Characterization of Residual Lignin Obtained by the Enzymatic Hydrolysis of Oil Palm Empty Fruit Bunch Pulps

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Residual lignin present in alkali pulps prepared from oil palm (*Elaeis guineensis*) empty fruit bunch was isolated using an enzymatic method and characterized successfully by pyrolysis-gas chromatography-mass spectrometry (Py-GC/MS). These pulps were prepared by soda-anthraquinone (AQ) and prehydrolysis (PH) soda-AQ cooking methods (pulp yields were 45.3 and 33.9%, respectively). Py-GC/MS of the residual lignin showed that two pyrolysis products which were indole and methylindole originating from the enzyme (contents 12 to 44%), in addition to eight pyrolysis products originating from syringyl (S) and guaiacyl (G) structure of lignin. Furthermore, palmitic acid was also detected in the residual lignin (contents 0.11 to 0.28%). The residual lignin was subjected to further purification by a cellobiose treatment method, and the quantity of enzyme present in the residual lignin was found to decrease after the treatment. Residual lignin in PH-soda-AQ pulp exhibited a higher S/G ratio (0.86 to 0.98) than that in soda-AQ pulp (0.76 to 0.97). This study showed that the contents of lignin (19 to 44%) and enzyme in enzymatically isolated lignin can be estimated by the Py-GC/MS method.

Keywords: Empty fruit bunch; Prehydrolysis soda-anthraquinone; Enzymatic hydrolysis; Cellobiose; Pyrolysis-gas chromatography-mass spectrometry; Syringyl/guaiacyl ratio

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INTRODUCTION

Non-wood lignocellulosic biomass is considered a highly promising alternative to wood. In particular, oil palm (*Elaeis guineensis*) empty fruit bunch (EFB) is abundantly available as a by-product of the palm oil industry. Indeed, the chemical and physical properties of oil palm EFB have been well examined (Law *et al*. 2007; Wan Rosli *et al*. 2011), and it has been utilized as a raw material for producing a range of fiber-based products, including pulp and paper. Wan Rosli *et al*. (1998) demonstrated that superior quality pulp with low shives content was obtained from EFB fibers through alkaline cooking using only sodium hydroxide, compared to that obtained by other chemical cooking methods. Biorefinery processes have also been used to produce cellulose derivatives, cellulose nanofibers, and other chemicals from lignocellulosic materials. Prehydrolysis (PH) soda cooking has been demonstrated to significantly improve delignification compared to that afforded by soda cooking (Wan Rosli *et al*. 2004). Hence, PH-soda-anthraquinone (AQ) cooking has been developed as an environmentally friendly

biorefinery process, because it involves prehydrolysis that aids in removing fragments of hemicellulose and in modifying lignin prior to cooking (Harsono *et al*. 2016).

Although many types of unbleached EFB pulps have been produced, the characteristics of the residual lignin in the EFB pulps have not yet been fully understood. Such an understanding could help in enhancing delignification in the bleaching process. Previous studies have mainly concentrated on the structure of residual lignin in wood chemical pulps (Jaaskelainen *et al*. 2003), and as such, similar studies are also required for non-wood residual lignin for future applications. In the 1980s, the enzymatic hydrolysis of pulp was introduced to study the characteristics of residual lignin (Yamasaki *et al*. 1981). It was found that no structural changes occurred in the isolated residual lignin, although carbohydrates were dissolved by the enzyme, leaving behind solid residues referred to as residual lignin. While this enzymatic method provides structurally intact lignin, the purities tend to be low, and traces of the mixed enzyme remain in the residue, although this has yet to be quantified.

The efficiency of enzymes in the enzymatic hydrolysis process for lignocellulosic materials depends on the characteristics of lignin remaining in the materials following pretreatment for the saccharification. This is an important consideration for designing enhanced bioethanol production processes for non-wood materials such as wheat straw and softwood (Morales *et al*. 2014; Yang *et al*. 2016). It is therefore important to enhance our understanding on the adsorption of enzymes on residual lignin when EFB is used as a raw material for bioethanol production. In a previous study (Tanifuji *et al*. 2011), the enzyme activity in terms of filter paper units (FPU) adsorbed onto residual lignin has been estimated by pyrolysis-gas chromatography-mass spectrometry (Py-GC/MS).

Lignin, which is often regarded as an undesirable component in the conversion of wood into bleached pulp, is a polymerized compound consisting of phenylpropane units known as *p*-hydroxyphenyl, syringyl (S), and guaiacyl (G) units. The most common method used to determine the S/G ratio is nitrobenzene oxidation that involves a number of steps and affords syringaldehyde and vanillin. However, this procedure is time consuming. Thus, an alternative analytical technique such as Py-GC/MS is recommended, as it is a faster, safer, and economical method for the determination of lignin (Kuroda *et al*. 2005). Py-GC/MS is based on the thermal degradation of lignin to produce pyrolysis compounds (G and S types), which can be detected by GC/MS. Previously, Py-GC/MS and nitrobenzene oxidation have been considered only in the context of characterizing lignin in wood and non-wood materials (Lima *et al*. 2008), but not for analyzing the residual lignin in non-wood chemical pulps.

Thus, we herein report the development of Py-GC/MS as a novel method to determine the enzyme quantity, the S/G ratio of lignin, and other components present in the residual lignin enzymatically isolated from EFB pulps. Furthermore, the residual lignin present in soda-AQ and PH-soda-AQ EFB pulps is also characterized.

EXPERIMENTAL

Materials

Preparation of EFB fibers, prehydrolysis fibers, and chemical pulps

EFB fibers were provided by the United Oil Palm Industries Sdn. Bhd, Nibong Tebal, Pulau Pinang, Malaysia. The EFB fibers were washed with filtered tap water to

remove any contaminants such as dust and sand. The washed fibers were then air-dried until the solid content reached over 90%. To obtain the PH-EFB fibers, the washed EFB fibers were treated with water (water/fiber ratio of 7/1) in a 4 L stationary stainless steel vessel at 165 °C for 60 min and air-dried until the solid content was over 90%.

Soda-AQ cooking was carried out at 160 °C for 120 min in a 4 L stationary stainless steel vessel with an AQ dosage of 0.1%, active alkali: (NaOH as Na_2O) (AA) dosages of 17–19%, and a water/fiber ratio of 7/1. The pulp slurry was subsequently screened on a TAPPI standard flat screen (slit width: 0.8 mm).

Preparation of the residual lignin and black liquor lignin

Enzymatic hydrolysis was carried out according to previous literature procedures (Chang 1992) using a mixture of cellulolytic enzymes (GC220, Genencore Kyowa Co. Ltd., Japan). The pulp was continuously shaken for 24 h in an acetate buffer at pH 4.5 and temperature 45 °C using 30 filter paper units per 1 g pulp (FPU/g) of GC220 and then for further 24 h with an additional 15 FPU/g. Subsequently, the residue was washed with distilled water and separated by centrifugation as the residual lignin. The residual lignins were repeatedly prepared twice from a pulp. These are referred to as soda-AQ lignin 1, PH-soda-AQ lignin 1, soda-AQ lignin 2, and PH-soda-AQ lignin 2. Further, the enzymatically isolated residue (80 mg) was treated with cellobiose (24 mg) in distilled water (50 mL) either once, twice, or thrice. The residual lignins were then freeze-dried.

Meanwhile, lignin was isolated from spent black liquor of soda-AQ and PH-soda-AQ cooking according to a previous literature procedure (Lin 1992). The black liquor was acidified to pH 2 using 2 M hydrochloric acid in one step. Subsequently, the mixture was centrifuged, and the precipitate was washed thrice with distilled water and then freeze-dried. These are referred to as soda-AQ black liquor lignin and PH-soda-AQ black liquor lignin.

Analytical Methods

Elemental analysis of the residual lignin

Elemental (CHN) analysis of carbon, hydrogen, and nitrogen in the enzymatically isolated residual lignin was carried out at the Chemical Analysis Division, Research Facility Center for Science and Technology, University of Tsukuba, Japan, using a Perkin-Elmer 2400 CHN Elemental Analyzer.

Estimation of lignin content by Py-GC/MS and acetyl bromide method

The Py-GC/MS conditions employed herein were as follows: Pyrolyzer, JHP-5 (Japan Analytical Industry Co. Ltd., Japan); pyrolysis conditions, 500 °C for 4 s; GC/MS system, QP-5050A (Shimadzu, Japan); column, HP 1-MS (30 m × 0.25 mm; film thickness: 1.0 µm). First, 100 µg of the enzymatically isolated residual lignin was subjected to Py-GC/MS with 0.1 µg of *n*-eicosane as an internal standard. Yields of lignin pyrolysis products were obtained using the parameters reported in the previous study (Nakagawa-Izumi *et al*. 2016), where the calibration lines authentic compounds with were made by Py-GC/MS. Black liquor lignins were also analyzed, and the lignin contents in the enzymatically isolated residual lignin were estimated using a conversion factor derived from the pyrolysis products yield and the lignin content in black liquor lignin. In addition, the acetyl bromide method (Dence 1992) was employed to estimate the lignin content in the enzymatically isolated residual lignin.

Determination of enzyme and fatty acid in the residual lignin by Py-GC/MS

The Py-GC/MS conditions were identical to those described previously. The parameter for estimating the enzyme content in the residual lignin was obtained from the calibration lines between the authentic compounds (indole and methylindole) and the freeze-dried GC220. The GC220 solution (1 mL) contained 426 mg of the freeze-dried solid, and exhibited an activity of 63.2 FPU. The nitrogen content of the solid was 5.6%, and 1 mL of the enzyme solution contained 149 mg of protein.

The parameter for estimating the fatty acid content in the isolated residue was obtained from the calibration line of an authentic palmitic acid sample.

Determination of acid-insoluble lignin and carbohydrate composition

The contents of the acid-insoluble lignin (Klason lignin), acid-soluble lignin, and ash were determined using TAPPI Test Method T 222 om-15 and T 211 om-02, and the amounts of glucose, xylose, and other sugars in the acid hydrolysate were determined using ion chromatography according to previously published procedures (Harsono *et al*. 2016).

RESULTS AND DISCUSSION

Fiber and Pulp Preparation

EFB fibers and pulps contained glucan, xylan, lignin, extractive, and ash (Table 1). The prehydrolysis process can increase the dissolution of lignin during alkaline cooking and thus reduce the ash content substantially (Wan Rosli *et al*. 2004).

Prehydrolyzed pulp exhibited a much lower kappa number (5.8) and rejects yield (4.2%) than those of the non-prehydrolyzed pulp (12.3 and 5.7%, respectively). In addition, the prehydrolyzed pulp gave a significantly lower screened yield than the non-prehydrolyzed pulp (Table 2).

Table 1. Chemical Composition of EFB Fibers and Pulps

	Acid-insoluble lignin (%)	Acid-soluble lignin (%)	Glucan (%)	Xylan (%)	Ash (%)	Other organics (%)
EFB material[a]	19.1	3.7	35.6	19.6	3.1	18.9[a]
PH-EFB material	18.2	2.0	35.2	9.2	0.6	5.8[b]
Soda-AQ pulp	1.3	0.6	31.0	14.1	0.8	7.1
PH-soda-AQ pulp	0.2	0.4	29.6	3.3	0.2	7.2

[a,b] Acetone extractives contents: [a] 2.1% and [b] 2.8%, respectively.

Table 2. Kappa Number and Pulp Yield of EFB Chemical Pulps

	Active alkali (%)	Kappa number	Rejects yield (%)	Screened yield (%)
Soda-AQ pulp	18	12.3	5.7	45.3
PH-soda-AQ pulp	18	5.8	4.2	33.9

Solubility of the Enzymatically Isolated Lignin

The yields of enzymatically isolated lignins from soda-AQ and PH-soda-AQ pulp were 1.6 and 0.8% based on pulp weights, respectively. Lignin isolated using the enzymatic method was insoluble in most standard solvents such as tetrahydrofuran, dimethyl sulfoxide, acetone, and chloroform (Chang 1992). This insolubility is mainly due to the inability of the enzymatic treatment to break the covalent bonds between carbohydrates and lignin; thus, the solubility of the resulting lignin-carbohydrate structure is low in these solvents. Indeed, previous studies have suggested protocols to alleviate these solubility problems of the enzymatically isolated lignin (Hortling *et al*. 1990; Fukagawa *et al*. 1992; Duarte *et al*. 2001). Furthermore, the enzymatically isolated lignin contained impurities originating from the enzyme, cellulose, and hemicellulose, which cannot be removed by prolonged and repetitive enzymatic treatments or by any purification procedure (Yamasaki *et al*. 1981). Therefore, a reliable method is required for determining the enzyme content in the isolated lignin.

Elemental (CHN) Analysis of the Residual Lignin

The enzymatically isolated lignin contained significant quantities of nitrogen (5.3 to 9.2%) because of the presence of enzyme protein, despite thorough washing and purification following enzymatic hydrolysis (Table 3). Typical nitrogen contents of enzymatically isolated lignin from unbleached pulps have been reported in the range 2.5 to 7.3% (Jiang *et al*. 1987). The enzyme amounts calculated from the values in this study using a nitrogen protein conversion factor of 6.25 (Hortling *et al*. 1990) were 33 to 58%, suggesting that more than one-third of weight for the isolated lignin is indeed protein.

Table 3. CHN Composition of Enzymatically Isolated Residual Lignin

	Carbon (%)	Hydrogen (%)	Others (%)	Nitrogen (%)	Nitrogen×6.25 (%)
Soda-AQ lignin 1	49.7	7.1	37.9	5.3	33
PH-soda-AQ lignin 1	50.4	7.3	35.7	6.6	41
Soda-AQ lignin 2	44.2	6.1	43.5	6.2	39
PH-soda-AQ lignin 2	47.0	6.4	37.4	9.2	58

Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance spectroscopy (NMR) have been used for the characterization of residual lignin and lignocellulosic materials (Fukagawa *et al*. 1992; Del Rio *et al*. 2007). In a supplemental experiment using FTIR, the enzymatically isolated lignin exhibited only one signal at 1650 cm^{-1}, which was reported to be corresponding to protein impurities (Hortling *et al*. 1990), whereas signals corresponding to an OH stretch (3400 cm^{-1}), palmitic acid (2920 cm^{-1}), CH_2 deformation (1460 cm^{-1}), S unit (1330 cm^{-1}), and G unit (1270 cm^{-1}) were observed in the EFB materials. Therefore, FTIR spectroscopy was unfortunately not useful for the characterization of the residual lignin. Because of poor solubility of the enzymatically isolated lignin in testing solvents, only minimal information could be obtained by NMR. Hence, the use of Py-GC/MS was examined.

Py-GC/MS of the Residual Lignin

In the total ion chromatograms (TIC), eight compounds (G1–G4, S1–S4) corresponding to lignin pyrolysis products and two compounds (indole: E1, methylindole: E2) corresponding to the cellulolytic enzyme in GC220 were identified (Fig. 1). The result

clearly indicates that the majority of the lignin pyrolysis products signals did not overlap with signals corresponding to cellulose and hemicellulose, and hence, determination of the chemical features of lignin was relatively straightforward by this method. In addition, although a number of cellulose and hemicellulose pyrolysis products could often be observed on TIC of the isolated lignin, their contents were very low compared to those of the EFB material.

For the lignin isolated from EFB pulps, an additional signal (P) was observed corresponding to a long-chain fatty acid. This long-chain fatty acid has been identified as palmitic acid (Harsono *et al*. 2016). However, as the EFB material does not actually have oil palm seed and long-chain fatty acids, it is suspected that this long-chain fatty acid originates from the crude palm oil processing process. On the other hand, the signal P was not observed for black liquor lignin. Palmitic acid was expected to be bound to the residual lignin of EFB pulps.

Determination of Enzyme and Palmitic Acid Contents in the Residual Lignin

Tanifuji *et al*. (2011) used Py-GC/MS to measure the enzyme activity in terms of FPU adsorbed to residual lignin. However, the amount of enzyme present in the form of protein was not estimated. We therefore expected that the enzyme protein and palmitic acid contents can be determined using Py-GC/MS based on the parameters that were obtained from the calibration lines (Fig. 2). The palmitic acid and enzyme contents were calculated using the ratio of total ion (TI) response area of peak P to that of an internal standard (IS, *n*-eicosane), or of peaks E1 and E2 to that of IS.

According to the following equations, palmitic acid and enzyme contents in the isolated residual lignins were calculated as 0.11 to 0.28% and 12 to 44%, respectively (Table 4). The cellobiose treatments resulted in a decrease in the enzyme contents (%) in the residual lignin.

$$\text{Palmitic acid content (\%)} = 100 \times \frac{[1.41 \times (\text{Peak P area ratio})] \times \text{Weight of IS}}{\text{Weight of the isolated residual lignin}}$$

$$\text{Enzyme content (\%)} = 100 \times \frac{[138 \times (\text{Peak E1 and E2 area ratio}) + 108] \times \text{Weight of IS}}{\text{Weight of the isolated residual lignin}}$$

Determination of Eight Lignin Pyrolysis Products

The lignin pyrolysis products yields based on material weight were determined using main ion (MI) intensity parameters (response factors of MI area to weight) according to the previous literature (Nakagawa-Izumi *et al*. 2016). The S/G molar ratio was also calculated using molar yields of S1–S4 and G1–G4 (Table 4). The yields of soda-AQ and PH-soda-AQ black liquor lignins were 7.4% and 7.8%, respectively. Meanwhile, the yields of enzymatically isolated residual lignin were only 1.7 to 4.2%.

Furthermore, lignin in the PH-EFB material has a higher S/G ratio (1.70) than that in the EFB material (1.27). In general, lignocellulosic materials containing higher S/G ratio lignin undergo easier delignification under alkaline cooking (Wallis *et al*. 1996; Del Rio *et al*. 2005). This is because the *β*-aryl ether-containing S units are easier to cleave during alkaline cooking than G units (Shimizu *et al*. 2012). Interestingly, the S/G ratios of PH-soda-AQ lignin (0.86 to 0.98) were also higher than those of soda-AQ lignin (0.76 to 0.97), and hence, it is the preferred material for the subsequent oxygen bleaching stage and the

production of bleached dissolving pulp. PH-soda-AQ black liquor lignin has a higher S/G ratio (1.70) than the soda-AQ black liquor lignin (1.44).

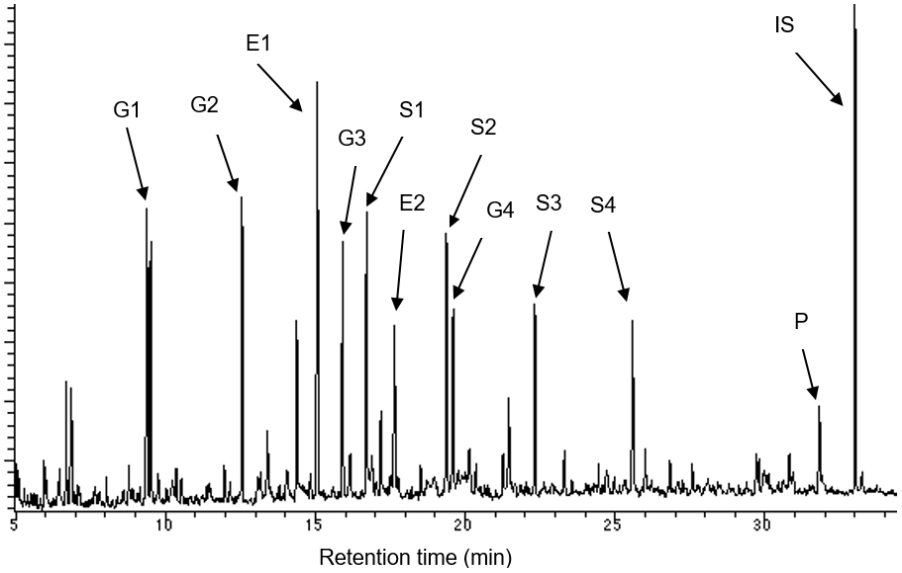

Fig. 1. Total ion chromatogram of enzymatically isolated residual lignin by Py-GC/MS

Note:

Peaks	Pyrolysis products	Main ions	Retention time (min)
G1	Guaiacol	124, 109	9.5
G2	4-Methylguaiacol	138, 123	12.5
E1	Indole	117	15.0
G3	4-Vinylguaiacol	150, 135	15.9
S1	Syringol	154, 139	16.7
E2	Methylindole	130	17.5
S2	4-Methysyringol	168, 153	19.6
G4	*trans*-Isoeugenol	164, 149	19.8
S3	4-Vinylsyringol	180, 165	22.3
S4	4-*trans*-Prophenylsyringol	194, 179	25.5
P	Palmitic acid	256	31.8
IS	*n*-Eicosane	282	33.0

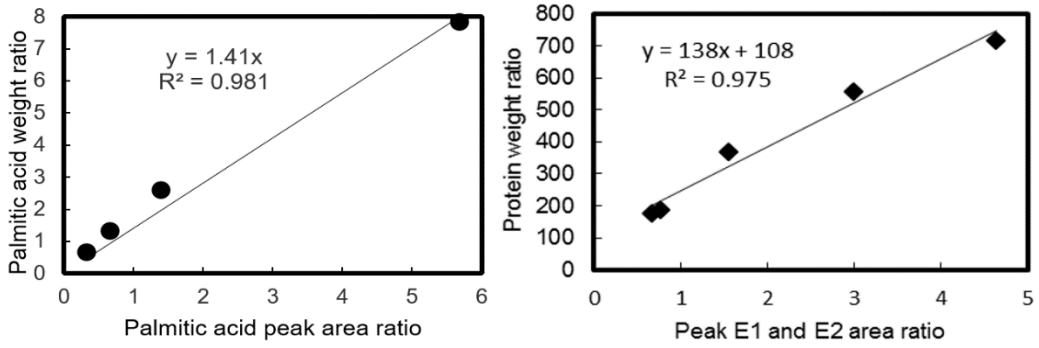

Fig. 2. Calibration line for palmitic acid and enzyme obtained by Py-GC/MS

Table 4. Determination of Palmitic Acid, Eight Lignin Pyrolysis Products, and Enzyme by Py-GC/MS

	Lignin pyrolysis products yield (%)	S/G ratio	Palmitic acid yield (%)	Enzyme yield (%)
EFB material	2.3	1.27	0.31	-
PH-EFB material	2.3	1.70	0.25	-
Soda-AQ lignin 1	4.0	0.91	0.28	22
Soda-AQ lignin 1-3[a]	4.2	0.97	0.10	12
PH-soda-AQ lignin 1	3.5	0.94	0.20	27
PH-soda-AQ lignin 1-3[a]	4.1	0.98	0.17	24
Soda-AQ lignin 2	3.1	0.76	0.11	34
PH-soda-AQ lignin 2	1.7	0.86	0.11	44
Soda-AQ black liquor lignin[b]	7.4	1.44	-	-
PH-soda-AQ black liquor lignin[c]	7.8	1.70	-	-

[a] Cellobiose treatment repeated in triplicate.
[b,c] Acid-insoluble lignin contents (%): [b] 82.4% and [c] 87.0%, respectively.

For native lignin in EFB material, the lignin pyrolysis products yield based on acid-insoluble lignin content (19.1% in Table 1) were 12.0% (2.3÷0.191). These are similar values to the yields for hardwoods (Nakagawa-Izumi *et al*. 2016). The yields for soda-AQ and PH-soda-AQ black liquor lignins were 9.0% and 8.9%, respectively, which were lower than 12.0% for native lignin in EFB materials.

The S1–S4 and G1–G4 yields based on acid-insoluble lignin contents are shown in Table 5. Interestingly, S2, S4, G2, and G4 were increased by the prehydrolysis while S1, S3, G1, and G3 were decreased. These changes should be probably due to the cleavage of lignin-carbohydrate bonds at Cα or Cγ positions by prehydrolysis. In addition to these data, the S1–S4 and G1–G4 yields based on materials for enzymatically isolated lignins are shown in Table 5.

Table 5. Determination of Eight Pyrolysis Products from EFB Materials, Black Liquor Lignins, and Enzymatically Isolated Lignin

	Yield of pyrolysis products (% based on acid-insoluble lignin)								
	S1	S2	S3	S4	G1	G2	G3	G4	Total
EFB materials	2.36	0.74	2.34	1.27	1.98	0.62	2.08	0.60	12.0
PH-EFB material	1.98	1.46	2.21	2.31	1.34	0.90	1.69	0.70	12.6
Soda-AQ black liquor lignin	2.46	0.92	1.42	0.53	1.55	0.56	1.29	0.29	9.0
PH-soda-AQ black liquor lignin	1.92	1.79	1.26	0.62	1.08	0.95	0.92	0.34	8.9
	Yield of pyrolysis products (% based on materials)								
	S1	S2	S3	S4	G1	G2	G3	G4	Total
Soda-AQ lignin 2	0.41	0.29	0.38	0.27	0.55	0.39	0.52	0.32	3.1
PH-soda-AQ lignin 2	0.27	0.18	0.21	0.14	0.26	0.24	0.31	0.12	1.7

Estimation of Lignin Contents in Enzymatically Isolated Lignin

The contents of lignin as acid-insoluble lignin in the enzymatically isolated residual lignin can be estimated when we assume that a conversion factor from the lignin pyrolysis products yield based on lignin (as acid-insoluble lignin) weight is 9.0% for the isolated residual lignin. As shown in Table 6, the lignin contents in the isolated residual lignins were 19 to 44%. Meanwhile, the acetyl bromide method indicated that the lignin contents in the isolated residual lignins were 25 to 49%.

Analysis of the carbohydrate composition indicated that the isolated residual lignins contained arabinose, galactose, and mannose in addition to glucose and xylose. The contents of glucan, xylan, arabinan, galactan and mannan in freeze-dried GC220 were 22.8, 1.0, 1.2, 1.2 and 44.8%, respectively. EFB material contains little amounts of arabinan, galactan and mannan. Relative increments of xylan, arabinan, and galactan in the residual lignins strongly suggest that the hemicelluloses should chemically combined with lignin.

Table 6. Chemical Composition of Enzymatically Isolated Residual Lignin

	Lignin (%)	Relative composition (%)				
		Glucan	Xylan	Arabinan	Galactan	Mannan
Soda-AQ lignin 1	44[a] (49[b])	13	11	4.6	2.9	2.1
PH-soda-AQ lignin 1	39[a] (48[b])	16	10	5.1	1.7	1.4
Soda-AQ lignin 2	34[a] (32[b])	14	11	3.1	2.0	1.8
PH-soda-AQ lignin 2	19[a] (25[b])	16	11	5.2	2.9	2.3

[a] Yield of lignin pyrolysis products (%) ÷0.09
[b] Acetyl bromide method.

CONCLUSIONS

1. Py-GC/MS was employed to identify lignin pyrolysis products, enzyme pyrolysis products, and palmitic acid, and to determine their respective contents of the enzymatically isolated residual lignin in oil palm EFB alkali pulps.
2. Residual lignin in PH-soda-AQ pulp exhibited a higher S/G ratio than that in soda-AQ pulp.
3. Palmitic acid was found to be bound to the residual lignin of EFB pulps.
4. The enzyme content adsorbed on the residual lignin was estimated, and it was reduced by cellobiose treatment.
5. The enzymatically isolated residual lignin still contained significant quantities of both carbohydrates and enzymes.
6. Black liquor lignin exhibited a lower total yield of lignin pyrolysis products than the native lignin in EFB materials.
7. Py-GC/MS was a useful method for estimating contents of lignin and enzyme present in enzymatically isolated lignin.

ACKNOWLEDGMENTS

We acknowledge the School of Industrial Technology USM for providing the raw materials and facilities through the Bio-resource, Paper, and Coatings Division. This work was in part supported by the RU grant (number 1001/PTEKIND/811088) and the Scholarship Donations of Hokuetsu Kishu Paper Co., Ltd. (ECF Bleached Pulp). This paper was presented in part at the 2014 Pan Pacific TAPPI Conference in Taipei, Taiwan, May 21–23, 2014.

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Article submitted: Jan. 26, 2015; Peer review completed: April 25, 2016; Revised version received and accepted: September 27, 2016; Published: November 11, 2016.
DOI: 10.15376/biores.12.1.183-194