Enzymatic biodelignification of Corncob by Laccase (Lac) from Cerrena Sp.B.Md.T.A.1.

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Abstract. Corn (Zea mays) is a commodity of food crops that are important after rice in Indonesia. Corn plantation produces waste in the form of corncob. Corncob is lignocellulosic biomass that can converted to cellulose, even sugar or another chemical like ethanol. The first step for utilization of corncob is delignification. The purpose of this study was to isolate and purify the enzyme laccase from white rot fungus Cerrena sp.B.Md.T.A.1 that inoculation in GDP media used for biodelignification of corncobs. Enzymatic biodelignification conduct with the variation of substrate was 5%, 10%, and 15% (w/v), while the time variation were 6 hours and 24 hours. The percentage of lignin concentration before pretreatment reached is 37.25%. The highest reduction of lignin content was achieved in 15% substrate for 24 hours incubation time around 42.71%.

1. Introduction
Ethanol is one of a group of chemical compounds from alcohols, that contain a hydroxyl group bonded to a carbon atom. The use of ethanol is very wide such as for solvents, disinfectants, antiseptics, and also to be developed as an alternative fuel. Ethanol can be produced from fermentation of sugar. The substrate of bioethanol can used foodstuffs such as sweet potato [1], cassava [2], and corn[3], that known as first generation bioethanol. But this raw material interferes with food security, another raw material for bioethanol production was lignocellulosic biomass. Lignocellulosic bioethanol or second-generation bioethanol used lignocellulosic biomass that contain lignin, cellulose and hemicellulose. The cellulose in lignocellulosic can converted to sugar and then fermented into ethanol. Some resource of lignocellulosic biomass are usually derived from agricultural waste such as corncob [4–6], Rice Husk [7] and oil palm empty fruit bunch [8,9].

Corn (Zea mays) is a commodity of food crops after rice. Corn plantation/industries produce waste in the form of corncob. Corncob is composed of lignin, cellulose, and hemicellulose. Corncob contain lignin 26.47%, hemicellulose 24.39%, and cellulose 31.69% [6]. Lignin is a part of plant cell walls and natural polymer after cellulose. Lignin serves as a water, nutrient, and metabolic carrier in plant cells. Lignin has a complex and heterogeneous structure that binds cellulose and hemicellulose in plant tissues
so it is difficult to be degraded, providing a sturdy structure for plants, and providing protection against insects and pathogens [10].

High lignin content causes a lack of cellulose utilization. Lignin inhibit enzyme for degradation cellulose. One way to overcome the levels of lignin in lignocellulose waste is biodelignification. Biodelignification can conduct by extracellular lignoclytic enzyme from white rot fungi of the Basidiomycetes class [11,12]. The extracellular lignoclytic enzyme is laccase (LAC), lignin peroxidase (LiP), and manganese peroxidase (MnP) [13]. The use of fungi in delignification has some disadvantages that require a longer time (at least 3 to 7 weeks) than the enzymatic pretreatment that makes it less profitable to use. The advantages of enzyme delignification are shorter processing time, did not require nutrient supplementation and no sugar consumption by fungi [14].

Cerrena sp. is a white rot fungus from a class of Basidiomycetes. These fungi have the ability to degrades lignin. Cerrena sp. B. Md. T.A.1. isolated from Mount Rinjani National Park has the potential to produce lignoclytic enzymes such as laccase (Lac) and manganese peroxidase (MnP) [15]. The laccase (Lac) has a higher enzyme activity compared to manganese peroxidase (MnP). This paper describes the isolation and purify the enzyme laccase from white rot fungus Cerrena sp. B.Md.T.A.1. to perform biodelignification of corncob.

2. Material and Methods

2.1. Materials
The strain used in this research are Cerrena sp. B.Md.T.A.1. Corncob was obtained from Indonesian Center for Agricultural Post Harvest R&D, Ministry of Agriculture. The corncob was reduced to the size below 1 cm granule then stored in a plastic bag. Other ingredients used include potato dextrose agar, ABTS (2.2'-Azino-bis(3-ethenthiazolin-6-sulfonic acid), ammonium sulfate (NH₄)₂SO₄, acetate buffer 0.5 M, H₂SO₄ and CaCO₃ are for synthesis and analytical grade was purchased from Merck.

2.2. Methods

2.2.1. Cultivation of fungi. Medium for inoculation was made by Potato Dextrose Agar (PDA) containing glucose (20 g L⁻¹), potato (200 g L⁻¹), and agar (15 g L⁻¹). Cerrena sp. B.Md.T.A.1. was taken from culture and transferred to a petri dish containing a PDA medium, then incubated at room temperature for 7 days.

2.2.2. Enzyme production and extraction. Cerrena sp. B.Md.T.A.1. from PDA was transferred to 20 ml sterile growth medium containing Potato Dextrose Broth (PDB). The cultivation was incubated for 6 days at 30 °C then filtered and to take filtrate for purification.

2.2.3. Purification of laccase. The purification of laccase was done in the time of maximum laccase activity. The extraction was conducted by grinding and followed by centrifugation at 10000 rpm for 20 min. Crude laccase extract are precipitated by ammonium sulfate salts. Salt is added gradually to crude laccase extract to gain saturation of 0-60%. The solution precipitate overnight at a temperature of 4°C, then centrifuged at 10000 rpm for 30 minutes. The sludge then dissolved into the 0.5 M acetate buffer pH 5.0 (10% w/v) for measure enzyme activity. The supernatants were decanted and the precipitant was dissolved in 0.5 M sodium acetate buffer (pH 5). This solution was stored at 4°C until further use.

2.2.4. Enzyme assay. Laccase activity was determined spectrophotometrically using 1 mM ABTS (2.2'-Azino-bis(3-ethenthiazolin-6-sulfonic acid) in 0.5 M sodium acetate buffer, pH 5 and measuring at 420 nm (ε420= 36,000 mol⁻¹ cm⁻¹). One unit of enzyme activity was defined as the amount of enzyme oxidizings 1 nmol ABTS min⁻¹. Enzyme activity was measured based on the following equation:

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\text{Enzyme activity (U/mL)} = \frac{(A_t-A_o) x V_{tot} x 10^6}{c_{max} x A x v_{ene}(mL)x t}
\]

(Eq. 1)
At = Absorbance at T-minute
Ao = absorbance in the 0 minute (value 0 (autozero measurement))
Ɛₘₐₓ = Absorption of molar tetrametoxy Azobismetilen Quinon (36000 M⁻¹cm⁻¹)
d = Cuvette thickness (cm)
Vₜₒ𝑡 = Total test Volume (mL)
Vₑنبيز = total added enzyme volume (mL)
t = Incubation time (minutes)

2.2.5. Analysis of corncob component. The lignin content in corncob was determined by NREL methods [16]. Corncob (300 mg, dry weight) was subjected to acid hydrolysis for lignin, cellulose, and hemicellulose content analysis. After hydrolysis, acid-insoluble lignin (AIL) was weighed using Sartorius BS224S, and acid-soluble lignin (ASL) was measured using UV/Vis Spectrophotometer (Optizen 2120 UV) at 205 nm. Total lignin was obtained from the sum of AIL and ASL. Ash content was measure by gravimetric after hydrolysis process. Hydrolizate was filtered use filter paper, residue and filter paper inserted to crucible cup and furnace at 575 º C for ± 3 hours. After finished the crucible is cooled in the desiccator ± 15 minutes then weighed for measure ash content. Lignin content was measured according to the following equation:

AIL [%] = \( \frac{W_{ks} - W_k - A}{W_s} \times 100 \) % \hspace{1cm} (Eq. 2)

ASL [%] = \( \left( \frac{Abs \times df \times 110}{W_s \times 1000} \right) \times 100\% \) \hspace{1cm} (Eq. 3)

AIL : Acid Insoluble Lignin [%]
ASL : Acid Soluble Lignin [%]
Wₛ : Dry sample weight (without moisture) [g]
Wₖ : Filter paper weight [g]
Wₖₛ : Filter paper weight and residue dried [g]
A : Ash weight [g]
Abs : Absorbance
df : Dilution Factor

2.2.6. Biodelignification of corncob. Corncob material was treated by extracted laccase from Cerrena sp. B.Md.T.A.1 in solid substrate culture. The substrate in variation 5%, 10% and 15% (w/v) was added by extracted laccase in the total volume 40 ml. Biodelignification was conducted in shaker incubator at 40°C, 100 rpm for 6 and 24 hours. Controls are done under the same conditions without using laccase. All experiments are made 2 times repeated. After treatment, the corncob carried out for measure lignin content after biodelignification.

3. Result and Discussion

3.1. Activity of enzyme laccase

Enzyme purification is an important step prior characterization. Purification was carried out by the salting out method using ammonium sulfate salt against the extract crude enzyme. The principle of ammonium sulfate precipitation is based on water-binding competition between salt and protein. Ammonium sulfate salt can separate enzymes from other proteins that did not precipitated during centrifugation. The advantage of using ammonium sulfate is that it has a high solubility of around 840 g/liter and exothermic process. Dissolved ammonium sulfate will ionize into an NH₄⁺ and SO₄²⁻ ions. This salt ion will attract water that binds to protein, so that protein solubility decreases then forms an aggregate and settles [17].
Ammonium sulfate concentrations are added to get the most diverse enzyme specific activities for each microorganism [18]. According to research conducted by Hidayat [15] the ammonium sulphate deposition fraction 40-60% produces the highest laccase activity. Therefore, the ammonium sulfate used in this study was 0-60%.

The measurement of laccase activity from fractionation was determined using ABTS. The change reaction rate in ABTS substrate into ABTS radical cation becomes a determinant of laccase activity in the optimum point. The measurement time interval on the laccase enzyme occurring at 0-15 minutes to reach the optimum point, exceeding that time will decrease the acceleration of the laccase reaction [15,19]. The results of laccase activity in this research was obtained at 0-15-minute intervals reached 242.76 U/mL. This result was slightly lower than Hidayat’s result in previous research that obtained 280 U/mL [15].

3.2. Lignin content of corncob

Corncob is one of the lignocellulosic biomass, that contain high lignin content. Lignin content in this study was 37.25%, while ash content was 1.54% (table 1). The lignin content in corncob was lower than rice straw and wheat straw, but higher than empty fruit bunch and sugarcane bagasse [20]. Due to the high lignin content, the delignification must be carried out prior utilize corncobs. The process of biodelignification (enzymatic pretreatment) is by mixing corn cobs with the enzyme laccase to degrade the lignin content in corncobs [21]. The ability of an enzyme in hydrolyzing lignocellulosic material is influenced by several factors, such as lignin content, hemicellulose, and the level of crystallization of cellulose [22].

| Biodelignification | Variations | Lignin Content [%] | Ash Content [%] |
|--------------------|------------|--------------------|-----------------|
| untreated          |            | 37.25              | 1.54            |
| pretreated         | 6 hours    | 24.53              | 1.39            |
|                    | 10%        | 23.40              | 0.80            |
|                    | 15%        | 22.47              | 0.97            |
|                    | 5%         | 23.08              | 0.92            |
|                    | 24 hours   | 21.89              | 0.66            |
|                    | 15%        | 21.34              | 0.94            |

3.3. Biodelignification of corncob

Biodelignification is one of the environmentally friendly lignin degradation techniques by microorganism such as fungi. The use of the enzyme laccase from the fungus Cerrena sp. B.Md.TA.1 aims to reduce the incubation time, thereby speeding up the biodelignification process. Laccase enzymes play an important role in lignin degradation. Laccase enzymes can induce oxidation reactions, producing unstable reactive phenoxy radicals that can lead to polymerization and depolymerization reactions [23].

Figure 1 showed the biodelignification result in corncobs using laccase from Cerrena sp. B.Md.TA.1. The result showed that Laccase could reduce lignin content in corncob. The longer the processing time the higher the delignification. This is caused by the optimal work of the laccase enzyme in degrade lignin. The highest delignification was obtained on a substrate of 15% (w/v) for 24 hours incubation time reached 42.71%. Similarly, the increasing percentage of substrate content the higher the biodelignification that occurs.
Figure 1. Biodelignification of corncob.

Delignification produced by the laccase is still smaller than delignification using chemicals. In our previous studies result, corn cobs after the delignification process using NaOH achieved a lignin reduction of 60.22%, but the biodelignification result closely to pretreatment process using spent black liquor [6]. Although enzymatic biodelignification results is lower than chemical delignification, this process didn’t produce another waste after process.

4. Conclusion
The activity of laccase obtained from isolation and purification using ammonium sulfate with 0-60% saturation reached 242.76 U/mL. The laccase from Cerrena sp. B.Md.T.A.1 can be used for enzymatic biodelignification of corncob. The laccase Cerrena sp. B.Md.T.A.1 used in delignification of 15% corn cobs for 24 hours can reduce the lignin content up to 42.71%. This process is promising for the process of removing lignin in lignocellulosic biomass without chemical and environmentally friendly.

Acknowledgments
The authors gratefully acknowledge to RC Chemistry Indonesian Institute of Sciences, and Forest Research and Development Centre, the Ministry of Environment and Forestry for support this research.

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