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Article

Laccase Functionalization of Flax and Coconut Fibers

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Abstract: Natural fibers have gained much attention as reinforcing components in composite materials. Despite several interesting characteristics like low cost, low density, high specific properties and biodegradability they show poor compatibility with the polymer matrix. We have shown that it is possible to use a laccase from *Trametes hirsuta* as a biocatalyst to attach different types of functional phenolic molecules onto the fibers. A 5% incorporation of the functional molecules was achieved as measured via X-ray photoelectron spectroscopy (XPS) in flax although it was lower in coconut fibers. In combination with different mediators it was possible to broaden the activation scope and graft hydrophobic molecules like dimer fatty amines. Among the different mediators tested 1-hydroxybenzotriazole (HBT), 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO) and 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), TEMPO were the most effective achieving a 10% increase in carbon as measured by XPS.

Keywords: laccase; flax; coconut; dimer fatty amine

1. Introduction

Laccases (E.C.10.3.2), *p*-diphenol dioxygen oxidorecductases, are multi-copper containing enzymes performing a one-electron oxidation of many structurally different aromatic substrates while simultaneously reducing oxygen to water. This family of enzymes has found numerous applications in diverse fields like environmental pollution control [1], organic synthesis [2,3] and several industrial areas like pharmaceutical [4,5], food [6,7], textile [8,9], *etc.*

The reaction catalyzed by laccases generates highly reactive radicals, which makes it difficult to control the process and an accurate prediction of the final reaction products. However, laccases have been successfully used to functionalize complex substrates, turning their high unspecifcity into a benefit as well as upgrading the properties of pulp.

For example, bio-bleaching of pulp with different laccase has been successfully achieved [10,11]. Laccase mediated grafting of different types of phenols is a common strategy [12,13] that can be used to introduce new properties onto cellulosic fibers, e.g., conferring antibacterial properties [14], increasing the pulp strength and swelling properties [15]. In addition to modification of pulp properties, laccases have also been successfully used to improve the hydrophobicity of wood veneers by grafting fluorophenols [16] or alkylamines [17].

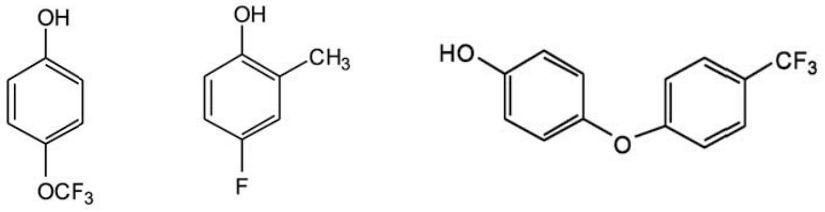
There is a huge potential for the production of novel materials through combining natural fibers with synthetic materials for the production of composites materials, with excellent properties. However, incompatibility issues with common materials like synthetic polymers [18] hinder a wider application. For example, the introduction of a hydrophilic material into a highly hydrophobic matrix as synthetic polymers, typically leads to a material with poor properties, due to the low adhesion between the fiber and the matrix. Depending on the nature of the fiber different chemical treatments followed by graft polymerization can be a suitable approach to improve the matrix-fiber interaction. There are several commercial coupling agents with different reactive chemistry like silane, isocianate, titanate or zirconate which can include a high variety of moieties into the fiber like vinyl, chloropropyl, epoxy, methacryl, amine, mecarpto, phosphate [18].

In this paper we show the effectiveness of two different approaches for the functionalization of coconut and flax fibers using laccase as environmentally friendly catalyst to effectively substitute traditional toxic chemicals. In the first approach laccase is used to oxidize different phenols, creating reactive radicals that can couple via formation of new C–C, β–O–5 or C–O bonds to the fiber. In the second approach using the same enzyme and in combination with some mediators it was possible to oxidize the lignin parts of the fiber generating phenol radicals that can easily reacted with amines. The functional molecules used were chosen based on previous mechanistic studies, which showed the possibility of oxidizing these molecules and coupling them onto lignin model substrates [16,17]. The study shows that, based on the properties of the phenolic molecules or amines used for grafting, it is possible to achieve a wide range of functional materials in an environmentally friendly way.

2. Experimental Section

Flax and coconut fibers were provided by DSM N.V. (Heerlen, The Netherlands), and the following dimer fatty amines (DFA) (Pripol^{TM} 1740, Croda, The Netherlands) was used. The fluorophenols, 4-(trifluoromehtoxy)phenol (4-F3MP), 4-fluoro-2-methylphenol (4,2-FMP) and 4-[4-(trifluoromethyl)phenoxy]phenol (4,4-F3MPP) (Figure 1) and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) and were of the highest grade available.

Figure 1. Structures of the fluorophenols used in the coupling reactions: (**a**) 4-F3MP; (**b**) 4,2-FMP; and (**c**) 4,4-F3MPP.



(**a**) (**b**) (**c**)

The laccase from *Trametes hirsuta* was produced and purified as previously described by Almansa *et al.* [8]. Laccase activity was determined spectrophotometrically by following the oxidation of, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) at 420 nm (ε = 36,000 M^{-1} cm^{-1}) as described by Greimel *et al.* [19] with some modifications. The reaction mixture contained 650 μL of enzyme diluted in 50 mM succinate buffer, pH 4.5 and 200 μL of ABTS (10 mM in 50 mM succinate buffer, pH 4.5). The absorbance was followed for 1 min in time scan mode with a spectrophotometer U-2001 (Hitachi, Tokyo, Japan). Enzyme activity is expressed in katal, whereas one katal (katal) is defined as the amount of enzyme that catalyzes the conversion of 1 mole of ABTS per second.

Direct and mediated functionalization of flax and coconut fibers was performed in 2 mL eppendorf tubes. In the case of the direct laccase fiber functionalization with the fluorophenols 15 mg of the corresponding fiber were placed in the tube with the corresponding fluorophenol in a final concentration of 2.0 mM with a final enzyme activity of 9 katal/mL in 50 mM succinate buffer pH 4.5 at 37 ℃ and 300 rpm for 3 h. The unbound fluorophenols were removed by washing with 75% ethanol for 16 h.

In the case of mediated coupling reaction of water insoluble DFA, a stock solution of 6 g/L in ethanol was produced; similarly a 20 mM stock solution of the mediators ABTS, 1-hydroxybenzotriazole (HBT) and 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO) in buffer (50 mM succinate buffer, pH 4.5) was prepared.

The reaction was carried out in 2 mL eppendorf tubes with 15 mg of fiber, 9 katal/mL of enzyme, 1 mM of the corresponding mediator and 2.7 g/L of the DFA. Incubation was carried out at 37 ℃, 3 h and 300 rpm using a Thermomixer (Eppendorf, Hamburg, Germany). The unbound amines were removed by with ethanol for 16 h.

The XPS measurements were carried out using a Quantera SXM X-Ray Photoelectron Spectrometer (XPS) from Ulvac-PHI (Q2) (Kanagawa, Japan). During the measurements the angle between the axis

of the analyzer and the sample surface was approximately 45 °; the information depth is then about 7 nm. The measurements were performed using monochromatic AlKα-radiation (100 Watt) and a measurement spot of 100 µm, scanned across an area of 1200 µm × 500 µm.

By means of wide-scan measurements the elements present at the surface were identified. Narrow-scan measurements have been performed to determine the concentrations and chemical binding states of the elements.

3. Results and Discussion

Oxidative enzymes have found wide applications in the field of polymer functionalization for different applications like personal care, textile industry, biosensors and organic synthesis [20].

In this study laccase oxidation of the lignin moieties present in coconut and flax had the goal of activating the lignin moieties present in the fibers, which in turn would allow covalent grafting of the functional fluorophenolic molecules. XPS analysis revealed the different reactivity of the flax and coconut fibers, which are most likely due to differences in their lignin content (Tables 1 and 2).

A slightly higher modification/incorporation of fluorophenols was achieved with flax fibers (Tables 1 and 2). Laccase oxidative coupling reactions involving different types of soluble molecules to lignin or lignin model substrates has previously been described for wood based materials [16,21]. In our previous mechanistic studies, the use of defined model substrates revealed that both 5–5 and 4–O–5 coupling reactions were possible [16,21–23] and this can be used to explain the couplings in this study.

Laccase activated functional molecules have not been only limited to lignocellulosic materials but also chitosan using different low molecular weight phenols like caffeic and gallic acid to improve the antioxidant and antimicrobial properties [24].

Similarly, laccases have been used to bleach pulps with leading to a decrease in residual lignin present in the pulp [25].

Table 1. XPS analysis of coconut fibers after laccase treatment with different fluorophenols.

Sample	% C	% F	% O	F/C	O/C
Flax + Laccase	65.6	–	27.2	–	0.415
Flax + Laccase + 4,2-FMP	64.5	–	30.2	–	0.468
Flax + Laccase + 4,-F3MP	65.6	0.7	27.6	0.011	0.421
Flax + Laccase + 4,4-F3MPP	61.0	0.5	31.3	0.0082	0.513

Table 2. XPS analysis of flax fibers after laccase treatment with different fluorophenols.

Sample	% C	% F	% O	F/C	O/C
Flax + Laccase	76.8	–	20.6	0	0.268
Flax + Laccase + 4,2-FMP	76.1	–	20.3	0	0.267
Flax + Laccase + 4,-F3MP	70.4	4.7	22.1	0.067	0.314
Flax + Laccase + 4,4-F3MPP	72.8	0.3	24.3	0.004	0.334

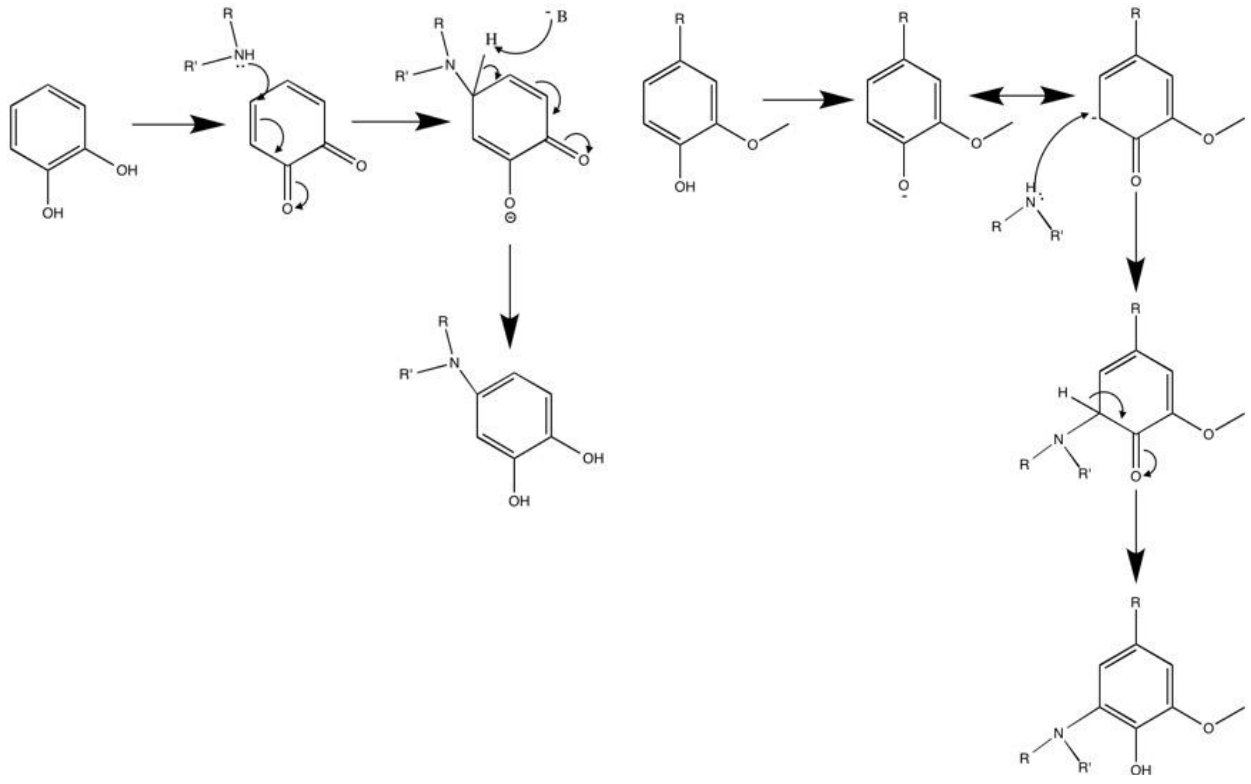
The results indicated a similar trend regarding the reactivity of the different fluorophenols for both fibers. The 4-F3MP was the most reactive since it was grafted to the highest extend, followed by 4,4-F3MPP, whereas 4,2-FMP did not attach neither to flax nor to coconut fibers. The latter result can

be attributed to the fact 4,2-FMP is a laccase substrate and intramolecular polymerization reaction in solution were most predominant [16]. Similarly Kudanga *et al.* [16] reported a lower reactivity in the case of 4,2-FMP, whereas they observed a higher amount of fluorophenol content for 4,4-F3MPP *vs.* 4-F3MP using as substrate beech veneers. The different content and distribution of lignin in the fibers compared with the beech veneers may also be the reason for the reactivity difference. Using different phenols, Schroeder *et al.* [26] showed that different phenols were grafted to different extents on flax as evidenced by color properties and antibacterial activities.

Although the results obtain for both fibers are promising they clearly flax fibers were more amenable to laccase mediated modification and consequently the further experiments were conducted with this material.

Amine based functional molecules were also grafted onto the flax fibers. This is based on the fact that laccase or laccase/mediator oxidized phenolic molecules can also be used to create cation radicals, that could then react with electron donating species like amines via either Michael addition [27] or radical coupling [28] as shown in Figure 2, leading to new C–N bonds and similar products [17].

Figure 2. Reaction mechanism for the coupling of amines to phenolic radicals [17]: (**a**) Michael addition to quinine; and (**b**) Radical coupling to form new C–N bond.



(**a**) (**b**)

In order to widen the substrate specificity and achieve a more efficient binding, three different mediators namely HBT, ABTS and TEMPO were also included in the experiment. Mediators are small molecules that act as electron shuttles between the laccase and the substrate broadening the oxidation range. In the particular case of flax fibers, the most effective mediator was TEMPO, which was able to clearly catalyse grafting of DFA onto flax fibers as shown by the significant increase of the total

amount of carbon, as well as the nitrogen content (Table 3). HBT and ABTS did not increase significantly the amount of bound DFA to the flax. In a similar fashion, tyrosyl silk residues were also successfully oxidized to their corresponding o-quinone, and subsequently reacted with amino groups of chitosan [29]. In addition, the peak position assigned to the aliphatic C (peak at 284.8 eV) increased its content from the 29.5% in the case of the sample treated with laccase and DFA compared with the 54.4% of the TEMPO treated sample (Table 3).

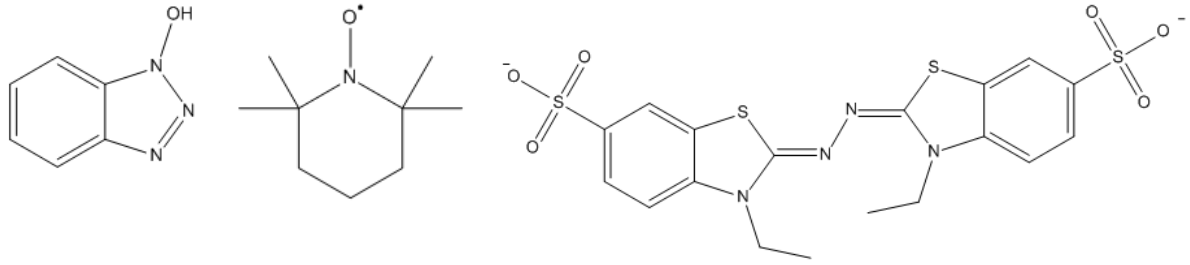
Table 3. XPS analysis of flax fibers after laccase treatment with dimer fatty amines (DFA).

Sample	% C	% N	% O
Flax + Laccase	72.5	3.9	23.3
Flax + Laccase + DFA	70.5	4.2	25
Flax + Laccase + DFA+HBT	73.7	4.4	21.5
Flax + Laccase + DFA+ABTS	71.9	4.5	23.3
Flax + Laccase + DFA+TEMPO	80.3	5.8	13.6

In addition, the peak position at 400.0 eV, assigned to the C–N bond also increased from 3.2% in the case of the blank with laccase and DFA to 4.9% for the TEMPO mediated reaction.

The mediators tested have different reaction mechanisms HBT and TEMPO oxidize via the generation of N–O* radicals while ABTS oxidizes via cation $ABTS^{+}$ radical (Figure 3). In agreement with previous results *N*-heterocycles and mediators carrying N–OH were more effective [30] while addition TEMPO lead the highest functionalization level.

Figure 3. Chemical structures of the mediators used.



The enzymatic coupling of hydrophobic molecules to natural fibers has substantial benefits when the fibers are used as reinforcement in polymer composites. The adhesion between the fiber and the polymer matrix is improved, leading to increases in the tensile strength and tensile modulus of the composite [31]. Interestingly from both N–OH mediators the most effective one was TEMPO. Natural materials typically have a great many functional groups, which when combined with a carefully selected right enzyme can turn be effectively be used in modifying polymer properties. Oxidative polymer activation for further functionalization has been successfully applied in the construction of biopolymer conjugates of chitosan and green fluorescence protein [32].

As shown in this study, this oxidative functionalization requires an activation step in which reactive groups are generated on the polymer surface or the functional molecule. For example polypropylene was treated with plasma to insert different methacrylate monomers to graft guaicaol sulfonic acid with a laccase in a subsequent step [26]. Hydrolytic enzymes have been also used for the to partially hydrolyze the surface of polyamide, generating free groups reactive groups in which phenols were

coupled in a similar fashion as described in this publication [33]. Nevertheless, when in the polymer structure there are appropriate reactive groups, direct laccase grafting of phenolics is also possible [34].

4. Conclusions

In this paper, we have clearly demonstrated the viability of using laccases as effective environmentally friendly catalyst to mediate the direct grafting of different phenols and amines on coconut and flax fibers. The different phenols showed distinctive reactivity and can be used as carriers of the different chemical moieties that could increase the compatibility with the desire polymer. Using mediators is possible to enlarge the substrate scope and effectively graft a hydrophobic molecule to improve the hydrophobicity of the fiber.

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Conflicts of Interest

The authors declare no conflict of interest.

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