Colour removal of an azo-textile dye and production of laccase by submerged cultures of *Trichoderma asperellum* LBKURCC1

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Abstract. Textile industry effluents containing synthetic dye waste, such as azo dyes, are harmful to health, and thus can present environmental hazard. Biological treatment of textile waste effluents can remove synthetic dyes. Here we present our results of colour removal from an azo dye solution, by mycelia of two Riau, Indonesia *Trichoderma* biocontrol isolates. Colour removal by *Trichoderma asperellum* LBKURCC1 was more rapid with higher maximums at pH 6.5, compared to removal at pH 2.5 and 4.5. Colour removal by *T. asperellum* LBKURCC1 was stable until the end of the experiments (120 hours). As comparison, colour removal by *Trichoderma asperelloides* LBKURCC2 was higher at the lower pH values, but was unstable after 60 hours, showing difference in dye removal mechanism between the two fungal species. Laccase are enzymes that degrade synthetic dyes, including azo dyes. In this paper, we show that submerged cultures of *T. asperellum* LBKURCC1 can produce laccase in media solution containing rice husk. LBKURCC1 also produced laccase in solid state fermentation (SSF) systems containing rice straw. Production of laccase can contribute to increased and more stable removal of azo dyes by live submerged *T. asperellum* LBKURCC1 mycelia, with appropriate supplement addition in waste effluents.

1. Introduction

Synthetic dyes are commonly used in the textile industry. Among the most used textile dyes are the azo dyes, which are polycyclic aromatic amine compounds. After the dyeing process, a large amount of azo dyes are still present in the textile effluent waste. If untreated, textile effluent waste containing azo dyes that are released to natural bodies of water are hazardous to the environment. This hazard is due to the toxic and mutagenic properties of aromatic amines that can cause dermatitis as well as induce cancerous tumor growth if ingested by humans or mammals [1][2][3]. It is therefore necessary to remove azo-dye waste as well as other types of textile dyes from textile waste effluents, before releasing them into natural waters.

Several physical and chemical methods to remove dye waste from textile production effluents exist, such as physical adsorption [4], chemical photocatalysis [5], or chemical coagulation and Fenton
oxidation [6]. These physical and chemical methods can be costly and can produce secondary pollutants, such as dye adsorbent waste and salts that need to be disposed [1]. Biological methods for removal of dye waste from textile production effluents, using microbes and enzymes, are considered more eco-friendly, since less toxic sludge is produced in these biological treatments [7]. Biological treatment of textile dye waste can be done using live microorganisms, such as microbe consortia [7], or life fungal monocultures [8]. Alternatively, biological treatment of textile dye waste can be done using enzymes, such as the laccase enzymes [9] [10].

Live cultures of various species of the fungus *Trichoderma*, have been reported to be able to remove a wide variety of azo, triphenylmethane and anthraquione dyes [8] [10][11][12][13]. Removal of dyes by species of *Trichoderma* sp. are suggested to be through biosorption into the fungal cells [13], or by the release of dye degrading enzymes, such as the laccase enzymes, manganese peroxidase, lignin peroxidase and 1,2- or 2,3-dioxygenase [8][10]. *Trichoderma asperellum* LBKURCC1 and *Trichoderma asperelloides* LBKURCC2 are two biocontrol *Trichoderma* strains isolated from plantation soil in Riau, Indonesia. *T. asperellum* LBKURCC1 was isolated from the rhizosphere of a cacao plantation in the Pekanbaru city area of Riau, Sumatra Indonesia, and originally identified as *Trichoderma harzianum* TNC52. *T. asperelloides* LBKURCC2 was isolated from the rhizosphere of a citrus plantation in the Kampar district of Riau, Sumatra Indonesia, and originally identified as *Trichoderma viride* TNJ63 [14]. The current identities of these *Trichoderma* strains have been revised, based on ITS1, 2 and 5.8s rRNA, and *tef1* gene sequences of the strains (GenBank access numbers KY203853, KY213959, EF467659, and MH017203). Based on phylogenetic analysis using those sequences, we now identify *T. harzianum* TNC52 as *T. asperellum* LBKURCC1, and *T. viride* TNJ63 as *T. asperelloides* LBKURCC2. Both strains are currently deposited at the Biochemistry Laboratorium Universitas Riau Culture Collection. In this paper we present our results of azo dye removal from a solution of Direct Violet 51 by both *T. asperellum* LBKURCC1 and *T. asperelloides* LBKURCC2 mycelia. We further show that *T. asperellum* LBKURCC1 can produce laccase in both submerged and solid state fermentation systems, suggesting a mechanism for increased degradation of the azo-dye using live cultures of *T. asperellum* LBKURCC1.

2. Material and methodology

2.1. Microorganism

The fungal strain *T. asperellum* LBKURCC1 was isolated from the rhizosphere of cacao plants in a cacao plantation in Kecamatan Rumbai, Pekanbaru city, Riau Province, Island of Sumatera, Indonesia. The fungal strain *T. asperelloides* LBKURCC2 was isolated from the rhizosphere of citrus plants in a citrus orchard located 30 Km away from Pekanbaru city, at the district of Kampar, Riau province, Island of Sumatra, Indonesia. Species identity of both strains were determined and confirmed by phylogenetic analysis using the ITS1 , 2 and 5.8S rRNA, and *tef1* gene sequences of the strains, and compared to sequences available in GenBank database by BLAST and sequence alignment methods (CLUTALX) [15]. GenBank Access numbers for ITS1,2 and 5.8S RNA sequences for *T. asperellum* LBKURCC1 and *T. asperelloides* LBKURCC2 are KY203853 and EF467659, respectively. GenBank Access numbers for *tef1* gene sequences for *T. asperellum* LBKURCC1 and *T. asperelloides* LBKURCC2 are KY213959 and MH017203, respectively. Both strains were cultured and maintained on potato dextrose agar slants supplemented with 0.05% (w/v) citric acid.

2.2. Chemicals and consumables

Liquid Direct Violet 51 azo dye, was obtained from Pekanbaru local textile small industry that uses the dye. Polyvinylpyrrolidone and Guiacol was from Sigma-Aldrich (St. Louis, USA, Cat. No. P6755 and G550, respectively). Glass fiber filters GF/C for separation of mycelia from crude enzymes was from Whatman™ Cat. No. 6780-2504, circle diameter 5.5 cm, pore size 1.2 μm. Sterile polyethersulfone syringe filters (low protein binding) for sterilization/clarification of enzymes was from Whatman™ Cat. No. 6780-2504. All other chemicals were of analytical grade.
2.3. Preparation of fungal mycelia for inoculation in dye solution

Strains were grown in 100 mL Erlenmeyer flasks in the following fungal growth medium (pH 6.5): 10 g KNO₃, 5 g KH₂PO₄, 2.5 g MgSO₄·7H₂O, 10 g Glucose, 10 g Polyvinylpyrrolidone, and 0.05 M citric-phosphate buffer (pH 6.5) to make 1 liter. A glass wool filtered spore suspension was inoculated to 25 mL of fungal growth media to give a final spore concentration of ~3x10¹¹ spore/mL, and the culture grown by shaking on a rotary shaker (150 rpm) at room temperature (approximately 30°C) for 2 weeks. After two weeks incubation, the fungal mycelia was filtered using a 0.45 µm GF/C (Whatman™) glass fiber filter, to separate it from the media. The filtered mycelia from one flask were directly inoculated without washing into the dye solution for experiments to determine dye removal by the fungi.

2.4. Dye colour removal experiments

25 µl of concentrated liquid Direct Violet 51 was added to a 100 ml buffer solution to give an initial absorbance at 570 nm of 0.39 to 0.41. Triplicate experiments were carried out in buffer solutions of pH 2.5; 4.5 and 6.5. Fungal mycelia prepared as described in section 2.3. were inoculated into the dye solution, and rotated at room temperature (30°C) on a rotary shaker at 150 rpm. As controls, dye solution at each pH buffer without addition of fungal mycelia were at the same time rotated at room temperature using the same rotary shaker. Dye solution was removed at various time intervals for determination of visible light absorbance at 570 nm. Percentage of dye removal by the fungal mycelia was calculated based on decrease of the absorbance of the fungal containing dye solution at the specific time point, corrected to decrease of the absorbance of the control dye solution at the same time point, using the following equation:

\[
\% \text{ Colour removal} = \left( \frac{A_{0f} - A_{tf}}{A_{0f}} \right) \times 100 - \left( \frac{A_{0c} - A_{tc}}{A_{0c}} \right) \times 100 \%
\]

2.5. Production of laccase in a submerged fermentation system

Laccase production by a submerged culture of *T. asperellum* LBKURCC1 was done using an optimized media suggested by Gao et al. [16], with modification, that is changing the wheat straw powder to rice husk powder. Every 100 mL of the media was inoculated with ~5.10¹⁴ spores of *T. asperellum* LBKURCC1, and incubated at room temperature (30°C) in 250 mL flasks with shaking at 150 rpm. Fermentation was carried out for 24, 36, 48, 60 and 70 hours. At the desired fermentation times, the fermentation was stopped by placing the fermentation flasks at 4°C for 1 hour. The media containing enzymes was separated from fungal mycelia, by 10 minutes cold centrifugation at 5-10°C and centrifugation speed of 9500 rpm. Crude enzymes extracts obtained from the filtrate was vacuum filtered using Whatman™ GF/C filters, to remove residual mycelia. The crude extract was filter sterilized using a 0.45 µm sterile polyethersulfone syringe filter. Crude extracts were stored at -20°C until further use. Production was repeated twice.

2.6. Production of laccase in a solid state fermentation tray reactor system

Laccase production by solid state fermentation (SSF) by *T. asperellum* LBKURCC1 was performed in a tray reactor system. Solid media for the fermentation system consisted of (NH₄)₂SO₄, MgSO₄, KH₂PO₄, CuSO₄ and powdered rice straw as the laccase inducer. To every 12 g of the solid media was added 30 ml of 5.5 pH buffer, and this mixture was sterilized by autoclave sterilization. The sterilized media was spread on sterilized fermentation trays to a bed thickness of 3 cm, and inoculated with 5 plugs (each plug having a diameter of 1 cm) of fungal lawns grown on potato dextrose agar. The trays were incubated at room temperature, and at time intervals, media samples were taken to extract any laccase produced within.

2.7. Extraction of laccase from SSF media

From each SSF tray reactor, 1 g media from 5 random spots on the tray were taken, and mixed together. Extraction was performed in pH 5.5 sodium acetate buffer following the method described by Hanung
et al. [17]. The enzyme filtrate obtained was filter sterilized using a 0.45 µm sterile polyethersulfone syringe filter. Crude extracts were stored at -20°C until further use.

2.8. Determination of laccase activity
Laccase activity was measured and calculated using guaiacol as substrate as described by Gao et al. [16]. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 µmol guaiacol per minute at the incubation temperature, pH 5.5. All enzyme assays were carried out in triplicates.

3. Results and discussions
3.1 Dye removal by fungal mycelia
Effluents waste from the textile industry containing azo-dyes should be treated for removal of the dyes before being released to the environment. Direct Violet 51 is an azo dye used widely in Indonesian textile industry due to the attractive violet colour it has. Therefore we used solutions of Direct Violet 51 as a model for azo dye removal in our experiments.

The use of fungal mycelia to absorb and degrade dyes have recently gained interest, since some have shown to be ecological friendly, efficient and simple to apply [8]. Species from the genus Trichoderma are very diverse, and several have shown ability to absorb and degrade textile dyes [11]. T. asperellum LBKURCC1 and T. asperelloides LBKURCC2 are two biocontrol strains isolated from the rhizosphere soil of two different plantations in the province of Riau, Indonesia. We used these two species in our dye removal experiments, as both have shown ability to produce a wide variety of extracellular enzymes [14][18] and are therefore good candidates to bioabsorb and enzymatically degrade dyes. Our results of dye removal experiments show that application of mycelia from these two different Trichoderma species to solutions of Direct Violet 51 could remove the colour with different patterns between the two species (Figure 1 and Figure 2). Colour removal by Trichoderma asperellum LBKURCC1 was more rapid with higher maximums at pH 6.5, compared to removal at pH 2.5 and 4.5 Colour removal by T. asperellum LBKURCC1 was stable until the end of the experiments (120 hours) (Figure 1). In contrast, colour removal by Trichoderma asperelloides LBKURCC2 was higher at the lower pH values, but was unstable after 60 hours (Figure 2), showing difference in dye removal mechanism between the two fungal species.

Stable colour removal by T. asperellum LBKURCC1 shows that the dye was not only absorbed by the mycelia, but that enzymatic degradation of the dye could also have taken place. Although the mycelia cultures were after two weeks prior growth in PDA already at the stationary or declining phase, the life cells that were still present in the mycelia mass could possibly be induced by the dye to excrete a low but effective amount of dye degrading enzymes. This would explain the better colour removal of T. asperellum LBKURCC1 at pH 6.5, than at the lower pH, since fungal cells would grow, metabolize and secrete enzymes better at pH 6.5, than at pH 2.5, or pH 4.5. The dye degrading enzyme secreted by T. asperellum LBKURCC1 apparently has a more optimum working pH at 6.5, than at the lower pH values (2.5 and 4.5). No significant difference can be observed in colour removal between pH 2.5 and 4.5.

Figure 2, shows the unstable colour removal by T. asperelloides LBKURCC2, where colour removal reached maximums after 48 hours treatment, but the colour came back gradually after 60 hours treatment (incubation). This shows that there was leaching back of the colour that was initially absorbed by T. asperelloides LBKURCC2 mycelia cells, possibly caused by lysis of the cells that absorbed the dye. It also shows that colour removal of the Direct Violet 51 dye by T. asperelloides LBKURCC2, was largely by biosorption, and possibly did not involve any enzymatic dye degradation. Biosorption as the main mechanism for colour removal by T. asperelloides LBKURCC2 is also supported by the fact that colour removal by T. asperelloides LBKURCC2 is better at the lower pHs (pH 2.5 to 4.5), compared to pH 6.5. Plasma membranes of the fungal cells would be more permeable to dye colour at lower pH, being less intact than at pH 6.5, which is closer to the physiological pH of most fungi. Apparently unlike T.aspererellum LBKURCC1, the fungal strain T. asperelloides LBKURCC2 does not produce effective enzymes that can degrade the azo-dye. This
causes the results shown in Figure 2, in which at longer incubation times (>60 hours), when fungal cells start to lyse due to cell death, un-degraded azo-dye leaching back to solution causes the solution to become coloured again.

Figure 1. Percentage of colour removal from solutions of Direct Violet 51 treated by T. asperellum LBKURCC1 mycelia as a function of incubation time at different pH levels.

Figure 2. Percentage of colour removal from solutions of Direct Violet 51 treated by T. asperelloides LBKURCC2 mycelia as a function of incubation time at different pH levels.
3.2. Production of laccase by *T. asperellum* LBKURCC1

Since the pattern of dye removal by *T. asperellum* LBKURCC1 suggest a mechanism involving enzymatic degradation of the Direct Violet 51 dye, we investigated possibility of laccase production by *T. asperellum* LBKURCC1. Laccase (EC 1.10.3.2), is the name of a family of enzymes that have oxidoreductase catalytic activity, and contain multi-copper ions at their reaction center [9]. They are among a few of enzymes, having a wide range of substrates that they are able to oxidize, and have been shown to be able to degrade a wide variety of synthetic dyes [19][20]. *T. asperellum* LBKURCC1 is a biocontrol strain of *Trichoderma*, having ability to protect plant cells from fungal pathogens [14]. Biocontrol *Trichoderma* has been shown to produce laccase as a defense mechanism against plant pathogenic organism [21].

To investigate if *T. asperellum* LBKURCC1 could produce laccase, we grew *T. asperellum* LBKURCC1 in submerged and solid state fermentation (SSF) systems using rice husk (submerged fermentation system), or rice straw (solid state fermentation system) as inducers of laccase. Our results show that *T. asperellum* LBKURCC1 can produce laccase in both fermentation systems. Figure 3 depicts production of laccase by submerged fermentation cultures of *T. asperellum* LBKURCC1, reaching peaks after 48 hours fermentation. The laccase activity produced by the submerged fermentation system was determined at 40°C, and at peak was relatively low, with activity of 0.21 U/L. In contrast, the activity of laccase produced by *T. asperellum* LBKURCC1 in the SSF system, was ~92 fold higher than the activity produced by the submerged fermentation system. But it should be noted, that activity of laccase obtained by the tray SSF system was determined at 30°C, which could be the more optimum temperature for laccase activity. Dye removal experiments were performed at room temperature, which was 30°C. If laccase were to contribute to degradation of dyes, the more accurate temperature to determine its activity would be at 30°C. Table 1 gives a brief summary of the laccase activity produced by both *T. asperellum* LBKURCC1 fermentation systems.

![Figure 3. Production of laccase activity by *T. asperellum* LBKURCC1 as a function of fermentation time in a submerged fermentation system.](image-url)
Table 1. Summary of laccase activity produced by T. asperellum LBKURCC1

| Fermentation system | Laccase inducer | Fermentation time to peak production (days) | Conditions for determination of laccase activity | Extracted laccase activity (U/L) |
|---------------------|-----------------|---------------------------------------------|-----------------------------------------------|-------------------------------|
| Submerged           | Rice husk       | 2                                           | 40°C, pH 5.5                                   | 0.2 ± 0.01                    |
| Solid State (tray reactor) | Rice straw          | 7                                           | 30°C, pH 5.5                                   | 19.3 ± 0.5                    |

SSF system is more favorable to produce fungal enzymes than submerged fermentation, since they provide environments that are closer to the natural fungal habitat where they were isolated [22]. SSF compared to submerged fermentation systems have been shown to give higher product yields, lower catabolite repression and due to having less moisture, is less prone to unwanted contamination. There is also higher contact between the enzyme inducers and the fungal cells [23].

Although production of laccase in our submerged fermentation systems by T. asperellum LBKURCC1 was relatively low, it may be enough to degrade azo-dyes over time, and result in stable colour removal. Our dye removal experiments did not add fungal growth media to the dye solution, so little growth and minimal metabolism of the fungal mycelia would be expected. However residual media, and presence of dye, could induce some laccase production by live T. asperellum LBKURCC1 cells that was enough to give stable degradation of Direct Violet 51. The ability of T. asperellum LBKURCC1 to produce laccase, can enable better and faster degradation of azo-dyes, by addition of appropriate media into dye waste effluents. Lignin containing agricultural waste, such as rice husk and rice straw used in our experiments, as well as fungal nutrition, could be added to the textile waste effluents, together with T. asperellum LBKURCC1 mycelia, to increase dye degradation and colour removal rates. Further research is still required to optimize the supplemental media that needs to be added to textile effluent waste for this purpose. Colour removal may also be maximized by optimal conditions using acclimated mycelia cultures that are at its growth phase.

Results in this study add to the growing list of application of T. asperellum LBKURCC1 in various biotechnology areas. T. asperellum LBKURCC1 can act not only as a biocontrol agent [14], but also producer of many extracellular enzymes, such as cellulase [24], and here as producer of laccase. Apart from the ability to degrade azo-dyes as shown in this study, T. asperellum LBKURCC1 (previously identified as T. asperellum TNC52) has also been shown to be able to degrade an anthraquinone dye, Colour Index Reactive Blue 5 [13]. Synthetic dyes are mainly from three chemical structure groups, that is the azo-, anthraquinone and triphenylmethane dyes. It still remains to be investigated if T. asperellum LBKURCC1 can also remove synthetic dyes from the triphenylmethane group. Having ability to degrade different chemically structured synthetic dyes, would enable the use of a single organism to be used to remove mixtures of synthetic dyes from textile waste effluents.

4. Conclusions
The results of this study show that both T. asperellum LBKURCC1 and T. asperelloides LBKURCC2 mycelia can remove colour from solutions of azo-dye Direct Violet 51, but with different mechanisms, shown by difference in stability of colour removal. Removal of colour by T. asperellum LBKURCC1 was stable, and at 30°C reached its maximum in 48 hours at pH 6.5, with (64 ± 4)% colour removal. Removal of colour by T. asperelloides LBKURCC2 was unstable, leaching out and returning the colour to the solution after 60 hours treatment, indicating that T. asperelloides LBKURCC2 is unable to degrade the azo dye. T. asperellum LBKURCC1 is able to produce laccase in both submerged and SSF systems. Production of laccase in the SSF system was more efficient than in the submerged system. Production of laccase in submerged systems, although relatively low, giving an extracted activity of 0.2 U/L, can contribute to increased and stable degradation of azo-dyes in solution. Further research is required to optimize the use of live cultures of T. asperellum LBKURCC1 for degradation of synthetic dyes in textile waste effluents.
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