Enhancement of laccase production in a new isolated *Trametes hirsuta* LBF-AA017 by lignocellulosic materials and its application for removal of chemical dyes

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Abstract. Laccase is one of the extracellular ligninolytic enzymes highly produced by white rot fungi (WRF) and widely used in industrial processes such as decolorizing of synthetic dyes. In this study, the enzyme was produced by a new isolated WRF (*Trametes hirsuta* LBF-AA017) through two types of fermentation: submerged and solid state fermentation. In the submerged culture, several local lignocellulosic materials (0.5% w/v): bagasse, palm kernel cake (PKC), sugar palm fruit cake (SPFC), corn cobs (CC), tobacco road (TR), were used as laccase inducers. Among 5 materials tested, treatment using SPFC resulted highest laccase production, 645 U L⁻¹ in the 9th day. Higher concentration of SPFC (5% w/v) enhanced laccase production by the fungus up to 2034.354 U L⁻¹ in 9 days. Addition of SPFC above 25% (w/v) into culture medium made solid state condition and significantly enhance the production of laccase by the fungus. The crude laccase could effectively decolorize three types of chemical dyes: congo red (CR), brilliant blue G (BBG), coomassie brilliant blue (CBB), in a short period. The laccase could effectively remove three kind of tested dyes with various rates: 45, 91, 48%, respectively for CR, BBG and CBB in one hour reaction. Rapid removal of dyes, especially for BBG, by the laccase *T. hirsuta* LBF-AA017 has initially proven the potential to be applied for environmental biotechnology.

Keyword: laccase, lignocellulosic materials, white rot fungi, dyes removal, enzyme inducers

1. Introduction
Laccase is useful oxidative enzyme for various biotechnological and industrial applications. Laccases (EC 1.10.3.2), also named p-diphenol: dioxide oxidor eductases, are blue multicopper oxidases (MCOs) that can catalyze the oxidation of a wide variety of organic aromatic compounds, concomitantly with the reduction of molecular oxygen to water [1]. Laccases contain four or more copper atoms and can reduce dioxygen completely to water. It is difficult to categorize laccases based on the substrate reduced because of the wide range of compounds that can be catabolized. Laccases can change their redox potential ($E^0$), and so substrate type can change from one laccase to another. In general, laccase has a highly specific binding pocket for oxygen, but the binding pocket for reducing substrates appears to be shallow and relatively non-stereospecific. In fact, the catalytic behavior of laccases on most reducible substrates depends on the electron acceptor $E^0$ Cu T1. Thus, laccases with greater $E^0$ T1 are of special interest in biotechnology due to their greater potential to oxidize substrates with greater $E^0$ such as PAHs or various organic synthetic dyes [1][2]
Consequently, laccases are considered “ideal green” catalysts because they employ O₂ as a co-substrate and generate H₂O as a byproduct. In addition, reactions of the phenol compound selected as a natural substrate can increase the redox status of PAHs, leading to their incorporation into cellular material. Clearly, the ability of laccase to catalyze non-specific one-electron oxidations is likely to be relevant in PAH remediation. However, the influence of co-substrates in laccase-catalyzed reactions is of even greater interest in the context of improving PAH accessibility for remediation [2].

Laccase can be applied in many industries, especially as a natural degrader in chemical industries and bioremediation. Laccase has been involved in bleaching processes in the pulp industry, decolorization of synthetic dyes such as azo dyes, anthraquinone, triphenylmethane, etc, and also biosensor for phenolic compounds [3][4]. As biodegrader for recalcitrant pollutants, laccase and other ligninolytic enzymes such as manganese peroxidase (MnP) and Lignin Peroxidase (LiP) have been reported for effectively removing of toxic pollutants such as Polycyclic Aromatic Hydrocarbons (PAHs) and Persistent Organic Pollutants (POPs) [5].

White Rot Fungi (WRF), a basidiomycota group, have been proved to have highly potential ability on ligninolytic enzymes production [6][7]. They can produce Laccase, MnP, and LiP to degrade lignin and cellulose in their natural substrates [7]. These enzymes also play a role in physiological activity of fungi such as pigmentation and photogenesis [3]. Kumari et al., [8] reported that among ligninolytic enzymes, laccase has most important role in the lignin degradation.

In the present study, we used a new isolated WRF, *Trametes hirsuta* LBF-AA017, for laccase production. Local lignocellulosic materials such as bagasse, palm kernel cake (PKC), sugar palm fruit cake (SPFC), corn cobs (CC), and tobacco road (TR). Local lignocellulosic materials (containing lignin with various concentrations) have been expected to induce laccase production by the WRF. Effect of various concentrations of lignocellulosic materials used in the culture was also observed and resulted two types fermentation: submerged and solid state fermentation. We observed most suitable fermentation condition for the selected WRF. In the last step, we applied the enzyme for dye decolorization of three different dyes: congo red (CR), brilliant blue G (BBG), coomassie brilliant blue (CBB). We found the enzyme could effectively remove all dyes in different decolorization rates. High potency of laccase produced by LBF-AA017 for industrial application has encourage low cost production of the enzyme such as using local biomass.

2. Materials and Methods

2.1. Materials and Chemicals

CR and CBB were purchased from Merck Co., Ltd. (Tokyo, Japan). BBG was purchased from Sigma Aldrich Co., Ltd. Agar, glucose, malt extract, peptone were purchased from Himedia. Lignocellulosic materials was obtained from biomass collection from Laboratorium of Biocatalyst and Fermentation (LBF).

2.2. Microorganisms

The WRF strain used in this study was *Trametes hirsuta* LBF-AA017. The fungus was isolated from decaying woods in Cibinong Science Center (CSC), Cibinong, West Java. The fungus was cultivated at 25°C on 2% malt extract agar (MEA) for 7 days in a disposable plastic Petri dish and maintained at 4°C prior to use.

2.3. Effect of different lignocellulosic materials on laccase production in submerged culture

The experiment was conducted in a liquid medium at pH 4.5 (ME). Five different of lignocellulosic materials: bagasse, palm kernel cake (PKC), sugar palm fruit cake (SPFC), corn cobs (CC), and tobacco road (TR) with concentration 0.5% (w/v) were added into the medium and sterilized at 121°C and 15 minutes. Three plugs of the WRF was then inoculated into the Erlenmeyer flask and incubated
under dark conditions at 25°C for 3, 6, 9 and 15 days. The cultures were centrifuged for 20 minutes at 10,000 rpm. The supernatant were used for laccase analysis.

2.4. Effect of various concentration of SPFC on laccase production
To investigate the effect of various biomass concentrations for laccase production by LBF-AA017 concentrations were used: 0.5, 2, 5, 10, 25, and 50 % (w/v). Concentration 0.5-10% (w/v) resulted a submerged state condition and concentrations 25-50% (w/v) resulted a solid state condition.

2.5. Enzyme assays
Laccase activity was analyzed using syringaldazine as the substrate and extracellular enzymes in sodium acetate buffer [5]. Enzyme activities were expressed in units per gram (U g⁻¹) for the solid state treatment and units per liter (U l⁻¹) for the liquid treatment, defined as the amount of enzyme required to oxidize 1 µmol of substrate in one minute.

2.6. Decolorization of dyes on a submerged culture by crude laccase produced by T. hirsuta LBF-AA017
The experiment was conducted in a reaction tube with distilled water (@ 5mL). The required amount of dyes (50 ppm) was eluted in distilled water. Three different treatments were assayed: addition of CuSO₄, as laccase mediator (A), only crude laccase (B), control treatment without laccase addition (C). All treatment were incubated in a shaking incubator at 150 rpm and 25°C. Sampling for 1, 4, and 24 hours was conducted to investigate decolorization rate of dyes by the enzyme. All samples were analyzed using spectophotometre UV–VIS in different wavelength based on the measured dye. CR, BB, and CBB were analyzed at 496, 590, and 552 nm, respectively. Decolorization rate were measured based on [9]

2.7. Molecular identification of the fungus
Fungal strains were maintained on PDA and incubated for 7 days. DNA was extracted using genomic DNA extraction with quick DNA™ fungal/bacterial miniprep kit (Zymo Research, D6005). The extracted DNA was used as a template for PCR to amplify the ITS1-F (specific for higher fungi) and ITS4-B (specific for basidiomycetefungi) regions. Products were then sequenced using PCR primers and and the results of sequencing were compared with the National Centre for Biotechnology Information (NCBI) GenBank database.

3. Result and Discussion
Trametes hirsuta LBF-AA017 is a new isolated WRF from decaying wood in germplasm garden (CSC). Based on initial screening using congo red agar medium, the fungus could grow fast and decolorize the dye indicating the production of potential ligninolytic enzymes (unpublished data). The fungal body and mycelium of T. hirsuta were shown in Figure 1. According to a molecular gene analysis, strain LBF-AA017 was similar (99–100%) to Trametes hirsuta. A gel photo of PCR product and phylogenetic tree of T. hirsuta is shown in Figure 2 and 3, respectively.
Figure 1. Fungal body (a) and fungal mycelium (b) of *T. hirsuta* LBF-AA017.

Figure 2. Gel photo of PCR produc (**T. hirsuta** LBF-AA017).
Figure 3. A Phylogenetic tree of *T. hirsuta* LBF-AA017.
3.1. Effect of different lignocellulosic materials on laccase production in submerged culture

To observe the most suitable lignocellulosic materials for fungus LBF-AA017, 5 types of lignocellulosic support were evaluated in submerged culture containing malt extract medium at pH 4.5: bagasse, palm kernel cake (PKC), sugar palm fruit cake (SPFC), corn cobs (CC), and tobacco road (TR). The fungus could grow well in all medium with different growth rates. Table 1 shows composition of materials such as cellulose, hemicellulose, and lignin based on some references. PKC has been reported having highest lignin content up to 31%. In this study, correlation of high lignin content and high laccase production can be observed.

Table 1. Composition of selected lignocellulosic materials.

| Biomass Source | Cellulose (%) | Hemicellulose (%) | Lignin (%) | References |
|----------------|---------------|-------------------|------------|------------|
| SPFC           | 53.4          | 7.45              | 24.9       | [10]       |
| PKC            | 22.0          | 4.0               | 31.0       | [11]       |
| Tobacco road   | -             | -                 | -          | -          |
| Bagasse        | 48.6          | 31.1              | 19.1       | [12]       |
| Corn cobs      | 39.1          | 42.1              | 14.0       | [13][14]   |

Figure 4 shows the laccase activity in various lignocellulosic inducers at 3, 6, 9 and 15 days. Laccase was enhanced in all treatments compared with control (no lignocellulosic materials addition). Among treatments, enzyme activity in SPFC treatment tends to increase up to 15 days. Laccase activity in SPFC treatment were 79.4 U L\(^{-1}\), 376.09 U L\(^{-1}\), 645 U L\(^{-1}\), and 663.1 U L\(^{-1}\) for 3, 6, 9, and 15 days, respectively. In other treatments, laccase tends to decrease after 15 days. Laccase activity for bagasse, TR, PKC, and control were 307.4U L\(^{-1}\),137. 2U L\(^{-1}\), 374.09U L\(^{-1}\), and 179.3U L\(^{-1}\), respectively for 3, 6, 9, and 15 days. Laccase could be enhanced effectively by selected lignocellulosic materials with the rate 2-3 fold. The highest enhancement was more than 3-fold in 15 days for SPFC treatment. SPFC was selected as the most suitable for the LBF-AA017 and will be used for scaling up production. SPFC provides a major source of energy for the fungus having a high growth. Moreover, the use of this material could be a convenient condition for the industrial production and application of the selected WRF. This high growth in this support promotes their spread in media that are not usually natural environments. Dzul-Puc et al. [15] demonstrated that the use of lignocellulosic residues as substrate depends on their chemical content (lignin, hemicellulose, cellulose, carbohydrates, etc.), type of components and their structure. The component of biomass such as lignin and cellulose has important role as laccase activator for WRF [16]. In the present study, we found that lignin content in the sample has no positive correlation with laccase production by the fungus. Rizal et al. [11] reported that PKC commonly has high lignin content about more than 30%. However, in this study we found that SPFC, which is reported with lower lignin content, has higher laccase activity. Murugesan et al [3] mentioned that phenolic compounds in the lignocellulosic materials might cause high laccase production by WRF.

Based on Levin et al. [6] malt extract medium has been reported on laccase enhancement by WRF. In the present study, we combined malt extract medium and local materials to observe their ligninolytic system. Malt extract provide more digestible nutrients for the fungus in the initial growth. WRF can produce extracellular laccase in both solid state and submerged culture. However, Ire and Ahuekwe [17] reported that submerged culture produced more laccase activity than using solid state fermentation. Separation of enzymes and mycelium in the submerged culture is also easier than solid state fermentation, by using centrifugation. However, laccase produced by solid state fermentation must be extracted prior to centrifugation.
3.2. Effect of various concentration of SPFC on laccase production

SPFC was selected as the most suitable biomass for laccase production by LBF-AA017. In the present study, we observed effect of various biomass concentration for laccase production by the fungus. Various concentrations of SPFC were added into culture medium: 0.5, 2, 5, 10, 25 and 50% (w/v). Concentration 0.5-5% (w/v) resulted submerged culture condition, concentration 10% (w/v) resulted semi solid state fermentation, concentration 25 and 50% (w/v) resulted solid state fermentation. The cultures were incubated for 3, 6, 9 and 15 days, except for solid state fermentation (only incubated for 15 days). Mycelium growing to cover all over medium in the solid state needs longer time than submerged culture.

Figure 4. Laccase activity using 5 lignocellulosic materials concentration 0.5% (w/v).

Figure 5. Laccase activity in various concentrations of SPFC.
Figure 5 shows highest laccase (more than 2500 UL⁻¹) in 15 days using 50% (w/v) biomass, a solid state fermentation. The laccase activity in 15 days for 0.5, 2, 5, 10 and 25% (w/v) in the order 66 3.1 UL⁻¹, 1562.03 UL⁻¹, 2034.4 UL⁻¹, 207.8 UL⁻¹ dan 1759.9 UL⁻¹. In the solid state fermentation, we diluted each 5 grams sample into 30 mL distilled water, and then measured the laccase activity (with dilution factor=6). The actual laccase activity for the treatment 25 and 50% (w/v) in the order are 15000 and 10559 U g⁻¹. In the present study, addition more lignocellulosic materials as inducer has been proved on enhancement of laccase activity. Ire and Ahuekwe [17] also reported that laccase production under static condition (solid state fermentation) produced higher laccase than under submerged culture condition. Solid state fermentation provide a natural condition for WRF to grow and the fungus can secrete the enzyme in optimum condition [18] Solid state fermentation also create more stabil condition for WRF to grow and produce more extracellular enzymes [19].

3.3. Decolorization of dyes on a submerged culture by crude laccase produced by LBF-AA017

Laccase belongs to oxidoreductase group and has important role in biotechnology industry such as for degradation of synthetic dyes in textiles, pulp, and cosmetics industries [20][21]. Synthetic dyes for industry are commonly xenobiotics compounds, recalcitrant, and toxic for ecosystem [20] than other physical or chemical methods [21].

The benefits of using laccase than other ligninolytic enzymes such as MnP and LiP are no need H₂O₂ for starting reaction and no specivity of substrate. Various organic pollutants such as diphenol, poliphenol, diamin, amino acids, aromatic dan benzenthiol can be degraded by the enzyme. In the present study, we used three different type of dyes: CR, BB and CBB [21] Table 2 shows the classification of the dyes. The appearances and structures can be seen in Figure 6 and 7.

| Name                  | Classification   |
|-----------------------|------------------|
| Panseu-S              | Diazo            |
| Methylene blue        | Heterocyclic     |
| Congo red             | Diazo            |
| Bromphenol blue       | Triphenylmethane |
| Commassie brilliant blue | Triphenylmethane |
| Remazol Brilliant Blue R (RBBR) | Anthraquinone |

Ref.: [21]

Figure 6. A) Coommasie Brilliant Blue, B) Congo Red dan C) Bromophenol Blue.
Azo is one of the biggest group of dye and has various colors. The dye is designed from a complex polymer and Azo dyes are hardly to degrade due to their complex structures. One of the azo dyes is congo red which reported as recalcitrant dye due to its amina aromatic structure [22].

![Chemical structure of Congo Red, Bromophneol Blue dan Coommasie Brilliant Blue](image)

Figure 7. Chemical structure of a) Congo Red, b) Bromophneol Blue dan c) Coommasie Brilliant Blue [23].

Figure 8 shows the result of decolorization in various incubation time for 3 different dyes. CR could be decolorized in 1, 4, and 24 h in the order 45, 49, and 75%. These results have no significant difference with no CuSO₄ addition. BB could be decolorized in 1, 4, and 24 h in the order 91.4, 92, 94.46%. These results were lower compared with no CuSO₄ addition treatment for 4 and 24 h: 93.16 and 95.27%, respectively. CBB resulted lowest decolorization among tested dyes, in 1, 4, and 24 h, in the order are 54, 56 and 53%. In control, there were no decolorization change after 24 h (decolorization rate=0%).

Based on the result, among three dyes tested, laccase from *T. hirsuta* LBF-AA017 could decolorize BB most optimum with the decolorization rate more than 91%. BB belongs to triphenylmethane group and well-known as recalcitrant dye group [21]. However, in the present study, BB could be effectively decolorized enzymatically by laccase from LBF-AA017. BB is easily to degrade related to its structure, difference in electron distribution and charge density [24]. Yang et al., [20] also reported that laccase could degrade BB in a short period, only 4 hours. CBB, similar with BB, is a triphenylmethane group. However, the decolorization rate was lower than BB. Degradation of CBB by
laccase needs longer time compared with BB due to its resistancy to enzyme [21]. CR could be decolorized up to 75% after 24 hours. CR is also one kind of dyes which is difficult to degrade enzimatically due to its amino aromatic functional group [22]. Laccase can degrade azo binding in CR and result clear solution after treatment [9].

![Figure 8](image)

**Figure 8.** Decolorization of 3 synthetic dyes by crude laccase from LBF-AA017.

The dye solution after treatment can be seen in Figure 9. In the present study, addition CuSO₄ to increase ability of laccase on dye decolorization could not work effectively. CuSO₄ contains Cu²⁺ ion that has important role as cofactor in the catalysis core of laccase. Addition of this ion has been expected on enhancement of laccase activity. However, in the present study, the effect did not occur. The phenomenon might be cause the utilization of crude type of laccase. The utilization of crude type of enzyme caused Cu³⁺ could not work properly [24].

![Figure 9](image)

**Figure 9.** Comparison of dye solution before and after treatment using crude laccase from *T. hirsuta* LBF-AA017.

4. Conclusion

Among 5 materials tested, treatment using SPFC resulted highest laccase production, 645 U L⁻¹ in the 9th day. Higher concentration of SPFC (5% w/v) enhanced laccase production by *Trametes hirsuta* LBF-AA017 up to 2034.354 U L⁻¹ in 9 days. Addition of SPFC above 25% (w/v) into culture medium made solid state condition and significantly enhance the production of laccase by the fungus up to
15,000 U/g. The crude laccase could effectively decolorize three types of chemical dyes: congo red (CR), brilliant blue G (BBG), coomassie brilliant blue (CBB), in a short period. The laccase could effectively remove three kind of tested dyes with various rates: 45, 91, 48%, respectively for CR, BBG and CBB in one hour reaction. Rapid removal of dyes, especially for BBG, by the laccase LBF-AA017 has initially proven the potential to be applied for environmental biotechnology.

5. References

[1] Rivera-Hoyos C M, Morales-Alvarez E D, Poutou-Pinales R A, Pedroza-Rodriguez A M, Rodriguez-Vazquez R, Delgado-Boada J M 2013 Fungal Biol Rev 27 67
[2] Gadd G M 2001 Fungi in Bioremediation (Cambridge University Press, The Edinburgh Building, Cambridge CB2 8RU, UK)
[3] Murugesan K, Manavalan A, Nam I, Kim Y, Chang Y S and Kalaichelvan P T 2006 Appl. Microbiol. Biotechnol. 72 939
[4] Yu G, Wen X, Li R and Qian Y 2006 Process Biochem. 41 1987
[5] Andriani A, Tachibana S and Itoh K 2016 World J. Microbiol. Biotechnol. 32 39
[6] Levin L, Herrmann C, and Papinutti VL 2008 Biochemical Eng. J 39 207
[7] Revankar MS and Lele SS 2006 Process Biochem. 41 581
[8] Kumari B, Upadhayay RC and Atri NS 2012 World J. Agric. Sciences 8 409
[9] Zille A, Gornacka B, Rehorek Aand Cavaco–Paulo A 2005 Appl. Environ. Microbiol. 71 6711
[10] Ishak M R, Sapuan S M, Lerman Z, Rahman M Z A and Anwar U M K 2012 Therm. Anal. Calorim. 109 981
[11] Rizal N FA A, Ibrahim M F, Zakaria M R, Abd-Aziz S, Yee P L and Hassan M A 2018 Molecules 23 1381
[12] De Souza A P, Leite D C C, Pattahil S, Hahn M G, and Buckeridge M S 2012 Bioenergy Research 6 564
[13] Kumar S, Jugmendra S U, and Negi Y S 2010 BioResources 5 1292
[14] Ashour A, Amer M, Marzouk A, Shimizu K., Kondo R and El–Sharkawy S 2013 Molecules 18 13823
[15] Dzul-Pue J D, Esparza-Garcia F, Barajas-Aceves M, Rodriguez-Vazquez R 2005 Chemosphere 58 1
[16] Osma J F, Moilanan U, Toca–Herrera J L and Rodriguez–Couto S 2011 FEMS Microbiol. Lett. 318 27
[17] Ire FS, Ahuekwe E F 2016 British Microbiol. Research J 15 1
[18] Rodriguez-Couto S, Sanromán M 2006 J. Food Eng. 76 291
[19] Mazumder S, Basu S K, Mukherjee M 2009 Eng. Life Sci. 9 45
[20] Yang X Q, Zhao X X., Liu C Y, Zheng Y and Qian S J 2009 Process Biochem. 44 1185
[21] Forootanfar H, Moezzi A, Aghaie–Khozani M, Mahmoudjanlou Y, Ameri A, Niknejad A and M, Ali Faramarzi MA 2012 Iranian J. Environ. Health Sciences Eng 9 27
[22] Bhattarcharya S, Das A, Mangai G, Vignesh K, and Sangeetha J 2011 Brazilian J. Microbiol. 42 1526
[23] Bumpus, JA 2004 Biodegradation of Azo Dyes by Fungi (Marcel Dekker, Inc.)
[24] Zhau R, Li M, Fan F, Gong Y, Wan X, Jiang M, Zhang X and Yang Y 2011 J. Hazardous Materials 192 855

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