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Exploitation of Laccase Producing *Trametes versicolor* (NBRC4937) for the Biodegradation of Triphenylmethanes (Crystal Violet) Dyes

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Abstract

Crystal Violet (CV) dyes are extensively used in various industries but pose significant environmental concerns owing to their persistence and potential toxicity, this necessitate the needs to search for organism capable of degrading CV. *Trametes versicolor* was cultivated on potato dextrose yeast agar supplemented with guaiacol to assess its growth and laccase production. A mycelium disk was then inoculated into an experimental broth culture containing Crystal Violet dye as the carbon source and agitated on a shaker for 15 days at 150 rpm. Fourier-transform infrared spectroscopy (FTIR) and Gas chromatography–mass spectrometry *(*GC-MS*)* were used for the characterization of the crystal violet dyes and its degraded products, and this showed significant variations in peak presence and positions. These significant changes in the FTIR spectrum of the degraded dye metabolite provided confirmation of the biotransformation of the dye into other compounds, which was supported by the observation of distinct peaks at specific wave numbers. Specifically, peaks were observed at 110.75 cm-1, corresponding to the Alkyl-substituted ether and C-O stretch, at 458.23 cm-1 for the Tertiary alcohol and OH-bend, at 1774.57 cm-1 for the Azo and N=N group, and at 1666.55 cm-1 for the Primary amine and NH stretch (C=C). GC spectral showed different peaks which represent some compounds formed during the biodegradation as interpreted by mass spectral data and fragmentation patterns from the NIST98 library. This demonstrated the capacity of *T. versicolor* to degrade CV dyes. The outcomes may lead to the development of efficient and environmentally friendly strategies for CV dye remediation, thus, reducing their environmental impact.

1.0 Introduction

Synthetic dyes have found wide-ranging applications across various industries such as textiles, plastics, pharmaceuticals, paper, printing, rubber, leather, paints, cosmetics, food, pulp and paper [1], [2]. However, due to the increased demand and use of these dyes in chemical-based industries, highly polluted wastewater has been released, contaminating the environment and posing a health risk [3].

Crystal Violet (CV), a dye belonging to the triphenylmethane class, is renowned for its recalcitrant properties, exhibiting long-lasting persistence in the environment and eliciting toxic effects [4]. It functions as a mitotic inhibitor, powerful carcinogenic agent, and clastogen, stimulating tumor development in specific species of fish [1]. In spite of its hazardous properties, CV continues to be widely employed in commercial textile processes, biological staining applications, as well as a dermatological agent [1], [5].

In severe cases, exposure to the dye can cause permanent blindness [6], [5], skin and digestive tract irritation, moderate eye irritation, painful light sensitization, respiratory complications, and kidney failure.Triphenylmethane (TPM) dyes, including CV, are challenging to degrade due to their complex molecular structures, resulting in the accumulation of these dyes in the environment [4].

Despite the existence of multiple physicochemical techniques like adsorption, coagulation, and ion-pair extraction for the removal of CV, these methods have proven inadequate for achieving complete elimination from industrial wastewater. Moreover, these approaches generate substantial quantities of sludge that may contain additional pollutants, and involve high investment and operating costs [1], [7], [8].

Adsorption is commonly used owing to its simplicity, minimal expense, high level of efficiency [9]. Nonetheless, the considerable expense associated with activating carbon significantly hampers its economic feasibility [10]. Consequently, researchers have been exploring alternative cost-effective materials such as Fe2O3@CeO2-ZrO2/ palygorskite composite catalysts, smectite-rich natural clays, graphene-based nanomaterials, modified tea leaves, biochar chitin, poplar sawdust, and roots of *Eichhornia crassipes* [11], [12], [13], [14], [15], [16], [17]. Biological treatment technologies offer environmentally friendly and cost-effective alternatives to traditional physicochemical methods, as they selectively degrade organic pollutants without causing collateral damage to the surrounding soil biota [1], [7], [8]. Considering the adverse impacts on human health, environmental pollution, and the limitations of current physicochemical techniques in addressing CV contamination, the development of efficient strategies for its removal and degradation is urgently to protect public health. As a result, laccases have found extensive applications in the domains of dye decolorization and detoxification. Consequently, the present study aims to assess the potential of *T. versicolor* and its laccase enzymes for biodegrading triphenylmethane (TPM) dye, specifically Crystal violet. The objective is to mitigate the adverse impacts of this pollutant and restore contaminated ecosystems.

2.0 Materials and Methods

2.1 Microorganism and Culture Conditions

A resuscitation medium was prepared by dissolving 24 g of potato dextrose, 5 g of yeast extract, and 15 g of agar in 1 liter of distilled water. This medium is referred to as Potato Dextrose Yeast extract agar (PDYA). A sterile loop or needle was used to transfer a small amount of the long-term stored *T.*

versicolor (NBRC4937) onto a fresh PDYA plate. The inoculum was spread on the surface of the agar using aseptic techniques. The PDYA plate inoculated with the strain was incubated at an optimal temperature for the growth of *T. versicolor*, typically around 25-30°C. The plate was observed regularly for the growth of the fungus. The resuscitation process can take several days or weeks, depending on the viability and condition of the stored strain. Once visible growth of *T. versicolor* was observed, a subculture was prepared by transferring a small portion of the fungus from the resuscitation plate onto a fresh PDYA plate. This ensures the availability of a pure and actively growing culture for further experimentation or storage. The resuscitated strain was stored for long-term use by transferring it onto fresh PDYA plates containing 10% PDY (Potato Dextrose Yeast extract) and storing them at a temperature of 4°C. In this procedure, the strain *T. versicolor* (NBRC4937) was successfully brought back to an actively growing state from long-term storage.

2.2 Chemical Reagents

The synthetic crystal violet dye used in this study was were bought from Merck-Sigma Aldrich Mumbai, India).

2.3 Screening for Laccase Production

T. versicolor was screened on Potato Dextrose Yeast extract agar (PDYA) (pH 6.5) containing 0.04% guaiacol for the detection of laccase activity. The culture was supplemented with 150 mM copper sulphate sterile solution as laccase inducer and was incubated at 37 °C for 3-5 days [18]. Laccase activity was visualized on plates containing 0.04% guaiacol since laccase catalyzes the oxidative polymerization of guaiacol to form reddish brown zones in the medium. Positive colonies oxidizing guaiacol by the formation of Reddish brown halos on the medium was selected.

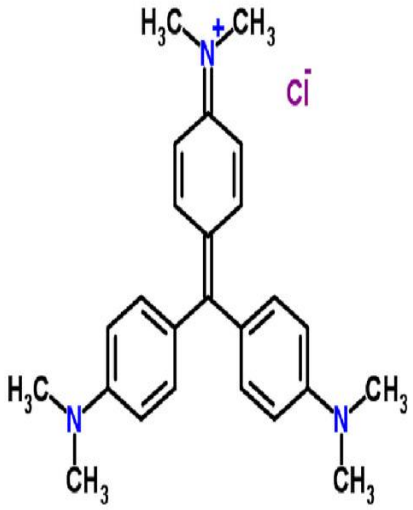


Figure. 1: Decolorization of Dyes by *T. versicolor*

Six plugs (diameter 3 mm) from 5-day-old fungal cultures on malt extract agar (MEA) plates were inoculated into each Erlenmeyer flasks containing 100 mg/ml of crystal violet dye. After fungal growth, the cultures were supplemented with 150 Mm $CuSO_4$ sterile solutions as a laccase-inducer. All the flasks were incubated in a shaker at 30^o C in 150 rpm for 12 days

2.4 Fourier-Transform Infrared (FTIR) Spectroscopy Analysis

FTIR analysis was performed using Shimadzu FTIR-8400S. A drop of the decolorized sample was taken and placed on the salt plate. The salt plate was then fixed to the sample holder. The spectrum was detected in scanning range of 650-4000 cm^{-1}. Initially, the FTIR underwent calibration by conducting background signal scanning in the absence of any sample (referred to as air blank). Following that, the experimental sample was scanned. Reports were generated by the IR solution software

through the process of subtracting the spectrum obtained from the air blank from the spectrum obtained from the experimental samples as described in the protocol manual of the FTIR

2.5 Analysis of CV biotransformation using Gas Chromatography Mass Spectrometry

The synthetic dye degradation product was firstly extracted with equal volume of ethyl acetate three times and the organic layer was collected. The organic layer was dried under nitrogen and the dried residue was dissolved in 100 µl ethyl acetate for analytical analysis. GC-MS analysis was carried out using Agilent 19091S-433 interfaced with a mass spectrometer detector. Compounds was separated with an HP-5MS column (30 m long, 0.25 mm id, non polar). Helium was used as a carrier gas at the flow rate of 1ml per minute. The injector temperature was maintained at 325°C with the following conditions: 100°C kept constant for 1 minute, increased to 230°C at a rate of 8°C/min and kept constant for 5 minutes and then raised to 280°C at a rate 10°C/minute and kept constant for 25 minutes. Compound was identified on the basis of mass value from the mass spectra.

2.6 Statistical analysis

Microsoft Excel package, 2013 version was used to carry out all statistical analysis. Results are expressed as mean ± standard deviation.

3.0 Results

3.1 Identification of extracellular Laccase Production

The current study utilized the reddish brown color zone surrounding the mycelial culture in the medium as an indicator of laccase production by the *T. versicolor* (Plate1)

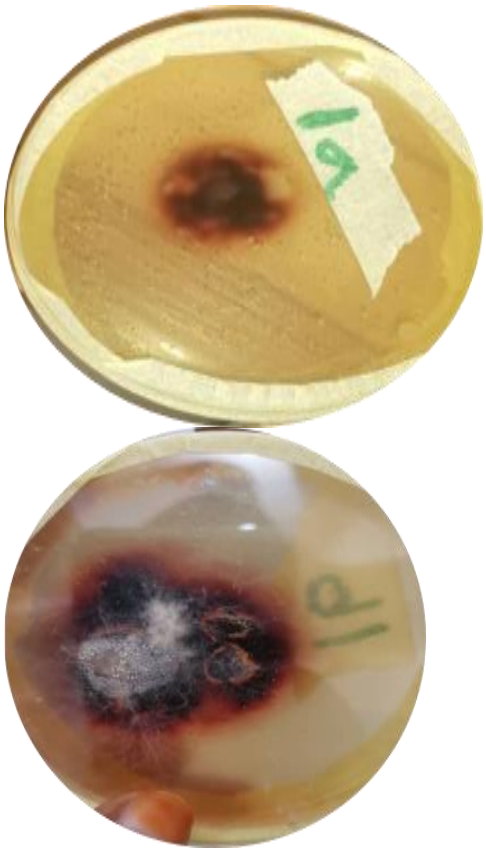


Plate 1: Detection of Laccase Production by *T. versicolor* A) Production of Laccase on the third day. B) Production of Laccase on the fourth day.

3.2: FTIR Analysis of Crystal Violet Dye Degradation

The degradation of Crystal Violet dye was assessed through FTIR analysis to identify the resulting metabolites. A comparison was made between the FTIR spectra of the dye control and the metabolite obtained after decolorization, revealing distinct peaks. In the FTIR spectra of the dye control, peaks were observed at 3500.00 cm^{-1} (O-H stretch), 2300.00 cm^{-1} (C≡C bend), 1500.00 cm^{-1} (C=C), 1150.00 cm^{-1} and 1050 cm^{-1}(C-O stretch) and the absorption bands below 900 cm^{-1} indicate aromatic nature of the compound 850 cm^{-1} (C-O bend), as presented in Figure 2

However, the FTIR spectra of the dye and its degradation products exhibited variations in peak presence and position (Figure 3). The

significant changes in the FTIR spectrum of the degraded dye metabolite confirmed the biotransformation of the dye into other compounds. The following peaks were observed at 110.75 cm^{-1}(Alky-substituted ether, C-O stretch), I458.23 cm^{-1} (Tertiary alcohol, OH-bend 1774.57 cm^{-1} (Azo, N=N group) and 1666.55 cm^{-1}(Primary amine, NH stretch (C=C)

3.3 GC-MS Analysis of the degraded Metabolites

In the control samples, a total of nineteen peaks were detected, whereas the degraded samples exhibited twenty peaks. Observable distinctions were noted in the size of the peaks, with some showing a decrease while others displayed elongation, particularly in peaks 5, 8, 9, 13, and 14. The metabolites were analyzed using mass spectral data and fragmentation patterns, and the fragments were classified based on comparison with the NIST98 Library, as outlined in Table 1. The predominant compounds were identified as amines and esters, which were further confirmed by the functional groups identified through FTIR analysis (Figures 3 &4). The GC-MS spectrum confirmed the presence of various components with different retention times as illustrated in Table 1. This affirmed the ability of *T. versicolor* to degrade CV to different fractions.

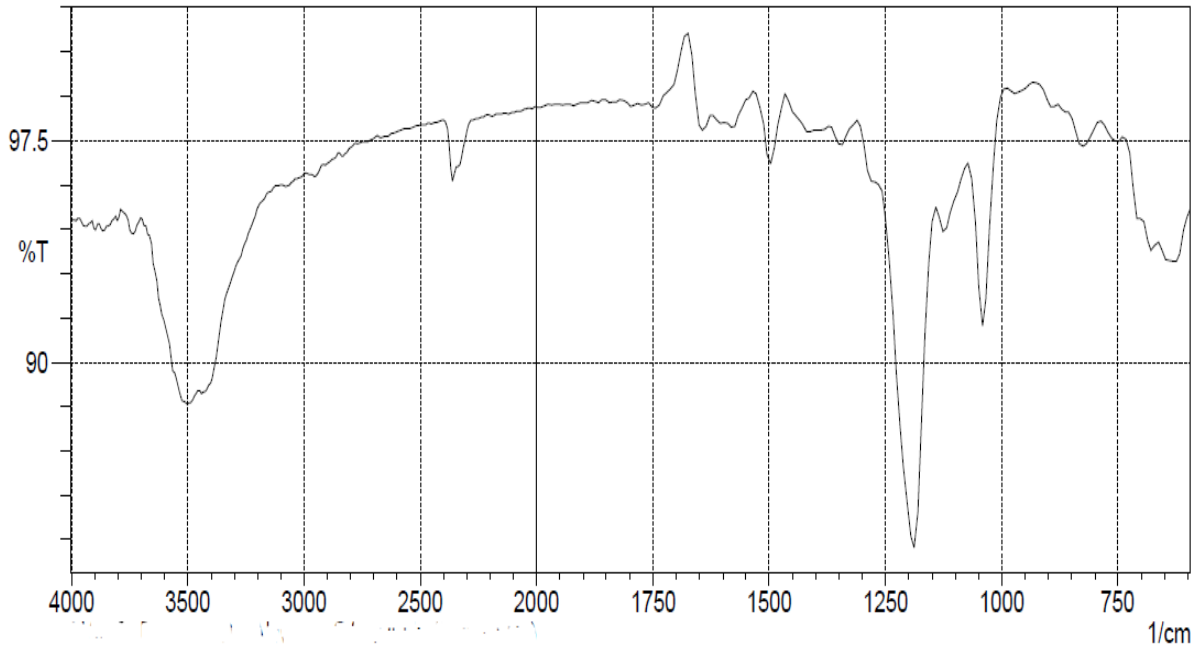


Figure 2: FTIR spectrum of the non-degraded crystal violet (Control)

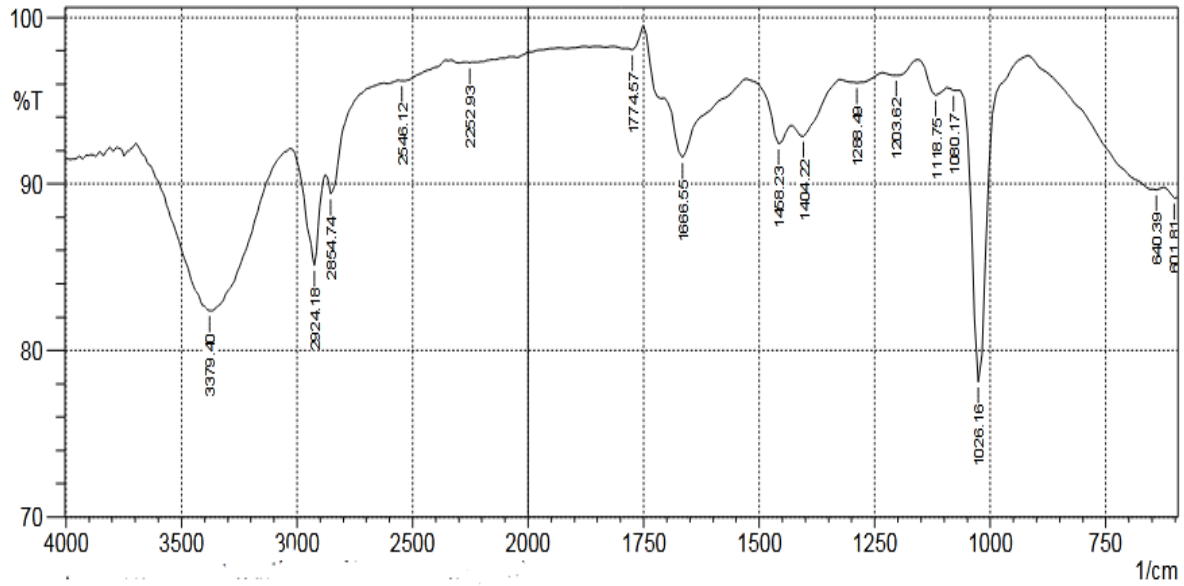


Figure 3: FTIR Spectrum of the Biodegradation of crystal violet dye by *T. versicolor*

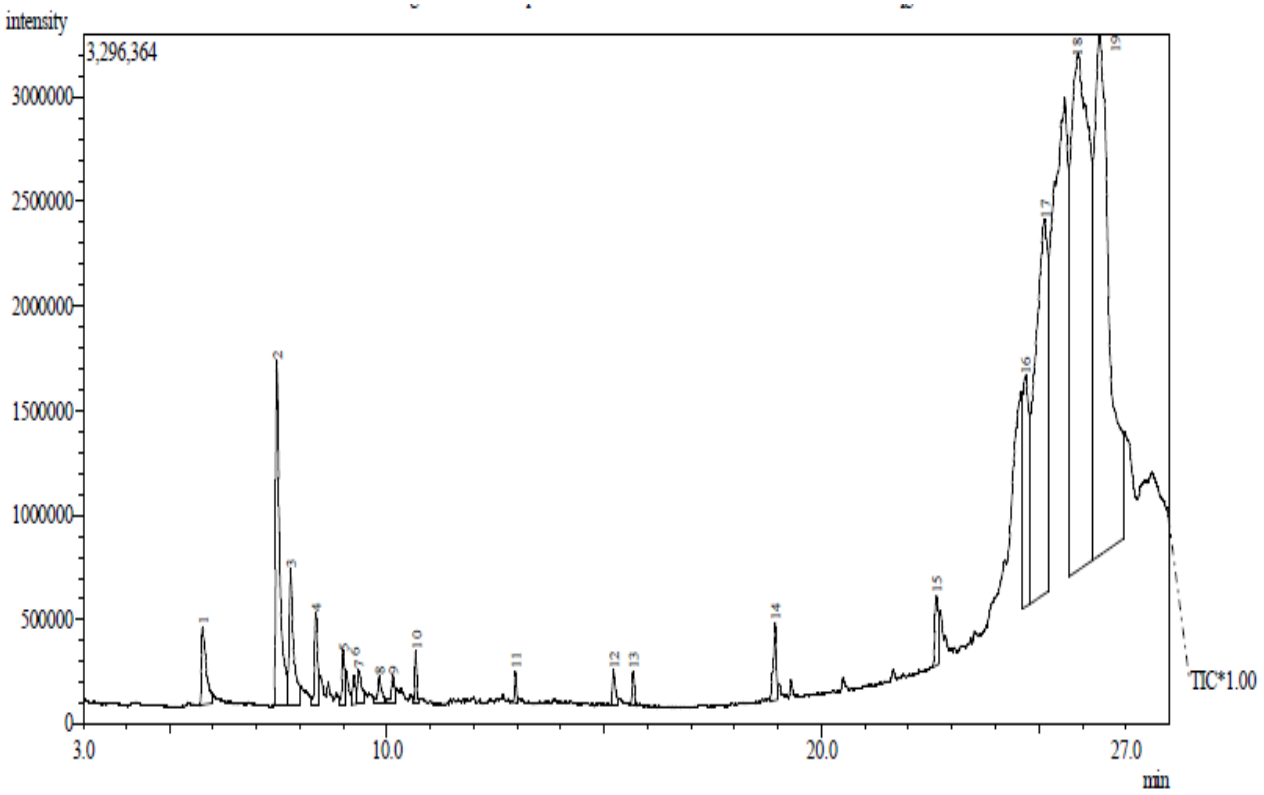


Figure 4: GCMS Spectral of crystal violet dye sample before degradation (Control)

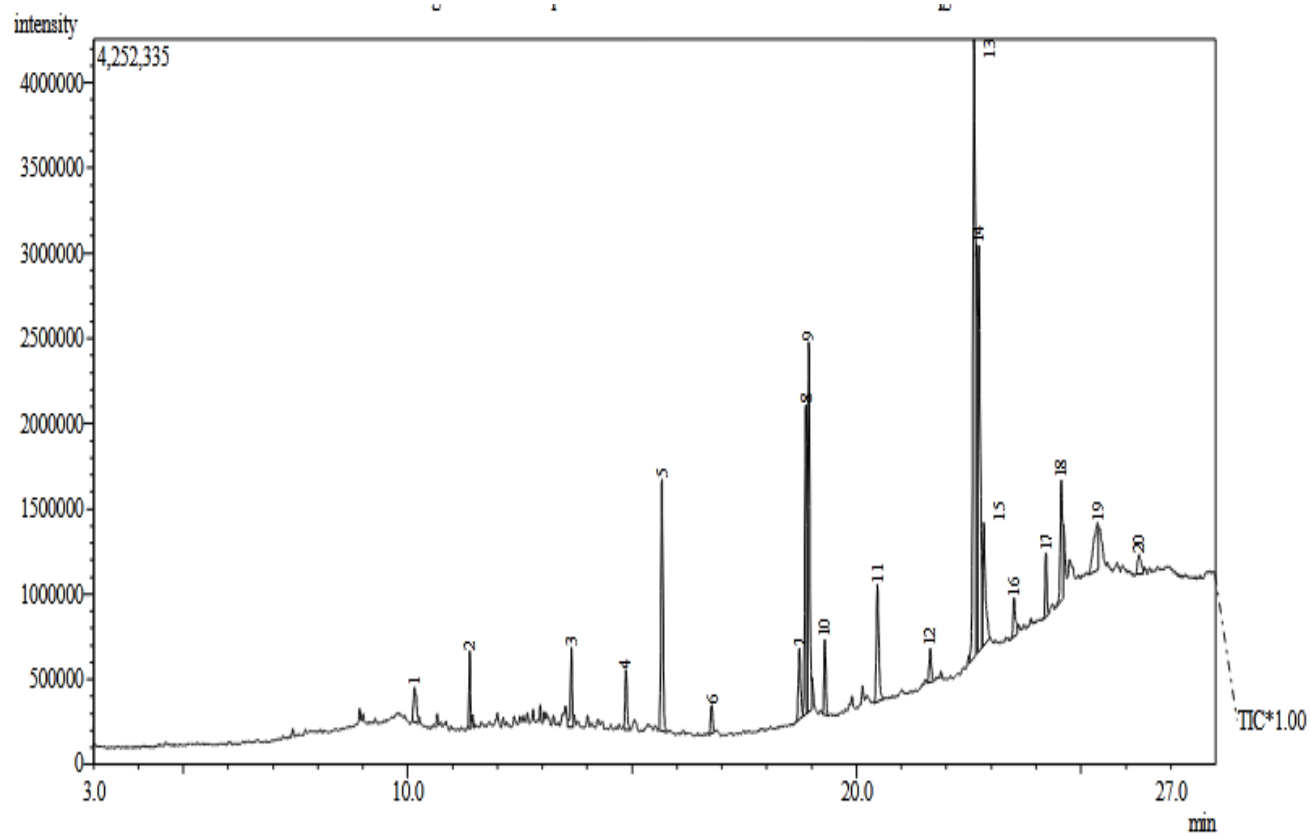
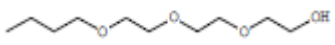
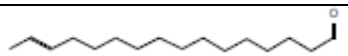
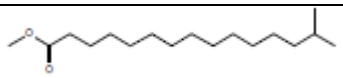
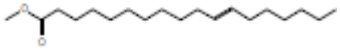
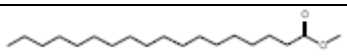
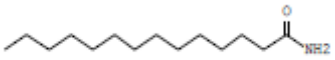
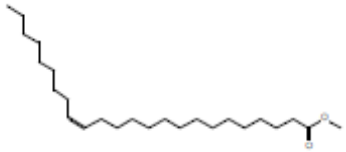
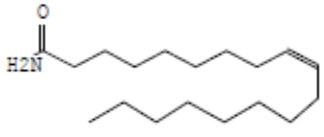
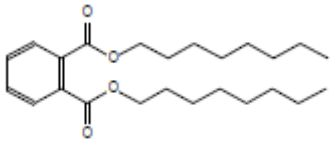
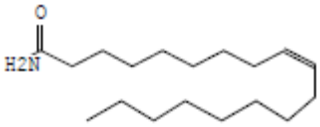
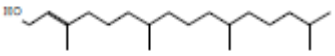
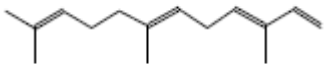


Figure 5: GCMS Spectral of the biodegraded crystal violet dye by *T. versicolor*

Table 1: Mass Spectral of the degraded Crystal violet by *T. versicolor* (NBRC4937)

Peaks	Retention Time	Compound Name	Molecular weight	Structure
1	10.153	Butoxytriethylene glycol	206	
2	11.383	1-Tetradecene	196	
3	13.650	E-14-Hexadecenal	233	
4	13.648	9-Heptadecanone	254	
5	15.667	Methyl 14-methylpentadecanoate	270	
6	16.775	1-Docosene	308	
7	18.725	1-Hexadecanol	242	
8	18.875	9,12-Octadecadienoic acid, methyl ester	294	
9	18.933	11-Octadecenoic acid, methyl ester	296	
10	19.292	Octadecanoic acid, methyl ester	298	
11	20.467	Tetradecanamide	227	
12	21.642	15-Tetracosenoic acid, methyl ester, (Z)	380	
13	22.617	9-Octadecenamide	281	
14	22.725	7-Nonenamide	155	

15	22.835	Tetradecanamide	22.833	
16	23.508	9,12-Octadecadienoyl chloride	298	
17	24.217	1,2-Benzenedicarboxylic acid, dioctyl ester	390	
18	24.558	9-Octadecenamide	281	
19	25.367	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl	296	
20	26.300	1,3,6,10-Dodecatetraene, 3,7,11-trimethyl	206	

The metabolites in Table 1 can be used to propose the degradation pathways of CV by *T. versicolor*.

4.0 Discussion

This study contributes to the existing body of knowledge by investigating the biodegradation of crystal violet using *T. versicolor*. This study highlights the ability of *T. versicolor* to secrete the laccase enzyme, as evidenced in Plate 1. Several studies have shown that the laccase enzyme facilitates the oxidative polymerization of guaiacol, resulting in the creation of reddish-brown regions within the medium. This phenomenon has been observed in studies conducted by [18], [19], [20], [21] and [22], the presence of these zones provides a convenient method for determining the microbial synthesis of laccase enzymes.

The remarkable ability of the laccase enzyme secreted by *T. versicolor* may have played a significant role in conferring the degradation properties observed in this study. Laccase enzymes are well-known for their capability to oxidize a wide range of aromatic compounds, including dyes like crystal violet, making them valuable in bioremediation processes [23].Numerous studies have documented the utilization of laccase enzymes derived from mushrooms for the bioremediation of various dyes. For instance, Bamigboye *et al*. [24] demonstrated the effective decolorization of trypan blue, Congo red, and textile dyes (red and blue) using *Pleurotus tuber-regium*. Kang *et al.* [25] also reported the successful decolorization of triphenylmethane dyes by several mushroom strains, including *P.*

coccineus, C. versicolor, G. lucidum, M. affinis, and *L. edodes*. Similarly, Yan *et al*. [26] and Kunjadia *et al*. [27] observed the decolorization of crystal violet dye through the action of laccase produced by *Pleurotus ostreatus.*

Interestingly, some of the end products identified in this study, such as amines and esters, have been previously reported by other researchers who explored the biodegradation of crystal violet by various white rot fungi [28]. These findings further validate the effectiveness of *T. versicolor* in degrading crystal violet and suggest that the organism follows similar metabolic pathways as other white rot fungi during the degradation process [29].

The identification of specific functional groups in the degraded samples aligns with previous studies. Amines and esters, which were identified in the degraded samples, have been reported as common degradation products of crystal violet by different microorganisms [30].These functional groups are typically associated with the breakdown of aromatic compounds and are indicative of the enzymatic transformation of crystal violet by *T. versicolor.*

The detection of these functional groups supports the notion that *T. versicolor* utilizes similar enzymatic mechanisms as other microorganisms during crystal violet degradation. The presence of amines and esters suggests the occurrence of alkylation or acylation reactions, respectively, which are commonly involved in the breakdown of aromatic compounds [31]. It is worth noting that the identification of these functional groups alone does not provide a comprehensive understanding of the complete degradation pathways involved. Crystal violet is a complex compound with multiple potential degradation routes, and the complete metabolic pathways[1] followed by *T. versicolor* for crystal violet degradation require further investigation.

The findings of this study contribute to the existing knowledge on crystal violet degradation and highlight the potential of *T. versicolor* as an efficient biodegradation agent for this dye. The ability of *T. versicolor* to produce laccase enzymes and degrade crystal violet adds to the growing body of evidence supporting the use of white rot fungi in environmental remediation applications.

Further studies could focus on elucidating the precise enzymatic reactions involved in crystal violet degradation by *T. versicolor*. Analyzing the gene expression patterns and enzyme kinetics of laccase production in the presence of crystal violet could provide valuable insights into the enzymatic mechanisms driving the degradation process.

Moreover, exploring the degradation efficiency of *T. versicolor* under various environmental conditions, such as pH, temperature, and substrate concentrations, would enhance our understanding of its applicability in practical dye remediation scenarios. Investigating the potential synergistic effects of combining *T. versicolor* with other microorganisms or optimizing the degradation process through co-culturing strategies could also be worthwhile avenues for future research.

5.0 Conclusion

In conclusion, this study provides evidence of the ability of *T. versicolor* to biodegrade crystal violet. The findings demonstrated that *T. versicolor* exhibited the ability to produce and secrete the laccase enzyme, which likely played a significant role in the degradation of CV. Analytical techniques such as FTIR revealed the presence of

diverse functional groups in the metabolites of CV, while GC-MS analysis confirmed the existence of amines and esters as end products. These functional groups and identified compounds underscored the biotransformation of CV by *T. versicolor*. Importantly, these findings hold potential implications for the development of effective and environmentally friendly strategies in the treatment of dye-laden wastewaters, thereby mitigating the environmental impact associated with CV dyes. Nonetheless, there remain several avenues that warrant further exploration. Subsequent studies should strive to elucidate the complete degradation pathways of CV. Additionally, optimizing the degradation conditions and upscaling *T. versicolor* culture systems are crucial steps for the practical application of this approach.

Declarations

Conflict of Interest

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

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Author contributions:

AAT Conceived and designed the experiments, Manuscript preparation. AWT Data analysis and interpretation, Manuscript preparation. JFA Contributed reagents/materials/analysis tools; all authors carried out laboratory experiment .All authors read and approved the final manuscript

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