Decolorization Assay of the Anthraquinone Dye Acid Blue 25 by Trichoderma asperellum LBKURCC1 Crude Laccase Extracts

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Abstract. One major concern of the textile industry waste is the health hazard imposed by textile dye waste effluents. Anthraquinone dyes are the second largest group of dyes produced and used annually worldwide, that is difficult to degrade naturally. Biological methods using enzymes for waste treatment is gaining popularity due to its eco-friendliness. Laccase is an enzyme with potential to degrade textile dyes, due to its wide ability to oxidize a wide range of substrates. The aim of this study was to evaluate the ability of T. asperellum LBKURCC1 laccase crude extract to decolorize the anthraquinone anionic dye Acid Blue 25 (AB25). A solution of 50 ppm AB25, pH 5.5, was treated with T. asperellum LBKURCC1 laccase crude extract and incubated at room temperature. Absorbance of the solution at 603 nm was measured daily and compared to buffer and heat denatured enzyme controls. No decolorization of AB25 was observed up to 6 days incubation in the enzyme treated samples, as well as the controls. Addition of 0.1 to 5 mM of 1-hydroxybenzotriazole hydrate (HBT) to the decolorization assay did not succeed in mediating the redox reaction of AB25 oxidation by the T. asperellum LBKURCC1 laccase.

1. Introduction
In the aim to become a global top-10 economy by 2030, the Republic of Indonesia government has in 2018 launched a road map to prioritize value-added manufacturing industries. The road map focuses on five chosen industries, which includes the textile and apparel industry [1]. This focus to prioritize growth in the textile manufacture leads to the prediction of increased textile industry waste in Indonesia that will need waste management attention. One major concern of the textile industry waste is the health hazard imposed by textile dye waste effluents. Approximately 10-15% of synthetic dyes used in the dying process of textile is disposed in its waste effluent [2]. Common synthetic dyes used in industry, are from the azo- and anthraquinone group of dyes. These dyes pose health hazards if untreated and thrown into the natural water body, as they are carcinogenic and mutagenic [3].
Anthraquinone dyes are the second largest group of dyes produced and used annually worldwide. Because of its structure with three fused benzene rings and two carbonyl groups on the middle ring, it is difficult to degrade naturally. The presence of sulfone groups in many anthraquinone dyes make them even more difficult to degrade [4].

Current waste water treatment plants by large scale textile manufacturers use physico-chemical methods, that are costly due to the use of chemicals and energy, and often produce sludge that need further treatment [5]. Biological methods for removal of dyes from textile wastewater offers an ecofriendly alternative. These biological methods include the use of life microbial cultures or the use of enzymes. However the use of life microbial cultures to degrade textile dyes require the addition of nutrition, and controlled or optimal growth conditions for the microbes [6]. On the other hand, degradation of anthraquinone dyes by the enzyme laccase has proven feasible and more versatile in its application [7]. Laccases catalyze the oxidation of phenolic and non-phenolic aromatics, using oxygen as the electron acceptor. Laccases work on a wide range of substrates, and it catalyze oxidation of substrates with and without redox mediators [8]. Most studies on the degradation of anthraquinone dyes by laccases, have involved laccase enzymes produced by basidiomycetes (fruiting body fungus), such as *Ganoderma lucidum* [9], *Trametes hirsuta* [10], *Marasmius cladoophylus* [11] and *Lentinus criticus* [2]. Degradation of anthraquinone dyes by cyanobacteria *Spirulina platensis* [12] and *Arthrospira maxima* [13], and by the bacteria *Streptomyces ipomoeae* [14] laccase have also been reported.

Decolorization of dyes, are a good indication of dye degradation. Nugroho et al. [15] reported that life cultures of *Trichoderma asperellum* LBKURCC1 (formerly identified as *Trichoderma asperellum* TNC52) could degrade the anthraquinone dye Color Index Reactive Blue 5 (CIRB5). *T. asperellum* LBKURCC1 is a filamentous ascomycetes fungal strain isolated from the rhizosphere of cacao at a cacao plantation in Riau, Sumatra, Indonesia [16]. *T. asperellum* LBKURCC1 produces laccase using rice straw as its inducer, in a solid state fermentation system [17]. Although producing laccase, no direct studies have been done showing the ability of *T. asperellum* LBKURCC1 crude laccase extracts to degrade anthraquinone dyes. In this paper we report our study on the activity of *T. asperellum* LBKURCC1 laccase to decolorize the anthraquinone anionic dye Acid Blue 25 (AB25) (figure 1). AB25 is used in many industrial processes to dye not only textile, but also leather goods and aluminum finishing. It is often used as a model system for anthraquinone anion dyes that contain sulfone groups [18]. The laccase activity on AB25 as a substrate was studied without and with the addition 1-hydroxybenzotriazole (HBT) as a redox mediator.

![Acid Blue 25 structure](image)

**Figure 1.** Acid Blue 25 structure
2. Methodology

2.1. Enzyme and chemicals
Laccase used in this study was a crude laccase extract produced by *T. asperellum* LBKURCC1 in a solid state fermentation system using rice stalk as the laccase inducer, with combined optimized parameters as described by Rahayu *et al.* [17] and Sellyna *et al.* [19]. The crude laccase extract was prepared following the method described by Rahayu *et al.* [17]. Acid Blue 25 (AB25) was from Sigma-Aldrich (Cat. No. 210684). Also purchased from SIGMA-Aldrich Inc. was the chemicals 1-hydroxybenzotriazole hydrate (HBT) (Cat. No. 54802) and 2,2’-azino-di-3-ethylbenzotiazol-6-sulfonate (ABTS) (Cat. No. G5502).

2.2. Laccase activity assay
Activity of crude laccase extracts were determined following the procedure as described by Agrawal and Verma [20], with ABTS as the laccase substrate. All enzyme activity assays were performed in 1 mL reactions, consisting of 5 mM ABTS as the substrate at a buffer pH of 5.5, and incubated for 5 minutes at room temperature (±30°C). As controls, heat denatured enzyme extracts were used. Absorbance of the reaction mixtures and controls were measured at 420 nm at times 0 (A_{0c} for enzyme reactions, A_{0e} for controls), and 5 minutes (A_{c} for enzyme reactions, and A_{e} for controls), and laccase activity was calculated using equation (1). \( V_{\text{tot}} \) is the total reaction volume, \( V_e \) is the crude enzyme volume added to the reaction mixture, \( D_f \) is enzyme dilution factor, \( d \) is the optical path length (1 cm), and \( \varepsilon_{420\text{nm}} \) is ABTS molar extinction coefficient at 420 nm (36,000 M\(^{-1}\) cm\(^{-1}\)).

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\text{Laccase Activity} = \frac{(A_{t} - A_{0t}) - (A_{c} - A_{0c}) \times V_{\text{tot}} \times D_f}{\varepsilon_{420\text{nm}} \times V_e \times d \times 5}
\] (1)

2.3. Acid Blue 25 decolorization assay.
Decolorization assay reactions were carried out in solutions of 50 ppm AB25 and laccase crude extracts giving a final activity concentration of 0.021 U/mL in 0.05 M sodium acetic buffer pH 5.5. The assay reaction was incubated at room temperature (±30°C), in the dark, without shaking. As controls, heat denatured laccase was used, and labelled as deactivated enzyme control. A buffer control was also incubated. The buffer control consisted of 50 ppm AB25 in buffer without addition of laccase crude extracts. At the indicated time points absorbance of the reaction assay solution was measured at AB25’s maximum wavelength (603 nm) with a UV-Vis Thermo Scientific Genesys 10S spectrophotometer. For assays with addition of a redox mediator, as mediator different concentrations of 1-hydroxybenzotriazole (HBT) was added to the reaction assay mixture. Each reaction assay was repeated twice. Relative absorbance at each time points to day zero was plotted against time.

3. Results and Discussion
Prior to the decolorizing assay the maximum absorbance wavelength of AB25 was determined by UV/Visible spectrophotometric scanning. The maximum absorbance wavelength of AB25 was determined to be 603 nm. This maximum wavelength was used for further AB25 decolorizing assays. Figure 2 shows the result of AB25 decolorizing assay by crude extracts of *T. asperellum* LBKURCC1 laccase without the addition of any mediators. As can be seen in the graph of Figure 2 there was no significant color decrease in any of the treatments up to day 6 of the assay. No significant change could be seen within treatments, shown as relative absorbance at 603 nm compared to day zero. Time points in which the relative absorbance slightly rises above 100% may be due to miniscule particulates that formed due to enzyme denaturation that was not removed by centrifugation prior to absorbance measurements. It can be concluded that crude *T. asperellum* laccase at the activity used in the experiment (0.021 U/mL) does not degrade AB25 without addition of mediators. Figure 3 panel A shows no visual color difference that can be seen in the enzyme treated AB25 solution to the AB25 treated by heat inactivated enzyme (denatured enzyme control), after six days of reaction.
Anthraquinone dyes have been reported to be more difficult to degrade by fungal laccases, compared to azo dyes. However, degradation of the anthraquinone dye Remazol brilliant blue R (RBBR) by the white-rot fungus *Marasmius scodonius* laccase could be achieved when 1 mM of the redox mediator 1-hydroxybenzotriazole (HBT) was added to the decolorizing reaction. Before addition of HBT, *M. scodonius* laccase was unable to degrade RBBR. After addition of 1 mM HBT, *M. scodonius* laccase could decolorize 61% of a 200 ppm RBBR solution [21]. To examine if HBT could also act as a mediator for decolorization of AB25 by *T. asperellum* LBKURCC1 laccase in this study, we added from 0.1 mM to 5 mM HBT to the decolorization assay. Figure 4 show results from the AB25 decolorization assays in the presence of several HBT concentrations. No significant change in relative absorbance of AB25 at 603 nm between treatments could be observed in a 6-day experiment. From Figure 4 it can be concluded that addition of HBT up to 5 mM did not mediate decolorization of AB25 by *T. asperellum* LBKURCC1. Figure 3 panel B shows no visual color difference in laccase plus 1 mM HBT treated dye solution compared to control solution (AB25 in buffer) after 6 days of incubation.

![Graph](graph.png)

**Figure 2.** Results of AB25 decolorization by *T. asperellum* LBKURCC1 laccase assay without addition of HBT.
Figure 3. Photograph of AB 25 solution at day 6 of the decolorization assay: A. Without addition of HBT; B. With addition of 1 mM HBT.

Figure 4. Results of AB25 decolorization by *T. asperellum* LBKURCC1 laccase assay with the addition of HBT at various concentrations as indicated on the graph.

In this study, we could not achieve decolorization of AB25 by *T. asperellum* LBKURCC1 laccase in the reaction conditions used. Of four anthraquinone dyes studied for degradation by *Trametes hirsuta* laccase, Yanto et al. showed that AB25 was the most difficult anthraquinone dye to degrade. Several factors determine the ability of a laccase enzyme to decolorize or degrade textile dyes [10]. Among the factors are structure of the laccase enzyme that can affect substrate specificity of the enzyme, total activity of the enzyme in the decolorization process, reaction conditions such as pH and temperature, and the requirement for redox mediators [8]. Due to these factors, the ability of laccase from a specific source may have different degradation, or decolorization activity for dyes that even belong to the same dye group or classification. As an example, laccase from *M. scorodonius* could
easily degrade the azo dye Malachite green without mediators, could degrade the azo dye Reactive orange 16 only when added 1 mM HBT, and could not degrade the azo dye Methyl red even after the addition of 1 mM HBT [21]. In this research, the addition of 1 mM HBT could not mediate the degradation of AB25 at pH 5.5. Other reaction conditions should be examined, such as optimizing the reaction pH, using more concentrated enzyme, or exploring the use of other types of redox mediators. Several researchers have reported that vanillin acts as a more effective redox mediator compared to HBT [9-10]. We will continue to explore if other reaction pH’s and redox mediators, than that used in this study, will make T. asperellum laccase able to catalyze the decolorization of AB25.

4. Conclusion
AB25 was not decolorized by T. asperellum LBKURCC1 laccase in the reaction conditions used in this study, that is pH 5.5, room temperature, laccase activity of 0.021 U/mL, and an initial AB25 concentration of 50 ppm. The addition of HBT from 0.1 mM to 5 mM as redox mediator, did not affect degradation of AB25 by T. asperellum LBKURCC1 laccase. Further investigation is required to explore if other reaction conditions or types of redox mediators can assist in AB25 decolorization by T. asperellum LBKURCC1.

Acknowledgment
This research is supported by the Direktorat Sumber Daya (Resources Directorate), Ministry of Education, Culture, Research and Technology of Indonesia based on amended contract for research grant scheme Penelitian Tahun Jamak Penelitian Dasar Dan Pembinaan Kapasitas (Multiple Year Basic Research and Capacity Building) number 1408/UN.19.5.1.3/PT.01.03/2021 to Titania Tj Nugroho as principle investigator.

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