Bioremediation of Triphenylmethane and Sulfonated Azo Dyes Using an Indigenous Strain of Trichoderma Asperellum: Mechanisms and Efficacy Assessment

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Abstracts: This study aimed to isolate a native fungal strain that was potent enough to curb the toxicity of azo dyes in textile effluent drain sludge. The native fungus, which has the potential to degrade several azo dyes under 'non-sterile' conditions, was isolated and identified as Trichoderma asperellum (TA). It was observed that the isolated strain was capable of decolorizing up to 71.42 %, 60.19 %, and 89.65 % of azo dyes (methyl orange, malachite green, and crystal violet, respectively) within a span of 14 days with an extensive range of temperatures (15°C-40°C) and pH (5-8) before optimization. The targeted dyes at concentrations of 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L, and 500 mg/L were decolorized after optimization. The maximum decolorization of methyl orange (MO), malachite green (MG), and crystal violet (CV) was 88.89 %, 71.09 %, and 92.75 %, respectively, at a dye concentration of 100 mg/L, pH of 6.5, and temperature of 25°C. The fungal isolate Trichoderma asperellum frequently contains chitinases, cellulose, and laccases. The findings of this study validated the potential of TA as an effective biocatalyst for the remediation of methyl orange, malachite green, and crystal violet in textile wastewater. The degradation products showed decreased toxicity to sprout seeds, such as Cicer arietinum (chickpea) and Vigna radiata (mung seeds), when compared to untreated wastewater.

Keywords: Textile Wastewater, Azo Dyes, Bio-Decolorization, Endophytic Fungi, Phytotoxicity, Optimization.

1. INTRODUCTION

Wastewater is an important natural resource responsible for the survival of various organisms. Clean water is not only a necessity, but also a livelihood opportunity, as well as the right of human beings. Various anthropogenic activities degrade the esthetic value of the water. The textile industry is among the most prominent sources of water pollution. According to (Wang *et al*., 2017,) the textile industry emits approximately 8×10^6 tons of dyes annually, and the textile and dye-stuff sectors contribute considerably (Obanan *et al*., 2022; Yaseen and Scholz, 2018). Each year, industrial effluents leak more than 280,000 tons of water into the aquatic environment, harming both the flora and fauna (Prasath *et al.,* 2019; Sen *et al*., 2016). dyes are visible in effluents from the textile industry, even at concentrations as low as 1 ppm (O Neil *et al*., 1999; Shailesh, R. Dave and Riddhi H. Dave, 2009), In general the average concentration of azo dyes in effluent is found to be 10 and 200mgL^{-1}. There are various techniques for treating azo dyes in wastewater; however, biological treatment is one of the most ecofriendly and cost-effective. Bioaccumulation, biosorption, and biodegradation are biological techniques. The process by which microorganisms obtain color from the environment and incorporate it into their cells as an essential element of metabolism is known as bioaccumulation, whereas adsorption of dye molecules onto microbial biomass is known as biosorption. Adsorption occurs through the interaction between dye molecules and functional groups on the cell walls (Mustafa et *al*., 2021, Hans's victor *et al*., 2020).

Biodegradation is the best possible solution for the complete removal and mineralization of azo dyes from wastewater, and can be utilized as a sustainable tool for the removal of azo dyes. Fungi are potent alternatives for the treatment of wastewater effluents, and azo dye molecules can be broken down into small compounds using enzymes secreted by microorganisms. In terrestrial and aquatic habitats, *Trichoderma* contains a prominent group

of filamentous fungi that are extensively used to control phytopathogenic organisms and industrial processes (Buchert *et al*., 1995; Rojo *et al*., 2007; Argumedo-Delira *et al*.,2020). O-Reductases, including laccases, manganese peroxidases, lignin peroxidases, and azo reductases are well-known enzymes that participate in biodegradation (Victor *et al*. 2020). The formation of less-toxic metabolites is predominantly caused by laccase-mediated enzymatic breakdown (Arunprasath *et al*., 2019). Another research has been carried out to degrade MO dye by utilizing the brown-rot fungus *Gloeophyllum trabeum* that can produce radical hydroxides through the Fenton reaction (A S Purnomo *et al*., 2020). After 14 days of incubation, G. trabeum was able to degrade47.53% of the MO (Purnomo *et al*., 2019; Purnomo *et al*., 2020). In the current study, isolated fungal species identified as *Trichoderma asperellum* were used to successfully decolorize and biodegrade azo dyes. There are very few reports on the degradation of azo dyes and decolorization of textile wastewater by *Trichoderma asperellum* under nonsterile conditions. This study bridges the knowledge gap regarding the role of fungi as dye degraders, the range of enzymes involved, and their efficacy in reducing the toxicity of triphenyl methane and sulfonated azo dyes. The aims of the present study were to evaluate the viability of Trichoderma *asperellum* as a bioagent, assess the toxicity of textile effluents, and ensure that treated wastewater is safe for the environment.

2. MATERIALS AND METHODS

2.1 Area of Study and Sample Collection

Samples were collected from the surface layer (15-20 cm) of an open drain from the textile industry in Bhadohi District (UP), India (25.3264^0N,82.4319^0N) in a sterilized plastic zipper (3 kg). The sampling sites are shown in fig.1. The untreated sludge sample contained native dye-decolorizing fungal strains. The physical appearance of the sample was light brown in color, and on-spot examination showed pH (6.09 to 9.8), EC (5.69-11.50 dSm-1). The samples were brought to the laboratory within 12hof collection and preserved at refrigerated temperatures for future studies.

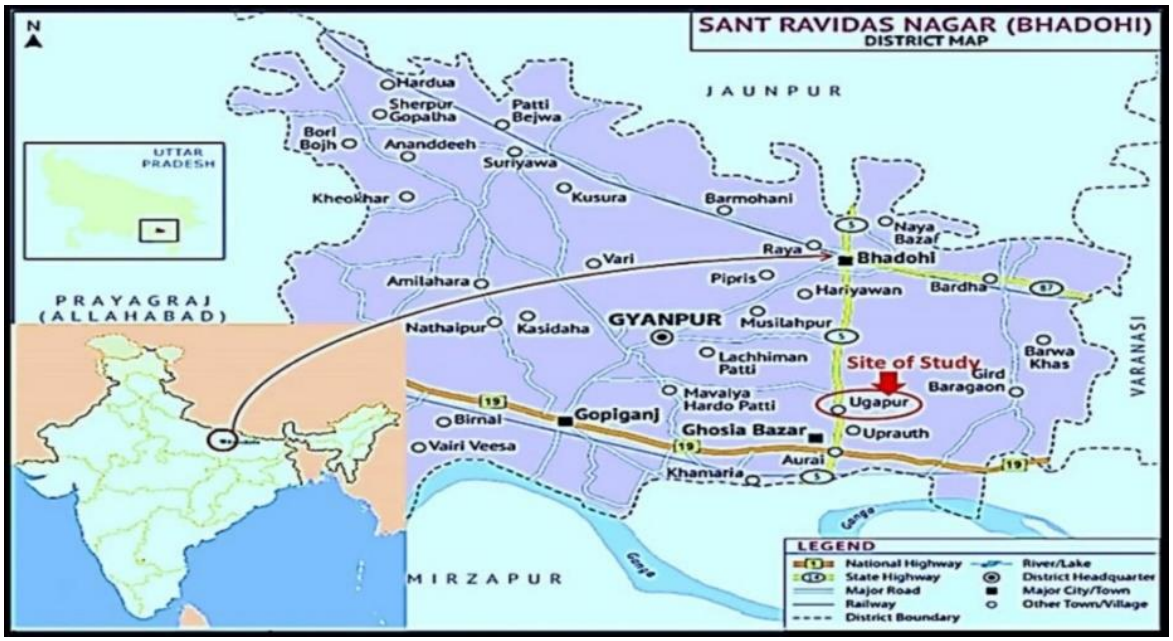

Fig 1: Study area of sampling

2.2. Chemicals, Reagents and Microbiological Media

The azo dyes MO, MC, and CV were procured from Himedia Laboratories Pvt. Ltd., and the media made from potato dextrose were purchased from Himedia Laboratories Pvt. Ltd. Sodium hydroxide (NaOH) was purchased from Sigma-Aldrich Pvt., Ltd.

2.3. Isolation and Screening of Dyes Decolorizing Fungus

Dye-degrading fungal strains were isolated from samples (untreated textile sludge). Different labeled fungal strains have been screened and assessed for their capacity to decolorize dyes using morphological, physiological, and biochemical analyses (Kasana & Pandey 2018). In summary, isolates that showed decolorization effectiveness were streaked on enriched PDA (2%) containing dyes. Isolated fungal colonies with a clear zone around them were collected and stored for future studies.

2.4. Culture Establishment

Trichoderma asperellum has been isolated from textile industry sludge (Ting *et al*., 2011). The isolate was regularly subcultured and maintained on Potato Dextrose Agar (PDA, Merck) at25°C. After seven days of incubation at 25°C, cultures that were seven days old were used to inoculate 100mL of Potato Dextrose Broth (PDB, Merck) to produce fungal biomass.

2.5. Colony Characteristics and Morphological

The colony features and morphology of the dye-decolorizing fungal strains were investigated. Colony parameters and cell shapes were studied using fresh isolate cultures. Colony characteristics were determined.

2.6. ITS rRNA Sequence Analysis

Isolated fungal strains were sent to Barcode Biosciences (Bangalore, India) for ITS rRNA sequence analysis (Pandey *et al*. 2018). The obtained sequence was submitted to the National Center for Biotechnology Information (NCBI) for identification and accession numbers.

2.7. Decolorization Experiments

The fungus was acclimated by cultivating the isolated strain in potato dextrose broth containing 2 mL each of MO, MG, or CV. The absorbance of the supernatant was measured regularly to calculate the percentage dye decolorization. The physiochemical conditions that affect decolorization, such as pH 6.5, temperature of 25°C, and minimum-to-maximum dye concentrations (100 mg/L, 200 mg/L, 300, 400, and 500 mg/L), were optimized and tested in triplicate with a negative control (Wanyonyi *et al*., 2017).

2.8. Ultraviolet–Visible (UV–Vis) Spectral Analysis

The decolorization of the dyes by *T. asperellum was investigated, and the* absorbance supernatants obtained from the dye decolorization tests were examined at wavelengths of 465 nm (MO), 624 nm (MG), and 590 nm (CV). The absorption spectra of the spectrum peaks assembled from azo dyes before and after treatment with Trichoderma asperellum were plotted and differentiated.

2.9. Toxicity Study

A phytotoxicity study of the MO, MG, and CV dyes and their after-degradation metabolites was conducted. The azo dyes were dissolved in 10 ml distilled water. In the final volume, the same dye concentration served as the control and was used to determine toxicity. The reaction was performed at room temperature and the seed surfaces were sterilized with mercuric chloride solution to prevent microbial growth. Ten *Cicer arietinum*(chickpea)and *Vigna radiata* (moong seeds) seeds were placed in a petri dish, and 10 ml of MO, MG, and CV standards (all at 1000 mg/L) were added. The breakdown products were dissolved in distilled water and 10 ml of each dye was used for a comparative study (Parshetti *et al*., 2006). Parallel controls of both seeds in distilled water were used for estimations. Root and shoot lengths, as well as germination rate were measured after1week.

3. RESULTS AND DISCUSSION

3.1. Colony Characteristics and Morphology Identification of The Fungus

The morphology and colony characteristics of the decolorizing fungal strain were investigated using freshly isolated cultures, and the colony parameters and cell shape were studied. The characteristics of the colonies were determined. Morphological analysis revealed that the unique isolate colony had a dark green (spore colour) front and a white (mycelium colour) back after five days of incubation. The mycelium had a rough shape, and dark green spores formed rapidly in the colony core. These initial morphological findings led to the identification of *Trichoderma asperellum*.

3.2. Optimization of Different Parameters for MO, MG and CV Decolorization

The strains identified based on morphology were used to remove color from the MO, MG, and CV dyes. The isolates from wastewater were named T. *asperellum*. The effectiveness of strain decolorization was calculated over the 14th day by using a UV-visible spectrophotometer with a double beam (Fig. 3). Its efficiency was at its highest level at pH 6.5 and 25°C. Decolorization was recorded with MO 88.89 % at 100 mg/L, MG 71.09%, and with CV 92.75 % in shaking incubation. This might be caused by the orientation and makeup of the Methyl Orange and Malachite Green and Crystal Violet functional groups. The results of the experimental observations demonstrate that as growth declines, the clearance efficiency decreases. This provides an examination of strain development at MO, MG, and CV decolorization concentrations of 100, 200, 300, 400, and 500 mg/L. This fungal strain was selected for sequencing because of its strong decolorization effectiveness. *Trichoderma asperellum* was identified by analysis of the ITS rRNA sequence acquired from the results, and the nucleotide sequence of the isolate was assigned accession number (EU021220.1). Fig. 2 depicts the phylogenetic tree for *Trichoderma asperellum*.

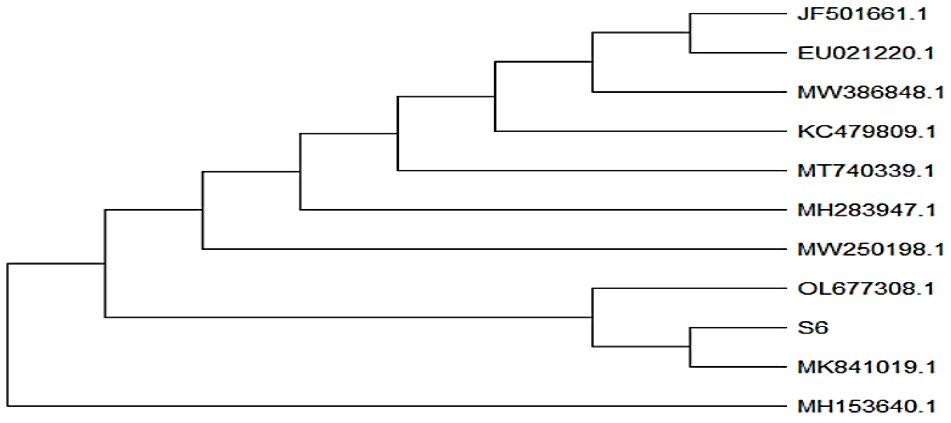

Fig 2: Molecular Phylogenetic analysis by Maximum Likelihood method

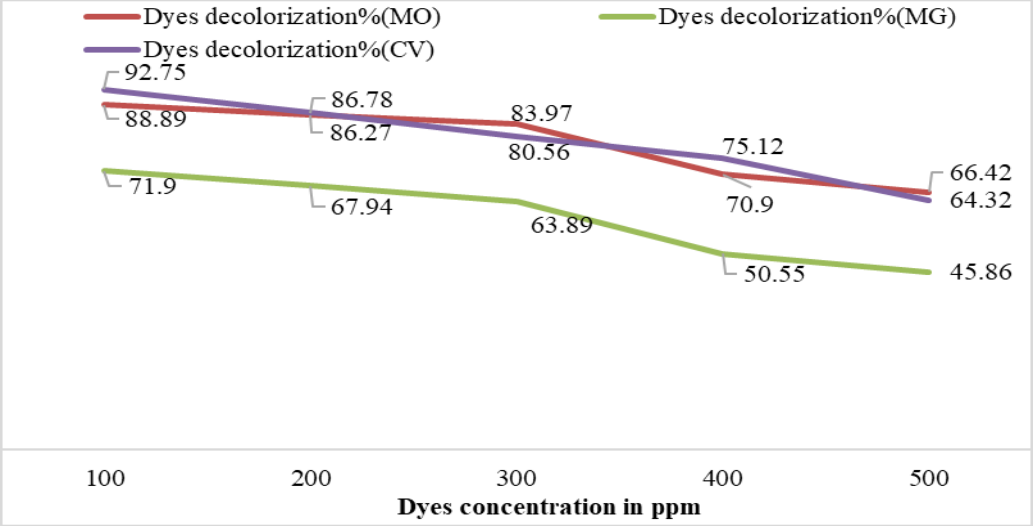

Fig 3: Decolorization % age of azo dyes in terms of concentration

3.3 Optimization of experimental condition pH

The effect of pH on the dye decolorization and degradation efficiency of TA is shown in (Fig.4). These numbers demonstrate that pH 6.5 has produced the maximum degradation. The sensitivity of fungi to pH can be linked to a decrease in the denaturation of azo dyes above this pH value.

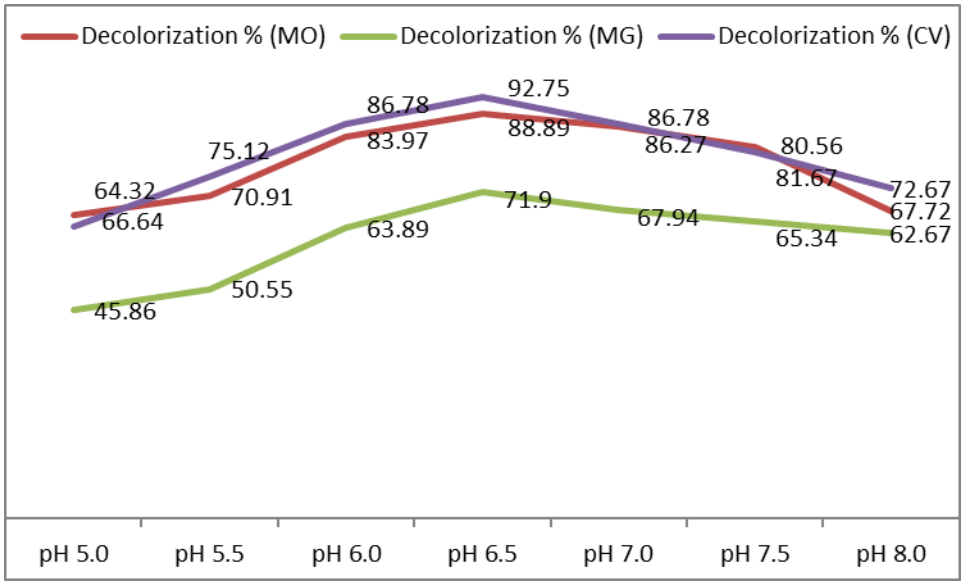

Fig 4: Decolorization % age of azo dyes in terms of pH

3.4 Optimization of Experimental Temperature

The degradation of azo dyes is a temperature dependent process. The optimal temperature for maximum activity of TA was determined by measuring azo dye removal at different temperatures, as shown in (Fig.5). An increasing trend in activity was observed until 25 °C, after which a decreasing trend was observed owing to the hindering of fungal activity at higher temperatures. The optimum temperature obtained was 25^0C.

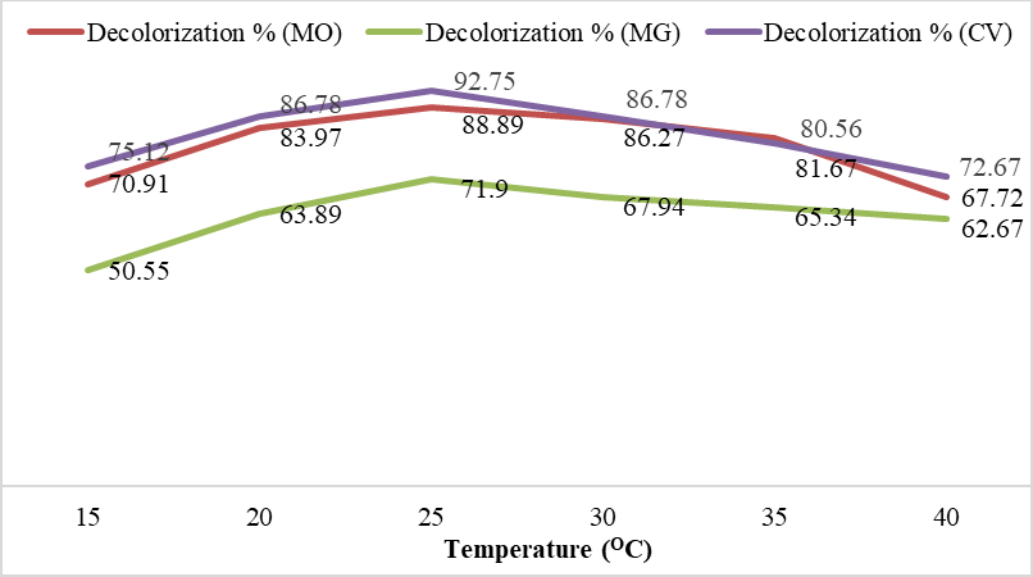

Fig 5: Percentage decolorization of azo dyes in terms of varying Temperature (^{0}C)

3.5. Decolorization Experiments

For MO, MG, and CV decolorization, 100 mg/L of dye was used to optimize the temperature, pH, and dye concentration. MO, MG, and CV decolorization was 61.42%, 50.19%, and 94.65%, respectively, on the 14th day of incubation under shaking conditions (120 rpm). Stagnant MO, MG, and CV decolorization rates were 88.89%,71.09%, and 92.75% on the 14th day. When there is shaking, there may be better oxygen transport and nutritional distribution than when there is shaking, which could account for the high decolorization observed in high decolorization. All optimization studies were performed under shaking conditions (120 rpm) at 25°C with 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L, and 500 mg/L concentrations (Fig. 6).

Fig 6: Selected fungus to decolorize azo dyes (A) Methyl orange (B) Malachite green (C) Crystal violet untreated and D, E, F treated by Trichoderma asperellum

3.5. Toxicity Study

The discharge of textile wastewater may have a direct effect on soil fertility, which is related to the crop yield. Owing to environmental concerns, toxicity assessment of dyes before and after degradation is important. The metabolites of azo dyes did not inhibit their growth. Chickpea and mung seeds were used to investigate the

phytotoxicity of dyes and their metabolites. Fifty % of dye-cultivated seeds grew into sprouted plants. The average plume (cm) and radicle length (cm) were measured. All seeds that had been exposed to deteriorated items germinated. The average plume and radicle of chickpeas were found to be 1.12 and 12.81 cm, respectively (Table 1). Close phytotoxic effects were observed in Triticum *aestivum*, *Oryza sativa* reactive red 141, *Vigna radiata* with methyl orange, and its degradation products (Maria Belen Ceretta et *al.,* 2018) Telke *et al*., 2008, Kriti Akanshaa, *et al*., 2019).

Table 1: Phytotoxicity assay of MO, MG, CV and its degraded metabolites by TA on Cicer arietinum and Vigna radiate

***Cicer arietinum* (Chickpea)**							
Parameter studied	**Distilled water (control)**	**MO Before treatment**	**MO After treatment**	**MG Before treatment**	**MG After treatment**	**CV Before treatment**	**CV After treatment**
Germination%	100	70	100	60	100	50	100
Plume (cm)	4.72±o.45	3.08±0.4	8.92±0.28	0.89.00	1.12. ±0	03±00	4.92±0.09
Radicle (cm)	12.81±0.34	3.01±0.74	5.2±0.54	1.23±01	2.13±0.88	4.09±00	7.2±0.54
***Vigna radiate* (Mung seed)**							
Germination%	100	80	100	60	100	60	100
Plume (cm)	5.72±o.21	2.09±0.46	6.92±0.01	1.12. ±0	3.12±0	1.78	4.92±0.09
Radicle (cm)	10.81±0.04	3.01±0.84	8.2±0.54	3.92±0.6	5.92±0.55	3.54±0.32	7.2±0.54

3.7 Statistical analysis

All statistical analyses were performed using Microsoft Excel and SPSS, version 26 (2019). Decolorization of dyes by TA for MO. The mean was 79.12% at a concentration of 100 mg/L with an incubation time of 14 days and a standard deviation less than 0.05. For MG, the mean was 59.86% at a concentration of 100 mg/L and the same incubation time, with a standard deviation of less than 0.05. In addition, for CV, with the same incubation time, the mean was 79.9% at 100 mg/L, with a standard deviation of less than 0.05.

4. DISCUSSION

The observations of these studies revealed that sulphonated and triphenyl azo dyes (MO, MG, and CV) were effectively decolorized by *Trichoderma asperellum, and* the decolorization of the dyes was comparable to that of other studies Marcharchand and Ting (2017), Similar studies reported that CV, MV, MG, and CB (100 mg/L) were decolorized by up to 11, 67, 76%, and 57%, respectively, by *Trichoderma asperellum* within 336 h (14 days). Other observations include *Penicillium species* such as *P. pinophilum* (Jasiska *et al.,* 2012) and *P. ochrochloron* (Shedbalkar *et al.,* 2008; Shedbalkar and Jadhav, 2011). *Phanerochaete chrysosporium* (Radha *et al*. 2005) and *Coriolopsis sp.* (Chen and Ting 2015a), The fungal strain *Aspergillus niger* (Ali *et al*., 2016), *Mucor mucedo* (Moturi and Singara Charya 2009), and *Trametes versicolor* are among the fungal species that may break down dye molecules (Casas *et al.,* 2009). Previous research has shown that non-white rot fungi can decolorize refractory colours in a manner identical to that of white rot fungi (e.g., *Coriolopsis sp*., *Phanerochaete sp., Trametes sp.) P. simplicissimum* decolorizes MO, MG, and CV dyes quickly, to decolorize the abilities of other *Penicillium species*. In accordance with *Shedbalkar et al*. (2008) and *Shedbalkar and Jadhav* (2011), *P. ochrochloron* decolored 50 mg L CB and MG at a rate of 93 % within 2.5 and 14 h, respectively; *P. pinophilum* removed 87.1 % of 10 mg L, MG within 48 h, and *P. janthinellum* decolored 150 mg/L CV at a rate of 56.9 % after 24 h of incubation (Jasinska *et al.,* 2012; Wang *et al*., 2015). According to Moturi and Singara Charya (2009), Mucor mucedo eliminated 0.02 % of CV (78 %) and MG (65 %) in 15 days. Although *Aspergillus niger* required 10 days to completely decolorize 80.9 % of 10 mg/L CV, *Aspergillus sp*. completely decolorized 20 mg/L MV in just one day (Ali *et al*., 2016; Kumar *et al*., 2011). On days 7, 7, 1, and 9, *Coriolopsis sp*. eliminated 97, 94, 91, and 52 % of MV, CV, CB, and MG, respectively (Chen and Ting 2015a). According to Jasiska *et al. (*2012), our study indicated that *Trichoderma asperellum,* when used with the chemical makeup of the dyes, and various experimental parameters (such as pH, temperature, shaking speed, nutrient availability, and dye concentrations), the targeted dyes with concentrations of 100, 200, 300, 400, and 500 mg/L were decolorized 65.2% at the same time after optimization. The maximum decolorization of methyl orange, malachite green, and crystal violet was 61.42 %, 50.19 %, and 94.65 %, respectively, at a dye concentration of 100 mg/L, pH of 6.5, and optimum temperature of 25°C, marked on the14th day of dye

decolorization, which was similar to the previous finding of Jasiska *et al.* (2012). It is possible that the entire experimental factor included in our study may have contributed to the non-identical textile azo dye removal rates in other studies compared with the current study.

CONCLUSION

Bioremediation is a promising technology for eliminating toxins from the environment, which helps improve the quality of ecosystems. Our observations showed that TA could decolorize MO, MG, and CV dyes. The physiochemical parameters also affected MO, MG, and CV decolorization by fungi, which became more tolerant to cultivation and decolorization of the dye over a wide range of pH and temperatures. The phytotoxicity assay showed preferable results for treated textile effluents. This suggests that fungal isolates can endure challenging environmental conditions and remove pollutants from the environment. The starting inoculum biomass was maintained constant throughout the investigation for all dye concentrations, and it was not discovered that the percentage of dye decolorization significantly increased with concentration. This suggests that dye decolorization may occur regardless of the original inoculum biomass. Additionally, as the dye content in the broth increased, the rate of decolorization gradually decreased, which explains the involvement of laccases in the decolorization and degradation processes, and the conversion of carboxylic acids may be converted into alkanes and alkenes by the lipase enzyme released by the fungus. This result reveals that *Trichoderma asperellum* might be exploited as a biological candidate for pollution control in effective environmental management because of the augmentation of nutrients as well as other biological components within the system.

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Author Contribution

KS (Assistant Professor) contributed to study design. VS (PhD Scholar) collected the data, contributed to data analysis and interpretation, and drafted the article with the help of all authors. Jaswant Singh(Professor and Head) and Abhishek Chauhan (Senior Scientist) critically reviewed the manuscript. All the authors have read and approved the final version of the manuscript.

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Ethical approval Not applicable

Data Availability Not applicable

Conflict of interest

The authors declare that they have no conflict of interest.

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