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Enhanced Levels of Laccase Synthesis by *C. Cladosporioides* and Its Potential as a Dye Decolorizing Agent

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Abstract

Laccases (EC 1.10.3.2) are multicopper oxidase enzymes with broad applicability in several industrial and environmental processes. In this study, laccase-producing fungi were systematically isolated from paper mill effluent samples. A total of 27 fungal isolates were obtained and identified through ITS rDNA sequencing. Among these, six isolates exhibited prominent substrate oxidation potential. The isolate GOJ7 showed the highest laccase activity, with a maximum yield of 52.00 U/mL. Molecular characterization based on ITS rRNA gene sequencing identified the isolate as *Cladosporium cladosporioides*. The decolorization efficiency of isolate GOJ7 was evaluated against various synthetic dyes in liquid culture. Malachite green showed the greatest decolorization at the concentration of the dye of 100 mg/L (81%), followed by methyl violet (74%), methylene blue (69%), and crystal violet (63%). Fourier Transform Infrared (FTIR) spectroscopy confirmed structural modifications in the dyes following treatment, indicating effective biodegradation. The results demonstrate that *C. cladosporioides* possesses strong potential as an efficient biosorbent for the remediation of dye-contaminated wastewater, offering an eco-friendly approach for reducing toxic industrial pollutants.

Keywords: Laccase; Laccase-producing bacteria; Dye Decolorization; Optimization; FT-IR

1. Introduction

Laccase (EC 1.10.3.2), commonly known as benzenediol, uses oxidoreductase activity to reduce oxygen and is an important oxidative enzyme that catalyzes the degradation of lignin and a wide range of phenolic compounds [1]. This enzyme is produced by diverse biological sources, including plants, fungi, and bacteria; however, fungi, particularly white-rot species, are the most prolific producers. Owing to their broad substrate specificity and strong oxidative capability, laccases have gained significant attention for applications such as textile dye decolorization, biopulping, degradation of recalcitrant pollutants, wastewater detoxification, and biosensor development.

Synthetic dyes commonly used in the textile industry, such as anthraquinone, azo, triphenylmethane, and heterocyclic dyes, are characterized by complex molecular structures that confer resistance to conventional microbial degradation. Traditional physicochemical treatment methods, including coagulation, flocculation, electrochemical oxidation, and adsorption, are often cost-intensive and may lead to the generation of secondary waste or toxic by-products [2].

In contrast, biological treatment strategies have emerged as sustainable and environmentally benign alternatives for dye removal. Fungal systems, particularly white rot and soft rot fungi, are recognized for their ability to degrade a broad spectrum of persistent organic pollutants through the action of extracellular oxidative enzymes [3]. These fungi are capable of mineralizing complex dye molecules rather than merely transferring them from one phase to another.

The soft rot fungus *Cladosporium cladosporioides* is known for efficient extracellular laccase production under submerged fermentation conditions [4]. Considering its enzymatic potential, the present study aimed to evaluate the

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dye decolorization ability of *C. cladosporioides* under optimized conditions using potato dextrose agar (PDA) medium, thereby assessing its applicability in the bioremediation of textile dye-contaminated effluents.

2. Materials and Methods

2.1. Chemicals, dyes, and fungal isolate

Guaiacol (o-methoxyphenol), malachite green, crystal violet, methyl violet, and methylene blue were obtained from HiMedia Laboratories (Hyderabad, India). All chemicals and reagents employed in the study were of analytical reagent grade. The fungal isolate *Cladosporium cladosporioides* was recovered from industrial effluent samples and maintained at 27 ± 1 °C on potato dextrose agar (PDA) plates on a regular basis. Pure cultures were preserved through periodic subculturing on PDA slants and stored at 4 °C until further experimentation.

2.2. Screening and assay of laccase activity

Laccase synthesis was initially screened using acetate-buffered agar medium (pH 5.8) supplemented with 0.1% (v/v) guaiacol, following the protocol outlined by [5]. The appearance of a reddish-brown halo surrounding the fungal colony was considered indicative of laccase activity.

Isolates showing positive results in the qualitative assay were further cultivated in potato dextrose broth supplemented with 0.025% (v/v) of 100 mM $CuSO_4$ as an inducer. Cultures were incubated at 27 ± 1 °C for six days under continuous agitation at 100 rpm. After incubation, the cultures were filtered to remove mycelial biomass and centrifuged for ten minutes at 10,000 rpm. The resultant cell-free supernatant was gathered and utilized to prepare the crude enzyme.

Quantitative estimation of laccase activity was carried out using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as the substrate. The reaction mixture consisted of 3 mM ABTS prepared in 100 mM citrate buffer, and the rise in absorbance at 420 nm was used to calculate the enzyme activity. One unit (U) of laccase activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of ABTS per minute at 30 °C.

2.3. Molecular identification of isolate GOJ7

The molecular identification of the fungal isolate GOJ7 with the highest laccase activity was done by producing and sequencing the internal transcribed spacer (ITS) area of ribosomal DNA. The resulting nucleotide sequence was aligned and compared with reference ITS sequences available in the GenBank database using the Clustal X software. The isolate's taxonomic affiliation was confirmed through phylogenetic analysis.

2.4. Protein estimation

The protein content of the crude enzyme extract obtained from isolate GOJ7 was determined using the Lowry method [6]. Bovine serum albumin (BSA) served as the standard, and a calibration curve was prepared using concentrations ranging from 0.1 to 2.0 mg/mL.

2.5. Dye decolorization assay and UV–Visible analysis

The potential of isolate GOJ7 to decolorize dyes was evaluated using four synthetic dyes: crystal violet, malachite green, methyl violet, and methylene blue. The experiment was carried out in 100 mL of extract broth amended with 10 mL of crude laccase preparation. Varying concentrations of each dye were introduced into the reaction mixture, which was then incubated under standardized conditions.

Dye decolorization was monitored spectrophotometrically by recording the decrease in absorbance at the specific maximum wavelength of each dye at defined time intervals. All treatments were conducted in triplicate to ensure experimental reliability. The extent of decolorization was expressed as a percentage and calculated using the method described by [7].

$$\text{Decolorization (\%)} = \frac{A_1 - A_2}{A_1} \times 100$$

where A_1 represents the initial absorbance of the dye and A_2 represents the absorbance post-decolorization.

3. Result and Discussion

3.1. Molecular Identification

3.1.1. Molecular identification of fungal isolate GOJ7

For molecular characterization, the fungal isolate GOJ7 was submitted to Barcode Bioscience, Bengaluru, Karnataka, India. Genomic DNA was isolated from approximately 20 g of fungal biomass. Mycelium cultivated in potato dextrose broth (PDB) was harvested and thoroughly rinsed with sterile distilled water to eliminate residual media components. The washed mycelium was then frozen with liquid nitrogen and finely powdered.

Approximately 100 mg of finely ground sample was suspended in 15 mL of extraction buffer containing 1.4 M NaCl, 0.1 M Tris–HCl (pH 8.0), 0.02 M EDTA, 2% cetyltrimethylammonium bromide (CTAB), and 200 µL of β-mercaptoethanol. The suspension was incubated at 65 °C for 60 min to promote efficient cell disruption. Following incubation, 10 mL of phenol:chloroform:isoamyl alcohol (20:19:1) was added, gently mixed, and centrifuged at 15,000 rpm to achieve phase separation. The upper aqueous layer was carefully transferred to a fresh tube, and genomic DNA was precipitated using chilled isopropanol at −20 °C for 1 h. After being collected by centrifugation, the DNA pellet was spun for 15 minutes at 15,000 rpm, then rinsed in 70% ethanol, dried in air, and suspended in 50 µL TE buffer. The extracted DNA was preserved at 4 °C for subsequent analyses.

The ribosomal DNA's internal transcribed spacer (ITS) region was subjected to amplification by means of universal primers, namely, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR reactions were performed in a thermal cycler under standard amplification conditions. The resulting amplicons were separated on a 2% agarose gel prepared in 1× Tris–borate–EDTA buffer and electrophoresed at 100 V. DNA fragments were visualized by ethidium bromide staining, and product sizes were determined using a molecular weight marker. To confirm reproducibility, PCR amplification was conducted in duplicate, and only consistent bands were selected for sequencing.

The amplified ITS regions were sequenced using a Genetic Analyzer (USA) at Barcode Bioscience. The BioEdit Sequence Alignment Editor was then used to modify and align the acquired nucleotide sequences (version 7.0.4.1). Sequence similarity searches were conducted using the BLAST program against fungal sequences available in the GenBank database. Based on comparative analysis of the 18S rRNA gene region, isolate GOJ7 was identified as *Cladosporium cladosporioides*. The validated sequence was submitted to GenBank and assigned the accession number OR649270.

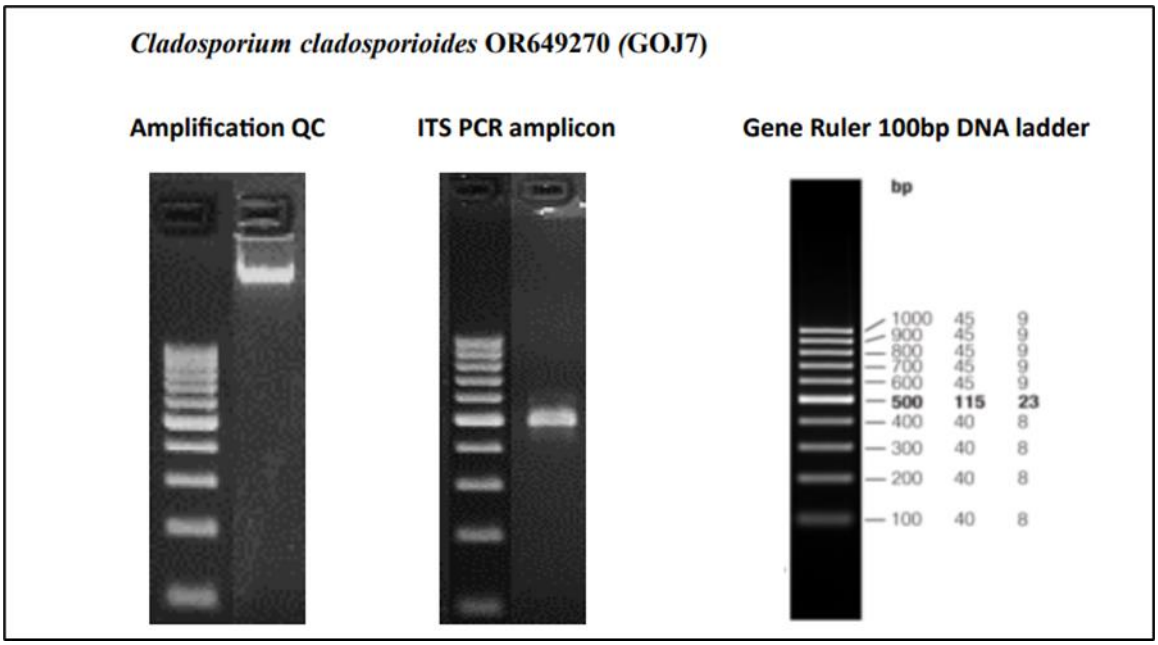


Figure 1 Represents the gDNA and ITS amplicon QC data (*Cladosporium cladosporioides* OR649270) (GOJ7)

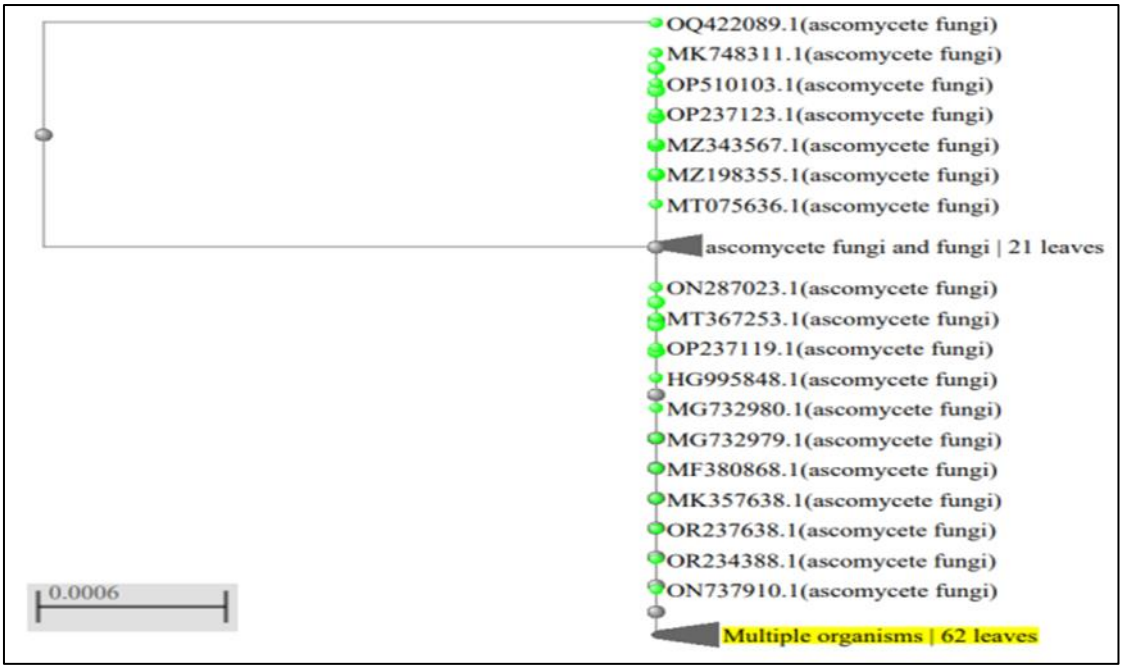


Figure 2 Represents the phylogenetic tree (*Cladosporium cladosporioides* OR649270) (GOJ7)

3.2. Qualitative Assay for Dye Decolourization

Soft rot fungus *C. cladosporoides* was cultivated on PDA plates supplemented with individual dyes at a concentration of 100 mg/L. The experimental isolate exhibited robust growth, indicating that the presence of dyes did not inhibit fungal development. Notably, the growth of *C. cladosporoides* on dye-amended PDA plates led to the formation of distinct decolorization zones. The extent of decolorization was visually assessed and quantified, with the results summarized in Table 1. Complete decolorization was observed on PDA plates containing malachite green and crystal violet.

Table 1 Decolorization of dyes using *Cladosporium cladosporioides* using qualitative method

Dyes	**Decolorization zone**
	C. cladosporioides (OR649270)
Methyl violet	++
Malachite green	++++
Crystal violet	++++
Methylene blue	++
(Decolourization scale: + 0-1 cm; ++ 1-3 cm; ++++ 3-4 cm; ++++ 4-5cm)	

3.3. Dye decolorization in liquid culture media

To quantitatively evaluate the dye decolorization efficiency of *Cladosporium cladosporioides* OR649270, individual dyes were introduced into the liquid culture filtrate. The extent of decolorization was monitored spectrophotometrically at 24-hour intervals over a period of 12 days. The isolate demonstrated the ability to decolorize all dyes tested, as illustrated in the corresponding figure and graph. Among the dyes examined, the highest decolorization efficiency was observed for malachite green (86%) and crystal violet (82%) on the 12th day of incubation. This was followed by methyl violet (71%) and methylene blue (69%). Notably, malachite green and crystal violet showed comparatively faster rates of decolorization than the other dyes. The dye decolorization visual representation is given in the Figure 3.

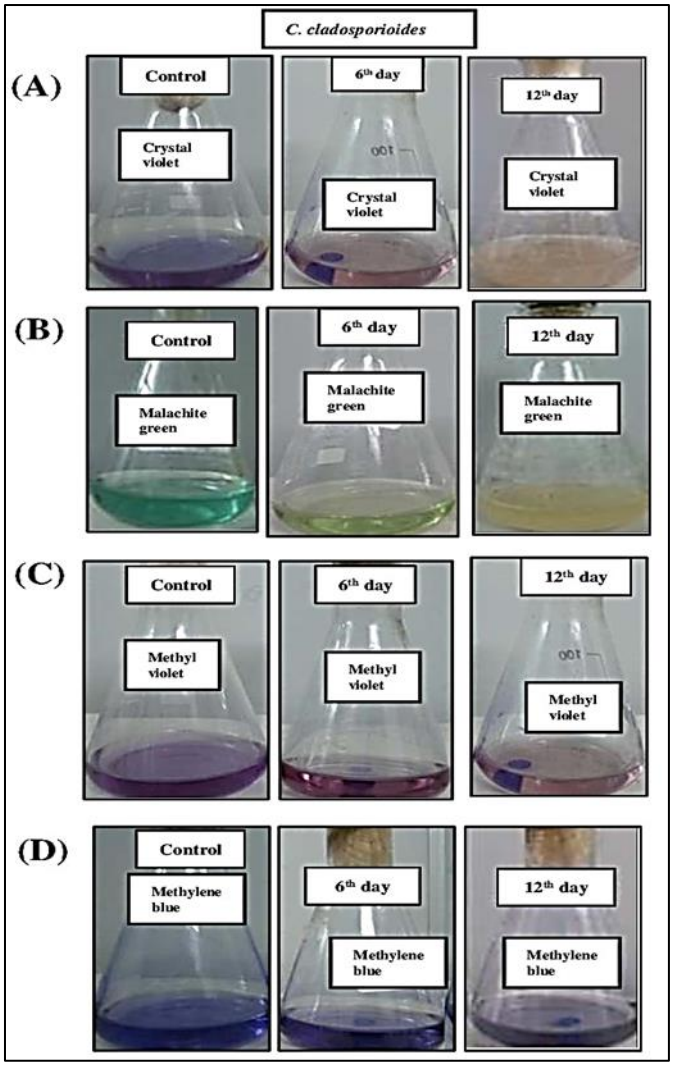


Figure 3 Represents the decolourization of (A) Crystal violet, (B) Malachite green, (C) Methyl violet and (D) Methylene blue in liquid media using *Cladosporium cladosporioides*

3.4. Optimization of dye decolorization

3.4.1. Effect of dye concentration

The present study evaluated the influence of initial dye concentration on the laccase-mediated decolorization of triphenylmethane dyes—crystal violet, malachite green, methyl violet, and methylene blue—by *Cladosporium cladosporioides*. The initial concentration of dyes was identified as a critical factor regulating the enzymatic degradation process. Increasing dye concentrations significantly inhibited laccase activity, thereby lowering decolorization efficiency. Experiments were conducted with initial dye concentrations of 25, 50, 100, 125, 150, and 200 mg/L using a fixed laccase dosage of 0.5 mL.*C. cladosporioides* exhibited a concentration-dependent reduction in decolorization efficiency, with optimal activity observed at 100 mg/L. At this concentration, the degradation efficiencies for methyl violet, malachite green, crystal violet, and methylene blue were 39.8%, 42.6%, 44.8%, and 45.7%, respectively. The highest decolorization was recorded for methylene blue (45.7%) after 60 minutes of incubation. The Graphical presentation of dye degradation at different incubations is given in Figure 4.

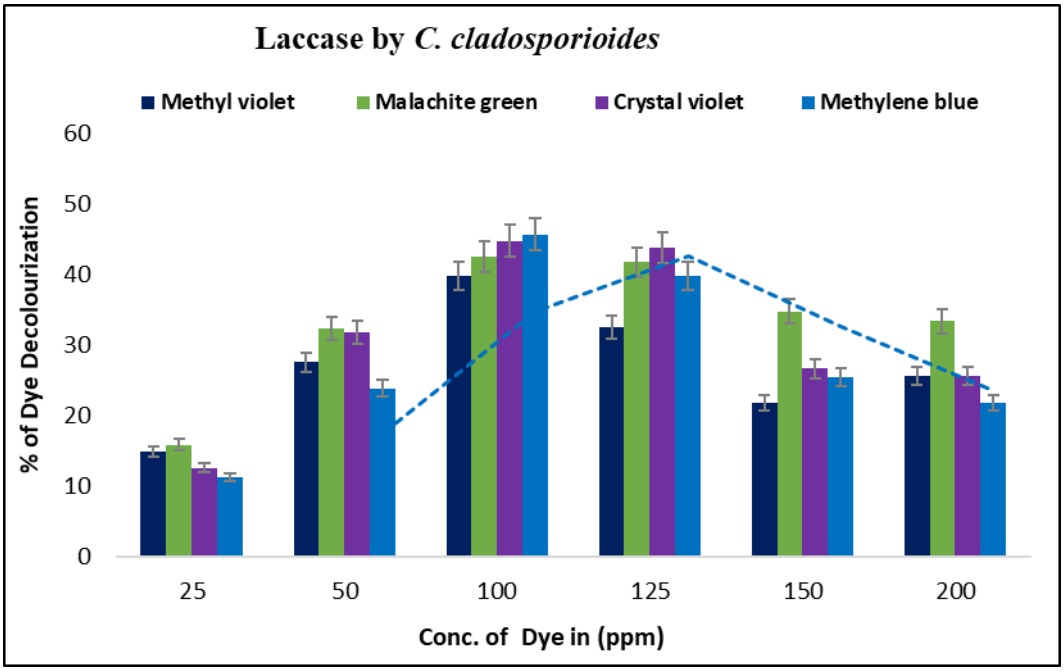


Figure 4 The effect of pH on dye decolourization with pure laccase by *C. cladosporioides*

The decline in degradation percentage at higher concentrations can be attributed to the toxic effects of excessive dye molecules, which interfere with fungal metabolism and enzyme function. Lower concentrations favored higher decolorization rates due to reduced substrate inhibition and improved accessibility of dye molecules to the enzyme active sites. The observed inverse relationship between dye concentration and decolorization efficiency indicates that *C. cladosporioides* possesses an effective enzymatic system capable of degrading triphenylmethane dyes under moderate dye loads.

This study demonstrated that *Cladosporium cladosporioides* could efficiently decolorize triphenylmethane dyes without requiring redox mediators or additional cofactors. The organism also showed tolerance to elevated dye concentrations up to 100 mg/L, maintaining significant degradation capability. These findings highlight the potential of *C. cladosporioides* as a promising biocatalyst for the eco-friendly treatment of dye-contaminated industrial effluents.

Supporting evidence from related research aligns with the present findings. Yan et al. [8] reported effective decolorization of triphenylmethane dyes—methyl violet, brilliant blue, crystal violet, and acid red—using fungal laccase from *Trametes trogii* Berk S0301, with malachite green exhibiting the highest removal rate without the use of redox mediators. Similarly, [9] observed that the laccase activity of *Ganoderma lucidum* decreased significantly at high concentrations of Remazol Brilliant Blue R (RBBR), indicating that excessive dye levels can inhibit enzymatic oxidation. [10] Further confirmed that increased concentrations of dyes such as Reactive Yellow, Acid Fuchsin Red, and Methyl Violet led to a substantial drop in decolorization efficiency, while [11] demonstrated that oxidoreductases from *Pleurotus sajor-caju* PS-2001 effectively removed color from disperse textile dyes. Collectively, these findings reinforce that the concentration of dye plays a decisive role in enzymatic dye degradation.

3.4.2. Effect of pH on dye decolourization

The pH of the reaction medium plays a crucial role in regulating laccase-mediated dye decolorization by *Cladosporium cladosporioides*. In this study, the decolorization efficiency of four triphenylmethane dyes—methyl violet, malachite green, crystal violet, and methylene blue—was evaluated across a pH range of 4.0 to 8.0. The optimal pH for maximum decolorization was found between 5.5 and 7.0, with the highest efficiency (70.5%) observed for malachite green at pH 5.5. A steady decline in decolorization occurred beyond pH 7.0, likely due to the decreased activity of laccase under alkaline conditions. The Graphical presentation of dye degradation at different pH levels is given in Figure 5.

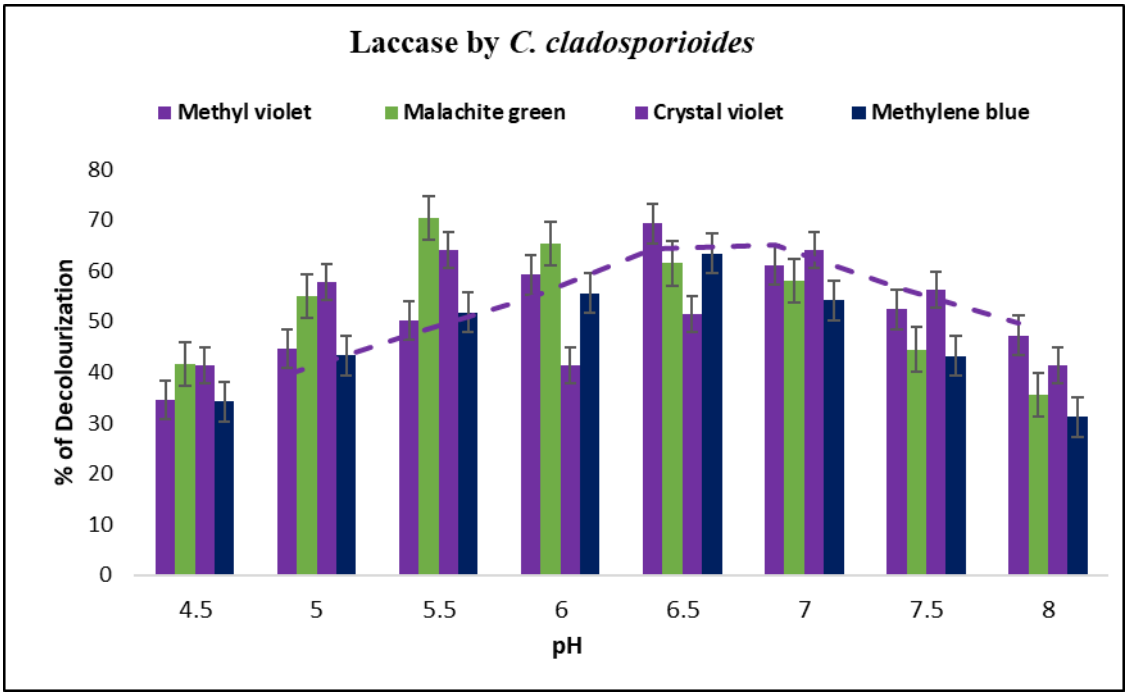


Figure 5 The effect of pH on dye decolourization with pure laccase by C. cladosporioides

Laccases typically perform best under mildly acidic conditions, as higher pH levels can disrupt the electron transfer between the T1 and T2/T3 copper centers due to anion interference [12]. In addition to acting as a regulator of the movement of dye molecules across the fungal cell membrane, the pH also affects the decolorization rate of dye molecules [13]. The present observations are consistent with the findings of [8], who reported maximum malachite green decolorization by laccase at an optimal pH of 6.0.

3.4.3. Effect of Temperature on Dye Decolorization

Temperature significantly influences laccase activity and dye degradation efficiency in *Cladosporium cladosporioides*. The organism exhibited effective decolorization of methyl violet, malachite green, crystal violet, and methylene blue between 25°C and 45°C, with the optimal temperature range being 30–35°C. The maximum decolorization (86.4%) was achieved for methyl violet at 35°C, while a marked reduction occurred at lower (25°C) and higher (above 40°C) temperatures. The Graphical presentation of dye degradation at different temperatures is given in Figure 6.

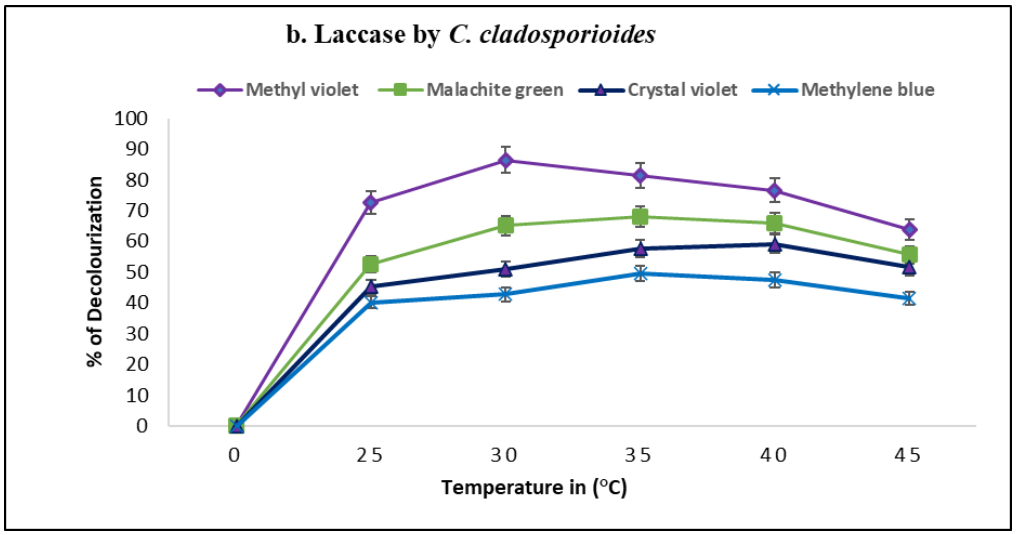


Figure 6 The effect of Temperature on dye decolourization with pure laccase by C. cladosporioides

At elevated temperatures, laccase activity declines due to enzyme denaturation, consistent with observations in other fungal systems [14,15]. Within the optimal temperature range, enzyme–substrate interactions and catalytic turnover are enhanced, promoting efficient dye degradation. Thus, *C. cladosporioides* demonstrates strong potential for bioremediation under moderate thermophilic and slightly acidic conditions.

3.5. Fourier Transform Infrared (FTIR) Spectroscopy Analysis of *Cladosporium cladosporioides*

The use of Fourier Transform Infrared (FTIR) spectroscopy was made to study the changes in structure and functional groups of the dyes that are treated with laccase from *Cladosporium cladosporioides*. The analysis was performed using a Shimadzu 8400S spectrophotometer operating in the mid-infrared region (400–4000 cm^{-1}) to compare spectra of untreated dyes and those subjected to enzymatic degradation [16]. Distinct variations in peak intensity and position indicated significant chemical transformations in the dye molecules following biodegradation [17].

For crystal violet, new absorption bands appeared around 1545 cm^{-1}, corresponding to N–O stretching vibrations, while the disappearance of a peak near 702 cm^{-1} associated with C=C bending suggested the cleavage of aromatic ring linkages. This indicates the breakdown of the chromophoric structure responsible for color Figure 7.

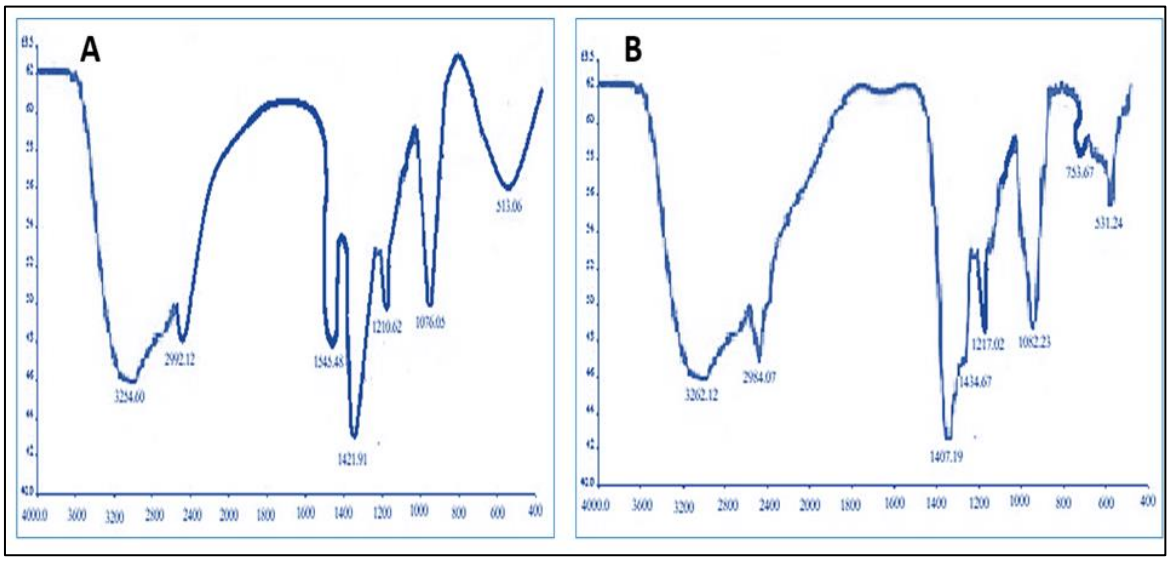


Figure 7 FTIR analysis of the dye reduced by crystal violet control (A) and *Cladosporium cladosporioides*

In malachite green, the disappearance of bands near 869 cm^{-1} and 742 cm^{-1}—related to C–H bending of disubstituted benzene rings—revealed the destruction of aromatic substituents and de-methylation of the dye. These changes point to the enzymatic transformation of complex aromatic rings into simpler intermediate compounds (Figure 8).

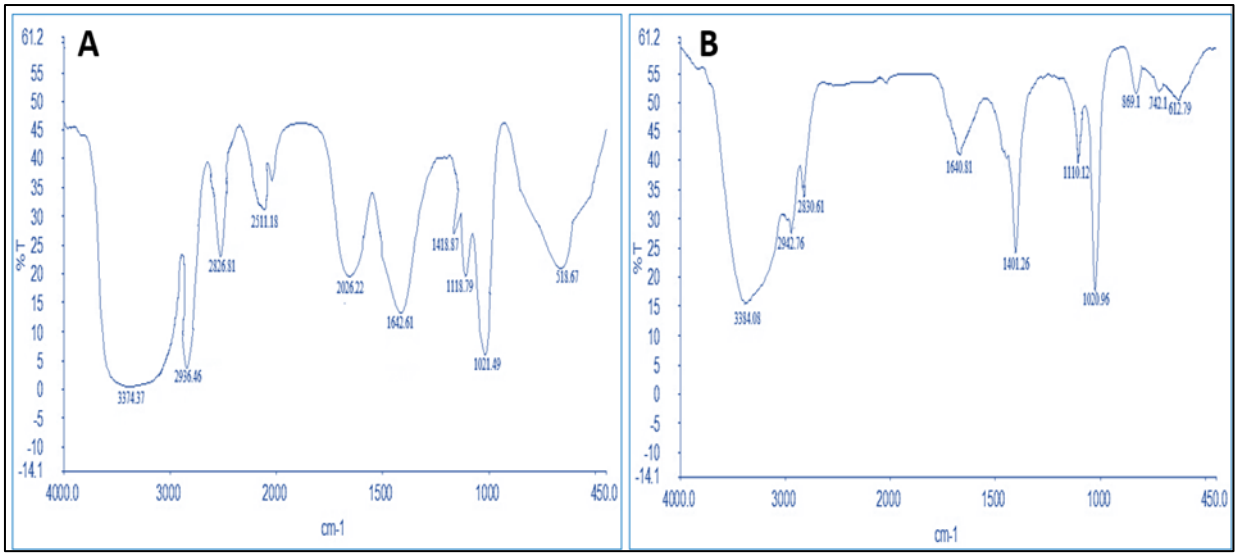


Figure 8 FTIR analysis of the dye reduced by Malachite green control (A) and *Cladosporium cladosporioides*

For methyl violet, the FTIR spectra showed a marked reduction and shift in the band at approximately 610 cm^{-1}, attributed to C–Br stretching vibrations. The loss or shifting of this peak confirmed the cleavage of halogen bonds and modification of amine-containing structures. Notably, no new absorption peaks were observed in the visible range, confirming that the degradation products were non-chromogenic, indicating complete color removal (Figure 9)

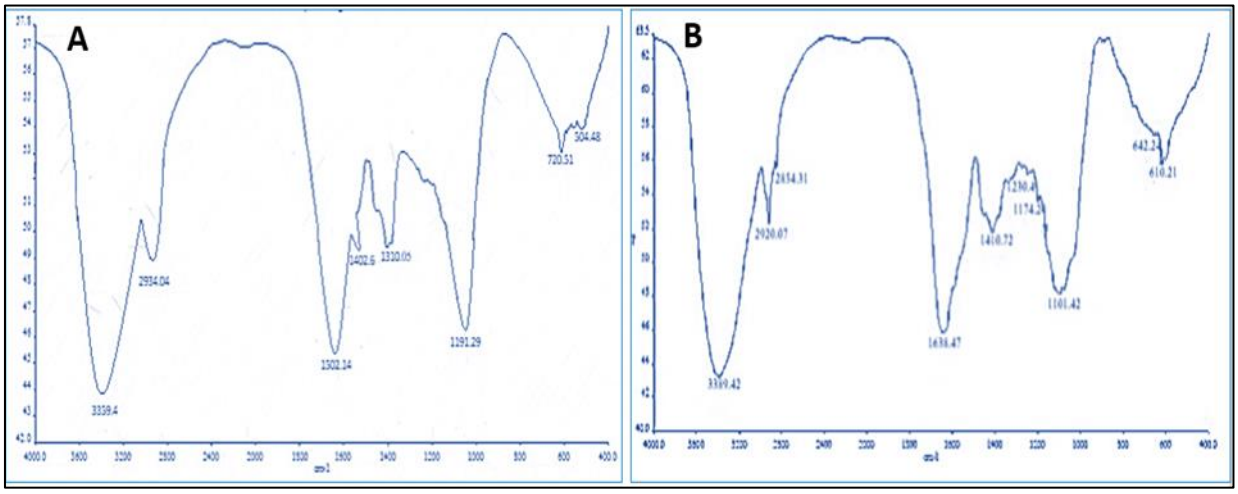


Figure 9 FTIR analysis of the dye reduced by Methyl violet control (A) and *Cladosporium cladosporioides*

In the case of methylene blue, the absence of the peak near 714 cm^{-1}, corresponding to C=C bending of the alkene group, suggested fragmentation of the triphenylmethane backbone and decomposition of the dye's conjugated system. The disappearance of characteristic chromophoric bands confirmed that enzymatic oxidation disrupted the electron-rich aromatic system, leading to decolorization (Figure 10)

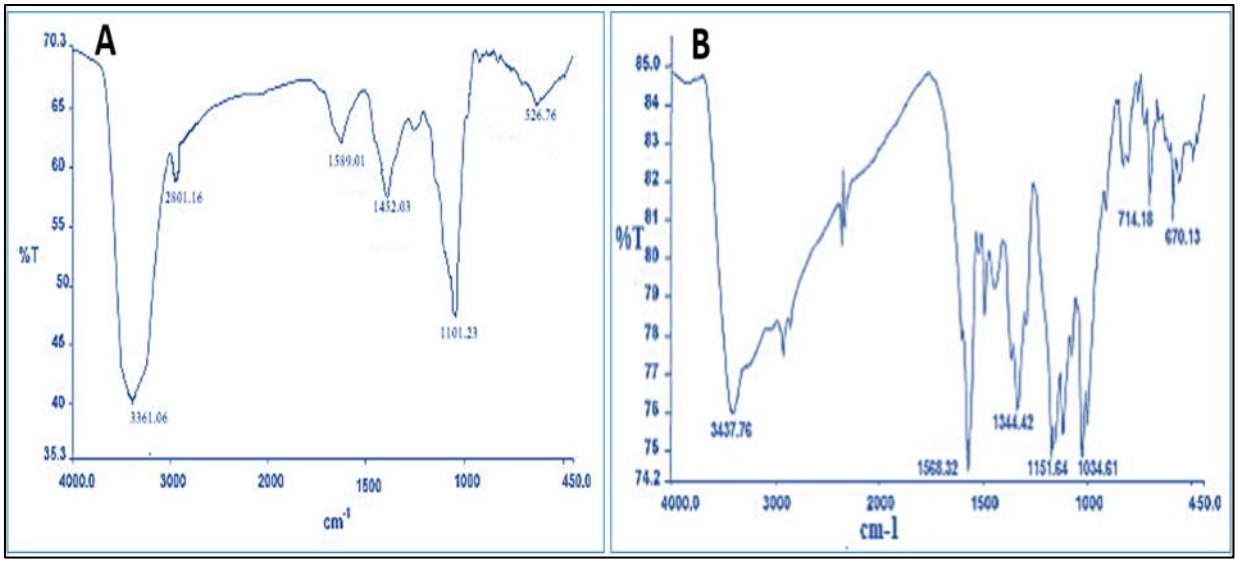


Figure 10 FTIR analysis of the dye reduced by Methylene blue control (A) and *Cladosporium cladosporioides*

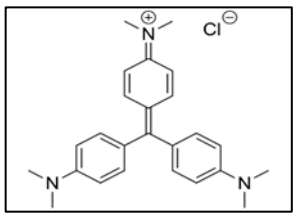
Overall, FTIR analysis confirmed that *Cladosporium cladosporioides* effectively degraded the triphenylmethane dyes through enzymatic oxidation, bond cleavage, and modification of functional groups such as hydroxyl, amine, and sulfonyl moieties. The loss or shift of key peaks across all treated samples validated the disintegration of complex aromatic structures and chromophores. These transformations explain the observed reduction in color intensity and confirm the organism's strong potential in the biodegradation of synthetic dyes [18]. The findings indicate that *C. cladosporioides* employs an oxidative enzymatic mechanism, primarily through laccase-mediated cleavage of C–N and C=C bonds, resulting in mineralization or conversion of dyes into less complex and non-toxic metabolites suitable for environmental applications.

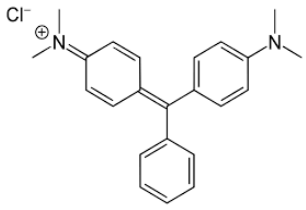
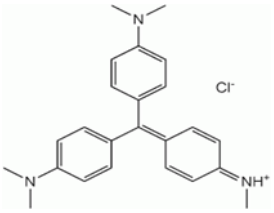
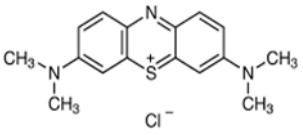
3.6. FT-IR analysis

The FT-IR technique, as well, was employed to identify modifications in the functional groups of the dyes in treated and untreated dyes, thus offering data about the new products generated by dye biodegradation. FTIR spectrum of crystal violet dye treated with *Cladosporium cladosporioides*, the peak was observed at 1545.48 cm^{-1} for nitro compounds of N–O stretching and the disappearance of peak at 702.27 cm^{-1} for alkanes C=C bending (Figure 7 (A)).

In FTIR spectra disappearance of peak indicates the decolorization of dyes [19]. FTIR spectrum of malachite green treated with *C. cladosporioides* showed complete disappearance of peak at 812.49 cm^{-1} for C–H bending of 1,3 disubstituted (and vibration observed at 747.23 cm^{-1} for 1,2 disubstituted. Methyl violet dye treated with *C. cladosporioides* exhibits the shifting of peak at site at 660 cm^{-1} for halo compounds of C–Br stretching. It indicates dye degradation. FTIR spectrum of methylene blue dye treated with *C. cladosporioides* the disappearance of peaks at 714 cm^{-1} for alkene of C=C bending reveals the degradation of dye. This study showcases the various dyes utilized, represented through their respective chemical structures, which are given in Table 2

Table 2 The dyes used in this study are represented by their chemical structures

Dyes	Structure	λ max (nm)	Chemical Formula	Chemical Class	Reference
Crystal violet		592	$C_25H_{30}ClN_3$	Tri-phenyl-methane	[20]

Malachite green		618	$C_{23}H_{25}ClN_2$	Tri-phenyl-methane	[21]
Methyl Violet		584	$C_{24}H_{28}N_3Cl$	Tri-phenyl-methane	[22]
Methylene Blue		664	$C_{16}H_{18}ClN_3S$	Tri-phenyl-methane	[23]

4. Conclusion

In this study, the crude enzyme laccase was successfully extracted from the mycelial cultures of *Cladosporium clodosporoides* and subjected to detailed characterization, including its enzymatic activity and stability under varying conditions. The findings revealed that the laccase produced by this fungal strain possesses a remarkable capacity for decolorizing synthetic dyes, with a particular efficacy observed for triphenyl methane dyes. The enzyme demonstrated optimal performance across a range of pH values (specifically between pH 4 to 8) and temperatures (ranging from 25°C to 50°C), indicating its robustness and versatility. These attributes suggest that laccases derived from *Cladosporium clodosporoides* hold significant promise for further research and development, particularly in applications aimed at the bioremediation of industrial effluents contaminated with toxic dye pollutants.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interest

Statement of ethical approval

This Study does not involve any human participants or identifiable

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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