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Effect of pH and Laccase Enzyme Concentration on the Properties of Syringaldehyde

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ABSTRACT

Syringaldehyde is recognized as a main component of monolignol in oil palm empty fruit bunch. The structure of syringaldehyde comprised of two methoxy groups, which have the potential to undergo demethoxylation process. Laccase enzyme from Trametes versicolor species have been reported to be able to demethoxylate at certain pH. Hence, the objective of this study is to characterize the effect of laccase enzyme concentration and pH of acetate buffer on syringaldehyde compound. Nine syringaldehyde preparation based on different pH and percentage of laccase enzyme treatment were studied. Phenolic acid and aldehyde compound were determined using HPLC and characterizations of the treated syringaldehyde were done using NMR. Based on HPLC results, no targeted compound of 4-hydroxybenzoic, vanillic, syringic, p-coumaric, trans-ferulic, 4-hydroxybenaldehyde, vanillin and guaiacyl were detected. However there was an uknown compound was detected for some of the sample. Concentration of syringaldehyde was found to be decreasing with increase of enzyme percentage starting from pH 4 and 5. A change of the carbonyl group was detected based on the presence of peaks at about 1.89 ppm using ^{1}H NMR and 173.33 ppm using ^{13}C NMR. The absence of methoxy groups in combination of pH 5 and 5% laccase enzyme sample were detected on ^{13}C NMR spectrum prove that this treatment appeared to give maximum changes in functional group compared to the other samples.

Keywords: Syringaldehyde, Laccase, Lignin, Demethoxylation, Hydrolysis

INTRODUCTION

The oil palm industry in Malaysia has been expanding from 400 hectares planted in 1920 and upto 5,000,000 hectares in 2011. The development of this crop produced large quantities of by products such as palm oil mill effluent, empty fruit bunches, mesocarp fibre and shell. The thermal treatment of Empty Fruit Bunches (EFB) for example, has been practiced traditionally to produce fertilizer in plantations. Due to pollution issues, this method has been discouraged. Instead EFB is being used as a mulching material in oil palm estate [1]. The waste of oil palm processing left EFB fibers which is categorized as lignocellulosic materials. Lignocellulosic materials are comprises of cellulose, hemicellulose and lignin that has the potential to be converted into beneficial chemicals [2].

Lignin is made of a complex structure and hydrophobic biopolymer consists of phenylpropanoid units. Typically, lignin constitutes 20-30% by weight of the lignocellulosic biomass [3]. It is also mainly synthesized from three monomeric precursors that is *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. The monomeric units will become p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units when incorporated into lignin polymers [4]. Lignin is an abundant natural resource and has started to gain more attention as renewable energy. However, the complex and highly methylated structure of lignin require the demethylation and demethoxylation process to increase lignin reactivity [5]. The production of waste from EFB lignocellulosic material can cause pollution to the environment if the material is not fully used.

One of the lignin derivative is syringaldehyde (Sy, 4-hydroxy-3,5-dimethoxybenzaldehyde). Studies reported that fractional isolation process of lignin from EFB gave syringaldehyde as a major component [6]. Syringaldehyde is found abundant in plant as a glycoside derivative. Oxidation of alkaline lignin from hard wood mostly produced syringaldehyde compared to vanillin. Syringaldehyde become an important compound that found in various plants after isolation process [7]. It can be synthesized from lignin depolymerization of hard wood [8]. Currently, syringaldehyde is mainly used directly as chemical and medication [9,10].

Depolymerization process of lignin is a complex process and involved different groups of enzyme such as peroxidase, oxidoreducatase and oxidase [11]. One of the enzyme is laccase (benzenadiol: oxygen oxidoreductase EC 1.10.2.2; AA) which can be found in fungi, plant and bacteria [12]. In earlier studies, author reported that the process of oxidation, demethoxylation and carbonyl group formation is occurred in milled wood lignin treated with laccase [13]. When lignin is oxidized by the removal of a single electron from a phenylpropanoid subunit, lignin structure will be changed. This is due to activation of the lignin surface by creating an active radical [14]. Activation of the lignin structure may cause bond cleavage, coupling and modification. Modification of functional groups includes acetylation and demethylation [15]. Thus, the aim of this study was to investigate the influence of different pH and laccase enzyme percentage on syringaldehyde properties.

The treated syringaldehyde were characterized using High Performance Liquid Chromatography (HPLC) and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) to identify the changes occurring in the syringaldehyde structure.

MATERIALS AND METHODS

Chemicals

Syringaldehyde and laccase from *Trametes versicolor* were purchased from Sigma-Aldrich (St. Louis. MO, USA). HPLC-grade methanol and acetic acid were obtained from JTBaker (Philipsburg, NJ, USA). All other reagents were obtained from commercial suppliers in analytical grade quality. Acetate buffer solutions were obtained by mixing acetic acid and sodium acetate trihydrate. The pH desired buffer solutions were adjusted to pH 3, 4 and 5.

Enzymatic reaction of laccase on syringaldehyde compound

The lignin monomer (syringaldehyde) underwent enzymatic hydrolysis using incubator shaker. About 1 g of syringaldehyde was dissolved in 10 mL acetate buffer (pH 3, 4 and 5) to observe the effect of pH on syringaldehyde. After that, the substrate solution was incubated for 2 h (40°C, 150 rpm) to increase the laccase enzyme activity [16]. Hydrolysis was initiated by adding different percentage of laccase that is 0%, 1% and 5% (w/v). Blank sample was prepared using syringaldehyde without buffer solution and enzyme, and replaced with deionized water as mediator. The experiment was carried out for 24 h at 40°C (150 rpm) to allow reaction occur between substrate and enzyme. Sample were withdrawn and boiled inside the water bath at 90°C for 20 min to deactivate enzyme [17]. Then, all the samples were centrifuged at 4000 rpm for 10 min and supernatant liquid was taken for further analysis [18].

Determination of phenolic and aldehyde compounds

Phenolic acid and aldehyde (4-hydroxybenzoic, vanillic, 4-hydroxybenzaldehyde, syringic, vanillin, syringaldehyde, *p*-coumaric, ferulic, guaiacyl) determination was performed using a HPLC system (Shimadzu) equipped with solvent delivery unit (LC-20AD), column oven (SPD-20AV), UV-VIS detector (SPD-20AV), autosampler (SIL-20AC) and LC solution Workstation. Separation was carried out using XBridge C18 column (4.6 × 250 mm; 5 µm, Waters, Ireland). The column temperature was maintained at 25°C. The pump was connected to a mobile phase system comprised of two solvents: 0.1% acetic acid (A) and MeOH (B). The flow rate was kept at 1.0 mL/min and the injection volume was 10 µL for each of the sample solutions. The detection was monitored at 280 nm. Peak identification was obtained by comparing the retention time and UV spectra of phenolic chromatograms with those pure standards. The gradient program was as follows: 0.01 min 100% A/0% B, 1.00 min 85% A/15% B, 4.00 min 80% A/20% B, 12.00 min 60% A/40% B, 14.00 min 40% A/60% B, 15.00 min 0% A/100%B, 15.20 min 40% A/60% B, 16.00 min 80% A/20% B, 18.00 min 60% A/40% B, 20.00 min 40% A/60% B, 24.00 min 20% A/80% B, 28.00 min 20% A/80% B, 31.00 min 10% A/90% B, 35 min 0% A/ 100% B, 36 min 100% A/0% B.

Characterization of treated syringaldehyde

The changes of functional group of syringaldehyde and treated syringaldehyde were studied using ^{1}H dan ^{13}C-NMR (JEOL / JNM-ECP 400). All sample were dissolved in DMSO-*d6*. Spectrum analyses were done using Bruker software.

Statistical analysis

ANOVA and DUNCAN's tests were carried out using the software Statistical Analysis System (SAS) Version 9.13. $p<0.05$ was selected as the level of statistically significant.

RESULTS AND DISCUSSION

Effect of pH and laccase enzyme on syringaldehyde

Calibration curves for 4-hydroxybenzoic, vanillic, syringic, p-coumaric, trans-ferulic, 4-hyroxybenzaldehyde, vanillin, syringaldehyde and guaiacyl were conducted. The result of all analytes showed an acceptable linearity and yielded correlation coefficients of 0.99 or better within the range of concentration investigated (Figure 1). Analytical parameters of standard compounds were used as method validation in the HPLC experiments were shown in Table 1. The retention time (min), linear equation between concentration and peak area, regression coefficient (R^2) and maximum absorption of wave length (nm) were studied. Quantitative analysis of syringaldehyde concentration form in treated syringaldehyde was shown in Table 2. The presence of syringaldehyde compound was consistent in all treatments whereas no targeted compound of 4-hydroxybenzoic, vanillic, syringic, p-coumaric, trans-ferulic, 4-hyroxybenzaldehyde, vanillin and guaiacyl was detected after the reactions. However, all treatment gave different peaks except for syringaldehyde. In addition, certain treatment showed the existence of an unknown compound in the chromatogram. When comparison was made between blank and treatment samples, it can be said that pH and laccase enzyme concentration did affect syringaldehyde concentration. In a qualitative aspect, samples that had a combined treatment of pH 4/5%, pH 5/1% and pH 5/5% shows the presence of an unknown compound. Figure 2 was an example of chromatogram with syringaldehyde compound only (pH 3/5%) whereas Figure 3 was an example of chromatogram of syringaldehyde compound detected with unknown compound (pH 5/5%).

Concentrations of syringaldehyde after modification were in the range of 8.50-102.9 mg/ml (Figure 4). The sample that had a combined treatment of pH 3 and 5% laccase enzyme gave the highest concentration of syringaldehyde ($p<0.05$) compared to other treatments. The lowest concentration of syringaldehyde ($p<0.05$) was the treatment that had a combination of pH 5 and 5% laccase enzyme. When laccase enzyme percentage was increase to 1% and 5%, concentration of syringaldehyde compound decreased significantly ($p<0.05$) in pH 4 and pH 5 samples. However, there was no significant difference ($p>0.05$) between blank sample and treatment of pH 4/1%. There was also no significant difference ($p>0.05$) between treatment of pH 4/5% and pH 5/1%. Different trend was observed for pH 3 samples where syringaldehyde concentration increased significantly ($p<0.05$) when laccase enzyme was added at 1% and 5% which exceeded syringaldehyde concentration in the blank sample. However, there were no significant differences ($p>0.05$) between pH 3/0% and pH 3/1% treatment.

Different concentration of syringaldehyde might occur due to important properties of laccase enzymology with phenolic substrate is dependent on pH [19]. Most of the enzyme activity followed a bell shaped curved [20]. Study reported that the pH of buffer used to study laccase activity was around 4.5 due to its proximity with the isoelectric point [21]. This may explained why different pH and laccase enzyme concentration that resulted in different concentrations of syringaldehyde during hydrolysis process.

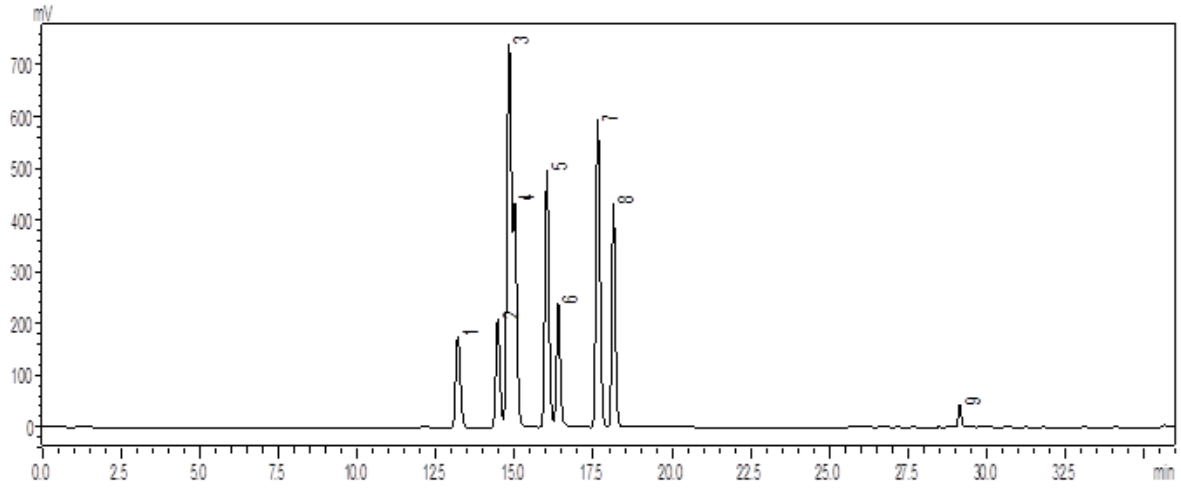


Figure 1: HPLC chromatogram of phenolic acid and aldehyde standards. The peak numbers correspond to: (1) 4-hydroxybenzoic, (2) vanillic, (3) 4-hydroxybenzaldehyde, (4) syringic, (5) vanillin, (6) syringaldehyde, (7) *p*-coumaric, (8) trans-ferulic, (9) guaiacyl

Table 1: Validation method of HPLC

Standards	Linear equation	Correlation coefficient (R^2)	Retention time (min)	UV (nm)
4-Hydroxybenzoic	$y=1.6512 \times 10^4x+11863$	0.9995	13.193	365
Vanillic	$y=1.8443 \times 10^4x+14533$	0.9999	14.461	365
4-Hydroxybenzaldehyde	$y=7.7504 \times 10^4x+71322$	0.9999	14.823	365
Syringic	$y=3.5264 \times 10^4x+29698$	0.9996	14.997	365
Vanillin	$y=4.3630 \times 10^4x+40080$	1	16.019	365
Syringaldehyde	$y=2.0282 \times 10^4x+46307$	0.9991	16.384	365
p-Coumaric	$y=5.0341 \times 10^4x+53898$	0.9999	17.636	365
trans-Ferulic	$y=3.3703 \times 10^4x+3923.6$	0.9992	18.134	365
Guaiacyl	$y=3.8172 \times 10^3x+1256.3$	0.9946	29.13	365

Table 2: Concentration of syringaldehyde compound (mg/ml) after being treated with different pH and laccase enzyme percentage

Sample	Syringaldehyde concentration (mg/ml)
Blank	50.49 ± 8.84
pH 3/0%	84.04 ± 1.51
pH 3/1%	84.51 ± 4.36
pH 3/5%	102.96 ± 5.67
pH 4/0%	79.39 ± 4.56
pH 4/1%	42.74 ± 1.57
pH 4/5%	26.80 ± 0.32
pH 5/0%	73.11 ± 1.42
pH 5/1%	24.01 ± 4.75
pH 5/5%	8.50 ± 0.39

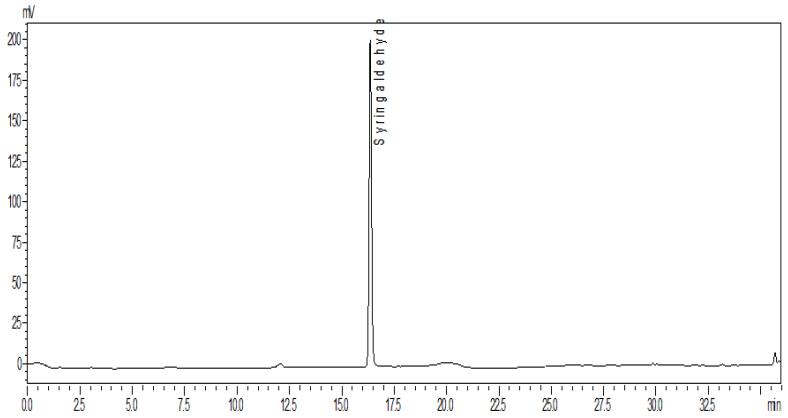


Figure 2: HPLC chromatogram of pH 3 / 5% laccase enzyme

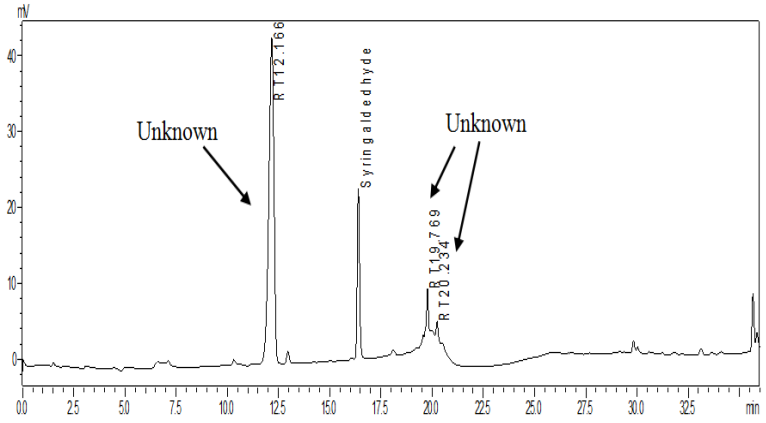
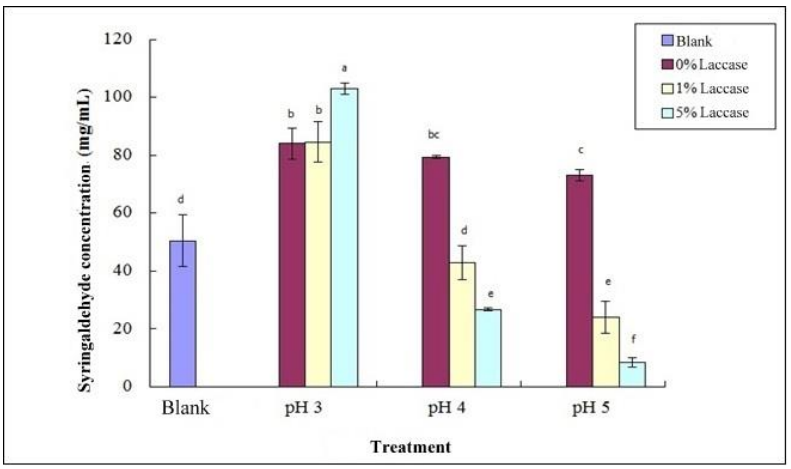


Figure 3: HPLC chromatogram of pH 5/5% laccase enzyme



[a-f]Mean with different alphabet have significant difference ($p<0.05$)

Figure 4: Concentration of syringaldehyde with different pH and laccase enzyme treatment

SPECTRAL DATA

Syringaldehyde

^{1}H-NMR (DMSO-*d6*) δ 9.77 (1H, H-7), 7.20 (2H, H-2, H-6), 3.84 (6H, 2 × OCH_3); ^{13}C-NMR (DMSO-*d6*) δ 127.57 (C-1), 107.43 (C-2, 6), 148.52 (C-3, C-5), 142.47 (C-4), 191.66 (C-7), 56.46 (OCH_3). The data were identical to previous study [22].

Treatment of pH 3, 0%; pH 3, 1%; pH 3, 5%; pH 4, 0%; pH 5, 0% and pH 5, 1%

^{1}H-NMR (DMSO-*d6*) δ 9.72 (1H, H-7), 7.18 (2H, H-2, H-6), 3.81 (6H, 2 × OCH_3). Additional signal found: δ 4.06 and 1.89. ^{13}C-NMR (DMSO-*d6*) δ 127.54 (C-1), 107.40 (C-2, 6), 148.45 (C-3, C-5), 142.37 (C-4), 192.23 (C-7), 56.45 (OCH_3). Additional signal found: δ 173.33 and 21.47.

Treatment of pH 4, 1%; pH 4, 5% dan pH 5, 5%

^{1}H-NMR (DMSO-*d6*) δ 9.70 (1H, H-7), 7.21 (2H, H-2, H-6), 3.69 (6H, 2 × OCH_3). Additional signal found: δ 8.24, 5.90, 4.25, 3.15 and 1.89. Absence of signal around 3.69 ppm involving methoxy group in pH 5, 5% sample. ^{13}C-NMR (DMSO-*d6*) δ 127.51 (C-1), δ 107.39 (C-2, 6), 148.37 (C-3, C-5), 142.22 (C-4), 192.73 (C-7), 56.41 (OCH_3). Additional signal found: δ 174.14, 166.59, 157.73 and 21.46.

CONCLUSION

The results showed that the combination treatment of pH 5/5% gave the highest effect on syringaldehyde. Studies on treated syringaldehyde characteristics exhibit different concentration of syringaldehyde and unknown compound formed in HPLC analysis. Results of ^{1}H and ^{13}C NMR shows the absence of methoxy group in pH 5/5% samples suggested that this treatment have a potential to give maximum changes compared to the other treatment.

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