Decolorization of synthetic textile dyes by laccase from newly isolated *Trametes hirsuta* EDN084 mediated by violuric acid

D H Y Yanto\(^1\), N Auliana\(^2\), S H Anita\(^1\) and T Watanabe\(^3\)

\(^1\) Research Center for Biomaterials, Indonesian Institute of Sciences (LIPI), Jl. Raya Bogor Km. 46, Cibinong Science Center, Cibinong, Bogor 16911, Indonesia
\(^2\) Departement of Biochemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University Dramaga, Bogor 16680, Indonesia.
\(^3\) Research Institute for Sustainable Humanosphere, Kyoto University Gokasho, Uji, Kyoto 611-0011, Japan

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

**Abstract.** Decolorization of synthetic dyes by laccase produced from newly isolated *Trametes hirsuta* EDN084 under in-vitro condition was investigated in this study. Partial purification was conducted using ultrafiltration Amicon 10K at 500 0 x g for 20 min. Laccase (0.1 U/mL) could decolorize 50% remazol brilliant blue R (RBBR), 47% reactive blue 4 (RBlue4), 51% acid blue 129 (AB129), 21% acid blue 25 (AB25), 40% acid blue 113 (AB113), 11% acid orange 7 (AO7), 2% reactive black 5 (RBlack5), 2% reactive red 120 (RR120), and 85% direct blue 71 (DBlue71) for 4 h. In order to improve the decolorization, the addition of violuric acid (VA), 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO), and 1-hydroxybenzotriazole (HBT) were individually assayed. The result showed that decolorization was improved significantly (2 to 30-fold) after the addition of 1 mM VA. This study suggests that mediator VA is suitable for the enhanced decolorization of synthetic dyes by laccase from *T. hirsuta* EDN084.

1. **Introduction**

The textile industry uses approximately 10,000 different dyes, pigments, and dye precursors. It is estimated that 7 x 10\(^5\) to 1.3 x 10\(^6\) tons of synthetic dyes are annually produced worldwide [1, 2]. The textile dyeing and finishing industry have created a huge pollution problem. The world bank estimates that 17 to 20 percent of industrial water pollution comes from textile dyeing and finishing treatment given to fabric. Up to 200,000 tons of these dyes are lost to effluents every year during the dyeing and finishing operations. Some 72 toxic chemicals have been identified in water solely from textile dyeing, 30 of which cannot be removed [2, 3].

Effluent treatment methods can be classified into physical, chemical, and biological methods [3]. Physical and chemical methods can improve air quality in a short time, but costly and produce large amounts of toxic iodine, aromatic amines, and residual secondary products [4]. Biological treatment methods have been more appropriate and widely used because of their cost-benefit ratio. Microbiological decolorization can occur through biosorption, enzymatic degradation, or a combination of both. The effectiveness of microbial decolorization depends on the adaptability and activity of the microorganisms selected [5].

Many reports have shown that bacteria can be used for dye decolorization and degradation. However, bacteria generate a reductive product of dye degradation such as aromatic amines which are potentially...
hazardous to a living organism. Bacteria are also unable to degrade the dyes efficiently due to the larger size of dyes. White rot fungi also can produce oxidoreductase enzyme including lignin peroxidase, manganese peroxidase, and laccase. These enzymes oxidize in a nonspecific way both phenolic and nonphenolic lignin derivatives. Therefore, these enzymes are a promising candidate for degradation of environmental pollutants, such as phenols, dyes, lignocelluloses, polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs). Nowadays, the oxidative enzymes, especially laccase are attributed to biodegradation of several persistent compounds such as dyes, pesticides, and lignin derivatives [6–8].

Thus, the objective of this research was to evaluate the ability of laccase enzymes from newly isolated *Trametes hirsuta* EDN084 to decolorize various dyes. The effect of enzyme mediators, such as violuric acid (VA), 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO), and 1-hydroxybenzotriazole (HBT and violuric acid (VA), on dye decolorization was also investigated.

2. Materials and methods

All experiment was conducted in the Laboratory of Biomass Conversion Technology and Bioremediation, Research Center for Biomaterials, LIPI. Malt extract, hipolypeptone, glucose, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), violuric acid (VA), 1-hydroxybenzotriazole (1-HBT), TEMPO, and acetate buffer (pH 4.5) were purchased from Wako Pure Chemical Industries, L Ltd. (Japan). The test dye substrates such as Remazol Brilliant Blue R, Acid Blue 25, Acid Blue 113, Acid Blue 129, Acid Orange 7, Reactive Blue 4, Reactive Red 120, Reactive Black 5, and Direct Blue 71 were purchased from Sigma-Aldrich.

2.1. Microorganism

*Trametes hirsuta* EDN084, newly white rot fungi isolated from Taman Eden 100, North Sumatera, Indonesia was used in this study. This strain was maintained on malt extract agar (MEA), stored at 4 °C and subcultured every two weeks.

2.2. Laccase production

*Trametes hirsuta* EDN084 was cultured in a medium consisting of malt extract 20 g/L, glucose 20 g/L, and hipolypeptone 1 g/L. The mixture was adjusted to pH 4.5 and autoclaved at 121 °C for 15 min. Three plugs of the fungus from the MEA were added to the mixture and incubated at room temperature for 7 d. After incubation, the mixture of medium and mycelial fungus was homogenized at 10,000 rpm for 10 min using an ACE AM-11 homogenizer (Nissei, Japan). Supernatant then was ultra-filtrated using Amicon 10,000 kDa at 5000 x g, 4 °C for 20 min.

2.3. Determination of laccase activity

Laccase activity was measured using UV spectrophotometer (UV-1800 Shimadzu) with 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate. The nonphenolic dye ABTS was oxidized by laccase to the more stable and preferred state of the cation radical. The concentration of the cation radical, which is responsible for the intense blue-green color, can be correlated to enzyme activity and is read at 420 nm. Purified laccase (100 μL) was mixed into 1 mL cuvette containing 500 μL ABTS 2 mM and 400 μL sodium acetate buffer 0.1 M (pH 4.5). The activity was measured for 5 min with triplicate measurement. Enzyme activity (U/L) was calculated according to Eq. (1) with molar absorptivity (ε) of 36,000 M⁻¹cm⁻¹.

\[
\text{Enzyme activity} = \frac{(Abs/ε) \times (V_{mixture} \mu L \times 10^6) \times 10^6 \times (60/t) \times (V_{enzyme} \mu L \times 10^3)}{V_{mixture} \mu L \times 10^6}
\]  

2.4. The effect of mediators on decolorization of dyes

Reactive black 5 (RB5) with a final concentration of 100 mg/L was mixed in the sodium acetate buffer 0.1 M pH 4.5. Three kinds of the mediator, 1-hydroxybenzotriazole (1-HBT), TEMPO, and violuric acid (VA) as much as 100 μL with final concentration 0.1, 0.5, 1, 2, 3, 4, and 5 mM in acetate buffer 0.1M pH 4.5 were added, respectively to the RB5 solution. Decolorization was applied to 500 μL of RB5
dyes, 300 μL of acetate buffer 0.1 M pH 4.5, and 100 μL of purified laccase. Decolorization other dyes was also analyzed using the best mediator in optimum concentration that was obtained before.

2.5. Decolorization assay

The decolorization process was measured after 1 h and 4 h incubation with UV spectrophotometer (UV-1800 Shimadzu) at the λ max of each dye (table 1). The maximum wavelength was obtained by scanning each dye at a wavelength between 300 to 700 nm. Each decolorization experiment was performed in triplicate, and the mean of the decolorization percentages was reported. The percentage of decolorization was calculated using equation (2).

\[
\text{Decolorization (\%)} = \frac{A_i - A_f}{A_i} \times 100
\]

| Dye                  | λ max (nm) | Dye                  | λ max (nm) |
|----------------------|------------|----------------------|------------|
| Remazol Brilliant Blue R/RBBR | 592.5      | Reactive Black 5 (RBlack5) | 598        |
| Acid Blue 25 (AB25)  | 603.5      | Reactive Red 120 (RR120) | 512        |
| Acid Blue 129 (AB129)| 629        | Acid Blue 113 (AB113)  | 543        |
| Reactive Blue 4 (RBlue4) | 599.5      | Direct Blue 71 (DBlue71) | 641.5      |
| Acid Orange 7 (AO7)  | 482.5      |                      |            |

3. Result and discussion

Laccase produced from newly isolated *Trametes hirsuta* EDN084 under in vitro condition was investigated in this study. The laccase enzyme activity after partial purification was 0.1 U/mL and used for decolorization various dyes including anthraquinone (RBBR, Acid Blue 25, Acid Blue 129, Reactive Blue 4), monoazo (Acid Orange 7), diazo (Reactive Black 5, Reactive Red 120, Acid Blue 113), and triazo (Direct Blue 71).

Decolorization of dyes by using laccase enzyme produced by *T. hirsuta* EDN084 was evaluated for 1 h and 4 h incubation. The result showed that the decolorization increased with time incubation. The decolorization of anthraquinone dyes was 50.54%, 21.23%, 52.20%, and 46.65% for RBBR, AB25, AB129, and RBlue4 respectively at 4 h incubation (figure 1). The optimum time required to decolorize dye significantly depends on the type and chemical structure of dyes [9]. The results showed that AB129 was the most rapidly decolorized, while AB25 was difficult to be decolorized. Decolorization level of anthraquinone dyes was different because of the chemical structure of dyes. Chulhwan et al. [10] explained that even small structural differences of dyes are expected to affect the decolorization level of dyes. Chemical structure of anthraquinone dyes used in this study is shown in table 2.

![Figure 1. Decolorization of anthraquinone dyes.](image-url)
Table 2. Chemical structure of anthraquinone dyes used in this study.

| Dyes       | Structure | References |
|------------|-----------|------------|
| RBBR       | ![RBBR Structure](image) | [11]       |
| Acid Blue 25 | ![Acid Blue 25 Structure](image) | [12]       |
| Acid Blue 129 | ![Acid Blue 129 Structure](image) | [13]       |
| Reactive Blue 4 | ![Reactive Blue 4 Structure](image) | [14]       |

Decolorization level of azo dyes was higher at 4 h incubation. Figure 2 shows that laccase enzyme produced by *T. hirsuta* EDN 084 decolorize AO7, RBlack5, RR120, AB113, and DBlue71 was as much as 11.50%, 1.98%, 2.41%, 39.58%, and 58.90%, respectively.

Decolorization of azo dyes was more difficult than anthraquinone dyes. Structural bonding in dyes can affect the ability of laccase enzyme in dye decolorization. Anthraquinone has a stable conjugate bond system. When laccase enzyme attacks the anthraquinone dyes, electrons derived from the enzyme are transferred to a structure that has conjugate bonds. It changes the configuration of the dye structure and reduces the color intensity. Whereas azo dyes have a very strong bond between the chromophore group and auxochrome groups so they are very difficult to be decolorized by laccase enzyme [15]. Chemical structure of azo dyes used in this study is shown in table 3.

![Decolorization of Azo Dyes](image)
Table 3. Chemical structure of azo dyes used in this study.

| Dyes                | Structure | References |
|---------------------|-----------|------------|
| Acid Orange 7       | ![Structure](image1.png) | [16]       |
| Reactive Black 5    | ![Structure](image2.png) | [17]       |
| Reactive Red 120    | ![Structure](image3.png) | [18]       |
| Acid Blue 113       | ![Structure](image4.png) | [19]       |
| Direct Blue 71      | ![Structure](image5.png) | [20]       |

Figure 3. Optimization of enzyme mediators.

Azo dyes have a complex chemical structure, so they are difficult to be degraded by laccase directly. The laccase reactivity decreased with the increase of the substrate size; therefore the limited substrate accessibility was overcome through the use of appropriate laccase mediators. A mediator is a small chemical compound that is continuously oxidized by the laccase enzyme and subsequently reduced by
the substrate. Mediators act as an electron shuttle for large substrates that cannot access the active site of the enzyme [21, 22]. Mediators such as VA, TEMPO, and HBT were used in this study. From the results, the decolorization of RB5 by addition of VA was higher than the one achieved with the addition of other mediators. The use of VA at concentrations above 1% did not increase the dye decolorization (figure 3).

The addition of 1 mM VA enhanced the decolorization rate of the dyes 2 to 30-fold compared to the rate without mediators. The decolorization of anthraquinone dyes significantly increased to 78.73%, 69.97%, 85.76%, and 83.57% for RBBR, AB25, AB129, and RBlue4 respectively at 4 h incubation (figure 4). VA could enhance the decolorization of azo dyes mainly for RBlack 5 and RR120 (figure 5). VA was one of the most effective redox mediators for laccase oxidation compared to the natural mediators [23]. Anita et al. [24] also reported that the addition of 2 mM VA could enhance the dye decolorization rate up to 10-fold.

4. Conclusion

Laccase produced from T. hirsuta EDN084 showed the capability to degrade anthraquinone and azo dyes. This study suggests that mediator VA is suitable for the enhanced decolorization of synthetic dyes by laccase from T. hirsuta EDN084. Decolorization improved significantly (2 to 30-fold) after addition of 1 mM VA.

Acknowledgments

Part of this research was supported by LIPI through DIPA of RC Biology 2018 at IBSAP (Indonesian Biodiversity Strategy and Action Plan) project and by RISH Kyoto – University, Japan through WP3 Japan ASEAN Science Technology and Innovation Platform (JASTIP) Project.

5. References

[1] Sengupta S, Singh BR 2003 Natural “green” dyes for the textile industry. Toxics Use Reduction Institute, University of Massachusetts Lowell, 12 page
[2] Chequer FMD, De Olivieira GAR, Ferraz ERA, Cardoso JC, Zanoni MVB, de Oliveira DP 2013 Textile Dyes: Dyeing process and environmental impact. In Ecofriendly textile dyeing and finishing. M. Gunay (ed.). Chapter 6 pp 151 ISBN 978-953-51-0892-4
[3] Kant R 2012 Textile dyeing industry an environmental hazard. Natural Science 4(1): 22-26
[4] Turhan K, Durukan L, Ozturkan SA, Turgut Z 2012 Decolorization of textile basic dye in aqueous solution by ozone. Dyes Pigments 92(3): 897-901
[5] Raphael LAS, Romeo MPBC, Glenda HGMSP, Felype TB, Raquel PB, Ana Lucia FP, Marcia VS 2018 Fungi of biotechnological interest in the discoloration of textile effluents. Trends in Engineering & Fashion Technology 4(3): TTEFT.000587
[6] Rodríguez E, Pickard MA, Vazquez-Duhalt R 1999 Industrial dye decolorization by laccases from ligninolytic fungi. *Current Microbiology* 38: 27-32

[7] Bergsten-Torralba LR, Nishikawa MM, Baptista DF, Magalhaes DP, da Silva M 2009 Decolorization of different textile dyes by Penicillium simplicissimum and toxicity evaluation after fungal treatment. *Brazilian Journal of Microbiology* 40: 808-817

[8] Singh MP, Vishwakarma SK, Srivastava AK 2013 Bioremediation of direct blue 14 and extracellular ligninolytic enzyme production by white rot fungi: *Pleurotus* spp. *Biomed Research International* Article ID 180156, 4 pages

[9] Sudiana IK, Sastrawidana DK, Sukarta IN 2018 Decolorization study of remazol black B textile dye using local fungi of *Ganoderma* sp. and their ligninolytic enzymes. *Journal of Environmental Science and Technology* 11(1): 16-22

[10] Chulhwan P, Lee Y, Kim T-H, Lee B, Lee J, Kim S 2004 Decolorization of three acid dyes by enzymes from fungal strains. *J. Microbiol. Biotechnol* 14(6):1190-1195

[11] Alrozi R, Anuar NS, Senusi F, Kamaruddin MA 2016 Enhancement of Remazol Brilliant Blue R adsorption capacity by using modified clinoptilolite. *Iranica Journal of Energy & Environment* 7(2): 129-136

[12] Badii K, Ardejani FD, Saberi MA, Lmaee NY, Shafaei SZ 2010 Adsorption of acid blue 25 dye on diatomite in aqueous solutions. *Indian Journal of Chemical Technology* 17: 7-16

[13] Palencia M, Martinez JM, Arrieta A 2017 Removal of acid blue 129 dye by polymerenhanced ultrafiltration (PEUF). *J. Sci. Technol. Appl.* 2: 65-74

[14] Becelic-Tomin M, Dalmacija B, Rajic L, Tomasevic D, Kerkez D, Watson M, Prica M 2014 Degradation of anthraquinone dye reactive blue 4 in pyrite ash catalyzed fenton reaction. *The Scientific World Journal* Article ID 234654, 8 pages

[15] Dewi RS, Lestari S 2010 Decolorization of textile wastewater using isolated indigenous fungi with different wastewater concentration (in Indonesian). *Molekul* 5(2): 75-82

[16] Akazdam S, Chafi M, Yassine W, Gourich B 2017 Removal of acid orange 7 dye from aqueous solution using the exchange resin amberlite FPA-98 as an efficient adsorbent: kinetics, isotherms, and thermodynamics study. *Journal of Materials and Environmental Sciences* 8(8): 2993-3012

[17] Nabil GM, El-Mallah NM, Mahmoud ME 2014 Enhanced decolorization of reactive black 5 dye by active carbon sorbent-immobilized-cationicsurfactant (AC-CS). *J. Ind. Eng. Chem.* 20(3):994-1002

[18] Bazrafshan E, Ferdos KM, Ali RH, Atoalah RK, Amir HM 2013 Decolorisation of reactive red 120 dye by using single-walled carbon nanotubes in aqueous solutions. *Journal of Chemistry* Article ID 938374, 8 pages

[19] Karimi A, Fatemeh M, Mohammadreza E 2012 Enzymatic in-situ generation of H2O2 for decolorization of acid blue 113 by fenton process. *Chemical Industry & Chemical Engineering Quarterly* 18(1): 89-94

[20] Maleki A, Nasseri S, Hadi M, Solaimany AM 2015 Discoloration of aqueous direct blue 71 solution using UV/H2O2/nano-SiO2 process. *Int. J. Environ.Res.* 9(2):721-734

[21] Christopher LP, Yao B, Ji Y 2014 Lignin biodegradation with laccase-mediator systems. *Frontiers in Energy Research* 12:1-8

[22] Mani P, Kumar VTF, Keshavarz T, Chandra TS, Kyazze G 2018 The role of natural laccase redox mediators in simultaneous dye decolorization and power production in microbial fuel cells. *Energies* 11(12):3455

[23] Hu MR, Chao YP, Zhang GQ, Xue ZQ, Qian S 2009 Laccase-mediator system in the decolorization of different types of recalcitrant dyes. *J. Ind. Microbiol. Biotechnol.* 36(1): 45-51

[24] Anita SH, Sari FP, Yanto DHY (2019) Decolorization of synthetic dyes by ligninolytic enzymes from *Trametes hirsuta* D7. *Makara Journal of Sciences* 23(1): 44-50