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Synthesis and Characterization of Dehydrogenation Polymers in *Gluconacetobacter xylinus* Cellulose and Cellulose/Pectin Composite

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To mimic the lignin polymerization process, mats of bacterial cellulose and of a pectin/cellulose composite were used as a host matrix for in vitro polymerization of coniferyl alcohol. A diffusion cell was used to allow the diffusion of both hydrogen peroxide and coniferyl alcohol into the peroxidase impregnated cellulose mats through dialysis membranes. The results indicate that significant polymerization occurs within the mats. The resulting binary and ternary blends were imaged by scanning electron microscopy (SEM) and characterized by chemical means. The presence of pectin induces a better dispersion of the synthetic lignin in the cellulose network and enhances the proportion of alkyl-aryl-ether in the polymer.

KEYWORDS: Lignin model compounds; bacterial cellulose; pectin; scanning electron microscopy; thioacidolysis

INTRODUCTION

Wood is a composite material of three main polymers: cellulose, hemicellulose, and lignin. Deposition of these polymers occurs according to a sequential pathway: polysaccharides are first deposited, followed by lignins (*1*). Thus, lignin building blocks are polymerized in a swollen polysaccharide gel, due to a reaction mechanism that remains unclear to this day. One of the key stages of lignin biosynthesis is the oxidative coupling of monolignols (**Figure 1**) (*2, 3*). During the process, delocalized phenoxyl radicals are generated upon phenol dehydrogenation of *p*-hydroxycinnamyl alcohol monomers and intermediate lignins units and coupled in a variety of ways to build up the lignin polymer (*4*−*8*). Former attempts at identifying the relevant parameters of the polymerization by the adjustment of reactions were made. For instance, several authors have emphasized the influence of the polysaccharides on the monolignol polymerization by running the polymerization in dilute or concentrated saccharide solutions (*4, 9*−*16*). Among all polysaccharides, pectin solutions were often used because in woody xylem the first lignified region (middle lamella) contains a large amount of pectin (*17*). Pectins are mainly composed of uronic acid, which can react with the quinone methide formed during the polymerization pathway of lignin, to form covalent bonds (*18, 19*). Although these model experiments in solution are useful tools in the understanding of lignin biosynthesis and structure, they are irrelevant in the study of other characteristics of plant cell walls such as mechanical properties or solid-state interactions. For decades, the bacterium *Gluconacetobacter xylinus* subsp. *xylinus* (formerly *Acetobacter Xylinum*) has been used to study cellulose structure and organization, alone or in the presence of various hemicelluloses (mannan, acetyl glucomannan, xyloglucan) (*20*−*24*). The resulting bacterial cellulose gels and composites were widely investigated and have helped the understanding of the cell wall assembly thanks to the elucidation of some of the interactions among cellulose, hemicelluloses, and pectin (*20*−*24*). Even recently, *Acetobacter* cellulose composites were used to investigate the organization of cellulose fibrils under mechanical stress demonstrating the efficiency of such tools to achieve a better understanding of the polymer assembly within the plant cell walls (*25*). However, all these previous studies were addressed only to unlignified primary cell wall. Thus, to investigate interactions among lignified cell wall polymers, we designed a new polymerization model system reflecting more closely the physical status of the secondary plant cell wall. In this study, we polymerized coniferyl alcohol, the precursor of the guaiacyl lignin, in two mats of (i) bacterial cellulose and (ii) a composite structure formed respectively by bacterial cellulose and pectin, to investigate the effect of cellulose and pectin on the polymerization process of coniferyl alcohol. The ultrastructure and the chemical structure of the resulting binary (cellulose/dehydrogenation polymers = DHPs = synthetic lignin) and ternary blends (cellulose/pectin/DHPs) were investigated and compared.

MATERIALS AND METHODS

Characterization of Pectin. Commercial pectin (Sigma) from citrus fruits with a low degree of esterification (28%) was used. The monomeric composition was determined using the method proposed by De Ruiter (*26*) and consisted of galacturonic acid (90%), galactose (7%), and rhamnose (3%). The molecular weight distributions of pectin

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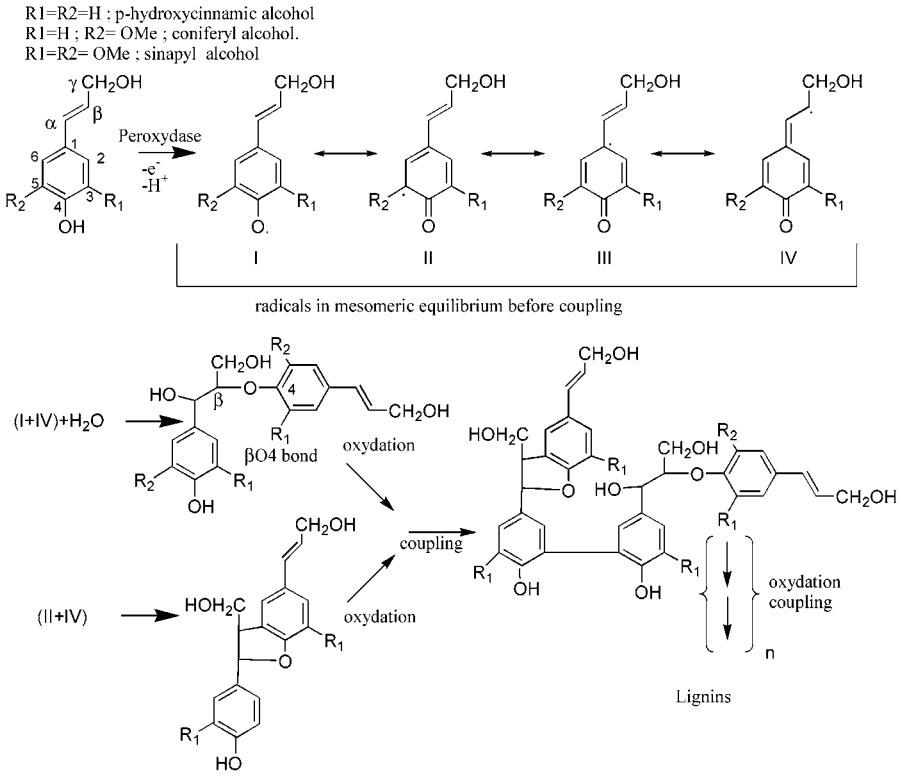

Figure 1. Schematic polymerization pathway of lignins. Monomers are oxidized by a peroxidase to form a radical stabilized as four mesomeric forms. Coupling occurs between radicals to form dimers which can be reoxidized to build up the lignin polymer.

samples were determined by size exclusion chromatography according to the procedure described by Chanliaud and Gidley (*21*). Weight-average (Mw) and number-average (Mn) molecular weights were found to be equal to 195 000 and 105 000 g/mol, respectively. The distribution of the methyl groups along the pectin chain was examined by ^{1}H NMR and ester groups are distributed according a sequential block structure as previously described (*27*).

Production of Bacterial Cellulose. Mats of bacterial cellulose were obtained by growing *Gluconacetobacter xylinus* subsp. *xylinus* (formerly *Acetobacter xylinus* subsp. *xylinus*) strain DSM 2004 on liquid Hestrin & Schramm medium pH 5 supplemented with Ca^{2+} (10 mM). The composition of the culture medium was (in g/L): bacto-Peptone 5 g; yeast extract 5 g; Na_2HPO_4 5 g; and citric acid 1.15 g. The pH was adjusted to 5 with concentrated HCl. The medium was sterilized by autoclaving at 121 °C for 20 min. Glucose 20 g/L final was added aseptically from a heat-sterilized 2 M solution. Cycloheximide 0.1 g/L final was added from a filter-sterilized 2% solution as a preservative against fungal growth.

Cultures were made in sterile flat polycarbonate boxes (21.5/15.5/3 cm) containing 250 mL of medium covered with aluminum foil and placed in a cooled incubator for 8−10 days at 28 °C. Inoculation was made with a 2% volume of a 5−8 days' old-culture with no need to disrupt nor blend the cellulose mat in the inoculum. Composites of cellulose/pectin were obtained by adding 0.5% pectin (low degree of esterification DM 28% Sigma) in the culture medium.

The mats were harvested and washed in five baths of phosphate buffer containing Ca^{2+} (10 mM) and 0.02% sodium azide. They were stored at 4 °C in this washing buffer.

Polymerization of Coniferyl Alcohol within the Cellulose Mats. A special glass device (diffusion cell) was used to perform the polymerization within the cellulose mat (**Figure 2**). It was made of two cylindrical halves, one flat flanged edge having a groove with an O-ring, the other a plain flat flanged edge. The two parts were secured together with a special metal clamp. Disks of 7.2 cm in diameter were cut into the wet cellulose mats and soaked overnight at 4 °C in 10 mL of sodium phosphate buffer (1/30 N; pH 6) containing 1 mg/mL horseradish peroxidase (Sigma). The disks were intercalated within two sheets of regenerated cellulose membranes (Zellu Trans, Roth, Karlsruhe, Germany) with a 4000−6000 molecular weight cutoff. The assembly was mounted in the middle of the glass diffusion cell and the reservoirs were filled, one with 100 mL of sodium phosphate buffer containing 2 mg/mL coniferyl alcohol, the other with 100 mL of sodium phosphate buffer containing 5 μL/mL hydrogen peroxide 30% w/w (Aldrich). The devices were held in a cooled incubator at 25 °C for about 10 days.

The cellulose disks were washed in tap water and stored at 4 °C in sealed plastic bags until used.

Scanning Electron Microscopy. The samples were fixed and stored in 1.25% glutaraldehyde solution. Small pieces (about 0.5 cm) were rinsed five times for 15 min in ultrapure water. They underwent further dehydration in a graded ethanol series (50, 70, 95, and 100% twice). The soaking in isopentyle acetate was performed before the critical-point drying in CO_2 medium using a EMSCOPE CPD 750 apparatus. The samples were coated with gold−palladium by cathodic spreading in a Polaron E 5100 coater and examined with a Hitachi S 3000N (Tokyo, Japan) scanning electron microscope operated at an accelerating voltage of 10 kV.

Chemical Analysis. *Sample Preparation.* Cellulose disks were freeze-dried and ground for 2 min in a ball mill oscillatory grinder (Retsch, MM 2000) at maximum amplitude. Powders were kept at room temperature in a desiccator over phosphorus pentoxide until analysis.

DHP content was determined according to the acetylbromide protocol proposed by Hatfield *et al.* (*28*) but without the addition of perchloric acid. For all the determinations, the acetyl bromide concentration was 25% (wt/wt) in glacial acetic acid. The detection was done at 280 nm (Lambda 14, Perkin-Elmer) after diluting 1 mL of the reaction mixture in sodium hydroxide (5 mL, 2 M) and 14 mL of glacial acetic acid.

Thioacidolysis was performed following Lapierre's procedure (*29, 30*). The main degradation products were analyzed by gas chromatography as trimethyl sililated derivatives using a J&W Scientific column (DB 1, 30 m × 0.25 mm, 0.25 μm film). Detection was made by FID using tetracosane as the internal standard.

RESULTS AND DISCUSSION

Polymerization Process. Previous studies have shown that the growth of *Acetobacter xylinum* in the presence of pectins

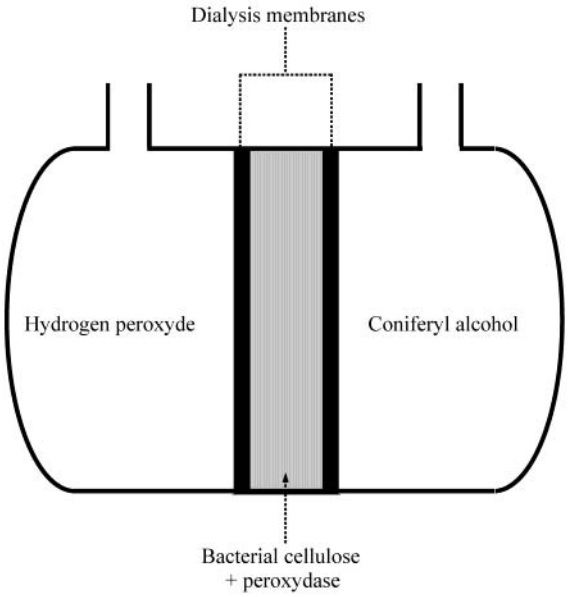

Figure 2. Schematic representation of the diffusion cell. Coniferyl alcohol diffuse into the mats and polymerize in the presence of peroxidase and hydrogen peroxide.

resulted in the formation of a interpenetrated network of bacterial cellulose and pectins (*21*). Accordingly, we have produced mats composed of bacterial cellulose and pectin/cellulose composite. Commercial pectin (Sigma) from citrus fruits with a low degree of esterification was used in this study (see materials and methods for details on pectin structure), because pectin with a low degree of methyl esterification was reported to afford the maximum level of incorporation in the mats (*21*) The content of pectin in the mat was 8% of the dry weight based on hydrolysis of the composite using a methanolysis/TFA method (*26*). This incorporated amount is quite low compared to those obtained by Chanliaud *et al.* (*21*), which reached 40% in similar conditions. However, during the *Acetobacter* culture, the incubation media were not submitted to agitation to favor the formation of the mat required for the polymerization step. However, this procedure results in the formation of a gel-like interface at the bottom of the pellicle. This may have limited the diffusion of the pectin within the cellulose network and will be examined in a future work. Another reason for the low incorporation of pectin might be that the *Acetobacter xylinum* species used was different from that of Chanliaud (*21*). When the mats were roughly 1.5 mm thick, they were soaked in a peroxidase solution and, after 24 h of peroxidase permeation, finally fitted into the diffusion device. The diffusion cell (**Figure 2**) consists of two identical chambers separated by the bacterial cellulose mat. The mat was enclosed in two dialysis membranes (4000−6000 molecular weight cutoff) which avoid the diffusion of the enzyme into the chambers, without limiting the diffusion of the reactants. One chamber was filled with a coniferyl alcohol solution and the other with hydrogen peroxide. When the reactants diffused through the membrane, the polymerization occurred within the mat. Thus, the supply of both reactants is only controlled by the diffusion process. Such an approach was already investigated in the 80s by Higuchi's team (*11*−*13*). They synthesized dehydrogenation polymers in dialysis tubes. The originality and the main difference of this present work (compared to these previous studies) are the use of a polysaccharide matrix as a polymerization medium and the production of a solid state complex between polysaccharide and dehydrogenation polymer. The disappearance of coniferyl alcohol in the first chamber was monitored by HPLC, and after 100 hours of reaction, coniferyl alcohol completely disappeared from the medium both in cellulose and cellulose−pectin experiments. The kinetic of the diffusion process of the coniferyl alcohol was found to be similar regardless the gel (cellulose or cellulose/pectin). This implies that the rate of supply of coniferyl alcohol to the polymerization medium—which is an important parameter of the reaction (*31*)—is identical for both polymerizations. Throughout all the experiments, coniferyl alcohol could not be detected in the hydrogen peroxyde chambers, suggesting that all the alcohol reacted within the mat. After a 10-day reaction, the creamy-brown colored mats were gently washed with phosphate buffer and then submitted to further analysis.

Microscopic Structure. Scanning electron microscope (SEM) was used to visualize the ultrastructural arrangement of DHPs within the cellulose mats and the cellulose pectin composite (**Figure 3**). During the polymerization, the deposition of the DHP was found to be asymmetric across the pellicles. Since the coniferyl alcohol diffuses from one end to the other one of the cell, the side of the pellicle which is contact with the coniferyl alcohol is nearly completely covered by DHP, and no conclusions can be drawn from these pictures. Thus, we present only the data concerning the side of the pellicles that was in contact with the hydrogen peroxide, where the DHP are present in lower quantity. Since the thickness of cellulose and composites mats, the rate of supply of coniferyl alcohol, and the amount of DHP incorporated are identical in both experiments, we assume that organization of the DHPs aggregates can be compared. DHPs appear in the cellulose as dispersed and irregularly aggregated ovoid globules, whereas the cellulose revealed well-defined fibrils similar to those described in previous studies (*21*). The size of the DHPs globule is about a few micrometers in diameter (**Figure 3b,c**), indicating a severe phase separation between the cellulose and the DHPs. Similar sizes and shapes were already reported for DHPs dispersed in water and examined by environmental scanning electron microscopy (*32*). This supports the assumption that the cellulose and DHPs behave separately. The spherical shape is only due to the hydrophobicity of amorphous DHPs because it represents the conformation of the lowest energy of an hydrophobic compound in an hydrophilic solvent. In contrast, DHPs synthesized in the cellulose/pectin mat present a more homogeneous dispersion. At the highest magnification (**Figure 3f**), the nodule of DHP present in cellulose/pectin composites is smaller than those observed in the cellulose mat (less than 1 μm). However, the nodule size of synthetic lignin still seems to be larger than those of the natural lignin—less than 100 nm (*33*). Nevertheless, a ternary blend (DHPs/cellulose/pectin) displays a dense and interpenetrated network pattern. It is obvious that pectin, acting as compatibilizer, induces an enhancement of the miscibility between cellulose and DHP. Similar effects have already been demonstrated in solution (*16*) and solid-state studies (*34*). In both cases, the effects observed were probably due to the occurrence in the medium of the lignin carbohydrate complex which is the copolymer of pectin (or the hemicellulose) and DHPs (or the lignin). This can be obtained by reaction of the galacturonic acid and the quinone methide formed during the polymerization of the coniferyl alcohol (*18*).

Chemical Analysis of the DHPs. To examine closely the effects of the pectin on the DHPs structure, DHPs were submitted to chemical analysis (see **Table 1**). All the determinations reported here are the duplicate results from two independent experiments. In both cases, the amount of DHPs incorporated within the mat was roughly similar. On the basis of the

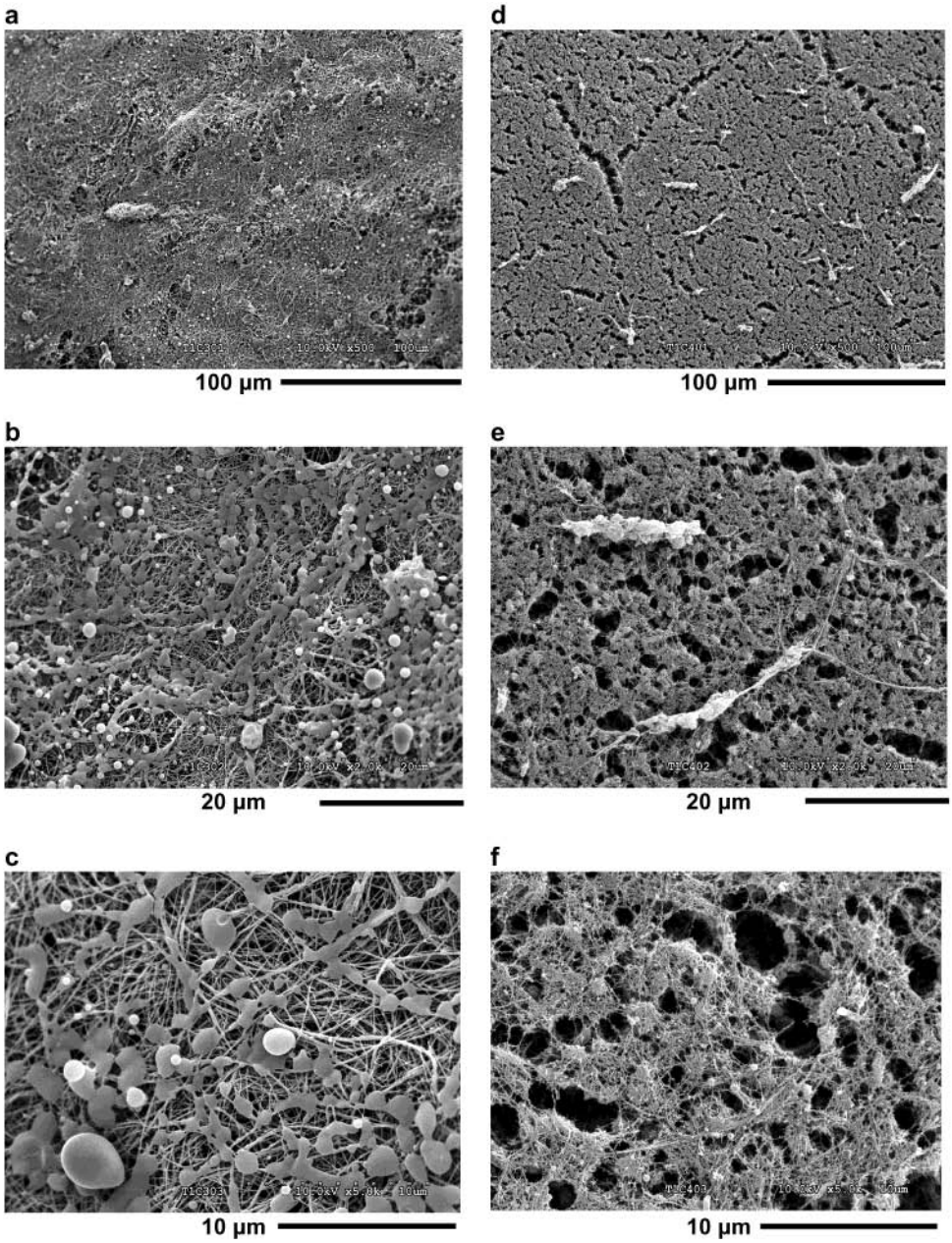

Figure 3. Micrograph of bacterial cellulose/DHP blends (a, b, c) and of bacterial cellulose/pectin/DHPs (d, e, f) blends at different magnification (side of the mats in contact with the hydrogen peroxide in the diffusion cell).

Table 1. DHPs Content, Thioacidolysis Yields of Cellulose/DHPs and Cellulose/pectin/DHPs composite and reference DHPs

	cellulose/DHPs composite	cellulose/pectin/DHPs composite
pectin content (% of dry weight)	0	8
DHPs content (% of dry weight)	27	26
thioacidolysis yield (μmol/g of DHP)	860 ± 30	1180 ± 80

amount of coniferyl alcohol the DHP yield was relatively low (25%); it seems that a large amount of DHP was lost during the washing of the mat at the end of the synthesis. The structure of those ≪unbound≫ DHPs will be examined in future work.

Subsequently, the milled mats were submitted to thioacidolysis, a specific degradation method that cleaves the alkyl−aryl−ether bonds (β-O-4, ≪uncondensed bond≫, see **Figure 1**) The DHPs synthesized in the cellulose/pectin composites present a higher thioacidolysis yield ranging around 1180 μmol/g of thioethylated monomers, a yield close to those observed on natural milled wood lignin of conifer. In contrast, the DHPs synthesized within the cellulose mat exhibited a lower yield similar to those observed on DHPs obtained in solution using a conventional ≪Zutropfverfahren≫ method (*31*, *35*). This finding is in agreement with the results of Terashima *et al.* (*10*), which synthesized DHPs in a concentrated solution of pectin, but not with our previous results (*16*) in which DHPs polymerized in dilute pectins solution presented a lower content of β-O-4 linkage. However, several parameters differ in both experiments, especially the degree of methyl esterification of the pectin. In the present study, to obtain the highest pectin incorporation in the mat as reported by Chanliaud and Gidley (*21*), a pectin with a low degree of methyl esterification (28%) was used, similar to that used by Terashima *et al.* (*10*), whereas in our previous study (*15*, *16*), we used pectin samples containing a large amount of methyl ester groups (67%). Moreover, some other synthesis parameters are also different,

namely, the polysaccharide concentration and the amount of hydrogen peroxide. This question will require an in-depth study to determine the relevant parameters and the underlying phenomena.

The increase of the amount of β-O-4 linkages in the presence of pectin emphasizes the fact that the physical status of polymerization media is critical for the lignin structure and organization. Indeed, since lignin polymerization is a process involving a mesomeric equilibrium, it is very sensitive to solvatation, polarity, and other physicochemical parameters of the reaction medium. These parameters can be controlled by the polymers (or other molecules) surrounding the monolignols. The underlying idea is that lignin polymerization can be partly controlled and/or affected not only by the monolignol biosynthetic pathway and the oxidizing enzyme system. Clearly, lignin structure and spatial organisation can be indirectly changed by a modification of the amount of pectin (for instance) without any variation of the polymerization systems (concentration of monolignols and enzyme). This information may be very helpful to investigate the physicochemical parameters involved in the interrelations of cell wall polymers along the biosynthesis of plant cell walls.

CONCLUSION

For the first time, solid state ternary blends of cellulose/pectin/DHPs—a model of lignified cell walls—were obtained using a diffusion cell. In the presence of pectin, DHPs were more efficiently dispersed in the cellulose mat, suggesting that the pectin may play the role of compatibilizer. The structure of the DHPs polymerized in the pectin/cellulose mat were found to have a higher amount of β-O-4 linkages than the DHPs synthesized in the cellulose mat. Thus, it is obvious that even a low content of pectin (8%) within the polymerization medium affects the structure and the ultrastructure of the lignin model compounds very significantly. Previous studies emphasized the interaction of the pectin with DHP via the lignin carbohydrate complex (LCC) (*4*, *15*, *16*, *34*, *36*). The effect observed here may be due to the formation of such linkages. Accordingly, the understanding of the plant cell wall assembly must take into account the lignin carbohydrate bond, not only because of their cross-linking effect but also because of their compatibilizing effect, which affects the interactions between the plant cell wall polymers and subsequently their mechanical and physicochemical properties. This hypothesis will be investigated soon by the study of the mechanical properties of binary and ternary blends.

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