

Laccase catalysed grafting of phenolic onto xylan to improve its applicability in films

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Abstract. Xylan can be tailored for various value-added applications. However, its use in aqueous systems is hampered by its complex structure, and small molecular weight. This research aimed at improving the xylan molecular weight and changing its structure. Laccase-catalysed oxidation of 4-coumaric acid (PCA), ferulic acid (FA), syringaldehyde (SD), and vanillin (VA) onto xylan was grafted to study the changes in its structure, tensile properties, and antibacterial activities.

A Fourier transform infrared (FTIR) spectrum analyser was used to observe the changes in functional groups of xylan. The results showed a band at 1635 cm^{-1} corresponding to the stretching vibration of conjugated carbonyl carboxy hemoglobin and a benzene ring structure were strengthened; the appearance of a new band between 1200 cm^{-1} and 1270 cm^{-1} corresponding to alkyl ethers on the aryl C-O stretching vibration was due to the fact that during the grafting process, the number of benzene ring structures increased and covalent connections occurred between phenols and xylan.

The reaction mechanism for the laccase-catalysed oxidation of phenol compounds onto xylan was preliminary explored by ^{13}C -NMR. The results showed that PCA-xylan, FA-xylan graft poly onto xylan by C_γ ester bond, SD-xylan graft poly onto xylan by ether bond and an ester bond, and VD-xylan graft poly onto xylan by ether bond.

The film strength of xylan derivatives has been significantly increased, especially for the PCA-xylan derivative. The increases in tensile stress at break, tensile strength, tensile yield stress, and Young's modulus were: 24.04%, 31.30%, 55.56%, and 28.21%, respectively. After laccase/phenolics were modified, xylan had a good antibacterial effect to *E. coli*, *Corynebacterium glutamicum*, and *Bacillus subtilis*. The SD-xylan, FA-xylan, and PCA-xylan showed a greater efficacy against *E. coli*, *Corynebacterium glutamicum*, and *Bacillus subtilis*, respectively.

1. Introduction

Lignocellulosic biomass is one of the most widely available, renewable, resources: it represents a promising low-cost raw material for the production of biofuel, bioenergy, and value-added

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biomaterials [1]. The main components of lignocellulosic biomass are: cellulose, hemicellulose, and lignin. Hemicelluloses, the second most abundant class of polysaccharides found in nature after cellulose, comprises roughly one-fourth to one-third of most plant materials [2]. In the pulp and paper making process, significant quantities of hemicellulose are dissolved and burned as fuel. The portion of hemicellulose burned can be roughly estimated at about 40 million tonnes per year [3]. How to make better use of the hemicellulose has become a hot topic among researchers. However, its use in aqueous systems is hampered by its complex structure and low molecular weight [4-6].

Hemicellulose contains many hydroxyl groups in the molecule. Many studies on hemicelluloses have been carried out by chemical modification (grafting, esterification, sulphonyl reaction, etherification, etc.). An active functional group is introduced to improve the film formation properties (such as solubility, hydrophilicity, barrier properties, and crystallisation) [7]. Chemical modification of hemicelluloses requires harsh reaction conditions, and will produce certain harmful by-products, while the use of bio-enzymatic modification, with its milder reaction conditions, can be more environmentally friendly. Thus, in recent years, using biological enzymes to catalyse biological graft-modified hemicellulose has become another focus of research attention. Some teams use laccase catalysed oxidation of aromatic groups for grafting onto galactoglucomannan, and the cross-linking treatment resulted in significant improvement of the mechanical properties of barrier films made with spruce galactoglucomannan [8]. However, the aromatic group best suited for grafting onto xylan remains to be studied. In addition, the main product for the modern paper-manufacturing industry is mechanical pulp, and its main raw materials are hardwood and grasses, which are important sources of hemicellulose.

Xylan is the main component of hemicellulose from grasses and hardwood: it comprises complex molecular polysaccharides based on the β -1, 4 xylosidic bond connecting D-xylose residues for its main chain [7]. In the presence of oxygen, a phenolic hydroxyl group can be oxidised to phenolic oxygen free radicals by laccase, and phenoxy radical mutual condensation generates macromolecules. Thus, laccase catalysing phenols were used as agents to be grafted onto xylan in this work.

In this research, phenols were oxidised to phenolic oxygen free radicals by laccase, and reacted with xylan by graft copolymerisation to improve the film-forming properties thereof: this also endowed the material with good antibacterial properties, thus expanding its potential areas of application. Infrared radiation (IR), and nuclear magnetic resonance (NMR), analyses proved the feasibility of the proposed graft modification method.

2. Materials and methods

2.1. Raw materials and reagents

Straw was sourced from Shandong; laccase, catalogue item number NOVOZYME 51003, with an enzyme activity of 1072 u/mL was provided by the Novozyme Company; phenols were purchased from the Sigma-Aldrich Corporation; two types of xylan were used: a) commodity-grade xylan: purchased from Shanghai Jing Chun Biochemical Technology Co., Ltd, with a molecular weight of about 30 kDa (200 sugar units); b) homemade xylan: laboratory preparation; other reagents were purchased from Tianjin Chemical Reagent Factory.

2.2. Experimental methods

2.2.1. Preparation of xylan. Straw powder was soaked in 60 g/L of NaOH solution with a solid-liquid ratio of 1:15, and the reaction took place at 100°C for 2 h, the filtrate was then obtained after filtering and pH adjustment to 7.0, the filtrate was then precipitated with the ratio of filtrate to ethanol being 0.8. After washing with anhydrous ethanol, the precipitate was obtained for use as the xylan sample [9].

2.2.2. Xylan with grafted phenols reaction. First, 90 ml of acetic acid buffer solution (pH 4.8) was

added to 1 g of homemade xylan in a beaker; then 10 ml of 5 mmol/L methanol solution of phenols was added to the beaker; thereafter, the beaker was stored in water at 40°C for 5 min and oxygen was supplied using an oxygen pump, then 200 µl of laccase was added and the reaction continued for 4 h in water at 40 °C. After the completion of this grafting reaction, absolute ethyl alcohol of a volume of three times of the reaction solution volume was added, and the resulting precipitate observed. After 24 h, the refined precipitate was obtained through repeated washing in absolute ethyl alcohol. The precipitate was then dried in vacuum at 60 °C for 24 h and the sample of xylan with grafted phenol was obtained.

2.2.3. FT-IR analysis. The VECTOR FTIR spectrometer (Brooke Instrument Company, Germany) was used on the xylan and its derivatives over 16 scans, capturing spectra between 500 and 4000 cm⁻¹, at a resolution of 4 cm⁻¹: samples were previously kept in the dark and dried for 24 h at room temperature in a desiccator.

2.2.4. NMR analysis. NMR spectra were captured by a Swiss Brook Baiesibin 400 M superconducting NMR spectrometer: 20 mg of xylan and graft product were dissolved in 0.6 mL of deuterated D₂O, the sampling conditions were as follows: sampling time 3.98 s, relaxation time 2.0 s, and accumulated 128 times, with a concentration of 4.70 ppm (using the solvent peak pattern) for correction. ¹³C spectra were taken from 100 mg samples dissolved in 0.6 mL of D₂O under the following sampling conditions: 30° (using the pulse sequence), sampling time 1.36 s, relaxation time 1.89s, and accumulated 30,000 times [10].

2.2.5. Preparation of the film from xylan and grafted product. Eight 0.6 g xylan or graft-modified product samples, and 0.4 g carboxymethyl cellulose sodium were each added separately to 35 ml of deionised water and stirred for 60 min at 80 °C, and then poured into a glass mould at room temperature (23°C, 50% humidity) for casting, and dry film manufacture [8, 10].

2.2.6. Tensile properties of film. A computer-controlled electronic universal testing machine (CMT4503, Shenzhen sans Testing Machine Co., Ltd, China) was used for film testing. The dried film was cut into 80 mm × 15 mm spindle-shaped samples, then their thickness was measured. The prepared film strip was placed on a microcomputer-controlled electronic universal testing machine for testing in accordance with Chinese Standard GB/T 1040.3-2006. The cross-head speed was 50 mm / min, and the test gauge length was 25 mm.

2.2.7. Antibacterial properties. The inhibition zone method was used for detecting the antibacterial activity of the film. First, indicator bacteria cultures were taken whose bacteria concentration was 105 to 106 CFU / mL, each with a volume of 0.1 mL which was added dropwise to beef extract peptone medium plates (diameter 90 mm), and the coating stick was then used to paint them evenly onto the flat surface before natural drying for 30 min. Then sterilised film sample wafers (diameter 5 mm) were placed evenly on the plate. The dishes were placed in an incubator for 24 h as 37°C, for measurement of the inhibition zone diameter. Each experiment was repeated three times per sample, and the results averaged.

3. Results and discussion

3.1. Xylan and grafted product FT-IR analysis

3.1.1. FT-IR characterisation of xylan. In the experiments, straw powder was used as a raw material in the alkali extraction method for preparing xylan. Firstly, compared with homemade xylan, the infrared absorption peaks of factory produced xylan are shown in figure 1.

Figure 1 shows that the infrared spectrum of homemade xylan had two distinct peaks at 1043 cm⁻¹

and 3396 cm^{-1} : these were from a sugar unit within a hydroxyl group and an ether linkage (C-O-C) absorption peak [8]. Spectrum (1) was the IR spectrum of commodity-grade xylan: it revealed that the band between 1170 cm^{-1} and 1000 cm^{-1} contained the typical absorption peaks of xylan, the bands at 1382 cm^{-1} , 1237 cm^{-1} , and 1249 cm^{-1} were -C-H stretching vibration, or -OH, or -C-O bending vibrations, the bands at 1157 cm^{-1} and 989 cm^{-1} were typical arabinose absorption peaks; and the band at 1045 cm^{-1} was a -C-C stretching vibration and hemicellulose -C-OH mixed-mode vibrations [10]. Spectrum (2) contained typical xylan absorption characteristics (1157.08 cm^{-1} and 1043.30 cm^{-1} peaks) that showed that homemade xylan contained the main structural elements of commercial-grade xylan: *i.e.* its pyran ring structure [11]. By this analysis, the structure of homemade xylan was shown to be consistent with commodity-grade xylan.

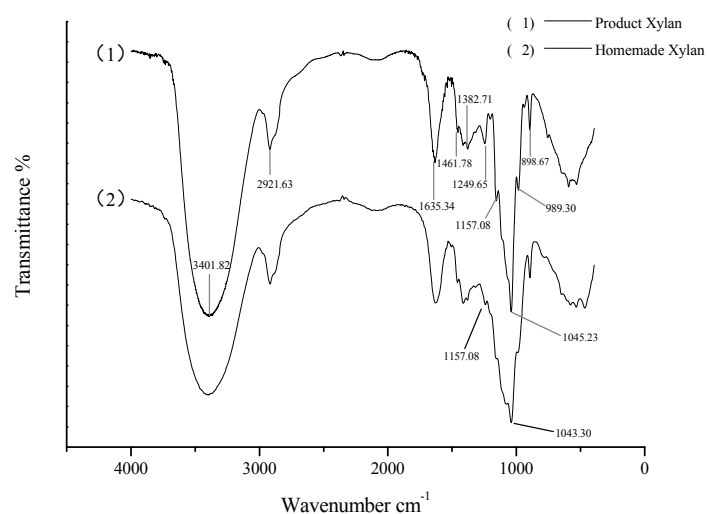


Figure 1. Commodity-grade xylan and homemade xylan blank FT-IR comparative analysis.

3.1.2. FT-IR characterisation of grafted product. The study aimed to improve the molecular weight of xylan and change its structure. 4-coumaric acid (PCA), ferulic acid (FA), syringaldehyde (SD), and vanillin (VA) were grafted onto xylan by laccase-catalysed oxidation. FTIR was used to observe the changes in functional groups, as shown in figure 2.

As can be seen from figure 2, a band at 1635 cm^{-1} came from conjugated carbonyl carbon and oxygen stretching vibration and the benzene ring structure absorption peak. This showed that the homemade xylan contained side groups with a benzene ring structure. A strong absorption peak near 1043 cm^{-1} came from a stretching vibration of the carbonyl carbon and oxygen in the xylan: this further explained that the hemicellulose prepared was, indeed, xylan.

The xylan after being laccase grafted, had a band at 1635 cm^{-1} corresponding to stretching vibration of conjugated carbonyl carboxyhemoglobin and a benzene ring structure which had been strengthened [12, 13]; a new band appeared between 1200 cm^{-1} and 1270 cm^{-1} corresponding to those alkyl ethers on the aryl C-O stretching vibration which showed that, during the grafting process [11], the benzene ring structure was stiffened and covalent connections occurred between phenols and the xylan.

Furthermore, in the infrared spectra of 4-coumaric acid-xylan and ferulic acid-xylan, new bands appeared at 1245 cm^{-1} and 1395 cm^{-1} , these two absorption peaks arose from carboxylic acid C=O stretching vibrations, and -OH bending vibrations, respectively [8]. In the infrared spectra of ferulic acid-xylan, syringaldazin-xylan, and vanillin-xylan, a new band appeared at 1384 cm^{-1} , this was the bending vibration of -CO-CH₃. By FTIR analysis, the phenols used were covalently grafted onto the xylan, and its benzene ring structure was stiffened.

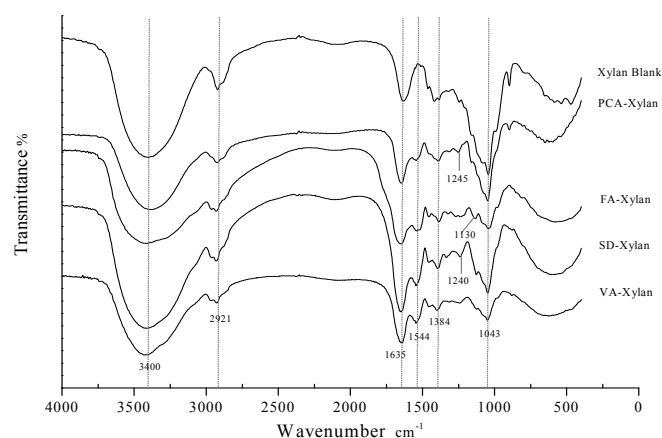


Figure 2. Homemade xylan and phenol-xylan FT-IR comparative analysis.

3.2. Xylan and grafted product ^{13}C -NMR analysis

To observe the xylan and grafted product functional group structure, ^{13}C -NMR analysis (see figure 3) was used.

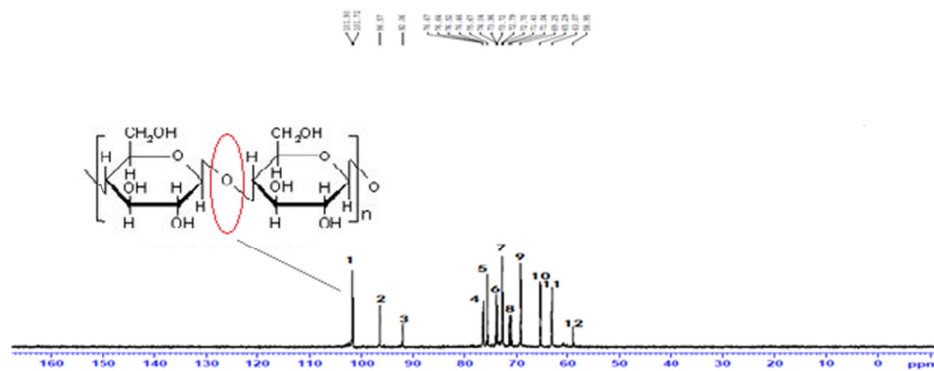
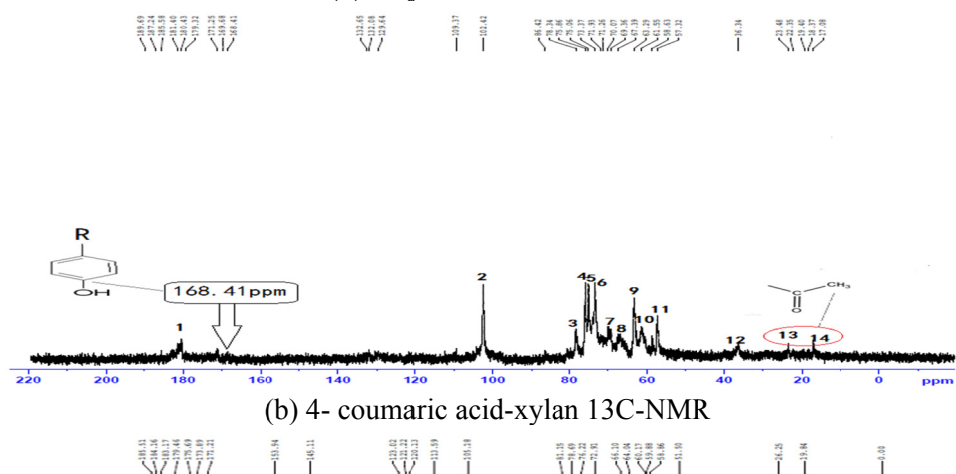
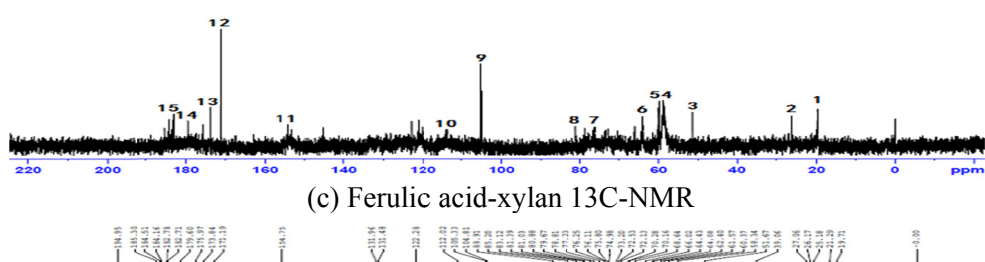
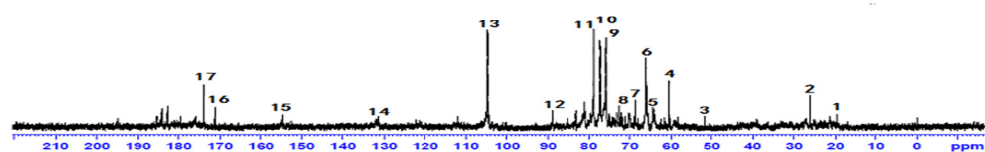
Xylan was the repeating structural unit represented by the inter-polymers of the monosaccharides which were mainly linked to each other by covalent bonding, hydrogen bonding, an ether bond, and an ester bond. Figure 3(a) shows anomeric carbon located at peak 1 (at 101.90 ppm) which showed that the xylan was based on β glycosidic linkage for its main chain [9].

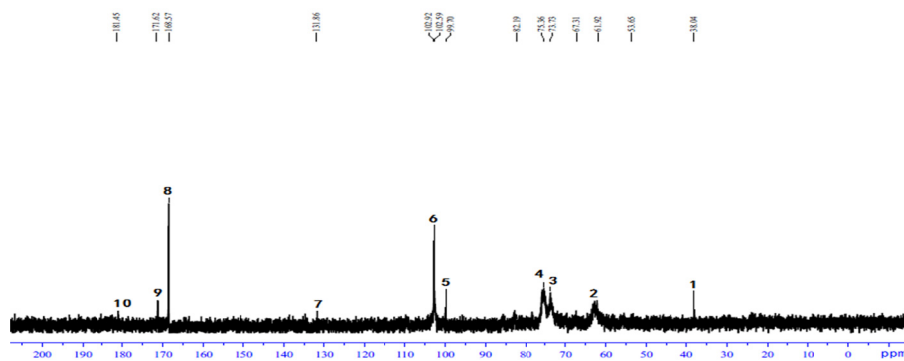
As seen from the comparison chart of figures 3(a) and 3(b), in figure 3(b), peak 13 (at 23.48 ppm), and 14 (at 18.37 ppm) was the methyl absorbent of the acetyl group [11]; a weak absorption peak appeared at 168.41 ppm which was the C-4 of the hydroxyphenyl; in addition, there was a weak absorption signal at 171.25 ppm which was the O-C=O absorption of the acetyl group and $\text{C}\gamma$ absorption of the cinnamic acid [14]. The hydroxyphenyl ring structure was found on the grafted xylan: the PCA was therefore grafted onto the xylan by C_γ ester bonding.

In figure 3(c), the peaks at 26.25 ppm, and 19.84 ppm were the methyl absorbent of the acetyl group; a weak absorption peak appeared at 145.11 ppm which was the C_α absorption of ferulic acid [11]; figure 3(b) showed strong absorption signals at 180 ppm and 173.89 ppm which were ester bond carbonyl and ferulic acid $\text{C}\gamma$ signals, respectively [14]. That showed that FA was grafted onto xylan by C_γ ester bonding.

In figure 3(d), an absorption peak appeared at 51.67 ppm which was the $-\text{OCH}_3$ absorption peak; a strong absorption band appeared at 75 ppm which formed the C2, C3, and C5 absorption signal for xylan, and the C3 and C4 absorption signal of a xylose structure; in addition, SD-xylan had a C_α peak (connected with the carbohydrate ether bond), etherified C_α (β -O-4) absorption signals at 83.12 ppm, and 85.20 ppm, an aromatic ring structure from C3/C5 (etherified) absorption at 154.75 ppm, and ester absorption signals at 171.19 ppm and 173.84 ppm [14]. That showed that SD-xylan grafted onto xylan by ether bonding and an ester bond.

Figure 3(e) shows an absorption peak at 38.04 ppm from the γ -methyl, and α and β -methylene absorption signals from lignin propane side chains; in addition, VA-xylan had a C_α peak (from carbohydrate ether bonding) at 82.19 ppm [11]; and a hydroxyphenyl C-4, vanillin C_α (etherified) absorption signal at 168.57 ppm [15]. That showed that VA-xylan was grafted onto xylan by ether bonding.

(a) Xylan blank ^{13}C -NMR(b) 4- coumaric acid-xylan ^{13}C -NMR(c) Ferulic acid-xylan ^{13}C -NMR(d) Syringaldazin-xylan ^{13}C -NMR

(e) Vanillin-xylan ^{13}C -NMR**Figure 3.** ^{13}C NMR spectrogram of homemade xylan and graft product.

3.3. Tensile properties of the film

We tested the tensile stress of the xylan and the grafted xylan films to characterise their mechanical strength, tensile yield stress, and Young's modulus (see results in table 1).

Table 1. Homemade xylan and its graft product film tensile properties.

Sample	Tensile Stress (σ_B) /MPa	Tensile Strength (σ_M) /MPa	Tensile yield stress (σ_Y) /MPa	Young's modulus (E) /MPa
Xylan	1.04	1.15	0.27	0.663
PCA-Xylan	1.29	1.51	0.42	0.850
FA-Xylan	1.21	1.43	0.33	0.771
SD-Xylan	1.13	1.47	0.35	0.758
VA-Xylan	1.18	1.33	0.37	0.761

Table 2. Changing material properties: homemade xylan compared to its graft products.

Sample	Increase in tensile stress/ %	Increase in tensile strength/ %	Increase in tensile yield stress/ %	Increase in Young's modulus/ %
PCA-Xylan	24.04	31.30	55.56	28.21
FA-Xylan	16.35	24.35	22.22	16.29
SD-Xylan	8.65	27.83	29.63	14.33
VA-Xylan	13.46	15.65	37.04	14.78

We measured the increase in strength and stiffness of the grafted xylan film (see table 2). The films produced with grafted xylan derivatives improved significantly with regards their tensile stress, tensile strength, tensile yield stress, and Young's modulus. Phenols were grafted onto the side chains of the molecular chain, the side groups induced steric hindrance, molecular chains of xylan underwent a relative separation, and reduced aggregation of molecular chains during film formation to improve its film-forming properties [16]. For testing, the grafted products selected were: PCA-xylose film, FA-xylose poly-film, SD-xylose poly-film, and VA-polyethylene film with xylose. The tensile stress increases at break were: 24.04%, 16.35%, 8.65%, and 13.46% respectively; the tensile strength increases were: 31.30%, 24.35%, 27.83%, and 15.65%, respectively; the tensile yield stress increases were: 55.56%, 22.22%, 29.63%, and 37.04% respectively; the Young's modulus increases were: 28.21%, 16.29%, 14.33%, and 14.78%, respectively. The results showed that the strength of films produced by xylan derivatives improved significantly, especially for the PCA-xylan derivative.

3.4. Antibacterial properties

In these experiments, 4-coumaric acid (PCA), ferulic acid (FA), syringaldehyde (SD), and vanillin (VA) were grafted onto xylan by laccase-catalysed oxidation, these phenolic compounds were commonly used in food preservatives, and their structures were similar to the structural phase in lignin (as found in paper). During the experiment, we mainly selected *E. coli*, *Corynebacterium glutamicum*, and *Bacillus subtilis* for antibacterial property evaluation.

Table 3. Homemade xylan and its graft product film antibacterial performance test results.

Sample	Bacterial inhibition zone diameter (mm)		
	<i>E. coli</i>	<i>Corynebacterium glutamicum</i>	<i>Bacillus subtilis</i>
Xylan	5	5	5
PCA-Xylan	10.0	10.5	10.0
FA-Xylan	11.0	11.5	8.5
VA-Xylan	9.0	10.0	7.5
SD-Xylan	12.0	11.0	8.0

Table 3 shows the antibacterial effect of xylan and grafted xylan on *E. coli*, *Corynebacterium glutamicum*, and *Bacillus subtilis*. As could be seen from the table, the untreated xylan film did not have any antibacterial activity, but after laccase/phenolic modification, xylan had good antibacterial effect on *E. coli*, *Corynebacterium glutamicum*, and *Bacillus subtilis*. The xylan grafted with different polyphenols exhibited different inhibitory effects. The SD-xylan showed a greater efficacy against *E. coli*; the FA-xylan showed a greater efficacy against *Corynebacterium glutamicum*; and the PCA-xylan had a greater efficacy against *Bacillus subtilis*.

Laccase catalysed the oxidation of phenols and grafted it onto xylan: after laccase/phenolic modification xylan had good antibacterial activity which was related to the phenolic itself exerting an inhibitory effect. After polyphenols had been grafted onto the side chains of the xylan, the intermolecular hydrogen bonds appeared to reduce in number, the re-stabilisation phenomenon began to weaken between molecules, at the same time, hydrophobic groups were introduced onto the modified xylan, and affinity between xylan and bacterial cells was enhanced [17], antibacterial activity centres increased in number, thereby enhancing the antimicrobial activity of xylan. Thus, xylan derivatives exhibited strong antibacterial properties under the synergistic effect of the phenolics.

4. Conclusion

This article revealed that grafting between phenols and xylan by laccase catalysis and oxidation represented a new approach to improving the molecular weight of xylan, changing the structure thereof, and to improving the applicability of xylan when used in barrier films.

Infrared spectra showed that, during the grafting process, benzene ring structures increased in stiffness and covalent connections occurred between the phenols and the xylan. ¹³C-NMR analysis showed that PCA-xylan, and FA-xylan, were grafted onto xylan by C_γ ester bonding, SD-xylan was grafted onto xylan by ether bonding and an ester bond, and VD-xylan was grafted by ether bonding.

The strength of films produced by xylan derivatives improved significantly, especially for the PCA-xylan derivative. After laccase/phenolic modification, xylan had good antibacterial effects on *E. coli*, *Corynebacterium glutamicum*, *Bacillus subtilis*, and SD-xylan, FA-xylan, and PCA-xylan showed a greater efficacy against *E. coli*, *Corynebacterium glutamicum*, and *Bacillus subtilis*, respectively.

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