Optimization of laccase production from a newly isolated
*Trametes* sp. EDN134

F Ningsih¹, D H Y Yanto²*, W Mangunwardoyo³, S H Anita² and T Watanabe⁴

¹Magister Program in Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas of Indonesia, Depok 16424, Indonesia
²Research Center for Biomaterials, Indonesian Institute of Sciences (LIPI), Jl. Raya Bogor Km. 46, Cibinong Science Center, Cibinong, Bogor 16911, Indonesia
³Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas of Indonesia, Depok 16424, Indonesia
⁴Research Institute for Sustainable Humanosphere, Kyoto University, Japan Gokasho, Uji, Kyoto 611-0011, Japan

*E-mail: dede@biomaterial.lipi.go.id*

**Abstract.** Laccase is one important enzyme in decolorization of textile dyes. This research aims to study the optimization of laccase production from *Trametes* sp. EDN134 under several variation of substrates (bagasse, bamboo, Jabon wood, oil palm empty fruit bunch (OPEFB), and sorghum fibers), time incubation (1–15 d), growth temperature (25, 27, 30, 32, 35 and 37°C), and concentrations of inducer CuSO₄ (1, 2, 3, 4 and 5 mM). The results showed that optimum laccase specific activity (14,838 U/g) detected in the OPEFB medium at 10 days incubation, temperature 30°C and CuSO₄ 2 mM. The study suggests that these parameters could be used for the optimization of laccase production from a newly isolated *Trametes* sp. EDN134.

1. Introduction

National enzyme needs in Indonesia still depend on imports from other countries such as China, India, Japan and most of Europe, and the demand for enzymes tend to increase year by year. Laccase is an enzyme that has great potential in the field of biotechnology and the market broad. Because of their capability of catalyzing the oxidation of phenols, fungal laccase is receiving increasing interest as potential industrial enzymes in various applications such as delignification of biomass [1], detoxification [2], decolorization of dyes [2], and polycyclic aromatic hydrocarbons [3].

Laccase (EC 1.10.3.2) is a multicopper blue oxidase that couples the four-electron reduction of oxygen with the oxidation of a broad range of organic substrates, including phenols, polyphenols, anilines, and even certain inorganic compounds by a one-electron transfer mechanism [4]. Laccase catalyzes the oxidation of a wide range of phenols and other substrates with the concomitant reduction of oxygen to water [5]. Laccase is widely distributed in higher plants, bacteria, fungi and some insects [6].

Production of laccase enzyme can be affected by several factors namely nutrition, pH, incubation time, temperature environment and concentration a substrate [7]. Cu (II) serves as the metal inducer on the establishment of an enzyme laccase [8]. Growth medium of fungi is a major influence in laccase
production. Utilization of biomass waste such as lignocellulose substrate for growth medium of fungi can hasten the fungal biomass due to this lignocellulose waste still containing lignin, cellulose and hemicellulose that can act as laccase inducer. The lignin content in the substrate can trigger the activity of the induction laccase in the production of the enzymes [9].

In this study, we used several lignocelluloses as substrates for laccase production of a newly isolated Trametes sp. EDN 134. Optimization of laccase production were also investigated under several condition of incubation time, temperature, and concentration of copper ions.

2. Materials and methods

2.1. Materials

Material used in this research including bagasse was provided by PG Rajawali II sugar factory, Subang, Indonesia, bamboo from Research Center for Biomaterials, Jabon wood from Garut, West Java, oil palm empty fruit bunch (OPEFB) fiber from oil palm plantation in Cikasungka, West Java, and sorghum fibers from Cibenong Science Center area. Trametes sp. EDN 134 a collection of the RC Biomaterials, LIPI, Cibenong, Indonesia was obtained from Taman Eden 100, North Sumatera. Malt extract [Himedia, India], glucose [Wako, Japan], peptone and CuSO4 [Merck, Germany] were used in growth media.

2.2. Methods

2.2.1. Preparation and analysis of raw materials. Trametes sp. EDN 134 was subcultured into malt extract (ME) medium and was incubated for 7 days at room temperature. Lignocellulosic fibers (3 g of each): bagasse, bamboo, Jabon wood, oil palm empty fruit bunch (OPEFB), and sorghum fibers with size 40-60 mesh were prepared. The chemical component analysis were conducted according to NREL LAP 003 for acid insoluble lignin [10], the Wise method for holocellulose [11], and the Rowell, et al. method for α-cellulose [12]. The hemicellulose content was calculated by subtraction of α-cellulose from holocellulose.

2.2.2. Selection of substrate for optimum fermentation. Each of 3 g lignocellulose substrates: bagasse, bamboo, Jabon wood, oil palm empty (OPEFB), and sorghum fibers were placed in 100 mL-Erlenmeyer flask, separately. Amount of liquid medium as showed in Table 1 were added to each substrate. The liquid media were contains 10 g/L malt extract; glucose 10 g/L; and peptone 1 g/L. The mixture then were sterilized using autoclave for 15 minutes, 121°C. After the sterilization finished and the mixture of substrate and medium were cool, the culture of fungi Trametes sp. EDN 134 were added and incubated at room temperature (27°C) for 15 days. Percentage of water retention was calculated as the grams of water added into substrate per grams of the wet substrate. Laccase activity was measured every day.

| Substrate   | Liquid media (g) | Wet substrate (g) | Water retention (%), w/w |
|-------------|------------------|-------------------|-------------------------|
| OPEFB       | 18.0             | 30                | 60                      |
| Bamboo      | 12.0             | 30                | 40                      |
| Bagasse     | 21.0             | 30                | 70                      |
| Sorghum     | 18.0             | 30                | 60                      |
| Jabon wood  | 16.5             | 30                | 55                      |

2.2.3. Optimization of incubation temperature. Optimization of incubation temperature were conducted at various temperature: 25, 27, 30, 32, 35 and 37°C. The selected lignocellulose fiber as much as 3 g with size 40-60 mesh was used as a substrate. After incubation at optimum time incubation, laccase activity was measured.
2.2.4. Optimization of CuSO₄ addition. Optimization of CuSO₄ addition as a laccase inducer was performed under optimum incubation time and temperature resulted from previous experiment. Concentration of CuSO₄ addition used in this study were 1, 2, 3, 4 and 5 mM with water retention 60% (w/w). After incubation, laccase activity was measured.

2.2.5. Enzyme extraction. After incubation, mixture of substrate was homogenized using ACE Homogenizer (Nisse AM 11, Japan) at 10,000 rpm and 4°C for 10 minutes. The filtrated was centrifuged and the supernatant was used for laccase and protein measurement.

2.2.6. Enzyme and protein assay. Laccase activity was measured using Spectrophotometer UV-Vis 1800 (Shimadzu, Japan). The reaction mixture contained 400 µL acetate buffer 0.1 M, 500 µL ABTS 2 mM and 100 µL dye solution. It was monitored at 420 nm using UV-Vis spectrophotometer and it was calculated from the molar extinction coefficient (ε) of 36.000 M⁻¹cm⁻¹ using Shimadzu UV Probe Software by following equation 1 [2].

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\text{Laccase activity (U/mL)} = \frac{\text{Abs} \times \frac{\text{Volution (µL)}}{10^6} \times \frac{(60/t)}{V_{\text{enzyme (µL)}}10^3}}{\varepsilon}.
\]  (1)

Protein concentration was determined by the dye-binding method of Bradford using bovine serum albumin (BSA) as the standard and the concentration was expressed in milligram per milliliter (mg/mL) [13]. Specific activity was calculated by the equation 2 according to Vrsanska et al. [14] with modification.

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\text{Specific activity (U/g)} = \frac{1}{\text{protein concentration (mg/mL)}} \times \text{enzyme activity (U/mL)} \times 10^3
\]  (2)

3. Results and discussion

Perennial grasses, agricultural by-products, agro-industrial by-products, wood, and vegetable residues are categorized as lignocellulosic biomass. This biomass contains lignin and polysaccharides such as cellulose, hemicellulose, pectin, ash, minerals, and salts, which are ideal raw materials for microorganism growth, enzyme production, and metabolite synthesis [15]. In this study, we use lignocellulosic biomass such as oil palm empty fruit bunch (OPEFB), bamboo, Jabon wood, bagasse, and sorghum fibers. Lignocellulose composition of this biomass varies depending on the source and should be evaluated before the waste is used as a fermentation substrate. The composition of lignocellulosic biomass used in this study can be seen in figure 1.

![Figure 1. Chemical components of lignocellulosic biomass for substrate of laccase production](image-url)
Lignocellulosic biomass in this study contains of 25–44% of cellulose, 25–29% of hemicellulose and 23–26% of lignin. Hemicellulose and cellulose component from this lignocellulosic biomass can be used as carbon sources for fungal growth. However, an exogenous carbon sources such as glucose was often added to the culture medium of laccase production. Besides serving as a carbon or nutrient source, certain lignocellulosic biomass also contain natural inductive substances, such as flavonoids and phenolic compounds, which can be applied directly in fermentation to enhance fungal laccase production. In addition, lignin content in the substrate can stimulate the laccase production of the fungus.

*Trametes* sp. EDN 134 was incubated for 15 days in bamboo, bagasse, Jabon wood, oil palm empty fruit bunch (OPEFB) and sorghum fibers with the addition of glucose as carbon sources at room temperature (27°C). All the lignocellulosic biomasses in this study can be potentially used to produce laccase enzyme. *Trametes* sp. EDN 134 grew on all substrates and produced laccase enzyme. As shown in figure 2, laccase production in the culture of *Trametes* sp. EDN 134 begin on day 5 of fermentation time. The result also indicate that OPEFB fiber is a potential substrate for production of laccase (7.22 U/mL) by *Trametes* sp. EDN 134 in comparison with other substrates on day 10 of incubation. Many investigators have reported different incubation periods for optimum production of crude laccases. Risdianto et al. [16] reported maximum laccase production on day 7 and on day 8 of incubation using *Trametes hirsuta* and *Trametes versicolor* with OPEFB fiber, respectively.

OPEFB is recommended as a substrate for microbial growth as it contains high cellulose, hemicellulose and also lignin. Bagasse (4.18 U/mL) and Jabon wood (4.09 U/mL) also showed reasonable laccase activities. The two lowest activities were recorded with sorghum (3.01 U/mL) and bamboo (2.86 U/mL). The different types of substrates have a notable effect on the production of laccase. Hence, OPEFB was selected as a solid substrate for further studies. The presence of OPEFB fiber has been reported to stimulate secretion of ligninolytic enzymes by *T. versicolor* U97 during degradation of DDT [17]. In addition, lignocellulosic biomass is a good candidate for laccase production by solid fermentation because it has a function as a support and nutrient source.

Temperature is one of an environmental factor that influences the fungal growth in SSF and also the production of enzymes and metabolites. In the biological cell system, temperature plays an important role since it determines several other factors, such as protein denaturation, acceleration and inhibition of enzyme production. Figure 3 showed that laccase activity increased as the temperature increased until it reached a maximum and then decreased. Maximum laccase production (9.29 U/mL) was recorded at 30°C on day 10 of incubation with the maximum protein (4.15 mg/mL) and highest specific activity (2242 U/g). Laccase production was recorded minimum (1.94 U/mL) at 25°C with maximum protein.
concentration (4.11 mg/mL) and the lowest specific activity (471 U/g) at day 10 of incubation. Varying results, based on microorganisms as well as types of substrate, have been reported by different researchers on production of laccase enzymes. Dhakar & Pandey [18] reported maximum production of laccase by Trametes hirsuta at 35°C on day 12 of incubation.

Figure 3. Laccase activity in variations temperature

CuSO₄ is known as inducer for enhancing the laccase activity. In the present investigation, addition of CuSO₄ increased laccase production (20.22 U/mL) almost 3-fold in comparison to control (without addition CuSO₄), maximum being in case of 2 mM concentration with the maximum protein (1.36 mg/mL) and highest specific activity (14,838 U/g). Further increment in CuSO₄ (up to 3 mM) resulted in decline of laccase production (figure 4). Laccase is blue copper oxidase that contains four copper atoms per molecule. The addition of copper may lead to the activation of the metal, resulting in the expression of laccase genes and synthesis of the laccase enzyme [19].

Figure 4. Laccase activity in variations concentration of CuSO₄

4. Conclusions
In the present study, OPEFB fibers was found to be a potential substrate for laccase production by Trametes sp. EDN 134. Maximum production of extracellular laccase and specific activity were 20.22 U/mL and 14,838 U/g, respectively. This value was obtained at 30°C on day 10 with addition of inducer...
(CuSO₄). The addition of 2 mM of CuSO₄ increased laccase production almost 3-fold in comparison to control (without CuSO₄).

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5. References
[1] Bagewadi ZK, Mulla SI and Ninnekar HZ 2017 Optimization of laccase production and its application in delignification of biomass Int. J. Recycl. Org. Waste Agricult. 6(4): 351–65
[2] Yanto DHY, Auliana N, Anita SH and Watanabe, T 2019 IOP Conf. Series: Earth and Environmental Science 374: 012005.
[3] Hidayat A and Yanto DHY 2018 Biodegradation and metabolic pathway of phenanthrene by a new tropical fungus, Trametes hirsuta D7 J. Environ. Chem. Eng. 6: 2454–60
[4] Piontek K, Antorini M and Choinowski T 2002 Crystal structure of a laccase from the fungus Trametes versicolor at 1.90-A resolution containing a full complement of coppers J. Biol. Chem. 40: 37663–9
[5] Jeon JR, Baldrian P, Murugesan K and Chang YS 2012 Laccase-catalysed oxidations of naturally occurring phenols: from in vivo biosynthetic pathways to green synthetic applications Microb. Biotechnol. 5(3): 318–32
[6] Gupta N and Singh D 2020 Microbial laccase: a robust enzyme and its industrial applications BioMecta https://doi.org/10.2478/s11756-019-00414-9
[7] Viswanath B, Rajesh B, Janardhan A, Kumar AP and Narasimha G 2014 Fungal laccases and their applications in bioremediation Enzyme Research 1–21
[8] Liu SH, Tsai SL, Guo PY and Lin CW 2020 Inducing laccase activity in white rot fungi using copper ions and improving the efficiency of azo dye treatment with electricity generation using microbial fuel cells Chemosphere 243: 125304
[9] Adekunle AE, Guo C and Liu CZ 2017 Lignin-enhanced laccase production from Trametes versicolor. Waste Biomass Valori. 8: 1061–6
[10] Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D and Crocker D 2012 Determination of structural carbohydrates and lignin in biomass–Laboratory analytical procedure (LAP) in: National Renewable Energy Laboratory Technical Report NREL/TP-510-42618: 1–15
[11] Wise LE, Murphy M and Addieco 1946 Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses. Pap. Trade J. 122(2): 35–43
[12] Rowell RM, Pettersen R, Han JS, Rowell JS and Tshabalala MA 2005 Cell wall chemistry Handbook Wood Chemistry and Wood Composites 1st ed CRC Press chapter 3 pp 71–2
[13] Bradford MM 1976 A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding Anal. Biochem 72: 248–54
[14] Vrsanska M, Voberkova S, Jimenez AMJ, Strmiska V and Adam V 2018 Preparation and optimisation of cross-linked enzyme aggregates using native isolate white rot fungi Trametes versicolor and Fomes fomentarius for the decolourisation of synthetic dyes Int. J. Environ. Res. Public Health 15: 23
[15] Wang F, Xu L, Zhao L, Ding Z, Ma H and Terry N 2019 Fungal laccase production from lignocellulosic agricultural wastes by solid-state fermentation: a Review. Microorganisms 665 (7): 1–25
[16] Risdianto H, Sofianti E, Suhardi SH and Setiadi T 2012 Optimization of laccase production using white rot fungi and agricultural wastes in solid-state fermentation. J. Eng. Technol. Sci. 44(2): 93–105
[17] Sari AA, Kristiani A, Tachibana S, Sudiyani Y and Abimanyu A 2014 Mechanisms and optimization of oil palm empty fruit bunch pre-grown source for white-rot fungus to degrade DDT J. Environ. Chem. Eng. 2: 1410–15

[18] Dhakar K and Pandey A 2013 Laccase production from a temperature and pH tolerant fungal strain of Trametes hirsuta (MTCC 1139) Enzyme Res 1–9

[19] Yang Y, Wei F, Zhui R, Fan F, Liu H, Zhang C, Ma L, Jiang M and Zhang X 2013 Enhancing the laccase production and laccase gene expression in the white-rot fungus Trametes velutina 5930 with great potential for biotechnological applications by different metal ions and aromatic compounds Plos One 8(11): e79307.