The identification of ethanol and aromatic compounds from delignification of cacao pod husk using *Phlebia sp. MG-60*

I Nurika, Z A N M Majid and Suprayogi

Department of Agroindustrial Technology, Faculty of Agricultural Technology, Universitas Brawijaya, Indonesia
E-mail: irnia@ub.ac.id

Abstract. The agricultural residue can be significantly transformed to both renewable energy and high value-added chemicals using biorefinery concept. Biological pre-treatment of lignocellulosic biomass and ethanol production from cacao pod husk can be done within a single unit-operation using a single microorganism white rot *Phlebia sp. MG-60*. The objective of this study was to identify the potential of white rot *Phlebia sp. MG-60* to produce ethanol and aromatic compounds under consolidated biological processing (CBP). The effect of different concentration of MnSO₄ on detecting of cellulose, hemicellulose, lignin and MnP activity were measured on 0, 14 and 28 days. The selected best concentration of MnSO₄ was then used for detecting of potential aromatic compound released and the yield of ethanol. By adding the inducer (MnSO₄), the results revealed that the addition of MnSO₄ could escalate the activity of manganese peroxidase (MnP) from *Phlebia sp. MG-60* in order to depolymerize lignocellulose. The yield of ethanol produced during the bioconversion process was 2g/L. Furthermore, several aromatic compounds such as 2,3-Dimethylphenol, trans-cinnamic acid, caffeic acid, and vanillin were potentially obtained from cacao pod husk incubated by the fungus for 28 days.

1. Introduction
Cacao pod is recognized as agricultural waste, which potentially used as feedstock for the production of bioenergy or biorefinery products. In 2014, the production of cacao in Indonesia was 703.3 M ton [1] which also potentially produced 75% cacao pod as residue [2]. The cacao pod contains 32.3% cellulose and hemicellulose and 21.4% lignin [3]. Therefore, it can be used as a feedstock for bioenergy source such as ethanol [4]. However, since lignin is a complex aromatic compound, which is not easy to be degraded, the bioconversion of lignocellulose needs pre-treatment to decay the structure of lignin. The delignification process can be conducted using physical, chemical and biological pre-treatments methods. However, the biological delignification using microorganism such as white rot fungi were reported as one of promising methods and environmental friendly for biomass pre-treatment [5].

Most of white rot fungi produced peroxidase enzymes, which are able to depolymerize lignin as it releases free radical compounds, and then disrupt lignocellulose structure [6,7]. *Phlebia sp. MG-60* was known as one of white rot fungi which has the ability to breakdown lignin as well as to produce...
ethanol in a single process. Phlebia sp MG-60 contains peroxidase enzymes such as lignin peroxidase (LiP), manganese-peroxidase (MnP) and laccase which are able to degrade lignin [8]. Furthermore Phlebia sp MG-60 is also able to convert glucose to ethanol directly using hexokinase, glucose-6-phosphate isomerase, 6-phosphofructokinase, fructose-biphosphate aldolase triosephosphate-isomerase, gliseraldehyde 3-phosphate dehydrogenase, phosgo-gliserat kinase, 2,3-biphosphogliserid-independen phosgo-gliserat mutase, enolase, and piruvat kinase [9].

In delignification process, the MnP enzyme plays an important role in increasing the ability to degrade lignin [10]. MnP is a peroxidase enzyme that is able to oxidize manganese (II) (Mn²⁺) ions to become more reactive Mn³⁺ [11]. The resulting Mn³⁺ will react with carboxylic acids such as oxalate, malonic, malate, tartrate, and lactate, which cause one electron oxidation of various substrates. In addition, Mn³⁺, which reacts with carboxylic acid, will produce alkyl radicals. These alkyl radicals then react with oxygen (O₂) to produce radical compounds [12] that play an important role in the delignification process. Several studies have shown that the delignification process using biological treatment can produce high-value chemical compounds such as vanillin [13] and ferulic acid [14]. An increase in MnP activity of Phlebia.sp MG-60 can be supported by the addition of an inducer. Several studies have shown that the addition of metal sources such as MnSO₄ to lignocellulose degradation process can improve the performance of white rot fungi [15]. The addition of MnSO₄ to rotting fungi can play a role in helping the MnP enzyme reaction to provide a source of Mn³⁺, which will later be oxidized by the MnP enzyme to Mn⁵⁺ [16,17]. The addition of 0.25 mM MnSO₄ was able to increase MnP enzyme activity in Anthracophyllum discolor Sp4 by 1.354 IU/L on the 13th days of incubation [18]. While Rajan et al. [19], reported that the highest MnP enzyme activity that could be achieved by Phanerochaete chrysosporium was 54.03 IU/L with the addition of 0.1 mM MnSO₄ concentration.

Therefore, in this study MnSO₄ was used as an inducer to increase the activity of MnP enzyme [19] in Phlebia sp. MG-60. This study was conducted to determine the effect of MnSO₄ in the change of lignin during delignification periods, cellulose and hemicellulose levels, and the activity of the MnP enzyme of Phlebia sp. MG-60. The released of potential aromatic compounds and ethanol production were also identified.

2. Materials and Methods

2.1. Fungal strain
The fungal isolate white rot Phlebia sp. MG-60 (MKFC40001) was obtained from Department of Forest and Environmental Sciences, University of Miyazaki Japan, which originally collected from National Institute of Technology and Evaluation, NITE, Chiba, Japan. The isolate was then regenerated in Laboratory Bioindustry, Faculty of Agricultural Technology, Universitas Brawijaya, Indonesia. Phlebia sp. MG-60 was maintained on Potato Dextrose Agar (PDA) medium for 7 days at 28°C, before applied to the biomass substrate.

2.2. Delignification of cacao pod husk
10 grams of cacao pod powder (42-100 mesh) [5,9,10], obtained from “Cacao village’ in Blitar city, was extracted using 99.8% of methanol (Sigma Aldrich) for 16 hours [5,20]. The moisture was maintained at 77% [5]. Certain concentrations of MnSO₄ (Sigma-Aldrich) (0 mM, 0.1 mM, dan 0.5 mM) were added. A mycellia disk 5mm of Phlebia sp. MG-60 was taken from the PDA medium and placed to the cacao pod substrate, following an incubation at 28 °C for 14 and 28 days [4,5].

2.3. The change of lignocellulose compounds (Chesson-Datta methods)
The change of lignocellulose compounds described based on the released of cellulose, hemicellulose and lignin analysis as followed: (a) 150 ml distillate water was added to sample and then heated at 100 °C for 2 hours. The solid part was dried in an oven (Memmert UF55) at 105 °C until constant and weighed, (b) 150 ml of 1N H₂SO₄ (Sigma-Aldrich) was added to sample and then heated at 100 °C for an hour. The solid part was then dried in an oven at 105 °C until constant and weighed, (c) 10 ml of
72% HSO₄ was added to sample and allowed to stand for 4 hours. Afterward the sample was diluted to 4% HSO₄ and heated at 100 °C in an oven at 105 °C until constant and weighed, (d) sample was heated at 600 °C for 4 hours and then weighed (d). The hemicellulose, cellulose and lignin concentration was calculated using the formula as reported by Datta [21], as follows:

\[
\text{Hemicellulose (\%) = \frac{a-b}{\text{sample}} \times 100%} \\
\text{Cellulose (\%) = \frac{b-c}{\text{sample}} \times 100%} \\
\text{Lignin (\%) = \frac{c-d}{\text{sample}} \times 100%}
\]

2.4. Fermentation conditions
20 mL of an autoclaved basal liquid culture (10 g/L yeast extract, 10 g/L KH₂PO₄, 1 g/L (NH₄)₂SO₄, 0.5 g/L MgSO₄.7H₂O) (pH 6.0) was prepared and added to the sealed honey jars to create semi-aerobic condition. The sealed culture was then dark-incubated at 28 °C for 28 days.

2.5. MnP activity assay
The crude enzyme of MnP were prepared by extracting the fungal treated cacao pod in aerobic condition for 14 and 28 days. The extraction was conducted in a waterbath (150 rpm, 4 °C) for an hour with addition of 10 mL sodium acetate buffer (50 mM, pH 5.0) (150 rpm, 4°C). The supernatant (crude enzyme MnP) was then separated using centrifuge (Thermo Fisher Scientific SL 40R) (12000 rpm at 37°C). The enzyme activity was measured at 37°C using spectrophotometry (Thermo Scientific Genesys 10 UV) at 469 nm and identified the changing of absorbance for 1 minute [22].

2.6. Ethanol assay
The fungal mycelium was separated by centrifugation with 12,000rpm at 4 °C for 10 min. The supernatant was then eluted with water at a flow rate 1mL/min. All cultures were analysed in quadruplicate. The ethanol concentrations were determined by GC Agilent 7890B using FID detector and HP-5 column.

2.7. Identification aromatic compounds
The extracted sample was added 100mL of methanol 99.8% (Sigma –Aldrich) and the mycelium was separated using centrifuge (Thermo Fisher Scientific SL 40R) at 12000 rpm for 10 min at 4 °C. The supernatant then was determined using LCMS/MS (Thermo Scientific Triple Quadrupole MS TSQ Quantum Access Max) kolom Hypersil Gold C18 (2.1 mm x 50 mm x 1.9 µm) for the identification aromatic compounds produced from derivative of lignin, which was based on the molecular weight [23].

2.8. Statistical analysis
All treatments were performed in triplicate and the obtained data was analyzed by multivariate analysis of variance (MANOVA) using Microsoft Excell 2010 with error bar p <0.05 and continued with least significant difference (LSD) test.

3. Results and Discussion

3.1. Changes in lignocellulose compounds (cellulose, hemicellulose and lignin)
Changes in lignocellulose composition of the cacao pod following pretreatment are shown in Figure 1. The percentage of cellulose, hemicellulose and lignin decreased significantly (p<0.05) in a time-dependent from the initial value. The composition of cellulose, hemicellulose and lignin on untreated cacao pod were 41.05%, 21.05% and 37.86%, respectively, which significantly decreased after 28 days incubation. The addition different concentration of MnSO₄ on substrate inoculated by Phlebia sp. MG-60 has also given similar effect on the reduction of cellulose, hemicellulose and lignin. Compare
to the initial value, the highest degraded of cellulose (58.86%), hemicellulose (37.01%) and lignin (45.35%) were obtained from samples incubated for 28 days with addition 0.1 mM MnSO₄. However, the change of lignin degraded between each treatment (0, 0.1mM and 0.5mM MnSO₄) was not significantly different, indicated that the addition of MnSO₄ has no effect on lignin degradation.

![Figure 1](image)

**Figure 1.** The effect of the addition MnSO₄ (0, 0.1, 0.5 mM) and time of incubation toward the change of lignocellulose compounds; (a) cellulose (%) (b) hemicellulose (%) (c) lignin (%).

### 3.2. Enzyme (MnP) activity

Manganese is a metal element acts as co-factor for some enzymes [24]. In this study, the addition of MnSO₄ as micronutrient or inducer aimed to stimulate the enzyme activity especially MnP. Figure2 showed that the addition of 0.1mM MnSO₄ significantly increased the enzyme activity, indicated that the addition of metal (Mn²⁺) in certain level can enhance enzyme activity. Another study using
Ceriporiopsis subvermispora showed that the highest MnP activity obtained from the sample with the addition of 0.2 mM MnSO₄, while the lowest enzyme activity revealed when 0.3 mM MnSO₄ was added [25]. Previous study also showed that the addition of 0.5 mM MnSO₄ to the substrate can inhibit the activity of MnP enzyme, suggested that the addition of metal ions in high concentration can be toxic to microorganisms [18].

![Figure 2](image2.png)

**Figure 2.** The effect of time incubation (days) and the addition of MnSO₄ in different concentrations to The MnP enzyme activity (U/mL).

The presence of Mn²⁺ in high concentration can cause the fungus to secrete more H₂O₂, and when the amount of MnP enzyme is out of balance to the H₂O₂, this will disrupt the MnP enzyme cycle in converting Mn²⁺ to Mn³⁺ [26].

### 3.3. Identification of aromatic compounds

![Figure 3](image3.png)

**Figure 3.** Identification of aromatic compound (using LCMS/MS) produced from pre-treated cacao pod using Phlebia sp. MG-60 incubated for 28 days with addition of 0.1 mM MnSO₄.

Lignin is a natural polymer compound that is used as a source of renewable aromatic compounds production [27]. The results of degraded lignin can be catagorized into several high value aromatic compounds. Some of these compounds are hydrocinnamic acid, 2,3-dimethyl phenol, 3-(2-hydroxyphenyl) propionic acid, veratraldehyde, benzoic acid, syringaldehyde, trans-cinamic acid, 4-methoxymandelic acid and caffeic acid [28,29].
The aromatic compound produced from selected sample was identified using LCMS/MS and showed that there are several peaks detected. Fig. 3 showed two dominant peaks (molecular weights 166 and 182) detected, indicated the breakdown of lignin released some aromatic compounds. Based on the molecular weight detected, those two peaks represent veratraldehyde (mw:166) and syringaldehyde (mw:182) [30–32].

3.4. Ethanol production

The ethanol assay was carried out on selected sample based on the best results of pretreatment experiment (above). The result showed that the addition 0.01M MnSO₄ caused an increased MnP enzyme activity. The results revealed that the samples released some sugars (0.09 mg/L of glucose and 0.08mg/L of xylose). The efficiency of ethanol production using white rot fungus Phlebia sp. MG-60 is very dependent on the amount of sugars released, as it needs as a substrate for ethanol production. However, since the sugars concentration both glucose and xylose produced from pretreated cacao pod is quite low, therefore the amount of ethanol released is also low (2g/L), means the releasing of sugars need to be improved by increasing enzymes activity. According to Wang et al. [9], white rot fungus Phlebia sp. MG-60 produces ethanol through the glycolytic pathway which is the same as the mechanism in Saccharomyces cerevisiae, and produces ethanol efficiently by involving all genes involved in glycolysis. Some enzymes involved in the glycolysis of Phlebia sp. MG-60 are hexokinase, glucose-6-phosphate isomerase, 6-phosphofructokinase, fructose-bisphosphate aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, enolase, and pyruvate kinase.

4. Conclusions

The addition of 0.01M MnSO₄ and 28 days incubation of pre-treated cacao pod using Phlebia sp. MG-60 revealed to enhance the enzyme activity which to be able to breakdown the structure of lignocellulose. The ethanol produced during 28 days culture was quite low (2g/L). Further analysis of the aromatic compounds using LCMS_MS needs to be conducted using the pure standard of the expected compounds in order to validate the results. The optimization fermentation condition is also need to be improved as well as the addition of nutrition, which then allowed the fungus to grow appropriately.

References
[1] BPS 2017 Statistik Kakao Indonesia (Indonesian Cocoa Statistics) 2016 (Jakarta: BPS - Statistics Indonesia)
[2] Cruz G, Pirilä M, Huuhtanen M, Carrión L, Alvarenga E and Keiski R L 2012 Production of Activated Carbon from Cocoa (Theobroma cacao) Pod Husk J. Civ. Environ. Eng. 2 1–6
[3] Vriesmann L C, de Mello Castanho Amboni R D and De Oliveira Petkowicz C L 2011 Cacao pod husks (Theobroma cacao L.): Composition and hot-water-soluble pectins Ind. Crops Prod. 34 1173–81
[4] Kamei I, Hirota Y and Meguro S 2014 Direct fungal production of ethanol from high-solids pulps by the ethanol-fermenting white-rot fungus Phlebia sp. MG-60 BioResources 9 5114–24
[5] Khuong L D, Kondo R, De Leon R, Kim Anh T, Shimizu K and Kamei I 2014 Bioethanol production from alkaline-pretreated sugarcane bagasse by consolidated bioprocessing using Phlebia sp. MG-60 Int. Biodeterior. Biodegrad. 88 62–8
[6] Wan C and Li Y 2011 Effectiveness of microbial pretreatment by Ceriporiopsis subvermispora on different biomass feedstocks Bioresour. Technol. 102 7507–12
[7] Yamagishi K, Kimura T and Watanabe T 2011 Treatment of rice straw with selected Cyathus stercoreus strains to improve enzymatic saccharification Bioresour. Technol. 102 6937–43
[8] Lundell T K, Mäkelä M R and Hilden K 2010 Lignin-modifying enzymes in filamentous
basidiomycetes - Ecological, functional and phylogenetic review. J. Basic Microbiol. 50 5–20
[9] Wang J, Suzuki T, Dohra H, Takigami S, Kako H, Soga A, Kamei I, Mori T, Kawagishi H and Hirai H 2016 Analysis of ethanol fermentation mechanism of ethanol producing white-rot fungus Phlebia sp. MG-60 by RNA-seq BMC Genomics 17 1–11
[10] Yamasaki Y, Yamaguchi M, Yamagishi K, Hirai H, Kondo R, Kamei I and Meguro S 2014 Expression of a manganese peroxidase isozyme 2 transgene in the ethanologenic white rot fungus Phlebia sp. strain MG-60 Springerplus 3 1–6
[11] Hofrichter M 2002 Review: Lignin conversion by manganese peroxidase (MnP) Enzyme Microb. Technol. 30 454–66
[12] Hatakka A 2001 Biodegradation of Lignin Biopolymers. Biology, Chemistry, Biotechnology. Applications. Vol 1. Lignin, Humic Substances and Coal ed M Hofrichter and A Steinbüchel (Weinheim: Wiley-VCH) pp 129–45
[13] Oliva-Taravilla A, Tomás-Pejó E, Demuez M, González-Fernández C and Ballesteros M 2016 Phenols and lignin: Key players in reducing enzymatic hydrolysis yields of steam-pretreated biomass in presence of laccase J. Biotechnol. 218 94–101
[14] Kaur B and Chakraborty D 2013 Biotechnological and Molecular Approaches for Vanillin Production : a review Appl. Biochem. Biotechnol. 169 1353–72
[15] Gomaa O M and Momtaz O A 2015 Copper induction and differential expression of laccase in Aspergillus flavaus Brazilian J. Microbiol. 46 285–92
[16] Tuor U, Wariishi H, Gold M H and Schoemaker H E 1992 Oxidation of Phenolic Arylglycerol β-Aryl Ether Lignin Model Compounds by Manganese Peroxidase from Phanerochaete chrysosporium: Oxidative Cleavage of an α-Carbonyl Model Compound Biochemistry 31 4986–95
[17] Brown J A, Alic M and Gold M H 1991 Manganese peroxidase gene transcription in Phanerochaete chrysosporium: Activation by manganese J. Bacteriol. 173 4101–6
[18] Acevedo F, Pizzul L, Castillo M del P, Rubilar O, Lienqueo M E, Tortella G and Diez M C 2011 A practical culture technique for enhanced production of manganese peroxidase by Anthracophyllum discolor Sp4 Brazilian Arch. Biol. Technol. 54 1175–86
[19] Rajan A, Kurup J G and Abraham T E 2010 Solid state production of manganese peroxidases using arecanut husk as substrate Brazilian Arch. Biol. Technol. 53 555–62
[20] Sluiter A, Ruiz R, Scarlata C, Sluiter J and Templeton D 2008 Laboratory Analytical Procedure (LAP): Determination of Extractives in Biomass (Colorado)
[21] Datta R 1981 Acidogenic fermentation of lignocellulose–acid yield and conversion of components Biotechnol. Bioeng. 23 2167–70
[22] Gassara F, Brar S K, Tyagi R D, Verma M and Surampalli R Y 2010 Screening of agro industrial wastes to produce ligninolytic enzymes by Phanerochaete chrysosporium Biochem. Eng. J. 49 388–94
[23] Deepa A K and Dhepe P L 2014 Solid acid catalyzed depolymerization of lignin into value added aromatic monomers RSC Adv. 4 12625–9
[24] Culotta V C, Yang M and Hall M D 2005 Manganese Transport and Trafficking: Lessons Learned from Saccharomyces cerevisiae Eukaryot. Cell 4 1159–65
[25] Mancilla R A, Canessa P, Manubens A and Vicuña R 2010 Effect of manganese on the secretion of manganese-peroxidase by the basidiomycete Ceriporiopsis subvermispora Fungal Genet. Biol. 47 656–61
[26] Wariishi H, Akileswaran L and Gold M H 1988 Manganese Peroxidase from the Basidiomycete Phanerochaete chrysosporium: Spectral Characterization of the Oxidized States and the Catalytic Cycle Biochemistry 27 5365–70
[27] Abdelaziz O Y, Brink D P, Prothmann J, Ravi K, Sun M, García-Hidalgo J, Sandahl M, Hultberg C P, Turner C, Lidén G and Gorwa-Grauslund M F 2016 Biological valorization of low molecular weight lignin Biotechnol. Adv. 34 1318–46
[28] Ko J J, Shimizu Y, Ikeda K, Kim S K, Park C H and Matsui S 2009 Biodegradation of high molecular weight lignin under sulfate reducing conditions: Lignin degradability and degradation by-products Bioresour. Technol. 100 1622–7

[29] Ibrahim M N M, Sriprasanthi R B, Shamsudeen S, Adam F and Bhawani S A 2012 A concise review of the natural existence, synthesis, properties, and applications of syringaldehyde BioResources 7 4377–99

[30] Grelier S and Koumbe Y G 2016 Process for Depolymerization of Lignin By Laccases

[31] Masai E, Yamamoto Y, Inoue T, Takamura K, Hara H, Kasai D, Katayama Y and Fukuda M 2007 Characterization of ligV essential for catabolism of vanillin by Sphingomonas paucimobilis SYK-6 Biosci. Biotechnol. Biochem. 71 2487–92

[32] Pérez-Pantoja D, González B and Pieper D H 2010 Aerobic Degradation of Aromatic Hydrocarbons Handbook of Hydrocarbon and Lipid Microbiology ed K N Timmis (Berlin, Heidelberg: Springer-Verlag Berlin Heidelberg) pp 799–837