enzymatic stage, antimicrobial tests demonstrated that the handsheets formed from the laccase/phenolic-treated pulp showed a greater efficacy against Gram-positive and Gram-negative bacteria compared to handsheet paper treated only with laccase and a control. Laccase in the presence of butyl *p*-hydroxybenzoate produced handsheets with the most significantly enhanced bactericidal effect. An analysis of scanning electron microscope images of the treated kraft pulps increased kappa numbers and surface features of the fibers. The reduction in the absolute value of the zeta potential of the pulps indicates that the phenolic compounds produce chemical cross-linking within the fiber surface. X-ray photoelectron spectroscopy shows that the lignin content of the pulp fiber surface increased after adding the laccase and phenolic compounds.

Keywords: Laccase; Phenolic compounds; Unbleached kraft pulp; Antimicrobial activity

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INTRODUCTION

Packaging is an important means of food preservation. It can provide the protection necessary for the food and prevent exposure of the food to microbial or other forms of contamination. It also retards food oxidation and other reactions. Active packaging, which involves changing the food packaging environment, is a new means of packaging. This new approach could prolong the shelf life of food, improve its safety, and thus maintain the quality of the food (Rooney 1995; Gutiéirrez *et al.* 2009; Ahvenainen 2003).

In recent years, as a kind of active packaging, antimicrobial packaging has attracted much attention. This is due to the increase in consumer demand, especially in the food industry, where there is a major need for packing systems that are able to hinder microbial development in the food (Bordenave *et al.* 2010). Currently, many various kinds of paper and board are used in the food and drink packaging field (Song *et al.* 2000; Triantafyllou *et al.* 2007). However, the shelf life of the food is still limited because of microbial spoilage. To date, the food industry has used several techniques to control microbial growth and thus improve the quality and shelf life of most of the major food products. These include: modified atmosphere packaging (MAP), the use of chemical preservatives, high pressure, electric or magnetic fields, and irradiation (Mainville *et al.* 2001; San Martin *et al.* 2001; Holley and Patel 2005). However, one of the most novel

and promising approaches is to use active, antimicrobial packaging material. To this end, there is increasing interest in fiber modification by enzymatically grafting natural antimicrobial organic molecules to lignocellulosic fibers. The technique is capable of meeting the growing expectation of consumers for higher standards of hygiene, ensuring safer products, and satisfying environmental protection concerns (Cha *et al.* 2004).

Laccase, a blue copper oxidase capable of reacting with a large variety of aromatic substrates (Leonowicz *et al.* 2001), is able to transform phenolic compounds through oxidative coupling reactions. It results in the production of polymeric products by self-coupling or cross-coupling with other molecules (Canfora *et al.* 2008; Chandra and Ragauskas 2001; Kenealy *et al.* 2003; Johannes and Majcherczyk 2000). In the last few decades, numerous studies have demonstrated the effectiveness of laccase mediator systems (LMS) in pulp bleaching. Some of these studies have focused on using LMS to bio-modify lignocellulosic fibers in order to improve their inherent properties or even create novel ones (Chandra and Ragauskas 2001; Kenealy *et al.* 2003). More recently, it has been demonstrated that some phenolic compounds can be used as natural laccase mediators (Rodriguez and Toca 2006). They allow the grafting of aromatic compounds onto the fibers, thus enhancing the fiber's properties (Chandra *et al.* 2004), and/or impart completely new properties to the fibers (Buchert *et al.* 2005).

Elegir *et al*. (2008) showed that handsheets treated with laccase/phenolic compounds had improved antimicrobial activity. Other studies have indicated that laccase is able to oxidize the phenolics naturally occurring in flax fibers and that the antimicrobial activity of the flax pulp could be further improved by adding laccase/ natural phenolic mediators (Fillat *et al.* 2008; Aracri *et al.* 2009; Fillat *et al.* 2009; Silva *et al.* 2011).

Nipagin esters are an acknowledged family of broad-spectrum preservatives. Their use is allowed in the food industries in America, Europe, Japan, Canada, Korea, and other developed countries (Burini 1994). Essential oils also represent a very well-known class of natural compounds that contain different phenolic structures that are particularly active towards bacteria (Burt 2004). In addition, *p*-coumaric acid and ferulic acid represent a very well-known class of natural compounds that have been used in the food industry as preservatives (Tsou *et al.* 2000; Weetall 1985).

In the current work, four natural mediators, butyl *p*-hydroxybenzoate (BPH), isoeugenol (ISO), *p*-coumaric acid (PCA), and ferulic acid (FA), are used to accomplish laccase-assisted grafting of fibers. The products are then compared in terms of their antimicrobial activity. This is achieved by using the shake flask method to evaluate the antimicrobial activity of the resulting paper. The aim of this work was to analyze the potential of laccase to couple phenolic compounds on fibers and to assess the ability of the modified surfaces to reduce bacterial growth. Furthermore, it provides a theoretical basis for investigation of the mechanism responsible for the antimicrobial activity of the paper.

EXPERIMENTAL

Materials

The unbleached kraft pulp (KP) was prepared from commercial pulp boards. The liquid laccase preparation (Novozym® 51003) with an activity of 1070 U/mL was provided by Novozymes A/S (Denmark).

The mediators employed: isoeugenol, butyl *p*-hydroxybenzoate, *p*-coumaric acid, and ferulic acid were purchased from Sigma-Aldrich (USA). The microbial strains were provided by the Culture Collection of Tianjin University of Science and Technology.

Enzymatic Treatment of the Pulp

The kraft pulp samples were refined using a PFI mill until their beating degree was 45°SR. The treatment with the laccase/phenolic compound system was carried out according to the following protocol: 4 g of KP (dry weight) at 4% consistency in 50 mM sodium tartrate buffer (pH = 5), 18 U/g laccase, and 3.5% phenolic compound (relative to the pulp dry weight) were shaken at 100 rpm, 50ºC, for 4 h. The control sample was prepared in the same way but without addition of mediator and laccase. After the enzymatic treatment of the pulp, handsheets (65 g/m^2) were prepared according to TAPPI Standard T 205 om-88.

Antimicrobial Analyses

The antimicrobial activities of the phenolic compounds were determined using an Oxford plate assay system. The Oxford cup was put on a plate containing 0.1 mL of the nutrient fluid of the experimental bacteria. Then, 0.2 mL of the sample liquid was added and the plate cultured in an incubator for 24 h at 37ºC. The circle of the inhibiting bacterium was then measured.

Determination of Dissolution Type

This experiment was conducted in accordance with the accepted standards (FZ/T73023-2006). The process is as follows. The sample (paper) was placed flat on the inoculated bacteria medium in a plate and pressed slightly with aseptic forceps to ensure close contact with the medium. Then the plate was inverted and incubated in a biochemical incubator for 16 h at 37ºC. After incubation, the total widths of the inhibition zone and sample were measured and their average values were calculated. The width of the inhibition zone *D* (mm) was calculated using Equation 1,

$$D = \frac{T - R}{2} \quad (1)$$

where *T* is the mean value of the external diameter of the inhibition zone (mm) and *R* is the diameter of the sample (mm). If the width of the inhibition zone *D* is greater than 1 mm, the paper is classified as a dissolution type antibacterial fabric. On the other hand, if *D* is less than or equal to 1 mm, then it is classified as a non-dissolution type antibacterial fabric.

Determination of the Antimicrobial Activity of Treated Pulp

The ASTM E2149-2001 test method was used to assess the antimicrobial activity of the pulp treated with the laccase/phenolic compounds. A fresh culture of experimental bacteria in sterile nutrient broth (shaken overnight) was diluted with KH_2PO_4 buffer until a final concentration of 1×10^5–1×10^6 CFU/mL was obtained. Then, 5 mL of the working bacterial dilution was added to a sterile 250 mL conical flask containing 70 mL PBS (phosphate buffer). The flask was placed on a shaker for 1 min. The concentration of the bacteria in the solution is approximately 1×10^4 to 2×10^4 CFU/mL and was used for the antibacterial tests. The test pulps and control specimen were placed in separate flasks and

shaken for 1 h at 37ºC with the 220 r/min shaking speed. Then, 0.5 mL aliquots of the liquid was placed in 90 mm petri dishes containing 18 mL of agar medium, mixed well, and incubated in an incubator for 24 h at 37ºC. After incubation, the colonies were counted. The percentage reduction of the organism resulting from contact with the specimen was calculated using the following formula,

$$Reduction = [(A - B)/A] \times 100\%, \quad (2)$$

where *A* is the average colony size of the control sample and *B* is the average colony size of the treated samples.

Zeta Potential Analysis

The zeta potential is used to characterize the surface charge of the fibers and filler. In our study, the zeta potential of the sample was carried out with an Mütek™ SZP-06 device (BTG, Germany).

Kappa Numbers and Fiber Morphology

The kappa number is an indication of the residual lignin content of the pulp and is estimated using a standard method (ISO 302-2004). The coarseness of the fibers was also tested using a fiber-tester. The morphology of the fibers was further investigated using a scanning electron microscope (SEM) to image the surface of the fibers.

XPS Analysis

X-ray photoelectron spectra (XPS) of the fibers surface were obtained using a Physical Electronics ESCALAB 250 XPS instrument. The detector was positioned at an angle of 45° to the sample surface and the area of analysis was 0.8 mm^2. A Gaussian curve fitting program was used to deconvolute the C1 carbon signal at 285 eV (C–C, C–H, and C=C functional groups) as an internal standard. The chemical shifts relative to C1 used in the deconvolution were 1.7 ± 0.2 eV for C–O (C2), 3.1 ± 0.2 for C=O or O–C–O (C3), and 4.3 ± 0.2 eV for O–C=O (C4) groups.

All KP samples for XPS analysis were subjected to sequential Soxhlet extraction with acetone and deionized water (4 h for each solvent). The samples were then placed on stainless steel plates and air-dried. The smooth side of the sample was used for XPS measurement. The lignin coverage Φ_{lignin} of the fiber surface was estimated according to the following equation developed by Ström and Carlsson (1992),

$$\Phi_{lignin} = \frac{N_O/N_C\,(\text{after extraction}) - N_O/N_C\,(\text{carbohydrates})}{N_O/N_C\,(\text{lignin}) - N_O/N_C\,(\text{carbohydrates})}. \quad (3)$$

RESULTS AND DISCUSSION

Antimicrobial Activity of the Phenolic Compounds

The *S. aureus*, *E. coli*, *Bacillus subtilis*, and *Salmonella* strains were chosen as the experimental samples to determine the antimicrobial activity of the phenolic compounds. The phenolic compounds were dissolved in dimethyl sulfoxide (DMSO). The diameter

of the inhibition zone produced using these mediators and DMSO was used to gauge the bacteriostasis effect (Table 1). The diameter of the inhibition zone of DMSO for each test strain was zero, which indicates that the DMSO had no antimicrobial activity. However, the diameters of the inhibition zones for the phenolic compounds were between 13.2 and 28.5 mm. BPH appears to exhibit the best overall antimicrobial activity as the average diameter of its inhibition zone was consistently greater than 20.0 mm. Even so, ISO had the best antimicrobial activity against *Bacillus subtilis* and *Salmonella*, and PCA had the best resistance to *E. coli*. Compared to other phenolic compounds, the antimicrobial activity of FA was the lowest.

Table 1. Susceptibility of Pathogens/Bacterial Species to Phenolic Compounds

Reagents*	Inhibition zone (mm)			
	S. aureus	*E. coli*	*B. subtilis*	*Salmonella*
DMSO	0.0	0.0	0.0	0.0
BPH	22.5	24.6	20.8	23.5
ISO	15.8	24.2	28.5	27.5
PCA	15.1	27.5	24.5	16.3
FA	16.3	16.9	14.0	13.2

*BPH = butyl *p*-hydroxybenzoate, ISO = isoeugenol, PCA = *p*-coumaric acid, and FA = ferulic acid

Evaluating the Dissolution Type of Unbleached Handsheets

In order to investigate whether the phenolic compounds produced chemical bonding in the fibers as opposed to simple physical adsorption in the presence of laccase, the type of dissolution of the unbleached kraft pulp was addressed.

The results of the dissolution analysis on the handsheets are shown in Tables 2 and 3. As can be seen from Table 2, the inhibition zones of the handsheets made with phenolic compounds only were all greater than 1.0 mm after contact with *S. aureus* and *E. coli*. However, those with added laccase and natural mediators were less than 1.0 mm after contact with *E. coli* and *S. aureus* (Table 3). The results show that pulp treated with laccase/phenolic compounds are non-dissolution types, while the pulp treated merely with phenolic compounds are dissolution types.

Table 2. Handsheet Dissolution Type with Phenolic Compound Only (number of trials, n = 3)

Treatment	Mean external inhibition zone diameter (mm)		Sample diameter (mm)		Inhibition zone width (mm)		Evaluation	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
Control	10.0	10.0	10.0	10.0	0.0	0.0	-	-
BPH	12.2	12.5	10.0	10.0	1.1	1.2	dissolution	dissolution
ISO	13.2	12.6	10.0	10.0	1.6	1.3	dissolution	dissolution
PCA	12.4	12.8	10.0	10.0	1.2	1.4	dissolution	dissolution
FA	12.2	12.8	10.0	10.0	1.1	1.4	dissolution	dissolution

Table 3. Handsheet Dissolution Type with Laccase and Phenolic Compound (number of trials, *n* = 3)

Treatment	Mean external inhibition zone diameter (mm)		Sample diameter (mm)		Inhibition zone width (mm)		Evaluation	
	S. aureus	*E. coli*	S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
Control	10.0	10.0	10.0	10.0	0.0	0.0	-	-
Laccase	10.0	10.0	10.0	10.0	0.0	0.0	non-dissolution	non-dissolution
L/BPH	10.2	10.0	10.0	10.0	0.1	0.0	non-dissolution	non-dissolution
L/ISO	10.0	10.2	10.0	10.0	0.0	0.1	non-dissolution	non-dissolution
L/PCA	10.0	10.2	10.0	10.0	0.0	0.1	non-dissolution	non-dissolution
L/FA	10.0	10.0	10.0	10.0	0.0	0.0	non-dissolution	non-dissolution

The study by Fillat *et al.* (2008) confirmed that PCA produced an increase in kappa number. This is partially due to a condensation reaction of the phenoxy radicals with the pulp. Thus, we infer that these phenolic compounds do not undergo physical adsorption in the fibers. Rather, they produce free radicals after being oxidized by the laccase and partake in a free radical condensation reaction with the lignin. Thus, there is little dissolution of the phenolic compound.

Antimicrobial Activity of the Pulp

The unbleached KP fibers were reacted with laccase in the presence of the different phenolic compounds. Untreated pulp was used as a standard control sample. In the experiments the washability of pulps was tested. The antimicrobial activity of isoeugenol and laccase/isoeugenol treated samples were tested after being washed two times, four times, and six times, respectively (Table 4).

Table 4. Inhibition of the Pulps after Repeated Washing

Washing times	*S. aureus*		*E. coli*		*Bacillus subtilis*		*Salmonella*	
	ISO	L/ISO	ISO	L/ISO	ISO	L/ISO	ISO	L/ISO
0	64.1	94.9	65.8	98.9	75.3	90.8	73.2	99.1
2	35.7	93.7	37.2	94.8	30.1	85.3	28.5	95.7
4	10.2	90.2	9.75	92.4	13.8	83.7	10.2	93.1
6	7.3	90.5	5.7	91.5	8.2	83.2	7.5	92.3

The results showed that the inhibition of the sample treated by isoeugenol merely decreased to 7.3%, 5.7%, 8.2%, and 7.5% of *S. aureus*, *E. coli*, *Bacillus subtilis*, and *Salmonella*, respectively, after washing six times. This indicated there was almost no antimicrobial activity against the strains and almost no phenolic compounds adsorbed on the fiber surface. The antimicrobial activity of pulps treated by L/ISO decreased a little

after different washing time compared with the unwashed pulps, and there were no obvious changes with the increasing of washing time.

In the next experiments, the samples for antimicrobial test were washed at least six times in order to remove the phenolic compounds adsorbed on the surface of the fibers. The antimicrobial activity of the KP treated with laccase/phenolic compounds were tested by determining their activity towards *S. aureus*, *E. coli*, *Bacillus subtilis*, and *Salmonella* (Fig. 1).

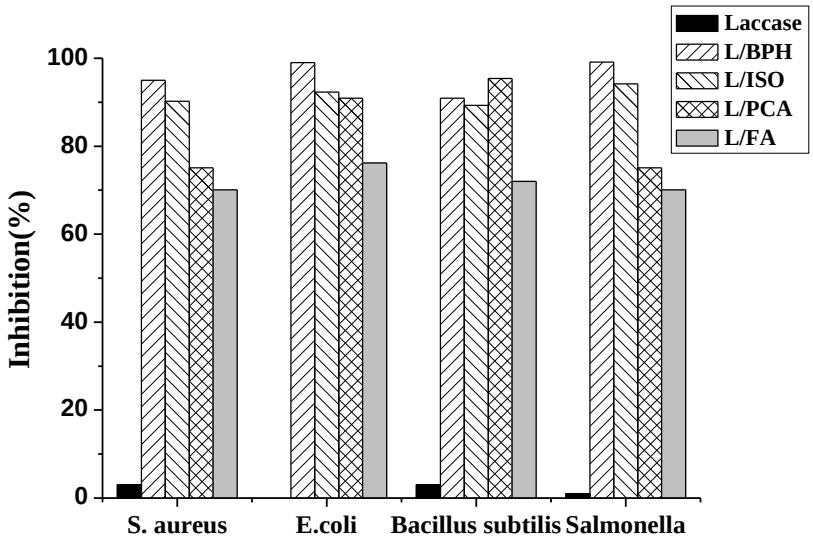


Fig. 1. Inhibition due to pulp treated with various laccase/phenolic compounds (%)

Compared with the samples treated by laccase separately, pulp obtained by treatment with laccase/phenolic compounds possessed higher antimicrobial activity towards growth of bacterial. Pulp treated with laccase/BPH produced the greatest reduction in the growth of bacteria after 24 h of cultivation (94.5%, 90.9%, 99.0%, and 99.1% inhibition of *S. aureus*, *Bacillus subtilis*, *E. coli*, and *Salmonella*, respectively). The average antimicrobial effect reached 90.9%.

The second best performer was the pulp treated with laccase/ISO, which also maintained good overall antimicrobial activity. Laccase/PCA had good resistance to *E. coli*, *Bacillus subtilis* and *Salmonella*, but especially towards *Bacillus subtilis* where the antimicrobial effect reached 95.0%. Laccase/FA also showed antimicrobial activity, but was inferior to the others. The results coincide nicely with the bacteriostatic efficacy of the phenolic compounds discussed earlier.

The laccase/phenolic-treated paper sheets showed a much greater efficacy against Gram-positive and Gram-negative bacteria than paper sheets treated only with laccase. Recently, Schröder *et al.* (2007) reported that laccase-assisted modification of flax fibres with ferulic acid or hydroquinone monomers improved their antibacterial activity toward the Gram-positive *Bacillus subtilis* and Gram-negative *E. coli* bacteria. Widsten *et al.* (2010) showed that direct grafting of hydrolysable tannins, particularly tannic acid and cationic mimosa tannin significantly inhibited the growth of *S. aureus* on wood veneer and pulp and also exhibited some antibacterial effect on *E. coli*. The grafting of the tannins was confirmed by studies with tannins and lignin model molecules, which showed covalent coupling only in the presence of laccase. Based on these results, we

conjecture that the phenolic compounds used in our study might be reacting to different extents with the laccase and, therefore, be potentially grafted onto the lignocellulose surface of the fibers, thus imparting highly effective antibacterial properties.

Morphological Parameters, Kappa Numbers, and SEM Analysis

Unbleached kraft pulp treated with laccase/phenolic compound clearly has an enhanced antimicrobial effect. Also, the dissolution type of the handsheets indicates that the phenolic compounds produce phenol oxygen free radicals during the laccase oxidation reaction. To further research the influence of phenols on the handsheets' antimicrobial activities, the kappa number of the pulps and their surface morphologies were investigated.

Figure 2 summarizes the results of the morphological tests made on the fibers. The tests indicate that the fibers treated with laccase/phenolic compounds were significantly coarser than those in the control sample and the pulp processed only with laccase. This is probably due to the phenolic compound producing free radicals during the catalytic oxidation process leading to condensation reactions with the lignin in the pulp.

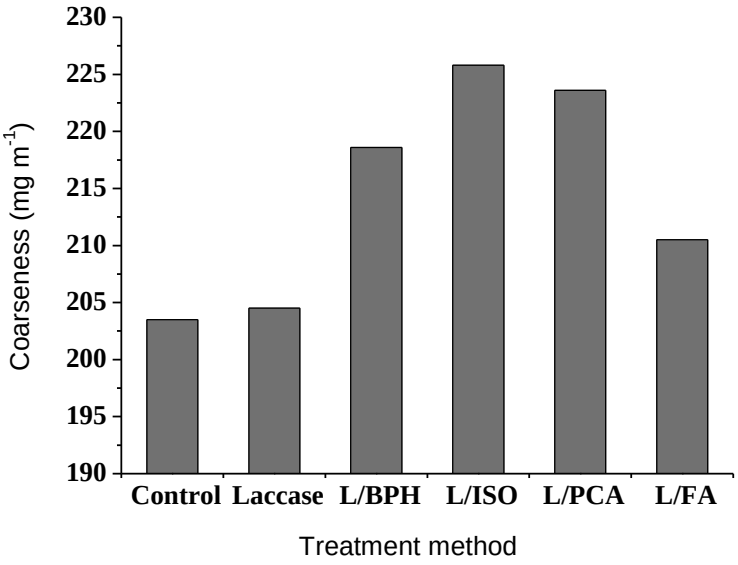


Fig 2. Effect of laccase/phenolic compound treatment on fiber quality

After the enzymatic treatment, the kappa numbers of the treated samples were increased with respect to the control (Fig. 3). This suggests that the laccase leads to a coupling of these phenolic compounds with the fibers. The highest degree of grafting was obtained with ISO, but the other phenols also produced kappa numbers larger than those of the control and laccase itself. This coincides with the conclusions of Aracri and Vidal (2009) and Fillat *et al.* (2009). This behavior implies that the mediators induce radical-coupling reactions with the pulp.

In order to further understand the changes occurring in the fibers, the morphology of the fiber surfaces after treatment with laccase/phenolic compound was studied using SEM. SEM images of the fiber surfaces in the control and samples treated with laccase and laccase/BPH are presented in Fig. 4.

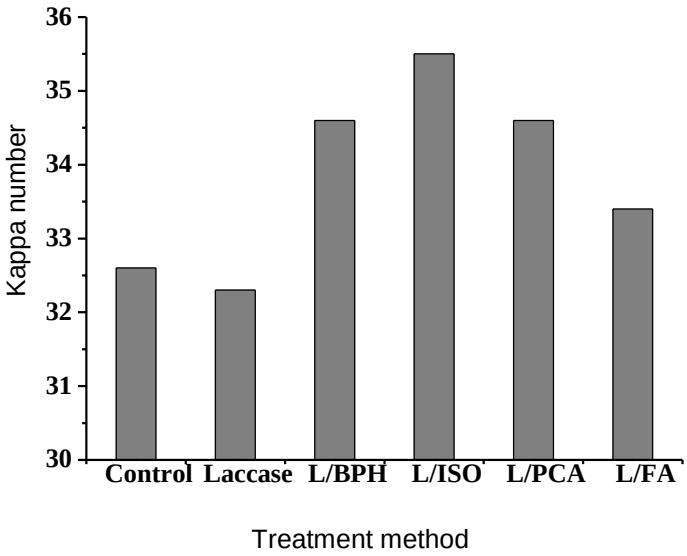
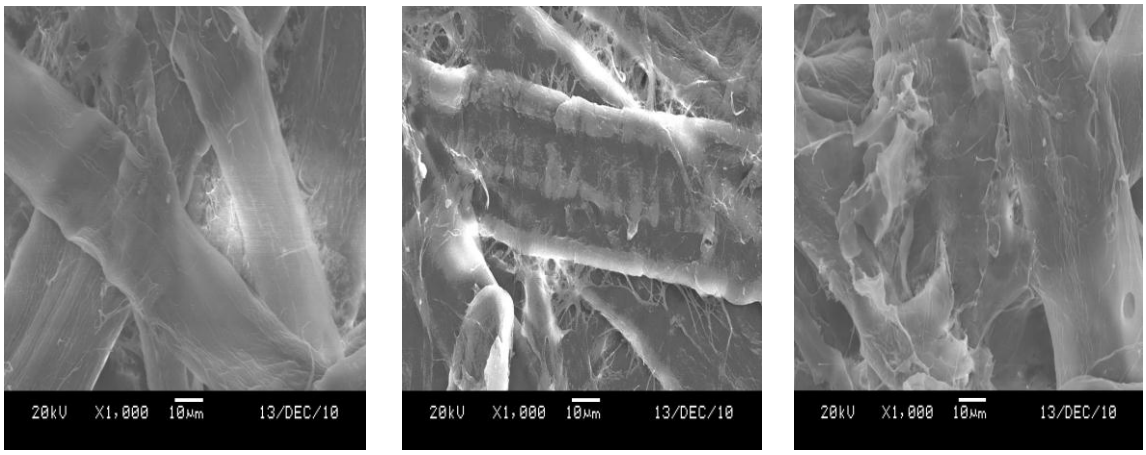


Fig. 3. The Kappa numbers of pulp treated with laccase/phenolic compounds



(a) (b) (c)

Fig. 4. SEM images of unbleached kraft pulp fibers, showing (a) control fibers, (b) laccase-treated fibers, and (c) laccase/BPH treated fibers

As can be seen in Fig. 4(a), the structures of the control pulp fibers were smooth and neat; the fibers were able to simply weave together. After laccase treatment, as shown in Fig. 4(b), adhesion between adjacent fiber surfaces occurred to some extent, and so the fiber surface became slightly rougher. Figure 4(c) reveals that the fibers treated with laccase/BPH became agglutinated over a wide area, which is in accordance with the increase in kappa number and coarseness of the fibers. As phenolic compounds were used in the treatment, their oxidation by laccase produced compounds that readily undergo polymerization. Therefore, we propose that with laccase only there is limited scope for bonding of the fibers in the pulp because of the potential use of hydroxide radicals from the lignin structure. However, with added phenolic compound, the supplied Ph-OH functional groups produce copious amounts of free radicals after oxidation by the laccase. These can then enter into the internal parts of the fibers because of sub-microscopic spaces in the structure and activate the surface of fibers, producing cross-linking from fiber to fiber and enhancing the antimicrobial effect of the paper.

Zeta Potential Analysis

The fibers produce a certain amount of carboxyl products during the cooking and bleaching process. Also, the raw materials have acidic groups, including the phenol hydroxyl groups on the residual lignin, *etc*. The hydrogen atoms on these acidic groups produce an electrically conductive medium in the presence of water and result in negatively charged pulp fibers. The zeta potential of the control sample was -43.2 mV (Table 5). However, as the table shows, the absolute values of the zeta potentials of the pulps treated with laccase/phenolic compounds were significantly reduced. We believe that the phenolic compounds produce cross-linking within the fibers in the presence of laccase and the phenolic compounds thus covering the fiber surface and reducing the absolute value of the negative charge on the fibers.

Table 5. Zeta Potentials of Differently Treated Pulp

Treatment	Zeta potential (mV)
Control	-43.2
L/BPH	-34.5
L/ISO	-37.1
L/PCA	-37.2
L/FA	-39.4

XPS Analysis

X-ray photoelectron spectroscopy (Fig. 5) has proven to be a useful tool for the study of the chemical structure of fiber surfaces. The core of the XPS method as far as fiber surface research is concerned, is centered on oxygen and carbon analyses. The surface lignin concentration can be inferred by determining the O/C ratio in low-resolution XPS spectra.

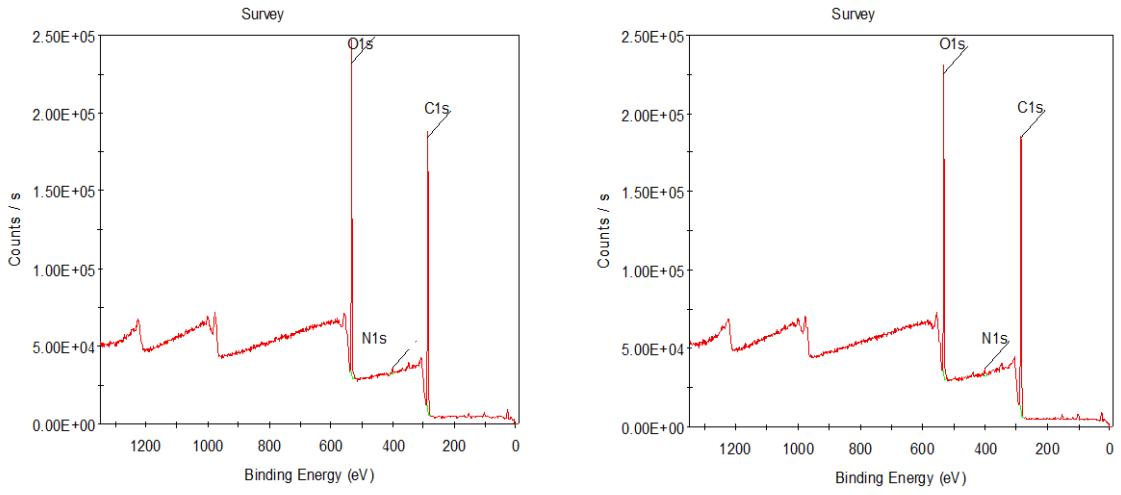


Fig. 5. XPS survey spectra of (a) control fibers, and (b) L/BPH treated fibers

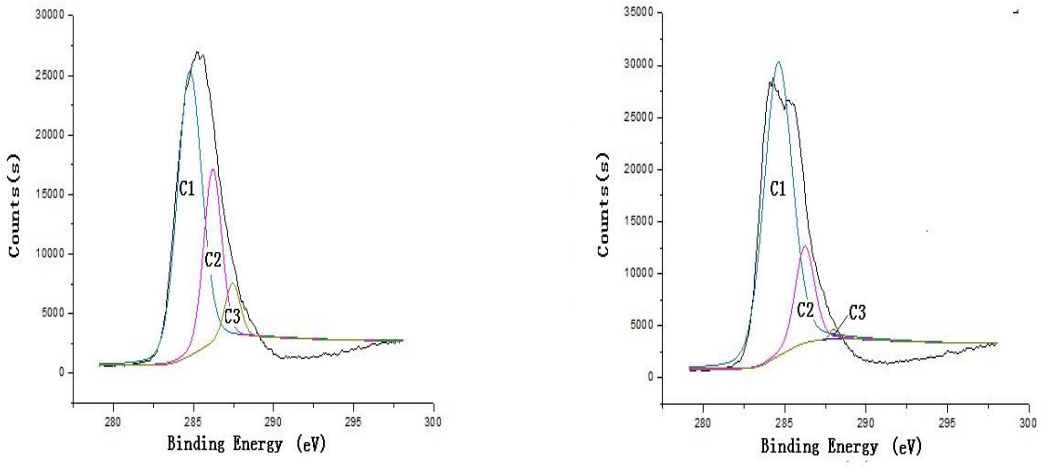
XPS can detect the oxygen and the carbon atom ratio at a shallow depth corresponding to the fiber surface, and the O/C ratio determined can represent the content of the lignin and extractive in the pulp fiber surface. If the O/C ratio is lower, the contents of the pulp fiber surface lignin and extractive are higher, and the middle layer covered by fiber surface may be higher. In contrast, the carbohydrate content of exposed fiber surface would be higher (Dorris and Gray 1978). Figure 5 shows wide scan XPS spectra of control fibers and L/BPH treated fibers. There are two prominent, sharp peaks due to the elements carbon and oxygen

As can be seen from Table 6, the O/C ratio of the fiber surface of the sample treated with laccase and BPH was lower than that in the control sample. Also, the surface lignin content increased from 72.8% to 78.3% after treatment with laccase and the phenolic compound.

Table 6. O/C Ratios and Surface Lignin Content of Two Different Samples

Samples	Control	L/BPH
C1s (%)	67.6	68.8
O1s (%)	31.5	30.2
O/C (%)	46.6	43.9
Surface lignin content (%)	72.8	78.3

Figure 6 shows high-resolution XPS spectra of the three different bands C1, C2, and C3 present in the pulp used in this study. The peak at electron binding energy 284.6 eV (C1) was from the fiber surface lignin content. That at 286.2 eV (C2) was mainly due to the cellulose hydroxyl absorption. The peak with an electron binding energy of 288.0 eV (C3) was principally from the carbonyl and O–C–O structure of the fibers. This latter characteristic peak would appear only after the wood fiber surface was oxidized. A C4 peak was not detected in any of the samples, which indicates that there are few carboxyl groups existing in the fiber surface.



(a) (b)

Fig. 6. High-resolution spectra of C1s peaks of (a) control fibers, and (b) L/BPH treated fibers

The data in Table 7 shows that the C1 ratio of the pulp increased when treated with laccase/phenolic compound compared with control samples. We infer from this that the concentration of the lignin in the surface of the fiber increased. However, the ratio of C2 and C3 decreased, which illustrates that the ratio of the hydroxide radical and carbonyl groups in the fibers decreased and the fiber surface lignin increased. We have speculated that the phenolic compound causes linking within the fiber surface in the presence of the catalytic oxidation of laccase and this improves the antimicrobial activity of the fibers. So, these XPS results provide some evidence for the grafting of phenolic compounds onto the fibers.

Table 7. Peak Areas and Binding Energies of Different Samples

Samples	Peak Area (%)			Electron Binding Energy (eV)		
	C1	C2	C3	C1	C2	C3
Control	63.5	28.6	7.9	284.6	286.2	288.0
Laccase/BPH	80.8	18.2	1.0	284.6	286.2	288.0

CONCLUSIONS

1. The enzymatic treatment of unbleached kraft pulp with laccase in the presence of different phenolic compounds (isoeugenol, butyl *p*-hydroxybenzoate, *p*-coumaric acid, and ferulic acid) resulted in their incorporation into the pulp. The results of antimicrobial tests indicate that the pulps produced by laccase/phenol treatment showed a greater efficacy against Gram-positive and Gram-negative bacteria than the pulps treated only with laccase and control samples. The pulp obtained with laccase/BPH had the best efficacy against the growth of bacteria, with resistance to *S. aureus*, *Bacillus subtilis*, *E. coli*, and *Salmonella* reaching 94.5%, 90.9%, 99.0%, and 99.1%, respectively.
2. Evaluation of the type of dissolution of the paper suggested that pulp treated with laccase/phenolic compounds belong to a non-dissolution type. From a determination of the fiber morphology and kappa number, the fiber coarseness when treated with laccase/phenolic compounds increased significantly compared with the control sample. Zeta potential data demonstrated that the change in the electronic charge on the fibers is due to the phenolic compounds produced cross-linking with the fibers. At the same time, when unbleached kraft pulp was treated with laccase, the fiber surface became rough. After treatment with laccase/phenol compounds, the fiber surface was agglutinated together over a wide area. Compared with the control sample, the zeta potential of the pulp treated with laccase and phenolic compounds was reduced significantly. These results demonstrate that the phenolic compounds form a chemical bond with the fibers rather than being simply physically adsorbed in the presence of laccase. The results of XPS indicate that the O/C ratio was decreased and the surface lignin content of fibers was increased after treatment with laccase and phenolic compounds.
3. Based on the results of this work, we speculate that the phenolic compounds could be assayed as mediators of laccase in order to confer new properties to pulp fibers for

use in practical applications. The mechanism underlying the antimicrobial activity of the paper is worthy of further study.

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REFERENCES CITED

Ahvenainen, R. (2003). *Novel Food Packaging Techniques*, Woodhead Publishing, UK, Cambridge, 384-400.

Aracri, E., Colom, J. F., and Vidal, T. (2009). "Application of laccase-natural mediator systems to sisal pulp: An effective approach to biobleaching or functionalizing pulp fibres," *Bioresour. Technol*. 100(23), 5911-5916.

Aracri, E., and Vidal, T. (2009). "Laccase- induced coupling of natural phenols onto sisal fibres," ISWFPC, P089.

Bordenave, N., Grelier, S., and Coma, V. (2010). "Hydrophobization and antimicrobial activity of chitosan and paper-based packaging material," *Biomacromolecules*. 11(1), 88-96.

Buchert, J., Gronqvist, S., Mikkonen, H., Oksanen, T., Peltonen, S., Suurnakki, A., and Viikari, L. (2005). "Process for producing a fibrous product," *European Patent* WO20050617.

Burini, G. (1994). "Determination of the alkyl esters of p-hydroxybenzoic acid in mayonnaise by high-performance liquid chromatography and fluorescence labeling," *J. Chromatogr*. 664(2), 213-219.

Burt, S. (2004). "Essential oils: Antibacterial properties and potential applications in food – A review," *Int. J. Food Microbiol*. 94(3), 223-253.

Canfora, L., Lamarino, G., Rao, M. A., and Gianfreda, L. (2008). "Oxidative transformation of natural and synthetic phenolic mixtures by *Trametes versicolor* laccase," *J. Agric. Food Chem*. 56(4), 1398-1407.

Cha, D. S., and Chinnan, M. S. (2004). "Biopolymer-based antimicrobial packaging: A review," *Crit. Rev. Food. Sci. Nutr*. 44(4), 223-237.

Chandra, R. P., and Ragauskas, A. J. (2001). "Laccase: The renegade of fiber modification," TAPPI Pulping Conference, TAPPI Press, Atlanta, GA, 1041-1051.

Chandra, R. P., Lehtonen, L. K., and Ragauskas, A. J. (2004). "Modification of high lignin content kraft pulps with laccase to improve paper strength properties.1. Laccase treatement in the presence of gallic acid," *Biotechnol Progress*. 20(1), 255-261.

Dorris, G. M., and Gray, D.G. (1978). "The surface analysis of paper and wood fibers by ESCA (Electron spectroscopy for chemical analysis). I. Application to cellulose and lignin," *Cellulose Chemistry Technology*. 12(4), 9-23.

Elegir, G., Kindl, A., Sadocco, P., and Orlandi, M. (2008). "Development of antimicrobial cellulose packaging through laccase-mediated grafting of phenolic compounds," *Enzyme Microbial Technology*. 43(2), 84-92.

Fillat, A., Colom, J. F., and Vidal, T. (2008). "Natural mediators in flax pulp delignification by laccase," International Pulp Bleaching Conference, Quebec (Canada), 247-248.

Fillat, A., Gallardo, O., Vidal, T., Pastor, F. J, Díaz, P., Colom, J. F., and Roncero, M. B. (2009). "Evaluation of natural mediators for developing antimicrobial properties of pulp fibers," ISWFPC, P046.

Gutiéirrez, L., Sácnchez, C., Battle, R., and Nerin, C. (2009). "New antimicrobial active package for bakery products," *Trends Food Sci. Technol*. 20(2), 92-99.

Holley, R. A. and Patel, D. (2005). "Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials," *Food Microbiol*. 22(4), 273-292.

Johannes, C., and Majcherczyk, A. (2000). "Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems," *Applied and Environmental Microbiology*. 66(2), 524-528.

Kenealy, W. R., Klungness, J., Tshabalala, M. A., Akhtar, M., Horn, E., Gleisner, R., and Buschle-Diller, G. (2003). "Modification of the physical properties of lignocellulosic materials by laccase," *Abstracts of Papers of the American Chemical Society*. 225, U270.

Leonowicz, A., Cho, N. S., Luterek, J., Wilkolazka, A., Wojtas-Wasilewska, M., Matuszewska, A., Hofrichter, M., Wesenberg, D., and Rogalski, J. (2001). "Fungal laccase: Properties and activity on lignin," *J. Basic Microbiol*. 41(3-4), 185-227.

Mainville, I., Montpetit, D., Durand, N., and Farnworth, E. R. (2001). "Deactivating the bacteria and yeast in kefir using heat treatment, irradiation and high pressure," *Int. Dairy J*. 11(1-2), 45-49.

Rodriguez Couto, S., and Toca Herrera, J. L. (2006). "Industrial and biotechnological applications of laccases: A review," *Biotechnology Advances* 24(5), 500-513.

Rooney, M. L. (1995). *Active Food Packaging*, Chapman & Hall, London.

San Martin, M., Harte, F. M, Lelieveld, H., Barbosa-Canovas, G. V., and Swanson, B. G. (2001). "Inactivation effect of an 18-T pulsed magnetic field combined with other technologies on *Escherichia coli*," *Innov. Food Sci. Emer*. 2(4), 273-277.

Schroeder, M., Aichernig, N., Guebitz, G. M., and Kokol, V. (2007). "Enzymatic coating of lignocellulosic surfaces with polyphenols," *Biotechnology journal*. 2(3), 334-341.

Silva, C., Matama, T., Kim, S., Padrao, J., Prasetyo, E. N., Kudanga, T., Nyanhongo, G. S., Guebitz, G. M., Casal, M., and Cavaco-Paulo, A. (2011). "Antimicrobial and antioxidant linen via laccase-assisted grafting," *React. Funct. Polym*. 71(7), 713-720.

Song, Y. S., Park, H. J., and Komolprasert, V. (2000). "Analytical procedure for quantifying five compounds suspected as possible contaminants in recycled paper/paperboard for food packaging," *J. Agric. Food Chem*. 48(12), 5856-5859.

Ström, G., and Carlsson, G. (1992). "Wettability of kraft pulps-effect of surface composition and oxygen plasma treatment, " *J. Adhes. Sci. Technol*. 6(6), 745-761.

Triantafyllou, V. I., Akrida-Demertzi, K., and Demertzis, P. G. (2007). "A study on the migration of organic pollutants from recycled paperboard materials to solid food matrices," *Food Chem*. 101(4), 1759-1768.

Tsou, M. F., Hung, C. F., Lu, H. F., and Wu, L. T. (2000). "Effects of caffeic acid, chlorogenic and ferulic acid on growth and arylamine N-acetyltransferase activity in *Shigella sonnei* (groupD)," *Microbios*. 101(398), 37-46.

Weetall, H. H. (1985). "Enzymic synthesis of gallic acid esters," *Appl. Biochem. Biotechnol*. 11(1), 25-28.

Widsten, P., Heathcote, C., Kandelbauer, A., Guebitz, G., Nyanhongo, G. S., Prasetyo, E. N., and Kudanga, T. (2010). "Enzymatic surface functionalisation of lignocellulosic materials with tannins for enhancing antibacterial properties," *Process Biochemistry*. 45(7), 1072-1081.

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