Journal of Tropical Biodiversity and Biotechnology
Volume 01, Issue 02 (2025): jtbb16121
DOI: 10.22146/jtbb.16121

Research Article

Bioremediation and Detoxification of the Textile Dye Methyl Blue by *Trichoderma asperellum* LBKURCC1 with Laccase Activity

Andi Dahliaty[1], Yuana Nurulita[1], Ismawati[2], Nuria Wulandari[1], Dwi Kurniati[1], Yanti[3], Titania Tjandrawati Nugroho[1]*

1)Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Riau, Kampus Bina Widya, Jl. Raya Soebrantas Km 12,5, Pekanbaru, Riau 28293, Indonesia
2)Faculty of Medicine, Universitas Riau, Jl. Diponegoro no. 1, Pekanbaru, Riau 28133, Indonesia
3)Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, Kampus III BSD, Jl. Raya Cisauk, Tangerang, Banten 15345, Indonesia
* Corresponding author, email: titania.nugroho@lecturer.unri.ac.id

Keywords:
Bioremediation
Laccase
Methyl blue
Trichoderma asperellum
Triphenylmethane
Submitted:
29 August 2024
Accepted:
26 November 2024
Published:
16 May 2025
Editors:
Miftahul Ilmi
Liya Audinah

ABSTRACT

Textile production generates effluent contaminated by 10-15 % dyes used. Fungal-mediated remediation presents an ecologically acceptable and inexpensive method for treating dye effluents. *Trichoderma asperellum* LBKURCC1 isolated from Riau cacao rhizosphere produces laccase. Laccase can catalyse the degradation of several textile dyes. This study aims to investigate *T. asperellum* LBKURCC1 ability to bioremediate and to detoxify the textile triphenylmethane dye, methyl blue (MB). *T. asperellum* LBKURCC1 cultures in minimal media with pHs of 4.5, 5.5 and 6.5 were incubated (24 hours, room temperature) with 50-ppm MB solutions. MB decolorisation analysis was conducted by examining the UV-Vis absorption spectra of MB after 24 hours treatment. It was also determined if the degradation was contributed by enzymatic biodegradation or by biosorption processes. The contribution of laccase activity to the bioremediation process was assessed by monitoring laccase activity in fungal treated MB solution. Acute toxicity of MB to *Artemia salina* larvae was determined pre- and post-fungal treatment. The results showed that decolorisation of MB by *T. asperellum* LBKURCC1 occurred at all tested pH levels, but at different rates for different pH. The highest decolorisation rate was at pH 4.5 (85 % per 24 hours). Enzymatic biodegradation was the higher contributor to the decolorisation, compared to mycelia biosorption. Laccase activity was induced by MB and the highest activity was found at pH 4.5. MB toxicity to *A. salina* larvae was eliminated by the fungal biodegradation. This study confirms the potential of *T. asperellum* LBKURCC1 life cultures in the degradation of MB, eliminating its toxicity.

How to cite:
Dahliaty, A. et al., 2025. Bioremediation and Detoxification of the Textile Dye Methyl Blue by Trichoderma asperellum LBKURCC1 with Laccase Activity. *Journal of Tropical Biodiversity and Biotechnology*, 10(2), jtbb16121. doi: 10.22146/jtbb.16121

INTRODUCTION

During textile dying, an average of 10-15 % of the dye goes to waste effluents (Juárez-Hernández et al. 2021; Boer et al. 2004). Synthetic dyes present in textile effluent pose a hazardous environmental threat. Water quality is degraded by dyes in the aquatic ecosystems. Dyes reduce the penetration of sunlight, thus hampering photosynthetic activity, and generation of oxygen. Synthetic dyes also increase the Chemical Oxygen Demand (COD) for degradation of these dyes, causing further reduction of oxygen availability for aquatic lives (Plácido et al. 2016). Members from the triphenylmethane family of synthetic dyes are also known to be mutagenic and difficult to degrade naturally (Sinha & Hanamghar 2019). A triphenylmethane dye widely used in the textile industry is methyl blue (MB) (Figure 1), also known as cotton blue, acid blue 93 or Helvetia blue, having the chemical formula $C_{37}H_{27}N_3Na_2O_9S_3$ (Singh et al. 2020). Due to the negative impacts on the environment, waste effluents containing methyl blue, or other triphenylmethane dyes must be removed or degraded before being released into natural waters (Dahlena et al. 2022).

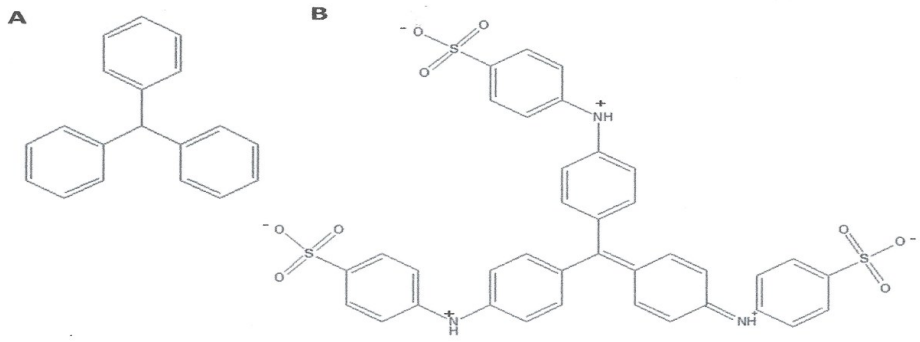

Figure 1. A) General structure of triphenylmethane dyes. B) Structure of methyl blue

Various chemical and physical methods have been developed to remove or to degrade synthetic textile dyes from effluents, among others adsorption methods (Eteba et al. 2023; Nadew et al. 2023; Procópio et al. 2023), chemical methods (Sandani et al. 2021), photocatalysis (Siraj et al. 2021), electrocoagulation (Mutambyi et al. 2023) and Fenton oxidation (Drăgoi et al. 2021). However, several methods are costly and can create secondary pollutants, such as sludge, which would require additional treatment (Sar et al. 2024). An ecologically acceptable and relatively inexpensive alternative for textile dye removal, decolourisation and/or degradation is using biological methods, employing life cultures of microorganisms, or enzymatic treatments (Shanmugam et al. 2017; Al-Tohamy et al. 2022; Kumar et al. 2024). Various fungal strains can decolorise textile dyes through enzymatic biodegradation and biosorption by fungal mycelia. *Aspergillus flavus* mycelia could decolorize 77 % of a 200 mg L^{-1} solution of the textile dye acid black 52 in 50 hours (Ghosh et al. 2017), and 100 % of a triphenylmethane dye malachite green (MG) solution (150 mg L^{-1}) within 6-8 days (Barapatre et al. 2017). Several laccase producing species from the genus *Trichoderma* have been reported to degrade synthetic textile dyes. A marine isolated *Trichoderma harzianum* strain TSK8 could degrade 89 % of MG in ten days (Saravanakumar & Kathiresan 2014). Live culture mycelia of *Trichoderma viride* and *Trichoderma virens* could decolorize 75 % to 93 % of 50 ppm MG and an azo dye congo red in 24 hours (Argumedo-Delira et al. 2021). A rapid growing species of *Trichoderma*, *T. tomentosum* could decolorise 99 % textile effluents containing 85.5 ppm of the azo-dye acid red 3R in three days (He et al. 2018). Other examples

are the bioremediation of the dyes methylene blue, malachite green and safranine by *Aspergillus terreus* YESM 3 (Mohammed & Mabrouk 2020), and direct black 22 by *Penicillium chrisogenum* ERK1 (Lanfranconi et al. 2022). The bioremediation of these dyes are closely linked to production and activity of ligninolytic enzymes such as manganese peroxidase (EC 1.11.1.13) (Ali et al. 2022), lignin peroxidase (EC 1.11.1.14) and laccase (EC 1.10.3.2) (Chatha et al. 2017; Arora et al. 2022). These enzymes catalyse oxidation reactions of toxic dyes, degrading them into less harmful compounds (Asses et al. 2018; Adenan et al. 2022; Anita et al. 2022).

Trichoderma asperellum strain BPLMBT1 has been reported to produce laccase, which when purified, effectively degrade the recalcitrant triphenylmethane dye crystal violet (Shanmugam et al. 2017). *Trichoderma asperellum* LBKURCC1 is a biocontrol strain, isolated from healthy cacao tree rhizosphere soil in a plantation located at Pekanbaru city area, Riau, Island of Sumatra, Indonesia (Nugroho et al. 2018). We then asked the question whether our Indonesian endogenous strain could also produce laccase and be developed for bioremediation purposes. In a previous study, we observed that the mycelia of this filamentous ascomycetes fungi effectively decolorise the azo dye direct violet 51 (Nugroho et al. 2018). Additionally, we found that *T. asperellum* LBKURCC1 produces laccase when cultured in a rice stalk solid-state fermentation medium (Rahayu et al. 2019; Sellyna et al. 2020). The crude laccase extracted from this solid-state fermentation medium demonstrated the ability to degrade methyl blue (Dahlena et al. 2022). Based on these findings, *T. asperellum* LBKURCC1 life cultures emerge as promising candidates for direct application in bioremediation of textile affluents containing methyl blue dye. Using life cultures directly offers advantages over isolated enzymes, eliminating the need for fermentation production and enzyme extraction, which can be costly. The use of fungal life cultures in bioremediation can be further optimised by employing immobilised cells (Couto 2009). However, before employing these life cultures, it is essential to assess whether the dye can induce extracellular laccase production, and the effectiveness of using life cultures in the bioremediation process with various types of dyes. Therefore, the objective of this research was to determine the efficacy of *T. asperellum* LBKURCC1 life cultures in decolorising a methyl blue solution, and to evaluate the dye's ability to induce extracellular laccase production, thereby accelerating the specific dye degradation and dye removal from the environment. Since some dye degradation products are still toxic or can cause acute toxicity in aquatic animals, in this study we also evaluated the toxicity of methyl blue before and after treatment with *T. asperellum* LBKURCC1 life cultures.

MATERIALS AND METHODS

Materials

Microorganism

The fungal strain *Trichoderma asperellum* LBKURCC1 was obtained from the Universitas Riau Biochemistry Laboratory Culture Collection facility in Pekanbaru, Indonesia, where it is maintained on PDA slants, supplemented with 0.005 % citric acid (Nugroho et al. 2023). Five days old, rejuvenated cultures on agar slants were used for bioremediation experiments, as described in the appropriate method subsection. This fungal strain was originally isolated from the rhizosphere of healthy cacao plants in a cacao plantation in Kecamatan Rumbai, Pekanbaru, Riau Province, Sumatera, Indonesia (Nugroho et al. 2018). The species identity of the strain was confirmed by phylogenetic analysis using ITS (Internal Transcribe Spacer region of the ribosomal RNA) and *tef*1 sequences. GenBank Accession numbers for these sequences are KY203853 (ITS) and KY213959 (*tef*1) (Nugroho et al. 2018).

Chemicals and consumables

Methyl blue or MB (also known as acid blue 93; cotton blue; water blue) was from Merck Sigma-Aldrich Co. (CAS No. 28983-56-4; MW 799.8). Glass fiber circles GF/C for filtering and separation of mycelia from culture media was from Whatman™ with the pore size of 1.2 µm (Cat. No. 6780-2504). For culture media chemicals were biochemical grade: Ammonium sulfate ($(NH_4)_2SO_4$), magnesium sulfate ($MgSO_4$), Potassium dihydrogen phosphate (KH_2PO_4), glucose, yeast extract, potato, dextrose, Tween-20, Copper sulfate heptahydrate ($CuSO_4.7H_2O$). Chlortetracycline was pharmaceutical grade. Other compounds employed in this study were analytical grade.

Methods

Bioremediation of the textile dye methyl blue by fungal life culture experiments

Trichoderma asperellum LBKURCC1 spores were obtained from five days old, rejuvenated agar slants, by suspending in sterile water, and filtered using sterile glass wool. Spore concentration was determined using a table previously made to correlate O.D. 660 nm to life spores (Sawitri 2010). Approximately 9 x 10^{12} spores were spread on PDA petri dishes and allowed to grow into lawns by incubation at room temperature (± 30 °C), for three days. Two plugs (1 cm in diameter) were aseptically plugged from the mycelium lawn and inoculated into 30 mL mineral media in each 100 mL flask. Mineral media (MM) for the experiments had the following composition for each 1L media: 1 g $(NH_4)_2SO_4$, 2 g $MgSO_4$, 0.6 g KH_2PO_4, 22.3 g yeast extract, 1 g Tween-20, 0.046 g $CuSO_4.7H_2O$ (Nugroho et al. 2023). The MM media were prepared in appropriate buffers for experiment at pH 4.5, 5.5 and 6.5. These flasks were assigned as fungal methyl blue bioremediation test flasks. The flasks were incubated for two days at room temperature (± 30 °C) with shaking at 120 rpm. At this point the logarithmic phase of fungal growth had been reached, and 750 µL of 0.002 g mL^{-1} methyl blue were added to each test flask, bringing the concentration of methyl blue to 50 ppm, following dye decolourisation experiments described for textile dyes of the azo, triphenylmethane and anthraquinone group of dyes (Adnan et al. 2017; Argumedo-Delira et al. 2021; Ben Ali et al. 2020; Nugroho et al. 2023). At such a concentration, methyl blue has an average absorbance at its visible light peak (594 nm), of 1 to 1.5 (depending on pH of the solution). Methyl blue was added together with 150 µL (5 g L^{-1}) chlortetracycline solution to prevent bacterial growth. The test flasks were then further incubated at room temperature (± 30 °C) with shaking at 120 rpm. For spectrophotometric and enzyme activity analysis, from each test flask aliquots were taken at different time points within a period of 24 hours. These aliquots were then centrifuged 10 minutes at 13,000 rpm, to separate any debris and fungal spores that can interfere with the supernatant spectrophotometric assays (Nugroho et al. 2023). Spectrophotometric analyses were done using a UV-Vis Agilent Cary 60 spectrophotometer, that measured the absorbance of the solution at 320 nm to 800 nm. Light photography of supernatant aliquots was taken for colour documentation and visual comparison between samples. Abiotic control methyl blue flasks were methyl blue dye in MM media without adding fungal plugs. For brevity these abiotic control flasks will further be revered to as controls. Controls had the same treatment as the fungal bioremediation test flasks. MM media alone served as blanks for the spectrophotometric assays. All were conducted in triplicate experiments.

Biosorption of methyl blue by fungal mycelia assay

To determine the quantity of the methyl blue dye that was absorbed by the

fungal mycelia after 24 hours of incubation in the test flasks, mycelia were filtered under vacuum with GF/C glass fibre filters, following methods described previously for the study of reactive black 5 bioremediation by life cultures of *Trichoderma asperellum* LBKURCC1 (Nugroho et al. 2023). To extract any of the dye absorbed by the fungal biomass, 30 mL of 1 M NaOH pH 8 was added to the collected mycelia in a flask and shaken for 7 hours at room temperature (± 30 °C). The NaOH extract was separated from the mycelia by centrifugation, and absorbance of the separated filtrate was measured at a wavelength of 320-800 nm. Mineral media alone served as blanks for the spectrophotometric assays.

Quantitative analysis of bioremediation, enzymatic biodegradation, and biosorption

Percentages of bioremediation (R), biosorption (S), and biodegradation by enzymes produced by life fungal cultures (D) were calculated using the equations (1), (2) and (3), as described previously by Adnan et al. (2017) and Nugroho et al. (2023), with slight modification, that is the area under peaks were calculated from 530 nm to 670 nm, as characteristics of methyl blue visible light absorption peaks.

$$R\ (\%)\ = \frac{A_c - A_t}{A_c} \times 100 \quad (1)$$

R: bioremediation percentages (%). A_c: area under the absorption spectrum curve of control flasks starting at the wavelength where the differences between controls and fungal bioremediation tests were observed. A_t: area under the absorption spectrum curve of fungal bioremediation test flasks at the same wavelength used to measure A_c (Nugroho et al. 2023).

$$S\ (\%)\ = \frac{A_s}{A_c} \times\ 100 \quad (2)$$

S: biosorption percentages (%). A_s: area under the absorption spectrum curve of NaOH extract supernatant at the same wavelength used to measure A_c.

$$D\ (\%)\ = R - S \quad (3)$$

D: enzymatic biodegradation percentages (%). *R*: total dye bioremediation is achieved through both absorption (S) and enzymatic degradation (D).

Determination of laccase activity

Enzyme activity assay was investigated to determine laccase activity during degradation. It was performed using the supernatant obtained from the culture media and measured at the indicated time points. Laccase activity was determined using a microplate reader (Berthold Technology TriStar LB 941, Germany), with ABTS as a substrate for laccase, in which the change of oxidized ABTS absorbance at 405 nm as a function of time was measured. The enzymatic reaction mixture contained 180 µl sodium acetate buffer (0.05M, pH 5.5), 10 µl of 5 mM ABTS and 10 µl of enzyme, and the reaction mixture was incubated at 30 °C for 5 minutes. The enzyme activity was calculated based on the extinction coefficient for oxidized ABTS at 405 nm (ε_{405}), that is 36.8 $mM^{-1}cm^{-1}$ (Wang & Subramani 2017). The quantity of enzyme required for the oxidation of one µmol of ABTS per minute (U L^{-1}) was defined as one unit of laccase activity.

Artemia salina toxicity tests

Acute toxicity tests were performed by adding the treated dye to *Artemia salina* (brine shrimp) nauplii. This Brine Shrimp Lethality Test (BSLT) is often used to test toxicity of dyes that are present in dye effluents, as a rapid and general screening of toxic compounds (Sani et al. 2018; Saravanakumar & Kathiresan 2014). Solutions of methyl blue (MB) dye in mineral media (MM)

treated with *T. asperellum* LBKURCC1 life cultures for 24 hours from the methyl blue bioremediation experiments (section 2.2.1), were added to vials containing freshly hatched nauplii (Instar I) in reconstituted sea water initially at varying concentrations. Blanks consisting only of MM media were also tested. Due to high salts in the MM media, 100 % of nauplii died in 24 hours when the concentration of blanks was higher than 0.4 % (v v^{-1}). Therefore, to test the toxicity of the MB before (undegraded) and after bioremediation (degraded), concentrations of 0.2 and 0.4 % (v v^{-1}) from the bioremediation test were used for BSLT. The treated nauplii were then incubated for 24 hours. After 24 hours, the number of still living and the number of dead larvae were each counted (Latha et al. 2007). The survival rate in percentages were calculated as percentages of life larvae to total number of larvae in each experiment. MB in MM treated with *T. asperellum* LBKURCC1 is denoted as "Degraded MB". The same experiments were done using MB in MM media denoted as "Undegraded MB" and likewise done with only MM media denoted as "Blanks". All toxicity tests were repeated 5 (five) times.

RESULTS AND DISCUSSION

Methyl blue or with the trade name cotton blue, Helvetia blue or acid blue 93 , is a member of the triphenylmethane group of dyes (Dahlena et al. 2022). The complex aromatic structure of triphenylmethane dyes make them difficult to degrade, so that they can easily accumulate in the natural environment, causing toxic effects on living organisms (Taharchaouche et al. 2023). The employment of fungi as bioremediation agents for biosorption and biodegradation of dyes is the appropriate choice as this method is ecologically acceptable, relatively inexpensive, and easy to implement (Munck et al. 2018). In this study we have investigated the ability of *T. asperellum* LBKURCC1 life cultures to de-colorize methyl blue.

Decolourisation of methyl blue by *T. asperellum* LBKURCC1 life cultures

Figure 2 illustrates the results of a study on methyl blue dye decolourisation using live *T. asperellum* LBKURCC1 mycelia after 24 hours at pH 4.5, 5.5, and 6.5. Decolourisation was compared among mineral media (MM) blanks, abiotic controls (MM with added dye only), and test reactions (MM with added dye and live mycelia). For brevity the abiotic controls will be referred to as controls. At pH 4.5, decolourisation occurred at a higher rate, evident from the disappearance of the blue color in the test reactions after 6 hours of incubation. The greenish and dark yellow color observed after 6 and 24 hours of incubation in the test reactions at pH 4.5 is due to the color of the MM media, which is yellow. Compared to controls, there could be seen mark differences in color at pH 4.5 after 6 hours incubation. At pH 5.5, the blue color disappeared after 24 hours of incubation in the test reactions, and mark difference between the test and control color could be observed. However, no visual difference could be observed by the naked eye at pH 6.5 in test reactions after 24 hours incubation, compared to time zero. There was also no visible difference at pH 6.5 that could be observed between the test reaction and the controls color from time zero, until 24 hours incubation. This is because at times zero of pH 6.5, methyl blue has already been partially oxidised and therefore changed colour from aqua blue to a greenish hue. It is therefore difficult to the naked eye to distinguish between controls and test reaction at pH 6.5.

To confirm the visual results, a more quantitative analysis of methyl blue decolorization by *T. asperellum* LBKURCC1 mycelia was conducted by examining the UV-Vis absorption spectra of methyl blue after 24 hours of treatment with the fungus at room temperature and compared to the controls. The absorption spectra (Figure 3) demonstrate that decolourisation of methyl

blue by *T. asperellum* LBKURCC1 life cultures occurred at all tested pH levels, but at different rates for different pH. This is evident from the differences in spectrum profiles of methyl blue at pH 4.5, 5.5, and 6.5 between controls (blue lines) and *T. asperellum* LBKURCC1 treated test solutions of the dye (orange lines). The absorption maximum wavelength of methyl blue at 594 nm, observed in controls, disappeared in the test solutions, indicating that the fungal life cultures successfully decolourised (bioremediated) methyl blue. The rate at which this 594 nm peak disappeared was higher at pH 4.5 compared to the other pHs.

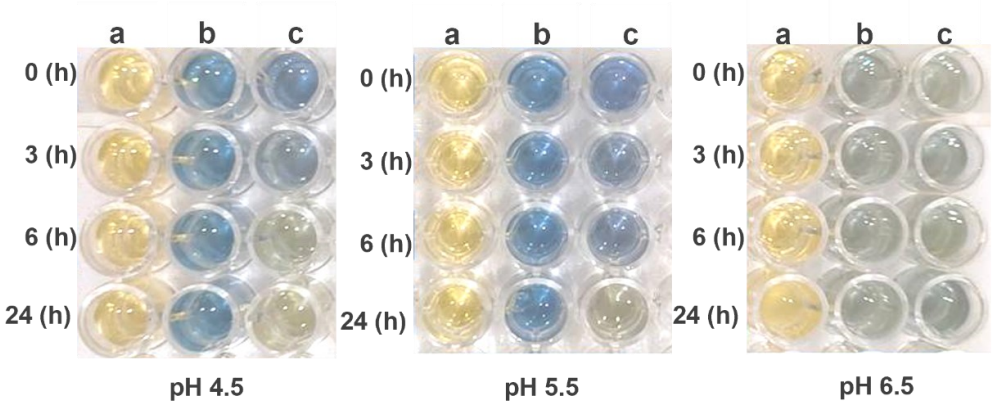

Figure 2. Visual colour results of methyl blue decolourisation at room temperature by *T. asperellum* LBKURCC1 at the indicated time points and pH. a = MM blanks; b = controls (methyl blue in MM); c = test reactions (methyl blue in MM with added *T. asperellum* LBKURCC1 live cells).

Bioremediation, biosorption and enzymatic biodegradation of methyl blue by *T. asperellum* LBKURCC1 live cultures

Dye bioremediation by fungal life cultures may result from biosorption by fungal mycelia or enzymatic biodegradation through secreted enzymes. To assess the contribution of biosorption and enzymatic biodegradation to methyl blue bioremediation by *T. asperellum* LBKURCC1 life cultures, we determined the bioremediation, biosorption and enzymatic biodegradation percentage after 24 hours of incubating the dye with fungal cells, as described in the Materials and Methods section.

Table 1 presents the percentages of methyl blue bioremediation, biosorption, and enzymatic biodegradation after 24 hours of incubation with *T. asperellum* LBKURCC1 life cultures. The highest bioremediation achieved after 24 hours was at pH 4.5, reaching an average of 85 % decolourisation. At pH 5.5, bioremediation did not reached half of that observed at pH 4.5 and was not significantly higher than that at pH 6.5. The results also indicated a minimal contribution of biosorption to the decolourisation process. It can be deduced from these results that more than 99 % of methyl blue bioremediation by *T. asperellum* LBKURCC1 cells was attributed to enzymatic biodegradation. Enzymatic biodegradation was preferably at pH 4.5 compared to the other pHs, which may reflect on the enzyme kinetic properties, and the fungal growth robustness.

Bioremediation in the decolourisation of a dye gives the total dye removal due to biosorption by cells or mycelia, and biodegradation due to enzymatic catalysed reactions, such as oxidation of the dye by laccase. A study conducted by (Arslantaş et al. 2022) investigated the decolourisation of basic red 18 using various fungal species, primarily through biosorption. When a high amount of dye removal is due to biosorption, this causes another problem because the fungal mycelia would have to be disposed and cannot be reused. In our study, the biosorption of methyl blue by *T. asperellum*

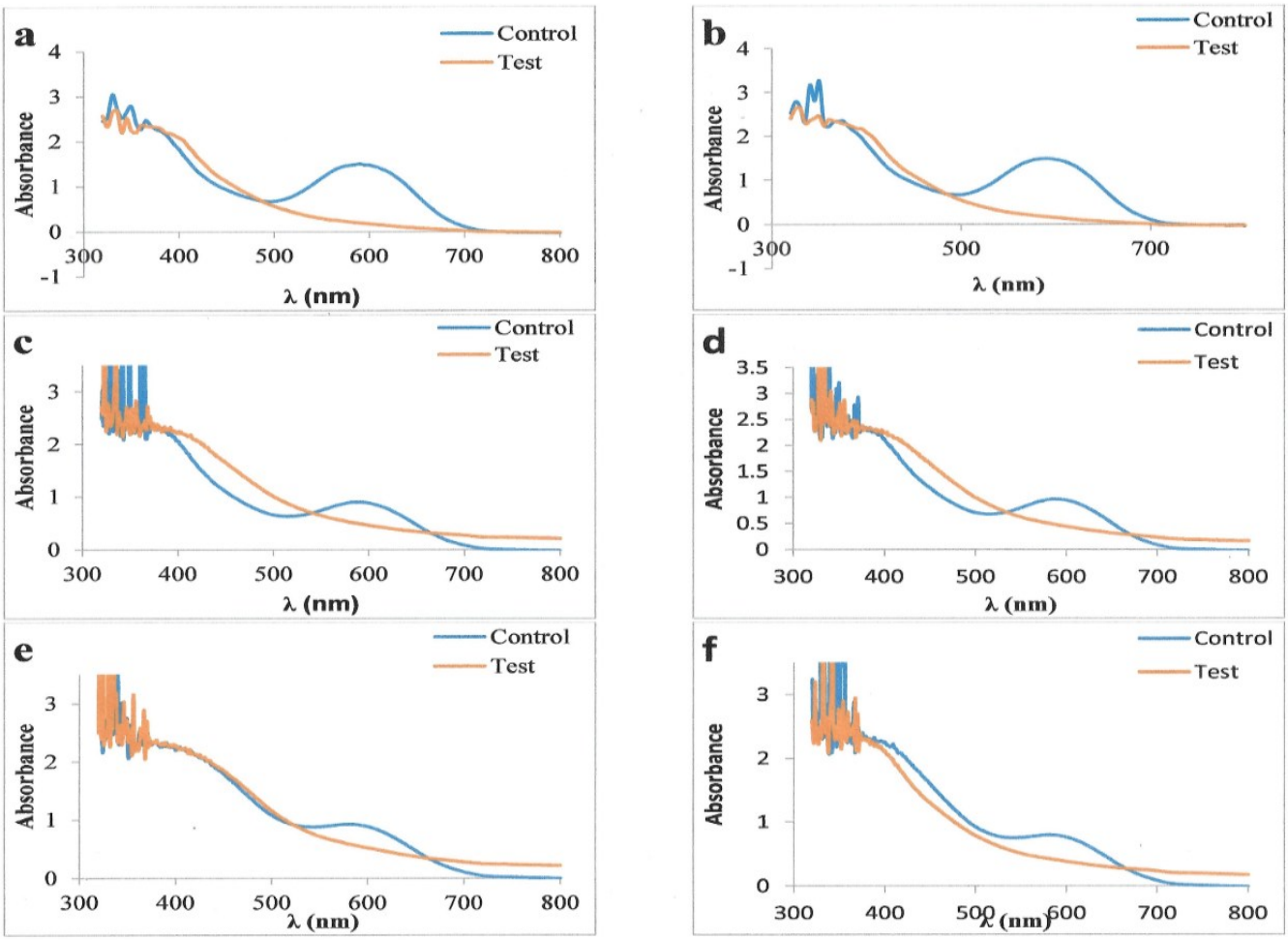

Figure 3. Duplicate results of UV-Vis scanning spectra of methyl blue after 24 hours treatment with *Trichoderma asperellum* LBKURCC1 cell, incubated at room temperature, and pH 4.5 (panels a & b); pH 5.5 (panels c & d); or pH 6.5 (panels e & f). Blue solid lines = Control solution of methyl blue in MM media. Orange solid lines = Test solution of methyl blue in MM media treated with *T. asperellum* LBKURCC1 life cultures.

Table 1. Average percentage values for bioremediation (R), biosorption (S), and biodegradation (D) of methyl blue by *T. asperellum* LBKURCC1 live cultures at various media pH, after 24 hours incubation†

pH	Bioremediation (R%)	Biosorption (S%)	Biodegradation (D%)
4.5	85 ± 1 (a)	0.2 ± 0.01 (b)	85 ± 1 (a)
5.5	36 ± 2 (b)	0.4 ± 0.01 (a)	35 ± 2 (b)
6.5	33 ± 10 (b)	0.0 ± 0.00 (c)	33 ± 10 (b)

†Average of triplicate experiments followed by standard deviation (SD) values. The mean value followed by the same letter in parentheses in a column is not statistically different according to the Duncan Multiple Range Test at $p > 0.05$ significance level.

LBKURCC1 was found to be minimal, as indicated in Table 1. Instead, biodegradation mediated by enzymatic activity played a predominant role in the decolourisation and bioremediation of methyl blue by *T. asperellum* LBKURCC1 cells. Since only a small amount of methyl blue is adsorbed by *T. asperellum* LBKURCC1 mycelia, the fungal mycelia can be effectively reused for the continuous biodegradation of methyl blue dye, particularly in an immobilized cell format.

Induction of *T. asperellum* LBKURCC1 laccase activity by methyl blue

A potential enzyme involved in the oxidation of methyl blue during the decol-

ourising process is the oxido-reductase laccase. To assess if *T. asperellum* LBKURCC1 excretes laccase into the media upon incubation with methyl blue, we measured laccase activity every hour for the first six hours and at 24 hours from the initiation of adding methyl blue to the fungal cultures in the MM media. Laccase activity at time zero was negligible at pH 4.5 but was detected at low activity in cultures grown at pH 5.5 and pH 6.5. The results (Figure 4) indicate that methyl blue induces production of laccase by the fungi at all pH tested, with a rise in activity at 1 hour after methyl blue addition to the fungal cultures in MM media. During the initial two hours after the addition of methyl blue, laccase production, as indicated by its activity, was highest at pH 4.5, followed by pH 5.5. Laccase activity at pH 4.5 experienced a slight decrease at 3 hours but then steadily increased, reaching 87 U mL^{-1} at 6 hours, and continuing to rise to 118 U mL^{-1} at 24 hours. Laccase activity at pH 5.5 peaked at 4 hours, reaching a maximum of 77 U mL^{-1} and remained present at 69 U mL^{-1} after 24 hours, suggesting relatively stable activity but at a lower level compared to pH 4.5. Laccase activity at pH 6.5 was relatively unstable, displaying negligible activity at various time points. At pH 6.5, the maximum laccase activity was only 37 U mL^{-1} after 6 hours of incubation with the dye, with no detectable laccase activity after 24 hours. These results show that methyl blue induces production of laccase by *T. asperellum* LBKURCC1 at all pH tested, however activity and stability of the enzyme differs at the different pHs. Activity and stability of *T. asperellum* LBKURCC1 laccase were highest at pH 4.5, and lower at pH 5.5 and pH 6.5, which is in line with the methyl blue bioremediation and enzymatic biodegradation percentages results shown in Table 1. Therefore, it can be deduced that the activity of *T. asperellum* LBKURCC1 laccase has a significant role in the enzymatic biodegradation and bioremediation of methyl blue by live cultures of *T. asperellum* LBKURCC1.

Our findings that production of *T. asperellum* LBKURCC1 is induced by a synthetic dye such as methyl blue, is in line with findings by other researchers of *Trichoderma* laccase induction by other kinds of textile dyes. Decolorization experiments of the triphenylmethane dye, malachite green, by the marine *Trichoderma harzianum* TSK8 revealed a strong correlation between dye degradation and laccase production, which was induced by the dye (Saravanakumar & Kathiresan 2014). Likewise, another study showed that the anthraquinone dyes alizarin red S and quinizarine green SS also induced production of laccase by *Trichoderma lixii* F21. Here too there was also a strong correlation between laccase production and degradation of these anthraquinone dyes. When the laccase inhibitor sodium azide was added to the decolourisation medium, there was a sharp decrease in both of the anthraquinone dye degradation by *T. lixii* F21 (Adnan et al. 2017).

Induction of *T. asperellum* LBKURCC1 laccase by methyl blue for 24 hours resulted in a much higher laccase activity (118 U mL^{-1}), when compared to the 24 hours induction of *T. harzianum* TSK8 laccase by malachite green (3.99 U mL^{-1}). This may explain why it took ten days to bioremediate 89 % of malachite green by *T. harzianum* TSK8 live cultures (Saravanakumar & Kathiresan 2014). In comparison, it took only 1 day for *T. asperellum* LBKURCC1 to achieve 85 % of methyl blue bioremediation. Likewise, induction of *T. lixii* F21 laccase by the anthraquinone dyes after 24 hours resulted in a laccase activity of only 15 U L^{-1} (0.015 U mL^{-1}), which again may explain the relatively slow degradation of the anthraquinone dyes by live cultures of *T. lixii* F21. It took 7 days to reach 78 % bioremediation of alizarin red S and 98 % of quinizarine green S by live cultures of *T. lixii* F21 (Adnan et al. 2017).

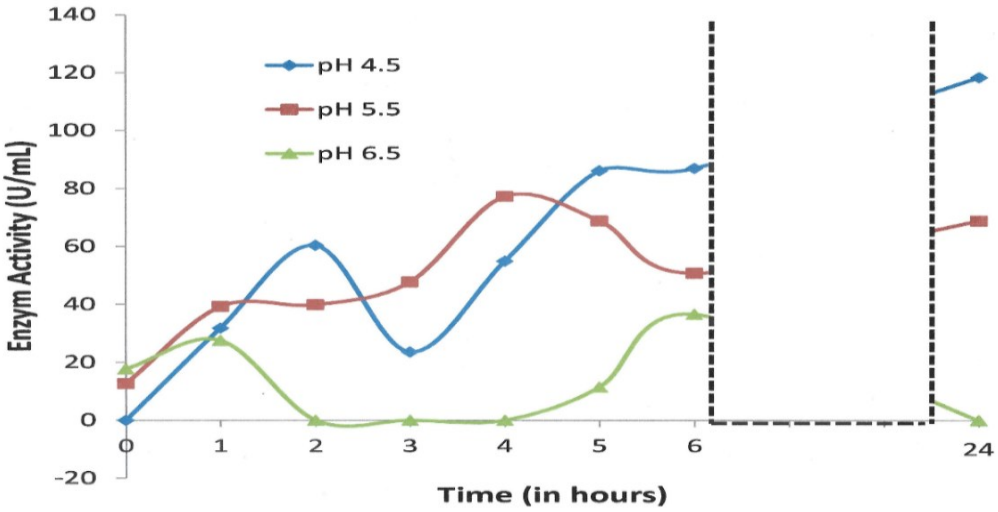

Figure 4. Laccase activity detected at the indicated time points in the culture media during bioremediation of methyl blue by *T. asperellum* LBKURCC1 at 30 °C and pH 4.5 (filled diamonds,♦), 5.5 (filled squares, ■), or 6.5 (filled triangles, ▲).

Removal of methyl blue toxicity by enzymatic biodegradation

The Brine Shrimp Lethality Test (BSLT) using *Artemia salina* nauplii is a well-established test for acute toxicity of various compounds. It is often used to test for compounds released into bodies of water that may harm aquatic life (Carballo et al. 2002). Compounds that are toxic against *A. salina* often indicate toxicity to other aquatic organisms, and so is often used to evaluate textile dye toxicity (Morshed et al. 2018; Ardiati et al. 2024). Toxicity tests of degraded and undegraded methyl blue (MB) dye were measured against brine shrimp (*A. salina*) nauplii and compared to blanks in which only minimal media was added to the reconstituted sea water. The results of the toxicity tests are shown in Figure 5. The survival rate of shrimp larvae in the blanks alone was in the average range of 70 % to 82 %, possibly due to some factor in the minimal media not compatible with the larvae metabolism. Survival rate of the larvae decreased significantly ($p<0.05$) when undegraded MB dye was exposed to it. In contrast, survival rate of the larvae exposed to degraded MB was the same ($p\geq0.05$) as that of the blanks, at all pH tested. It thus can be concluded MB dye is toxic to *A. salina* larvae, and that treatment of MB dye solution with live cultures of *T. asperellum* LBKURCC1 can detoxify MB. Apparently 30 to 40 % bioremediation of MB at pH 5.5 and 6.5 by *T. asperellum* LBKURCC1 cells is sufficient for the detoxification of MB concentration used in the study (50 ppm).

The findings of this study align with prior research demonstrating the correlation between bioremediation of the triphenylmethane dye malachite green and the production of laccase by a marine *Trichoderma harzianum* strain TSK8 in media containing malachite green. *T. harzianum* TSK8 achieved an 89 % degradation of a 750-ppm malachite green solution within ten days (Saravanakumar & Kathiresan 2014). Likewise, removal of phenolic substances from pharmaceutical waste waters by the fungi *Trametes versicolor* was also linked to the presence of laccase activity (Bernats & Juhna 2018). These results confirm the presence of laccase is important for the bioremediation of various dyes and chemicals by fungi.

Fungi are recognized producers of other ligninolytic enzymes, other than laccase, that have been implicated in textile dye bioremediation. These other enzymes are lignin peroxidase (LiP; EC 1.11.1.14) (Singh et al. 2021) and manganese peroxidase (MnP; EC 1.11.1.13) (Pedroza et al. 2007; Kumar & Arora 2022). However, the utilization of these enzymes necessitates the presence of peroxide. In a study on the bioremediation of anthraquinone dyes,

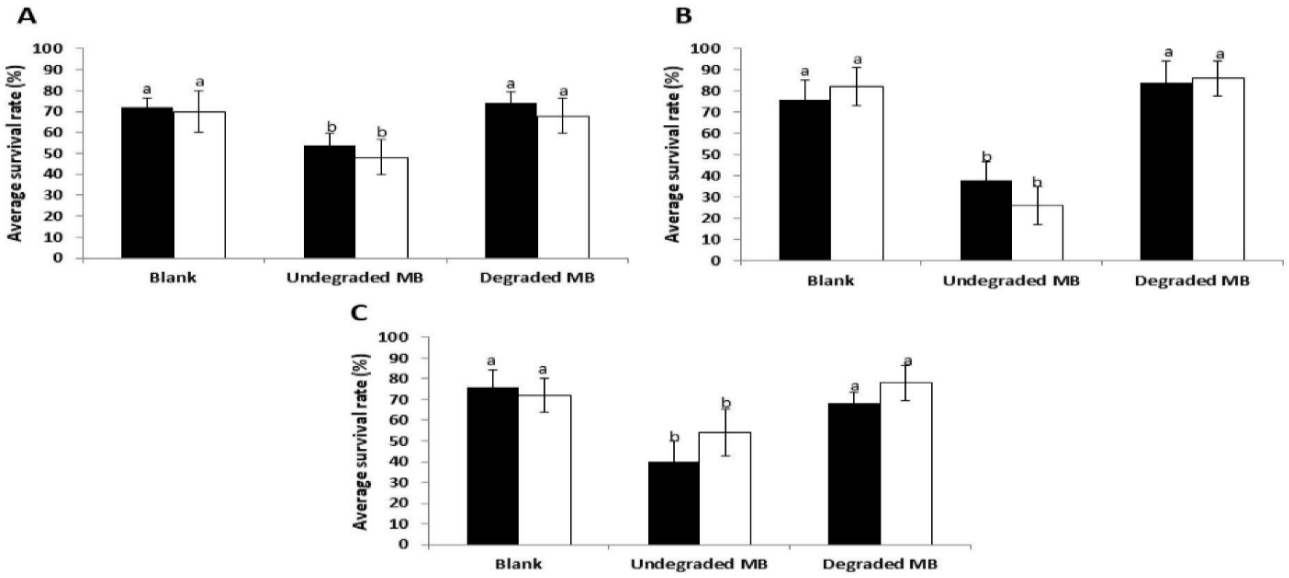

Figure 5. Average survival rate of *A. salina* in salt water added with MM media (Blank), MM media with MB dye (Undegraded MB) or MM media with MB dye treated by *T. asperellum* LBKURCC1 cells (Degraded MB) at different pHs: A) pH 4.5; B) pH 5.5; C) pH 6.5. Concentration of sample: 2 mL L^{-1} (black bars); 4 mL L^{-1} (white bars). Difference in alphabet depicted on top of each bar of the same color shows a statistically significant difference in averages (p<0.05) as determined by the Duncan Multiple Range Test. Standard deviation of each average is shown by error bars.

Adnan et al. (2017) found that *Trichoderma lixii* F21 life cultures exhibited no LiP or MnP activity, with laccase being the dominant enzyme factor. Another study on *Marasmius cladiphyllus* UMAS MS8 demonstrated that laccase activity induced by the azo dye remazol brilliant blue R contributed to its degradation, while no MnP activity was observed, and only trace amounts of LiP activity were detected (Sing et al. 2017). Given these findings, LiP and MnP activity were not assessed in the methyl blue bioremediation experiments in this study.

Our prior research established that *T. asperellum* LBKURCC1 produces laccase in a solid-state fermentation system using rice stalks as the inducer (Rahayu et al. 2019; Sellyna et al. 2020) . Crude laccase extracts (0.014 U mL^{-1}) from this system degraded 60 % of a 50-ppm methyl blue solution after a 4-day reaction (Dahlena et al. 2022). However, enzyme production and extraction, even utilizing agricultural waste, can be time-consuming and relatively expensive. This study demonstrates the feasibility of directly employing *T. asperellum* LBKURCC1 cultures for methyl blue bioremediation, thus eliminating the need for separate enzyme production and extraction. The cell culture system also exhibits a higher methyl blue biodegradation rate than our previous study using crude laccase enzyme extracts.

This study contributes valuable data to the database of fungi and their capabilities to decolourise or biodegrade specific dyes, focusing on laccase induction. There is a diverse efficiency of laccase activity from different sources towards a wide range of dyes (Jeon & Lim 2017), as well as a diverse efficiency of various fungi in bioremediation of various dyes (Tang et al. 2022). As an example, in this study *T. asperellum* LBKURCC1 at pH 4.5 in 24 hours could bioremediate 85 % of a 50-ppm methyl blue solution. This is significantly higher than our previous study in which the same fungal strain at pH 4.5 after 24 hours could only bioremediate 5 % of a 50-ppm solution of the azo-dye reactive black 5 (Nugroho et al. 2023). Considering this, the selection of an appropriate strain for a specific dye biodegradation necessitates a comprehensive database for various applications. In this study we have shown that *T. asperellum* LBKURCC1 can produce laccase by induction of methyl blue dye.

Methyl blue dye is then degraded by the laccase produced in the media, causing decolourisation. The degradation products of methyl blue by *T. asperellum* LBKURCC1 laccase apparently is non-toxic, as demonstrated by the BSLT assay.

The cost-effectiveness of utilizing live microorganism cultures for textile dye effluent biodegradation can be further optimized by employing immobilised cells instead of free cells. Cell immobilisation enables cell reuse, reducing cell recycling and recovery costs (Alam et al. 2021). Additionally, immobilised cells allow the utilisation of microbial consortiums, or mixtures of fungi and bacteria for bioremediation of various substances simultaneously (Pereira et al. 2021). Future developments involving immobilised *T. asperellum* LBKURCC1 cells in conjunction with other dye-degrading fungi hold promise for cost-effective and efficient systems to biodegrade mixtures of various textile dyes.

CONCLUSIONS

T. asperellum LBKURCC1 Life cultures effectively bioremediate the triphenylmethane textile dye methyl blue. Following a 24-hour incubation of the life cultures with 50 ppm of methyl blue at pH 4.5, an average of 85.2 % of the dye was successfully remediated. Notably, over 99 % of methyl blue bioremediation by *T. asperellum* LBKURCC1 mycelia can be attributed to enzymatic biodegradation, specifically through laccase activity. The induction of *T. asperellum* LBKURCC1 laccase production in response to methyl blue occurs across a pH range of 4.5 to 6.5. However, the highest and most stable laccase activity was observed at pH 4.5. Toxicity testing on *Artemia salina* showed that degradation of methyl blue by *T. asperellum* significantly ($p<0.05$) eliminated its toxicity. These findings underscore the potential of *T. asperellum* LBKURCC1 as a promising candidate for direct fungal-mediated bioremediation of wastewater containing the triphenylmethane dye methyl blue.

AUTHOR CONTRIBUTION

A.D.: Methodology, validation, visualisation, data collection and analysis, and original draft writing. Y.N.: Supervision and formal analysis. I.: Methodology and validation. N.W. and D.K.: Data collection, analysis, and graphical production. Y.: Supervision and validation. T.T.N.: Conceptualisation, research designation, drafts and final paper revision, review, and supervision, Principal Investigator of project and corresponding author. The final manuscript was read and approved by all authors.

ACKNOWLEDGMENTS

This work was financially supported by the Directorate of Research, Technology, and Community Service, Ministry of Education, Culture, Research and Technology of the Republic of Indonesia, through implementation of the research grant contract, scheme basic research year 2023 under contract no. 11321/UN19.5.1.3/AL.04.2023 awarded to Titania Tj Nugroho as Principal Investigator.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest regarding the research or the research funding.

REFERENCES

Adenan, N.H., Lim, Y.Y. & Ting, A.S.Y., 2022. Removal of triphenylmethane dyes by *Streptomyces bacillaris*: A study on decolorization, enzymatic reactions and toxicity of treated dye solutions. *Journal of Environmental Management*, 318, 115520. doi: 10.1016/j.jenvman.2022.115520.

Adnan, L.A. et al., 2017. Rapid bioremediation of Alizarin Red S and Quinizarine Green SS dyes using *Trichoderma lixii* F21 mediated by biosorption and enzymatic processes. *Bioprocess and Biosystems Engineering*, 40 (1), pp.85–97. doi: 10.1007/s00449-016-1677-7.

Al-Tohamy, R. et al., 2022. A critical review on the treatment of dye-containing wastewater: Ecotoxicological and health concerns of textile dyes and possible remediation approaches for environmental safety. *Ecotoxicology and Environmental Safety*, 231, 113160. doi: 10.1016/j.ecoenv.2021.113160.

Alam, R. et al., 2021. Biodegradation and metabolic pathway of anthraquinone dyes by *Trametes hirsuta* D7 immobilized in light expanded clay aggregate and cytotoxicity assessment. *Journal of Hazardous Materials*, 405, 124176. doi: 10.1016/j.jhazmat.2020.124176.

Ali, S.S. et al., 2022. Exploring the potential of a newly constructed manganese peroxidase-producing yeast consortium for tolerating lignin degradation inhibitors while simultaneously decolorizing and detoxifying textile azo dye wastewater. *Bioresource Technology*, 351, 126861. doi: 10.1016/j.biortech.2022.126861.

Anita, S.H. et al., 2022. Decolorization of Synthetic Dyes by Tropical Fungi Isolated from Taman Eden 100, Toba Samosir, North Sumatra, Indonesia. *HAYATI Journal of Biosciences*, 29(4), pp.417–427. doi: 10.4308/hjb.29.4.417-427.

Ardiati, F.C. et al., 2024. Evaluation of batch and fed-batch rotating drum biological contactor using immobilized *Trametes hirsuta* EDN082 for non-sterile real textile wastewater treatment. *Journal of Environmental Chemical Engineering*, 12(4), 113241. doi: 10.1016/j.jece.2024.113241.

Argumedo-Delira, R., Gómez-Martínez, M.J. & Uribe-Kaffure, R., 2021. *Trichoderma* biomass as an alternative for removal of congo red and malachite green industrial dyes. *Applied Sciences (Switzerland)*, 11(1), 448. doi: 10.3390/app11010448.

Arora, S. et al., 2022. *Innovations in Environmental Biotechnology*, Singapore: Springer Singapore. doi: 10.1007/978-981-16-4445-0.

Arslantaş, C. et al., 2022. Basic Red 18 and Remazol Brilliant Blue R biosorption using *Russula brevipes*, *Agaricus augustus*, *Fomes fomentarius*. *Water Practice and Technology*, 17(3), pp.749–762. doi: 10.2166/wpt.2022.008.

Asses, N. et al., 2018. Congo Red Decolorization and Detoxification by *Aspergillus niger*: Removal Mechanisms and Dye Degradation Pathway. *BioMed Research International*, 2018, 3049686. doi: 10.1155/2018/3049686.

Barapatre, A., Aadil, K.R. & Jha, H., 2017. Biodegradation of Malachite Green by the Ligninolytic Fungus *Aspergillus flavus*. *Clean - Soil, Air, Water*, 45 (4), 1600045. doi: 10.1002/clen.201600045.

Ben Ali, W. et al., 2020. Screening of five marine-derived fungal strains for their potential to produce oxidases with laccase activities suitable for biotechnological applications. *BMC Biotechnology*, 20(1), 27. doi: 10.1186/s12896-020-00617-y.

Bernats, M. & Juhna, T., 2018. Removal of phenols-like substances in pharmaceutical wastewater with fungal bioreactors by adding *Trametes versicolor*. *Water Science and Technology*, 78(4), pp.743–750. doi: 10.2166/wst.2018.340.

Boer, C.G. et al., 2004. Decolorization of synthetic dyes by solid state cultures of *Lentinula (Lentinus) edodes* producing manganese peroxidase as the main ligninolytic enzyme. *Bioresource Technology*, 94(2), pp.107–112. doi: 10.1016/j.biortech.2003.12.015.

Carballo, J.L. et al., 2002. A comparison between two brine shrimp assays to detect *in vitro* cytotoxicity in marine natural products. *BMC Biotechnology*, 2, 17. doi: 10.1186/1472-6570-2-17

Chatha, S.A.S., Asgher, M. & Iqbal, H.M.N., 2017. Enzyme-based solutions for the textile processing and dye contaminant biodegradation-a review. *Environmental Science and Pollution Research*, 24, pp.14005–14018. doi: 10.1007/s11356-017-8998-1.

Couto, S.R., 2009. Dye Removal by Immobilized Fungi. *Biotechnology Advances*, 27(3), pp.227–235. doi: 10.1016/j.biotechadv.2008.12.001.

Dahlena, M. et al., 2022. Preliminary kinetic studies on the degradation of the textile dye methyl blue by *Trichoderma asperellum* LBKURCC1 laccase without mediators. *AIP Conference Proceedings*, 2638, 100005. doi: 10.1063/5.0104615.

Drăgoi, M. et al., 2021. Degradation of Methyl Blue By Fenton Process. *Annals of the University of Craiova, Series Chemistry*, 27(2), pp.53–59. doi: 10.52846/aucchem.2021.2.06.

Eteba, A., Bassyouni, M. & Saleh, M., 2023. Utilization of chemically modified coal fly ash as cost-effective adsorbent for removal of hazardous organic wastes. *International Journal of Environmental Science and Technology*, 20 (7), pp.7589–7602. doi: 10.1007/s13762-022-04457-5.

Ghosh, A., Dastidar, M.G. & Sreekrishnan, T.R., 2017. Response surface modeling of bioremediation of acid black 52 dye using *Aspergillus flavus*. *Water Science and Technology*, 75(12), pp.2864–2874. doi: 10.2166/wst.2017.167.

He, X. ling et al., 2018. Efficient degradation of Azo dyes by a newly isolated fungus *Trichoderma tomentosum* under non-sterile conditions. *Ecotoxicology and Environmental Safety*, 150, pp.232–239. doi: 10.1016/j.ecoenv.2017.12.043.

Jeon, S.-J. & Lim, S.-J., 2017. Purification and Characterization of the Laccase Involved in Dye Decolorization by the White-Rot Fungus *Marasmius scorodonius*. *Journal of Microbiology and Biotechnology*, 27, pp.1120–1127. doi: 10.4014/jmb.1701.01004

Juárez-Hernández, J. et al., 2021. Isolation of Fungi from a Textile Industry Effluent and the Screening of Their Potential to Degrade Industrial Dyes. *Journal of Fungi*, 7, 805. doi: 10.3390/jof7100805.

Kumar, A. & Arora, P.K., 2022. Biotechnological Applications of Manganese Peroxidases for Sustainable Management. *Frontiers in Environmental Science*, 10, 875157. doi: 10.3389/fenvs.2022.875157.

Kumar, A., Kumar, S. & Ratna, S., 2024. *Nature-Based Wastewater Treatment Systems:Emerging Approaches with Potential Resource Recovery Options*, Boca Raton: CRC Press. doi: 10.1201/9781003441144.

Lanfranconi, I. et al., 2022. Textile dyeing wastewater treatment by *Penicillium chrysogenum*: Design of a sustainable process. *Water Science and Technology*, 86(2), pp.292–301. doi: 10.2166/wst.2022.204.

Latha, L.Y. et al., 2007. Antibacterial activity and toxicity of *Psophocarpus tetragonolobus*. *Pharmaceutical Biology*, 45(1), pp.31–36. doi: 10.1080/13880200601026317.

Mohammed, Y.M.M. & Mabrouk, M.E.M., 2020. Optimization of methylene blue degradation by *Aspergillus terreus* YESM 3 using response surface methodology. *Water Science and Technology*, 82(10), pp.2007–2018. doi: 10.2166/wst.2020.476.

Morshed, M.H. et al., 2018. Cytotoxicity of Four Active Dyes Against *Artemia salina* Leach. *Journal of Engineering Science*, 9(2), pp.55–59.

Munck, C. et al., 2018. Biofilm formation of filamentous fungi *Coriolopsis* sp. on simple muslin cloth to enhance removal of triphenylmethane dyes. *Journal of Environmental Management*, 214, pp.261–266. doi: 10.1016/j.jenvman.2018.03.025.

Mutambyi, V. et al., 2023. Removal of Reactive Black 5 from simulated textile effluents by an electrocoagulation process: optimization by response surface methodology. *Water Practice and Technology*, 18(12), pp.3048–3064. doi: 10.2166/wpt.2023.210.

Nadew, T.T. et al., 2023. Synthesis of activated carbon from banana peels for dye removal of an aqueous solution in textile industries: optimization, kinetics, and isotherm aspects. *Water Practice and Technology*, 18(4), pp.947–966. doi: 10.2166/wpt.2023.042.

Nugroho, T.T. et al., 2023. Bioremediation , Biosorption , and Biodegradation of the Textile Dye Reactive Black 5 by Life Cultures of *Trichoderma asperellum*. *Jurnal Natur Indonesia*, 21(2), pp.93–99. doi: 10.31258/jnat.21.2.93-99.

Nugroho, T.T. et al., 2018. Colour removal of an azo-textile dye and production of laccase by submerged cultures of *Trichoderma asperellum* LBKURCC1. *Journal of Physics: Conference Series*, 1116(4), 042027. doi: 10.1088/1742-6596/1116/4/042027.

Pedroza, A.M. et al., 2007. Sequential treatment via *Trametes versicolor* and UV/TiO2/RuxSey to reduce contaminants in waste water resulting from the bleaching process during paper production. *Chemosphere*, 67(4), pp.793–801. doi: 10.1016/j.chemosphere.2006.10.015.

Pereira, J.C.V., Serbent, M.P. & Skoronski, E., 2021. Application of immobilized mycelium-based pellets for the removal of organochlorine compounds: A review. *Water Science and Technology*, 83(8), pp.1781–1796. doi: 10.2166/wst.2021.093.

Plácido, J. et al., 2016. Degradation and detoxification of synthetic dyes and textile industry effluents by newly isolated *Leptosphaerulina* sp. from Colombia. *Bioresources and Bioprocessing*, 3, 6. doi: 10.1186/s40643-016-0084-x.

Procópio, A.M. da S. et al., 2023. Removal of 17α-ethinylestradiol (EE2) from aqueous solutions by peanut shells (*Arachis hypogaea*): adsorption kinetic, isothermal, and thermodynamic studies. *Water Practice and Technology*, 18(11), pp.2543–2560. doi: 10.2166/wpt.2023.183.

Rahayu, A.G. et al., 2019. Surfactant , Nitrogen and Carbon Media Optimization for *Trichoderma asperellum* LBKURCC1 Laccase Production by Flask Solid State Fermentation of Rice Straw. *Journal of Physics: Conference Series*, 1351, 012030. doi: 10.1088/1742-6596/1351/1/012030

Sandani, Y. et al., 2021. The effect of hydrogen peroxide on catalytic activity of manganosite MnO/ oil palm fly ash catalyst for degradation methylene blue. *Journal of Physics: Conference Series*, 2049(1), 012038. doi: 10.1088/1742-6596/2049/1/012038.

Sani, Z., Abdullahi, I. & Sani, A., 2018. Toxicity Evaluation of Selected Dyes Commonly used for Clothing Materials in Urban Kano, Nigeria. *European Journal of Experimental Biology*, 8(4), 26. doi: 10.21767/2248-9215.100067.

Sar, T. et al., 2024. Resource recovery and treatment of wastewaters using filamentous fungi. *Science of the Total Environment*, 951, 175752. doi: 10.1016/j.scitotenv.2024.175752.

Saravanakumar, K. & Kathiresan, K., 2014. Bioremoval of the synthetic dye malachite green by marine *Trichoderma* sp. *Springer Plus*, 3, 631. doi: 10.1186/2193-1801-3-631.

Sawitri, N., 2010. *Penentuan beberapa parameter produksi kitinase Trichoderma asperellum T.N.C52 dan T.N.J63 pada berbagai substrat kitin*. Universitas Riau.

Sellyna, N. et al., 2020. Optimalisasi waktu fermentasi, kadar air dan konsentrasi Cu2+ pada produksi lakase Trichoderma asperellum LBKURCC1 secara fermentasi padat batang padi dalam reaktor labu. *Chimica et Natura Acta*, 8, pp.7–16. doi: 10.24198/cna.v8.n1.26730.

Shanmugam, S. et al., 2017. *Trichoderma asperellum* laccase mediated crystal violet degradation-Optimization of experimental conditions and characterization. *Journal of Environmental Chemical Engineering*, 5(1), pp.222–231. doi: 10.1016/j.jece.2016.11.044.

Sing, N.N. et al., 2017. Decolourisation Capabilities of Ligninolytic Enzymes Produced by *Marasmius cladophyllus* UMAS MS8 on Remazol Brilliant Blue R and Other Azo Dyes. *BioMed Research International*, 2017, 1325754. doi: 10.1155/2017/1325754.

Singh, A.K. et al., 2021. Lignin peroxidase in focus for catalytic elimination of contaminants — A critical review on recent progress and perspectives. *International Journal of Biological Macromolecules*, 177, pp.58–82. doi: 10.1016/j.ijbiomac.2021.02.032.

Singh, N.J. et al., 2020. Alkali-cation-incorporated and functionalized iron oxide nanoparticles for methyl blue removal/decomposition. *Nanotechnology*, 31, 425703. doi: 10.1088/1361-6528/ab9af1.

Sinha, S. & Hanamghar, M., 2019. Acute Toxicity Of Triarylmethane Dye, Crystal Violet To Indian Major Carpfish, *Labeo rohita*. *International Journal of Research and Analytical Reviews*, 6(2), pp.288–300.

Siraj, Z. et al., 2021. KIT-6 induced mesostructured TiO2 for photocatalytic degradation of methyl blue. *Environmental Science and Pollution Research*, 28(38), pp.53340–53352. doi: 10.1007/s11356-021-14442-z.

Taharchaouche, D. et al., 2023. Degradation by hydrolysis of three triphenylmethane dyes: DFT and TD-DFT study. *Theoretical Chemistry Accounts*, 142(1), 10. doi: 10.1007/s00214-022-02950-1.

Tang, K.H.D. et al., 2022. Biological Removal of Dyes from Wastewater: A Review of Its Efficiency and Advances. *Tropical Aquatic and Soil Pollution*, 2(1), pp.59–75. doi: 10.53623/tasp.v2i1.72.

Wang, W. & Subramani, S., 2017. Assays to Monitor Pexophagy in Yeast. In *Methods in Enzymology*. Academic Press Inc., pp. 413–427. doi: 10.1016/bs.mie.2016.09.088.