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**Elucidation of Xylan Function in Lignin Formation
Using Artificial Wood Cell Wall**

(人工木材細胞壁を用いたリグニン形成におけるキシラ
ンの機能解明)

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Doctoral Thesis

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ABBREVIATIONS

aq. dioxane	90 % dioxane aqueous solution
BC	Bacterial cellulose
CA	Coniferyl alcohol
DHP	Dehydrogenation polymer
DHP-FBC	DHP-deposited FBC
DHP-HPBC	DHP-deposited HPBC
DHP-Xyl-FBC	DHP and xylan-deposited FBC
DHP-Xyl-HPBC	DHP and xylan-deposited HPBC
EDXA	Energy dispersive X-ray analyzer
FBC	Flat bacterial cellulose film
FE-SEM	Field emission scanning electron microscope
<i>G. xylinus</i>	<i>Gluconacetobacter xylinus</i> (ATCC53582)
GC	Gas chromatography
GPBS	Glycine/phosphate-based saline
HPBC	Honeycomb-patterned bacterial cellulose film
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HS medium	Hestrin-Schramm medium
MOE	Modulus of elasticity
NMR	Nuclear magnetic resonance
PBS	Phosphate buffer saline
PCL/CAP	Polycaprolactone and acrylate copolymer

PDMS	Polydimethyl siloxane
SEM-EDXA	FE-SEM equipped with EDXA
TLC	Thin-layer chromatography
UV	Ultraviolet
Xyl	Xylan
Xyl-FBC	Xylan-adsorbed FBC
Xyl-HPBC	Xylan-adsorbed HPBC

CHAPTER 1

General Introduction

1.1 General introduction of wood and wood cell wall

Tree is the most abundant, long-lived, and largest plant on earth. Since the early history of human-beings, wood has been used not only as an energy resource, but also as a construction material for house and furniture. In addition, wood is a very important feedstock of fibers for pulp and papermaking industry. In 2014, total production of paper and paperboard was 2.65×10^7 metric tons in Japan (Japan Paper Association 2014). In this century, with increasing demand of renewable energies alternative to fossil resources, wood or lignocellulose has also attracted much attention as a feedstock for biomass power generation, and a second generation feedstock for bioethanol.

Wood mainly consists of elongated cells oriented along the axis of wood stem (Watanabe et al 2002, Barnett and Bonham 2004), and it has a hierarchical structure from molecular level to tissue level. Here, I explain the structure in bottom-down.

At first, a tree includes tissues of bark, phloem, vascular cambium, xylem, pith and so on (Plomion et al 2001, Daniel 2009). Wood is from the xylem part. Xylem of adult trees can be divided into outer part (sapwood) and inner part (heartwood). In an annual ring of wood, there are two parts, earlywood and latewood.

The major cells of earlywood and latewood have hexagonal morphology, and array like as honeycomb when we see softwoods in the cross section. Thereof, Mod é n and Berglund (2008) proposed a two-phase annual ring theory to elucidate

the deformation anisotropy of wood responded to external stress on the basis of a honeycomb structure of the cells. Their theory was constructed by a combination of the bending model (Gibson et al 1982) and the stretching model (Masters and Evans 1996), taking the contribution of earlywood and latewood to the response into consideration. In the paper, they demonstrated that the bending deformation was predominant for earlywood, while the stretching for latewood. However, their theory has not been empirically proved. Here, I point out that it is **the first subject** to prove the validity of this theory in near future. In this section, I would like to point out unknown and unclear subjects as well.

In turn, a wood cell wall of tracheids or wood fibers is composed of primary cell walls (P) and secondary cell walls (S), and intercellular region is termed as middle lamella (ML) (Figure 1-1 A). The S layer has a “three-ply structure”, which is divided into S1 (outer), S2 (middle), and S3 (inner) (Brändström 2001, Higuchi 2002, Harris 2006). The layered structure of wood cell wall can be clearly observed by transmission electron microscopy (TEM; Figure 1-1 B, Joseleau et al 2004). The thickness of S2 layer ranges from 1 μm to 10 μm , which is much larger than that of S1 (0.1 μm to 0.35 μm) and S3 layers (0.5 μm to 1.1 μm) (Plomion et al 2001).

P, S1, S2, and S3 layers differ in cellulose microfibrils organization. In P layer, microfibrils are not oriented uniformly. The microfibrils in both S1 and S3 have opposite helical orientation (S and Z helices), while S2 has microfibrils in Z helix (right handed) only (Sjöström 1993, Barnett and Bonham 2004). Microfibril angles (MFAs), defined as the angle between the long axis of the fiber and the cellulose microfibrils, is used for describing the orientation of microfibrils in these

S layers. MFA in S1 is between 50 ° and 70 °, while the MFA in S2 varies between 20 ° and 30 ° in earlywood, and between 5 ° and 10 ° in latewood. MFA in S3 is from 50 ° to 90 °. (Barnett and Bonham 2004, Donaldson 2008, Jyske et al 2014). It is thought that the longitudinal modulus of elasticity of wood is very dependent on MFA in S2, in contrast, S1 and S3 layers have a crucial role in keeping wood lateral mechanical strength (Donaldson 2008). Here, I point out that relationship between MFA and mechanical properties is **the second unclear subject**.

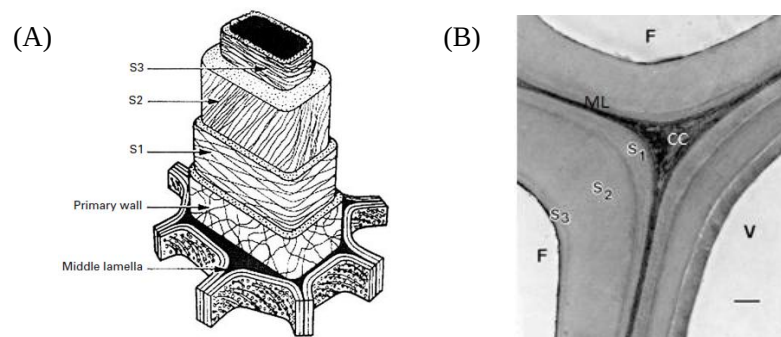


Figure 1-1. Structure of a wood fiber or tracheid cell wall (A) as illustrated by Barnett and Bonham (2004) and a TEM image of thin transverse section of *Populus deltoides* wood (B) adapted from Joseleau et al (2004). The striations in image (A) represent cellulose microfibrils which have different orientation (microfibril angles) in different layers of S1, S2, and S3. Image (B) was taken after staining with PATAg. F is fiber, V is vessel, ML is middle lamella, CC is cell corner, bar = 0.5 μm .

Wood secondary cell wall is composed of three main biopolymers, cellulose, hemicellulose, and lignin. Table 1-1 shows the amount of each component. Cellulose is the principal constituent, and occupies in approximately 40-45 % of dry wood. Most cellulose is distributed in the S2 cell layer. A predominant hemicellulose in softwood is galactoglucomannan, while glucuronoxylan is a major hardwood hemicellulose. The content of galactoglucomannan increases from the outer part of tracheid cell walls toward inside (lumen) in softwood, while

most glucuronoxytan presents in outer S2 and S1 layers of wood fibers in hardwood. The amount of lignin in softwood is about 26-32 %, while 20-25 % in hardwood. Lignin in middle lamella has the highest concentration, but most lignin (at least 70 %) locates in the secondary cell wall of the fiber cells. Other wood components, such as pectin, protein, and extractives, usually have a minor amount in wood with contents less than 10 %.

Table 1-1. Components in wood cell wall (Sjöström 1993).

	Cellulose, %	Hemicellulose, %	Lignin, % ^a
Softwood	40-45	Galactoglucomanan, ca. 20	26-32
		Arabinoglucuronoxytan, 5-10	
Hardwood		Glucuronoxytan, 15-30	20-25
		Glucomanan, 2-5	

%, based on the weight of dry wood; ^a, determined gravimetrically by the Klason method.

1.2 Wood cell wall formation

Wood formation involves a very complex process. Generally, it is divided into cell division, cell expansion, cell wall thickening, and programmed cell death. The formation of wood cells originates from cambium, and once cells expansion is completed, the formation of the secondary cell wall begins. This step, called cell wall thickening, involves the successive biosynthesis and deposition of main secondary cell wall components: cellulose, hemicelluloses, and lignin. (Higuchi 1990, Plomin 2001)

1.2.1 Cellulose

The main function of cellulose is to provide mechanical enforcement of wood (Heriksson and Lennholm 2009). Cellulose is a linear homopolysaccharide.

It consists of β -D-glucopyranose (β -D-Glcp) units which are linked together by covalent β -1,4-glycosidic bonds (Figure 1-2). The native cellulose in wood has a cellulose I polymorphism with two different crystalline allomorphs, I_α and I_β (Atalla and VanderHart 1984, Heiner et al 1995, 1998). The degree of polymerization (DP) of wood cellulose is about 10000 (Sjöström 1993).

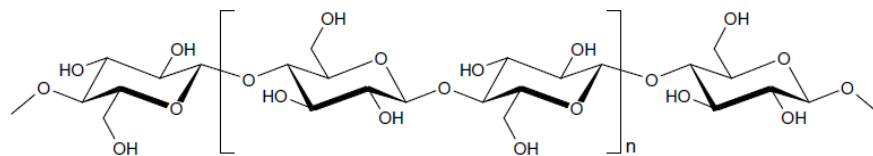


Figure 1-2. Cellulose structure and a cellubiose repeating unit linked by β -1,4-glycosidic bonds.

Cellulose is biosynthesized from its precursor UDP-D-glucose in plasma membrane by cellulose synthases complexes (CSC, initial name is terminal complexes, TC). In higher plants, CSC is arranged in a hexagonal array called rosette, and every rosette contains 36 CesA proteins (Cosgrove 2005, Somerville 2006, Lerouxel et al 2006). The glucosyl residue in UDP-D-glucose is transferred to growing β -1,4-glucan chain with release of UDP by CesA. The growing glucan chain moves through a channel formed by the eight transmembrane domains (TMDs) of CesA and then merges with other glucan chains to form a microfibril. The orientation of microfibrils is thought to be controlled by cortical microtubules, which guides the move of rosettes. (Paredez et al 2006, Somerville 2006, Lerouxel et al 2006)

1.2.2 Hemicelluloses

Hemicelluloses are heteropolysaccharides composed of different pentoses and hexoses, and amorphous polymers with a low degree of polymerization (ca.

200, Sjöström 1993). Hemicelluloses in hardwood are different from those in softwood (Table 1-1). In my research, I only focused on xylan, the main hemicellulose in hardwood, and therefore, I would like to introduce xylan here.

A representative structure of xylan is shown in Figure 1-3, the backbone is comprised of β -D-xylopyranose unit, linked by covalent β -1,4-bonds. About seven O-acetyl groups per ten xylose residues are presented at xylose C-2 or C-3 position. The side chain is 4-O-methyl- α -D-glucopyranosyluronic acid residues linked with xylose backbone by α -1,2-bonds. On the average, about ten xylose residues contain one uronic acid (Burton et al 2010, Sjöström 1993).

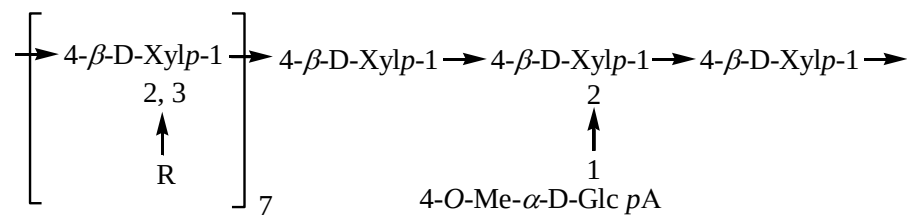


Figure 1-3. Abbreviated formula of glucuronoxylan (from Sjöström 1993). Xylp is β -D-xylopyranose; 4-O-Me- α -D-Glcp A is 4-O-methyl- α -D-glucopyranosyluronic acid; R is an acetyl group (CH_3CO —).

Different from cellulose biosynthesis, hemicelluloses are biosynthesized in the Golgi apparatus and then exported to the plasma membrane via Golgi-secretory vesicles through an exocytosis mechanism (Burton et al 2010). The biosynthesis of the hemicellulose backbones has been widely assumed to be controlled by cellulose synthase like (CSL) genes, while the side chains of hemicellulose are added to the backbones by glycosyltransferases. However, only xylosyltransferases and galactosyltransferase for the sidechains biosynthesis of xyloglucans and glucomannans, respectively, have been identified in *Arabidopsis* (Cosgrove 2005, Lerouxel et al 2006, Pauly et al 2013, Scheller and Ulvskov

2010).

The function of the hemicelluloses in wood cell wall is assumed to act as binders connecting cellulose microfibrils and lignin, and also to control the spaces between microfibrils (Terashima et al 2009, 2012), and/or to act as a ‘host structure’ for lignin deposition (Reis et al 1994, Reis and Vian 2004).

1.2.3 Lignin

Lignin is a complex biopolymer with three main precursors (monolignols), called *p*-coumaryl, coniferyl, and sinapyl alcohols (Figure 1-4).

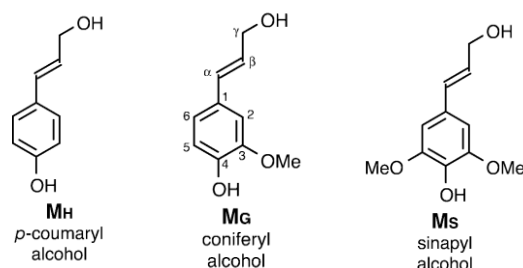


Figure 1-4. Three main monolignols as precursors of lignin (Ralph et al 2004a).

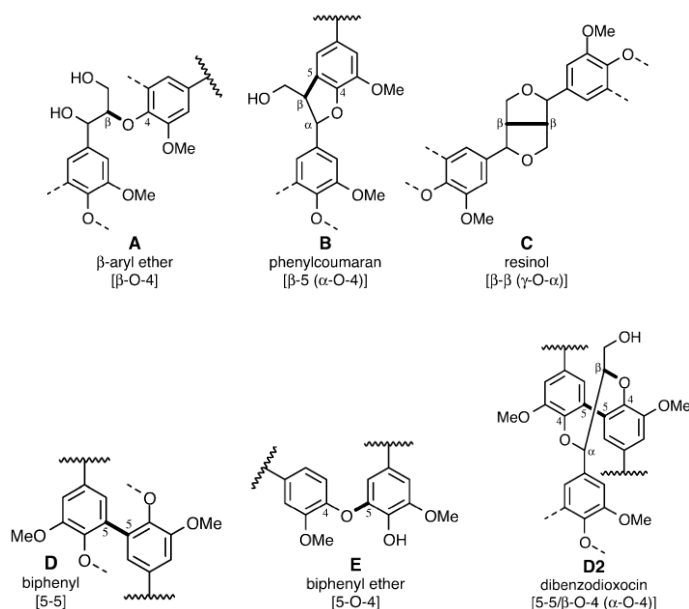


Figure 1-5. Major interunitary linkages in lignin. The bolded bonds are the ones formed in the radical coupling reactions (Ralph et al 2004a).

The monolignols are linked through different types of ether bonds (C—O—

C) and carbon-carbon bonds (C—C), forming a series of interunitary linkages as shown in Figure 1-5 (Ralph et al 2004a). Native lignin in wood is rich in β -O-4 linkage (Henriksson 2009). These linked units may form a branched structure. Lignin in softwood is composed almost exclusively of coniferyl alcohol, while lignin in hardwood consists both coniferyl and sinapyl alcohols. Only minor amount of *p*-coumaryl alcohol is presented in wood.

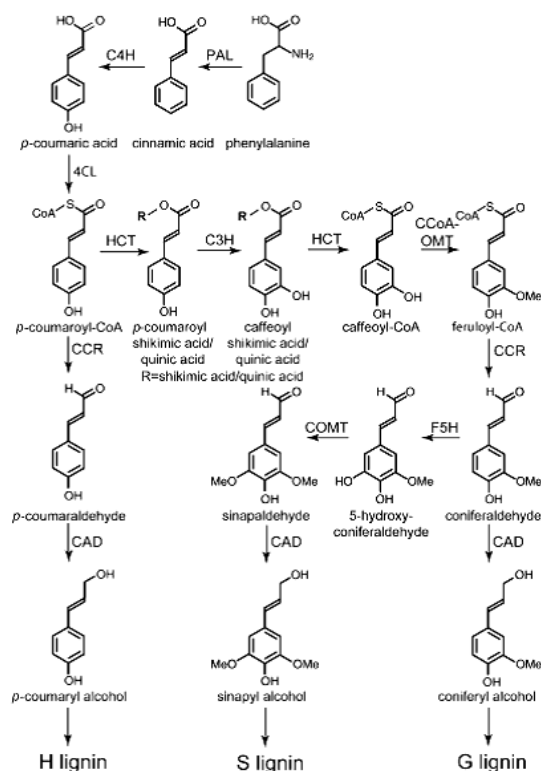


Figure 1-6. The main routes of lignin monolignols biosynthesis. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; HCT, *p*-hydroxycinnamoyl-CoA; C3H, *p*-coumarate 3-hydroxylase; COMT, caffeic acid O-methyltransferase; F5H, ferulate 5-hydroxylase; 4CL, 4-coumarate:CoA ligase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; SAD, sinapyl alcohol dehydrogenase. (Vanholme et al 2010)

The biosynthesis of lignin involves three processes: monolignols biosynthesis, monolignol transportation, and lignification. The lignin monolignols

are biosynthesized from their precursor, phenylalanine (Phe), synthesized from shikimate pathway (Bentley 1990, Cho et al 2007, Roberts et al 1998, Herrmann and Weaver 1999, Rippert et al 2009). The most possible route for monolignols biosynthesis is shown in Figure 1-6. Phe undergoes deamination, aromatic ring C4 position hydroxylation to form *p*-coumaric acid. To form coniferyl alcohol, the *p*-coumaric acid undergoes phenolic hydroxylation and methylation at the aromatic C3 position. To form sinapyl alcohol, the hydroxylation and methylation reactions at the aromatic C5 position occur at the aldehyde level. Recently, a catechyl lignin (C lignin) was found in seed coats of vanilla orchid (Chen et al 2012), cactus species (Chen et al 2013), and angiosperm (Tobimatsu et al 2013), which suggests that lignin can be synthesized from caffeyl alcohol as a precursor.

The synthesized monolignols are released in cytoplasm (Alejandro et al 2012, Vanholme et al 2010, Liu et al 2011), and then transported into cell wall for monolignols polymerization. Here, I point out **the third unknown subject** about coniferyl and sinapyl alcohols transportation. There are two theories: One is that coniferyl and sinapyl alcohol are passively diffused or transported by an unknown ABC transporter through membranes. The other is they are converted into monolignol glucosides by glucosyltransferase firstly and then transported into vacuole by some ABC transporters (Liu et al 2011, Sibout and Höfte 2012). The only indentified ABC transporter gene is AtABCG29, which exports *p*-coumaryl alcohol across the plasma membrane (Alejandro et al 2012).

The polymerization of monolignols (lignification) is an enzymatically catalyzed radical coupling reaction. The phenoxy radicals are initiated by enzymatical dehydrogenation in the presence of oxidase, peroxidase/H₂O₂ and/or

laccase/O₂. (Higuchi 1985, Higuchi 1990, Higuchi 2002, Ralph et al 2004b, Ralph et al 2009, Sjöström 1993) A typical formation of radicals from coniferyl alcohol is shown in Figure 1-7. The main products from dimerization are β -O-4 (Figure 1-5 A), β - β (Figure 1-5 C), and β -5 (Figure 1-5 B, only for coniferyl alcohol), while when a monolignol is coupled with a preformed oligomers, β -O-4 and β -5 with minor 5-5 (Figure 1-5 D) and 4-O-5 (Figure 1-5 E) are preferred products, when the radical coupling occurs between two oligomers, 5-5 and 4-O-5 are most produced. (Boerjan et al 2003, Ralph et al 2004a, Ralph et al 2008, Ralph et al 2009)

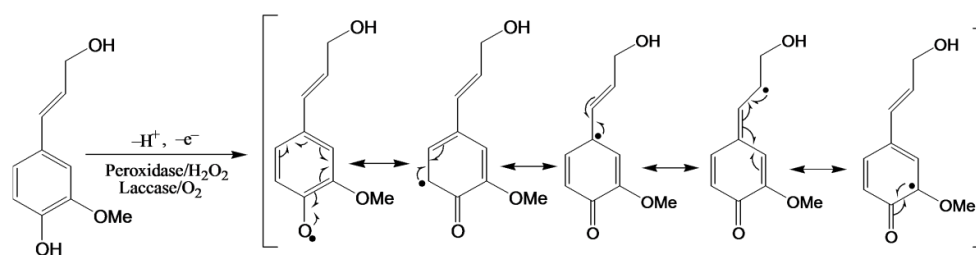


Figure 1-7. Phenoxy radicals from coniferyl alcohol by enzymatical dehydrogenation. (adapted from Higuchi 1990)

The mechanism of the radical coupling reaction, taken the formation of β -O-4 unit for example, is shown in Figure 1-8. Monolignol and lignin radicals undergo β -O-4 coupling to form quinone methide intermediate, this intermediate is stabilized by the nucleophilic addition of water to the α -carbon to form a β -aryl ether structure. The phenolic group in this structure can be further oxidated to form radicals and then undergoes next radical coupling. (Grabber 2004, Holmgren et al 2006, Ralph et al 2009, Tobimatsu et al 2013)

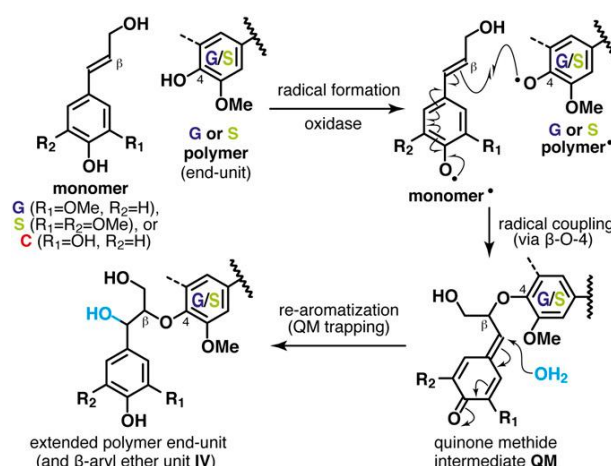


Figure 1-8. The mechanism of the radical coupling reaction for the formation of β -O-4 unit. (Tobimatsu et al 2013)

The function of lignin in wood cell wall is to provide mechanical strength and the hydrophobic surface of a fiber needed for the transport of water, and to play a role in protecting plants against pathogens (Boerjan et al 2003, Chabannes et al 2001, Higuchi 2002, Jones et al 2001, Plomion 2001, Sarkanen and Ludwig 1971, Wan et al 2012).

The formed wood cell wall, composed of cellulose, hemicellulose, and lignin, are extremely intermixed (Bergander and Salmén 2002, Salmén 2004, Burgert 2006). The organization of wood components with cell wall is still unknown. Here, I point out that **the fourth subject** is the relationships between cell wall components, for example, the arrangement of these polymers and the interactions among them. In the following parts, I would like to introduce several proposed models related to this relationship.

1.2.4 Relationship between wood cell wall components

Several wood cell wall models were reported at ‘ultrastructural’ level (in a nanometer scale, Brändström 2001) to display the interrelation of cell wall

components within wood cell wall. The first ultrastructural model was proposed by Kerr and Goring (1975), as shown in Figure 1-9 A. S2 layer consists of many concentric lamellae. Based on this model, wood cell wall architecture is thought to be analogous to rods-concrete (Bidlack 1992, Higuchi 2002): Cellulose microfibrils are embedded in lignin, much as rods are embedded in concrete. Hemicelluloses bind tightly to cellulose microfibrils via hydrogen bondings (Bacic et al 1988, Bidlack 1992, Keegstra 2010), and link to lignin probably by the covalent linkages of benzyl ester bond, benzyl ether bond, phenyl glycoside, and/or acetal (or hemiacetal) (Achyuthan et al 2010, Albersheim et al 2010, Grabber 2004). Lignin is distributed with hemicelluloses in the spaces of inter-cellulose microfibrils and acts as a cementing component to connect and harden the cell walls.

Fengel (1971) gave a two hierarchical structure (Figure 1-9 B) of the organized wood cell wall components. Cellulose elementary fibrils aggregate into microfibrils, hemicelluloses are located in the space between the microfibrils, while lignin is deposited around the hemicelluloses-decorated cellulose fiber but not between the microfibrils. By using polarized imaging FTIR microscopy, Salmén and coauthors demonstrated that both hemicellulose (Stevanic and Salmén 2009, Simonovi et al 2011, Olsson et al 2011) and lignin (Jurasek 1998, Åkerholm and Salmén 2003, Simonovi et al 2011, Olsson et al 2011, Salmén et al 2012, Chang et al 2014) were oriented parallel to cellulose microfibrils. Atalla and Agarwal (1985) also gave evidences by Raman microscopy, which lignin is orientated in tangential direction in the plane of the cell wall surface. Similarly, lignin was reported to deposit in the spaces between the pre-formed

polysaccharides matrix with a preferential alignment along the cellulose microfibrills (Donaldson and Knox 2012, Faulon and Hatcher 1994, Reis and Vian 2004, Reul et al 2002, Reul et al 2006, Scallan 1974). All these observations and findings imply that the localization of the hemicellulose and lignin is spatially restricted by cellulose.

Lawoko et al (2005) and Du et al (2014) characterized the structure of lignin-hemicelluloses fractions isolated from the spruce, and concluded that lignin bound with xylan was richer in condensed structure (less β -O-4 linkages) than that bound to glucomannan. Thus, probably two types of lignin, surrounded by xylan and surrounded by glucomannan as proposed by Lawoko et al (2005) exist in wood cell wall. Salmén and Olsson (1998) also hypothesized that most glucomannan was bound with cellulose, while predominant xylan was associated with lignin. According to these findings, Henriksson (2009) proposed a molecular architecture model of wood secondary cell wall as shown in Figure 1-9 C. In his model, lignin covalently links with different polysaccharides (glucomannan and xylan), forming a complex matrix.

A recent proposed model (Figure 1-9 D) by Terashima et al (2009, 2012) showed the different role of glucomannan and xylan in wood secondary cell wall assembly process: glucomannan was successively deposited around cellulose elementary fibrils after the biosynthesis of cellulose. Thus, the size of the elementary fibrils aggregates may be controlled by glucomannan; while xylan was deposited after the aggregation of cellulose microfibrils, so the distance between the microfibrils is controlled mainly by xylan; lignin was formed between the microfibrils, thereof it was more directly bound to xylan. The model of the formed

wood cell wall, as shown in Figure 1-9 D–a and –b, is similar to that from Henriksson (2009) shown in Figure 1-9 C.

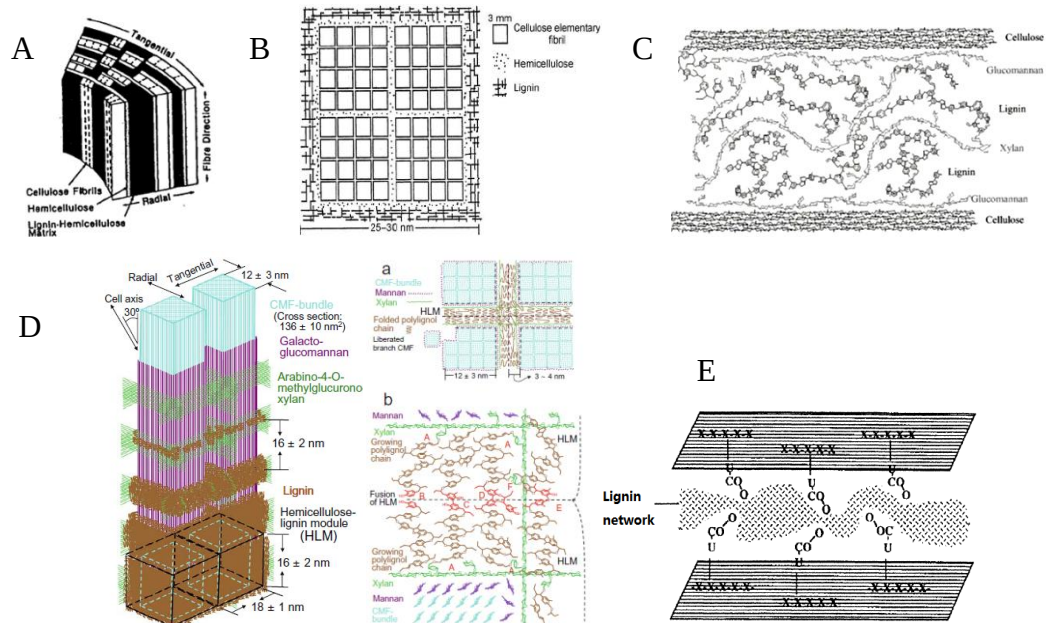


Figure 1-9. Proposed ultrastructural models of wood cell wall. A, cell wall architecture from the citation by Higuchi 2002; B, cell wall cross section from the citation by Klemm et al 2011; C, model of S2 layer from Henriksson 2009; D, model for wood secondary cell wall assembly process (from top to bottom), and its cross-sectional view (a), and the deposition of lignin (b) (Terashima 2009); E, lignin intercalation into the space between xylan-coated cellulose microfibrils (Reis et al 1994, Reis and Vian 2004).

Especially, Vian and coauthors (Vian et al 1992, Vian et al 1994, Reis et al 1994, Reis and Vian 2004) emphasized the function of xylan in wood cell wall. Spaces between microfibrils were controlled by the electrostatic repelling force between the negative charges in uronic acid group on the side chain of glucuronoxylan. In their model (Figure 1-9 E), lignin was inserted into or filled the gaps between the microfibrils. Consequently, the glucuronoxylan-coated cellulose microfibrils were speculated to act as a ‘host structure’ for lignin formation.

There have been some empirical results which support the hypothesis that xylan affects lignin formation. In fact, xylan exists in the lignified cell wall layers (Awano et al 1998, Maeda et al 2000, Ruel et al 2006, Kim et al 2010, Nakagawa et al 2014). Salmón and Burgert (2009) reported that highly substituted xylan was closely associated with lignin with less condensed structure in hardwood. In addition, the frequency of β -O-4 bond in an *in-vitro* lignin, dehydrogenation polymer (DHP), was increased by the presence of xylan (Barakat et al 2007). Thus, xylan is assumed to contribute to lignin deposition in cell wall and steric regulation of lignin polymerization.

On the other hand, there have been opposite findings. Donaldson and Knox (2012) reported that xylan was not closely related to the degree of lignification in Radiata pine. In addition, an isolated xylan-lignin fraction had less β -O-4 linkages than other hemicellulose-lignin fractions (Miyagawa et al 2012, 2013, Lawoko et al 2005, Du et al 2014). Here, I point out that the function of xylan in the lignin formation as the unknown **subject 4-1**. Meanwhile, the function of another important hemicellulose, glucomannan, in lignin formation is as the unknown **subject 4-2**.

1.2.5 Mechanical strength of wood cell walls

The mechanical strength of each cell wall component was reviewed by Gibson (2012), and displayed in Table 1-2. Cellulose, as a crystalline material, has a distinctly higher modulus of elasticity and tensile strength than hemicellulose and lignin, implying cellulose is the major contributor to the cell wall strength. The values for hemicellulose and lignin are much lower than that of cellulose, suggesting that hemicellulose and lignin have only little contribution to

mechanical strength.

Table 1-2. Mechanical properties of cell wall components (Gibson 2012).

Material	Density, Kg m ⁻³	MOE ^b , GPa	T.S. ^c , MPa
Cellulose ^a	1450-1590	120-140 ^d	750-1080
Lignin	1200-1250	2.5-3.7	25-75
Hemicellulose	n.d.	5-8	n.d.

a, along the length of cellulose fiber; b, modulus of elasticity; c, tensile strength; d, data for the MOE of the crystalline cellulose; n.d., no data available.

On the other hand, lignin acts as ‘concrete’ in cell wall, implying that lignin can harden cell wall. In addition, since cell wall components are highly intermixed in wood cell wall, it is thought that the mechanical properties are not strongly dependent on an individual component, but more importantly, on the interactions between wood components and the arrangement of cell wall (Salmén 2004, Salmén and Burgert 2009, Terashima et al 2009). Accordingly, hemicellulose and lignin are supposed to have contribution to the mechanical properties of cell wall (Salmén and Burgert 2009, Terashima et al 2009).

However, the experimental data on the contributions of hemicellulose and lignin to wood cell wall mechanical properties are scarce. **The fifth subject** is the influences of hemicellulose and lignin on the mechanical strength of wood cell wall.

In summary, the above-mentioned unclear subjects in items of wood cell wall in this chapter are (1) the validity of wood cell wall deformation theories, (2) the relationship between MFA and mechanical properties, (3) which are transported by ABC transporter, monolignols or monolignol glucosides? (4) relationships between cell wall components in cell wall, (4.1) the functions of

xylan in the lignin formation, (4.2) the functions of glucomannan in the lignin formation, (5) the influences of hemicellulose and lignin on the mechanical strength of wood cell wall.

To elucidate these unknown subjects of wood cell wall formation, *in-vitro* mimicking approach for wood components and wood cell wall formation process has drawn much attention to the previous researchers.

1.3 Model-mimicking of wood cell wall structure and formation

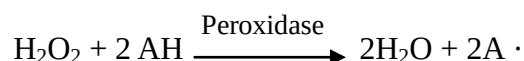
1.3.1 Bacterial cellulose

Bacterial cellulose (BC) is extensively used as a wood cellulose bio-mimetic material for two reasons: one is that BC has the same polymorphism (cellulose I) with the woody cellulose (Uraki et al 2007, 2011), the other one is BC has high purity without any other components like hemicellulose and lignin (Teeri et al 2007). BC, secreted by bacteria of the *Gluconacetobacter* genus, can be obtained from the culture medium. The biosynthesis of cellulose in bacteria involves the genes of CesA, CesB, CesC, and CesD. CesA is for the transfer of the glucosyl residue in UDP-D-glucose to the growing β -1,4-glucan chain, and CesB has a cyclic dimeric guanosine monophosphate (c-di-GMP) binding domain and provides the regulatory subunit. CesC involves in the export of the glucan chains across the bacterial cell wall, while CesD is proposed to have a role in the release of the growing glucan (Wong et al 1990, Ross et al 1991, Saxena et al 1994, Rönling 2002). The resultant BC is usually used as framework for assembly of other wood cell wall components.

1.3.2 Artificial lignin

Lignin formation in trees is an extremely complex process, thus, elucidation of the reaction mechanism of lignin precursor polymerization *in-vivo* is too difficult to carry out. For this reason, dehydrogenation polymers (DHP) as an artificial lignin were prepared *in-vitro*. DHP closely resembles wood lignin in almost all aspects in functional group contents, spectral characters and yields of various degradation products. (Tanahashi and Higuchi 1981) Therefore, DHP is thought to be a good bio-mimicry of wood lignin.

DHP can be prepared in two ways: “Zutropfverfahren” (endwise polymerization) or “Zulaufverfahren” (bulk polymerization). Horseradish peroxidase (HRPC) together with H_2O_2 , were used to produce phenoxy radicals *in-vitro*. The mechanism for the peroxidase catalysis, as proposed by Henriksen et al (1999), shows a three-step cyclic reaction. Firstly, the native state of peroxidase (Fe^{3+}) is oxidized into Compound I (Fe^{4+}) by H_2O_2 with a release of water. Secondly, one phenoxy radical is formed, and Compound I (Fe^{4+}) is converted to Compound II (Fe^{4+}). Third, Compound II (Fe^{4+}) is reduced into native enzyme state, generating one more phenoxy radical and one water molecule. The overall chemical reaction formula is shown below. One mole of H_2O_2 and two moles of substrate (AH) are consumed, while two moles of phenoxy radicals ($A\cdot$) as well as two moles of water are produced.



The structure of the synthetic DHP is variable upon the changes in reaction conditions, for instance, pH of the reaction medium (Terashima et al 1996,

Fournand et al 2003, Grabber et al 2003, Tobimatsu et al 2010), monomers adding rate (Grabber et al 2003), concentration of monolignols (Terashima et al 1996), and peroxidase (Tanahashi and Higuchi 1981). In addition, the presence of the polysaccharides such as pectin (Terashima et al 1995, 1996, Higuchi et al 1971, Touzel et al 2003), extracted hemicellulose (Higuchi et al 1971), arabinoxylan (Barakat et al 2007), and cyclodextrin (Nakamura et al 2006), also affect the structure of DHP.

1.3.3 Fabrication of wood cell wall mimicking models using BC and DHP

Natural assembly process of wood cell wall is generally mimicked by assembly of non-cellulosic polysaccharides and artificial lignin on a BC framework. This bio-mimetic model was used for elucidating the interrelation of components in cell wall. At first, BC/hemicellulose composite was prepared for mimicking the unlignified primary cell wall. For instance, Whitney et al (1999), Chanliaud et al (2002), and Cybulska et al (2010), respectively, cultured *Gluconacetobacter xylinus* in the presence of xyloglucan and/or pectin in the Hestrin-Schramm (HS) medium. They explained the role of each polysaccharide in mechanical properties of cell wall analogues. In addition, Dammström et al (2009) prepared BC/glucuronoxylan nanocomposites by adding glucuronoxylan solution into the microfibril suspension. Their material was used for elucidating the interactions between cellulose and xylan in hardwood cell wall.

Secondly, BC/hemicellulose/DHP composite was prepared for mimicking the lignified cell wall. Touzel et al (2003) performed dehydrogenative polymerization of coniferyl alcohol in the presence of pectin-BC blends. By using this model, they investigated the effects of cellulose and pectin on the

polymerization of coniferyl alcohol.

All of these biomimicking models can help us to understand the real situations in native cell wall. However, the cross section of wood cell walls exhibits a honeycomb-like alignment (Scallan 1974, Ando et al 1999). The BC frameworks used in all aforementioned cell wall models have no controlled morphology, suggesting these models did not mimic the real architecture of plant cell wall. Thus, in this research, a bacterial cellulose film with hexagonal honeycomb patterns will be used to mimic the honeycomb morphology of wood cell wall cross section.

1.3.4 Honeycomb-patterned cellulose film

The honeycomb patterns of cell wall presumably play a role in wood properties, especially the physical strength of wood cell walls (Scallan 1974, Ando et al 1999, Uraki et al 2011). In addition, due to this honeycomb-like alignment aforementioned, all mathematic theories used for wood cell wall deformation mechanism, viz. bending model (Gibson and Ashby 1982, Gibson et al 1982), stretching model (Masters and Evans 1996), bending-stretching model (Masters and Evans 1996), and two-phase annual ring model (Mod é n and Berglun 2008), were established based on a honeycomb unit. As demonstrated by these models, the modulus of elasticity (MOE) of wood cell wall is affected by the geometry of the honeycomb, namely the thickness, length and cell shape angle of the honeycomb hexagonal skeleton. Accordingly, with the objectives of elucidating the validity of these cell wall deformation theories, developing an *in-vitro* bio-model which approximates the real architecture of native cell wall (termed artificial wood cell wall) attracts much attention in our laboratory.

Uraki et al fabricated two kinds of honeycomb-patterned cellulose film with cellulose I and II polymorphisms, honeycomb-patterned bacterial cellulose film (HPBC) and honeycomb-patterned regenerated cellulose film (HPRC), respectively (Uraki et al 2007, Uraki et al 2011). HPBC was produced by the culture of cellulose-producing bacteria, *Gluconacetobacter xylinus*, on an agarose film as a template, which has regular honeycomb array. HPRC was fabricated from cellulose acetate by the transcription with a PDMS template with honeycomb pores on surface.

Uraki et al also attempted to assemble HPBC with an isolated lignin, acetic acid lignin (Uraki et al 2010). The mechanical strength of HPBC was found to be improved by the lignin adsorption. Furthermore, the validity of wood cell wall deformation models (bending, stretching, bending-stretching, and two-phase annual ring model) was evaluated by HPRC (Uraki et al 2015). They demonstrated that the mechanical property, MOE, predicted from the two-phase annual ring model were highly consistent with the results from the mechanical test using the cellulosic film. Therefore, the honeycomb cellulose is thought to be a potential material for better understanding of wood cell wall. However, in these previous works, hemicellulose has not been assembled onto honeycomb cellulose films yet. My work is to fabricate an “artificial wood cell wall” model by assembling both hemicellulose and lignin into HPBC framework.

1.4 Objectives of my research

In this thesis, there are two research objectives. The first objective is to clarify xylan function in wood cell wall formation, lignification in particular. Two

opposite hypotheses on the function were proposed: One hypothesis was that xylan affects lignin formation (Salmén and Burgert 2009, Nakagawa et al 2014, Reis and Vian 2004, Barakat et al 2007), the other one was that xylan does not (Donaldson and Knox 2012, Miyagawa et al 2012, 2013, Lawoko et al 2005, Du et al 2014). The second objective is to elucidate the contribution of xylan and lignin to the mechanical strength of the wood cell wall.

To achieve these objectives, I made a research strategy as follows. At first, I fabricated an artificial wood cell wall composed of cellulose, xylan, and DHP (as an artificial lignin). Such components were stepwise assembled, by which the fabrication process mimics the assembly process of native wood cell wall formation. The xylan function for lignification and the role of lignin on mechanical strength of the cell wall were investigated. These research results will be described in Chapter 2 and 3, and general discussion on the basis of the results will be described in Chapter 4. An outline of each chapter is briefly described as follows:

Chapter 2: To fabricate artificial wood polysaccharides matrix.

I attempted to prepare HPBC as the starting material for creation of artificial wood cell wall. Then, xylan was adsorbed onto HPBC. The distribution of xylan was observed under a fluorescence microscope by a combination with immunolabeling. The resultant xylan-HPBC binary blend would be used as the artificial wood polysaccharides matrix to elucidate the xylan function for lignification.

Chapter 3: To fabricate artificial wood cell wall and to investigate the effects of xylan on the lignin formation.

I attempted to perform the dehydrogenative polymerization of a lignin precursor, coniferyl alcohol, in the artificial wood polysaccharides matrix. The resultant DHP-deposited xylan-HPBC tertiary blend was used as the artificial wood cell wall.

Chapter 4: General discussion

I attempted to summarize the conclusions obtained in this research, and generally discuss the formation process of DHP in the fabrication process of artificial wood cell wall.

CHAPTER 2

Fabrication of Artificial Wood Polysaccharides Matrix

2.1 Introduction

The objective in this chapter is to fabricate artificial polysaccharides matrix composed of cellulose and hemicellulose. To achieve it, cellulose film as a framework is prepared for the first step. Flat bacterial cellulose film (FBC) from the bacteria-produced pellicles has been widely used as a host for the deposition of non-cellulosic polysaccharides (Chanliaud et al 2002, Cybulska et al 2010, Touzel et al 2003, Whitney et al 1999), and/or DHP (Touzl et al 2002). However, most cellulose fibrils of this FBC film are not exposed outside, indicating low response of cellulose filaments to guest molecules or external stimuli. To overcome the problem, I fabricated another material, honeycomb-patterned bacterial cellulose film (HPBC), in addition to the FBC as a BC pellicle. HPBC has two advantages in comparison with FBC, (i) most fibrils of HPBC are assumed to be exposed to the outer circumstance, and thus they would have high accessibility to external guests; (ii), since the hexagonal alignment, HPBC mimics the cross-section of native wood cell wall arrangement. Based on these merits of HPBC, I consider it as a better framework for artificial wood polysaccharides matrix. After the preparation of HPBC, as a second step, xylan as a major hemicellulose in wood is attempted to adsorb onto the cellulose frameworks.

2.2 Materials and Methods

2.2.1 Materials

An amphiphilic copolymer (CAP) was purchased from Wako Pure Chemicals (Osaka, Japan), which was synthesized with dodecylacrylamide and ω -carboxyhexylacrylamide as reported previously (Nishikawa et al 2002). Polydimethylsiloxane (PDMS) and its curing agent (SYLGARD 184 Silicone Elastomer Kit; Dow Corning Corp., Midland, MI, USA) were purchased from Hokkaido Wako Pure Chemicals, Sapporo, Japan. Agarose (melt point at 65 °C) and xylan isolated from beechwood were purchased from Wako Pure Chemicals (Osaka, Japan) and Sigma-Aldrich (Steinheim, Germany) respectively. Primary antibodies, LM 10 and LM 11, for xylan recognition were got from PlantProbes, Leeds, UK. A secondary antibody, goat anti-rat IgG Alexa Fluor 568 was purchased from Invitrogen, Carlsbad, CA. Other used chemicals were purchased from Wako Pure Chemicals, Osaka, Japan. All commercial chemicals and reagents were used as received.

2.2.2 Fabrication of honeycomb-patterned bacterial cellulose film (HPBC)

A schematic procedure to prepare HPBC is shown in Figure 2-1. The first step for preparing a HPBC film was the fabrication of an agarose gel with honeycomb-patterned grooves on the surface, which was prepared according to the transcription method reported by Uraki et al (2007a, 2011). To fabricate it, three templates were prepared as follows. The first template as a convex honeycomb-patterned film was prepared from a mixture of polycaprolactone and CAP (weight ratio, 9:1) by blowing humid air onto the mixture/chloroform solution. Poly(dimethyl siloxane) (PDMS) together with curing agents was poured onto the first template. After carefully removing air bubbles in PDMS by vacuum, the PDMS was allowed to stand at room temperature for 48 h, and then peeled off

from the first template to give a second template with concave honeycomb-patterned surface. By repeating transcription of the second template with PDMS, the third template with convex honeycomb morphology was obtained. Finally, 1.5 % of hot agarose aqueous solution containing nutrients of Hestrin-Schramm (HS) medium (2.0 % of glucose, 0.27 % of anhydrous Na_2HPO_4 , 0.115 % of citric acid, 0.5 % of polypeptone and 0.5 % of yeast extract, pH 6.0; Hestrin and Schramm 1954) was poured onto the third PDMS template. After cooling for coagulation of agarose, agarose gel with concave honeycomb-like grooves was generated and peeled off from the third template to give the concave honeycomb agarose template.

The second step for preparing a HPBC film was culture of cellulose-producing bacteria on the honeycomb-patterned agarose template. The concave honeycomb-like agarose film was placed on a flat agarose gel as a support in a Petri-dish with a diameter of 90 mm. *Gluconacetobacter xylinus* (ATCC53582), which was pre-cultured in HS medium for 3-4 days, was inoculated in 250 μL and cultured on the agarose template at 28 °C for several days under a CO_2 atmosphere with a concentration of more than 90%, where CO_2 concentration was monitored on a Cosmotector XP-314, New Cosmos Electric Co., Osaka, Japan. The culture was carried out in a chamber that equipped two tubes for gas inlet and outlet, and the petri-dish for the culture was placed on a float of polystyrene foam on water in the chamber. Relative humidity inside the chamber was kept at almost 100%. After the culture, the agarose template with HPBC was immersed in hot distilled water at 70 °C, resulting in agarose dissolution. As a result, HPBC remained in the water. The resultant HPBC films

were scooped up, and then soaked in 1 M aqueous sodium hydroxide at 65 °C for 1 h. After washing the films with distilled water repeatedly until pH of the washings became 7, the films were air-dried, and kept in a refrigerator until use.

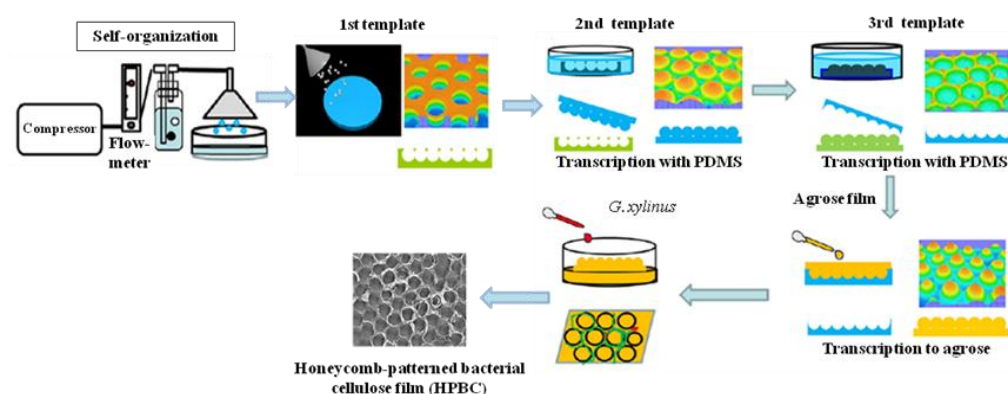


Figure 2-1. Preparation of HPBC (adapted from Uraki et al 2007a).

2.2.3 Fabrication of flat bacterial cellulose film (FBC)

FBC was prepared by inoculating 250 μ L of bacterial suspension, in which *G. xylinus* was pre-cultured, into a fresh HS medium for 12 h (Figure 2-2). The obtained FBC were also soaked in 1 M aqueous sodium hydroxide, and then washed, dried as described in the HPBC preparation.

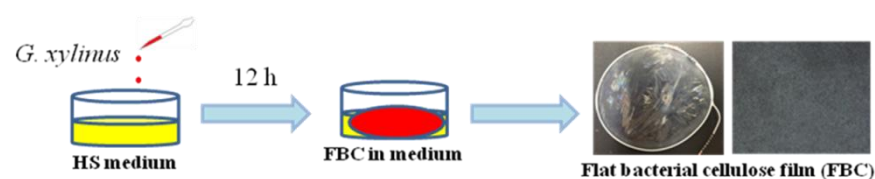


Figure 2-2. Preparation of FBC.

2.2.4 HPBC and FBC morphologies observation

The morphologies of the obtained HPBC films were observed on a VK-9500 violet-laser color 3D profile microscope (Keyence, Osaka, Japan), while that of the obtained FBC films were observed on a JSM-6301F field emission-scanning electron microscope (FE-SEM; JOEL Ltd., Tokyo, Japan)

operated at an accelerating voltage of 5 kV. The samples for SEM observation were coated with gold-palladium by cathodic spreading type of an E101 ion sputter (Hitachi, Tokyo, Japan).

2.2.5 Characterization of beech xylan

Beech xylan was subjected to acid hydrolysis by Klason method (Dence 1992) for measuring its lignin content, and neutral sugar compositions in the hydrolysate were analyzed (Winarni et al 2013). At first, 500 mg of xylan was immersed in 7.5 mL of 72 w% sulfuric acid for 4 h at room temperature. Afterwards, the sulfuric acid was diluted to a concentration of 4 w% with distilled water, and the suspension was allowed to react for 1.5 h at 121 °C in an autoclave. The hydrolysis suspension was filtered after the reactor was cooled down. The residue of filtration was thoroughly washed with distilled water, dried in an oven at 105 °C. Klason lignin content in beech xylan was obtained from the weight of the dry residue. Meanwhile, the filtrate was neutralized with barium hydroxide, and then centrifuged at 4800 g. The supernatant was subjected to a high performance liquid chromatograph (HPLC) (Shimadzu VP series system, Kyoto, Japan) for the measurement of the neutral sugar compositions. One milliliter of this supernatant was filtered by an advantec syringe filter (0.45 µm, Toyo Roshi Kaisha Ltd., Tokyo, Japan) before injection. The injection volume was 20 µL. The column used in the HPLC system was a Shodex Sugar SP 0810 (Showa Denko K.K., Tokyo, Japan) with 300 mm in length × 7.5 mm i.d., together with a guard column (Shodex Sugar SP-G; 50 mm in length × 7.5 mm i.d.). Column oven temperature was set at 80 °C, and MilliQ water as an eluent and a Corona detector (ESA Bioscience Inc., Chelmsford, MA, USA) were used. Calibration lines were

made by using authentic D-xylose, D-galactose, D-mannose, L-arabinose and D-glucose. An authentic D-mannitol was added to the sample solution as an internal standard.

Uronic acid content in xylan was determined according to the method reported by Blumenkranz and Asboe-Hansen (1973). $\text{Na}_2\text{B}_4\text{O}_7$ solution (1.2 mL, 12.5mM in conc. H_2SO_4) was added into 0.2 mL of aqueous beech xylan solution (0.4 mg/mL) in an ice bath. After cooling, the reaction was carried out in an oil bath at 100 °C for 5 min. After cooling of reactants, 20 μL of m-hydroxydiphenyl (0.15% in 0.5% of aqueous NaOH) was added into the reactor. The absorbance at 520 nm was measured on a UV spectroscop (Shimadzu UV-1800, Tokyo, Japan). The content of uronic acid was calculated from its absorbance with a calibration line ($y=0.083x+0.06$) made by using an authentic glucuronic acid. The effects of xylose on this measurement were estimated by a calibration line of $y=0.427x-0.001$.

2.2.6 Xylan adsorption on cellulosic films

A sheet of cellulosic film (2.5 mg of FBC and 0.8 mg of HPBC with 90 mm in diameter) was immersed in 10 mL of aqueous beech xylan solution at different concentrations of 0.1 – 1.0 mg/mL for 1 d at room temperature. Then, the films were picked up and washed with 1 mL of distilled water, and then air-dried to yield xylan-adsorbed FBC and HPBC. The residual xylan solutions were subjected to HPLC analysis for the measurement of xylan adsorption.

2.2.7 Immunolabeling of xylan after xylan adsorption on HPBC and FBC

To confirm the distribution of xylan inside the HPBC and FBC films, the films after xylan adsorption were embedded in epoxy resins. After curing in an

oven (35 °C for 1 d, followed by 45 °C for 1 d and 65 °C for 3 d), the resultant resins were trimmed and sectioned with a diamond knife on a rotary microtome (Reichert-Jung ULTRACUT E, Heidelberg, Germany) to get the cross sections of films (Figure 2-3). Immunofluorescent labeling of xylan in the cross sections of both HPBC and FBC was preformed according to the procedure described by Kim et al (2010). Cut sections (1.0 μ m in thickness) were mounted on super frost aminosilane (MAS)-coated slides (Matsunami S9441, Matsunami Glass Ind. Ltd., Kishiwada City, Japan). After treating with 50 mM glycine/phosphate-based saline (GPBS) solution for 15 min at room temperature, the slides were suspended in a blocking buffer (GPBS solution containing 3% skim milk) for 30 min, followed by washing with GPBS buffer solution three times for 10 min each. After that, sections were incubated in two kinds of primary antibodies for xylan, LM 10 and LM 11 (PlantProbes, Leeds, UK; 1:20 dilution in GPBS buffer) at 4 °C for 2 days. After washing three times with GPBS buffer, sections were incubated in the secondary antibody, goat-anti-rat IgG Alexa Fluor 568 (Invitrogen, Carlsbad, CA, USA; 1:50 dilution in GPBS buffer) at 35 °C for 2 h. After three times washing as above, sections were mounted with Pristine (Falma, Tokyo, Japan). Sections as control specimens were incubated only with the secondary antibody without the primary antibodies. Micrographs of immunolabeling specimens were taken with a BX50 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a Semrock TxRed-4040C filter set (E_x 470–490 nm, E_m 515–550 nm, Opto-Line Inc., Tokyo, Japan).

2.2.8 Xylan adsorption analysis

The concentration of xylan solution before and after xylan adsorption on

cellulosic films was measured by using the above HPLC system with a different column (Shodex Asahipak GF 7M HQ with 300 mm in length $\times$ 7.5 mm i.d. together with a guard column (Shodex Asahipak GF 1G 7B with 50 mm in length $\times$ 7.5 mm i.d.; Showa Denko K.K., Tokyo, Japan). The column oven temperature was 40 °C. The xylan concentration was calculated from total area of xylan peak by using a calibration line. The adsorption of xylan was expressed as weight percent of cellulose according to the following equation:

$$W = \frac{10 \times (C_0 - C_1)}{M} \quad \text{Equation 2-1}$$

where W (g/g of cellulose) is the adsorption of xylan on a cellulosic film, 10 (mL) is the volume of xylan solution, C_0 (mg/mL) is xylan concentration before adsorption, C_1 (mg/mL) is xylan concentration after adsorption, and M (mg) is the weight of a cellulosic film.

The xylan-adsorbed FBC and HPBC films were washed once with 1 mL of distilled water and air-dried, and then were subjected to the experiment of DHP formation in the Chapter 3.

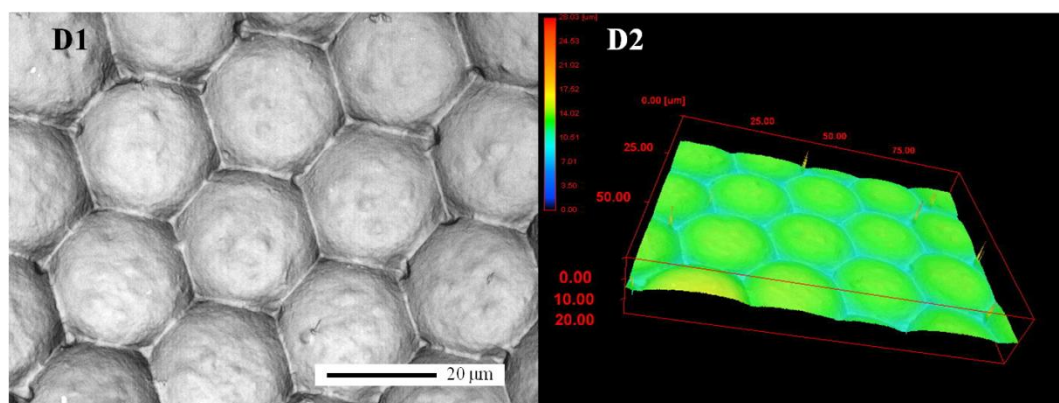
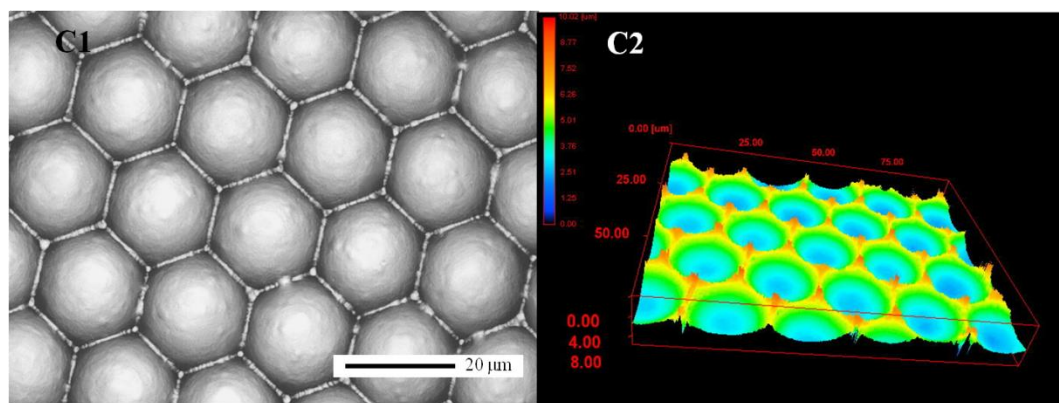
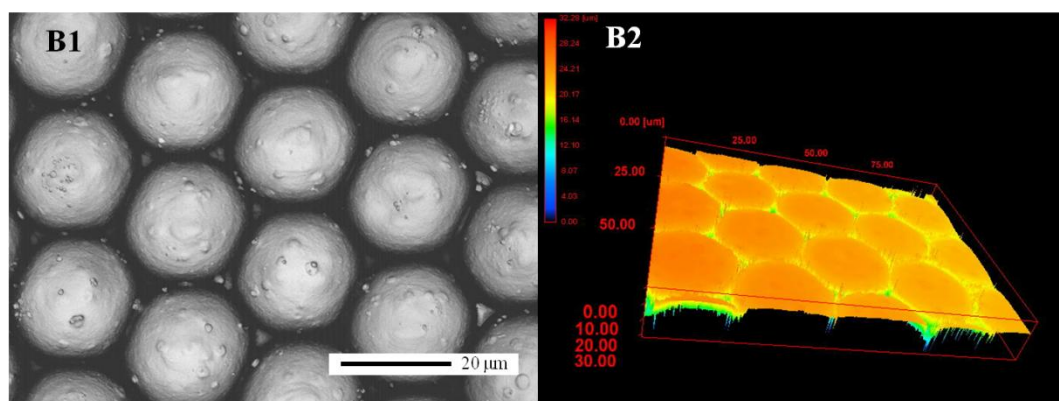
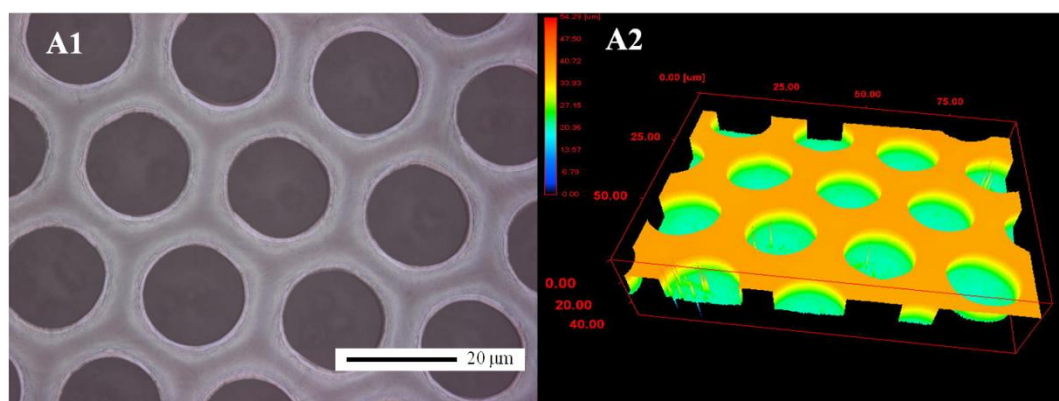
2.3 Results and discussion

2.3.1 Fabrication of BC

2.3.1.1 Fabrication of HPBC

My strategy to prepare HPBC with cellulose I polymorphism similar to the structure of native cellulose was to cultivate a cellulose-producing bacterium, *Gluconacetobacter xylinus* (ATCC53582), on a convex honeycomb-patterned agarose film as a template for bacterial moving. Therefore, the first step was the preparation of this agarose film as the template. A film of PCL/CAP mixture was

prepared as a first mold to prepare the agarose template. The fabrication of honeycomb-patterned material was performed on the basis of the self-organization of amphiphilic polymer, CAP, with the assistance of water droplet as a hexagonal template (Widawski et al 1994). The resultant film prepared has a regular honeycomb-patterned pores with diameters about 20 μm (Figure 2-3 A1). These hexagonal array of honeycomb is a concave type of wall (Figure 2-3 A2). However, this template has two serious drawbacks: one is that honeycomb-patterned wall is formed inside the film, and the other is that the polymers of film are very easy to dissolve in organic solvent, such as chloroform and acetone. Therefore, it cannot be directly used as a mold for the preparation of agarose template. Hence, I transcribed the morphology of honeycomb-patterned PCL/CAP film with PDMS in order to get a second template with opposite surface morphology (Figure 2-3 B1). This template is un-dissolvable in any organic solvent because of cured PDMS rubber. Therefore, it can be repeatedly used as a template. This convex type of PDMS honeycomb (Figure 2-3 B2) was, in turn, further transcribed with PDMS into a concave type of third template (Figure 2-3 C1 and C2). Finally, a hot agarose aqueous solution containing nutrient for bacteria was poured onto the third template (concave PDMS) for transcription of the surface morphology to give an agarose film with a convex type of surface (Figure 2-3 D1 and D2). The 3-D microscopic image of Figure 2-3 D2 clearly demonstrated that this agarose film had regular honeycomb-patterned valleys with the depth of ca. 3 μm .



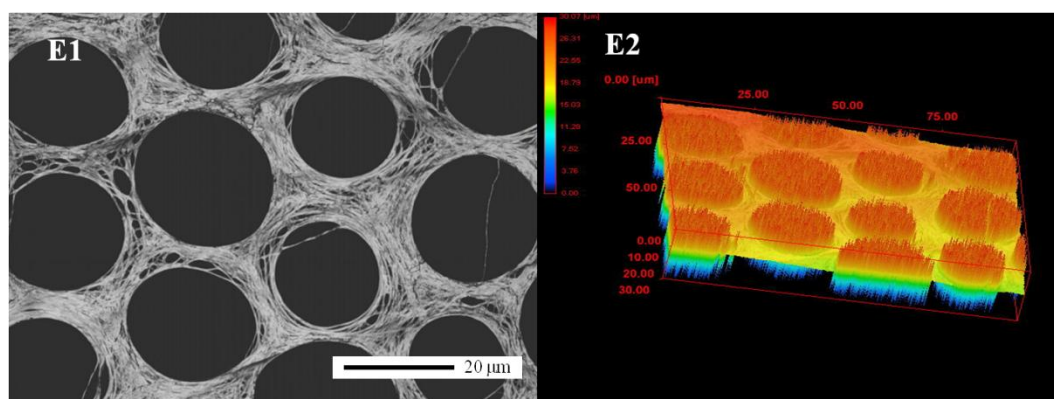


Figure 2-3. 3D microscope images of 1st template of PCL/CAP film (A1), 2nd template of PDMS (B1), 3rd template of PDMS (C1), agarose film (D1), and HPBC film (E1). A2-E2 are corresponding 3D images of A1-E1.

A final process of HPBC preparation was culture of a cellulose-producing bacterium, *G. xylinus*, which has been reported to move along the grooves of agarose together with secretion of cellulose under the unique conditions, that is humid and CO₂-rich atmosphere up to 90 % (Uraki et al 2007a, 2011). Although most of *G. xylinus* cannot be alive under the conditions, the survived ones were found to move along the hexagonal grooves of agarose template, and produce cellulose more effectively than those incubated under conventional conditions. Surface morphology of the resultant HPBC film is shown in Figure 2-3 E1. In the figure, white parts are cellulose filaments, and the black parts are hollow pores with the diameter about 20 μm (Figure 2-3 E2). Thus, honeycomb patterns of cellulosic filaments were successfully prepared by the method above mentioned.

2.3.1.2 Fabrication of FBC

As a reference of non-porous cellulosic film, FBC was prepared by cultivating *G. xylinus* (ATCC53582) in liquid HS medium. As shown in Figure 2-4 A, FBC was non-porous in this magnification under the 3D microscope. The

3D image of this FBC (Figure 2-4 B) shows a uniform color (blue or greenish blue), which means FBC has a flat surface. However, these images were not enough to demonstrate the structure of FBC in detail because of a low magnification. Thereby, SEM images were, in turn, taken to observe its morphology.

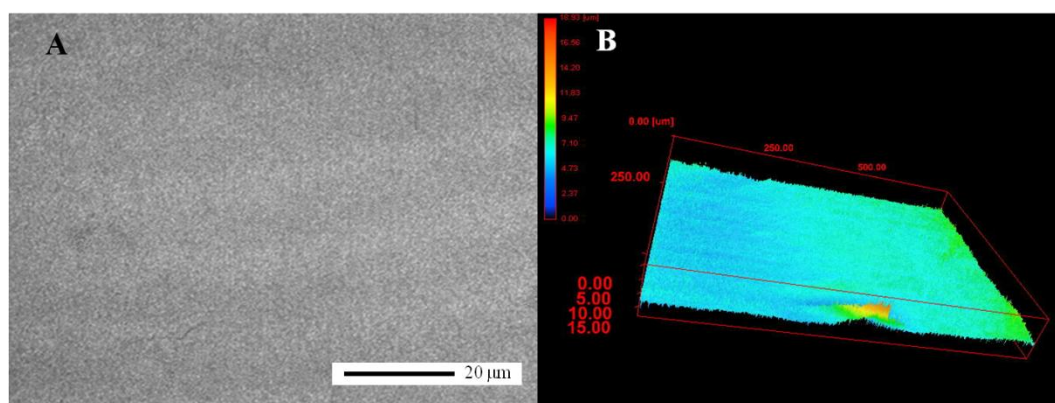


Figure 2-4. 3D microscope image of FBC (A) and its 3D image (B).

In Figure 2-5 A, the surface of FBC was apparently very flat but with some small pores. To check these pores, we observed them at higher magnifications. As shown in Figure 2-5 B, these pores are not arrayed like honeycomb. Probably, they were formed between the spaces of microfibrils. From the cross section of FBC (Figure 2-5 C), some spaces or gaps between microfibrils could also be observed inside the films. These spaces seem to provide transfer routes for guest molecules (such as xylan and coniferyl alcohol in the following experiment) to enter the film, or serve as deposited sites for guest molecules localization inside film.

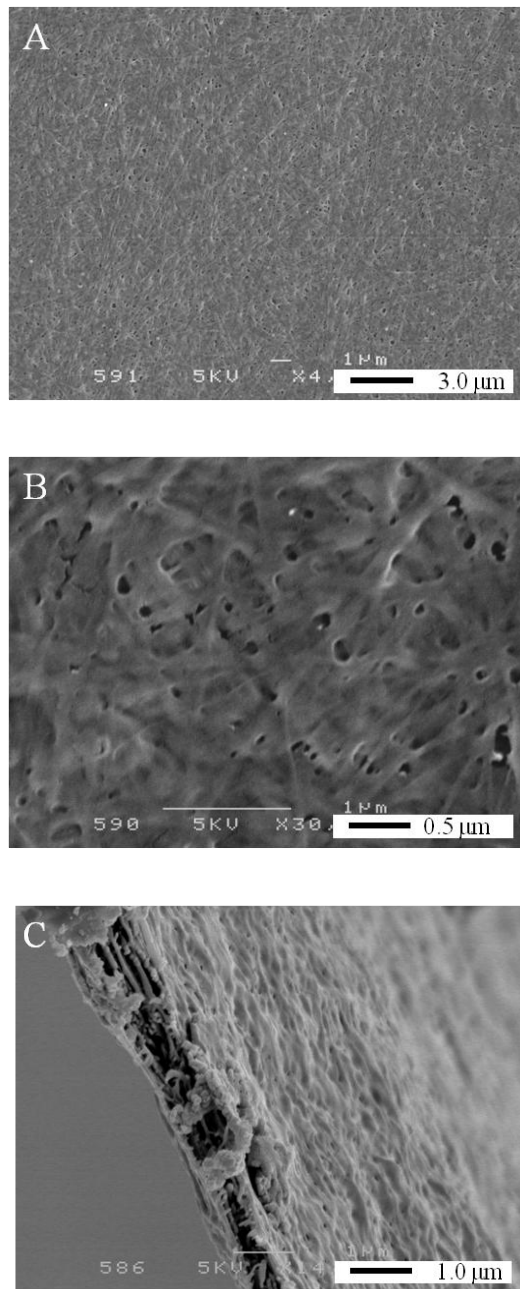


Figure 2-5. SEM images of FBC. A, surface morphology of FBC; B, magnified image of A; C, cross section of FBC.

2.3.1.3 Comparison in cellulose microfibrils between HPBC with FBC

Both HPBC and FBC have been confirmed to be composed of cellulose microfibrils with cellulose I polymorphism as BC pellicles (Uraki et al 2007a, 2011). However, the arrangement of microfibrils in HPBC is different from that in

FBC. As shown in Figure 2-3 E1, the microfibrils in HPBC are highly oriented, this is because microfibrils are aggregated in a narrow space of agarose hexagonal valleys (Uraki et al 2007a). In the case of FBC, bacteria could move freely in the HS liquid medium, thus, the secreted microfibrils in FBC appear to randomly aggregate, and interlaced with each other without any orientation (Figure 2-5 B). This difference in the microfibrils arrangement is displayed as models represented for HPBC and FBC in Figure 2-6. Additionally, I can found that most parts of microfibrils in FBC faces pellicle inside, and only a few of microfibrils at the surface are exposed outwards (Figure 2-5 B and C), whereas most cellulose microfibrils in HPBC are exposed outwards (facing air and solvent) (Figure 2-3 E1). These structural differences between HPBC and FBC would probably affect the amount of xylan adsorption or lignin deposition, which will be discussed in the section of “Xylan adsorption onto HPBC and FBC” in this Chapter and DHP formation in the Chapter-3.

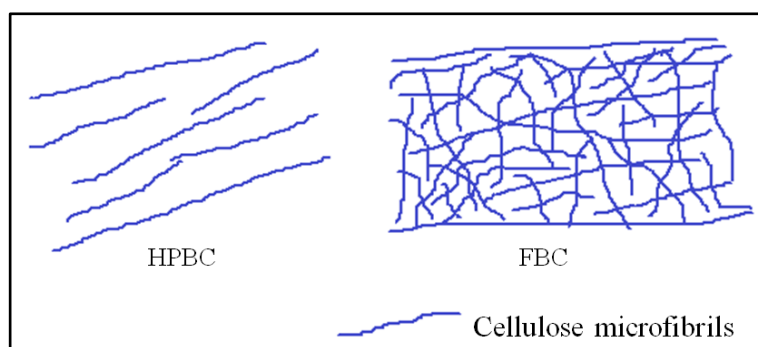


Figure 2-6. Models of HPBC and FBC.

2.3.2 Xylan adsorption onto HPBC and FBC

2.3.2.1 Adsorption isotherms of xylan on HPBC and FBC

Constituent sugars of beech xylan were released as monosaccharides by sulfuric acid hydrolysis in the Klason method, which was used for determination

of lignin content (Dence 1992). Thereby, Klason lignin and constituent sugars can be determined from the filtration residue and the filtrate in the method. As a result, the Klason lignin content was estimated as 1.3% based on the weight of xylan. Sugar compositions in the filtrate was found to give 86.0% xylose, 1.2% galactose, 0.5% mannose, 0.5% glucose, and 11.8% glucuronic acid (Table 2-1).

Table 2-1. Sugar compositions of beech xylan

Xylose	Galactose	Mannose	Arabinose	Glucose	Uronic acid
86.0 %	1.2%	0.5 %	0	0.6 %	11.8 %

Adsorption isotherms of xylan on HPBC and FBC are shown in Figure 2-7. The adsorption amount of xylan on HPBC at 1.0 mg/mL was 0.77 g/g of cellulose, while that on FBC was 0.27 g/g of cellulose. Likewise, the adsorption of xylan on HPBC was much higher than that on FBC at any concentration. This result indicated that HPBC had a larger accessible area to xylan than FBC did. The reason may be attributed to the difference in cellulose fibrils: most cellulose fibrils in HPBC are exposed outwards (facing air and solvent), whereas most part of fibrils in FBC faces pellicle inside, and only the fibrils at the surface are exposed outwards. Thereby, xylan could easily interact with most of the cellulose microfibrils in HPBC as compared with those in FBC. I thus considered that HPBC was a suitable platform for the evaluation of interaction between cellulose microfibrils and guest molecules.

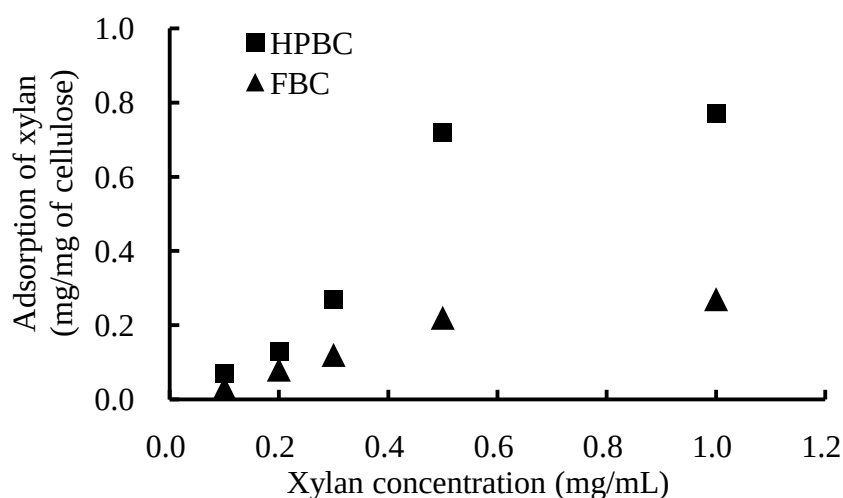


Figure 2-7. Adsorption isotherms of xylan on HPBC and FBC films.

2.3.2.2 Xylan distribution inside HPBC and FBC after xylan adsorption

After immersing cellulosic films into xylan aqueous solution, I investigated the distribution of xylan in the films by a combination of immunolabeling and fluorescent microscopy. The films after xylan adsorption were embedded into epoxy resins, and then the specimens were trimmed and microtomed to give 1 μm thick cross sections after curing the resins. Figure 2-8 shows the microtomed positions for HPBC and FBC series. At least two cross sections at the first and the second lines were cut out.

Xylan in the cross sections was immunolabeled with antibodies, LM 10 and LM 11, and then immunolabeled specimens were, in turn, labeled with the second antibody with fluorescent chromophore. The second immunolabeled specimens were observed under a fluorescence microscope. LM 10 can recognize low-substituted and/or un-substituted xylan, while LM 11 can recognize not only low-substituted and/or un-substituted xylan, but also arabinoxylan (McCartney et al 2005). Both antibodies have been widely used for immunolabeling xylan in

both hardwood and softwood, for instance, birch (McCartney et al 2005), poplar (Kaneda et al 2010), pine (Donaldson and Knox 2012), and cedar (Kim et al 2010). The control specimens (with the secondary antibody only) omitting incubation of primary antibodies (LM 10 and LM 11) did not show obvious fluorescence including self-fluorescence of lignin (A1 and B1 in Figures 2-9), whereas the other test specimens clearly indicated red fluorescence, which revealed the existence of xylan. In Figures 2-9 A2 and A3, xylan distributes in the both outside and whole inside of xylan-adsorbed FBC. In the case of xylan-adsorbed HPBC (Figures 2-9 B2 and B3), xylan was also found to deposit inside the film. Hereafter, xylan-deposited HPBC and FBC films were abbreviated as Xyl-HPBC and Xyl-FBC, respectively.

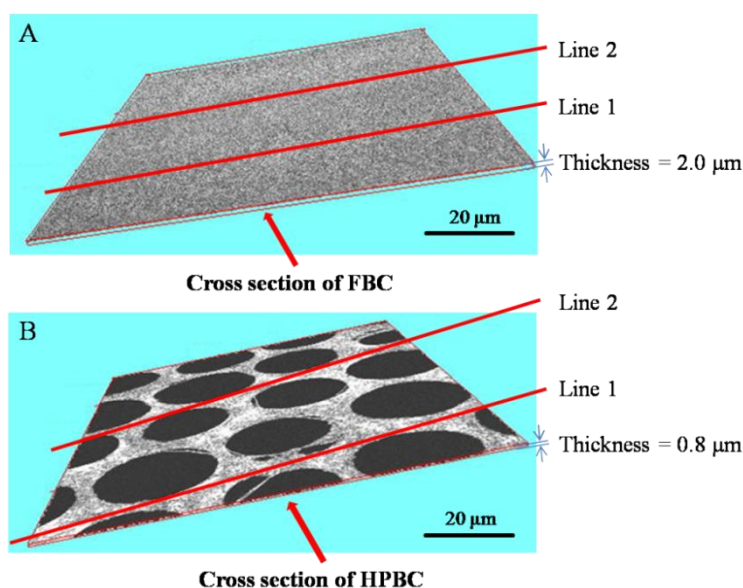


Figure 2-8. Cutting positions for the cross section of FBC (A) and HPBC (B). Images were taken under the violet-laser color 3D profile microscope.

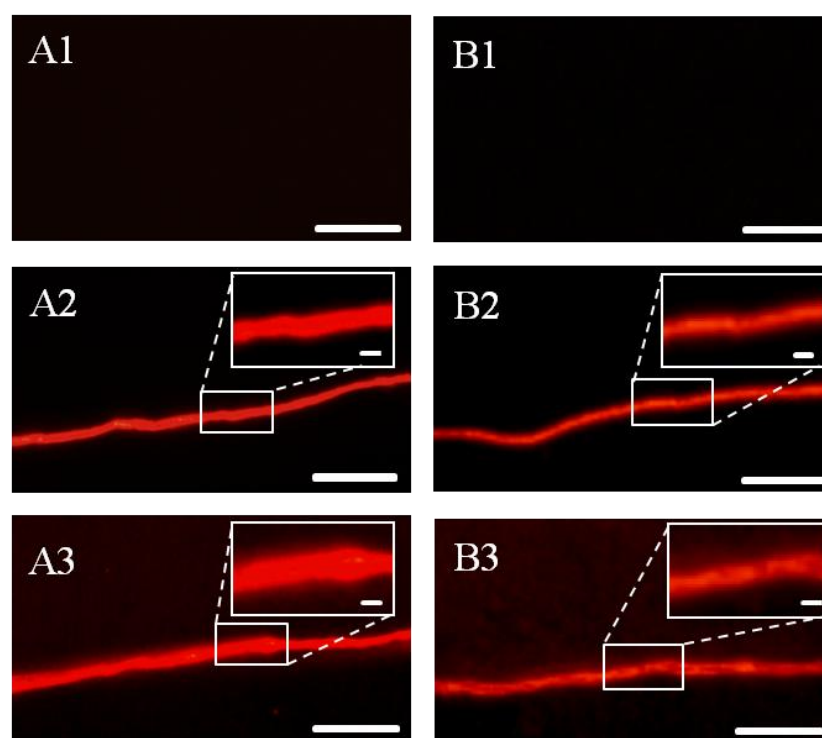


Figure 2-9. Immunofluorescent labeling of xylan in the cross sections of xylan-deposited FBC series (A1-A3), xylan-deposited HPBC series (B1-B3). A1 and B1, controls without primary antibodies labeling; A2 and B2, immunolabeling with LM 10; A3 and B3, immunolabeling with LM 11. Longer bars are 20 μm ; shorter bars in magnified images are 2 μm .

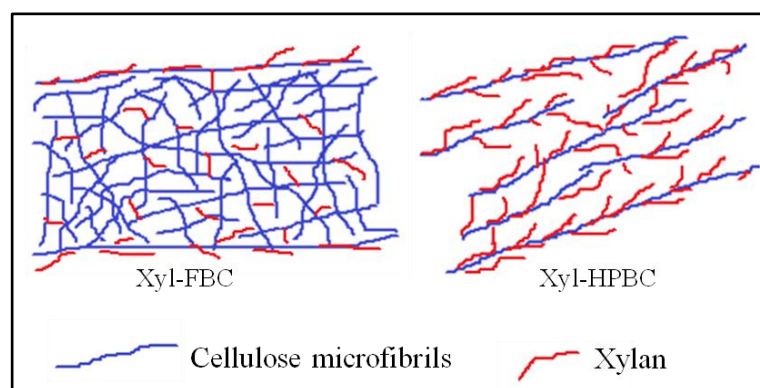


Figure 2-10. Models of Xyl-FBC, and Xyl-HPBC.

Here, I propose speculated structures of Xyl-HPBC and Xyl-FBC in Figure 2-10, where xylan chains (red lines) bind with cellulose microfibrils (blue lines) outside and inside, thereafter, deposit on film surface and in the gaps between

microfibrils inside films. However, due to the lower adsorption of xylan in FBC than in HPBC, xylan deposited inside FBC is supposed to be less than that deposited inside HPBC (indicated by the number of red lines).

2.4 Summary

An artificial solid polysaccharides matrix composed of hemicellulose and cellulose has been prepared by the fabrication of HPBC and FBC films as frameworks followed by xylan adsorption onto them. For both HPBC and FBC, xylan was observed to distribute not only on the surface but also inside the films after the xylan adsorption. However, HPBC showed a much higher adsorption of xylan than FBC did. The reason for this was deduced to be less interaction of xylan with cellulose microfibrils inside FBC. The prepared artificial polysaccharides matrix, termed Xyl-HPBC and Xyl-FBC, will be used as polysaccharides matrix or polymerization medium for lignin (DHP) formation in the next experiment described in Chapter 3.

CHAPTER 3

Dehydrogenation Polymer (DHP) Formation in the Artificial Wood Polysaccharides Matrix

3.1 Introduction

The second step for preparing the artificial wood cell wall is to assemble the lignin into the artificial polysaccharides matrix. In this chapter, I synthesized dehydrogenation polymer (DHP) of coniferyl alcohol as a monolignol in the Xyl-BC polysaccharides matrix. Objectives of this chapter are (i) to empirically evaluate validity of two opposite hypotheses for the effects of xylan on the lignin formation in trees; one is that xylan affects lignin formation (Awano et al 1998, Reis et al 1994, Reis and Vian 2004, Salmén and Burgert 2009, Nakagawa et al 2014), the other one is that xylan does not (Donaldson and Knox 2012, Du et al 2014, Lawoko et al 2005, Miyagawa et al 2012, 2013), and (ii) to clarify the influences of xylan and DHP on the mechanical properties of cellulose framework.

3.2 Materials and Methods

3.2.1 Materials

Horseradish peroxidase (100 units/mg) was purchased from Wako Pure Chemicals, Osaka, Japan. Primary and secondary antibodies are the same as ones used in Chapter 2. All of the other chemicals or reagents were purchased from Wako Pure Chemicals, Osaka, Japan, and used as received.

3.2.2 Preparation of dehydrogenation polymer (DHP)

3.2.2.1 Synthesis of coniferyl alcohol

Coniferyl alcohol, one of the precursors of lignin termed as monolignols, was synthesized in two steps, synthesis of monoethyl malonate from diethyl malonate and synthesis of coniferyl alcohol with vanillin and monoethyl malonate (Figure 3-1).

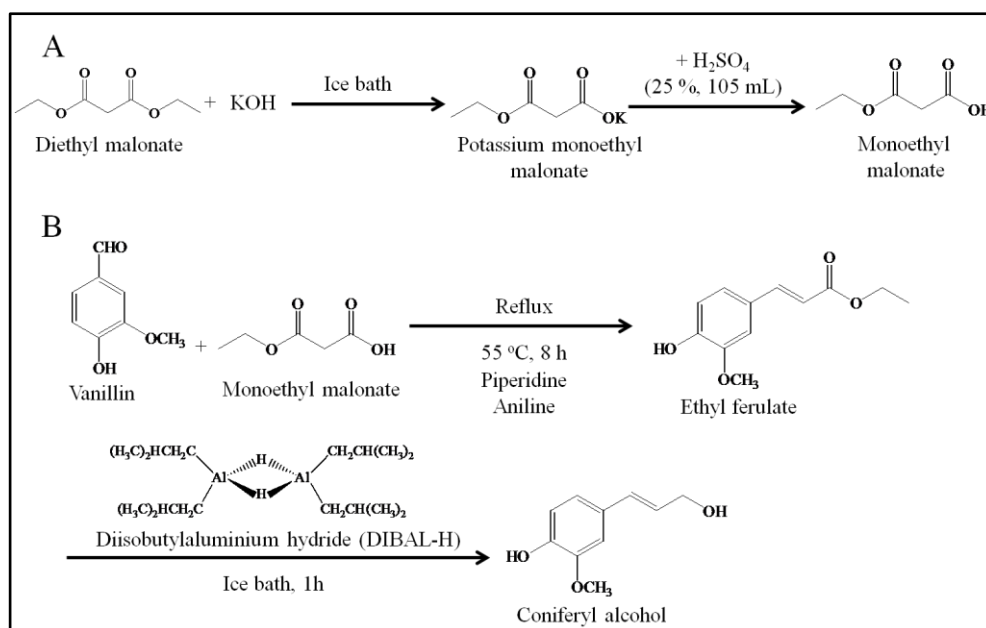


Figure 3-1. Synthesis of coniferyl alcohol. A, synthesis route of monoethyl malonate from diethyl malonate; B, synthesis route of coniferyl alcohol with vanillin and monoethyl malonate.

As the first step, 227 mL (1.5 mol) of diethyl malonate and 98.4 g (1.5 mol) of potassium hydroxide (85.5% purity) were separately dissolved in 800 mL ethanol (99.9% purity). Diethyl malonate/ethanol solution in a flask was placed in an ice bath, potassium hydroxide/ethanol solution was dropwise added into diethyl malonate for 1-2 h with stirring. When a crystallized product was formed during the addition, stirring speed was gradually increased. After the complete addition, stirring was further continued for about 1 h. Afterwards, the reaction

mixture was filtrated using a Büchner funnel. The residue was washed with ca. 50 mL of cold ethanol for 3 times, and then air-dried overnight followed by dried *in-vacuo* at 50 °C for one day. 25% sulfuric acid was added to 100 g (0.58 mol) of aqueous solution of the obtained potassium monoethyl malonate until pH 1-2. The mixture was stirred for 1 h. Then, monoethyl malonate was extracted with ca. 500 mL of diethyl ether for 3 times. The organic layer was washed with 300 mL of distilled water for 3 times, and 300 mL of saturated saline for 3 times (until pH 3). The organic layer after washing was dried over anhydrous sodium sulfate for 2 h, and then the solvent was evaporated to give crude product. Pure monoethyl malonate was collected by reduced pressure distillation (collected when steam temperature was higher than 100 °C). Purity and structure of monoethyl malonate were confirmed by thin-layer chromatography (TLC, eluent: chloroform) and ¹H NMR (solvent, CDCl₃ with 0.05% v/v TMS; measured by 270 Hz-NMR spectrometer). δ-1.29 (t, 5-H, *J* = 7 Hz), 3.44 (s, 2-H), 4.25 (q, 4-H, *J* = 7 Hz), 10.22 (s, COOH1). The mole yield of monoethyl manolate based on diethyl manolate was 12.7%.

As the second step, 20 g (0.13 mol) of vanillin was dissolved in 10 mL of pyridine. Twenty three mL (0.19 mol) of synthesized monoethyl malonate was added into vanillin solution, and 3 drops of piperidine and 3 drops of aniline were also successively added. The reaction was carried out under nitrogen atmosphere at 55 °C for 8 h. The mixture was dissolved in 100 mL of chloroform, and then washed with 100 mL of hydrochloric acid at 1 mol/L once, and with 100 mL of hydrochloric acid at pH 3 (HM-5S pH meter, TOA Electronics Ltd., Tokyo, Japan) twice. After washing, the pH of water layer reached less than 3. The organic layer

was then dried over anhydrous sodium sulfate for 2 h, and the solvent was concentrated by evaporation. Afterwards, the reaction product was purified by silica-gel column chromatography with ethyl acetate/hexane (1:2, v/v) as an eluent. The collected product fraction was concentrated by evaporation. In this process, crystalline ethyl ferulate was generated. After completely evaporation, the obtained crystalline products were dried *in-vacuo* at 40 °C overnight. The purity of ethyl ferulate was checked by TLC (eluent, ethyl acetate/hexane, 1:2, v/v) and ^1H NMR (CDCl_3 with 0.05% v/v TMS, 270 Hz). δ -1.30 (t, 11-H, $J = 7$ Hz), 3.87 (s, OCH_3 3'), 4.25 (q, 10-H, $J = 7$ Hz), 5.92 (s, OH4), 6.30 (d, 8-H, $J = 10$ Hz), 7.05 (m, 2-H, 5-H, 6-H, overlap), 7.60 (d, 7-H, $J = 15$ Hz). A yield of ethyl ferulate based on vanillin was 64.7%.

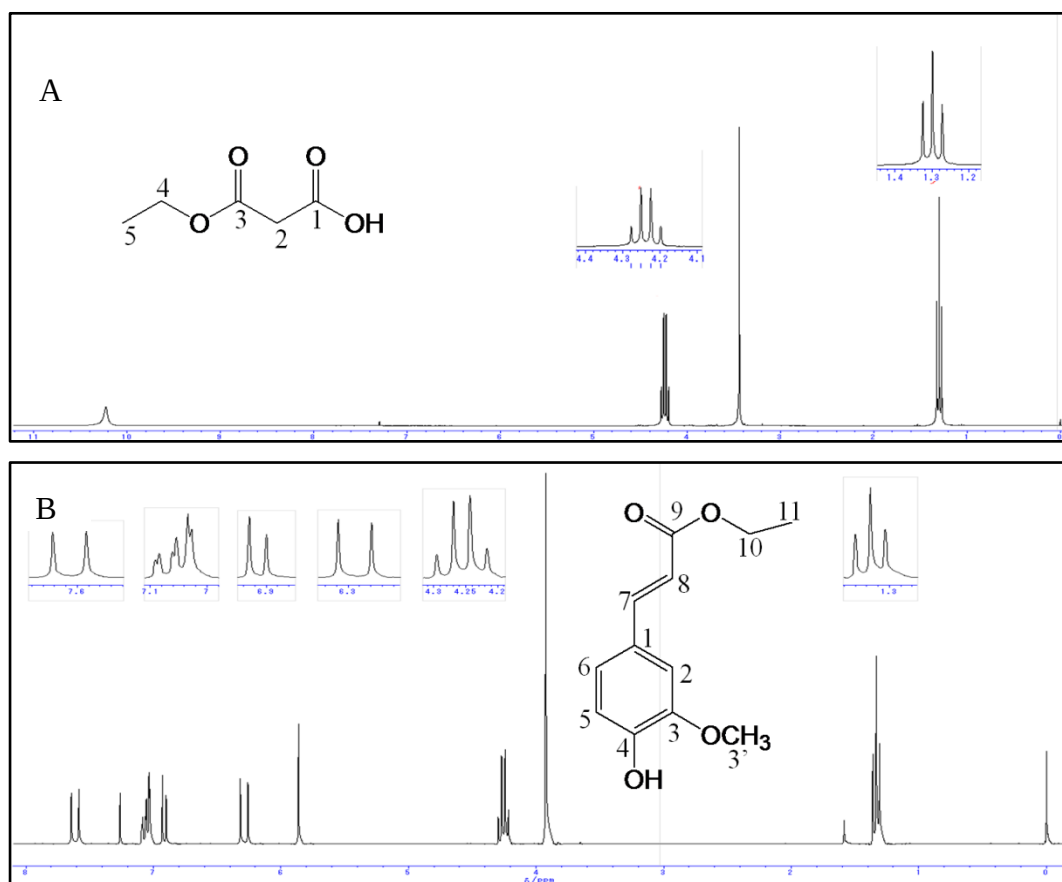


Figure 3-2. ^1H -NMR spectrum of monoethyl malonate (A), ethyl ferulate (B).

Reduction of ethyl ferulate to coniferyl alcohol was performed according to the method reported by Quideau and Ralph (1997). Synthesized ethyl ferulate (5.3 g, 0.024 mol) was dissolved in 250 mL of freshly distilled toluene, and then 100 mL of diisobutylaluminium hydride (DIBAL-H) was added dropwise under a nitrogen atmosphere with cooling using an ice bath. The reaction mixture was further stirred for 1 h. Then, 25 mL of cold ethanol was carefully added to quench the reaction. Two hundreds mL of distilled water was added, and the resultant precipitate of aluminum salt was filtrated off on a celite cake followed by washing with distilled water and ethyl acetate. The filtrate and the washings were combined, and the product was extracted with 200 mL of ethyl acetate for 3 times. The extraction organic layer was washed again with 100 mL of saturated saline 3 times, and dried over anhydrous sodium sulfate for 2 h. Crude crystal solid can be obtained by evaporating the solvent. Re-crystallization was carried out with chloroform/hexane to yield pure coniferyl alcohol. TLC (eluent, toluene/ethyl formate/formic acid, 5:4:1, v/v) and ^1H NMR (CDCl_3 with 0.05% v/v TMS, 270 Hz) were used to confirm the purity.

3.2.2.2 Dehydrogenative polymerization of coniferyl alcohol with enzyme in the presence of cellulosic films

For the polymerization of coniferyl alcohol with enzyme (horseradish peroxidase), the following three solutions were prepared (Cathala et al 1998). Solution I, 100 mg of coniferyl alcohol was dissolved in 2 mL of acetone, and then mixed with 48 mL of phosphate buffer saline solution (PBS; pH 6.1, 0.01 M phosphate containing 0.8 w/v% of NaCl and 0.02 w/v% of KCl). Solution II, 5 mg of horseradish peroxidase was dissolved in 50 mL of PBS. Solution III, 0.25 mL

of 30 wt% aqueous hydrogen peroxide solution (14.6 mmol) was mixed with 50 mL of PBS. As shown in Figure 3-3, HPBC and FBC with/without adsorption of xylan were separately immersed in solution II. Solution I and solution III were injected dropwise into solution II from separate two syringes by using syringe pumps (YSP-101, YMC Keyboard Chemistry, Kyoto, Japan) at a flow rate of 5 mL/h for 10 h. The mixture was allowed to stand for further 16 h. The resultant films were picked up and immersed in distilled water two times for 5 min each, and then air-dried.

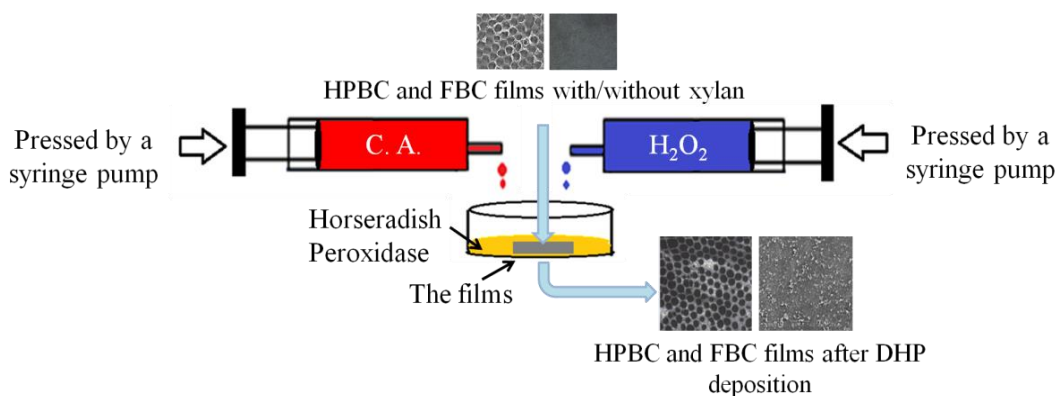


Figure 3-3. Apparatus for dehydrogenation polymer preparation. C.A. is coniferyl alcohol.

The resultant DHP suspended in the PBS was collected by centrifugation (2000 g, 10 min), and washed two times with distilled water followed by lyophilization. The collected DHP was used as a lignin standard for the acetyl bromide method.

3.2.3 Observation of film morphology

The morphologies of the obtained films were observed under the violet-laser color 3D profile microscope or the field emission-scanning electron microscope mentioned in the Chapter-2.

3.2.4 Lignin bromination and SEM-EDXA analysis

DHP deposited on the cellulosic films was brominated with bromine vapor in a sealed desiccators. The cellulosic films and bromine (5 mL, 99% purity) in a glass Petri dish were separately placed in a desiccator, and allowed to stand for 1 day at room temperature after tight sealing the desiccator with a glass cap. Afterwards, the brominated films were placed in air for half a day to evaporate the un-reacted bromine, and then embedded in epoxy resin. After curing the resin by successive heating (35 °C for 1 day, followed by 45 °C for 1 day, and then 65 °C for 3 days) in an oven, the resins were trimmed and microtomed by using Leica RM2255 (Leica Biosystems Nussloch GmbH, Nussloch, Germany) to obtain the cross sections of films. The location of bromine on the cross sections of films was analyzed on a FE-SEM (Hitachi S-800, Tokyo, Japan) equipped with an energy dispersive X-ray analyzer (EDXA, Horiba EMAX-2000, Tokyo, Japan) after specimens were coated with carbon (VC-100, Vacuum-Device Ltd., Ibaraki, Japan). SEM analysis was operated at an accelerating voltage of 10 kV. The EDXA images were represented as an inversion mode of black and white using the software of Microsoft Powerpoint 2007.

3.2.5 Immunolabeling of xylan in the DHP-deposited films

The cellulosic films (FBC and HPBC) and xylan-adsorbed cellulosic films after DHP deposition were embedded in epoxy resin, and cut by using the microtome mentioned in Chapter 2. The small section was immunolabeled by xylan-antibodies, LM 10 and 11, and the second antibody, as described in the section of 2.2.7 in Chapter 2.

3.2.6 Characterization of DHP

All films (HPBC, FBC, Xyl-HPBC, and Xyl-FBC) after DHP deposition were cut into small pieces, and then washed once by immersing them into 5 mL of 90% 1,4-dioxane aqueous solution (aq. dioxane) for 10 min with gentle stirring. After that, the films were carefully collected and dried *in vacuo* at 50 °C overnight. DHP contents in DHP-deposited cellulosic films before and after washing were determined by acetyl bromide method, and an alkaline nitrobenzene oxidation for only aq. dioxane-washed films was performed to estimate β -O-4 frequency in DHP (Figure 3-4) as follows.

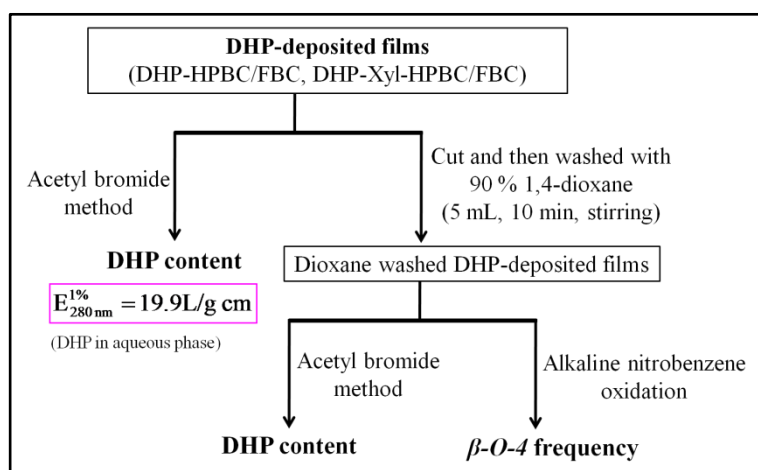


Figure 3-4. Experiment scheme for DHP characterization.

3.2.6.1 Acetyl bromide method

Determination of DHP content by acetyl bromide method was according to Hatfield et al (1999). Freshly prepared acetyl bromide [2.5 mL of 25 % (v/v) solution in acetic acid] was added to 5 mg of sample, and then the mixture was heated at 50 °C for 4 h. If the sample did not completely dissolve after heating, 0.1 mL of perchloric acid was added to the reactor, and it was allowed to stand for more 0.5 h. The reaction medium was diluted with 10 mL of 2 M aqueous NaOH

and 12 mL of acetic acid (99%). Absorbance between 250-360 nm was measured by a UV spectrophotometer (Shimadzu UV-1800, Tokyo, Japan). DHP content was calculated from the absorbance at 280 nm using an absorptivity of acetylated DHP of 19.9 L/g cm at 280 nm, which was obtained from absorbance of the DHP recovered from the suspension mentioned in 3.2.2.2.

3.2.6.2 Alkaline nitrobenzene oxidation

Alkaline nitrobenzene oxidation was conducted according to the method reported by Chen (1992). Four milliliter of aqueous NaOH (2 M) and 0.24 mL of nitrobenzene were added to 10-15 mg of sample in a 10 mL stainless steel vessel. The reaction was performed at 160 °C in an oil bath for 2 h. Afterwards, the vessel was cooled down by placing on ice, and 0.2 mg of acetovanillone as an internal standard was added and mixed with the reaction mixture. The mixture was transferred to a flask, and the vessel was washed 5 times with 5 mL of aqueous NaOH (0.1 M). The reaction mixture and washings were combined, and filtered with a glass filter. Reaction products in the filtrate were extracted with diethyl ether (30 mL × 3 times). The aqueous layer was acidified to pH 2-3 with 2 M aqueous HCl followed by further extraction with diethyl ether (30 mL × 3 times). The organic layer was collected and dried over anhydrous sodium sulfate for 2 h followed by evaporation. Reaction products (vanillin and vanillic acid) were determined by gas chromatography (Shimadzu GC-2010, Kyoto, Japan) under the following conditions: detector, FID; column, InertCap 1701 (30 m in length, 0.25 mm I. D., 0.25 µm of film thickness; GL Science, Tokyo, Japan); split ratio, 5.0; carrier gas, He (110 mL/min); injector temperature, 250 °C; detector temperature, 280 °C; column temperature program, 150 °C (5 min), 5 °C/min to 230 °C (1 min),

10 °C/min to 250 °C (10 min). All experiments described above were performed in duplicates, and the average value was shown as a result.

3.2.7 Mechanical test

Films were gently pressed under a 300 g load for 10 min, and then cut into 1-2 mm in width and 15 mm in length. The cut films were then fixed on double-side adhesive placed on a specimen holder, which was made of printing paper with two 5 mm wide slots (Figure 3-5). Film thickness was measured by using 3D profile microscope (Keyence VK-9500, Osaka, Japan), where a film was placed perpendicular to a stage of microscope. The measurement was repeated for at least five different positions, and their average was reported as a result.

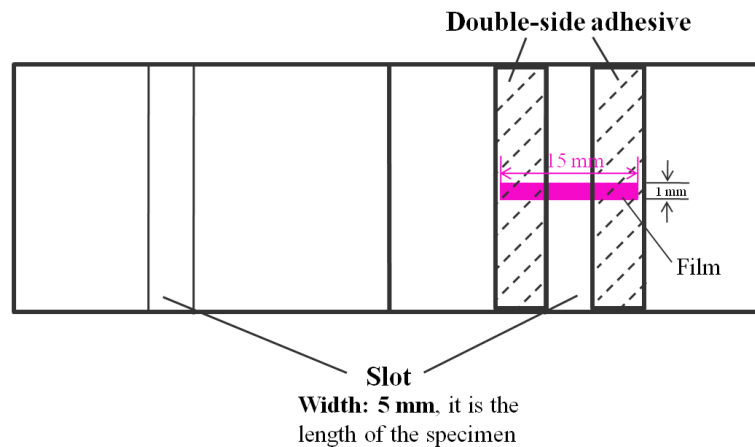


Figure 3-5. Specimen preparation for the mechanical test.

The specimens were then allowed to stand at 50% relative humidity and 22 °C for 1 day, and subjected to tensile test under the same ambient conditions. A specially designed testing apparatus (Figure 3-6) reported previously was used (Uraki et al 2010). Specimens in the paper sample holder were placed on sample support of the apparatus, and both sides of the paper holder were cut to make the film free supporting. The tensile loading (force) and the corresponding

displacement were monitored synchronously during measurement.

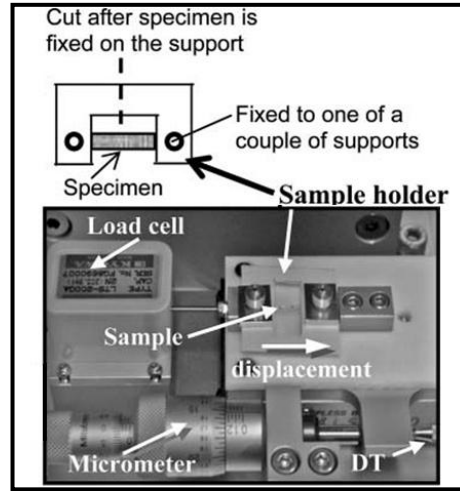


Figure 3-6. Mechanical testing apparatus. DT is a displacement transducer (Uraki et al 2010).

The definitions of each mechanical property are shown in Equation 3-1 to 3-4 (Burgert 2006). MOE is estimated from slope of stress–strain curve in the elastic deformation region.

$$\sigma = F / A \quad \text{Equation 3-1}$$

$$\varepsilon = \delta / L \quad \text{Equation 3-2}$$

$$\text{MOE (MPa)} = \sigma' / \varepsilon' \quad \text{Equation 3-3}$$

$$\text{Elongation (\%)} = (\delta' / L) \times 100 \quad \text{Equation 3-4}$$

Where σ is stress, MPa; F is the force applied on film specimen, N; A is the cross section area of film specimen, mm^2 ; ε is strain; δ is the displacement (change in film specimen length) produced by the force, mm; L is the original length of film specimen, mm; σ' is the stress at elastic deformation region, MPa; ε' is the strain at elastic deformation region; MOE is modulus of elasticity, MPa; δ' is the displacement at fracture, mm.

Mechanical test was carried out for at least 20 specimens cut from four different pieces of films. Results of mechanical test were reported as mean $\pm$ standard deviation.

3.3 Results and discussion

3.3.1 Synthesis of coniferyl alcohol

Reduction of coniferyl alcohol from ethyl ferulate gave a mole yield of 51.1 %. ^1H -NMR of coniferyl alcohol is shown in Figure 3-7. δ -1.47 (s, OH9), 3.90 (s, OCH_3 3'), 4.30 (t, 9-H, $J = 5$ Hz), 5.64 (s, OH4), 6.20 (m, 8-H, $J = 7$ Hz), 6.50 (d, 7-H, $J = 16$ Hz), 6.87 (m, 2-H, 5-H, 6-H, overlap). Coniferyl alcohol with high purity was successfully synthesized.

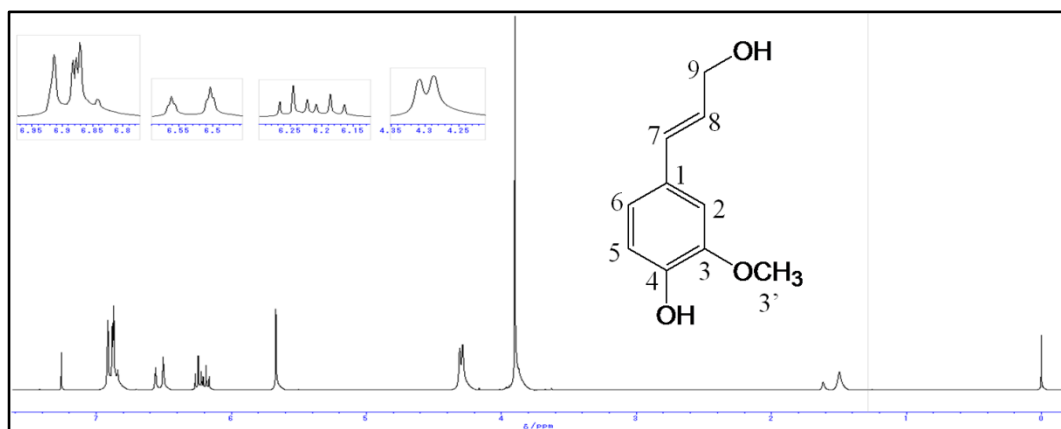


Figure 3-7. ^1H -NMR spectrum of coniferyl alcohol.

3.3.2 Dehydrogenative polymerization of coniferyl alcohol in the presence of cellulosic films with/without xylan

As a final step for preparing artificial cell wall, an endwise polymerization of coniferyl alcohol (Cathala et al 1998) was attempted in the presence of cellulosic films to obtain DHP-deposited cellulosic films. Surface images of the resultant films are shown in Figure 3-8. Before DHP deposition, no white regions were observed in the images of HPBC and FBC, Figure 2-3 E1 and Figure 2-5 A, respectively. After DHP deposition, white regions were observed on the surfaces of HPBC (Figures 3-8 A and C) and FBC (Figures 3-8 B and D), respectively.

These white regions on the HPBC film, as indicated with white arrows, was like to distribute along with the cellulose hexagonal skeleton, whereas they were randomly accumulated as particles with irregular shapes on the FBC film. Additionally, the number of white regions in Xyl-FBC (Figure 3-8 D) was much larger than that in FBC without xylan (Figure 3-8 B). It was presumed that white regions deposited on HPBC and FBC were DHP. To prove this assumption, a following experiment was attempted using SEM-EDXA.

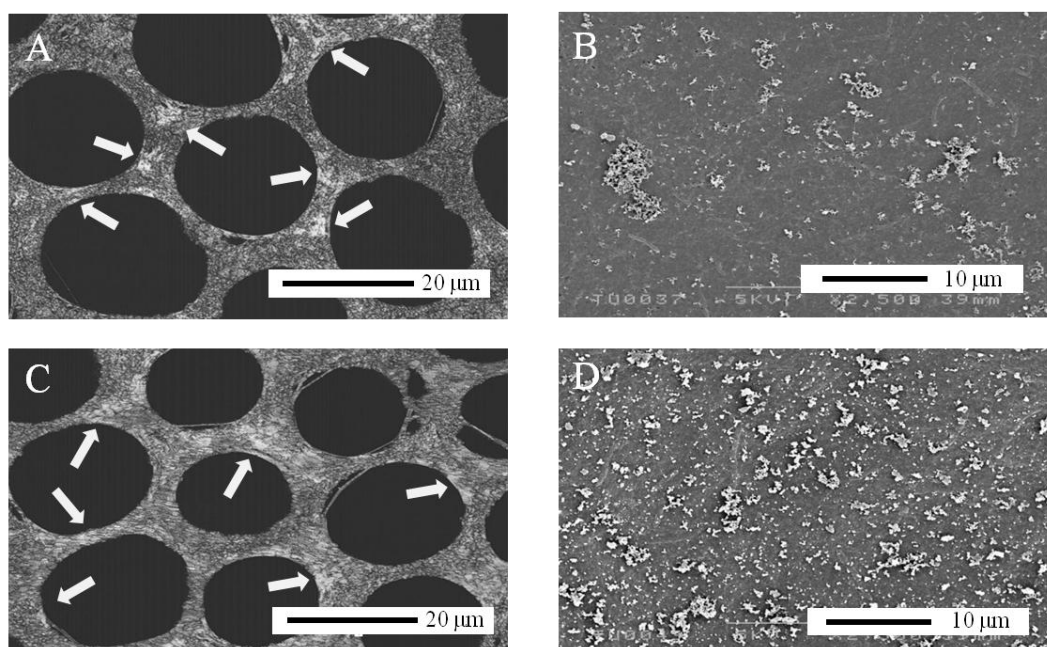


Figure 3-8. 3D microscopic images of HPBC series (A, C) and SEM images of FBC series (B, D). A and B are images of both cellulosic films after DHP preparation. C and D are images of xylan-deposited films after DHP preparation. White arrows show “white region” in HPBC.

3.3.3 Identification of DHP by SEM-EDXA

Since lignin can react with bromine, a combined method of lignin bromination and SEM-EDXA technique is widely used for labeling and detecting lignin (Saka et al 1978, Saka et al 1982). To confirm the formation of DHP on the

surface of HPBC and FBC films, after bromination of film specimens, the surface of the resultant films was observed under a SEM-EDXA. Br elements were depicted as white dots in a black background of original EDXA image, as shown in Figure 3-9 C. However, this image is very unclear for checking the Br dots. To make clear image, the brightness and contrast of the image was changed by using a software of Microsoft PowerPoint 2007, as shown in Figure 3-9 D, but still unclear. Therefore, black and white was switched by the software, and got the image of Figure 3-9 E. As a result, we can see Br location and distribution in the image.

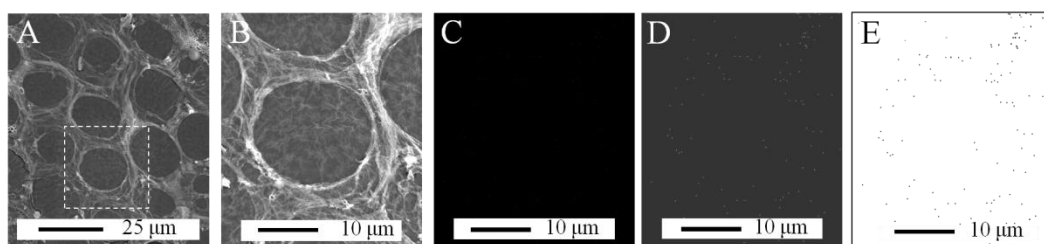


Figure 3-9. Comparison of Br mapping images after changing their brightness and contrast. A, SEM image of a honeycomb unit in Xyl-HPBC after DHP deposition. The area in the dotted square in image A was magnified under SEM to give image B. C, the original EDXA image of Br mapping; D, the image after changing the brightness and contrast of the image C; E, the image after switching black and white color of image D.

Figure 3-10 shows the SEM-EDXA images corresponded to high magnification images of Figure 3-8. In the case of FBC-based films, single white particle was focused on to take Br mapping image. The location of bromine dots in Figures 3-10 a3, b3, c3, and d3 was consistent with the location of white regions in Figures 3-10 A3, B3, C3, and D3, respectively. Consequently, DHP deposition on the surface of cellulosic films was confirmed by a combined analysis of bromination and SEM-EDXA. Hereafter, the obtained films were

abbreviated as follows: DHP-deposited HPBC and FBC, DHP-HPBC and DHP-FBC, respectively; DHP-deposited Xyl-HPBC and FBC, DHP-Xyl-HPBC and DHP-Xyl-FBC, respectively.

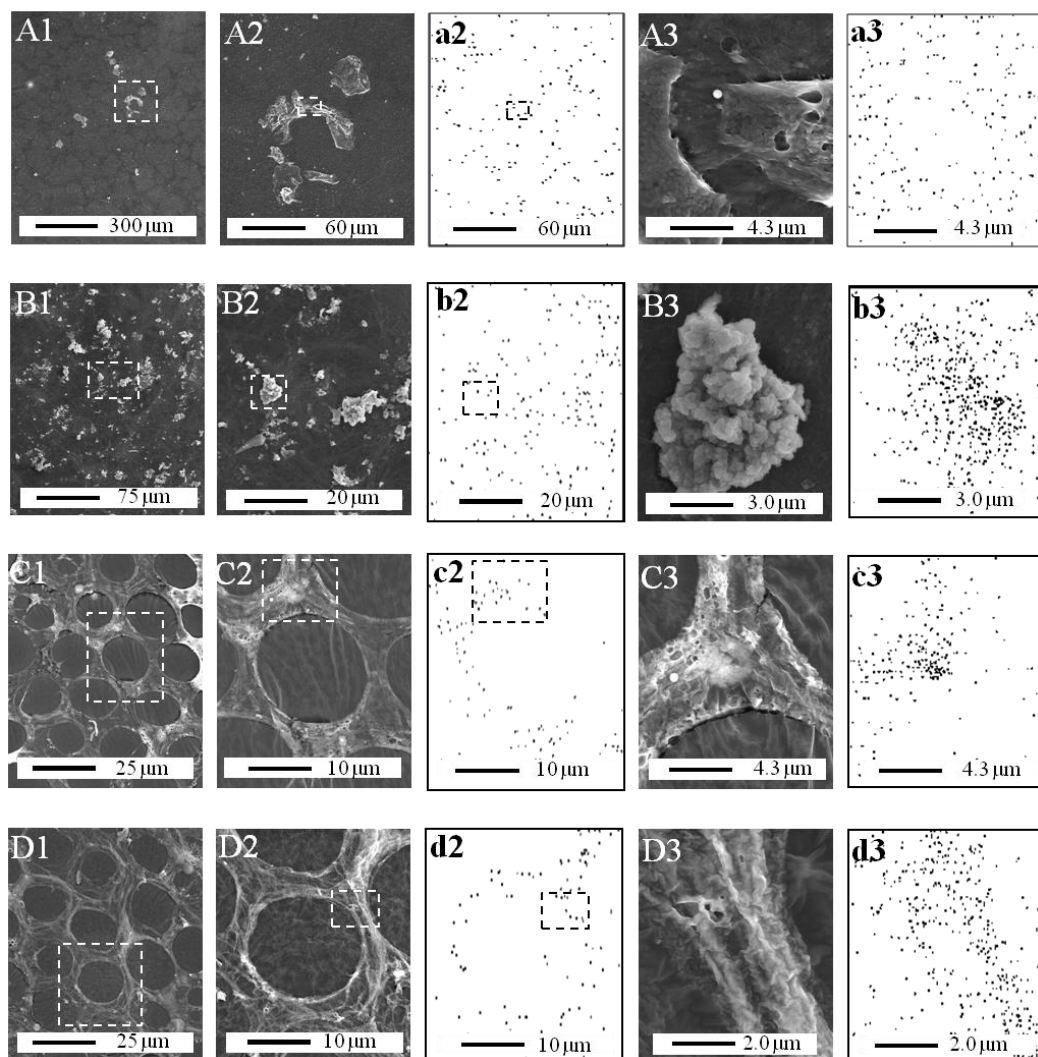


Figure 3-10. SEM-EDXA images of the surface of DHP-deposited FBC and HPBC films. A series, FBC after DHP preparation without xylan; B series, xylan-deposited FBC after DHP preparation; C series, HPBC after DHP preparation without xylan; D series, xylan-deposited HPBC after DHP preparation. The area in the dotted square was magnified under SEM and used as another photo with an increased number by one (e.g. A1 to A2). The small letters, a, b, c, and d, are Br-mapping images by EDXA for the corresponding large letters.

The distribution of DHP on films surface can be observed from SEM

images in Figure 3-10. Lignin was located on honeycomb skeletons in both DHP-HPBC and DHP-Xyl-HPBC film, whereas randomly distributed on the surface of DHP-FBC and DHP-Xyl-FBC. This observation is consistent with that on the 3D microscope (Figure 3-8). Accordingly, a model of lignin distribution on the surfaces of films was presented in Figure 3-11. DHP, aggregated in different size, was randomly deposited on the surface of flat film, and was deposited along with the framework of honeycomb-patterned films.

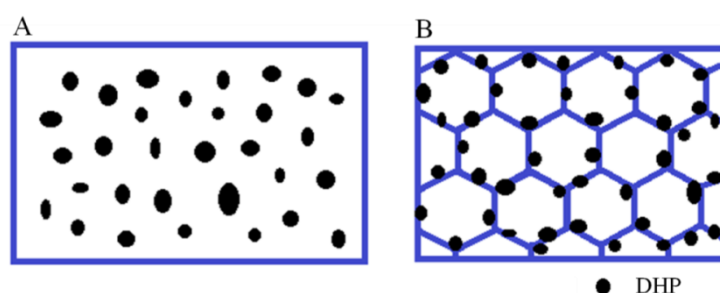


Figure 3-11. Models of DHP distribution on the surface of FBC (A) and HPBC (B).

3.3.4 DHP distribution inside HPBC and FBC films

To further investigate the DHP location inside the films, the Br-stained films were embedded into epoxy resins, and then the specimens were trimmed and microtomed after curing the resins. The microtomed positions for HPBC and FBC series are shown in Figure 2-8. Two cross sections at the first and the second lines were observed on the SEM-EDXA. Figure 3-12 shows the SEM-EDXA images of the cross sections of DHP-deposited cellulosic films at the first cut-line (Line 1 in Figure 2-8). In the Figure 3-12 a3 of DHP-FBC without xylan, Br dots or DHP were observed only at the outside of the film, but not in the inside. However, when xylan was adsorbed on FBC (DHP-Xyl-FBC), DHP was located in both sides of the film (Figure 3-12 b3). The same phenomena (Figure 3-13) were

observed for the cross sections at the second cut-line (Line 2 in Figure 2-8). From these images, I anticipated that xylan induced DHP formation into the inside of the FBC films.

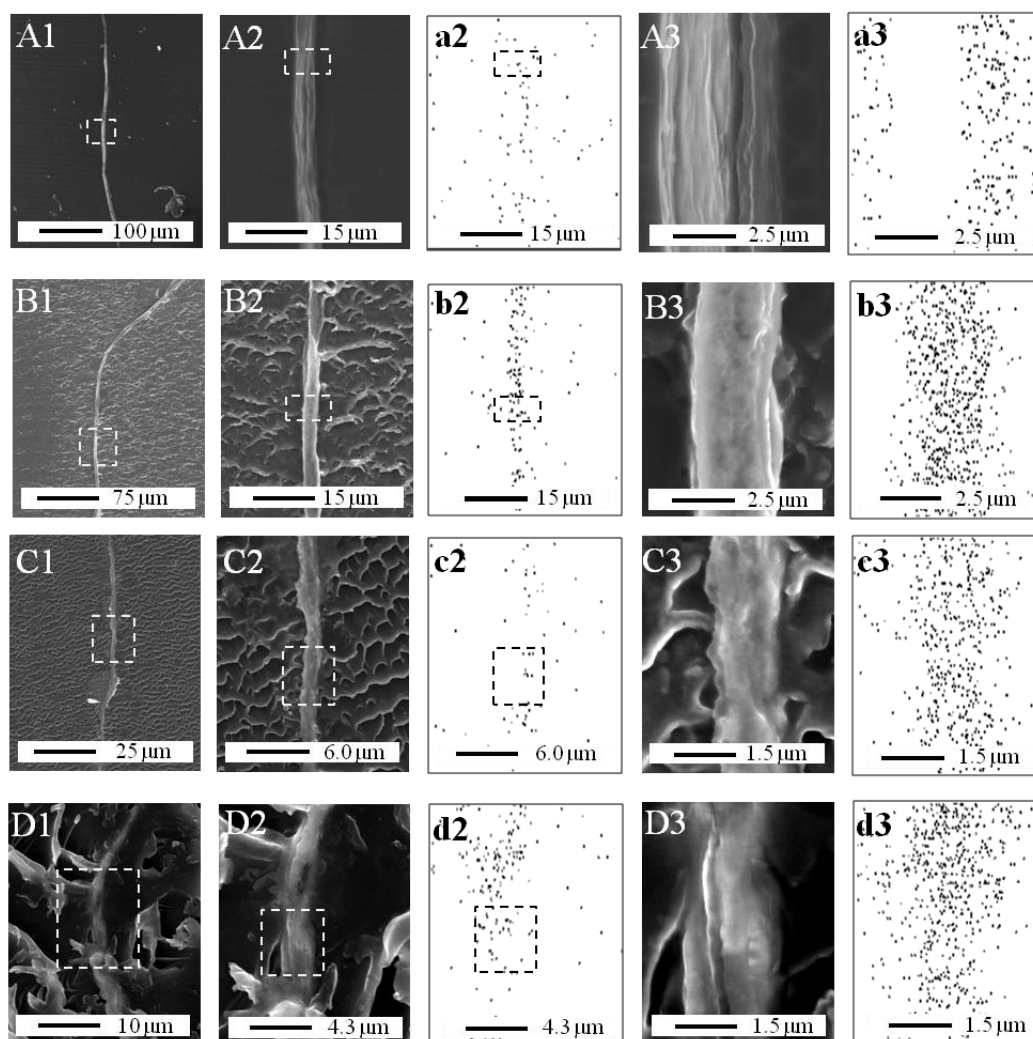


Figure 3-12. SEM-EDXA images of DHP-deposited FBC and HPBC films at the cross section from the first cut line. A series, FBC after DHP preparation without xylan; B series, xylan-deposited FBC after DHP preparation; C series, HPBC after DHP preparation without xylan; D series, xylan-deposited HPBC after DHP preparation. The area in the dotted square was magnified under SEM and used as another photo with an increased number by one (e.g. A1 to A2). The small letters, a, b, c, and d, are Br-mapping images by EDXA for the corresponding large letters.

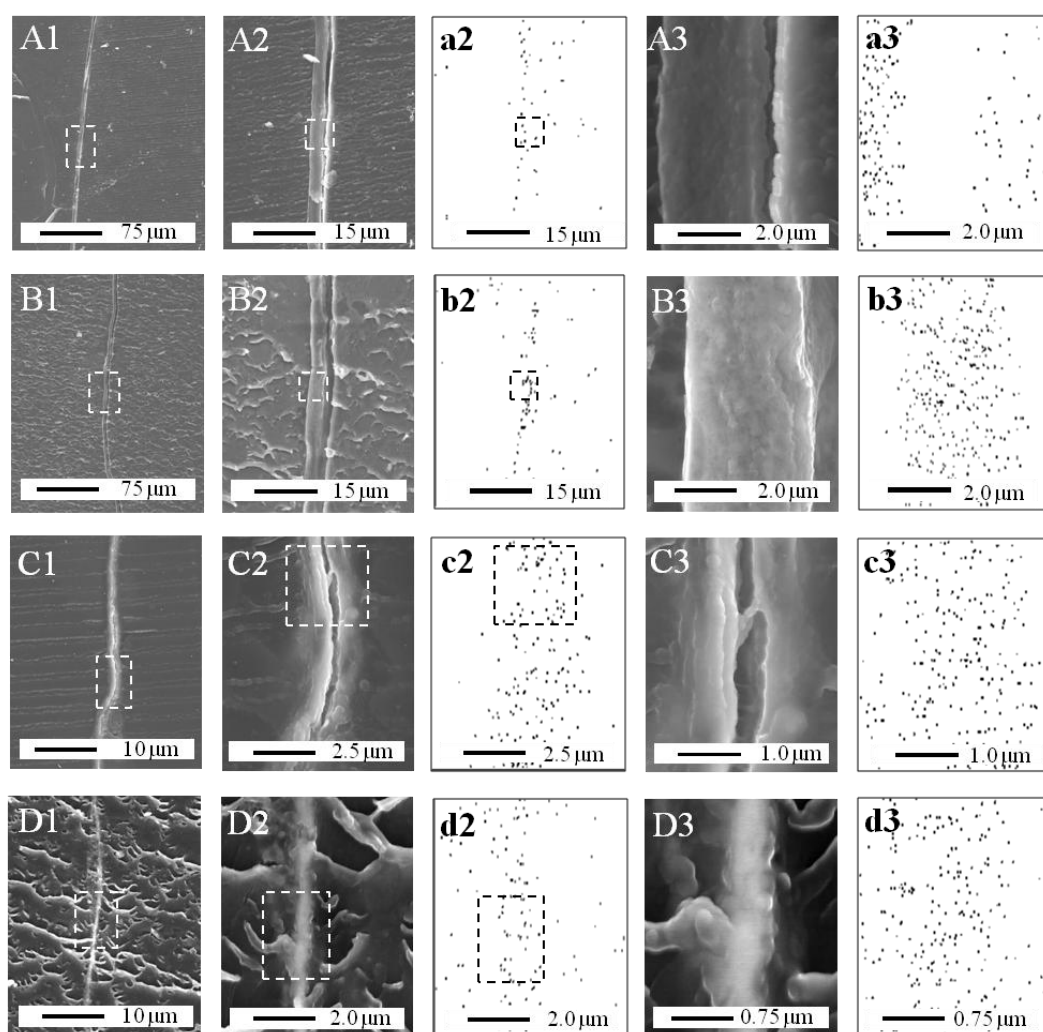


Figure 3-13. SEM-EDXA images of DHP-deposited FBC and HPBC films at the cross section from the second cut line. A series, FBC after DHP preparation without xylan; B series, xylan-deposited FBC after DHP preparation; C series, HPBC after DHP preparation without xylan; D series, xylan-deposited HPBC after DHP preparation. The area in the dotted square was magnified under SEM and used as another photo with an increased number by one (e.g. A1 to A2). The small letters, a, b, c, and d, are Br-mapping images by EDXA for the corresponding large letters.

To confirm this assumption, xylan distribution in the DHP-deposited films was observed by a combination of immunolabeling and fluorescent microscopy. As control experiments, the specimens were immersed in a buffered solution

containing only a second antibody (anti-rat IgG Alexa Fluor 568), which recognized the first antibodies. The control specimens did not show obvious fluorescence including autofluorescence of lignin (Figures 3-14 A1 and B1). On the other hand, the other test specimens clearly displayed red fluorescence, which revealed the existence of xylan. In Figures 3-14 A2 and A3, the xylan distribution was confirmed in the whole inside of DHP-Xyl-FBC. Thus, together with the result shown in Figures 3-12 and 3-13 (a3 and b3), colocalization of xylan and DHP inside FBC has been distinctly confirmed by the combined microscopic observations. This result suggests that xylan deposited on cellulosic framework acts as a good scaffold material for lignin deposition onto/into the cellulosic substances.

In the case of HPBC, Br dots or DHP were observed at insides of both DHP-HPBC and DHP-Xyl-HPBC films (c3 and d3 respectively in Figures 3-12 and 3-13). This Br distribution in DHP-HPBC was different from that in DHP-FBC; location of DHP in DHP-FBC was only outside of the film, while that in DHP-HPBC was inside and outside (c3 in Figures 3-12 and 3-13). This may suggest that coniferyl alcohol can easily penetrate inside of HPBC because most bundles or fibrils composed of several microfibrils in HPBC are exposed outwards. Thus, HPBC can provide a better scaffold model for the assembly of lignin than FBC.

Comparing DHP-Xyl-HPBC (Figures 3-12 d3) and DHP-HPBC (Figures 3-12 c3), the density of Br dots was increased by the xylan adsorption. In addition, xylan deposition at the inside of DHP-Xyl-HPBC film was also observed in Figures 3-14 B2 and B3. These observations suggest that migration of coniferyl

alcohol into HPBC films is accelerated by xylan deposition. Consequently, more DHP was observed in Figures 3-12 d3 than in Figures 3-12 c3.

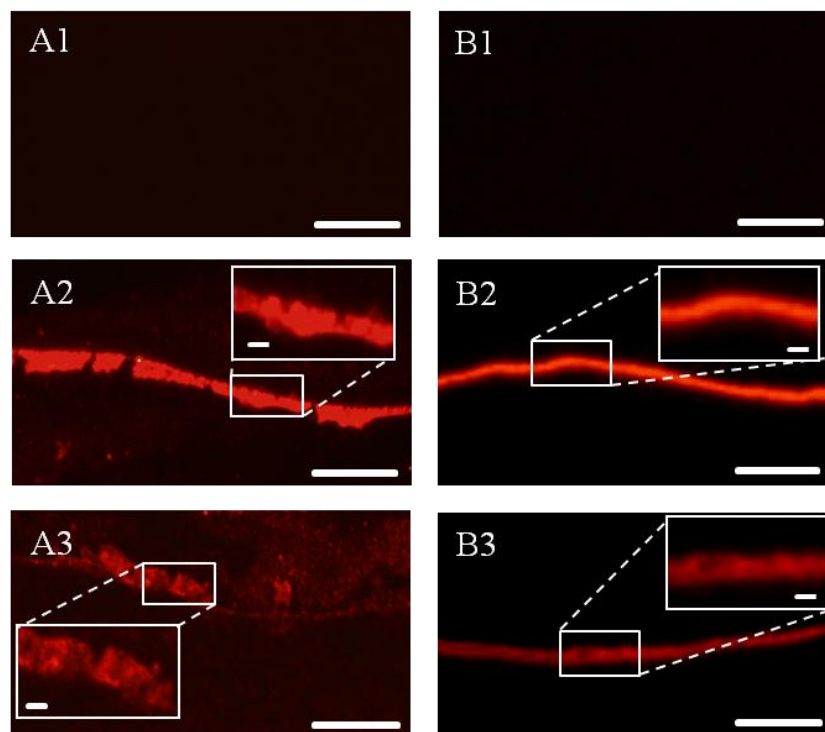


Figure 3-14. Immunofluorescence labeling of xylan in the cross sections of DHP-xylan-FBC series (A1-A3) and DHP-xylan-HPBC series (B1-B3). A1 and B1, controls without primary antibodies labeling; A2 and B2, immunolabeling with LM 10; A3 and B3, immunolabeling with LM 11. Longer bars are 20 μm; shorter bars in magnified images are 2 μm.

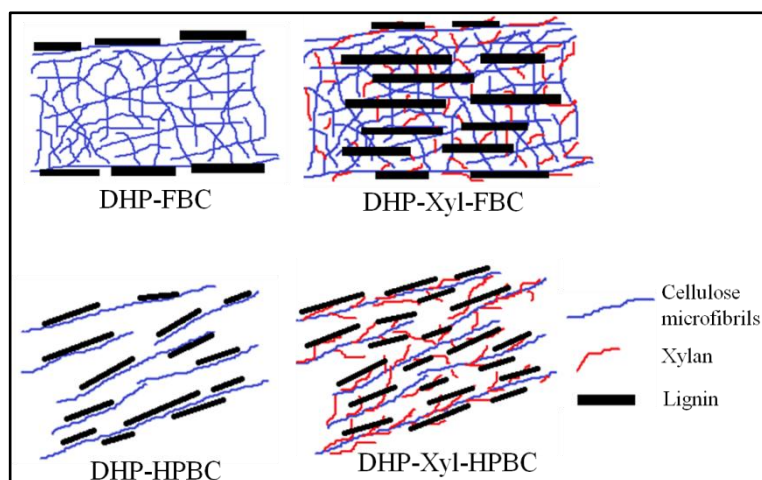


Figure 3-15. Supposed models for DHP-deposited FBC and HPBC films.

Here, I propose speculated model for DHP-deposited FBC and HPBC films to elucidate chemical structure in molecular level on the basis of the microscopic observations with the SEM-EDXA and fluorescent microscope in Figures 3-15. In the figure, when xylan is present, DHP (black bar) can deposit inside FBC, and more DHP is supposed to deposit inside HPBC (indicated by the number of black bars).

3.3.5 Effects of xylan on DHP content

To quantitatively examine the effects of xylan on DHP formation, DHP content in the films was estimated by the acetyl bromide method (Hatfield et al 1999). Figure 3-16 indicated that a small amount of DHP was deposited on the cellulosic films, while most of the formed DHP was precipitated in the PBS buffer solution. The former DHP was probably derived by enzymatic polymerization of coniferyl alcohol in the polysaccharide solid matrix, whereas the latter one was formed by the polymerization in aqueous buffer medium.

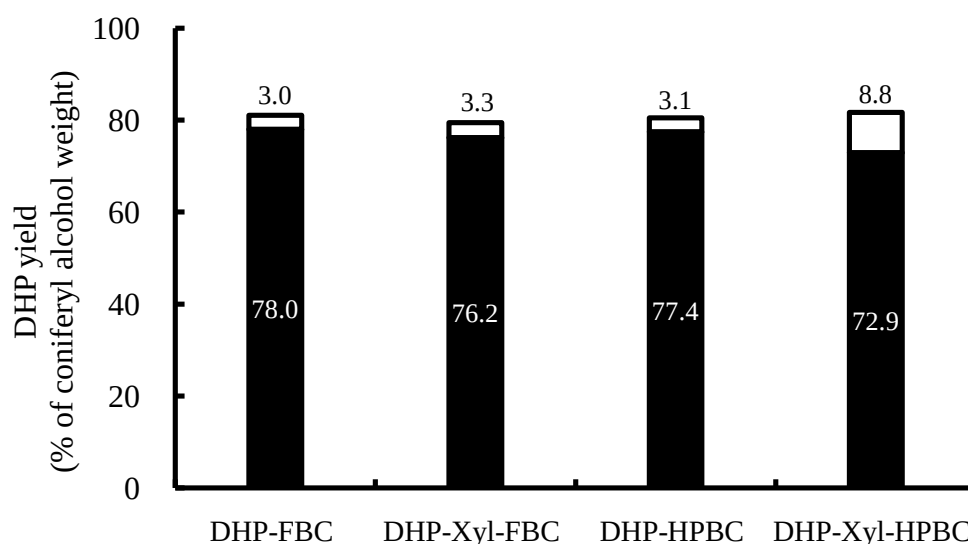


Figure 3-16. DHP yield based on the weight of coniferyl alcohol. ■, DHP precipitated in PBS buffer solution; □, DHP deposited on/in the films.

Figures 3-17 A and B show DHP contents in the cellulosic films based on the total weight of DHP-deposited films (empty columns) and the initial weight of cellulose (filled columns) before and after washing with aq. dioxane, respectively. The contents of DHP in both FBC and HPBC, based on the initial weight of cellulose, were increased by the xylan adsorption on cellulosic films in Figures 3-17 A and B. This result also suggests that xylan enhances the amount of DHP deposition in the films. Consequently, xylan affected DHP formation as a scaffold.

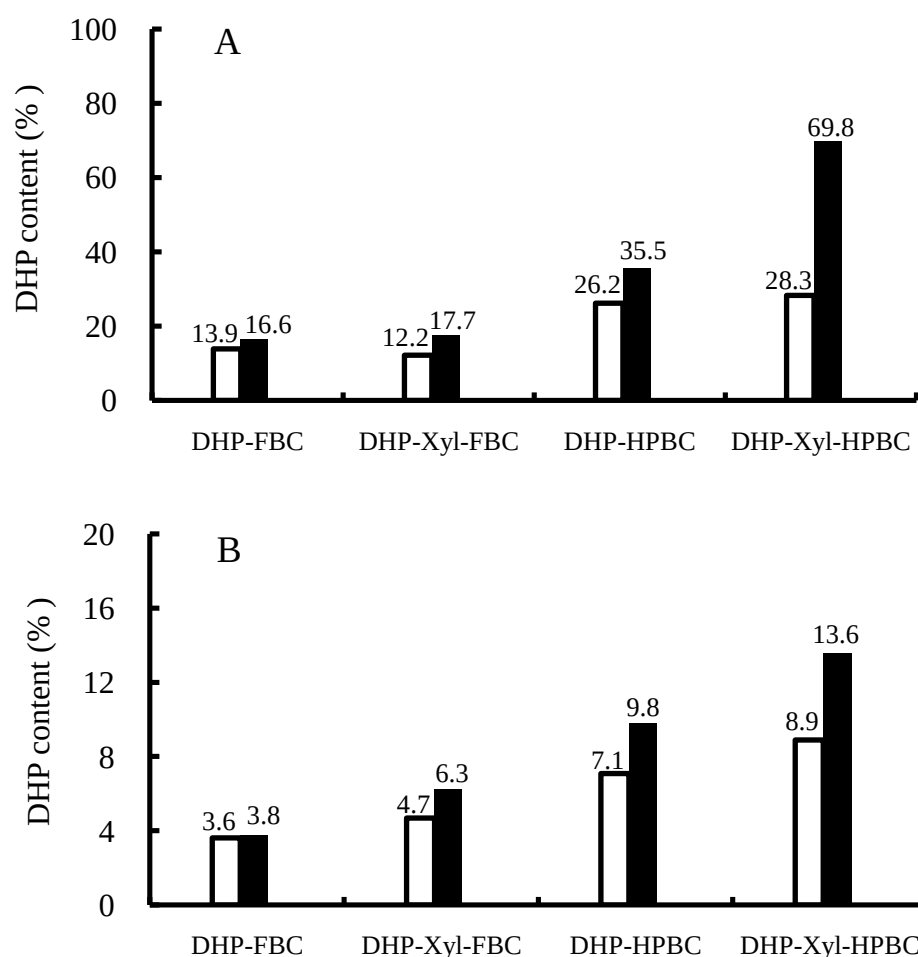


Figure 3-17. DHP content in the cellulosic films before (A) and after (B) washed with 90 % 1,4-dioxane aqueous solution. , based on the total weight of DHP-deposited films. , based on the initial weight of cellulose.

However, it seemed that this conclusion somewhat contradicted the

previous findings by Touzel *et al* (2003), who reported that the DHP content of the pectin-deposited BC films was not affected by the polysaccharides. Higuchi *et al* (1971) also reported that the yield of DHP was not significantly affected by the presence of hemicelluloses (pectin, pectic acid, and dilute alkali-extracted hemicellulose). However, the DHP in the former paper was formed in solid media together with pectin but not with xylan, and the DHP in the latter paper was formed with the hemicelluloses in liquid media. Therefore, their target DHP was quite different from my target.

By washing with aq. dioxane, DHP content was remarkably decreased (comparing Figure 3-17 B with A). This suggests that the DHP unbound and/or weakly bound with polysaccharides was removed by dissolving in aq. dioxane solution. However, a significant amount of DHP remained in the films, HPBC in particular (Figure 3-17 B). The remained DHP fraction was thought to be tightly associated with polysaccharides matrix, suggesting that this aq. dioxane-unwashable DHP fraction might be a lignin-carbohydrate complex.

3.3.6 Effects of xylan on β -O-4 interunitary linkage

To further investigate the effects of xylan on the interunitary linkage frequency of DHP in the films, the DHP-deposited films washed with aq. dioxane, were then subjected to alkaline nitrobenzene oxidation. In this reaction, oxidative products, vanillin and vanillic acid, are generated from non-condensed structure of lignin, and therefore, the yield of the oxidation products is one of the measures to estimate the frequency of aryl ether interunitary linkages in lignin, mainly β -O-4 linkage (Chen 1992, Schultz and Templeton 1986).

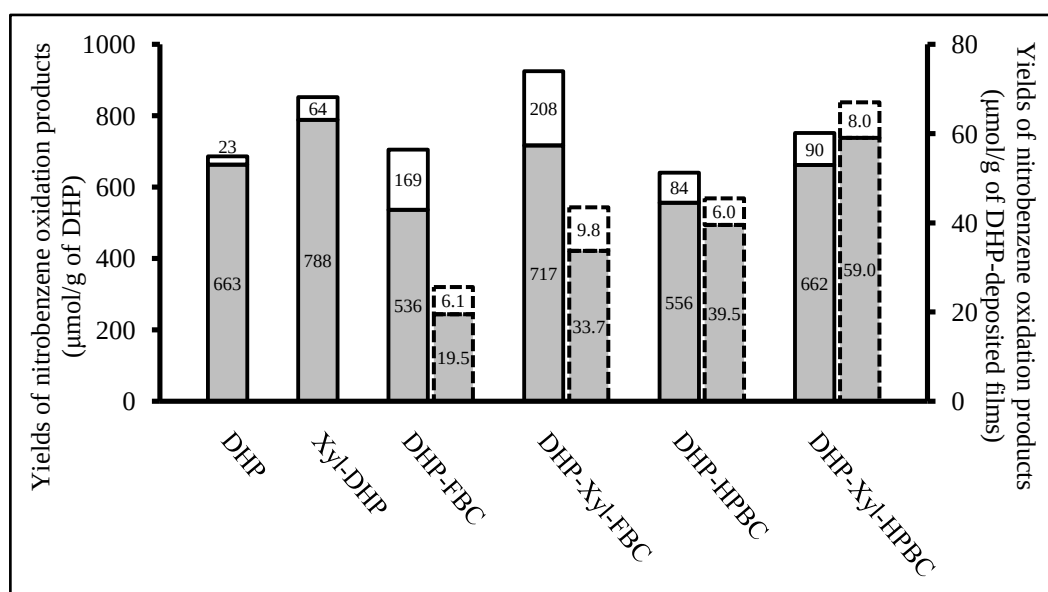


Figure 3-18. Yields of nitrobenzene oxidation products (vanillin and vanillic acid) from DHP deposited on cellulosic films after washed with 90% 1,4-dioxane solution. Filled columns, vanillin yield; empty columns, vanillic acid yield. Columns of solid lines are represented as a yield based on the weight of DHP (left axis). Columns of dash lines are represented as a yield based on the total weight of DHP-deposited films (right axis).

The yield of the oxidation products from DHP prepared in liquid media (without cellulosic films) was increased by the addition of xylan into the PBS buffer solution, as shown in Figure 3-18. Similarly, the yield from DHP formed in both HPBC and FBC films was increased by the xylan adsorption. These results suggested that xylan enhanced the frequency of aryl ether linkage in DHP, probably resulting in the predominant formation of β -O-4 linkage. These observations are consistent with the findings by Barakat *et al* (2007), who reported that the higher concentration of arabinoxylan was added to the DHP reactant, the higher yield of thioacidolysis products was obtained. Therefore, we conclude that xylan can act not only as a scaffold for lignin deposition, but also as a structure-regulator of lignin, especially formation of aryl ether linkage.

3.3.7 Effects of xylan and DHP deposition on the mechanical properties of cellulosic films

Cellulose as a crystalline material mainly contributes to the mechanical strength of wood cell wall (Burgert 2006, Gibson 2012). In general, hemicelluloses and lignin are supposed to affect the mechanical properties of wood cell wall or cellulosic framework (Salmén 2004, Salmén and Burgert 2009, Terashima 2009). To confirm these hypotheses, the mechanical properties, viz. tensile strength at fracture, modulus of elasticity (MOE) and elongation (Equation 3-1 to 3-4), were measured for the specimens prepared in this study.

At the onset of the mechanical test, thicknesses of cellulosic films were measured by using the 3D microscope. The values are shown in Table 3-2. Cross section area (parameter A in Equation 3-1) of the films were estimated from such thickness, and stress (unit of MPa) was also calculated using A and the load to sample. The stress-strain curves were depicted in Figure 3-19.

HPBC is much thinner than FBC. The reason is as follows. When cellulose microfibril deposition on the agarose gel exceeds honeycomb groove, HPBC become FBC. Therefore, the thickness of HPBC depends on the depth of hexagonal grooves in the surface of agarose films, which is ca. 3 μm (Figure 2-3 D1 and D2). On the other hand, secretion of microfibrils or pellicle formation of *G. xylinus* in the HS liquid medium depends on the depth of liquid medium. Therefore, a thick FBC film is easily generated by the culture of such bacterium. After xylan deposition and the followed DHP formation, both HPBC and FBC had no significant change in their thicknesses.

The density of HPBC (0.17 mg/mm^3) is less than that of FBC (0.36

mg/mm³), thus the amount of cellulose in HPBC is much smaller than that in FBC with same thickness. Tensile strength and MOE of HPBC were found to be lower than those of FBC, as shown in Figure 3-20. These weak mechanical properties of HPBC must be attributed to a smaller density of cellulose in HPBC than that in FBC (Uraki et al 2010).

Table 3-2. Thickness of HPBC and FBC films.

	Intact	Xyl-	DHP-	DHP-Xyl-
HPBC	0.77 ± 0.03	0.79 ± 0.05	0.76 ± 0.06	0.77 ± 0.05
FBC	2.0 ± 0.6	2.0 ± 0.4	2.1 ± 0.3	1.9 ± 0.4

Unit is μm .

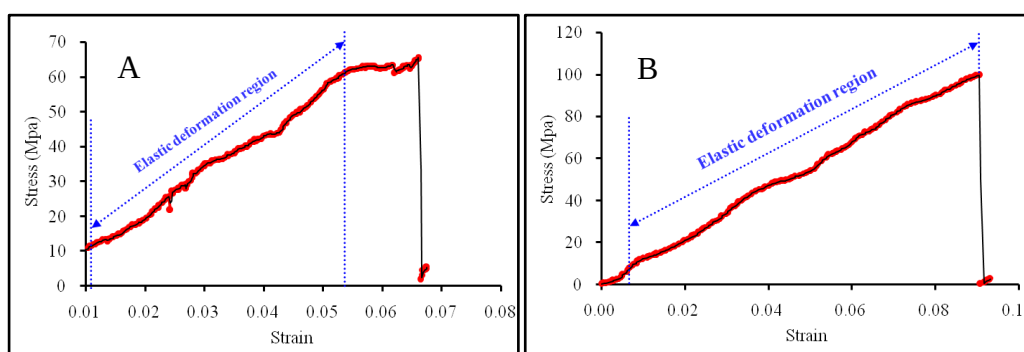


Figure 3-19. Typical stress-strain curves for DHP-Xyl-HPBC (A) and DHP-Xyl-FBC (B).

Figure 3-20 shows the mechanical properties of cellulosic films after deposition of xylan and/or DHP. At first, I attempted to evaluate the changes of tensile strength (Figure 3-20 A) and MOE (Figure 3-20 B). Tensile strength of FBC and HPBC was increased 1.6 and 1.9 times, respectively, by xylan deposition followed by DHP deposition. MOE of FBC and HPBC was augmented to 2.4 and 3.0 times, respectively, by the deposition of xylan and DHP. Thus, both xylan and DHP strengthen the cellulosic films. Secondly, these increments in

tensile strength and MOE were compared. MOE increment was larger than that of tensile strength upon the deposition of both xylan and DHP on FBC and HPBC, especially DHP deposition on HPBC. It seems that DHP gives cellulose more rigidity. Thirdly, the elongation (Figure 3-20 C) was evaluated. For both HPBC and FBC, the elongation did not change even after depositing either xylan or lignin, which means cellulose, but not xylan or lignin, is important to prevent the deformation of cell wall. From these results, I would like to anticipate that the functions of xylan and lignin in cell wall mechanical properties are (i) to enhance the cell wall strength, (ii) to give cell wall rigidity, especially brought about by lignin.

In addition, when the mechanical properties of HPBC and FBC were compared, the increments of tensile strength and MOE were clearly observed for HPBC rather than FBC in Figure 3-20 A and B. Therefore, HPBC is thought to be a better model for the investigation on the mechanical effect of wood components than FBC.

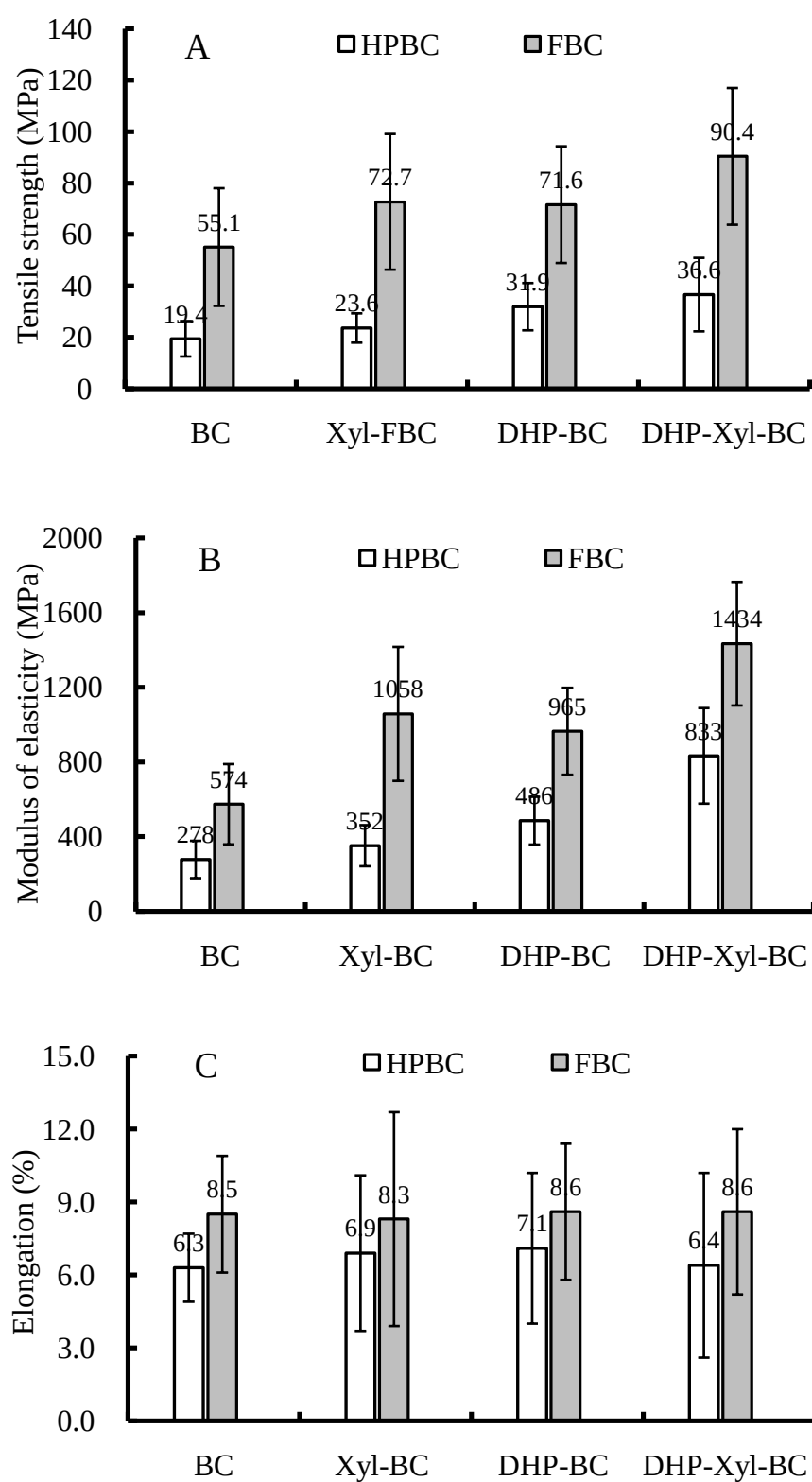


Figure 3-20. Mechanical properties of BC films. A, tensile strength; B, modulus of elasticity; C, elongation.

3.4 Summary

In this chapter, a wood secondary cell wall-mimetic system termed as “artificial wood cell wall” was established by enzymatic polymerization of coniferyl alcohol in the artificial polysaccharides matrix composed of xylan and cellulose. Using this artificial wood cell wall, the effects of xylan on lignin formation could be elucidated as follows: first, xylan accelerated DHP to deposit inside cellulosic films, and more DHP deposited on cellulose; second, xylan induced more β -O-4 substructures in DHP. Thereby, I concluded that xylan acts as a scaffold for lignin formation and as a structure-regulator for lignin, especially the formation of aryl ether linkage.

The role of xylan and lignin in cell wall mechanical performance was also elucidated with this artificial wood cell wall model. Both xylan and lignin improve cell wall rigidity, especially the lignin, thus, both xylan and lignin are concluded to enhance the cell wall strength to some extent.

CHAPTER 4

General Discussion

A study using *in-vitro* model is one of important approaches to elucidate complicated natural phenomena by simplified embodiment. In this research, I have fabricated an *in-vitro* model, termed as “artificial wood cell wall”, using honeycomb-patterned cellulose film as its framework. This model mimics the alignment of cross section of wood cell wall and the assembly process of wood components for the formation of wood cell wall.

I have pointed out five unknown subjects in items of wood cell wall in Chapter 1. In my study of PhD course, I elucidated two subjects using the fabricated artificial wood cell wall: **Subject 4-1**, the function of xylan in lignin formation, and **Subject-5**, the effects of both xylan and lignin on the mechanical strength of cellulose as the framework of wood cell wall.

The highlighted results from my study are as follows. (I) Xylan induced more lignin to deposit inside the cellulose framework. (II) Xylan increased the formation of the main lignin linkage, β -O-4. Accordingly, the function or the role of xylan in lignin formation was concluded as a scaffold for lignin deposition and a structure-regulator for lignification. (III) Both xylan and lignin enhanced the mechanical strength of cellulosic framework, particularly rigidity upon lignin deposition. In this chapter, I would like to give more detailed discussions on DHP formation process during the assembly of artificial cell wall.

In the previous research of our laboratory, monolignols were found to have a higher affinity to xylan than their corresponding glucosides (Uraki et al 2007b).

This indicates that when xylan-adsorbed BC films are immersed in the solution of coniferyl alcohol (CA, the monolignol used in my case), CA is easily adsorbed on the surface of the films, and then probably migrates into the films. As a result, CA binds with xylan to form a CA-Xyl-BC matrix. When lignin-polymerized enzymes, oxidase (laccase) and peroxidase exist in the matrix together with coenzymes, oxygen and hydrogen peroxide, respectively, CA is enzymatically polymerized into lignin. These processes are depicted in Figure 4.1. I empirically confirmed the speculated adsorption process of CA and lignin formation as follows.

In Chapter 3, the SEM-EDXA and fluorescent microscopic observations demonstrated location and distribution of DHP and xylan in both FBC and HPBC. In the case of intact FBC films without xylan, DHP formed only on the surface (a3 Figure 3-12 and 3-13), which means that CA was adsorbed only on the film surface, and was polymerized at the position by the action of horseradish peroxidase with the help of hydrogen peroxide. On the other hand, colocalization of DHP and xylan inside a film was observed for xylan-deposited FBC. This observation resulted from the migration of coniferyl alcohol into xylan-based matrix. Thus, the observation is an obvious evidence to support the speculated adsorption and polymerization process of CA.

In addition, I confirmed that xylan regulated the formation of interunitary linkages in DHP, mainly β -O-4 linkage formation, resulting from the yield of the alkaline nitrobenzene oxidation. The xylan functions were observed for HPBC films. Although the xylan effect on DHP deposition inside the HPBC film was not drastic as compared to FBC, much more β -O-4 linkage was formed in

xylan-deposited HPBC than in xylan-deposited FBC. The difference in DHP formation between FBC and HPBC is illustrated in Figure 4-2.

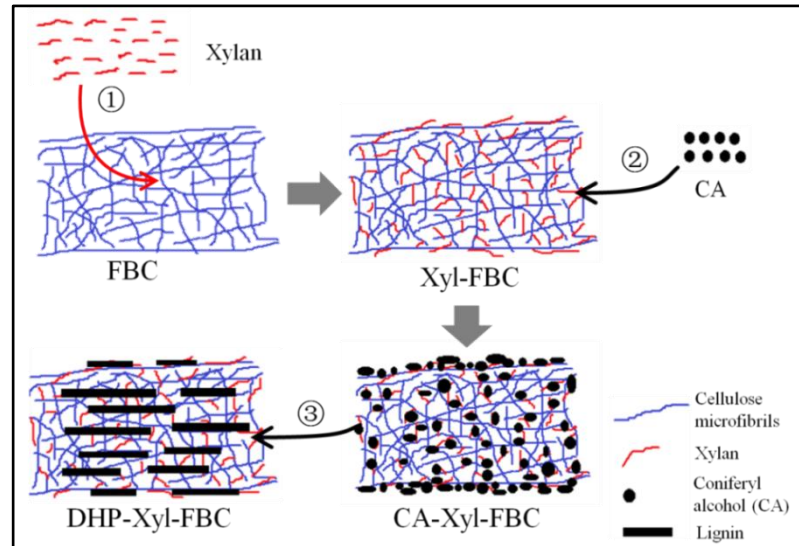


Figure 4-1. A supposed DHP formation process in BC films with xylan. The first step (①), xylan penetrates into the cellulosic film. The second step (②), coniferyl alcohol migrates inside the film. Finally (③), coniferyl alcohol polymerization. The formation process of DHP in HPBC is essentially the same as that in FBC in the presence of xylan. CA-Xyl-FBC, the supposed coniferyl alcohol bound to Xyl-FBC.

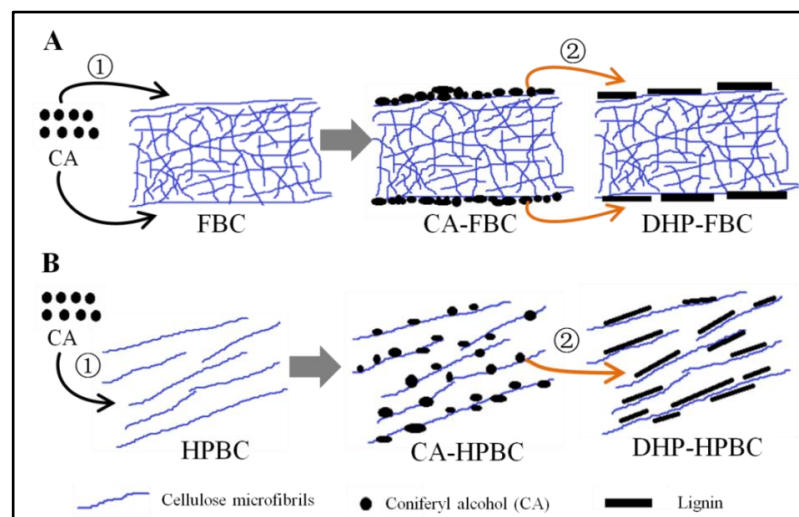


Figure 4-2. A supposed DHP formation process in FBC (A) and HPBC (B) films without xylan. Step ①, coniferyl alcohol deposition; step ②, enzymatic polymerization.

In conclusion, my study has elucidated the functions of xylan in lignin formation. However, the migration of coniferyl alcohol into xylan-adsorbed polysaccharides matrix was not observed directly. Therefore, this migration phenomenon should be clarified in the near future to completely interpret monolignol deposition inside the polysaccharides matrix of trees.

Additionally, in my research, I did not elucidate other unknown subjects about wood cell wall, which were proposed in Chapter 1 of General Introduction. I convince that these unclear items will be elucidated in future work by using “artificial wood cell wall” with honeycomb-patterned cellulose as its framework. The subjects and the reason why they can be clarified are listed as follows:

Subject 1, to evaluate the validity of wood cell wall deformation theories.

The theories on wood cell wall deformation were proposed based on honeycomb units (Section 1.1 in Chapter 1). Therefore, honeycomb-patterned cellulose is a suitable real model for evaluating validity of the theories.

Subject 2, to clarify the relationship between MFA and cell wall mechanical properties.

A model for cellulose microfibrils with different MFA will be able to be fabricated by controlling deposition process of orientated microfibrils in honeycomb films on agarose.

Subject 3, to observe the transportation of monolignols and their corresponding glucosides from outside to inside the cell wall.

Transportation of monolignols and their glucosides into inside HPBC can be monitored by a combination of isotope-labeled monolignols and the

corresponding glucosides with a TOF-SIMS (Time of flight-secondary ion mass spectroscopy) or isotope microscopy.

Subject 4-2, to elucidate the functions of glucomannan in lignin formation.

HPBC is a suitable framework for assembling guest molecules including glucomannan as well as xylan (Section 2.3.1.3 in Chapter 2).

Thus, I consider “the artificial wood cell wall” as a promising tool to clarify the wood cell wall formation and functions of wood cell wall components. This study is a first step to elucidate them. I hope that unclear subjects proposed in this study will be clarified in the near future by using the models *in-vitro*.

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