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Appraisal of malachite green biodegradation and detoxification potential of laccase from *Trametes cubensis*

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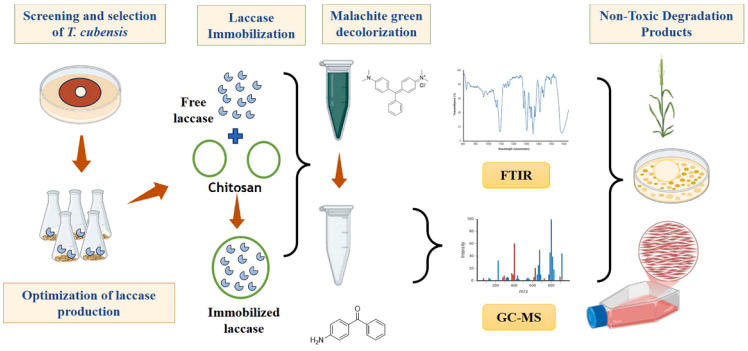
HIGHLIGHTS

- First report on malachite green degradation by *T. cubensis* laccase.
- Maximum laccase production by SSF was 6577.00 ± 14.32 U/g at 8 d.
- 89 and 86 % decolorization in 2 h by free and chitosan immobilized laccase.
- Non-toxicity of malachite green degradation confirmed by toxicological studies.
- Pathway for malachite green degradation by laccase deduced by GC–MS analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

The laccase from the newly isolated *Trametes cubensis* was investigated for its potential to degrade malachite green (MG) dye. Optimized solid-substrate fermentation enhanced laccase production by 8.8-fold, reaching an activity of 6577.0 ± 14.3 U/g. Proteomic characterization identified enzyme with 4 % sequence coverage, molecular weight of 43.1 kDa, and alignment with multicopper oxidases. Using one-factor-at-a-time optimization, MG decolorization was maximized at 89 % under optimal conditions: 20 U/mL enzyme dose, 0.1 mg/mL dye concentration, pH 5.0, and 2 h incubation at 50 °C. Crosslinking the laccase onto chitosan beads resulted in 82 % immobilization efficiency, with high recyclability and reusability, retaining over 52 % activity after 7 cycles and demonstrating similar ($p < 0.05$) dye degradation potential. MG degradation products exhibited significantly reduced phyto-, cyto-, and microbial toxicity. The degradation pathway was elucidated using gas chromatography-mass spectrometry analysis. Thus, both free and immobilized laccase from *T. cubensis* offer sustainable tool for effective MG degradation with reduced toxicity.

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1. Introduction

The discharge of various recalcitrant and toxic organic or inorganic industrial pollutants excessively contaminates the environment (Vignesh et al. 2020). The large-scale application of synthetic dyes in fabric coloring processes makes textile wastewater highly rich in such dyes, as a relatively large proportion of the dye remains unbound to the fabric. It is estimated that synthetic dyes contaminate wastewater globally at levels crossing 80,000 tons per year (Yadav et al. 2021). In India, around 280,000 tons of textiles dyes are discharged into the effluents annually (Madamwar et al., 2019). Malachite green (MG) is a common, synthetic, cationic, triphenylmethane dye with high water solubility. It is used in dyeing of fabrics (silk, jute, wool, and cotton), leather, and paper, as well as in staining of biological samples (Abu-Hussien et al. 2022; Vignesh et al. 2020). Some of its prohibited applications include its use as a colorant for food adulteration and as an antimicrobial therapeutic agent in aquaculture (Ashok et al. 2014). MG has effective fungicidal and parasiticidal activity in aquatic systems, often leading to elevated concentrations in waterbodies.

Like many other textile dyes, MG is known for its toxicity, mutagenicity, and carcinogenicity in various life forms (Abu-Hussien et al. 2022). Therefore, there is an urgent need for targeted research on MG's degradation, as its persistence and accumulation in waterbodies pose significant environmental challenges. Removal of MG from water has been attempted via adsorption, photolysis, phytoremediation, chemical precipitation, chemical oxidation–reduction, and electrochemical treatments (Haleem et al. 2023; Sarathi et al. 2023). The photocatalytic activity of LaFeO3 has resulted in 80 % degradation of MG in 80 min (Jabeen et al. 2023). A study using rice husk-derived biochar via the adsorption method achieved 96.9 % removal of MG (Tsai et al. 2022). In contrast, cell suspension cultures of *Blumea malcolmii Hook.* achieved 93.4 % MG decolorization in 24 h through phytodegradation (Kagalkar et al. 2011). However, the generation of toxic by-products and high sludge as secondary pollutants, along with high-cost makes such physico-chemical methods less attractive than biological methods of dye removal. Enzymes can also be applied to completely biodecolorize and biodegrade dyes like MG into non-toxic products. Examples of these enzymes include azoreductases for the decolorization of methyl red (Qi et al. 2016), laccase for MG (Himanshu et al. 2023), dye-decolorizing peroxidase for various anthraquinone and azo dyes (Santos et al. 2014), manganese peroxidase for malachite green (Yang et al. 2016), and lignin peroxidase for several textile dyes such as reactive red M5B, direct red 5B, direct brown MR, and disperse red DK (Ghodake et al. 2009).

Among enzymes, laccases (EC 1.10.3.2) have specifically gained significant attention due to their high oxido-reductive potential and ability to catalyze the ring cleavage in several aromatic compounds. This is attributed to the presence of connected cupredoxin-like domains, which are twisted into a tight globule (Wang et al. 2019). Laccase production has been reported under both solid-state fermentation (SSF) and submerged fermentation (SmF); however, SSF gained attention due to its low cost, high yield, and effective use of agro-industrial by-products. Moreover, microorganisms grow on a solid substrate with sufficient moisture to sustain microbial metabolism and offer economic and technical advantages over conventional SmF (Martau et al. 2021). Although both crude and purified laccases have been extensively employed for dye degradation, immobilizing laccases can significantly reduce operational costs and improve enzyme stability, thereby, enhancing their suitability for large-scale applications. Among various matrices used to enhance immobilization efficiency (IE) of laccase, application of chitosan is well-documented. The biobased nature of chitosan (2-amino-2-deoxy-β-D-glucose moieties linked via β-1,4-glycosidic linkages), biodegradability, biocompatibility, physiological inertness, strong affinity for proteins and cost-effectiveness of chitosan makes it ideal for enzyme immobilization. Cross-linking with glutaraldehyde can further enhance the stability and repeated use of the chitosan immobilized enzyme (Bilal et al. 2019).

White rot fungi (WRF), such as *Lentinus tigrinus, Trametes versicolor, Polyporus ciliates, Phanerochaete chrysosporium, Trametes hirsuta, Phlebia tremellosa, Ganoderma lucidum,* and *Coriolopsis gallicia* are among the highest laccase producers (Jadhav et al. 2011). Laccase from *Trametes* sp. laccase finds extensive applications in bioremediation, including MG degradation, with reported decolorization activities ranging from 73.7 to 95.1 % (Abbas et al. 2024). Attributes like higher enzyme production and high substrate conversion efficiency are crucial for large scale applications. Thus, the immense bioremediation potential of white rot fungal laccase, in general, and that of the *Trametes* genera, in particular, makes bioprospecting for novel white rot fungal laccases worthy of investigation.

This study aimed to exploring the potential of crude and chitosan immobilized laccase from *T. cubensis* in the biodegradation of triphenylmethane dye MG, deducing the possible degradation mechanism via Gas Chromatography Mass Spectrometry analysis (GC–MS) and exploring the toxicity of the generated metabolites. Previously, *T. cubensis* laccase has been reported for medicinal applications (Li et al. 2021), but its bioremediation potential, especially in decolorization and degradation of MG to non-toxic products, remains unexplored.

2. Materials and methods

2.1. Materials

Wheat bran was purchased locally, thoroughly cleaned, and oven-dried at 50 °C until reaching a constant weight. All the chemicals used were of analytical grade or better. MG was purchased from Sigma Aldrich (India), while 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), hydroxybenzotriazole (HOBT), and guaiacol from Merck Life Science Pvt. Ltd. and Sisco Research Laboratories Pvt. Ltd., respectively. Dulbecco's Modified Eagle Medium (DMEM) was procured from GIBCO (Life Technologies).

2.2. Microorganisms and medium

The fungal culture used in this study was isolated from Lepa Rada (Arunachal Pradesh), India. It was grown on malt extract agar (MEA) with a pH of 5.5 by incubating at 30 °C for 7 d, preserved at 4 °C and subcultured monthly. Bacterial cultures (*Bacillus thuringiensis* and *Escherichia coli*) were procured from culture collection of Microbiology Department at the Central University of Haryana and maintained on nutrient agar slants at 4 °C. The mineral salt solution (MSS) used for laccase production had a pH of 5.0 and contained KH_2PO_4, $MgSO_4$, $(NH_4)_2SO_4$ each at 0.05 % (w/v).

2.3. Molecular identification

The molecular identification of the fungus was carried out by amplifying and sequencing the conserved region of the ITS-5.8S rRNA gene, using the primer pair ITS-1 (forward) and ITS-4 (reverse). This was followed by aligning the obtained sequence with those from National Center for Biotechnology Information (NCBI) GenBank and constructing the dendrogram using MEGA-11 for phylogenetic analysis (Tamura et al. 2021). The isolated fungal strain was deposited in the National Agriculturally Important Microbial Culture Collection (NAIMCC), at the ICAR-National Bureau of Agriculturally Important Microorganisms (ICAR-NBAIM), Mau, India. It has been assigned the accession number NAIMCC-TF-3609 within the repository.

2.4. Laccase production

Laccase was produced in 250 mL conical flasks under SSF conditions, using 5 g of wheat bran as the solid substrate saturated with 15 mL MSS. Inoculation of the SSF flasks was done with 4 mycelial discs (8 mm

diameter) taken from periphery of 1 week old culture on MEA. After incubation at 30 °C for 1 week, the fermented substrate was supplemented with four volumes of 0.1 M citrate–phosphate (CP) buffer. The mycelial mat was disrupted using a sterile spatula, followed by incubating at 30 °C for 1 h with gentle shaking at 150 rpm. Thereafter, solids were separated by squeezing through double-layered muslin cloth and centrifuging the filtrate at 10,000 rpm for 10 min at 4 °C. The extracted crude enzyme was subjected to a laccase activity assay.

2.5. Enhanced laccase production by One-Factor-at-a-Time optimization

To maximize laccase production by SSF, following combinations of influential factors were tested: carbon source (cotton stalk, mustard stalk, wheat bran and saw dust, each separately at 5 g), amount of biomass (5–20 g) in a 250 mL Erlenmeyer flask, volume of enzyme extraction buffer (10–60 mL), incubation time (3–15 d), incubation temperature (15–35 °C), pH (3.0–8.0), inoculum dose (1–8 mycelial discs), inoculum age (3–13 d), substrate-to-moisture ratio (1:1–1:7), and inducers (ethanol, vanillin, $CuSO_4$, guaiacol, veratryl alcohol, each at 2 mM separately).

2.6. Estimation of laccase activity

Laccase activity was analyzed by adding 0.2 mL of appropriately diluted enzyme extract to 1.8 mL of 0.01 M guaiacol (dissolved in 0.1 M CP buffer with pH 5.0) followed by incubation at 25 °C and measuring the absorbance at 470 nm after 20 min. One enzyme unit (U) was defined as "the change in the absorbance of 0.01 per min per mL at 470 nm under standard assay conditions" was considered as (Vasdev et al. 1994).

2.7. Protein identification by LC-MS/MS

The crude laccase sample was subjected to native and sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) (Gaur et al. (2018). For zymogram analysis, the gel after native PAGE was incubated in citrate phosphate buffer (100 mM, pH 5) containing 10 mM guaiacol for 20 min (Himanshu et al., 2023). The molecular weight of laccase was determined by comparison with the pre-stained protein molecular weight markers (10–250 kDa; BioRad, USA). The development of a brown-coloured band corresponded to laccase. The estimated corresponding band on SDS PAGE was excised (1x1 mm), and further subjected to reduction, alkylation, and trypsinization (15 ng/µl trypsin). Thereafter, the digested peptides were analyzed by Easy-nLC-1000 mass spectrometer system (Thermo Fisher Scientific, Massachusetts, USA) coupled with Orbitrap Exploris (Thermo Fisher Scientific) and separated on an Acclaim PepMap 100 C18 analytical column (Thermo Fisher Scientific, 2 µm particle size, 75 µm inner diameter × 25 cm length). For precursor mass tolerance (MS1) and fragment mass tolerance (MS2), maximum ion transfer time was kept 60 ms and 120 ms, respectively. Full-scan MS spectra was recorded with mass scan range of *m/z* 375–1500. Raw MS data files were analyzed in proteome discoverer software (ver. 2.5) against the constructed database of *Trametes* available in UniProt (https://www.uniprot.org/). For dual Sequest and Amanda search, the precursor and fragment mass tolerances were set at 10 ppm and 0.02 Da, respectively. Carbamidomethyl on cysteine as fixed modification and oxidation of methionine and N-terminal acetylation were considered as variable modifications for database search. Both peptide spectrum match (PSM) and protein false discovery rate were set to 0.01 to filter out protein with high confidence. Multiple sequence alignment of identified proteins was performed using ClustalW in Uniprot and the Blastp tool in NCBI. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD056936.

2.8. Immobilization of laccase

A chitosan suspension was prepared by dissolving 25 g/L chitosan in 1.5 % (v/v) acetic acid solution with constant stirring at 50 °C. The suspension was then used to make chitosan beads by dropping it into 1 M potassium hydroxide and the resultant beads were allowed to harden at 4 °C for 16 h. Uniform-sized beads were sorted and incubated at 4 °C in 2 % (v/v) glutaraldehyde solution for their activation, followed by removal of unbound glutaraldehyde by washing in CP buffer (pH 5.0). The activated beads were then incubated with laccase solution (20 U/mg protein, 18.55 mg/mL) for 1 d in a refrigerator and the unbound laccase was removed from the beads by washing with 0.1 M CP buffer (pH 5.0), followed by storage under refrigeration (Bilal et al. 2019). The activity of bound laccase was quantified to determine % immobilization efficiency (IE) using eq. (1).

$$IE(\%) = \frac{Total\ activity\ of\ immobilized\ enzyme(U/mL)}{Total\ activity\ of\ free\ enzyme(U/mL)} \times 100 \quad (1)$$

2.9. Reusability of immobilized laccase

Enzyme reusability was estimated by quantifying residual laccase activity after each cycle of use, which involved filtration of beads, washing with CP buffer and incubation with 10 mM guaiacol dissolved in 0.1 M CP buffer (pH 5.0). The % residual activity after each cycle of reuse was calculated as a percentage of the laccase activity during the first cycle, which was considered as 100 %. Storage stability of immobilized and free enzyme was assayed by storing the enzyme in CP buffer (pH 5.0) for three weeks at 4 and 25 °C. The enzyme activity during 1st cycle was considered as 100 %.

2.10. Dye decolorization study

Dye decolorization experiments with free laccase were performed in 100 mL capped flasks by adding appropriate enzyme dose to a total volume of 30 mL of dye solution prepared in 0.1 M CP buffer (pH 5.0) under shaking at 150 rpm. Process variables such as enzyme dose (2–120 U/mL), decolorization time (30–1440 min), temperature (20–60 °C), pH (4.0–9.0, maintained with sodium citrate, CP, phosphate and Tris-HCl buffers, respectively, for pH 2.0–4.0, 5.0–6.0, 7.0–8.0 and 9.0) and dye concentration (0.1–0.5 mg/mL) were varied to maximize the MG decolorization using an OFAT approach. Redox mediators ABTS and HOBT (0.1–1.0 mM) were also used to study their impact on the decolorization process. Dye solutions with or without mediator (control) were added with enzyme and incubated at the previously optimized parameters. The decolorization of MG was monitored spectrophotometrically (Specord210 Plus Spectrophotometer, Analytik Jena, Germany) at 614 nm and reported as the dye decolorization (%), according to eq. (2):

$$Dyedecolorization(\%) = \frac{A(i) - A(f)}{A(i)} \times 100 \quad (2)$$

where, A_i and A_f are the absorbances of dye samples without or with laccase treatment, respectively.

Similarly, MG dye (0.1 mg/mL) was subjected to decolorization by immobilized laccase at 50 °C and 150 rpm for 120 min and the aliquots were collected at different times to determine % decolorization (eq. (1). To exclude the possibility of adsorption of MG on to the beads, control sets employed chitosan beads without laccase. Immobilized laccase was recycled and reused for decolorization of MG dye by filtration of beads after the 1st cycle, followed by washing of beads with CP buffer (10 mM, pH 5.0).

2.11. Characterization of dye degradation products

Toluene (0.6 mL) and $CaCO_3$ solution (1 mL of 50 % w/v) were

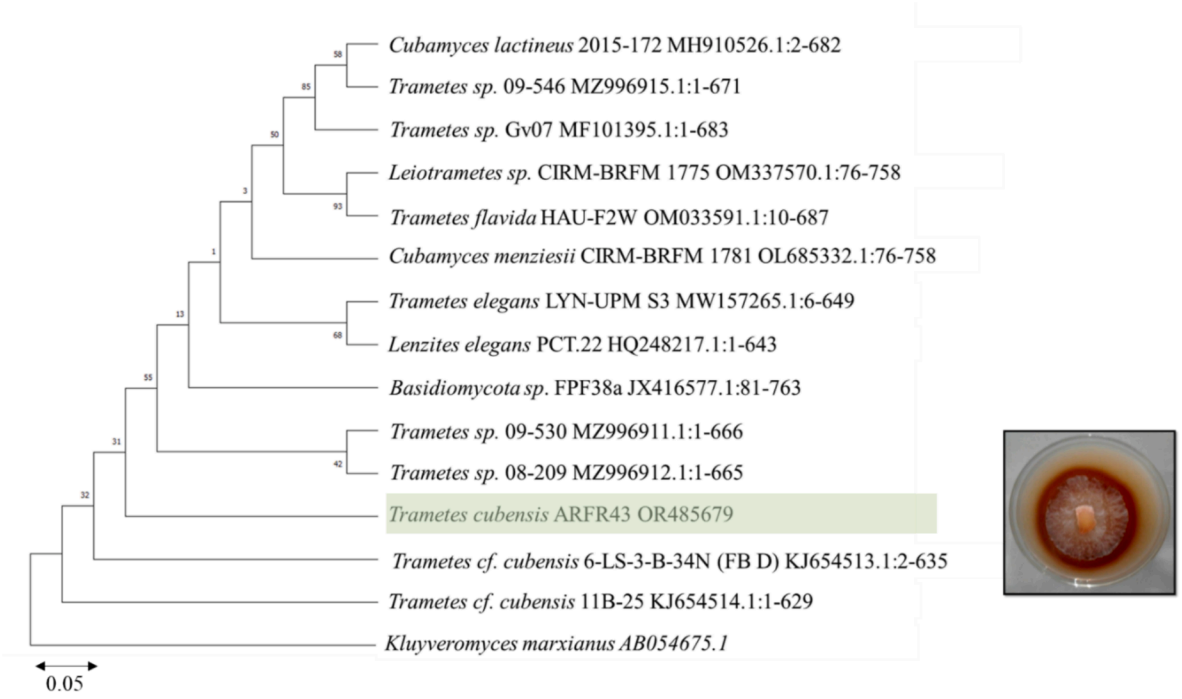


Fig. 1. Dendrogram illustrating the phylogenetic relationship of *T. cubensis* ARFR43 with other white rot fungi. MEGA software with the N-J method and 1000 bootstrap replication was used. Inset shows the halo zone formation by the *T. cubensis* ARFR 43 in presence of guaiacol.

added to untreated or treated MG dye solution (3 mL) in conical flasks (working volume of 30 mL) and shaken at 150 rpm and 25 °C for 1 h. Thereafter, the flasks were kept under static condition for 1 h and the organic layer containing the dye and degradation products was recovered and subjected to Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) and GC–MS analyses. FTIR spectra (Bruker IFS 66v/s, Karlsruhe, Germany) were recorded from 4000 to 500 cm^{-1}. For GCMS (QP2010 Ultra, Kyoto, Japan) analysis of MG degradation, a DB-5 column was used with Helium (flowing at 1.21 mL/min) as the carrier gas (Wanyonyi et al. 2017).

2.12. Toxicity assessment

For phytotoxicity studies, ten seeds each of *Triticum aestivum* and *Pennisetum glaucum* were placed on a filter paper in a Petri dish and incubated with 5 mL untreated or treated MG dye solution or distilled water (control) at 30 °C. After one week, seed germination %, and the elongation of the roots and shoots were determined. The microbial toxicity of the original and degraded dye was reported in terms of the halo zone of growth inhibition obtained by agar disc diffusion against *B. thuringiensis,* and *E. coli,* following the protocol of Vignesh et al. (2020).

The cytotoxic impact of dye (6.25–200 μg/mL), degraded dye, enzyme control and control devoid of test solution on the survival of human foreskin fibroblast (HFF) cells (National Centre for Cell Science, Pune, India) was assessed by 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. The HFF cells were cultured in DMEM containing 10 % FBS and antibiotics (1 % each of penicillin and streptomycin) at 37 °C under CO_2 environment (5 %). Nutrient replacement was done daily, and cell detachment was facilitated using 0.25 % trypsin-EDTA. The percentage of MTT reduction was reported as cell viability (%), considering the absorbance of the control assay (without any treatment) as 100 %. The susceptibility of cells to untreated or treated MG, and enzyme solution was characterized by IC_{50}, which was calculated using GraphPad Prism 9 software. The evaluation of toxic effects of MG degraded products was done by comparing them with untreated MG sample.

2.13. Statistical analysis

Three replications of the experiments were conducted and results were reported as mean ± standard deviation. ANOVA, Dunnett's or Tukey's multiple comparison tests ($p \leq 0.05$) were carried out using GraphPad Prism version 9.

3. Results and discussion

3.1. Identification of laccase producing fungus

An efficient laccase producing WRF was isolated from Lepa Rada, Arunachal Pradesh, India. It exhibited high laccase activity during qualitative and quantitative screening (746.6 ± 7.01 U/g) after 8 d under SSF conditions. The fungus was identified as *Trametes cubensis* ARFR43 based on its phylogenetic relatedness (99.36 % homology) to *T. cubensis* (GenBank accession no. KJ654514.1) (Fig. 1). The obtained sequence of the ITS-5.8S rRNA gene was deposited in NCBI GenBank (accession no. OR485679). WRFs, such as *T. cubensis* belong to Basidiomycetes and produce unique oxidative and extracellular ligninolytic enzymes with the capability to biodegrade numerous man-made contaminants and render them non-toxic (Zhuo and Fan, 2021). The genus *Trametes* is well known for its medicinal and edible properties and has also been investigated for the removal of several environmental pollutants (Li et al. 2021).

3.2. Enhanced laccase production by T. cubensis

Optimization of SSF led to 8.8-fold enhancement in laccase production with the following combination of process variables: wheat bran (5 g) as the carbon source, extraction buffer volume of 50 mL, incubation time of 8 d, pH 5.0, incubation temperature of 30 °C, inoculum size of 4 mycelial discs, inoculum age of 6 d, substrate-to-moisture of 1:2 and copper sulfate (2 mM) as an inducer. The most influential factors affecting the laccase fermentation process were the volume of the enzyme extraction buffer, incubation period, inoculum age, substrate moisture ratio, and inducers. The highest activity of 6577.0 ± 14.3 U/g

by *T. cubensis* was better than the reported 77.9 U/g using farnesol-pineapple waste (Backes et al. 2023).

3.3. Proteomics analysis and characterization of laccase

A single brown-colored band observed during zymography confirmed the enzyme's monomeric nature, corresponding to a molecular weight of approximately 40 kDa as determined by SDS-PAGE (unpublished data). However, mass spectrometric analysis predicted the molecular weight of 43.1 kDa and this discrepancy could be likely due to glycosylation of the protein. The identified peptide sequence SAGSSVYNYDNPIFR showed 100 % sequence identity with laccases from *Pycnoporus cinnabarinus* and *Trametes cubensis*, as confirmed through sequence alignment using ClustalW with the Uniprot database. Additionally, a search in the NCBI conserved domain database predicted that the identified protein belonged to the cupredoxin superfamily (Cl9115), which harbors type I copper centers characteristic of multicopper oxidases (EC 1.10.3.2). This alignment further supports the relatedness of the identified laccase to well-characterized enzymes involved in oxidative processes. Furthermore, the sequence coverage was 4 %, supported by one peptide and two peptide-spectrum matches (PSMs), confirming the presence of a unique peptide. The identified laccase comprised 394 amino acids, with an isoelectric point (pI) of 5.31, indicating a slightly acidic nature. Confidence in the identification was validated by search scores of 4.47 in Sequest HT and 440.26 in MS Amanda. The laccase was confirmed as an extracellular enzyme, consistent with its known role in lignin degradation and other oxidative processes relevant to environmental and biotechnological applications.

3.4. Enhanced dye decolorization by free laccase

The optimization of MG decolorization by free laccase enzyme was performed using the OFAT approach. With different enzyme doses (2–120 U/mL), the minimum laccase quantity of 20 U/mL appeared to be optimum for maximum 62.5 % decolorization of the dye within 1440 min. Prior studies have also reported maximum decolorization with minimum laccase enzyme quantities of 30 U/mL (Sharma et al. 2015) and 25 U/mL (Murugesan et al. 2007). Varying the incubation time from 30 to 1440 min revealed that a minimum of 120 min was needed to achieve maximum decolorization of 63 %. These results were consistent with those of Qin et al. (2019), where the maximum decolorization of Remazol Brilliant Blue (RBB) R dye reached 60.3 % within 150 min. A positive correlation between temperature and the decolorization process from 20 to 50 °C was observed, with a maximum of 71.7 % decolorization, followed by a sharp decline at 60 °C. The dependency of dye decolorization on pH was determined by adjusting the reaction pH between 4.0 and 9.0. Acidic pH was more suited for MG decolorization by laccase, with a maximum decolorization of 70.7 % at pH 5 and a sharp decline in decolorization activity in the alkaline range. Acidic pH is more favourable for laccase activity as alkaline pH interrupts the electron transport between the T1 and T2/T3 centers due to linkage of OH^- to the T2/T3 copper sites of laccase (Yadav et al. 2021). Backes et al. (2022) reported similar pH and temperature profiles for laccase mediated decolorization of MG, with the highest decolorization in the pH range of 4–5 and temperatures of 40 to 50 °C, respectively. The decolorization efficacy of laccase is also dependent upon the amount of the dye (Arunprasath et al. 2019; Qin et al. 2019), therefore, the influence of the dye concentration (0.1–0.5 mg/mL) on decolorization was determined with a fixed laccase dose of 20 U/mL at 50 °C. The lowest concentration of dye exhibited the highest 70.9 % decolorization. The decline in decolorization with increasing dye concentration could likely be due to the saturation of the dye binding/decolorization capacity of laccase at a particular dye concentration. Arunprasath et al. (2019) revealed the maximum 96.9 and 89.2 % decolorization with 0.05 and 0.5 mg/mL of MG dye, respectively, in 24 h. Similarly, the highest decolorization (96.8 %) of RBBR occurred when the dye concentration was at a minimum of 25 mg/L (Yadav et al. 2021).

The oxidation–reduction (O-R) potential of fungal laccase (0.5–0.8 V) is sufficient for the direct biodegradation of chemical moieties with low O-R potential. Redox mediators can broaden the degradation capability of laccases by assisting in electron movement between the enzyme and substrate (Sharma et al. 2015). The addition of redox mediators enhances the reaction rate, as they act as electron carriers between enzyme and the target pollutant. The enzyme oxidizes the mediators, generating stable radicals with high redox potential, which then oxidize the substrates and are reduced during the process. The investigation of the effect of two redox mediators (ABTS and HOBT) on dye decolorization revealed a positive impact of ABTS (concentration of 0.1 mM) in enhancing the effectiveness of decolorization process (89 % decolorization) compared to 70.8 % decolorization observed in the control experiment (without any mediator) at 120 min. In contrast, HOBT supplementation led to a decrease in decolorization to 55 %. ABTS has been earlier reported to successfully enhance the decolorization of Brilliant Blue R to 100 % within 30 min (Kagalkar et al. 2015). It has been stated that the action of laccase is not affected by the stable $ABTS^{\bullet+}$ cation generated from ABTS, whereas the 1-HOBT radical gets converted into its derivative form, benzotriazole, which could possibly inactivate the laccase, thereby decreasing its decolorization efficiency (Chhabra et al. 2009).

Dye decolorization time significantly impacts the overall cost-effectiveness of the process, with shorter times being preferable. Previously, 73.7 % decolorization in 24 h by laccase from *Trametes* sp. G31 (Ado et al. 2019) and 32 % decolorization in 48 h by laccase from *T. pubescens* MB89 Abbas et al. (2024) have been reported, which were significantly lower than that observed under this study. The results of dye degradation reported here are significant due to the application of a lower enzyme dose as well as a shorter incubation time compared to earlier reports. For example, free laccase from *Fusarium oxysporum* took a relatively longer time (20 h) for 90 % decolorization of MG dye (0.1 mg/mL) (Thoa et al. 2022).

3.5. Immobilization of laccase on chitosan

Efficient industrial application necessitates the cost-effective immobilized enzyme. Thus, the cost, availability of a suitable support matrix, and simplicity of the immobilization operation with minimal steps for the support activation through chemical treatment are some key requirements during enzyme immobilization (Aslam et al. 2021; Bilal et al. 2019). In this study, laccase was effectively immobilized on chitosan that was previously functionalized by glutaraldehyde, resulting in a high IE of 82 % (67.76 mg/g of chitosan). These findings were similar to previously reported IE of 84.7 % (Bilal et al. 2019). In comparison, Jeong and Choi (2020) achieved laccase loading-rate of 18.5 mg/g of chitosan-tripolyphosphate beads using a laccase solution of 2 mg/mL. Zheng et al. (2016), achieved a higher IE (95 %) of *T. pubescens* laccase during the decolorization of Acid black 172, after the statistical optimization of immobilization process parameters. Other matrices have also been used for laccase immobilization, such as activated biochar made from rice husk (IE of 66 %) (Imam et al. 2021) and crosslinked calcium alginate (IE of 72 %) (Lassouane et al. 2019), but the reported IE were lower. Thus, glutaraldehyde-activated chitosan beads appear to be a biocompatible support for optimal laccase immobilization, primarily due to the generation of aldehyde groups on the chitosan bead surface after glutaraldehyde treatment. The attractive forces between laccase and glutaraldehyde, along with Schiff base formation as a result of the reaction between the amino groups of laccase and glutaraldehyde, lead to effective and enhanced binding of laccase to chitosan, with glutaraldehyde acting as a bridge (Aslam et al. 2021; Bilal et al. 2019).

The recyclability of immobilized laccase is crucial for reducing the cost of industrial enzyme applications and it was assessed by repeated substrate (guaiacol) oxidation. Immobilized laccase retained 85 % of its activity after three consecutive cycles and more than 50 % activity after

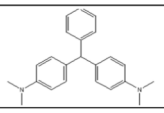
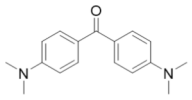
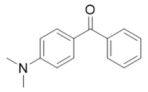
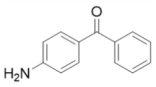
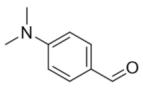
Compound name	Retention time	Molecular weight (m/z)	Chemical structure
p,p'-Benzylidenebis (N,N-dimethylaniline)	22.8	330	
Methanone, bis(4-dimethylamino) phenyl	11.8	268	
Methanone, (4-aminophenyl) phenyl	9.3	197	
Methanone, (4-(dimethylamino) phenyl)phenyl	15.2	225	
Benzaldehyde, 4-(dimethylamino)	19.4	149	

Fig. 2. Intermediates in the malachite green degradation as characterized by GC–MS.

seven cycles, indicating significant retention of laccase activity after multiple cycles. Previously, Pandey et al. (2022) also reported that 53 % activity of immobilized laccase could be retained after six cycles. Thus, laccase immobilization on activated chitosan beads provided significant resistance, thereby enhancing its catalytic stability after multiple oxidation cycles. The decline in activity after prolonged utilization of immobilized laccase could be attributed to enzyme leaching from chitosan after washing steps. Additionally, conformational changes induced by nearby molecules could also cause the loss of enzyme activity (Bilal et al. 2019; Lassouane et al. 2019).

Storage stability is also an important parameter for assessing immobilization performance, as enzymes are more susceptible to a decline in activity over extended storage periods. After three weeks storage at 4 °C, immobilized laccase remained more active (retaining 90 % of its original activity) than free laccase (which retained 80 % activity). Chitosan formed a protective microenvironment, providing extra resistance to laccase inactivation and extending its shelf life (Pandey et al. 2022). Similarly, Zheng et al. (2016) reported the retention of

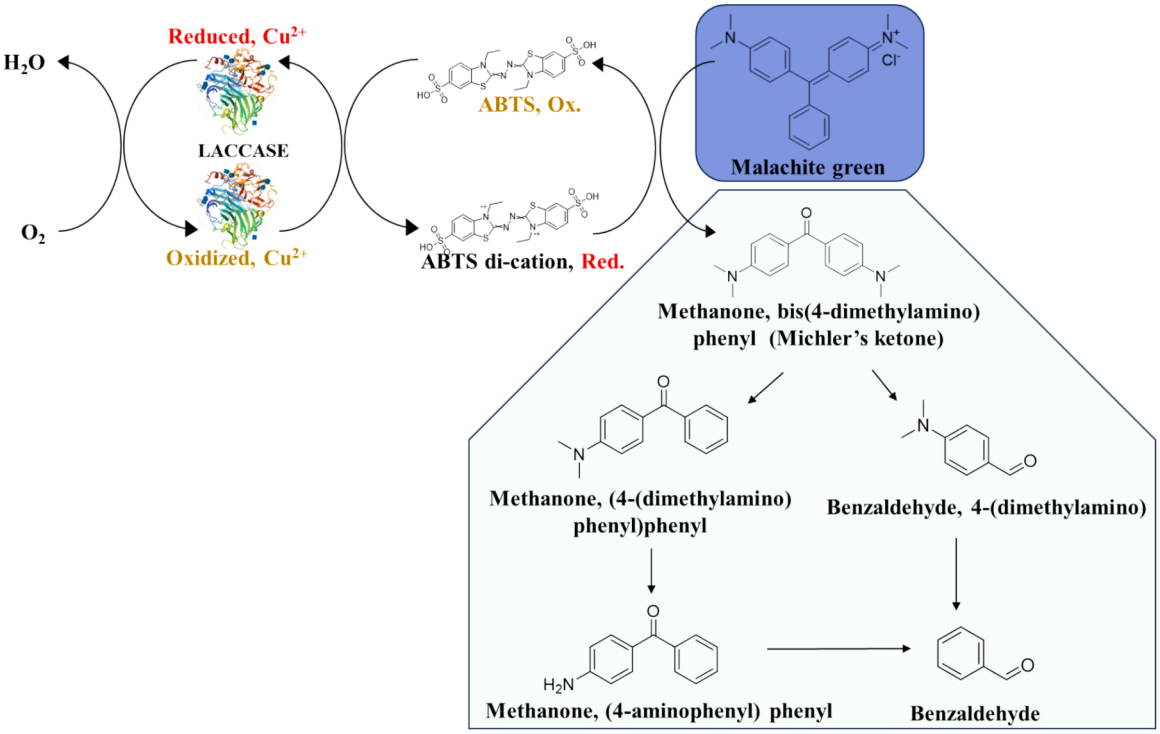


Fig. 3. Proposed pathway of laccase-catalyzed malachite green degradation and detoxification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Phytotoxicity of untreated or laccase treated MG dye solution on *P. glaucum* and *T. aestivum.*

Parameters studied	*P. glaucum*			*T. aestivum*		
	Water	Untreated	Treated	Water	Untreated	Treated
Germination (%)	100	20***	90*	100	30***	100
Shoot length (cm)	6.07 ± 0.40	1.91 ± 0.25***	4.43 ± 0.20***	7.60 ± 0.27	1.64 ± 0.25***	7.32 ± 0.38
Root length (cm)	7.58 ± 0.42	0.00***	5.37 ± 0.26***	7.57 ± 0.39	1.06 ± 0.27***	5.34 ± 0.11***

Statistical analysis was carried out using one-way ANOVA, followed by Dunnett *t*-test (*** denote significantly different results in comparison to control at P-value < 0.01).

nearly 30 and 40 % activity for free and immobilized laccase, respectively, after a similar period.

3.6. Decolorization of malachite green by immobilized laccase

A high dye decolorization of 85.6 % was seen after 120 min of treatment with chitosan-bound laccase. The effectiveness of the immobilized laccase was evident from the 50 % decolorization observed after the third cycle of continuous use. This indicated that the enzyme, after immobilization, could be used to decolorize a greater quantity of dye cumulatively. The catalytic activity of immobilized enzymes generally decreases with repeated use due to the loss of active sites. Pandey et al. (2022) reported 85 % decolorization using laccase immobilized on pine needle biochar in 300 min when the concentration of MG was 0.05 mg/mL of the reaction mixture. Another study documented 90 % decolorization of 0.02 mg/mL MG within 360 min, using laccase-biotitania biocatalyst (Zhang et al. 2018). These results indicated that both free and immobilized enzymes were effective for the decolorization process.

3.7. Characterization of dye degradation products

FTIR-spectroscopic analysis of laccase mediated MG degradation revealed changes in the absorption pattern, indicating the modification of various functional groups during the decolorization process. The FTIR spectrum showed the fingerprint region of MG between 1500–500 cm^{-1}, characterized by discrete peaks of mono- and di-substituted aromatic rings. Significant changes in the fingerprint region after laccase treatment were observed. A major broad peak between 3025 and 3710 cm^{-1} in the untreated sample corresponded to N-H stretching vibration of 1° amines; however, this peak was undetected in the degraded dye (Alaya et al. 2021). Peak shifting, from 874 to 894 cm^{-1}, after laccase treatment corresponded to C-H bending of a 1,2-disubtituted benzene derivative. The peak at 1635 cm^{-1}, corresponding to N-H bending of amines in the untreated sample, disappeared after the degradation of the dye. Additionally, the peak at 1030 cm^{-1} corresponded to amines (N-C), while the peaks at 1457 and 2916 cm^{-1} indicated aromatic and alkene (C-H) groups, respectively, in the IR spectrum of the degraded dye. Overall, the difference in the IR spectra of the original and treated dye confirmed its degradation into simpler metabolites (Arunprasath et al. 2019; Chaturvedi and Verma, 2015).

GCMS analysis was employed to understand the degradation products and mechanism of MG degradation by laccase. (Fig. 2). The dye was eluted at 22.850 min (330 *m/z*), and a significant decrease in its peak area was observed after laccase-mediated degradation. The intermediate compounds generated during MG degradation were detected in the GC–MS chromatograms and were further used to deduce the biodegradation pathway (Fig. 3). The starting point of degradation was the hydroxylation of the central carbon atom of MG dye, leading to the formation of Methanone, bis(4-dimethylamino)phenyl, also known as Michler's ketone. Subsequent deamination and oxidative cleavage of Michler's ketone formed Methanone, (4-(dimethylamino)phenyl)phenyl and benzaldehyde, 4-(dimethylamino). Further oxidative cleavage of these identified compounds resulted in the formation of end products i.e., Methanone, (4-aminophenyl)phenyl and benzaldehyde subunits, which may be converted to fatty acids. The deduced pathway for laccase-mediated MG degradation aligns with several previous studies (Zhang et al. 2018; Shanmugam et al. 2017). This could be the first report on MG biodecolorization and degradation by *T. cubensis* laccase and the proposed mechanism for the same.

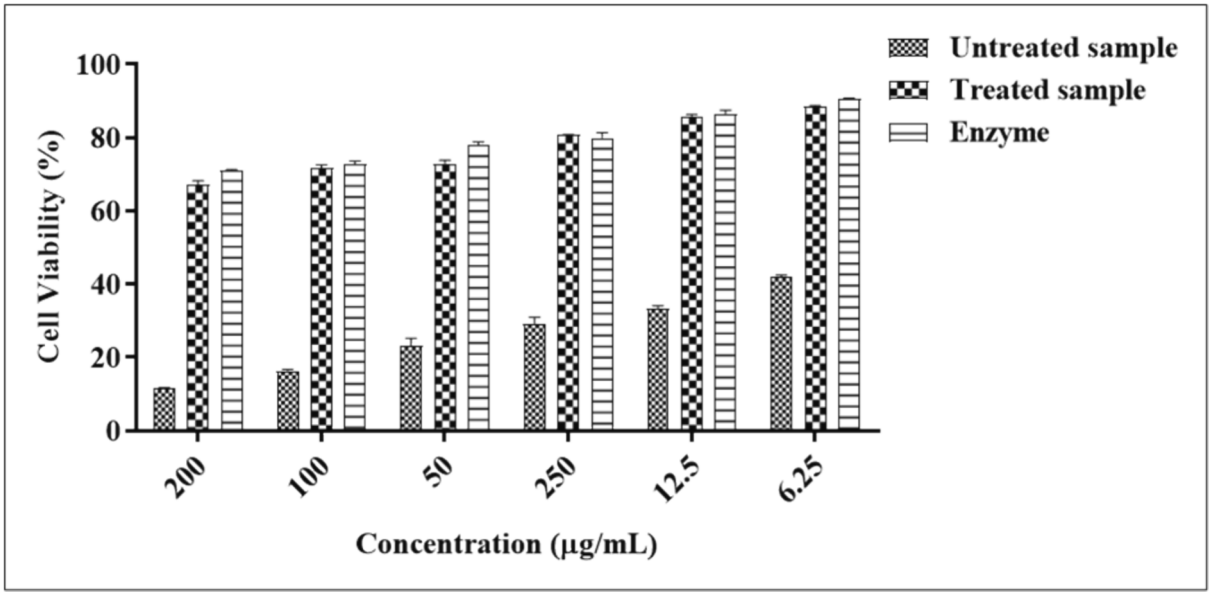


Fig. 4. Effect of biodegraded malachite green dye on viability of Human foreskin fibroblast cells.

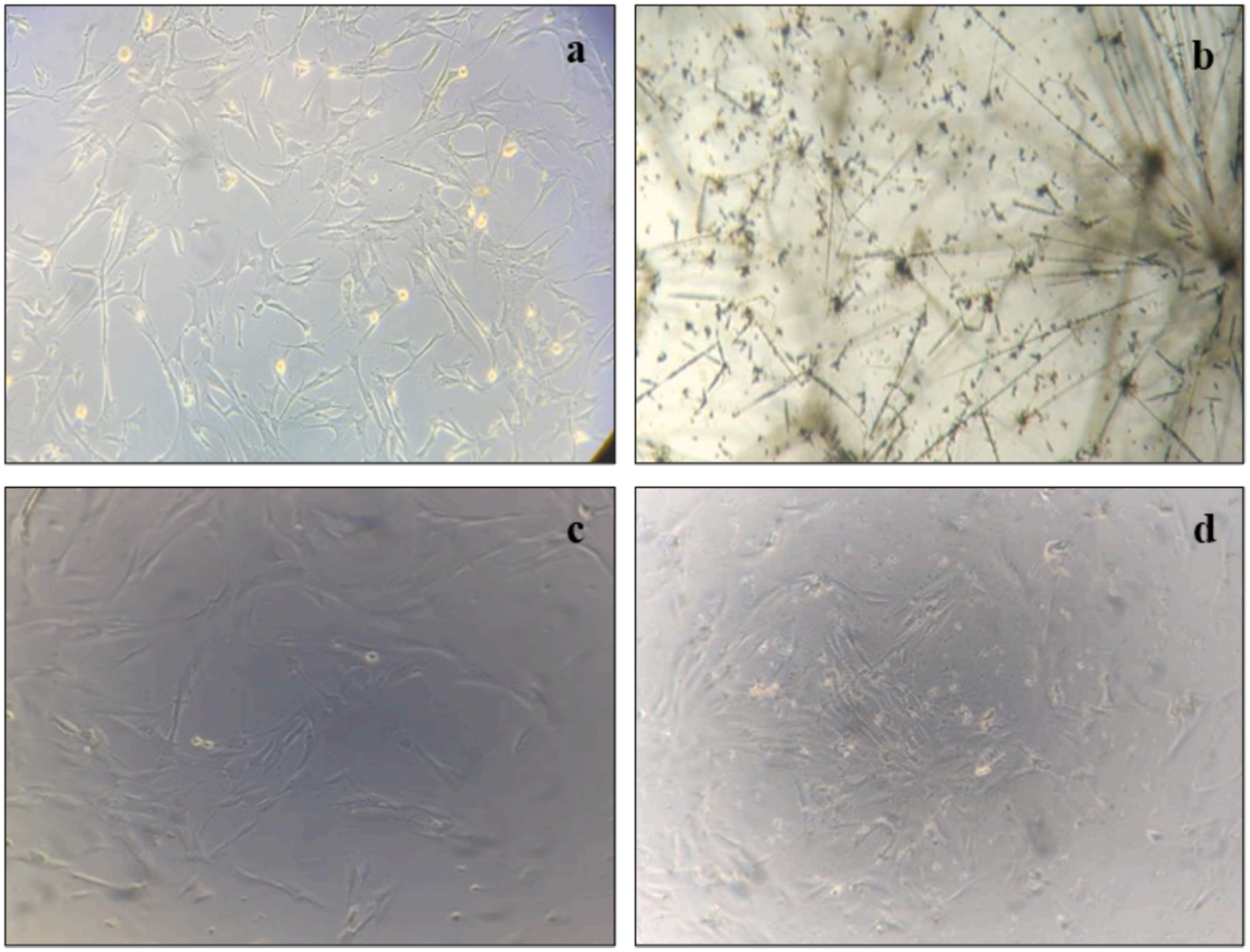


Fig. 5. Morphology of Human foreskin fibroblast (HFF) cells after exposure to malachite green and its metabolites. (a) Control (cells without treatment), (b) untreated MG dye solution, (c) laccase treated dye solution, and (d) enzyme solution. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.8. Toxicity assessment of degraded products

The treated industrial effluents should not pose harmful effects on natural flora and fauna when disposed of in the water bodies or used for irrigation. Therefore, the assessment of the phytotoxicity, microbial toxicity, and cytotoxicity of the completely or partially degraded dye is crucial (Vignesh et al. 2020; Sahasrabudhe et al. 2014). The toxic nature of MG and its degraded products was studied in *P. glaucum* and *T. aestivum,* taking seed germination rate and elongation of roots and shoots as critical parameters. A significantly lower rate of germination was observed in the dye exposed seeds compared to higher germination rates (>90 %) of the seeds exposed to degraded dye (Table 1). MG toxicity significantly impaired the elongation of roots (0 and 1.06 ± 0.27 cm) and shoots (1.91 ± 0.25 and 1.64 ± 0.25 cm) in *P. glaucum* and *T. aestivum,* respectively, compared to the water control. Laccase treatment alleviated the toxicity of the dye, as indicated by the higher average lengths of seedling roots (5.37 ± 0.26 and 5.34 ± 0.11 cm) and shoots (4.43 ± 0.20 and 7.32 ± 0.38 cm) for *P. glaucum* and *T. aestivum,* respectively. Partial restoration of the root and shoot length in *P. glaucum* and that of root length in *T. aestivum* revealed the sensitivity of root and shoot elongation to dye degradation products. Previously, a lower phytotoxicity of MG degradation products on *P. glaucum* and *Cyamopsis tetragonoloba* through the application of white rot fungal laccase has been reported (Himanshu et al. 2023). Similarly, the decolorization of Direct Red 81 by laccase from *Enterococcus faecalis* reduced the phytotoxicity in *P. mungo* as well as *S. vulgare* (Sahasrabudhe et al. 2014). Microbial toxicity of the degraded dye solution was assessed against *B. thuringiensis* and *E. coli.* The absence of growth inhibition around discs containing treated dye solution confirmed the nontoxic nature of the degraded dye against the test microorganisms. In conclusion, MG degradation products would be nontoxic to natural microorganisms following *T. cubensis* laccase treatment, which is in harmony with the findings of Vignesh et al. (2020).

The cytotoxicity of the degraded dye on HFF cells was studied by MTT assay based on death, survival and metabolic activities of the cells before and after MG treatment with laccase. There is a scarcity of reports on the use of the HFF cell line for toxicity testing against MG and its metabolites. Fibroblasts play a crucial biological role and are central in wound healing (Nadalutti and Wilson, 2020). Moreover, the culture of foreskin fibroblasts has been reported as a suitable in vivo prototype for investigating cell proliferation and differentiation, toxicological studies, and inhibition of normal cells by different compounds, including anticancer drugs (Nadalutti and Wilson, 2020; Oliveira et al. 2018). It was observed that MG significantly affected cell viability even at the lowest concentration tested, revealing its elevated toxic effects on human cells, as evident from the lower IC_{50} of untreated dye (7.7 μg/mL). These results were similar to the IC_{50} of 6.4 μg/mL reported for L929 epithelial fibroblast cells (de Almada Vilhena et al. 2023). In contrast, high (>60

%) cell viability was observed after exposure of the HFF cells to laccase-treated dye with a higher IC_{50} of 257.8 μg/mL (Fig. 4). These findings correlated with the reported IC_{50} of > 200 μg/mL for *S. exfoliatus* treated MG using human splenic fibroblasts (Abu-Hussien et al. 2022). The cells exposed to untreated dye solution exhibited significant morphological changes, such as elongation, flattening, granularity, and cell shrinkage, pointing to dye induced apoptosis (Fig. 5). However, HFF cells exposed to both control and treated dye were morphologically similar, indicating the reduced toxicity of the treated dye.

Overall, laccase of *T. cubensis* exhibited industrial potential and future studies may involve scale-up of laccase production, exploration of bioremediation of other dyes and pollutants and integration into sustainable wastewater treatment systems. Future work could investigate alternative immobilization matrices or further optimize immobilization conditions to enhance enzyme stability and efficiency under varied environmental or industrial conditions. This could extend its use in continuous-flow systems for wastewater treatment.

4. Conclusions

Trametes cubensis exhibited enhanced laccase production of 6577.0 ± 14.3 U/g under solid-state fermentation. For the first time, *T. cubensis* laccase was explored for the biodegradation of malachite green, achieving a maximum of 89 % decolorization within 2 h. Upon crosslinking with chitosan, the laccase exhibited 82 % immobilization efficiency, retained > 52 % activity after 7 consecutive cycles and demonstrated similar dye degradation efficacy to that of the free enzyme. FT-IR and GC–MS analyses supported the dye decolorization. The detected metabolites were useful in proposing a possible dye degradation pathway and showed significantly reduced phyto-, cyto- and microbial toxicities.

CRediT authorship contribution statement

Himanshu: . **Baishali Behera:** Methodology. **Neetu Kumari:** Methodology. **Mulaka Maruthi:** Methodology, Formal analysis. **R.K. Singh:** Resources, Project administration, Funding acquisition. **J.K. Saini:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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