Enzymatic Degradation of Lignin Extracted from Oil Palm Empty Fruit Bunch Using Laccase and Cutinase

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Enzymatic degradation products of lignin, having potential for added value, were obtained by extraction and subsequent enzymatic treatments of oil palm empty fruit bunch (OPEFB). The objective was to optimize the production of OPEFB lignin degradation products and study the effects of different enzymes and reaction media. Powder of OPEFB lignin was recovered from organsolv black liquor by using methanol, acidified water, and deionized water, respectively. OPEFB lignin was later subjected to enzymatic hydrolysis in an incubator shaker for 24 h using laccase and cutinase in various reaction media, including phenol, water, and acetate buffer. Nine compounds were recovered as OPEFB lignin degradation products, namely hydroxybenzoic acid, hydroxybenzaldehyde, vanillic acid, vanillin, syringic acid, syringaldehyde, coumaric acid, ferulic acid, and guaiacyl alcohol. When laccase was used in water, the product with the highest concentration was syringaldehyde (4061.1 ± 89.9 mg/L), and followed by hydroxybenzoic acid (1029.8 ± 50.2 mg/L). Vanillic acid was the product with the highest concentration (126 ± 97.5 g/L) found when laccase was used in phenol. When cutinase was used in water, products with the highest concentrations in the medium were syringaldehyde (4837.6 ± 156.4 mg/L) and syringic acid (2387.7 ± 105.3 mg/L). High performance liquid chromatography (HPLC) was used to quantify the OPEFB lignin degradation products.

Keywords: Lignin; Cellulose; Laccase; Biotransformation; Biodegradable; Renewable

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INTRODUCTION

Approximately 17 million tons of oil palm empty fruit bunch (OPEFB) are produced every year in Malaysia, and this lignocellulosic biomass is a potentially useful renewable resource (Ahmadzadeh and Zakaria 2007; Akhtar *et al.* 2015). The OPEFB is composed of 67% holocellulose (hemicellulose and cellulose) and 24% lignin (Serrano *et al.* 2008). Many researchers have tried to establish valuable products (lignin and cellulose derivatives) from OPEFB (Ahmadzadeh and Zakaria 2007; Akhtar *et al.* 2015; Rahman *et al.* 2007; Cui *et al.* 2014; Wanrosli *et al.* 2011; Piarpuzan *et al.* 2011). Lignin is the second most plentiful biomass that is associated with cellulose and hemicellulose in plant cells (Wang *et al.* 2011; Luo *et al.* 2013). Through chemical or bioprocessing technology, lignin and cellulose from OPEFB can be separated (Hassan *et al.* 2013). This material can be used as a renewable feedstock for the production of bio-products, ethanol, and industrial chemicals (Chen *et al.* 2015). Since lignin is a complicated aromatic polymer with high molecular weight and is rather resistant to microorganisms, its utilization is quite limited

(Wang *et al.* 2011; Luo *et al.* 2013). Thus, there is an urgent need for the optimization of separation of lignin from biomass sources and the subsequent production and isolation of lignin degradation products that are cost effective and environmentally friendly.

In lignin biodegradation, white-rot and brown-rot fungi are well known for degradation and functional extracellular oxidative enzymes. These ligninolytic enzymes mainly include laccase (Lac), lignin peroxidase (LiP), and manganese peroxidase (MnP). Ligninolytic enzymes mediate an effective degradation of lignin, and the degrading ability significantly depends on the interactions of ligninolytic enzymes with lignin and mediators. Therefore, lignin degradation is a complex process with synergism among many enzymes (Chew and Bhatia 2008; Wang *et al.* 2013). Currently, environmentally friendly technologies using oxidoreductive enzymes are being developed for the treatment of lignin, including the pretreatment of lignocellulose for de-lignification, in order to improve the processing and conversion of the biomass.

Laccases (EC.1.10.3.2) primarily catalyze the oxidation of phenolic hydroxyl groups. Laccases can act on hydroxyl groups of monophenols and related compounds, using oxygen as the electron acceptor. Accordingly, laccase activity does not require the presence of peroxidases, *e.g.*, lignin peroxidases (EC 1.11.1.14) and manganese peroxidase (EC 1.11.1.13) (Munk *et al.* 2015). The use of laccase with appropriate mediators is anticipated to produce lignin degradation products with high yields. Cutinases are hydrolytic enzymes that degrade cutin, the cuticular polymer of higher plants. Cutin is a polyester composed of hydroxy and epoxy fatty acids (Ferrario *et al.* 2016). Ester bonds predominate in the cutin, although peroxide bridges and ether linkages are also present. Therefore, cutinase is also expected to degrade the lignin.

A variety of monomers, oligomers, and polymers may be produced as products of lignin degradation, depending on the feedstock biomass and the pretreatments. Vanillin, syringaldehyde, syringic acid, ferulic acid, 4-hydroxybenzaldehyde, 4-hydroxyaceto-phenone, or cinnamic acid derivatives are common lignin-derived monomers, and these compounds also have various applications in food and pharmaceutical industries (Kolb *et al.* 2013). The aim of this study was to produce degradation products of lignin extracted from OPEFB in high yield with the use of laccase and cutinase. The effect of the OPEFB lignin treatments on the yield was studied. It was anticipated that the separation of lignin from cellulose would enhance the lignin and cellulose utilization for high value products.

EXPERIMENTAL

Raw Materials

OPEFB fiber was collected from a local palm oil mill (United Oil Palm Industries Sdn Bhd, Malaysia), sun dried, and ground to a particle size of < 1 mm. The OPEFB fiber was then oven dried at 105 °C overnight, and standard methods were used to determine its composition.

Chemicals and Enzymes

Solvents and chemicals were obtained from Sigma-Aldrich (Sunway, Malaysia) and R & M Chemicals (Subang, Malaysia). All enzymes were obtained from Novozymes Malaysia Sdn Bhd (Kuala Lumpur). Novozyme 51032 ‘cutinase’ and laccase from *Trametes versicolor* (Kuala Lumpur, Malaysia) were used.

Composition of OPEFB

The measurement of the OPEFB composition was conducted using TAPPI T222 om-11 (2011). As a comparison, the National Renewable Energy Laboratory (NREL) method was also carried out (Sluiter *et al.* 2008).

OPEFB Lignin Extraction

In the first stage, autohydrolysis pretreatment was conducted to remove hemicellulose from OPEFB fibers. This method was developed in this study. OPEFB fibers (30 g) was loaded into a stainless-steel reactor (4L, 98 kPa, 120 °C) and supplemented with an appropriate amount of deionized water. The autohydrolysis was carried out for 2 h. Autohydrolyzed OPEFB fibers (10 g) were milled and mixed with 80% aqueous ethanol ($EtOH/H_20$: 8/2 v/v) and 0.2% w/w sulphuric acid as a catalyst. The mixture was heated at 120 °C for 1 h, filtered, and washed with methanol (Hage *et al.* 2010). This was followed by treatment with hydrogen peroxide (H_2O_2) 2% for 4 h at 5 °C to obtain OPEFB cellulose. The black liquor of the lignin, 'the filtrate', was collected, from which the powder of the OPEFB lignin was recovered by three different methods. In the first method, methanol was used to wash the OPEFB lignin recovered from the solvent, and this was followed by drying. In the second method, H_2SO_4 was introduced into the black liquor, and the precipitated OPEFB lignin was later recovered by filtration. The last method was carried out by adding deionized water three times the volume to reduce the concentration of the ethanol, leading to OPEFB lignin precipitation. This was followed by centrifugation, filtration, and drying.

Degradation of OPEFB Lignin

Enzymatic hydrolysis was carried out in a 250 mL shaking flask at 40 °C and 150 rpm in an incubator shaker. In a typical laccase enzymatic reaction, 1 g of OPEFB lignin powder was added to 10 mL of media (water, phenol, and acetate buffer) and incubated for 2 h (40 °C; 150 rpm). After this pre-incubation step, hydrolysis was initiated by adding 5% laccase of total OPEFB lignin (activity 0.5 u/mg). The same procedure was followed when using the cutinase enzyme (Novozyme 51032) as a comparison. The type and amount of lignin powder, mediators, and enzymes were varied. After 24 h, the sample and reaction medium were withdrawn, incubated at 90 °C for 20 min to deactivate the enzyme and centrifuged (Yeh *et al.* 2010). The sample was then diluted in ultra-pure water and filtered (0.2 μm) prior to analysis using high performance liquid chromatography (HPLC). For this analysis, a Waters HPLC (Herts, UK) was used with a MERCK-RP-Cromolith-Rp-18e column and UV-Vis spectrometer with 280 nm detector.

Material Characterization

Fourier Transform Infrared spectra of raw OPEFB, treated OPEFB, and OPEFB lignin were recorded using a Perkin Elmer, Fourier Transform Infrared (FTIR) model GX (London, UK). Thermogravimetric analysis (TGA) was carried out using a Mettler Toledo model TGA/SDTA 851^e (Columbus, OH, USA). Samples of approximately 6 mg were placed in an alumina pan and heated from 30 to 800 °C at 10 °C/min, under a dynamic flow of nitrogen (50 mL/min) to study the thermal stability of the samples. The morphology of each sample was examined by using Zeiss, Field Emission Scanning Electron Microscope (FESEM), model Supra 46VP (Oberkochen, Germany). The SEM images were recorded using an accelerating voltage of 3-5 kV. HPLC was used to determine the quantity of lignin degradation products obtained.

RESULTS AND DISCUSSION

Composition of OPEFB Fibers

The composition of raw OPEFB fibers is shown in Tables 1 and 2. The total lignin content obtained by these two methods were roughly 14 to 19%. Tables 1 and 2 also show the yield of cellulose and hemicellulose. The total amount of lignin from the OPEFB analyzed by using the TAPPI method and the NREL method were 18.4 ± 1.8% and 13.9 ± 1.2%, respectively (Table 1). The results were different due to the different treatments of the samples and different methods of analysis. The data obtained from the TAPPI method was based on the weight calculation, whereas the NREL data were obtained from HPLC analysis. Previously, the total lignin content of OPEFB was reported as 20.4% (Khalid *et al.* 2008) and 11.7% (Rahman *et al.* 2006). The different values of the OPEFB composition depends on the treatment of the samples, the method of cultivation, and the source of the plant (Wicke *et al.* 2008).

Table 1. Composition of OPEFB Based on Sluiter *et al.* (2008)

Composition	Raw OPEFB (%)
Total Solids	89.0 ± 1.3
Ash	4.1 ± 0.5
Acid Insoluble Lignin	0.4 ± 0.1
Acid Soluble Lignin	13.9 ± 1.2
Glucose	31.6 ± 2.1
Xylose	49.8 ± 1.5

Table 2. Composition of OPEFB Based on TAPPI T222 om-11 (2011)

Composition	Raw OPEFB (%)
Total Solids	89.2 ± 1.5
Ash	3.2 ± 0.3
Lignin	18.4 ± 1.8
Holocellulose	82.8 ± 2.8
Alfa-Cellulose	32.3 ± 2.2
Hemicellulose	50.3 ± 0.8

OPEFB Lignin Extraction

OPEFB lignin obtained in this study was extracted using the organosolv method where ethanol and water were used as solvents. Prior to extraction, the autohydrolysis pre-treatment was carried out to break up the OPEFB fibers into smaller pieces as well as to start the hemicellulose removal process. OPEFB fibers (30 g) was loaded into 4 L stainless steel reactor (98 kPa, 120 °C) and the mixture was supplemented with appropriate amount of deionized water. The autohydrolysis was carried out at 120 °C for 1 to 2 h. The organosolv treatment separated the cellulose from lignin. OPEFB cellulose was recovered as a solid product, whereas the OPEFB lignin was in a solution. The yield of the OPEFB lignin and cellulose produced was 15.1 ± 1.30% (isolated by deionized water) and 63 3± 1.8%, respectively. This amount was high enough compared to using conventional cellulose extraction, which would be around 50 to 60% (Nazir *et al.* 2013). The lignin and cellulose purity were determined using TGA.

Thermal Analysis of OPEFB Cellulose and Lignin

Figure 1 presents the TGA thermograms and the corresponding DTG curves of the raw OPEFB, pre-treated OPEFB, and OPEFB cellulose. A clear 'shoulder' at around 250 to 300 °C is usually assigned to the thermal decomposition of hemicellulose (Zhao *et al.* 2009). The high temperature "tails" around 400 to 600 °C were ascribed to the degradation of lignin. For the raw OPEFB (Fig. 1c TGA and DTG), hemicelluloses shoulder peaks were not obvious because they were overlapped with the main peaks of cellulose. In the thermogram for pre-treated OPEFB (Fig. 1 b TGA and DTG), the first peak appeared at around 300 °C (shoulder) resembling the thermal decomposition of hemicellulose. There were no tails that appeared within the range of 400 to 600 °C, indicating that the delignification process was successful. In the case of cellulose obtained from H_2O_2 treatment (OPEFB cellulose), there were no broad shoulders and tails present in the thermogram range 400 to 600 °C, indicating that the lignin and hemicellulose were successfully removed (Fig. 1a TGA and DTG). The thermal decomposition at around 310 °C showed that the OPEFB cellulose was successfully obtained (Fig. 1a TGA and DTG).

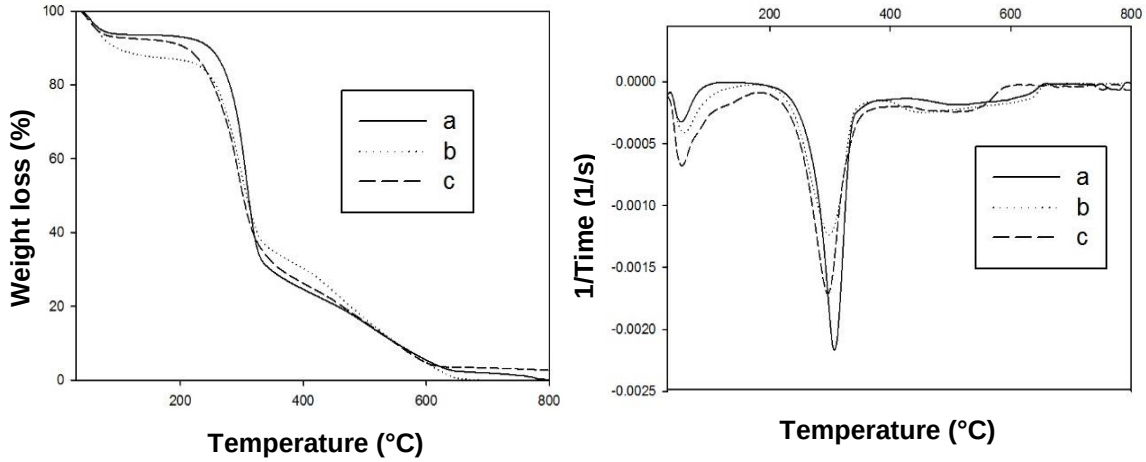

Fig. 1. TGA thermograms (left) and DTG curves (right) of (a) OPEFB cellulose, (b) pre-treated OPEFB (autohydrolysis and organosolv), and (c) raw OPEFB

Figure 2 shows the TGA thermograms and the corresponding DTG curves of the commercial and OPEFB lignin produced in this study. There were broad shoulders in both thermograms, indicating that the degradation of the lignin occurred over a rather broad temperature range of 400 to 600 °C. In Fig. 2, there are peaks appear between 300 to 600 °C, which are usually ascribed to the thermal decomposition of the lignin (Zhao *et al.* 2009). Thus, the TGA and DTG thermograms show that lignin was successfully extracted by the organosolv method.

FTIR Spectroscopy

Figure 3 shows the FTIR spectra of cellulose extracted from the OPEFB (OPEFB Cellulose), raw OPEFB, and pre-treated OPEFB. The peaks ascribed to the lignin appeared at 1501 and 1512 cm^{-1} and are due to C=C stretching and the C=C aromatic skeletal vibration of lignin, respectively (Pavia *et al.* 2009). As expected, two peaks of the lignin were not observed in the FTIR spectra of the OPEFB cellulose (Fig. 3c).

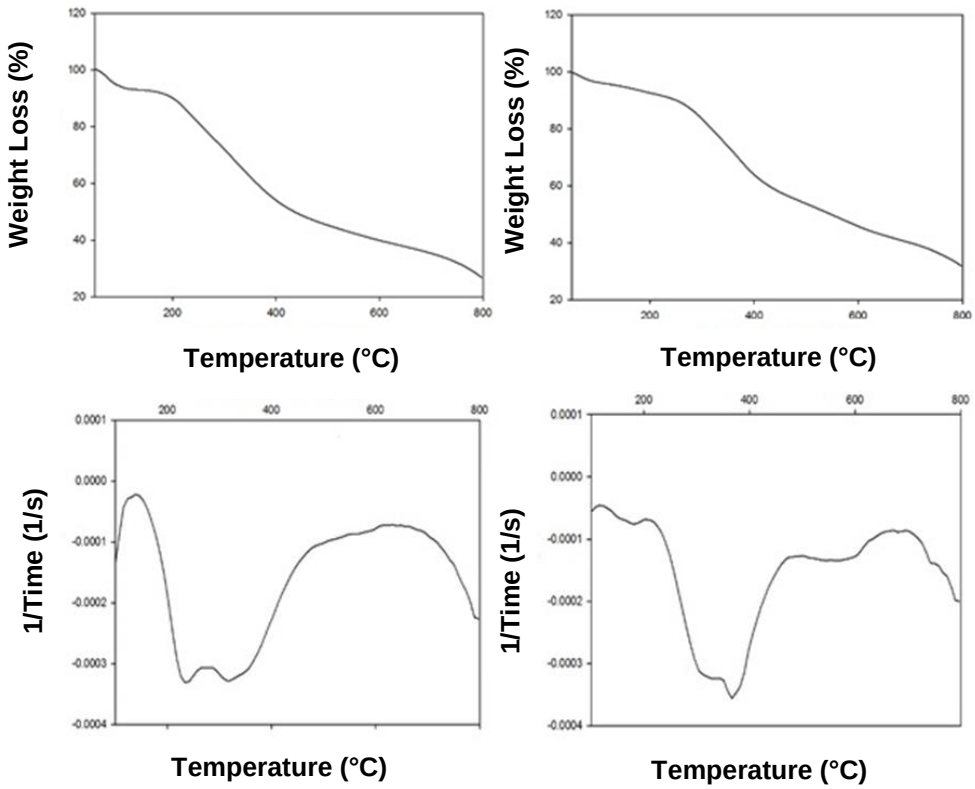

Fig. 2. TGA thermograms (top) and corresponding DTG curves (bottom) for OPEFB lignin (left) and Commercial lignin (right)

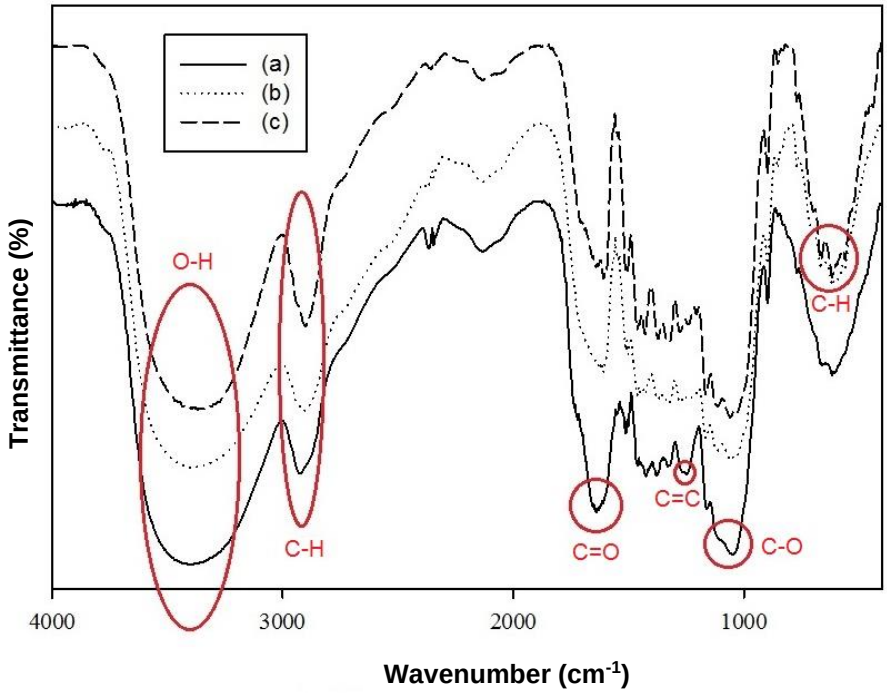

Fig. 3. FTIR spectra for (a) Raw OPEFB (b) pre-treated OPEFB (organosolv and autohydrolysis) (c) OPEFB Cellulose

Meanwhile, the peak attributed to hemicellulose was observed in the spectra of the raw OPEFB and pre-treated OPEFB (Fig. 3b) at 1732 to 1735 cm^{-1} due to C=O stretching. However, the corresponding peaks disappeared from the spectrum of the OPEFB cellulose (Fig. 3c). This observation indicated that the organosolv followed by hydrogen peroxide treatments were able to remove lignin as well as hemicellulose from the OPEFB fibers. The peak observed at 898 cm^{-1} was attributed to the presence of the β-glucoside linkage between glucose units in the cellulose (Sampedro *et al.* 2012). All FTIR spectra in this study showed a β-glucoside linkage peak including the FTIR spectrum for the obtained OPEFB cellulose (Fig 3c). Furthermore, Fig. 4 shows the FTIR spectra of the OPEFB lignin powder that was successfully separated from cellulose and hemicellulose.

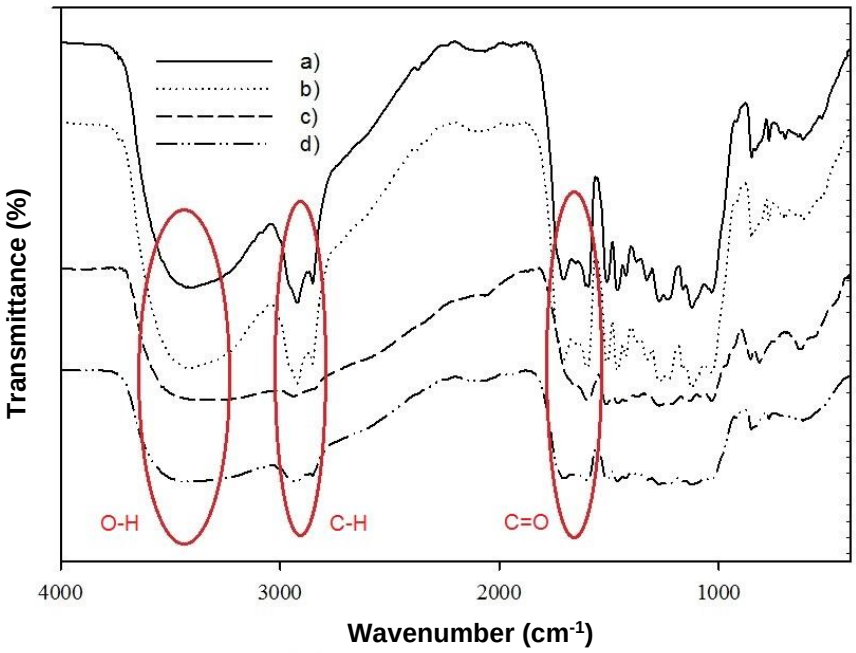

Fig. 4. FTIR spectra of lignin for (a) Lignin isolated by deionized water (b) Lignin isolated by acidified water (c) Commercial lignin (d) Lignin isolated by methanol

Figure 4 shows the FTIR spectra for all isolated lignin samples. All spectra showed broad peaks around 3400 cm^{-1}, which were due to the O-H bond from the phenolic compound of lignin. The peak that appeared at 2943 cm^{-1} was due to C-H stretching of methyl groups, whereas the peak at 1460 cm^{-1} was due to the stretching of methylene C-H. The peak that attributed to the aromatic rings of the lignin observed at 1596 cm^{-1} and 896 cm^{-1} were due to C-H deformation and ring vibration.

Morphology of OPEFB Fibers and Lignin

The SEM micrographs of the raw OPEFB fibers, pretreated OPEFB, and obtained cellulose (OPEFB cellulose) are shown in Fig. 5. Raw OPEFB fibers had a rigid appearance (Fig. 5a). OPEFB fibers exhibited stiff and hard surfaces. Autohydrolysis of OPEFB fibers reduced the stiff appearance, and some parts of the fibers were split and became more refined. The images also show that some fibers were broken. Figure 5c shows the SEM image of the obtained cellulose (OPEFB cellulose). The appearance is quite different, and diameter of the fibers was reduced. This is probably due to the decrease in the spiral angle around the fiber axis and the increase in the molecular orientation after the pre-treatment.

A fair amount of randomness is introduced to the orientation of the crystallites due to the removal of the non-cellulosic matter (Yeh *et al.* 2010), leading to the formation of fibers with smaller diameter and length.

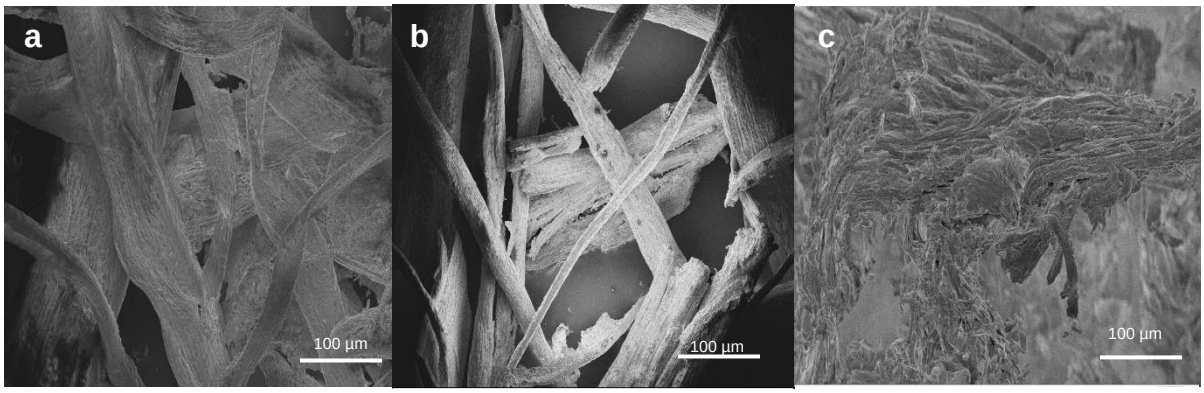

Fig. 5. SEM images of (a) raw OPEFB fibers, (b) pre-treated OPEFB (organosolv and autohydrolysis), and (c) OPEFB cellulose (Mag = 100 X)

Figure 6 shows the morphology of the OPEFB lignin recovered using several isolation methods. Figures 6a and b show the commercial lignin and OPEFB lignin isolated by deionized water, respectively.

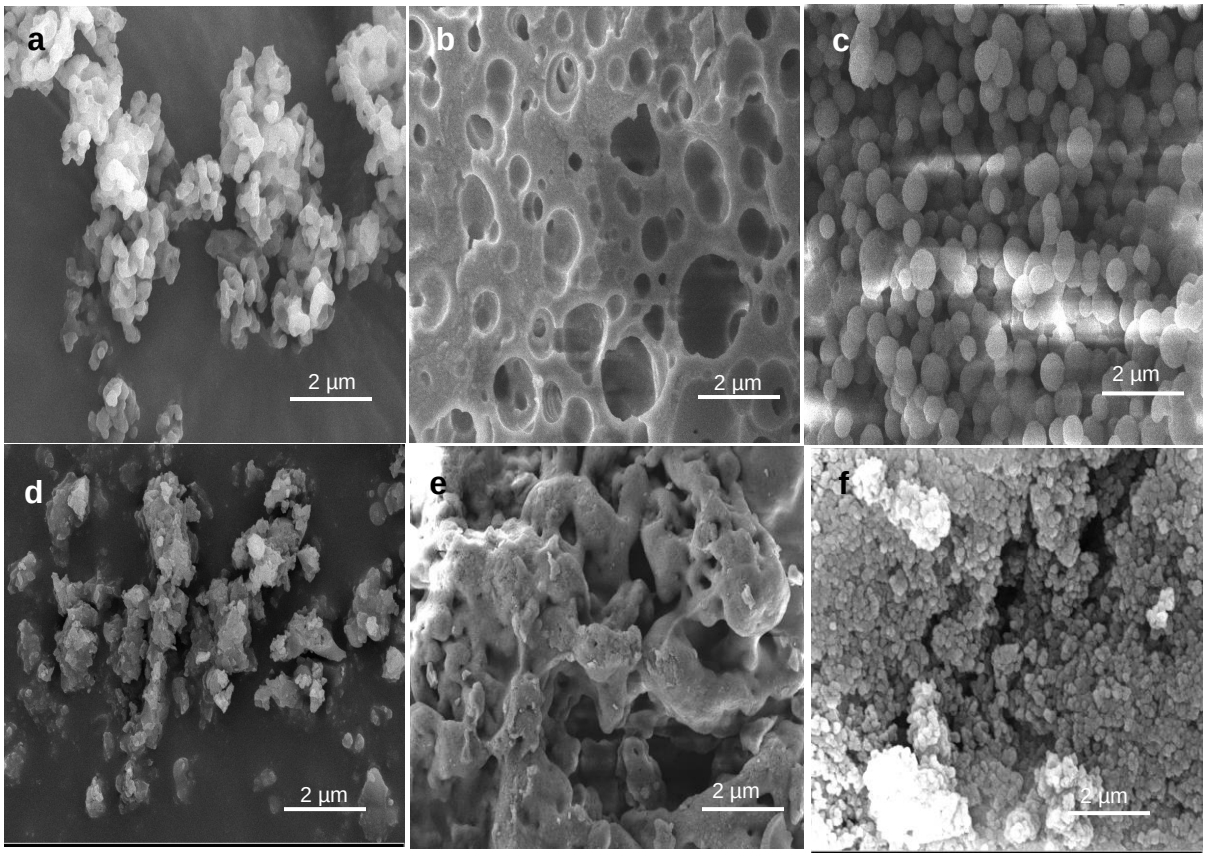

Fig. 6. SEM images of (a) Commercial Lignin, (b) OPEFB fibers after autohydrolysis treatment, (c) OPEFB fibers after autohydrolysis and organosolv treatments, (d) OPEFB Lignin isolated by deionized water, (e) Lignin isolated by acidified water and (f) Lignin isolated by methanol (Mag = 5000 X)

A round shape with stiff surfaces was observed in commercial lignin. Lignin isolated using methanol (Fig. 6c) shows small particles of round shapes while some parts remain intact, whereas OPEFB lignin showed a perfect round shape with small particle size (Fig. 6e). Figures 6d and 6e show a rather extensive porous structure, indicating that the lignin sample was partially degraded.

Effect of Reaction Medium on Enzymatic Degradation of OPEFB Lignin

There were nine compounds recovered from OPEFB lignin degradation by enzymes (Fig. 7). Different reaction media and treatments somewhat affected the yield of the products. The nine compounds were hydroxybenzoic acid, hydroxybenzaldehyde, vanillic acid, vanillin, syringic acid, syringaldehyde, coumaric acid, ferulic acid, and guaiacyl alcohol. The yield of degradation products from the different media and treatments on the enzymatic hydrolysis of lignin is shown in Table 3. Syringyl, guaiacyl and p-hydroxyphenyl units were oxidized into corresponding syringic acid, syring-aldehyde, vanillic acid, vanillin, p-hydroxybenzoic acid and p-hydroxybenzaldehyde.

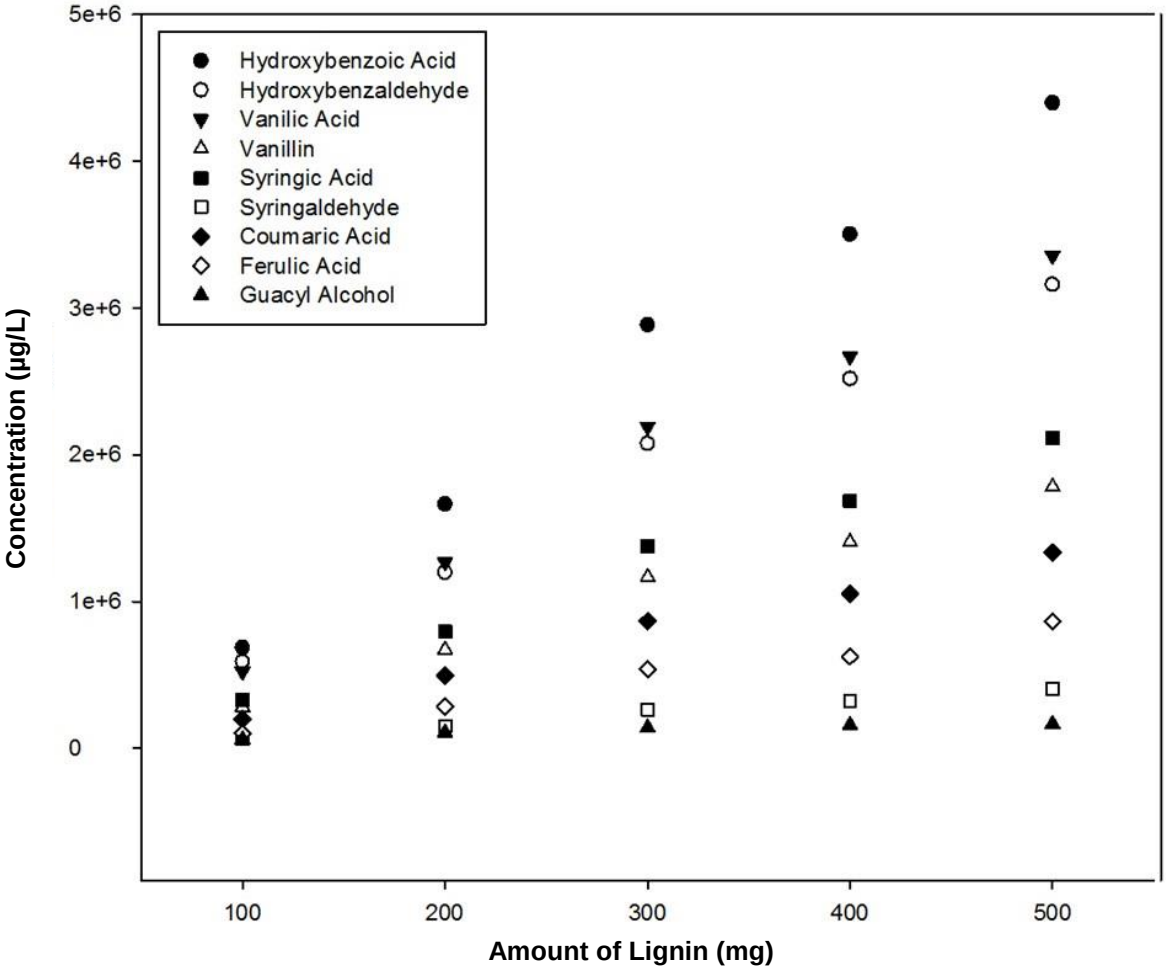

Fig. 7. Degradation products of OPEFB lignin

The reaction with laccase in phenol produced the highest amount of vanillic acid, 126006.1 ± 97.5 mg/L. Hydroxybenzoic acid and syringaldehyde were obtained in high yield when laccase was used in water with concentrations of 1029.8 ± 50.2 mg/L and

4061.1 ± 89.9 mg/L, respectively. When cutinase was used, all compounds were obtained; however, the yield was rather low, particularly the yield of hydroxybenzoic acid. Syringaldehyde and syringic acid were produced the most in this case, with concentration of 4837.6 ± 156.4 mg/L and 4837.6 ± 156.4, respectively.

Lignin degradation via enzymatic process in this study showed higher yields than alkaline nitrobenzene oxidation that is commonly used (Hussin *et al* 2013). The cited authors reported below 1 % yield of each products produced.

Table 3. Effect of Reaction Medium on Enzymatic Degradation of Lignin

Name of Samples	Concentration of Degradation Products (mg/L)			
	(Laccase; Water)	(Laccase; Phenol)	(Laccase; Buffer)	(Cutinase; Water)
Hydroxybenzoic Acid	1029.8 ± 50.2	-	38.0 ± 10.2	140.0 ± 25.0
Hydroxybenzaldehyde	54.6 ± 23.0	34.7 ± 20.1	31.5 ± 8.8	115.3 ± 16.7
Vanillic Acid	56.1 ± 15.2	126006.1 ± 97.5	31.4 ± 5.9	288.5 ± 29.5
Vanillin	673.9 ± 83.0	-	-	138.9 ± 24.1
Syringic Acid	-	377.7 ± 30.3	120.9 ± 20.4	2387.7 ± 105.3
Syringaldehyde	4061.1 ± 89.9	-	-	4837.6 ± 156.4
Coumaric Acid	224.5 ± 24.5	-	-	713.0 ± 36.0
Ferulic Acid	549.0 ± 38.0	-	120.1 ± 18.6	490.5 ± 46.5
Guaiacyl Alcohol	-	-	-	93.4 ± 17.6

Lignin is a peculiar biopolymer. Unlike other natural polymers such as cellulose, proteins, and nucleic acids, lignin does not have readily hydrolysable bonds along the backbone. Instead, lignin is a three-dimensional, amorphous polymer with a seemingly random distribution of stable carbon-carbon and ether linkages between monomeric units. The structure is not amenable to normal modes of biological hydrolysis (Crawford 1981).

CONCLUSIONS

1. Two enzymes and three reaction media were used to produce lignin degradation products. The reaction with laccase in water produced high yields of syringaldehyde and hydroxybenzoic acid.
2. The reaction with cutinase enzyme in water produced a lower yield for hydroxybenzoic acid and vanillin, however a higher yield for syringic acid and syringaldehyde was obtained.
3. These results can be used to utilize and optimize the lignin degradation products for further applications.

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