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Nepalese White-Rot Fungus *Ganoderma lucidum*: A Potential Source of Ligninolytic Enzymes for Aromatic Dye Degradation

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Abstract

This study evaluates aromatic dyes degradation in solid and liquid media and the ligninolytic enzymes production by the white-rot fungus *Ganoderma lucidum*. The results demonstrated that *G. lucidum* degraded Congo red (CRD) and methylene blue (MBD) more rapidly than malachite green (MGD) ($p < 0.0001$). In the solid medium containing CRD and MBD, the fungus showed a high decolorization index, while its mycelial growth remained unaffected by the presence of dye. Similarly, in liquid media, *G. lucidum* effectively decolorized CRD and MBD. Similarly, the fungus was cultured in naphthalene-supplemented liquid media to assess the ligninolytic enzyme production. Laccase activity peaked at 8 days (985 U L^{-1}), while lignin peroxidase reached maximum activity at 14 days (1192 U L^{-1}), followed by a decline in both enzymes. These results suggest the potential of *G. lucidum* for selective bioremediation of dye-contaminated environments.

Keywords: Decolorization; Aromatic dyes; *Ganoderma lucidum*; Enzyme activities; Biodegradation; Fungal bioremediation; Textile dye

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Introduction

Environmental pollution remains one of the major global challenges, where industrial waste is the major contributor. Among the various industries, the textile sectors stand out as a major polluter, utilizing more than 100,000 different kinds of commercial dyes and producing nearly one million tons annually. Out of which, around 10% of the dyes are released into natural ecosystem as untreated waste (1,2). The widespread application of these dyes makes them major pollutants in textile effluents. Furthermore, the extensive water consumption in textile processing generates large volumes of dye-laden wastewater, causing severe ecological harm (3). Industries like paper and pulp mills, dyes-related manufacturers, distilleries, tanneries, and pharmaceuticals also contribute to environmental degradation by generating intensely colored wastewater (1). Additionally, untreated effluents from dyeing and finishing processes substantially elevate biological oxygen and chemical oxygen demand, and total organic carbon, further stressing aquatic systems (4).

Out of the various synthetic dyes used, aromatic dyes (a significant portion of which are azo dyes) are of particular concern, which are mutagenic and carcinogenic (5). These compounds were shown to induce frameshift mutations in human hepatoma cell lines and pose risks to other organisms (4,5). Reduction of these azo dyes generates aromatic amines that resist metabolic breakdown, compounding their toxicity and persistence, challenging conventional removal technologies (6). Thus, the urgent need to eliminate these hazardous compounds is clear. The physical and chemical methods currently used in decolorization of the textile industry effluent face limitations due to high cost and inefficacy against a broad range of dyes (7). Microbial decolorization methods emerge as a cost-effective, and environmentally friendly alternative, as these organisms are capable of not only removing color but also breaking down dyes into simpler, non-toxic compounds (8). However, bacterial-based approaches have shown limited success (9). In contrast, white-rot fungi degrade recalcitrant organo-pollutants through oxidative enzymes such as lignin peroxidase, lignin, and manganese peroxidase, which efficiently decompose lignin and aromatic compounds under aerobic conditions (10). Furthermore, these fungi also mineralize persistent organic pollutants and diverse dyes, positioning them as promising candidates for bioremediation approaches (11,12)

Ganoderma lucidum (*G. lucidum*), a white-rot basidiomycete polypore fungus, is renowned for its unique medicinal properties and distinctive morphology;

Table 1: Details of dyes used for decolorization assay

Dye	Class	Molecular formula	Molecular weight (g/mol)	λ_{max} (nm)
Congo red	Diazo	$C_{32}H_{22}N_6Na_2O_6S_2$	696.67	562
Methylene blue	Heterocyclic	$C_{16}H_{18}ClN_3S$	319.85	644
Malachite green	Triphenylmethane	$C_{23}H_{25}ClN_2$	364.92	617

a glossy red, kidney-shaped fruiting body with brown spores (13). This fungus produces various ligninolytic enzymes, enabling it to degrade xenobiotics and synthetic compounds, making it a candidate for environmental applications (14). The high-altitude regions of Nepal, characterized by low oxygen levels, strong UV radiation, fluctuating temperatures, and soil composition, create a unique ecological niche that may give rise to *G. lucidum* strains with improved metabolic activity and stress resilience (15). This study evaluates the potential of wild *G. lucidum* from the mountainous region (2500 meters above sea level) of Nepal to decolorize key synthetic classes of dyes-Congo Red (CRD), Methylene Blue (MBD), and Malachite Green (MGD)-in both solid and liquid media (**Table 1**). In addition, the ligninolytic enzyme production was quantified. The findings may advance the use of this understudied Nepalese white rot fungus in developing sustainable decolorization processes for textile effluents.

Materials and methods

Chemicals

All chemicals utilized in this study were of analytical grade. Congo Red (CRD), Methylene Blue (MBD), and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich (Germany) and Malachite Green (MGD) from Qualigens Fine Chemicals Ltd. (India). ABTS was used as the substrate for enzyme assays. All media components were of high purity grade and obtained from Hi-Media Lab. Pvt. Ltd. (Mumbai, India).

Collection, Identification, and Inoculum Development

Fruiting bodies of *G. lucidum* were collected in August from various locations on Chandragiri Hill, Kathmandu, Nepal. The mushrooms were identified and confirmed by Dr Mahesh Kumar Adhikari, a senior mycology taxonomist at the Department of Plant Resources, Kathmandu, Nepal. After identification, the mushrooms were cleaned, and the inner tissue from the fresh fruiting bodies was excised and used to isolate mycelium on standard potato dextrose agar (PDA) medium (containing 4 g potato Infusion, 20 g dextrose, and 15 g agar-agar per liter, adjusted pH 5.6) under aseptic conditions. The cultures were incubated at 25 ± 1 ℃ for 5 days. Pure mycelial cultures were maintained by subculturing on freshly prepared PDA and kept at 4 ℃ for downstream experiments.

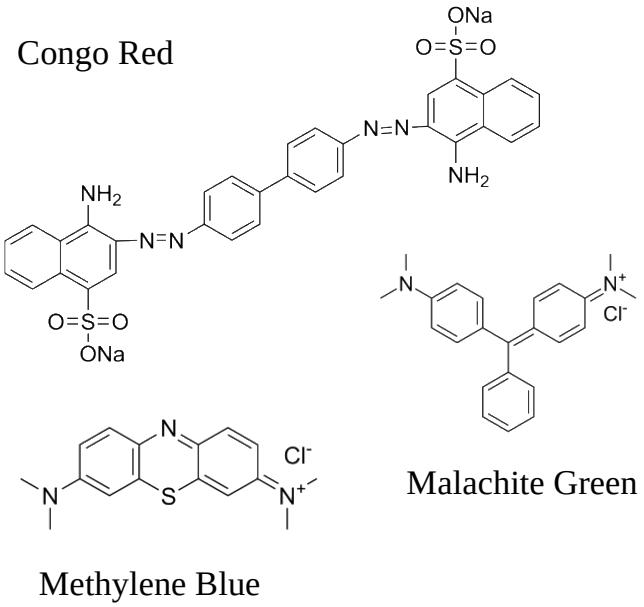

Figure 1. Chemical structures of Congo Red, Methylene Blue, and Malachite Green, showing their functional aromatic rings and key functional groups

Solid Phase Decolorization

The solid-phase decolorization ability of *G. lucidum* was conducted using the method described by (16,17). Three dye classes were tested: azo (Congo Red (CRD)), heterocyclic (Methylene Blue (MBD)), and triphenylmethane (Malachite Green (MGD)) (**Table 1**). Each dye was added into a PDA plate at a concentration of 100 mg L^{-1}. Each plate was inoculated with a 5 mm diameter mycelial disc at the center, while uninoculated dye-containing plates served as controls, and all plates were incubated at 25 ℃ incubation for 10 days. After incubation, mycelial diameter (MD) and decolorization diameter (DD) were measured. The decolorization index (DI) was calculated as:

DI=DD/MD

Experiments for each dye were conducted in three biological replicates.

Liquid State Decolorization

Liquid-state decolorization was evaluated in Potato Dextrose Broth (PDB) enriched with 100 mg L^{-1} of each dye, following the method described by (17,18). Erlenmeyer flasks containing 160 mL of PDB were inoculated with a single 10 mm diameter agar plug of mycelia, excised from the edge of 7-day-old actively growing cultures. The flasks were maintained in a shaking incubator at 25 ℃ and 140 rpm for 16 days. At regular intervals, 2 mL aliquots were withdrawn, and filtered through Whatman® Grade 1 qualitative filter paper, and the resulting filtrate was centrifuged at 4000 rpm for 2 minutes. The absorbance was measured at the dye-specific λ_{max} (644 nm for Methylene Blue, 617 nm for Malachite Green, and 562 nm for Congo Red) using an SSI-2101 UV spectrophotometer (hereafter spectrophotometer). Dye-containing media without mycelia was used as controls. The decolorization percentage was calculated using:

(%) decolorization = $(A_o - A) / A_o \times 100$

where A_o represents the initial absorbance and A is the absorbance at the time of measurement. Experiments for each dye were conducted in three biological replicates.

Ligninolytic Enzyme Assay

Pure *G. lucidum* mycelia was cultivated in 20% potato broth medium enriched with 1% naphthalene and 1% glucose. The cultures were filtered every two days using Whatman® Grade 2 qualitative filter paper to remove fungal mycelia. The resulting supernatant was then collected for enzyme activity analysis.

Using ABTS as the enzymatic substrate, laccase activity was determined following the protocol outlined by (19). For the assay, 0.5 mL of the culture filtrate was combined with 1.2 ml phosphate buffer (0.1M, pH 6.0) and 0.5 mL ABTS (0.45 mM). This reaction mixture was then incubated at 30 ℃ for 2 minutes. The enzymatic oxidation of ABTS was assessed by monitoring the increase in absorbance at 420 nm with a spectrophotometer.

Lignin peroxidase activity was determined using the same procedure as for laccase assay, with the inclusion of 0.5 mL hydrogen peroxide (2 mM) to mixture to initiate the reaction. For both laccase and lignin peroxidase, one unit of enzyme activity was defined as the quantity of enzyme needed to oxidize one micromole of ABTS per minute at 25 ℃.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 10 software (GraphPad Software, Inc., USA). Each experiment was conducted in three biological replicates, and the data are presented as mean values ± standard deviation.

Results and Discussion

Solid Phase Decolorization

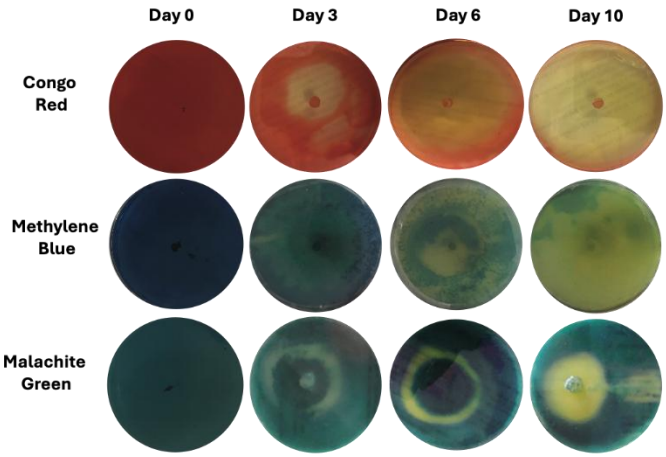

Figure 2. Solid state decolorization of aromatic dyes by G. lucidum over a 10-day incubation period. Representative images showing the progression of decolorization for each dye at days 0, 3, 6, and 10

Table 2: Decolorization of dyes on solid media by *G. lucidum* mycelium after 10 days of incubation.

Dyes	MD (mm)	DD (mm)	DI= (DD/MD)
Congo red	78 ± 5.5	72 ± 3.1	0.924 ± 0.02 $^{\alpha}$
Methylene blue	79 ± 5.2	71 ± 3.5	0.913 ± 0.02 $^{\alpha}$
Malachite green	56 ± 1.4	27 ± 2.5	0.484 ± 0.03 $^{\beta}$

MD: Mycelial diameter, DD: Decolorization diameter, DI Decolorization index. For each dye listed in the DI column values sharing same symbol are not significantly different while different symbol indicate statistically significan differences ($p < 0.0001$). Differences in DI among the dyes were analyzed using one-way ANOVA followed by Tukey's post-hoc test, with significance set at $p < 0.05$.

The mycelial growth and degradation efficiency of *G. lucidum* on aromatic dyes in solid media were evaluated based on the decolorization ability of the dyes. The visual fading of color on the plates was used as an indicator of dye decolorization (**Figure**. 2). Over 10 days, *G. lucidum* showed higher decolorization activity for both Congo Red (0.924 ± 0.02) and Methylene Blue (0.913 ± 0.02) compared to Malachite Green (0.484 ± 0.03) (**Table 2**). This pattern was consistent with the observed mycelial growth, where media containing Congo Red and Methyl Blue exhibited more rapid mycelium growth than those containing Malachite green.

Interestingly, the decolorized zones on the media plates were observed to be smaller than the mycelial outgrowth, suggesting that the fungal mycelium extended beyond the region where the enzymatic activity was primarily concentrated. This discrepancy may be attributed to the faster mycelium growth compared to the slower rate of

enzymatic degradation of the dye, as enzyme diffusion from the fungal hyphae into the deeper layers of the medium might be limited. This observation was consistent with previous studies where enzymatic degradation seems to be slower than growth resulting in the gap between the growth front and the decolorized zone (10).

A similar trend was reported by (17), where *G. lucidum*, along with other fungi including *Trametes suaveolens, Fomitopsis rosea*, and *Pleurotus pulmonarius* exhibited comparable Methylene blue decolorization rates, albeit with relatively lower mycelial growth compared to our study. The breakdown of the aromatic compounds likely occurs during the secondary metabolism of white-rot fungi (20,21), a process that provides both carbon and energy sources for fungal growth and thus positively influences their growth (22,23).

Liquid State Decolorization

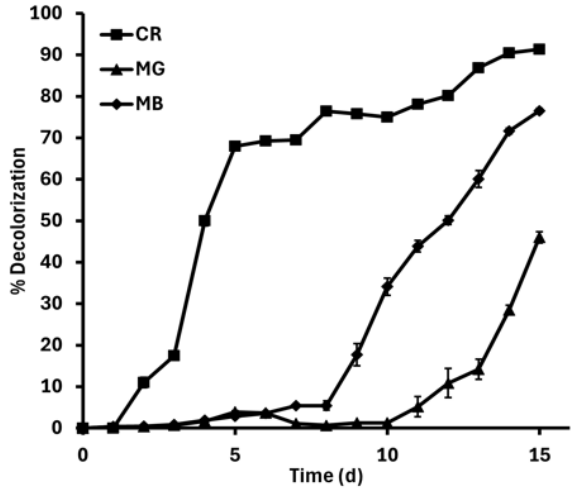

Figure 3. Decolorization of aromatic dyes in the liquid phase by *G. lucidum* from day 0 to day 15. Results are presented as the mean of three biological replicates. CR: Congo Red, MB: Methylene Blue, MG: Malachite Green.

The cultural filtrate obtained after incubating *G. lucidum* in a liquid medium containing 100 mg L^{-1} of different dyes for varying time intervals (2, 4, 6, 8, 10, 12, 14, and 16 days) was assayed for decolorization activity. Visual observation of the liquid medium revealed a notable reduction in color, which was further quantified using spectrophotometric measurements at the specific maximum absorbance wavelength for each dye (**Table 1**). After 16 days of incubation, *G. lucidum* effectively decolorized all the dyes tested; however, CRD and MBD showed the highest decolorization efficiency, with decolorization percentages of 91.5 ± 0.1 and 75.9 ± 0.8, respectively (**Figure**. 3). In contrast, MGD exhibited significantly lower decolorization, with around 46% of the dye removed (**Figure**. 3). These results indicate that the decolorization efficiency increased with longer incubation periods. Furthermore, this trend indicates that the dye structure and the redox potential of the enzymes produced by *G. lucidum* contribute to the decolorization process (24,25).

The higher decolorization rates observed for CRD and MBD suggest that *G. lucidum* is more efficient in breaking down these dyes compared to MGD. This is consistent with findings from similar studies, where *Naematoloma spp.* (17,18) and *Ganoderma spp.* (26,27)demonstrated efficient CRD decolorization in liquid media. Furthermore, studies involving *Pycnoporus cinnabarinus* and *G. lucidum* also reported similar decolorization patterns for CRD, MBD, and MGD. However, in other mushroom species, *Trametes suaveolens, Fomes fomentarius, Coriolus versicolor*, *Stereum ostrea* and *Pycnoporus coccineus, only* around 40% of decolorization of MBD was observed within 20 days (17)

A separate study (26,27) reported that laccase from *G. lucidum* achieved 80% decolorization of Congo Red within 6 hours under conditions of pH 4.0 and 40 °C. This rapid decolorization was likely due to the alterations in the chromophore and other structural components of the CRD, with enzymatic activity inducing observable changes in its molecular structure.

Malachite green, a triphenylmethane aromatic dye, showed the least decolorization in both solid and liquid state experiments. This reduced effectiveness may be due to its well-documented antifungal properties, commonly used in the aquaculture industry, which may hinder *G. lucidum* mycelial growth, thus reducing its ability to decolorize the dye (28–30). Additionally, the rigid and stable aromatic structure of Malachite green compared to Congo Red and Methylene Blue poses a significant challenge to enzymatic degradation (31). The stability of the dye's molecular structure makes it more resistant to oxidation and breakdown by the ligninolytic enzymes produced by *G. lucidum.*

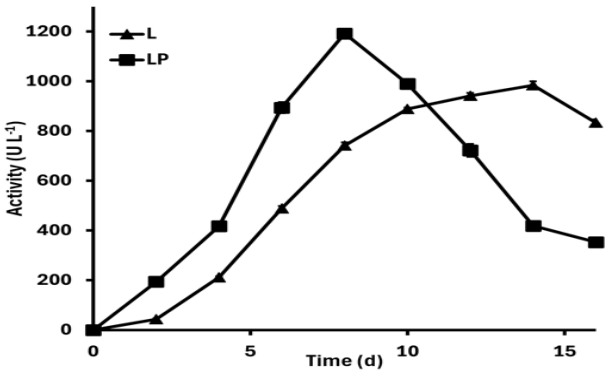

Figure 4: Production of laccase and lignin peroxidase by *G. lucidum* measured at different time points (Day 0 to Day 16) in potato broth containing 1% naphthalene and glucose. Results are presented as the mean of three biological replicates

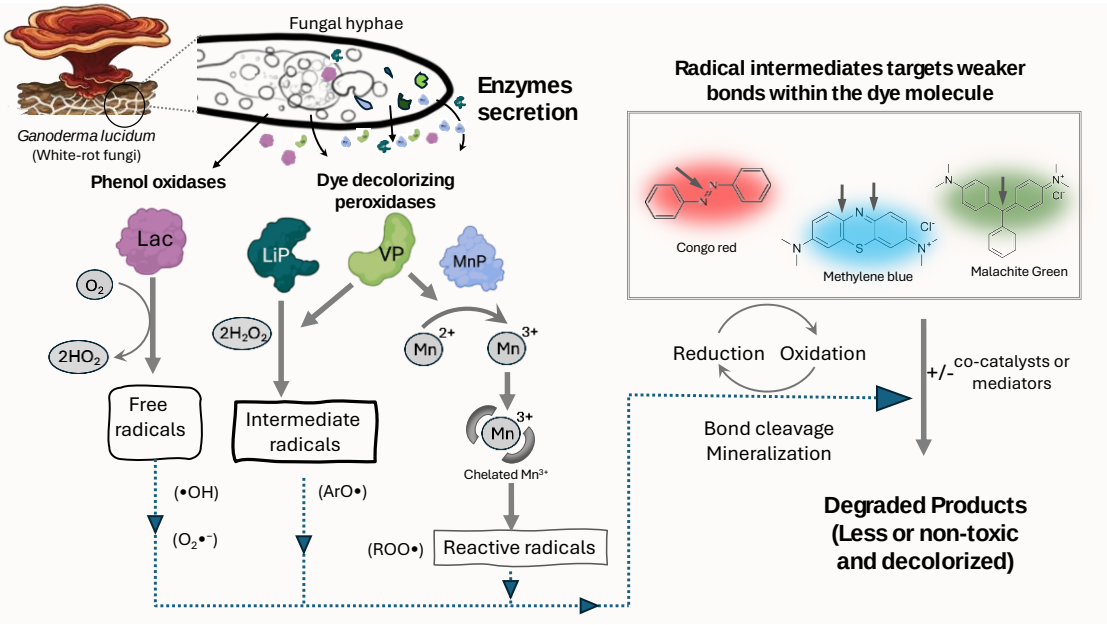

Figure 5: Illustration of proposed mechanisms of aromatic dye degradation by white-rot fungi such as *G. lucidum* through series of steps. Fungal hyphae secrete extracellular ligninolytic enzymes, including laccase (Lac), lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP), which work synergistically to degrade dyes. LiP initiates oxidation by generating hydroxy radicals, MnP enhances the process by producing Mn^{3+} and additional free radicals. Lac and VP further contribute by oxidizing both phenolic and non-phenolic compounds. The free, intermediate, and reactive radicals target weaker bonds within the dye molecule, a process that is further enhanced in the presence or absence of co-catalysts and mediators. These enzymes actions ultimately lead to the formation of decolorized, less toxic aromatic amines and other by-products. ($O_2•^-$): Superoxide radical; (•OH): Hydroxyl radical; (ArO•): Phenoxy radical; (ROO•): Peroxyl radical

Production of Ligninolytic Enzymes

Potato broth (20%) supplemented with 1% naphthalene and 1% glucose was used for the growth of *G. lucidum* mycelium, and laccase production was determined through the ABTS assay. The highest activity of laccase (Lac) (985.3 ± 4.4 U L^{-1}) was observed on the 14th day, while lignin peroxidase (LiP) activity peaked at 1192.2 ± 12.7 U L^{-1} on the 8th day under the same condition (**Figure**. 4). The enzyme production followed a normal distribution pattern with a gradual increase in activity up to the 8th and 14th days, followed by a subsequent decline (**Figure**. 4). This pattern is characteristic of fungal cultures, where enzyme synthesis is closely tied to the metabolic demand and nutrient availability (**Figure**. 4). The asynchronous peak activities of LiP and Lac observed in our study suggest differences in their regulatory mechanisms, expression timelines, and functional roles in fungal metabolism. The early peak in lignin peroxidase (LiP) likely corresponds to the early-to-mid exponential growth phase, indicating its role in the initial breakdown of complex lignin-like or aromatic compounds. In contrast, the delayed peak in Lac activity suggests expression during the late exponential or stationary phase, where it may function to complement earlier oxidative enzymes by further degrading intermediate products.

The production of ligninolytic enzymes is influenced by various factors, including temperature, pH, nutrient composition, ratios of carbon-to-nitrogen, and aeration rate (32–34). Aromatic compounds are commonly used as inducers to enhance the synthesis of these enzymes. Therefore, to improve the production, as suggested by (18,35), incorporating small amounts of aromatic hydrocarbons, such as naphthalene, has been shown to enhance the production of ligninolytic enzymes, thus promoting more efficient degradation by the fungi. This supports the rationale for supplementing the broth with naphthalene in our experiments.

The high activity of laccase and lignin peroxidase produced by *G. lucidum* may explain its strong decolorization efficiency. These enzymes can catalyze the oxidation of variety of inorganic substrates and organic compounds through a one-electron process, including aromatic amines, ascorbates, phenols (mono-, di-, and poly). This oxidation reaction is subsequently couple with four-electron reduction of molecular oxygen into water (36). Furthermore, studies (24,37) reported the significant potential of laccase from *Lentinus polychrous* for the treatment of waste dyes, emphasizing the role of extracellular ligninolytic enzymes in the decolorization process carried out by white-rot fungi.

Potential mechanism of degradation dyes by white-rot fungi, *G. lucidum*

High-altitude environments, such as mountainous regions of Nepal, are extreme due to year-round consistently low temperatures, reduced oxygen levels, intense UV radiation, and limited nutrient availability. These factors can significantly influence the physiology and metabolism of organisms, including fungi (38). Research suggests that nutrient limitation, a common feature of high-altitude ecosystems, induces the ligninolytic enzymes production in white-rot fungi as a survival strategy to efficiently utilize available resources (39–41). The breakdown of dyes by white-rot fungi like *G. lucidum* is facilitated by complex ligninolytic enzymes, including Lac (laccase), LiP (lignin peroxidase), MnP (Manganese peroxidase), and VP (versatile peroxidase) (40,42,43) These enzymes are secreted extracellularly through the fungal hyphae, targeting weaker bonds in dye molecules and reducing them to less harmful by-products (41). LiP, for instance, is particularly effective in the oxidative cleavage of aromatic substances, initiating a non-enzymatic chain reaction that produces highly reactive hydroxy radicals (•OH). These radicals, in turn, specifically target the azo bonds in dyes like Congo Red, resulting in less toxic aromatic amines formation, as supported by studies (43–45). This degradation mechanism involves the oxidation of aromatic rings of the dyes by LiP's heme-containing active site, extracting electrons and generating •OH, a process required for breaking down these recalcitrant structures (44) MnP further complements LiP by oxidizing Manganese (Mn^{2+} to Mn^{3+}) in the presence of hydrogen peroxide (H_2O_2) to produce a powerful oxidant capable of catalyzing the breakdown of phenolic compounds and aromatic amines. Mn^{3+} can specifically diffuse and react with substrates, generating additional free radicals such as superoxide ($O_2^{\bullet -}$) and hydroxyl (OH) radicals, which further enhance the degradation process. This dual role of MnP in direct oxidation and radical production though is versatile, it's efficiency hinges on hydrogen peroxide availability, which can very likely be impacted by specific environmental (46).

Laccase, a multicopper oxidase, on the other hand, operates through a four-electron oxidation reaction, generating semiquinone radicals that primarily target phenolic compounds (47). Its ability to degrade non-phenolic compounds via a mediator system is particularly noteworthy. Mediators, such as expand laccase's substrate range by acting as redox shuttles, through the oxidation of otherwise recalcitrant dyes. This mediator system is important for decolorization and structural breakdown, leading to the formation of smaller, less toxic intermediates, including aromatic amines (43).

Versatile peroxidase (VP), however, exhibits combined characteristics of both LiP and MnP, generating both •OH and $O_2^{\bullet -}$ radicals that interact with dye molecules, specifically attacking azo bonds and through the oxidative cleavage of the chromophore (48). This dual capability of VP makes it effective across a broad range of substrates; however, its activity is pH-dependent. Since its optimal activity is near neutral conditions, which may limit its application in acidic or alkaline wastewaters (45). These radical-driven reactions decolorize the dye and also lead to the formation of smaller, less toxic environmentally benign by-products (49,50).

The degradation products, typically aromatic amines, are generally less toxic than the original parent dyes. These products are further metabolized to final by-products by *G. lucidum*, such as carbon dioxide and water, supporting the fungus's role in bioremediation (51). This could be a sustainable approach to environmental detoxification, particularly for textile wastewater, where synthetic dyes are prevalent. However, challenges including scaling up fungal cultures, and optimizing enzyme activity under varying pH and temperature, need to be addressed. Some limitations of the current study and potential future directions include: a) We selected a representative dye concentration of 100 mg L-1; however, future studies should include a gradient dose providing deeper insights into degradation thresholds, potential substrate inhibition, and to determine EC50 values. b) Incorporating additional negative controls, such as heat-killed fungal control and enzyme inhibitors, would clarify the relative contributions of enzymatic degradation and passive dye adsorption. c) Byproduct characterization using advanced analytical tools could provide valuable insights into the degradation pathways and profiling of intermediate products.

Conclusion

The biodegradation potential of *G. lucidum* for aromatic dye removal was successfully demonstrated in this study. *G. lucidum* can rapidly and efficiently decolorize various dyes, demonstrating its effectiveness for practical application. Utilizing fungi for dye degradation offers a cost-effective, efficient, and eco- friendly alternative to conventional chemical methods used for treating industrial effluents. Future research should focused on comprehensive investigations involving multiple fungal species, addressing scaling challenges in scaling through optimization of enzyme activity by evaluating the effects

of pH, temperature, substrate concentration, and degradation thresholds. Such efforts could lead to the development of robust fungal-based biodegradation technologies suitable for industrial-scale application.

Conflict of Interests

None.

Ethical issues

The study did not involve any human or animals; therefore, ethical approval was not required.

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