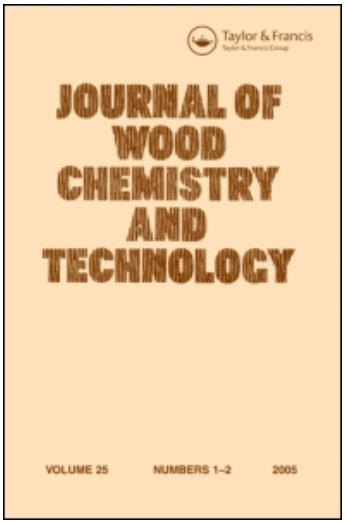

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XYLANASE-RESISTANT XYLAN IN UNBLEACHED KRAFT PULP

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ABSTRACT

A water-soluble chromophoric xylanase-resitant xylan fraction (LF-D) was separated from a hardwood unbleached kraft pulp (UKP) after hydrolysis with a cellulase/ xylanase-membrane bioreactor. LF-D contained over 70% unremovable inorganic atoms including Si, Na, and S, together with a β-1,4-linked xylan. A nucleus exchange reaction and a nitrobenzene oxidation showed that LF-D contained a trace amount of a lignin component abundant in quinoid structures which had been partly demethylated during the course of kraft pulping. On the other hand, a higher molecular weight residual lignin fraction (HF-P), which was obtained from an impermeable part of the enzymatic digests, was found to have a diphenylmethane structure. LF-D was partially decolorized by *Coriolus versicolor* and bacterial microflora without action of extracellular lignin peroxidase, Mn-peroxidase, laccase or xylanase.

INTRODUCTION

During kraft cooking, uronic acid and arabinose side chains of xylans are cleaved and the xylans are redeposited on the cellulose fibrils as the alkaline concentration of the cooking liquor decreases[1-3]. A part of the redeposited

xylans can be degraded with xylanase and the enzymatic treatment enhances the bleachability of the pulp[4-5]. Another type of xylan in the kraft pulp is resistant to xylanase[6,7]. Although no special attention has been paid to the structure and microbial degradation of the xylanase-resitant xylan, microbial or enzymatic decolorization of the xylanase-resitant xylan is important to avoid substantial weight loss of the pulp during the conventional bleaching processes, including alkali-extraction, and also to minimize bleaching effluents containing chlorine. In addition, it is still an open question why this fraction is not hydrolized by xylanase even though xylans are lacking in their side chain groups, and it is unclear whether ligninolitic enzymes are really effective for decolorization of the xylanase-resistant xylans. In this study, we focused on the chromophoric xylanase-resitant xylans involved in a hardwood unbleached kraft pulp. To characterize this fraction, unbleached kraft pulp was hydrolyzed with a cellulase/ xylanase membrane bioreactor, and the xylanase-resistant xylan and residual lignin fractions were isolated from the unbleached kraft pulp. Chemical properties and microbial treatment of these fractions are reported.

RESULTS AND DISCUSSION

Redeposited xylans during kraft cooking have been categorized into two types: (i) a solvent-extractable xylan which is bound weakly on the surface of cellulose fibrils, and (ii) a xylan which is co-crystallizing with cellulose and insoluble in alkali and DMSO[6,7]. The residual lignin in kraft pulp has been shown to associate with the latter type of xylan. As S.-Jørgensen reported, the solvent-extractable xylans are not hydrolyzed with xylanase[7]. In this study, a water-soluble chromophoric xylanase-resitant xylan fraction (LF-D) and a residual lignin fraction (HF-P) were separated from the hydrolysis products of a hardwood unbleached kraft pulp (UKP) by a cellulase/ xylanase-membrane bioreactor. As shown in FIGs.1,2, a permeable fraction (LF) was fractionated into four fractions (LF-A -D) by silica gel chromatography. As shown in FIG. 2, LF-D was extensively colorized and strong UV absorption was observed in this fraction. However, NMR signals from lignin components such as the methoxyl group and aromatic ring were below the background noise level. A commercial xylanase, Pulpzyme™, was unable to hydrolize this chromophoric xylan. In FIG. 3, the main signals of LF-D were assessed as those from

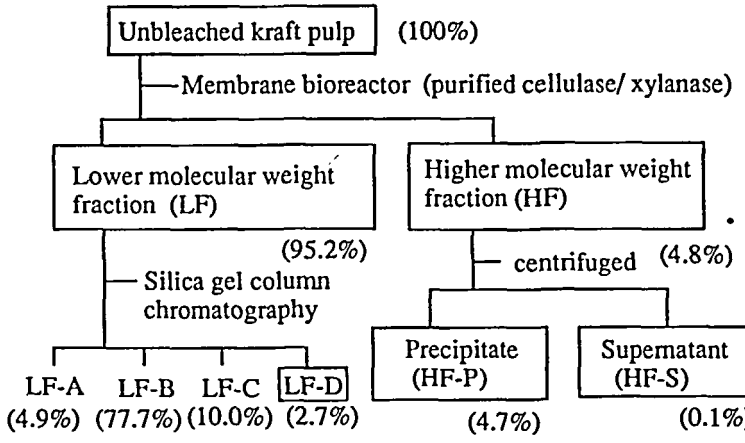

FIGURE 1 Enzymatic degradation and fractionation of unbleached kraft pulp.*

*Values in parentheses represent relative yield based on the dry weight of original UKP.

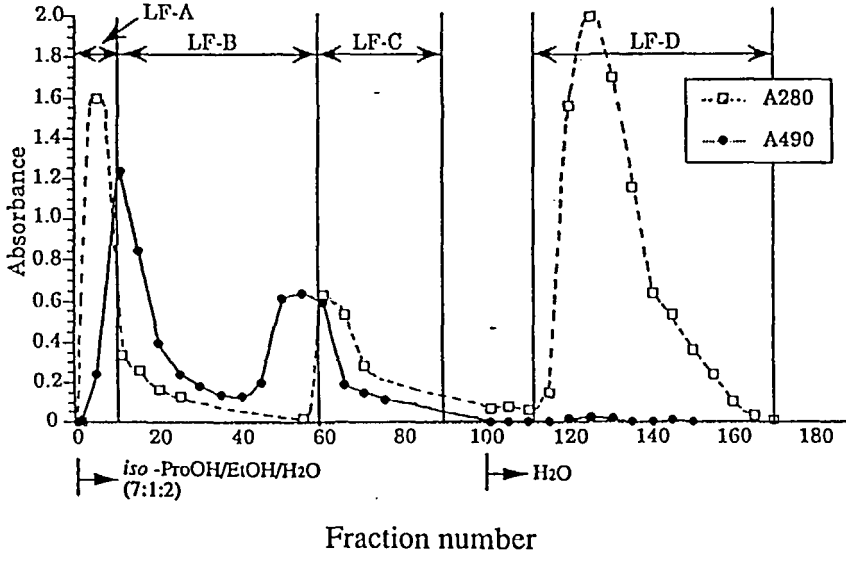

FIGURE 2 Elution profile of the enzymatic hydrolyzates (LF) of unbleached kraft pulp by silica gel column chromatography.*

*Absorptivity of the eluate at 400 nm was as follows; 0.12 (LF-A), 0.10 (LF-B), 0.12 (LF-C) and 0.74 (LF-D) (l, g^{-1}, cm^{-1})

*Elution of the carbohydrate component was monitored by phenol-sulufuric acid method at 490 nm.

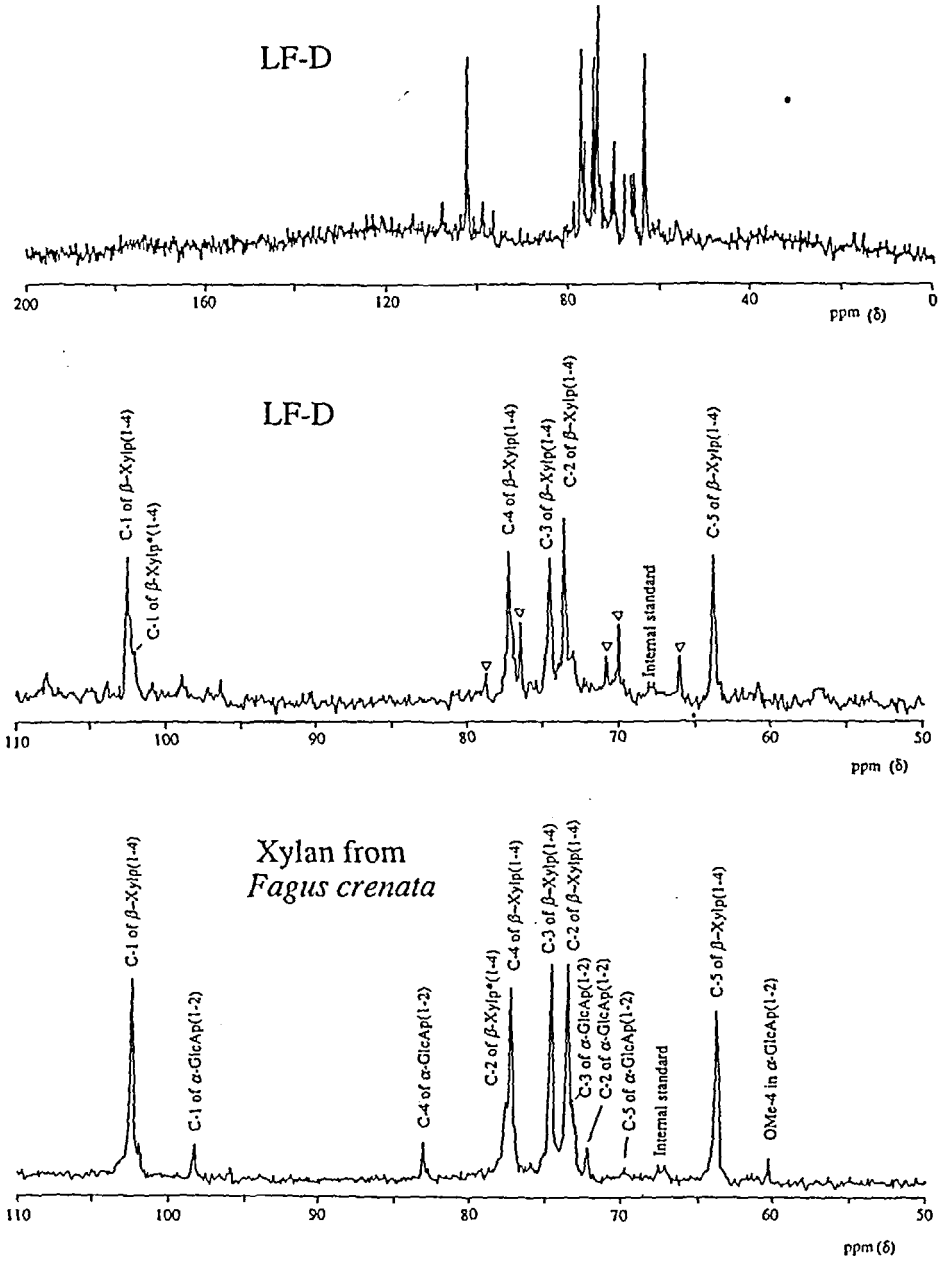

FIGURE 3 ^{13}C-NMR spectra of LF-D and xylan from *Fagus crenata*.*

*A signal originating from the methoxyl group was below the background noise level. ▽: Unknown signals.

TABLE 1
Elemental analysis of LF-D by X-ray fluorometry*[1]

Element	Al	Br	Ca	Cl	Cr	Cu	Fe	K	Mg
Content (%)	0.02	0.0	1.0	0.7	0.01	0.005	0.5	0.2	0.03
Element	Na	Ni	P	S	Si	Sr	V	Zn	Total
Content (%)	30.0	0.8	2.0	10.0	30.0	0.02	0.06	0.005	75.5

*[1] Values are expressed as weight percentage of LF-D

β-1,4-linked xylan with less side chains than native hardwood xylans. In addition, unknown NMR signals were observed in the carbohydrate regions of the spectra. Recently, Teleman reported that the 4-*O*-methylglucuronic acid side group in xylan is converted to a hexenuronic acid side group during kraft pulping[8]. In the ^{13}C-NMR spectrum of LF-D, no signals from the olefinic carbons of hexenuronic acid were detected. Elemental analysis revealed that LF-D contained a large amount of unremovable inorganic atoms including Si, Na, and S (TABLE 1). The carbon content of LF-D was only 14%. Neutral sugars other than xylose were not detected by Glc in LF-D (TABLE 2). Although NMR signals from lignin components were not detected in ^{13}C-NMR spectra, existence of a small amount of lignin components was confirmed by nitrobenzene-oxidation and a nucleus exchange reaction. The nucleus exchange reaction revealed that a part of lignin nuclei in LF-D had been demethylated during the course of kraft pulping because LF-D produced a l arge amount of catechol and pyrogallol in a nucleus exchange reaction at 110°C (TABLE 3). A nitrobenzene oxidation of LF-D yielded vanillic acid but yields of vanillin and syringaldehyde were very low, suggesting that quinoid structures are involved in LF-D (TABLE 4). In any case, the high UV absorptivity of LF-D cannot be ascribed entirely to the quantity of the lignin component. This observation is consistent with the fact that carbohydrate-derived material in short chain xylans is one of the origins of the chromophores of UKP[10] although the contribution of inorganic materials to the colorization should be taken into account.

A higher molecular weight residual lignin fraction (HF-P) was obtained from an impermeable part of the enzymatic digests. Although Yamasaki reported

TABLE 2
Chemical analysis of enzymatic hydrolysis products of UKP

	Neutral sugar composition (wt%)		Total neutral sugar (wt%)*1	Uronic acid (wt%)*2	Lignin (wt%)*3
	D-Xyl	D-Glc			
LF-A	26.0	74.0	90.3	1.7	4.5
LF-B	16.0	84.1	16.2	0.4	0.7
LF-C	51.1	48.9	39.0	1.8	3.7
LF-D	100.0	0.0	7.3	3.4	15.4
LF	21.1	78.9	86.7	2.2	3.8
HF-P	9.5	90.5	-	-	13.6
UKP	18.8	81.2	-	-	-

*1 Phenol sulfuric acid method, *2 *m*-Hydroxybiphenyl method
*3 Acetyl bromide method; the values were calculated on the basis of absorptivity (44.6 at 280nm) for mixed hard wood kraft lignin[9]. However, the lignin content of LF and its subfractions is estimated to be much lower than that of the calculated values because UV absorption of these fractions originates not only from residual lignin but also from inorganic substances, etc.

TABLE 3
Nucleus exchange reaction of wood meal and chromophoric fractions from UKP

Sample	Temp.	NEP*1 yield (% sample weight)				
		Guaiacol	Catechol	1,3-Pyro-gallol*2	1-Pyro-gallol*3	Pyro-gallol
Beech wood	180°C	0.4	1.7	0.04	1.6	1.7
	110°C	0.9	0.3	2.6	0.7	0.02
UKP	180°C	0.03	0.3	0.03	0.03	trace
	110°C	0.05	0.02	0.02	0.05	trace
UKPWS*4	180°C	0.1	0.3	0.04	0.1	0.05
	110°C	0.1	0.06	0.08	0.02	trace
LF-D	180°C	trace	0.2	0.09	0.2	0.5
	110°C	0.01	0.2	0.1	0.2	0.5
HF-P	180°C	0.07	1.1	0.3	0.4	0.5
	110°C	0.1	0.1	0.2	0.2	0.2

*1 NEP: nucleus exchange products, *2 1,3-Pyrogallol: Pyrogallol-1,3 dimethyl ether, *3 1-Pyrogallol: Pyrogallol-1-methyl ether, *4 UKPWS: water-insoluble fraction from ball-milled UKP

TABLE 4
Nitrobenzene oxidation of wood meal and chromophoric fractions from UKP

Sample	NOP*[1] yield (% sample weight)						
	V*[2]	S*[3]	VA*[4]	SA*[5]	Total NOP (wt %)	S+SA/ V+VA	SA+VA/ NOP
Beech wood	2.62	8.04	0.27	1.78	12.71	3.39	0.16
UKP	0.10	0.05	0.02	0.37	0.52	3.63	0.75
UKPWP*[6]	0.06	trace	0.17	0.81	1.01	3.52	0.97
UKPWS*[7]	0.29	0.23	0.11	0.51	1.10	1.85	0.56
HF-P	0.44	0.24	0.15	1.87	2.69	3.57	0.75
LF	trace	trace	0.07	0.53	0.59	7.57	1.00
LF-A	0.05	0.25	0.13	0.53	0.96	7.23	0.69
LF-B	0.01	trace	0.02	0.18	0.21	7.26	0.95
LF-C	0.07	0.07	0.07	0.96	1.17	7.19	0.88
LF-D	trace	trace	1.77	0.07	1.84	0.04	1.00

*[1]NOP: nitrobenzene oxidation products , *[2]V: vanillin, *[3]S: syringaldehyde, *[4]VA: vanillic acid, *[5]SA: syringic acid, *[6] UKPWP: water-insoluble fraction from ball-milled UKP, *[7]UKPWS: water-soluble fraction extracted from ball-milled UKP

that residual lignin was extracted from enzymatic hydrolizates of UKP with 96% and 75% aqueous dioxane[11], none of compounds were extracted from the impermeable part with the aqueous dioxane solutions nor with 1 N NaOH. CP/MAS-NMR and carbohydrate analysis revealed that the main component of this fraction is cellulose; however, the existence of a lignin component was confirmed by nitrobenzene oxidation and a nucleus exchange reaction. The amount of nitrobenzene-oxidation and nucleus-exchange products indicates that lignin in HF-P exhibits a diphenylmethane structure (TABLE 5).

The chromophoric xylanase-resistant xylan fraction (LF-D) was more effectively decolorized by bacterial microflora from soils than by selected white rot fungi such as *P. chrysosporium* and *B. adusta* which can partly decolorize the original unbleached kraft pulp (UKP). However, *C. versicolor* was found to decolorize not only the UKP but also the isolated xylanase-resistant

TABLE 5
Indexes based on NEP and NOP of wood meal and chromophoric fractions from UKP

Sample	NEPSy*[1]	Total NEP	NOP*[2]/NEP
Beech wood	5.98	8.03	1.58
UKP	0.32	0.62	0.83
UKPWS	0.25	0.69	1.60
LF-D	1.10	1.32	1.40
HF-P	2.77	3.92	0.69

*[1]NEPSy: Nucleus exchange reaction products from syringyl units
*[2]NOP: Nitrobenzene oxidation products from syringyl units

TABLE 6
Activities (U/ml) of extracellular ligninolytic enzymes and xylanase during cultivation of white-rot fungi and bacterial microflora in the presence of xylanase-resistant chromophoric xylans.

Day		1	2	3	4	5	6	7
KUC-10*[1]	LiP*[3]	-	-	-	-	-	-	-
	MnP	-	-	-	-	-	-	-
	Lac	-	0.02	-	-	-	-	-
	Xylanase	-	-	-	-	-	-	-
KUC-11*[1]	LiP*[3]	0.01	-	-	-	-	-	-
	MnP	-	-	-	-	-	-	-
	Lac	-	-	-	-	-	-	-
	Xylanase	-	-	-	0.1	-	-	-
SOP-14*[1]	LiP*[3]	-	-	-	-	-	-	-
	MnP	-	-	-	-	-	-	-
	Lac	-	-	-	-	-	-	-
	Xylanase	-	-	0.1	0.1	-	-	-
B. adusta	LiP*[3]	-	-	-	-	-	-	-
	MnP	-	-	-	-	0.05	0.8	2
	Lac	0.02	-	0.06	-	-	-	-
	Xylanase	-	0.1	-	-	0.05	-	-
*C. versicolor**[2]	LiP*[3]	-	0.01	0.02	0.03	0.02	-	0.01
	MnP	-	0.04	0.01	0.02	0.2	0.09	0.07
	Lac	0.05	-	0.02	-	-	-	-
	Xylanase	-	-	-	-	-	-	-
P. chryso-sporium	LiP*[3]	-	-	-	-	-	-	0.01
	MnP	-	-	-	-	-	-	-
	Lac	-	-	-	-	-	-	-
	Xylanase	0.03	0.06	0.08	0.2	0.06	-	-

-: Not detected. *[1]Bacterial microflora from soil partially decolorized the xylanase-resistant chromophoric xylans. *[2]*C. versicolor* also partially decolorized the xylans. However, activities of the extracellular ligninolytic enzymes were trace. *[3]One unit of Lip activity is defined as the amount of enzyme which oxidizes 1 μmol of veratryl alcohol to veratraldehyde in 1 min.

chromophoric xylan fraction. The activities of extracellular lignin peroxidase, Mn-peroxidase, laccase and xylanase in the culture filtrates of *C. versicolor* and the bacterial microflora were very low during the cultivation with the chromophoric xylans (TABLE 6). In contrast with the oxidizing enzymes, reducing activity of sodium 2,6-dichlorophenol-indophenol was detected in the cultures of the bacterial microflora. Thus, chromophores of the xylanase-resistant xylans are different from typical residual lignin in UKP, and their biochemical decolorization requires enzymes different from the extracellular ligninolytic enzymes. Although the xylanase-resitant xylans can be removed by alkali-extraction from UKP, their microbial decolorization is important for environmentally safe high yield bleaching[12].

EXPERIMENTAL

General Methods

Cellulases (Celluclast, Novo Nordisk Co. Ltd.) were purified by salting-out from 70% saturated ammonium sulfate and gel filtration on a Pharmacia Superdex 200 column (16 mm X 60 cm). Activities of the purified cellulase preparation used were as follows: Avicelase 111 U/mL, β-glucosidase 314 U/mL, xylanase 95 U/mL. Nitrobenzen oxidation was carried out at 170°C for 3 hr. An nucleus exchange reaction was performed at 110 and 180°C for four hours as described in the literature[13]. GLC was carried out with a Shimadzu GC-14A Gas Chromatograph equipped with a flame ionization detector using helium as a carrier gas on a silicon OV-101 (50 m x 0.25 mm). NMR spectra were measured on a Varian XL-200 FT spectrometer (^{1}H: 200 MHz). X-ray fluorometry was carried out with Rigaku RIX 3000 at 50 kV and 50 mA.

Enzymatic Degradation of Kraft Pulp

Unbleached kraft pulp (10 g, dry weight) from mixed hardwoods (kapper Number 17.8) was hydrolized with the purified enzyme (1340 U for Avicellase activity) at 50°C for 24 hr using a 20mM sodium acetate buffer (pH 5.0) in a shaker flask. The hydrolizates were transferred to a membrane bioreactor (Mw cut off: 10,000) and further hydrolized until the sugar component was not detected in the permeable fraction. The impermeable fraction (HF) was washed with distilled water in the bioreactor. HF was recovered and then centrifuged at 9,000 rpm for 30 min. to separate the supernatant (HF-S) and precipitate (HF-P). HF-P was washed three times with water by the centrifugation. The permeable fraction (LF) was concentrated, and then applied to a silica gel column (6 x 50 cm). The elution was carried out first with a 2-propanol, ethanol and water mixture (7:1:2, v/v/v) and then with water (Fig. 1). Elution of the carbohydrate component was monitored by phenol-sulufuric acid method at 490 nm.

Microbial Treatment of Chromophoric Xylans

White rot fungi, *Coriolus consors* (K-1198, K-1227), *Irpex lacteus* (IFO-5367), *C. hirsutus* (IFO-4917), *C. versicolor* (K-2912), *C. vellereus* (K-1957), *Bjerkandera adusta* (K-2679), *Phanerochaete chrysosporium* (ATCC 34541) *and Pleurotus ostretus* (WRI-1) were incubated stationary in a glucose-peptone medium in the presence of the xylanase-resitant chromogens and Kirk's salt at 37°C for *P. chrysosporium* and 30°C for all others[14]. Bacterial microflora from a paper-making mill, subtropical regions, etc. were also cultured in a glucose-peptone medium containing the xylanase-resitant chromogens at 30°C. During the cultivation, activities of lignin peroxidase (LiP), Mn (II) peroxidase (MnP), laccase (Lac) and xylanase were measured periodically as described previously[15]. A reduction test of sodium 2,6-

dichlorophenol indophenol dihydrate was carried out by incubating 10 mg of the chemical with 1 ml of each culture filtrate at 30°C for 30 min.

CONCLUSION

A water-soluble xylanase-resitant chromophoric xylan fraction (LF-D) contained a large amount of unremovable inorganic atoms including Si, Na, and S, together with a non-branched β-1,4-linked xylan chain. LF-D contained lignin as a minor component. A nucleus exchange reaction of LF-D revealed that a part of the lignin nuclei in LF-D had been demethylated during the course of kraft pulping. A nitrobenzene oxidation of LF-D yielded vanillic acid but yields of vanillin and syringaldehyde were very low, suggesting that quinoid structures are involved in LF-D. On the other hand, HF-P exhibited a diphenylmethane structure. Thus, the molecular structure of lignins in LF-D and HF-P was different. LF-D was more effectively decolorized by bacterial microflora from soil than by selected white rot fungi including *P. chrysosporium* and *B. adusta* which can partly decolorize the original unbleached kraft pulp (UKP). However, *C. versicolor* was found to decolorize not only the UKP but also the isolated xylanase-resistant chromophoric xylan fraction. Furthermore, activities of extracellular lignin peroxidase, Mn-peroxidase, laccase and xylanase in the culture filtrates of *C. versicolor* and the bacterial microflora were very low during the cultivation with the chromophoric xylans. Thus, chromophores of the xylanase-resistant xylans are different from typical residual lignin in UKP, and their biochemical decolorization requires enzymes different from the extracellular ligninolytic enzymes.[12]

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