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COMPARATIVE ASSESSMENT OF CONGO RED DECOLORIZATION WITH AND WITHOUT CU^{2+} USING WILD FUNGAL ISOLATES

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ABSTRACT
The Congo Red decolorization potential of three native wood-rotting fungal isolates from Mumbai (wild isolates A, B, and C) was investigated for a period of 15 days, with particular emphasis on their ability to function under heavy metal stress induced by the addition of 1.5 mM Cu^{2+} (~95 ppm). Under un-supplemented conditions, wild isolate A possessed the highest inherent ability with a decolorization zone diameter of 6.3 ± 0.2 cm, which was significantly higher than the 3.5 ± 0.2 cm and

3.6 ± 0.2 cm diameters of wild isolates B and C, respectively ($p < 0.05$). The addition of 6 Cu^{2+} ions had a differential inhibitory effect, with wild isolate A being the most sensitive, as its decolorization zone was reduced to a mere 1.2 ± 0.1 cm ($p < 0.001$). Wild isolate B, on the other hand, was determined to be highly tolerant of metals, as the diameter of the decolorization zone increased to 3.0 ± 0.2 cm, with no significant difference in growth from the control for the first 10 days. Wild isolate C displayed consistent susceptibility, with its diameter reduced to 3.0 ± 0.2 cm. These findings indicate that fungal efficiency is sharply compromised when Cu^{2+} concentrations exceed the catalytic threshold of approximately 6 ppm, Medinformatics Vol. XX Iss. XX yyyy where the metal acts as a metabolic inhibitor rather than an enzymatic cofactor. Effective bioremediation strategies must therefore be tailored to the specific heavy metal profiles of local industrial effluents.

Keywords: *lignolytic, decolorization efficiency, wood rotting fungi, laccase, Sustainable wastewater treatment.*

1. INTRODUCTION
The efficiency of fungal-assisted systems is greatly influenced by physicochemical factors, especially pH and temperature, which affect enzymatic activity (Gugel et al., 2024). The importance of metal ions, particularly Cu^{2+}, has been recognized as an important aspect of improving bioremediation.

It is also important to have a more detailed comparison of decolorization radius and colony diameter in the presence of Cu^{2+}, particularly for Congo Red on solid media (Shah et al., 2023). This study evaluated the Congo Red decolorization capacity of three Mumbai wild isolates in the presence and absence of Cu2+.This strategy offered a way to screen fungal strains for textile wastewater bioremediation.

Hypothesis:The efficiency of Congo Red decolorization varied significantly among different wild wood-rotting fungal isolates, as reflected by measurable differences in decolorization halo diameter.

Objectives:The primary objective of this study is to evaluate the Congo Red decolorization efficiency of three wild wood-rotting fungal isolates using plate-based decolorization assays.

The secondary objectives are to (i) Quantitatively measure Congo Red decolorization using halo (decolorization zone) diameter as the primary evaluation parameter.

(ii) Assess the influence of copper ion (Cu^{2+}) supplementation on Congo Red decolorization by comparing treated and untreated plates.

(iii) Perform a comparative analysis of the decolorization efficiency among the selected fungal isolates based on decolorization zone diameter.

2. LITERATURE REVIEW
Indigenous wood-rotting isolates, Balinese fungi like *Trametes hirsuta*, *Microporus xanthopus*, and *Ganoderma applanatum*, decolorizes Turquoise Blue dye with a maximum efficiency of 85.84% (Sudiana et al., 2022). Likewise, *Pleurotus ostreatus* was recognized as a potent tool for decolorizing azo dyes like Congo Red (Fiana et al., 2023). Qualitative analysis is done by estimating the zone of decolorization around the fungal colonies on solid media (Fiana et al., 2023).

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pH of the culture medium was an important parameter in fungal decolorization of dyes and the optimal pH range for decolorization was slightly acidic to neutral (Rajhans et al., 2021). Research on *Pleurotus ostreatus* and *Agaricus bisporus* showed that maximum decolorization of azo dyes was achieved at pH

6.0 and 7.0, respectively (Mumbaikar et al., 2023). Investigations into *Aspergillus quadrilineatus* indicated that Congo Red decolorization was highest at pH 5.0 (Yusuf et al., 2023).

Temperatures between 35°C and 37°C supported peak metabolic activity and ligninolytic enzyme secretion in *P.ostreatus* and *A. bisporus* (Mumbaikar et al., 2023), while optimisation studies on

A. quadrilineatus demonstrated maximum decolorization at 30°C (Yusuf et al., 2023). Excessive heat beyond 45°C led to enzyme denaturation, reduced cell viability, and impaired fungal growth (Mumbaikar et al., 2023; Yusuf et al., 2023).

The presence of metal ions was another factor; it was observed that while high concentrations of heavy metals could be toxic, certain fungi, like *Penicillium sp.*, showed the ability to preserve cell integrity while performing biosorption of copper (Oliveira et al., 2023). Moreover, Cu^{2+} was investigated as an enzyme mediator. In certain experiments, the addition of Cu^{2+} enhanced the biodegradation of complex hydrocarbons by over 40% when combined with laccase extracts of *Trametes versicolor* (Fatima et al., 2025). In some cases, the presence of Cu^{2+} at concentrations up to 6 ppm was found to enhanced decolorization process of Congo Red, whereas other metals, such as iron and arsenic, acted as inhibitors (Yusuf et al., 2023).

It was estimated that azo dyes, characterised by the recalcitrant azo bond (N=N) and aromatic rings, constituted approximately 60 to 70% of all synthetic colourants (Carrascal-Hernández et al., 2025). Among these, Congo Red was identified as a particularly problematic pollutant. These dyes are very important in the textile industry because of their stability and low cost (Carrascal-Hernández et al., 2025). The inefficiency of the industrial fixation process results in the discharge of up to 50% of these dyes into wastewater, hence the need for effective remediation strategies (Carrascal-Hernández et al., 2025).

Biological waste treatment is a superior alternative to physicochemical processes, offering greater cost-effectiveness and safety (Yusuf et al., 2023). Fungi demonstrates greater potential for bioremediation than bacteria due to their ability to overcome environmental toxicity and persistent organic pollutants (Gugel et al., 2024). In particular, the saprotrophic Basidiomycetes were acknowledged as the most effective decomposers of dead plant material, using their enzymatic apparatus to colonize the substrates quickly (Eichlerová et al., 2020). In previous screening experiments where 150 strains of Basidiomycetes were involved, the white-rot fungi were found to be the most reliable in decolorizing azo dyes (Eichlerová et al., 2020).

Laccase, a multicopper oxidase enzyme, facilitates the oxidation of aromatic amines and phenolic compounds, meanwhile manganese peroxidase catalyzes the oxidative cleavage of aromatic rings (Eichlerová et al., 2020). Genomic analysis of fast-decomposing species showed an enrichment of Class II peroxidases and DyP-type peroxidases, which facilitated the direct decolorization of dye molecules (Yu et al., 2023).

Results revealed that the dye decolorization potential was highly influenced by the growth rate of the fungus, since biomass was needed to ensure the required concentration of enzymes (Yu et al., 2023). Nevertheless, some studies showed that the growth rate was not necessarily linked to decolorization, indicating that even slow-growing fungi could be potent decomposers if they were capable of high titers of specific peroxidases (Yu et al., 2023).

The current study was therefore intended to bridge the above-mentioned research gaps by conducting a comparative assessment of Congo Red decolorization by three wild wood-rotting fungal isolates This study evaluates whether metal ion supplementation can enhance the efficiency of native strains.

3. RESEARCH METHODOLOGY

A] Isolation and Cultivation of Mycelial Cultures

Three wood-rotting fungal isolates (wild isolates A, B, C) were obtained from naturally decaying hardwood in Mumbai and preserved in aseptic conditions. These isolates were considered to be separate strains from the environment and labeled as wild isolate A, B, and C. Molecular identification was not required since the aim of the study was the functional screening comparison of decolorization ability. In this study, decolorization refers to the visible loss of dye colour on solid agar plates, measured as clearance zone formation and expansion, whereas degradation denotes the biochemical breakdown of dye molecules. Since the current study is plate-based visual and metric analysis, the results obtained are more indicative of decolorization than mineralization. To remove surface contaminants, the samples were washed thoroughly under water and sterilized with a 1% sodium

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hypochlorite solution. After sterilization, small tissue samples were removed and inoculated onto agar plates The agar medium used (1.0 g KH_2PO_4, 0.2 g $MgSO_4.7\ H_2O$, 0.1 g $CaSO_4$, 0.01 g $Fe_2(SO_4)_3$,

10.0 g glucose, tap water 1 litre; 20.0 g agar) for this process was adapted from the formulation described by Ginterová and Janotková (1975). The inoculated plates were incubated at 28 ± 2 °C for 4 days to facilitate the development of pure mycelial cultures.

B] Preparation of Dye-Amended Assay Plates

Experimental agar plates were set up as described by Ginterová and Janotková, (1975) and supplemented with Congo Red dye at a final concentration of 50 mg/L. Two different types of assay plates were set up to test decolorization abilities: (1) Congo Red plates inoculated with mycelial plugs, and (2) Congo Red plates supplemented with copper and inoculated with mycelial plugs. A Cu^{2+} stock solution was made by dissolving analytical-grade $CuSO_4 \cdot 5H_2O$ in sterile distilled water to achieve a

100 mM Cu^{2+} working concentration. For copper-supplemented plates, 0.3 mL of 100 mM $CuSO_4 \cdot 5H_2O$ stock solution was combined with ~20 mL of molten agar (50-55°C), resulting in ~1.5 mM Cu^{2+} (~95 ppm) of an elevated stress concentration to test tolerance for bioremediation. The concentration of copper ions in textile wastewater has been reported to be in the low ppm range (0.01-3.73 ppm) (Dwi Astuti et al., 2023). The concentration used in this experiment is a stress test to determine the upper tolerance limits for bioremediation. This concentration is in the millimolar range that has been shown to affect fungal growth and metal resistance, with some environmental isolates able to tolerate up to 5.4 mM in solid media (Fu et al., 2025).The medium was gently mixed, poured into sterile Petri dishes, and allowed to solidify. Fresh mycelial plugs were inoculated simultaneously onto both types of plates.

C] Incubation and Periodic Monitoring

All experimental plates were incubated at a temperature of 28 ± 2 °C for 15 days. Systematic observation was carried out at 5-day intervals (Day 5, Day 10, and Day 15) during the incubation period. During the observation phase, the radial growth of the mycelium and the color change in the dye-supplemented agar were measured.

D] Measurement and Comparative Assessment of Decolorization Zone

Decolorization zones were measured by adding up the diameter (cm) of the clear zone from edge to edge of the plate passing through the center of the fungal colony using a ruler in transmitted light as per standard protocols for solid media analysis [Shah et al., 2023; Fiana et al., 2023]. The measurements were recorded on day 5, 10, and 15 post-inoculation from the underside of the plates to note the clear zones devoid of any interference caused by the aerial mycelium. Unlike the Plate Volume Method (PVM), which measures the decolorization percentage based on the geometry of the plate [Shah et al., 2023], this study measured linearly.All experiments were performed in triplicates (n = 3). The radial mycelial growth and decolorization zones were measured for each plate, and the values are expressed as mean ± standard deviation (SD). Comparison between the control and Cu^{2+}-treated samples was done using t-test. A p-value < 0.05 was considered statistically significant. All statistical analyses were carried out using Microsoft Excel (Microsoft Corporation, USA).

4. RESULTS AND DISCUSSION

Table 1. Radial mycelial growth (cm) of wild isolates under control and Cu^{2+} treatment at different incubation periods (mean ± SD, n = 3). $p < 0.05$ (significant). NS (not significant).

Isolate	Day	Radial Growth (cm) – Without Cu^{2+}	Radial Growth (cm) – With Cu^{2+}	p-value	Significance
A	5	3.0 ± 0.1	1.0±0.1	<0.001	Significant
A	10	4.5±0.2	1.2 ± 0.1	<0.001	Significant
A	15	6.3 ± 0.2	1.2 ± 0.1	<0.001	Significant
B	5	2.0 ± 0.2	2.5 ± 0.1	0.428	NS
B	10	2.5 ± 0.2	3.0±0.2	0.264	NS
B	15	3.5 ± 0.2	3.0±0.2	0.004	Significant
C	5	1.3±0.1	1.0±0.1	0.025	Significant
C	10	2.5±0.1	2.2±0.1	0.021	Significant
C	15	3.6 ± 0.2	3.0 ± 0.2	0.002	Significant

Radial mycelial growth (diameter) increased progressively across all wild isolates over 15 days on non-supplemented plates. Wild isolate A grew from 3.0 cm (day 5) to 4.5 cm (day 10) and 6.3 cm (day 15). Wild isolate B grew from 2.0 cm to 2.5 cm and 3.5 cm, respectively. Wild isolate C went from1.3 cm to 2.5 cm and

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3.6 cm. Cu^{2+} supplementation significantly inhibited growth across all isolates (see Figures 1,2 and 3). Upon Cu^{2+} supplementation, wild isolate A reached only 1.0 cm (day 5) and

1.2 cm on (day 10 and day 15); wild isolate B grew to 2.5cm (day 5) and 3.0 cm on (day 10 and 15); wild isolate C expanded from 1.0 cm (day 5) to 2.2 cm (Day 10) and 3.0 cm (Day 15). Therefore, Cu^{2+} was able to suppress radial growth compared to the controls at all time points. Statistical analysis by Student's t-test revealed that the addition of Cu^{2+} significantly suppressed radial growth of wild isolate A at all time points ($p < 0.001$). Wild isolate B did not show any significant difference between the control and Cu^{2+} treatment at day 5 and day 10 ($p = 0.428$ and $p = 0.264$, respectively), but showed significant inhibition at day 15 ($p = 0.004$), which indicates delayed sensitivity. On the other hand, wild isolate C showed significant growth inhibition at all times during the incubation period (day 5: p

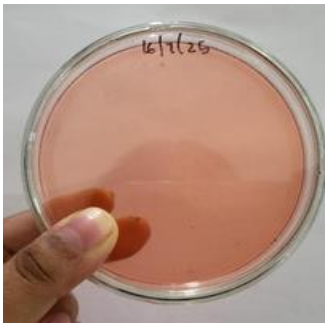
= 0.025; day 10: p = 0.021; day 15: p = 0.002), which indicates consistent sensitivity to copper stress.

Wild Isolate A

Wild Isolate B

Wild Isolate C

Observation: Standard Plate (Day 1)



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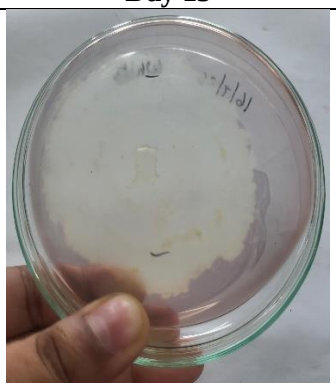
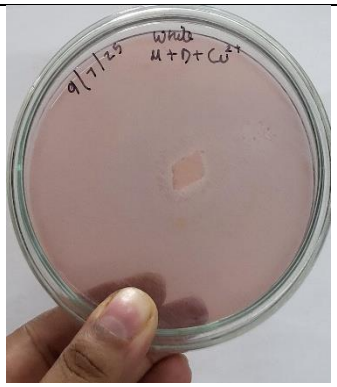
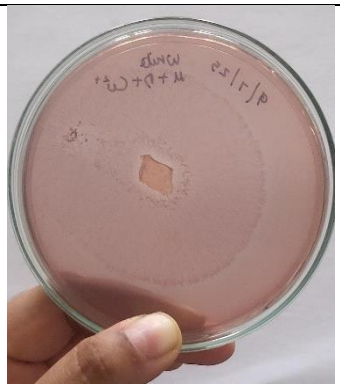
	Day 5	Day 10	Day 15
Congo Red+ Mycelium	 **3cm**	 **4.5cm**	 **6.3cm**
Cu^{2+} supplemented	 **1cm**	 **1.2 cm**	 **1.2cm**

Table 2.Decolorization zones of wild isolate A on Congo Red agar after 15 days.

Wild isolate B

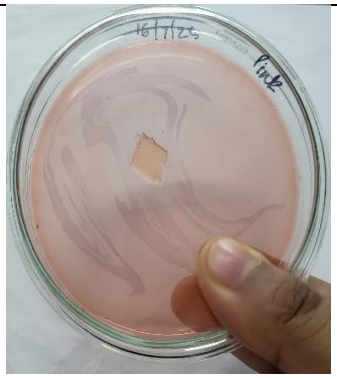
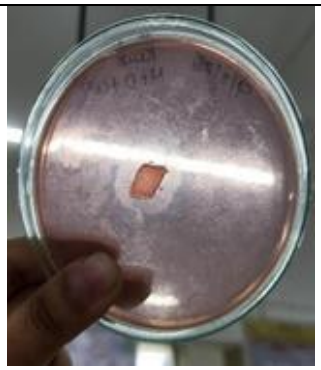
	Day 5	Day 10	Day 15
Congo Red+ Mycelium	 **2cm**	 **2.5cm**	 **3.5cm**
Cu^{2+} supplemented	 **2.5cm**	 **3cm restriction zone created**	 **No change in 3cm restriction zone**

Table 3. Decolorization zones of wild isolate B on Congo Red agar after 15 days.

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Wild isolate C

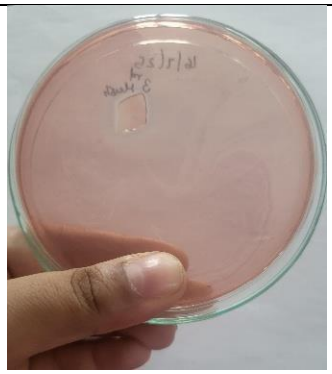
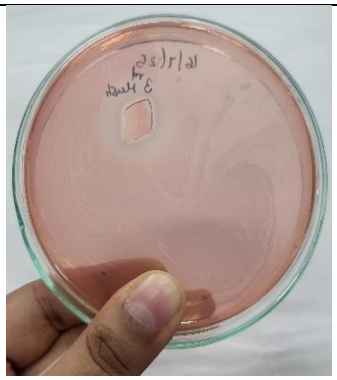
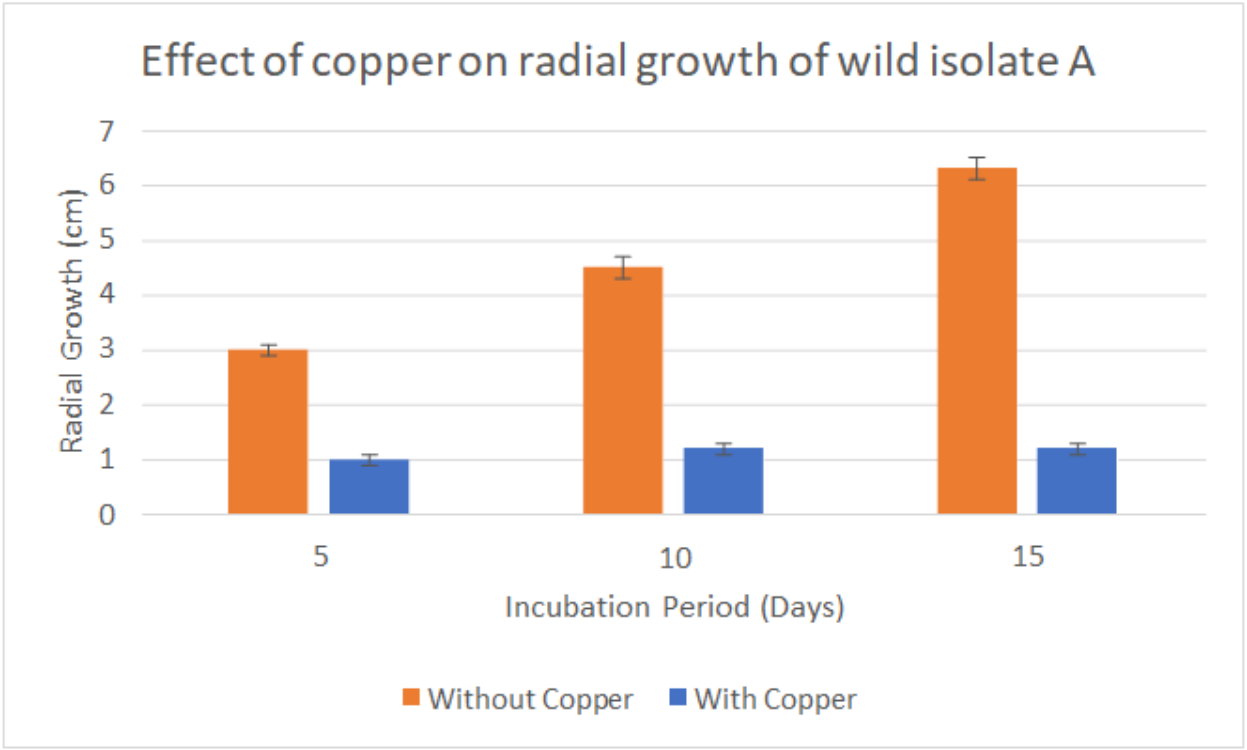
	Day 5	Day 10	Day 15
Congo Red+ Mycelium	 **1.3cm**	 **2.5cm**	 **3.6cm**
Cu^{2+} supplemented	 **1cm**	 **2.2cm**	 **3cm**

Table 4. Decolorization zones of wild isolate C on Congo Red agar after 15 days.

Error bars



Graph 1. Radial growth of wild isolate A under control and Cu^{2+}-supplemented conditions at 5, 10, and 15 days of incubation. Bars represent mean radial growth (cm) ± SD (n = 3).

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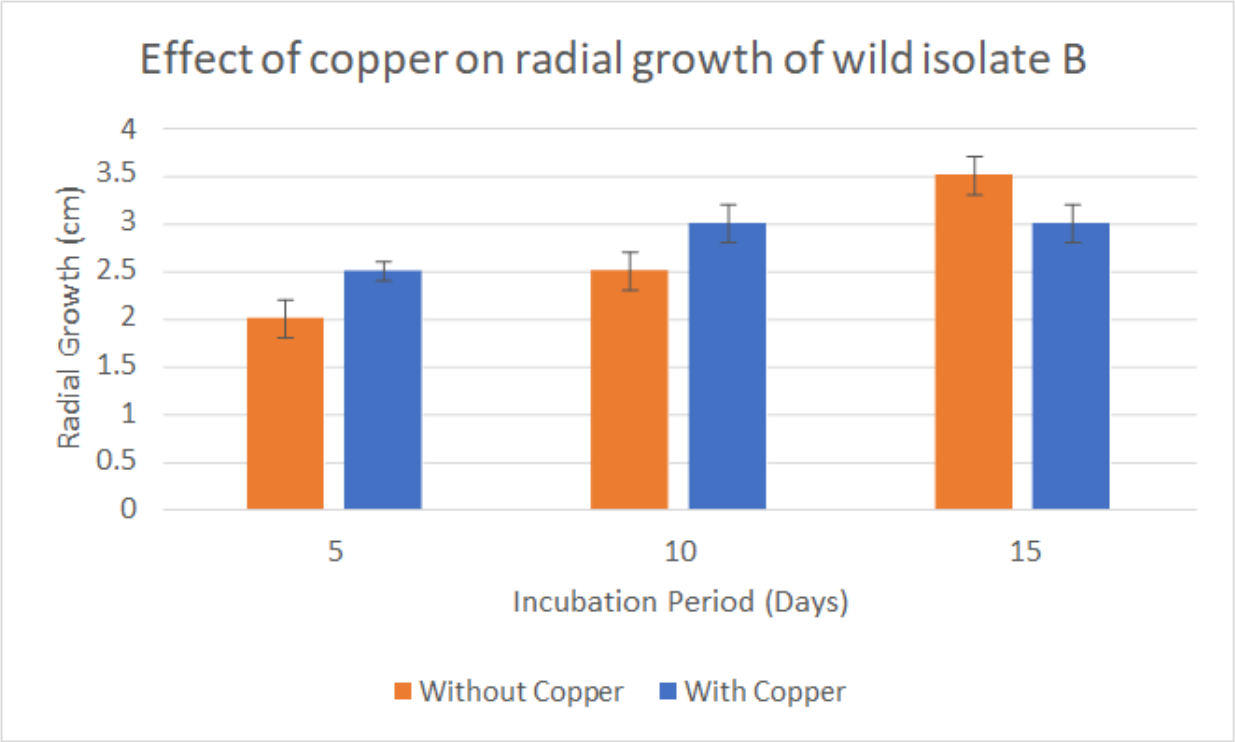
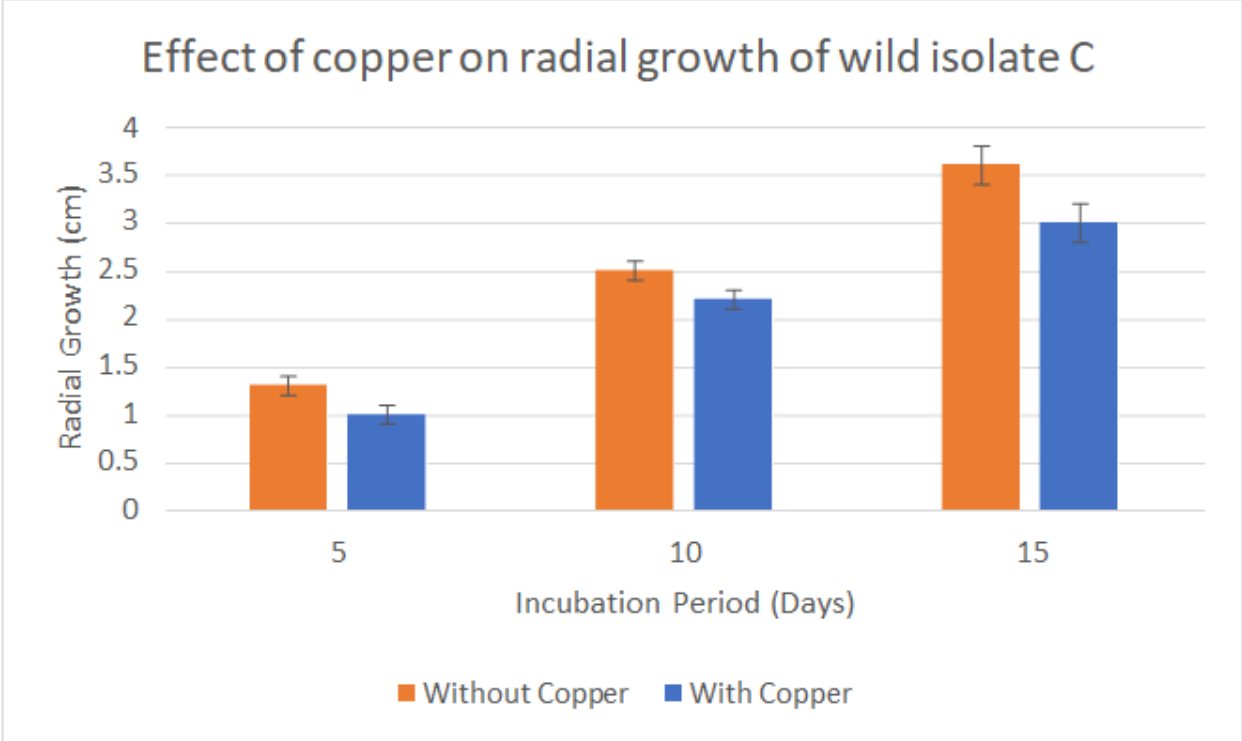


Graph 2. Radial growth of wild isolate B under control and Cu^{2+}-supplemented conditions at 5, 10, and 15 days of incubation. Bars represent mean radial growth (cm) ± SD (n = 3).



Graph 3. Radial growth of wild isolate C under control and Cu^{2+}-supplemented conditions at 5, 10, and 15 days of incubation. Bars represent mean radial growth (cm) ± SD (n = 3).

Although all three isolates showed progressive radial growth under normal conditions, the addition of 1.5 mM Cu^{2+} (~95 ppm) caused considerable growth inhibition, especially in wild isolate A, with only 1.2 cm growth (Table 1). This dramatic inhibition of growth indicates that high concentrations of copper caused massive reactive oxygen species (ROS) production and ergosterol synthesis inhibition, resulting in loss of hyphal membrane integrity and subsequent inhibition of extracellular enzyme secretion (Ibarra-Laclette et al., 2022; Robinson et al., 2021). Wild isolate B initially resisted the copper challenge (days 1-10, $p > 0.05$) through chelation/biosorption (Oliveira et al., 2023; Fu et al., 2025; Calvillo-Medina, 2021; Gundurao et al., 2022).

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Fungi have several overlapping tolerance mechanisms to adapt to heavy metal stress, such as biosorption, bioaccumulation, biotransformation, metal ion efflux, intracellular chelation, and activation of antioxidant defense mechanisms (Priyadarshini et al., 2021). The wild isolate C showed statistically significant growth inhibition at all time points, indicating immediate and constant sensitivity to Cu^{2+} exposure (Table 4). The early onset of inhibition indicates a quick intracellular stress response and a low adaptability to copper exposure. At the high concentration used in this study (~1.5 mM, ~95 ppm), copper is expected to interfere with ergosterol biosynthesis, stimulate reactive oxygen species production, and impact cell wall integrity, hence inhibiting hyphal growth and decolorization capacity (Ibarra-Laclette et al., 2022). In fungal biology, Cu^{2+} is a dual-purpose compound; it acts as a vital catalytic cofactor for laccase enzymatic activity at a high concentration (typically up to ~6 ppm), but changes to a strong metabolic inhibitor at high concentrations (Eichlerová & Baldrian, 2020; Robinson et al., 2021; Priyadarshini et al., 2021). The 1.5 mM (~95 ppm) concentration utilized in this study represents a 16-fold increase over stimulatory levels, thereby establishing a stress-testing environment that identifies the upper tolerance limits of these indigenous Mumbai strains (Fu et al., 2025; Calvillo-Medina, 2021; Gundurao et al., 2022).

5. CONCLUSION

These findings suggested that wood-rotting fungi are effective candidates for Congo Red degradation. The comparative analysis of three wild isolates showed that wild isolate A had the strongest natural potential for biodecoloration, with a decolorization diameter of 6.3 cm, significantly outperforming wild isolate B and wild isolate C. Although progressive growth was noticed in all controls, the presence of Cu^{2+} consistently inhibited radial growth, with wild isolate A being the most inhibited, as its growth was only 1.2 cm (Calvillo-Medina, 2021; Ibarra-Laclette et al., 2022). Notably, the wild isolate B exhibited a characteristic pattern of metallo-tolerance, suggesting that certain indigenous strains could have developed novel attributes to bind or sequester copper in their cell walls (Gundurao et al., 2022).The findings clearly suggest that the efficacy of fungal-based systems is highly dependent on the chemical environment. Decolorization activity was reduced at Cu^{2+} concentrations above ~6 ppm Cu^{2+}, which is consistent with earlier findings that high concentrations of copper are inhibitory to the process (Yusuf et al., 2023). The inhibitory effect may have been related to molecular disturbances, including the inhibition of ergosterol biosynthesis, the generation of reactive oxygen species (ROS), and direct damage to the fungal cell wall (Ibarra-Laclette et al., 2022). Therefore, effective bioremediation approaches must consider the particular heavy metal composition of industrial effluents in the local environment, as even highly effective strains such as wild isolate A could be less effective in the presence of environmental contaminants (Eichlerová et al., 2020; Rajhans et al., 2021).

The drawback of the current study is the lack of molecular identification of the fungal isolates. Though species-level identification would be more beneficial for the reproducibility and taxonomic resolution, the primary aim of the present study was to compare the decolorization capability of the wild fungal isolates in a plate-based system, which is a common approach in functional bioremediation research using indigenous wood-rotting fungi (Sudiana et al., 2022). Future studies should include ITS sequencing of the most effective isolates (wild isolates A, B) along with enzyme activity analysis (laccase/peroxidase activity) and wastewater consortium experiments. Future studies aimed at the deliberate manipulation of parameters such as pH, temperature, incubation time, and other natural accelerators could potentially increase the degradation rates and confirm their usability. Specifically, studies on the combined action of wood-rotting fungal enzymes such as laccase and manganese peroxidase and low-concentration metal mediators could provide more effective treatment methods (Fatima et al., 2025; Yu et al., 2023). Moreover, additional studies on the genomic diversity of the wild isolates could reveal more effective detoxifying pathways that are currently unused in commercial strains (Yu et al., 2023). In conclusion, this study places the wild fungal isolates as promising low-cost agents for large-scale wastewater treatment approaches when aided by environmental optimization. By filling the gap in comparative data regarding wild fungal isolates under heavy metal stress, this research provides a vital foundation for developing sustainable and circular bioeconomy solutions for textile industry pollution (Gugel et al., 2024; Yusuf et al., 2023).

6. ACKNOWLEDGEMENT

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7. ETHICAL STATEMENT

This study does not contain any studies with human or animal subjects performed by any of the authors.

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8. CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest in this work.

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