Determination of the mechanism and optimization of the conditions of the process of removing colored aromatic compounds by selected Basidiomycota

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1. Introduction

Rapid urbanization and industrialization have driven the widespread use of synthetic dyes across industries such as textiles, leather, paper, pharmaceuticals, food, and cosmetics [1,2]. Since the introduction of synthetic dyes in 1856, more than 10,000 dye types have been developed, with global production exceeding 1 million tons annually [3]. The textile industry alone consumes nearly 80% of these dyes and plays a crucial role in the economies of many developing countries by generating employment and export revenue [4].

However, the environmental cost of this growth is substantial. Only 10-15% of dyes bind effectively to textile fibers, while the remainder is discharged into wastewater. As a result, approximately 100,000-280,000 tons of dyes enter aquatic environments each year [5]. Countries with highly concentrated textile industries, such as Bangladesh, India, and China, generate billions of tons of dye-containing wastewater annually, much of which is released untreated [6]. These effluents severely impair water quality, hinder light penetration, disrupt aquatic ecosystems, and pose serious risks to human and ecological health, including toxic, allergenic, and carcinogenic effects [7].

Synthetic dyes are particularly problematic due to their complex aromatic structures, high chemical stability, and resistance to biodegradation. Dye pollution significantly undermines sustainable development and exacerbates global water scarcity. Consequently, the treatment of dye-containing wastewater has become a major research priority and a key objective of international environmental policies [8], including the United Nations Sustainable Development Goals (notably SDG 6: Clean Water and Sanitation, and SDG 3: Good Health and Well-Being) and the EU Green Deal.

Conventional wastewater treatment methods-such as coagulation, advanced oxidation processes, adsorption, membrane filtration, and microbial degradation-have been widely applied for dye removal [3]. However, their efficiency varies depending on dye structure, solubility, and operational conditions, and many approaches are costly, energy-intensive, or unsuitable for decentralized applications. Therefore, there is an urgent need to develop environmentally friendly, cost-effective, and decentralized treatment technologies specifically tailored to dye-containing wastewater [9].

Biological treatment using microorganisms offers a promising alternative due to its operational flexibility and environmental compatibility. Among biological agents, fungi - particularly white-rot fungi - have gained increasing attention for their exceptional dye-removal capabilities [10]. Through a synergistic combination of biosorption and enzymatic biodegradation, white-rot fungi can transform complex dye molecules into less toxic or mineralized products. Mycoremediation is especially attractive due to its low infrastructure requirements, scalability, and sustainability [11].

Despite growing interest in fungal-based dye remediation, significant knowledge gaps remain in understanding and optimizing the mechanisms governing dye removal by white-rot fungi.

In particular, the synergistic contributions of biosorption and extracellular enzymatic biodegradation are not yet sufficiently elucidated, limiting the predictability and scalability of fungal treatment systems. There is a pressing need to evaluate the feasibility of integrating white rot fungi into small-scale, decentralized bioreactor systems tailored to specific dye classes, especially for regions lacking centralized wastewater infrastructure [12]. Furthermore, insufficient attention has been given to the ecotoxicological safety of treated effluents, which is critical for environmental protection and regulatory acceptance. At present, the absence of mechanistic insight linking gene expression, enzyme secretion, and dye degradation performance hinders rational process optimization [13]. Addressing these gaps through advanced multi-omics approaches - including transcriptomics, proteomics, and metabolomics - is urgently required to enable predictive modelling, improve treatment efficiency, and support the development of next-generation fungal-based wastewater treatment technologies.

2. Research Statement

2.1 Research Gap

Based on the extensive literature survey, the present research identified the following key research gaps in dye decolorization with mycoremediation technique:

1. Limited Mechanistic Understanding,
2. Scope for Optimization Studies,
3. Limited Integration of Multi-Omics,
4. Lack of Comprehensive Toxicity Assessment,
5. Demand of Decentralized Wastewater Treatment.

2.2 Research hypothesis

The hypothesis of this study is derived from these gaps that WRF such as *T. versicolor* and *P. ostreatus* exhibit dye removal capabilities via a synergistic mechanism of biosorption and enzymatic biodegradation, which can be markedly improved by optimizing critical physicochemical parameters and effectively applied in compact, decentralized bioreactor systems designed for specific dye categories. Moreover, this treatment can produce effluents with minimal ecotoxicological impacts, rendering the method environmentally viable. Multi-omics studies may help to decipher the connection between enzyme profile and gene expression which may provide base for predictive modelling of interaction between fungal enzymes and different class of dyes.

2.3 Objective of Research

Based on the delineated research scope, specific objectives have been systematically formulated to address each aspect in a comprehensive and practically feasible manner (Figure 1).

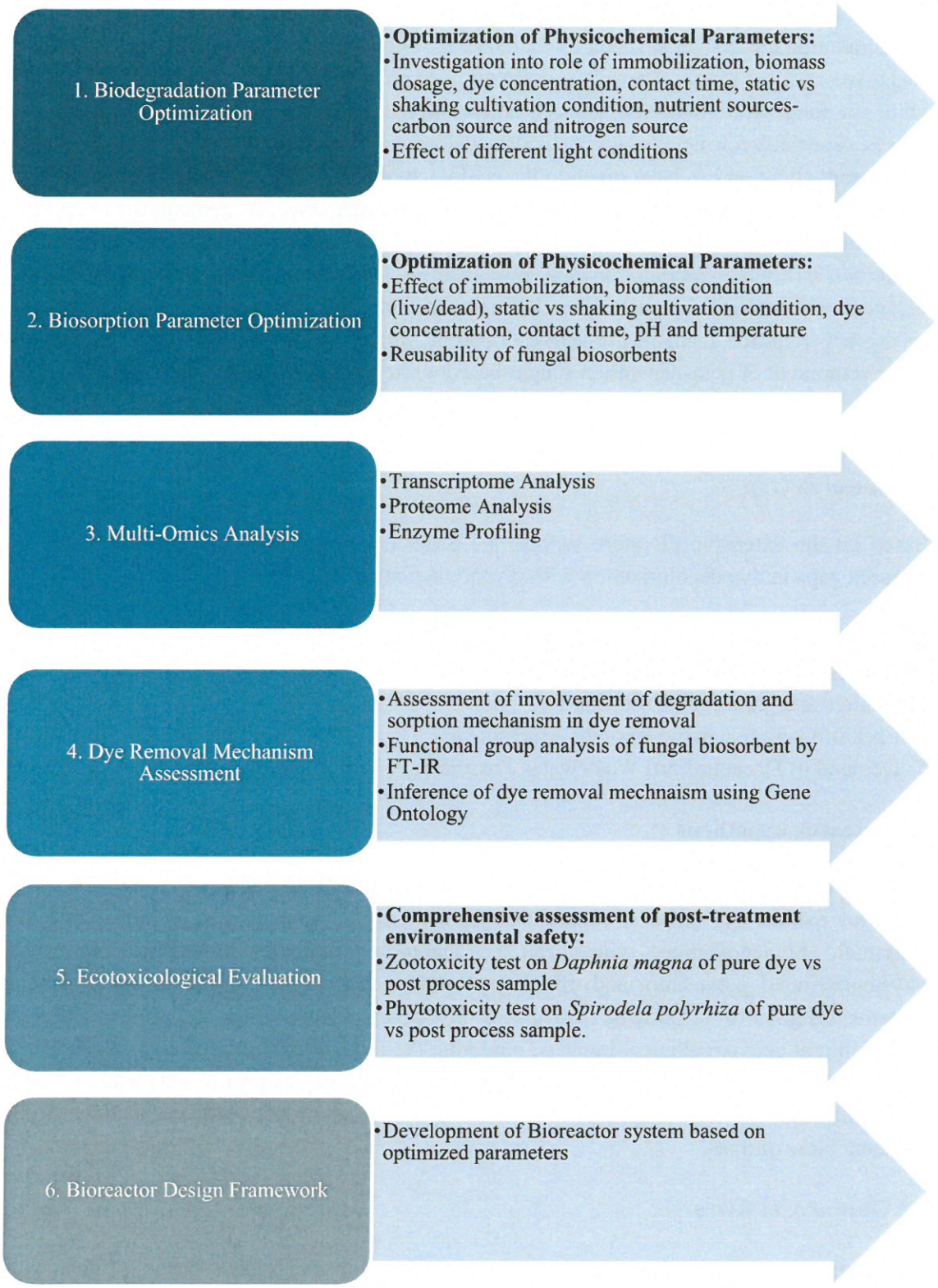


Figure 1. Schematic of Research Objectives

3. Materials and Methodology

3.1 Test substances - synthetic dyes

In this study, a range of synthetic dyes representing three major structural classes –azo (Congo Red (CR), Evans Blue (EB)), triphenylmethane (Brilliant Green (BG), Crystal Violet (CV)), and anthraquinone (Remazol Brilliant Blue R (RBBR) - were selected for the decolorization by white-rot fungi. Analytical-grade dyes were used to prepare sterile stock solutions (1000 mg/L) in deionized water and working solutions of desired concentrations were freshly prepared under aseptic conditions.

3.2 Selection of fungal strain and carrier for immobilization

The pure cultures of white-rot fungi (WRF), *Pleurotus ostreatus* (strain BWPH), and *Trametes versicolor* (strain CB8) were collected from the depository of Fungal Strain Collection of Environmental Biotechnology Department, Silesian University of Technology, Gliwice, Poland. The isolation and identification of the fungal species were previously described by Jureczko et al. [34]. Both strains were maintained in liquid organic medium, which termed as regular medium (RM), containing glucose - 5 g/L, peptone - 1 g/L, $MgSO_4 \cdot 7H_2O$ - 1 g/L, and KH_2PO_4 - 1 g/L (pH-5.7).

The immobilization of fungal biomass was employed to enhance the structural stability and reusability of the fungal cultures during bioremediation processes. Support S1: Dishwasher-grade polypropylene and support S2: Sponge - Commercial polyurethane were selected because of cost effectiveness, mechanical stability and environmental neutrality.

3.3 Fungal Biomass preparation and immobilization

The 7 day old cultivated biomass was transferred to 250 mL Erlenmeyer flask containing 150 mL of liquid organic medium and cultivated at different growth parameters in order to obtain different fungal biomass variants (Table 1). For biodegradation experiments, dye were added directly to growth medium containing extra cellular enzymes. For biosorption experiments, fungal biomass were separated from growth medium and then only fungal biomass pellets were added to dye.

Table 1. Growth conditions for producing Fungal biomass variants

Growth Parameters	Description	Free Fungal Biomass	Self-immobilized Fungal Biomass	Carrier immobilized Fungal Biomass
Static	-	X	-	-
Shaking	150 rpm	-	X	X
Carrier for immobilization	dishwasher-S1 or sponge-S2	-	-	X
Temperature	20°C ± 2°C	X	X	X
Abbreviation for biodegradation experiments	Fungal biomass + extra cellular extract	CB8/ST, BWPH/ST	CB8/SH, BWPH/SH	CB8/S1, CB8/S2, BWPH/S1, BWPH/S2
Abbreviation for biosorption experiments	Only Fungal biomass	-	CB8-BS, BWPH-BS	CB8/S2-BS, BWPH/S2-BS

(Note: "X" means application of parameter**)**

3.4 Evaluation of dye decolorization by biodegradation

The preliminary biodegradation experiments were carried out with five selected dyes as mentioned previously. The effect of initial dye concentration were assessed (100-400 mg/L). The one piece of 7-day-grown mycelium pellets with or without immobilizer was transferred to 2 mL of medium containing an appropriate concentration of each dye and incubated in static condition at room temperature for 96 h. The biotic and abiotic controls were prepared and incubated in the same manner without inoculation. The dye decolorization percentage (%) was calculated using equation 1.

$$DP[\%] = \frac{C-S}{C} \times 100 \qquad \text{(Equation 1)}$$

Where, C is the current concentration of dye in a control sample with support or only medium (mg/L), and S is the current concentration of dye in samples with immobilized or free fungal biomass (mg/L).

After preliminary experiments, RBBR, EB and CV were selected for further optimization as it resulted in more than 90% decolorization. The dye biodegradation efficiency was assessed at different parameters:

- Impact of fungal culture agitation on dye decolorization: Static and shaking
- Impact of Carbon source and agitation on dye decolorization: Sucrose medium (SM), Glucose medium (GM)
- Impact of Nitrogen source and agitation on dye decolorization: Ammonium nitrate medium (ANM), Yeast extract medium (YEM)

- Impact of Immobilization on dye decolorization: sponge immobilization
- Evaluation of Dye mixture decolorization efficiency: RBBR, EB and CV mixture in 1:1:1 proportion

3.5 Enzyme profiling and Multi-omics analyses

The study of Laccase, Manganese Peroxidase (MnP) and Lignin Peroxidase (LiP) enzyme activity patterns under non-immobilized, self-immobilized, and carrier-immobilized circumstances were performed to clarify the impact of physical and biochemical parameters of both fungal systems during RBBR, EB and CV dye degradation. *T.versicolor* (CB8) grown in shaking condition and sponge immobilized condition proven to be superior for dye decolorization hence they were selected for transcriptomic and proteomic analysis to understand the underlying mechanism of dye degradation.

For transcriptomic analysis, 24 h RBBR and EB dye exposed CB8/SH fungal pellets were collected for RNA extraction. RNA extraction was performed by utilizing Qiagene RNeasy Plant Mini kit, purchased from Qiagen polska Sp. z o.o., Wrocław, Poland. RNA quantification was performed using Nanodrop and high quality RNA samples were sent to Macrogen, Netherlands for sequencing and bioinformatic analysis. For Gene Ontology (GO) and KEGG pathway analyses, differentially expressed genes (DEGs) with an absolute fold change ≥ 2 ($|FC| \geq 2$) and nbinomWaldTest raw p-value <0.05 were selected and functional annotation was performed using eggNOG-mapper based on functional orthologs.

For proteomic analysis, extracellular culture filtrates of CB8/SH exposed to RBBR, EB, and CV dyes (48 h) were collected and concentrated using 10 kDa MWCO ultrafiltration units (VIVASPIN TURBO). Protein concentration was estimated using a BSA-based colorimetric assay. The lyophilized samples were analyzed by LC-MS at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw. Raw mass spectrometry data were processed using MaxQuant (v2.7.5.0). Trypsin was specified with one missed cleavage allowed; minimum peptide length was 7 amino acids, and PSM FDR was set to 0.01. HCD was used for data acquisition, with methylthio (C) as a fixed modification and oxidation (M) as a variable modification; LFQ was disabled due to the lack of biological replicates. Data were further processed in Perseus (v2.1.6.0), excluding reverse hits, contaminants, and site-only identifications. Protein intensities were $\log_2$-transformed, $\log_2$ fold changes were calculated relative to controls, and proteins with $\log_2 FC \geq 0.3$ were considered differentially abundant for GO and KEGG analyses.

3.6 Ecotoxicological assessment

Zootoxicity was assessed for pure dye and fungal-treated dye samples using the DAPHTOXKIT F™ MAGNA (Tigret Sp. z o.o., Warsaw, Poland). Pure dye and post-process samples were serially diluted (100-6.25%) in a two-fold geometric series using standard freshwater. Five neonates were added per well, and plates were sealed with Parafilm and incubated in the dark at 20 °C. Mortality and immobilization were recorded after 24 and 48 h.

EC_{50} values were determined from concentration-mortality curves, converted to toxicity units using equation 2, and classified according to Persoone toxicity classes.

$$TUa = 100/EC_{50} \qquad \text{(Equation 2)}$$

To assess the toxic effects of pure dye and fungal-treated dye samples at the producer level, a growth inhibition microbiotest was performed using the freshwater duckweed *Spirodela polyrhiza* (SPIRODELA DUCKWEED TOXKIT, TIGRET Sp. z o.o., Poland). Control, pure dye, and treated samples were set at 100% and serially diluted to 50%, 25%, 12.5%, and 6.25%. One germinated turion was placed in each cup containing Steinberg growth medium (control) or toxicant solution, and plates were incubated for 72 h at 25 °C under continuous 6,000 lux illumination. Images taken at 0 and 72 h were analysed using ImageJ to measure frond area, and growth was calculated as the difference between final (72h) and initial (0h) frond areas. Percentage growth inhibition was determined for each concentration, and the 72 h EC_{50} was calculated by Probit analysis and expressed as Toxicity Units (Equation 2).

3.7 Evaluation of dye decolorization by biosorption experiments

The biosorption potential of *T. versicolor* (CB8) and *P. ostreatus* (BWPH) for the triphenylmethane dyes Brilliant Green and Crystal Violet was evaluated under batch conditions. Fungal biosorbents were prepared as mentioned in 3.3. Live fungal biomass was used to study the effect of initial dye concentration and contact time on sorption efficiency. The influence of immobilization and static growth conditions on dye removal was assessed and compared with freely suspended biomass. To distinguish between passive and metabolically driven uptake, dye removal by autoclaved (dead) biomass was also examined. The effects of pH and temperature on the biosorption of both dyes were systematically investigated to determine optimal operational conditions. Multivariate analysis using Principal Component Analysis (PCA) was applied to identify key factors governing dye sorption behaviour. Structural and surface characteristics of the mycelial matrix before and after dye uptake were analysed by FT-IR and SEM to elucidate biosorption mechanisms. Finally, the reusability and stability of immobilized mycelial pellets were evaluated over successive sorption cycles to assess their practical applicability.

3.8 Bioreactor study

Previous bench-scale studies identified CB8/SH and CB8/S2 as the most efficient and rapid RBBR decolorization systems; therefore, these configurations were selected for bioreactor experiments. Bioreactor decolorization was conducted under optimal conditions using 0.2 L RM, over four consecutive dye-addition cycles, with appropriate abiotic controls. Following a 7-day incubation to establish fungal biomass, reactors were dosed with 125 mg/L RBBR per cycle without nutrient supplementation or aeration. Dye concentrations were measured at 596 nm after each addition and after 48 h, and removal efficiencies were calculated relative to the controls using equation 1.

4. Result and Discussion

The biological elimination of synthetic dye in wastewater treatment relies on the biosorption and biodegradation capabilities of the selected organism. Both fungal strains showed optimal growth in RM and immobilized successfully on both supports, more optimally on sponge (70-100%). As a result of preliminary experiments, *T. versicolor* and *P. ostreatus* effectively degraded structurally diverse dyes from different classes such as azo, triphenylmethane and anthraquinone group with reasonably high efficiency at higher dye load. *T. versicolor* achieved up to ~98%, 91%, 98%, 97%, 97% dye removal and *P. ostreatus* achieved up to ~88%, 57%, 92%, 84%, 97% at higher dye load (400 mg/L) for Evans Blue, Congo Red, Brilliant Green, Crystal Violet and Remazol Brilliant Blue R respectively [14].

4.1 Optimization of dye biodegradation parameters and enzyme profiling

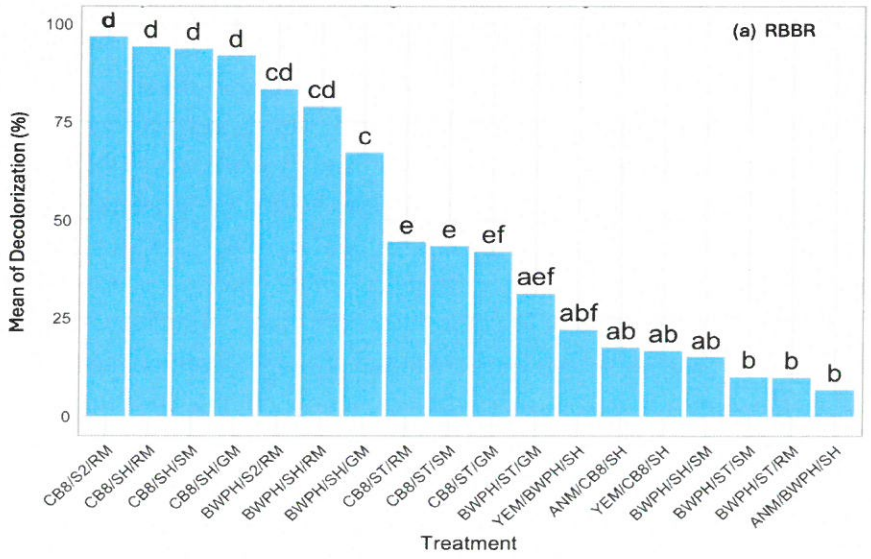
Impact of fungal culture agitation, carbon source, nitrogen source and immobilization was assessed. A three-factor ANOVA was performed to identify optimal nutrient conditions for dye decolorization, evaluating the effects of carbon source, nitrogen source, and fungal species on dye removal efficiency. All three factors had statistically significant effects. Nitrogen source was the most influential factor ($F = 226.64$, $p < 0.001$), followed by fungal species ($F = 18.85$, $p < 0.001$), while carbon source had a smaller but significant effect ($F = 4.81$, $p = 0.0125$). The relatively low residual variance indicates a good model fit. These patterns reflect established fungal physiology: nitrogen sufficiency favors primary metabolism while suppressing secondary processes such as lignin modification and dye degradation. In contrast, nitrogen limitation shifts fungi toward secondary metabolism, markedly increasing extracellular oxidative enzyme production for degrading complex aromatic compounds [15,16]. Overall, nutrient composition-particularly nitrogen source-and fungal selection are critical for process optimization.

Tukey's post hoc test was applied to compare mean dye decolorization efficiencies across agitation conditions, immobilization strategies, media composition, and fungal biomass types ($p < 0.05$) (Figure 2). For RBBR, *T. versicolor* (CB8) with sponge-immobilized biomass in regular medium (CB8/S2/RM) achieved the highest decolorization (~98%), significantly outperforming other treatments. Other CB8 treatments in regular medium also showed high efficiency (92–95%), while nitrogen-rich media resulted in the lowest removal. Immobilization generally enhanced dye removal, and agitation improved *P. ostreatus* (BWPH) performance. For Evans blue, the highest efficiencies were obtained with CB8 grown in regular medium under sponge-immobilized or shaking conditions (97.6-98.4%), which formed the top statistical group. BWPH showed moderate efficiency, while glucose-, sucrose-, and nitrogen-rich media significantly reduced decolorization for both fungi. Similarly, for crystal violet, immobilized CB8 in regular medium represented the optimal condition, forming the leading statistical group. These results further support the ANOVA finding that nitrogen source has a stronger influence than carbon source. BWPH achieved its highest removal under shaking or immobilized conditions in regular medium but remained less effective than CB8. Overall, CB8 cultivated in regular medium under immobilized or shaking conditions was consistently

identified as the statistically superior system for decolorization of RBBR, Evans blue, and crystal violet dyes. When these biomass formulations were used to decolorize a ternary dye mixture in a 1:1:1 ratio, *T. versicolor* (CB8/SH and CB8/S2) achieved more than 70% decolorization. This indicates its strong potential for treating mixtures of dyes from different classes, as commonly found in real wastewater.

Other researchers have also highlighted the benefits of immobilized cultures, showing that white-rot fungi immobilized on carriers such as polyurethane foam, loofa sponge, or alginate beads exhibit enhanced and more stable enzyme production, protection from shear stress and toxic dye intermediates, dense biofilm formation, and improved dye degradation through sustained activity and additional adsorption capacity [17,18].

The incorporation of RBBR, EB, and CV elicited a distinct induction of laccase activity in both *T. versicolor* (CB8) and *P. ostreatus* (BWPH), with the extent of enzyme production differing based on the dye, fungal species, and growth conditions. Highest laccase enzyme production was noted for CB8/S2 in RBBR dye treating condition (20 U/L).CB8/SH has overall high production of MnP (~80 U/L). LiP production was lower in dye treating condition as compared to control. To conclude this section, statistical analyses were conducted to determine the significance of each enzyme and to assess the correlation between enzyme activities and the observed decolorization percentages under static, shaking, and sponge-immobilized fungal biomass conditions. Spearman correlation showed a strong positive association between RBBR decolorization and both laccase and MnP activities ($\rho = 0.771$, $p = 0.072$), whereas LiP exhibited a negligible correlation ($\rho = 0.086$, $p = 0.872$). These findings indicate that although extracellular ligninolytic enzymes are important for dye oxidation, enzyme activity alone is insufficient to reliably predict decolorization efficiency in complex fungal systems. This complexity highlights the need for systems-level approaches, such as transcriptomic and proteomic analyses, to clarify the molecular mechanisms underlying dye decolorization.



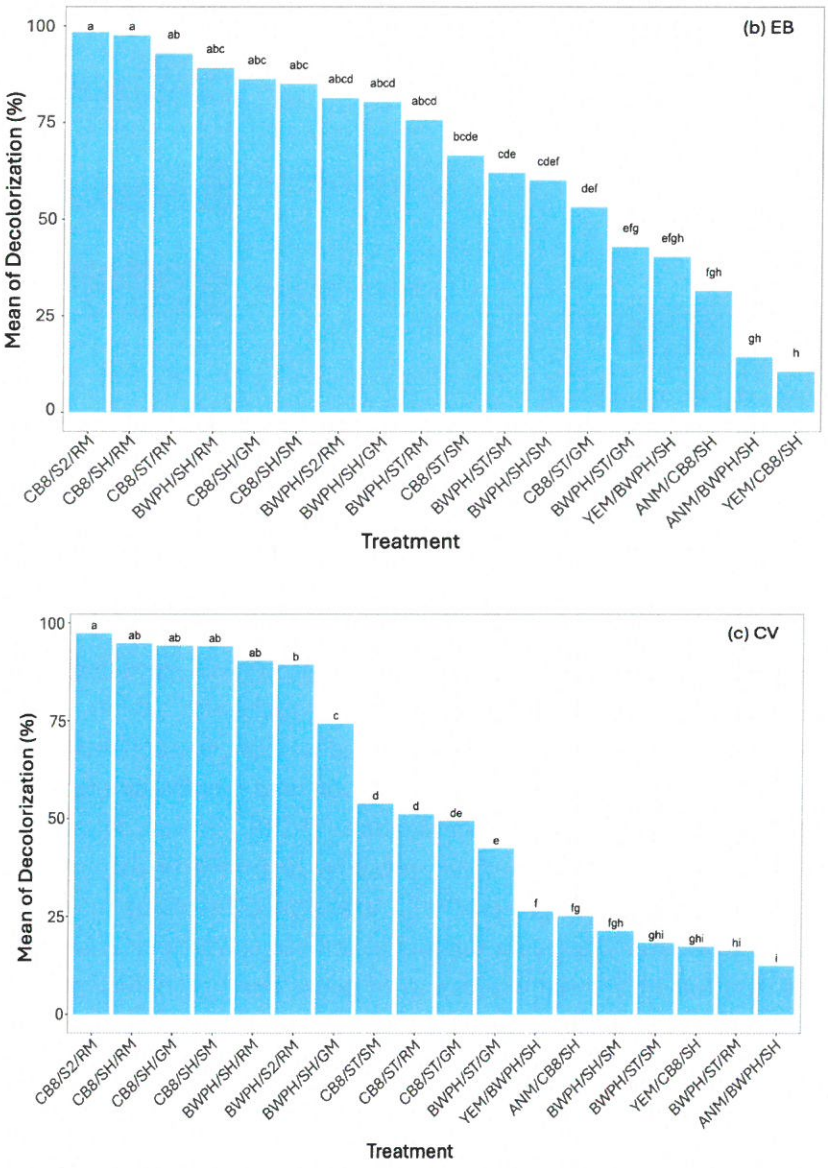


Figure 2. Tukey's post hoc significant grouping test for choosing optimal dye decolorization condition among agitation, immobilization and nutrient source for (a) Remazol Brilliant Blue R (b) Evans Blue and (c) Crystal Violet by *T. versicolor* (CB8) and *P. ostreatus* (BWPH)

4.2 Molecular Insights into Dye Degradation Mechanisms

In this study, whole-transcriptome sequencing of *T. versicolor* (CB8 strain) was conducted to identify differentially expressed genes (DEGs) and proteomics by LC/MS was performed to identify differentially expressed proteins (DEPs). The objective was to perform Gene Annotations (GO) and uncover previously uncharacterized genes and proteins potentially involved in dye decolorization by white-rot fungi. Overall, transcriptomic and proteomic analysis revealed that high proportions of genes and proteins were downregulated as seen in Figure 3.

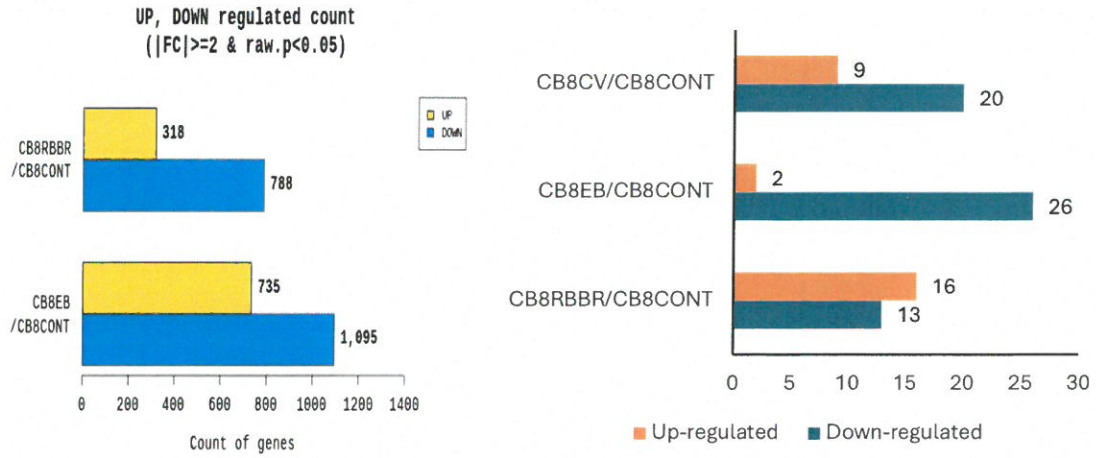


Figure 3. Differential expression of (a) Genes ((|fc|>=2) and nbinomWaldTest raw p-value <0.05) and (b) Proteins (log2fc>0.3) of comparison pair of control versus dye treated samples of *T. versicolor*

While studying these genes and proteins in detail, it was revealed that intracellular detoxification pathways, primarily involving cytochrome P450 monooxygenases, oxidoreductases, dehydrogenases, glutathione-dependent enzymes, and membrane transporters collectively mediate dye transformation, detoxification, and metabolite transport. Intracellular pathways are major contributors to dye degradation [19,20]. Downregulation of cell division and cytoskeletal genes indicates growth suppression to prioritize detoxification and stress tolerance [21]. Several genes and proteins with unassigned functions may contribute to dye degradation. Gene annotations based on functional orthologs were performed in detail. A brief overview of the data for differential expression of genes during RBBR dye degradation by *T. versicolor* is presented here in Table 2.

Table 2. Gene Ontology for DEGs during RBBR dye degradation by *T. versicolor*

Gene Ontology (GO) Enrichment category- Molecular Functions (MF)					
Protein ID	**Putative function**	**COG cate-gory**	**GO Description**	**GO ID**	**Fold Change**
39887	cytochrome P450	IQ	monooxygenase activity	4497	147.4
35158	Aldo keto reductase	C	Catalytic activity	3824	126.3
34024	oxidoreductase	C	Catalytic activity	3824	4.0
39879	Fatty acid hydroxylase superfamily	I	Catalytic activity	3824	3.2
34426	Iron hydrogenase	Y	Catalytic activity	3824	3

32719	Belongs to the mitochondrial carrier (TC 2.A.29) family	C	transporter activity	5215	3
36733	MFS general substrate transporter	U	transporter activity	5215	2.8
42056	Manganese peroxidase isozyme MP2	T	Mn-dependent (NADH-oxidizing) peroxidase activity	16689	-2.1
44196	Lignin peroxidase	T	Diarylpropane peroxidase activity	16690	-2.1
32737	Laccase I	Q	Oxidoreductase activity	16682	-2.5
32773	Fungal specific transcription factor domain	K	transcription regulatory region nucleic acid binding	1067	-5.5
39667	RF-1 domain	J	nucleic acid binding	3676	-84.3

4.3 Toxicity Reduction and Environmental Safety

Zootoxicity tests using *Daphnia magna* showed that pure RBBR, EB, and CV dyes were classified as class IV after 48 h exposure, which decreased to class III following bioremediation by *T. versicolor* and *P. ostreatus*. Phytotoxicity assays with *Spirodela polyrhiza* indicated that pure RBBR, EB, and CV were classified as IV, III, and IV, respectively, and their toxicity was reduced to classes III, II, and II after *P. ostreatus* treatment. Fungal treatment reduced residual toxicity, confirming ecotoxicological safety of treated effluents [22].

4.4 Optimization of Dye Biosorption Parameters

The biosorption aspect of this study revealed that sponge-immobilized live fungal biomass provides quick, high-capacity, and reusable dye removal, especially for triphenylmethane dyes (Brilliant Green and Crystal Violet), greatly surpassing traditional self-immobilized biosorbents. Maximum sorption in 6 h-379.4 mg/g for BG and 48.9 mg/g for CV at 400 mg/L. Our biosorbent has a markedly higher capacity than previously reported biological or physicochemical sorbents for BG dye removal. It also demonstrates superior efficiency and faster uptake than earlier fungal sorbents. For comparison, loofa sponge-immobilized *Phanerochaete chrysosporium* achieved 101.06 mg/g for Remazol Brilliant Blue R [23], while dried *Sarocladium* sp. reached 58.48 mg/g for Remazol Black dye [24]. The table summarizes the biosorption capacities of developed biosorbents in both live and dead biomass states. Research on the optimization of the sorption process has shown that BG removal is the highest at 20.85-32.17 °C, pH 3.4-6 and for CV sorption is the most efficient at 30 °C, pH 6.5-7.5; sorption data fit quadratic model ($p < 0.05$). Fungal pellets also showed reusability potential without additional treatment.

Table 3. Biosorption capacities of *T. versicolor* and *P. ostreatus* variants for BG and CV in batch treatment (conditions: C_0= 200 mg/L, biomass dose of self-immobilized biomass= 0.5 g wet/ 10 mL, biomass dose of sponge-immobilized biomass= 1 piece/ 10 mL, Biomass type= Live/Autoclaved-Dead, T = 22.5 °C, t = 6 h)

Biosorbent	Biomass Type	Brilliant Green Sorption (%)	Crystal Violet Sorption (%)
Trametes versicolor (CB8-BS)	Live	14.2	15.6
Immobilized *Trametes versicolor* (CB8/S2-BS)	Live	90.3 ****	43.9 **
Immobilized *Trametes versicolor* (CB8/S2-BS-A)	Autoclaved dead	48.4 ***	22.8 *
Pleurotus ostreatus (BWPH-BS)	Live	23.9	12.1
Immobilized *Pleurotus ostreatus* (BWPH/S2-BS)	Live	81.7 *	39.3 *
Immobilized *Pleurotus ostreatus* (BWPH/S2-BS-A)	Autoclaved dead	30.3^{-}	18.9^{-}
Sponge (S2)	No fungal biomass	49.9 ***	6.5^{-}

(Note: **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, $^{-}p \geq 0.05$ for one tail *t*-test compared with self-immobilized biomass).

4.5 Bioreactor Performance and Practical Implications

Previous bench-scale studies showed that self-immobilized and sponge-immobilized *T. versicolor* (CB8/SH and CB8/S2) achieved rapid and high decolorization of RBBR under optimized conditions; therefore, these biomasses were selected for bioreactor experiments. Decolorization performance was evaluated over four successive dye-addition cycles (125 mg/L per cycle) to assess operational stability relative to abiotic controls. The abiotic control containing only sponge fragments exhibited negligible removal (2.4–8.2%), confirming minimal physical adsorption and the necessity of fungal activity (Figure 4). Both fungal reactors showed high decolorization from the first cycle, with CB8/SH and CB8/S2 removing 95.3% and 96.0% of dye within 48 h, respectively. Throughout four cycles, 75-80% decolorization efficiency were retained. Overall, *T. versicolor* sustained effective decolorization across cycles, with sponge immobilization providing comparable or improved performance, particularly during early operation. Laboratory-scale findings successfully translated to bioreactor-scale, showing feasibility for compact, decentralized treatment systems. The results of research provides a basis for developing customized fungal-based systems tailored to specific dye types and wastewater characteristics.

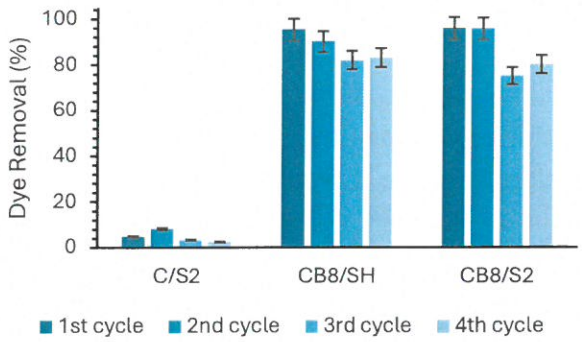


Figure 4. RBBR dye removal percentage in bioreactors containing self-immobilized and sponge immobilized biomass of *T. versicolor* (CB8/SH and CB8/S2)

5. Conclusion

This PhD research conclusively demonstrates that white-rot fungi, particularly *Trametes versicolor* (CB8), are highly effective, environmentally sustainable, and scalable biological agents for the remediation of synthetic dye-contaminated wastewater. Through systematic optimization of physicochemical parameters, strategic biomass immobilization on cost-effective sponge carriers, and validation at both laboratory and bioreactor scales, the study achieved high and consistent removal efficiencies across structurally diverse dye classes, even at elevated dye loads and in mixed-dye systems. The findings reveal that dye removal is governed by a synergistic interplay of rapid biosorption, extracellular ligninolytic enzyme activity, and-critically-intracellular detoxification pathways, as elucidated by integrated transcriptomic and proteomic analyses, which highlight the central role of cytochrome P450-mediated metabolism, redox enzymes, and transport mechanisms. Ecotoxicological assessments further confirmed that fungal treatment substantially reduces residual toxicity, ensuring the environmental safety of treated effluents. Collectively, this work advances mechanistic understanding of fungal dye degradation, establishes a robust framework for designing customized, decentralized fungal bioreactors, and positions *T. versicolor* as a biotechnologically viable solution for sustainable wastewater management, directly supporting the objectives of the United Nations Sustainable Development Goals 6, 12, and 14.

6. Future prospects of Research

The findings of this study point toward clear future directions for translating fungal dye remediation into industrial practice, including the development of continuous, modular bioreactor systems with immobilized white-rot fungal biomass, validation using real industrial effluents, and integration with existing treatment technologies to enhance efficiency and sustainability. Omics-driven mechanistic insights provide a strong basis for targeted strain improvement through metabolic and genetic engineering-such as enhancing cytochrome P450–mediated detoxification, oxidoreductase activity, and transporter function-to generate dye-specific or "designer" fungal systems with improved tolerance and degradation capacity. Complementary life-cycle assessments (LCA), long-term ecotoxicological evaluations, and studies on biomass reuse and valorization will be essential to confirm environmental safety and circular bioeconomy potential. Together, these advances in bioreactor engineering, strain optimization, and sustainability assessment offer a coherent pathway for establishing white-rot fungi as robust, scalable, and environmentally responsible solutions to industrial dye pollution.

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