



Fungal-mediated Bioremediation of Textile Dyes Assesses the Decolorization Efficacy of *Penicillium singorense* and *Aspergillus ochraceus*

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

The textile sector significantly contributes to environmental pollution, mainly attributed to the release of dye effluents. These effluents contain toxic, recalcitrant compounds that adversely impact on aquatic ecosystems, soil integrity, and human well-being. This study intends to screen and investigate the possibilities of locally isolated river-derived fungi as agents for bioremediation of dye pollution and human health. Fungi obtained from the surface water of the Noyyal river in Tiruppur were evaluated for their capacity to decolorize synthetic dyes using the tube agar overlay technique. Of the 15 isolates tested, 2 isolates demonstrated complete dye decolourisation of 0.01% Congo red and Rose Bengal in two media (PDA and SMS). Results showed that the gene

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sequence analysis verified the identities of these dye decolorising fungi as *Penicillium singorense*. (NRS2), and *Aspergillus ochraceus*. (NRS15). Further assays in liquid media with varying dye concentrations (50 mg/l, 100 mg/l, 150 mg/l, and 200 mg/l) revealed that *Penicillium singorense* decolorized Congo Red by 71% (50 mg/l), 81% (100 mg/l), 92% (150 mg/l), and 91% (200 mg/l). Decolorization of Rose Bengal exceeded 95% across all concentrations. *Aspergillus ochraceus* decolorized Congo Red by 83% (50 mg/l), 89% (100 mg/l), 91% (150 mg/l), and 96% (200 mg/l), while Rose Bengal decolorization efficiency decreased with increasing concentration. Phytotoxicity studies using *Triticum aestivum* demonstrated reduced toxicity of dye decolourisation products compared to untreated dyes. These findings suggest the possible application of fungal isolate for sustainable bioremediation of textile dye wastewater.

Keywords: Textile dye effluent; fungal isolates; decolorization; detoxification; bioremediation; solid media; liquid media; fungal screening.

1. INTRODUCTION

Dyes are essential in multiple industries, including textiles, paint, nutrition, cosmetics, and paper and pulp industries. (Islam et al., 2023; Varjani et al.,). The rapid expansion of industrial activities and population growth necessitates developing novel dyes with diverse properties. Annually, hundreds of tons of synthetic dyes are manufactured worldwide to meet the requirements of various industries, particularly the textile sector (Selvaraj et al., 2021a). At present, water pollution due to the failure of textile industries to adequately manage their wastewater is a significant issue impacting globally. (Al-Tohamy et al., 2022a) Textile industries are among the most significant water consumers, utilizing approximately 100- 200 liters to process just 1 kilogram of fabric. (Jahan, N et al.,) However, nearly 60–65% of this water is discharged as effluent, often laden with numerous pollutants, including persistent organic compounds, heavy metals, salts, softeners, phenolic compounds, and, most notably, dyes (Selvaraj et al., 2021b; Varjani et al.,). An estimated 10 -15% of total dyes utilized in the dyeing procedure are released, contributing to severe water contamination and the discoloration of river systems (Berradi et al., 2019; Wang et al., 2022).

Dyes possess significant solubilizing properties in water, complicating their removal by conventional methods (Lellis et al., 2019). Due to their intricate and stable chemical structures, synthetic dyes exhibit high resistance to degradation. Their persistence in the environment leads to severe ecological and health concerns, as they are often toxic, mutagenic and carcinogenic (Al-Tohamy et al., 2022b; Ardila-Leal et al., 2021). Textile dye contains pigments that inflict artistic harm and

impede light transmission in water, resulting in reduced dissolved oxygen levels adversely impacting the aquatic organisms' photosynthesis rate and it can destroy the physiochemical quality of water bodies. (Ajaz et al., 2020; Alsukaibi, 2022; Lellis et al., 2019; Shi et al., 2021). These pigments can enter the food chain, disrupting food webs and ecosystem dynamics, thus posing significant risks to human and environmental health (Berradi et al., 2019). Water contamination from synthetic dyes has emerged as a significant concern for human health and presents major environmental risks. Due to rampant industrialization, the unregulated use of dyes has led to human exposure to these harmful substances. (Jankowska et al., 2022; Sarkar et al., 2022) Given these concerns, it is imperative to create effective and sustainable approaches for the treatment of dye-laden water.

Considering the constraints of traditional wastewater treatment methods, biological strategies have garnered significant interest as an effective and environmentally friendly option (Sen et al., 2016). Fungi exhibit notable potential for dye decolorization, attributed to their capacity to produce extracellular enzymes like lignin peroxidases, laccases, and manganese peroxidases are capable of degrading complex dye molecules (Buratti et al., 2022; Chatterjee et al., 2020). Filamentous fungi demonstrate significant biomass production and the ability to thrive in challenging environmental conditions. They are also essential in breaking down persistent pollutants, such as synthetic dyes and hydrocarbons (Chatterjee et al., 2020) In addition to bacterial cultures, various fungal species have been studied for their capacity to biodegrade azo dyes due to traits like high biomass production and rapid growth. Fungi exhibit a higher resistance to inhibitory substances compared to bacterial organisms. The fungal hyphal

development offers enhanced protection for their delicate organelles. The cell walls of fungi, an additional layer of polysaccharide matrix, shield them from harmful compounds through absorption. (Subasri et al., 2020) Bioremediation, particularly through fungal-associated degradation, removes colour and also breaks down intricate dye molecules into simpler, harmless components (Lopes et al., 2020).

Furthermore, fungi demonstrate a distinct benefit compared to bacterial bioremediation systems, as they possess the capability to withstand and break down intricate aromatic dye structures in both aerobic and anaerobic environments. White-rot fungi have demonstrated impressive effectiveness in breaking down a variety of structurally diverse dyes, positioning them as a key area of focus in wastewater treatment studies (Lellis et al., 2019). The ability of fungi to adapt their metabolism allows for the production of secondary metabolites that aid in degrading of dye contaminants, leading to the thorough mineralization of harmful compounds.

The objectives of the study are –

- To screen and select fungal isolates capable of decolorizing textile dyes in both solid and liquid media.
- To evaluate their detoxification potential.

2. MATERIALS AND METHODS

2.1 Samples Collection and Isolation of Fungi from Surface Water

The water samples were collected from 10 sample points in clean sterile bottles of 1 l capacity at a depth of 15-20 cm from the water surface. The samples were collected by grab sampling (Bhuyan et al., 2017). The samples were collected at different locations with a stretch of approximately 10km. The distance between each sampling location is taken as 1000m. A composite mixture of the 10 water samples taken from Noyyal River, Tiruppur, was thoroughly mixed. The composite mixture was filtered by Whatman 42, CAT No. 1442-110.1 ml of water sample was diluted in 9 ml of distilled water to create serial dilutions (10^{-1} to 10^{-5}) (Alsohaili et al., 2018; Kaiding et al., 2018).

1 mL from each dilution was cultured onto Potato dextrose agar (PDA) enriched with chloramphenicol. The petri dish was maintained at a temperature of 28 °C. in darkness. The

plates were monitored for 1 week. Pure fungal isolates were further sub-cultured based on their observed morphology.

2.2 Microbial Media and Inoculum Preparation

The Mineral Salt Medium (MSM) was prepared following the modified procedure outlined by (Yang et al., 2006), with the following concentrations (g/l): K_2HPO_4 – 1.5, KH_2PO_4 – 0.5, $MgSO_4 \cdot 7H_2O$ – 0.2, $CaCl_2$ – 0.05, $FeSO_4$ – 0.02. For a carbon source, 0.5 g/l of glucose is augmented and the pH was calibrated to 5 for fungal growth. MSM was utilised in solid form by incorporating 1.5% agar for preservation and as a liquid for evaluating the decolourisation capacity.

A suspension of inoculum was prepared using fresh cultures of selected fungal species. The inoculum was obtained by gently scraping the colony with a sterile loop in 5 ml of sterile distilled water; the vial with the isolate was mixed, and the inoculum size was adjusted to 1.0×10^6 spores.

2.3 Dye Selection

The following textile dyes were selected for decolorization and detoxification studies:

- Congo Red dye (azo dye) –Molecular formula: $C_{32}H_{22}N_6Na_2O_6S_2$, Molecular weight - 696.68 g mol⁻¹, here abbreviated as CR, dyecontent $\geq 95\%$ (high cert) and was purchased from S D FINE- CHEM Limited, India.
- Rose Bengal dye (xanthene dye) Molecular formula: $C_{20}H_4Cl_4I_4O_5$, Molecular weight–1017.64 g mol⁻¹here abbreviated as RB, dye content $\geq 75\%$ and was purchased from S D FINE- CHEM Limited, India.

The concentration of dyes was measured spectrophotometrically before and after the treatment to quantify decolorization.

2.4 Screening of Fungi for Dye Decolorization Activities on Solid Media

Fifteen isolated fungal isolates were evaluated for their capability to decolorise dyes utilising the tube overlay technique(Rani et al., 2014). Initially, fungal isolates were cultured on PDA

plates for four weeks at ambient temperature. After incubation, mycelial agar plugs (~5 mm²) were excised from the edge of the colony at a distance of 5 mm and seeded into test tubes (in triplicate) with 5 ml of PDA and 1 ml of PDA enriched with 0.01% (w/v) selected dyes. All experimental tubes were cultured in darkness at ambient temperature (~28 °C) and monitored every week for up to 4 weeks. The removal of the overlying dye signifies complete decolourisation (+++). The reduced intensity of the dye relative to the control evidenced partial dye decolourisation (++) . The control consists of uninoculated PDA topped with PDA and 0.01% dye. All fungal isolates were chosen based on complete or maximum (+++) decolourisation.

2.5 Fungal Identification

After the screening of fungal isolates on solid media, selected isolates were identified and proceeded with the other assays. The genomic DNA of strains NRS2 and NRS15 was obtained utilizing Nucleo Spin® Plant II Kit (Macherey-Nagel) DNA Isolation Kit. For NRS2, the internal transcribed spacers (ITS) region of genomic DNA was amplified with PCR using primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3'). The PCR was conducted with the amplification cycle as follows: denaturation at 95 for 5 minutes; then 40 cycles of 30 seconds at 95°C, 30 seconds at 58°C, 30 seconds at 72°C; and concluding with a final extension of 5 minutes at 72°C.

For NRS15, the LSU rRNA gene was amplified by utilizing universal primers LROR 5'-ACCCGC TGAAGTAAAGC-3' and LR7 5'-TACTACCA CCAAGATCT-3'. The program for the polymerase chain reaction (PCR) was set as: 1 cycle at 98°C for 30 seconds, followed by 40 cycles of 98°C for 5 seconds, 54°C for 10 seconds, and 72°C for 15 seconds, concluding with 60 seconds, a final extension at 72°C.

PCR product was sequenced by Rajiv Gandhi Centre for Biotechnology (RGCB), (Trivandrum, India).

Sequences were compared in the NCBI database GenBank. The phylogenetic analysis was conducted using MEGA 11.0

2.6 Dye Decolourisation Activities of Isolated Fungi in Liquid Medium

Dyes were incorporated into the 30 ml Mineral Salt liquid media in a 100 ml Erlenmeyer flask to specified concentrations of 50, 100, 150, and 200

mg/l. Each flask was added with 1 ml of the fungal inoculum and kept in the dark at 28 °C and 150 r.p.m, at a specific pH of 5. A flask containing the appropriate dye without any fungal inoculum served as a control. Triplicate cultures of each condition were prepared. Dye decolourisation (%) was measured in temporal sequence every alternate day, up to 8 days considering the day of inoculation as "0" day.

The isolated fungus's dye decolourisation was assessed by tracking the absorbance peak of each dye in the medium at its specific wavelength (490 nm for Congo red and 550 nm for Rose Bengal) with a UV-Vis spectrophotometer (Monica *et al.* 2024).

The decolourisation percentage was calculated using:

$$\text{Dye decolourisation (\%)} = \frac{A_c - A_s}{A_c} \times 100,$$

Where A_c indicates the absorption peak of dye in the control at time t , and A_s indicates the absorption peak of dye in the sample at time t . (Duygu & Ali Mazmanc, n.d.).

2.7 Effect of Shaking and Stationary Conditions

To assess the impact of static and shaking conditions on dye decolourisation %, the study was conducted under two varying conditions (0 rpm and 150 rpm, respectively) by incorporating dyes into the 30 mL Mineral Salt liquid medium within a 100 mL Erlenmeyer flask at concentrations of 50, 100, 150, and 200 mg/l. Inoculated with 1 ml of the fungal inoculum and cultured in the dark at 28 °C while maintaining other experimental conditions constant.

2.8 Fungal biomass Analysis

Throughout the sampling process, every culture was collected and centrifuged for 10 minutes at 6000 rpm to isolate the fungal biomass from the cultured media. Biomass was assessed bypassing the culture through pre-weighted Whatman No.1 filter paper. The mycelia were washed with distilled water and kept at 70°C in a hot air oven for 48 h and reweighted. Mycelium DW was calculated by

$$DW \text{ (mg/30ml)} = (W_c - W_i),$$

Where DW represents the total dry weight of biomass, W_c is the weight of the culture along with the filter paper, and W_i refers to the initial

weight of the filter paper. Results are presented in mg/30 mL of submerged culture.

2.9 Phytotoxicity Studies

Phytotoxicity assessments were conducted to assess the toxicity of the dyes prior to and following decolourisation. Triplicate sets of 10 sterilized seeds (*Triticum aestivum* L.) were utilized for every experiment. The wheat seeds underwent sterilisation with a 0.1% HgCl₂ solution for 50 seconds and were subsequently rinsed 6 to 7 times using distilled water to eliminate any residual HgCl₂. Petri plates contained sterile filter paper saturated with treated dye solutions at concentrations of 50, 100, 150 and 200 mg/l, untreated dye solution and filter paper soaked in sterile distilled water as controls. The experiment was carried out at an ambient temperature of 25 ± 1 °C. The percentage of seed germination, relative root growth, and shoot growth were recorded for seeds included in untreated, treated, and control (distilled water) samples.

The rate of germination was determined in the following way:

$$GR = G_s/G_c \times 100$$

Where G_s and G_c represent the number of seeds germinated in the sample and control groups, respectively.

The germination index (GI) was determined as follows:

$$GI = L_a/L_c \times GP$$

GP signifies the percentage of seeds germinated relative to control values, L_a represents the average length of the root in the dye solutions, and L_c symbolizes the average length of the root in the control group (Nouren & Bhatti, 2015).

2.10 Statistical Analysis

Every experiment was conducted in triplicate. The data underwent statistical analysis through Analysis of Variance (ANOVA), succeeded by post-hoc Tukey's test for multiple comparisons.

3. RESULTS AND DISCUSSIONS

3.1 Fungal Morphology, Growth and Screening of Fungal Isolates for Decolorization in Solid Media

Fifteen fungal isolates were subculture and observed according to their colour, traits, margin and Colony size % (Table 1). The fungal isolates

exhibited diverse colony morphology and growth rates. NRS6 exhibited the highest growth percentage (96.48%), while NRS9 had the lowest (45.37 %). In solid media, among the 15 tested isolates grown in two different culture media with 0.01% Congo red and Rose Bengal, NRS15 demonstrated complete decolorization (++++) for both dyes under both conditions, making it a strong candidate for bioremediation (Table 2). NRS2 also showed strong performance, particularly with Rose Bengal. Thus, for Screening in liquid media after 4 weeks, NRS2 and NRS15 were selected for identification. For all the fungal strains, growth primarily started on the fourth day of incubation.

3.2 Identification of Selected Fungal Isolate

Multiple isolates were evaluated for their dye decolorizing capacity, with isolates NRS2 and NRS15 exhibiting high decolorizing ability. As a result, isolates NRS2 and NRS15 were chosen for additional research. The ITS and LSU analysis revealed that strain NRS2 was categorized as *Penicillium singorense*, whereas NRS15 was classified as *Aspergillus ochraceus*. Identification was done by analysing the gene sequence data and the types of isolates present in the GenBank databases through the BLASTn sequence matching methods. MUSCLE program was employed for sequence alignment and assessed using the molecular evolutionary genetics analysis software (MEGA11). (Kumar et al., 2004; Tamura et al., 2011). For isolate NRS2, the phylogeny construction was carried out using the neighbour-joining method based on evolutionary distances obtained from sequence-pair similar characteristics, implemented in MEGA, using Tamura-3 parameter model with bootstrap numbers computed from 1000 replicates in the MEGA software (Fig. 1a). After molecular identification, the sequence was uploaded to GenBank under accession number PV000959.

For the NRS15 sample, phylogenetic reconstruction was performed utilizing the neighbour-joining method for evolutionary distances based on sequence-pair similarities, as executed in MEGA, employing the Kimura-2 parameter model with bootstrap values computed from 1000 replicate runs in the MEGA software (Fig. 1b). Following molecular identification, the sequence was uploaded to GenBank with the accession number PV172353.

Table 1. Morphology and colony size of different fungal isolates

Isolate code	Colour Surface	Characteristics	Edge	Colony size%
NRS 1	Brown	powdery	Irregular	67.03704
NRS 2	White turn grey	Velvety	White, circular	90.92593
NRS 3	white	cottony	grey circular	51.40741
NRS 4	White with little green	velvety	circular	67.22222
NRS 5	Black	Velvety	circular	93.51852
NRS 6	Grey	cottony	circular	96.48148
NRS 7	White	cottony	white circular	55.55556
NRS 8	White	smooth	irregular	78.7037
NRS 9	Black	Granular	irregular	45.37037
NRS 10	White	velvety	circular	72.48148
NRS 11	Black	Velvety	circular	78.51852
NRS 12	Mint green	velvety	circular	70.37037
NRS 13	Black	powdery	White, Irregular	52.03704
NRS 14	white	cottony	circular	48.74074
NRS 15	Brown	Granular	Irregular	84.81481

Table 2. Dye decolourisation activity of fungal isolates on different media (PDA and SMS) using two dyes, Congo red and Rose Bengal

Fungal Isolates	Dye decolourisation			
	Congo red		Rose Bengal	
	PDA	SMS	PDA	SMS
NRS1	+++	++	-	-
NRS2	++	+++	+++	+++
NRS3	-	-	-	++
NRS4	-	-	-	-
NRS5	-	-	-	-
NRS6	++	+++	-	-
NRS7	++	++	-	-
NRS8	-	-	-	-
NRS9	+++	-	-	-
NRS10	-	-	-	-
NRS11	++	-	-	-
NRS12	+++	-	++	-
NRS13	-	++	-	-
NRS14	-	-	-	-
NRS15	+++	+++	+++	+++

3.3 Screening of Fungal Isolates for Decolorization in Liquid Media

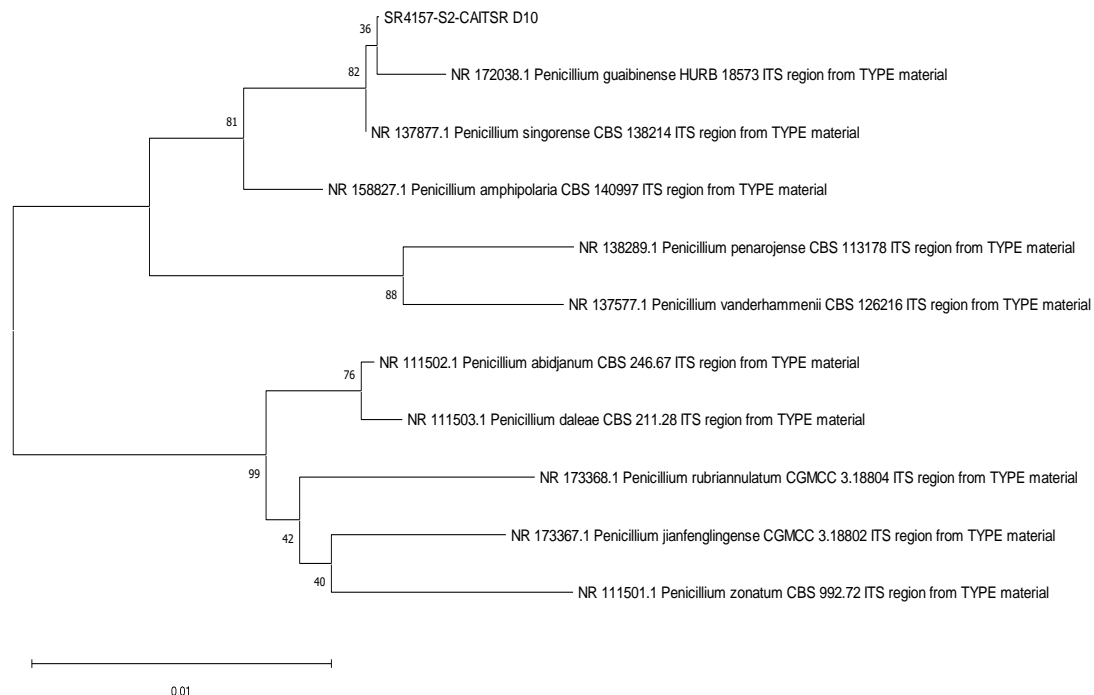
In the experiment of dye decolourisation in textiles dyes, wavelength within the visible light spectrum was chosen for Congo red, with maximum absorption noted at 490 nm, linked to the azo double bond and extensively conjugated system present in the entire dye molecule (Asses et al., 2018), the absorbance peak of Rose Bengal dye, i.e. 550nm, indicates the conjugation of aromatic rings with the basic xanthene group (Alshammari et al., 2024). Chromophores associated with these chemical groups determine the hue of these molecules. Numerous microorganisms are utilized to

eliminate such substances that results from industrial discharges. Certain fungi are under investigation for such purposes. The capacity of fungi for the bioremediation of various substances like industrial slurry, diesel, and herbicides has been shown, and concerning textile industry contaminants, microorganisms, particularly fungi, are being researched as biodegrading/ bio remediating agents (Bulla et al., 2017). The findings regarding the decolouration capabilities of *Penicillium singorense* and *Aspergillus ochraceus* indicate that both were initially successful in decolourisation of Congo red and Red Bengal dyes (Fig. 2), and confirming this effectiveness over a period, it was noted that *Penicillium*

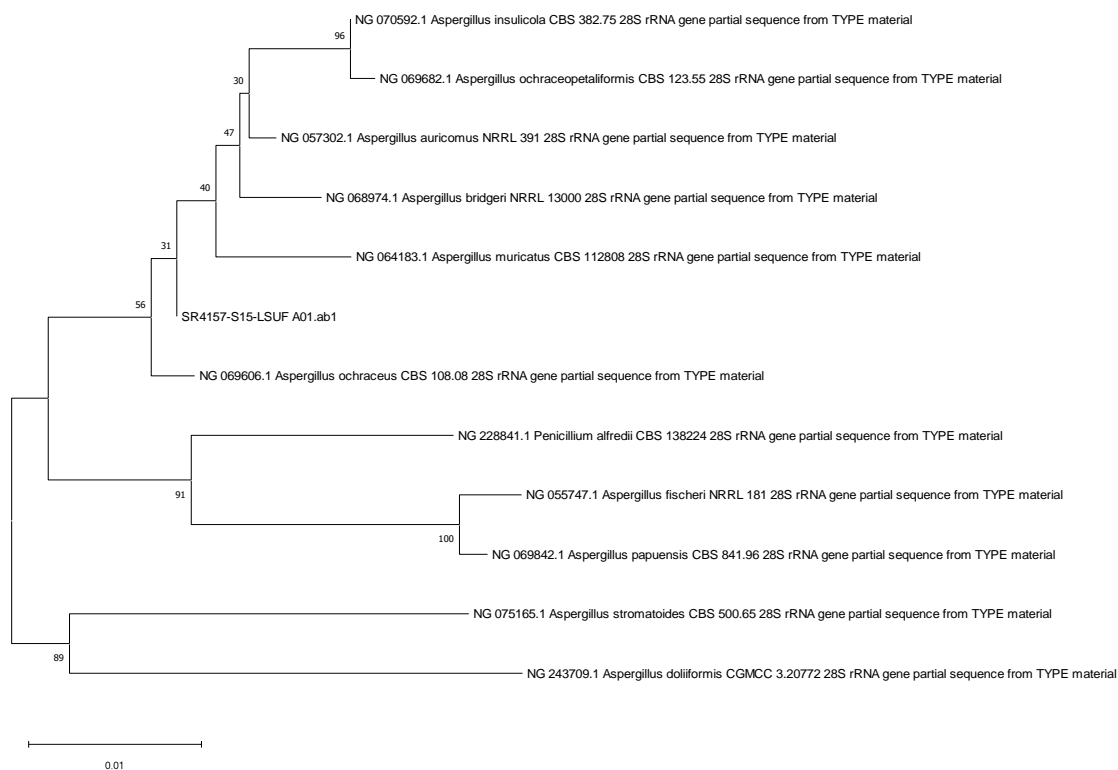
singorensis decolourised 92% of 50mg/l, 91% of 100mg/l, 81% of 150mg/l and 71% of 200mg/l of Congo red after 8th day (Fig. 2 a). Whereas for Rose Bengal dye, the decolourization stabilized after 6th day, decolourizing more than 95% of the different concentrations. (Fig. 2b). Treatment with *Aspergillus ochraceus*, the dye decolorization percentage of Congo red after 8 days resulted in a reduction of 83% of 50 mg/l, 89% of 100mg/l, 96% of 150 mg/l, and 63% of 200mg/l concentrations (Fig. 3a). Thus, it is observed that there is an increase in decolorization percentage as we increase the dye concentration (50 – 150 mg/l) and reduce at 200 mg/l, this may be attributed to the potential formation of by-products during the remediation process or other extracellular substances that can absorb light in the visible spectrum employed to monitor the decolourisation of the dye.

The composition and concentration of dyes significantly affect their efficiency in decolorisation and decolourisation. (Rajhans et al., 2021) Whereas for Rose Bengal dye the dye decolorization percentage is 93% of 50mg/l, 67% of 100mg/l, 67% of 150mg/l and 43% of 200mg/l, a noticeable reduction in decolorization efficiency was observed as we increased the concentration (Fig. 3b). This decline could be attributed to substrate inhibition, where higher dye

concentrations interfere with enzymatic function or exert toxic effects on microbial cells, thereby limiting their decolourisation potential. Enzymes released by dye-degrading fungi may fail to identify low dye concentrations. Conversely, a strong dye concentration is harmful to fungi and may hinder dye decolorisation by obstructing the enzyme's active sites. Likewise, it is straightforward to remove colour from dyes that have a low molecular weight and simple structure. Dyes that possess intricate structures and a higher molecular weight, on the other hand, exhibit a low rate of decolourisation (Kumar Sani & Chand Banerjee,). The elevated concentration of dye hampers dye decolourisation and, or decolorisation (Liu et al., 2017). Furthermore, comparative analysis of microbial performance revealed that samples treated by *Aspergillus ochraceus* consistently achieved higher decolorization percentages than samples treated by *Penicillium singorensis*, across all concentrations. This suggests that *Aspergillus ochraceus* possesses superior adaptability to the dye or an enhanced metabolic capacity for breaking down the dye molecules. The graph suggests that the percentage of decolourisation increased as the incubation period lengthened, though the rise in the final stages was somewhat lower than that in the initial stages.



(a)



(b)

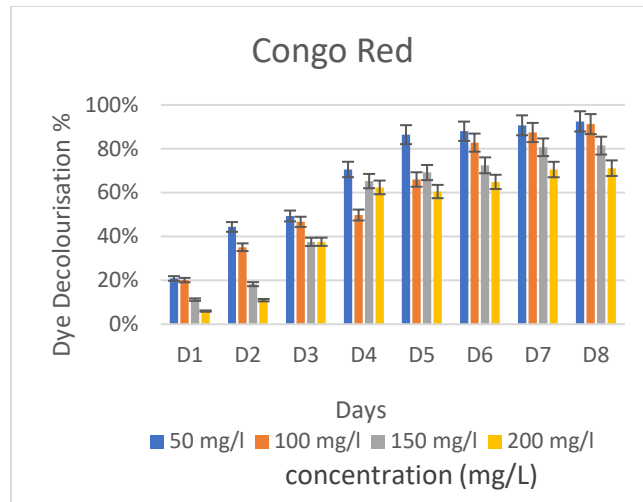
Fig. 1. Phylogenetic tree constructed through the neighbour joining method using MEGA 11. (a) The phylogenetic tree of NRS2 identified as *Penicillium singorense* and (b) the phylogenetic tree of NRS15 identified as *Aspergillus ochraceus*

3.4 Effect of Agitation and Stationary Conditions

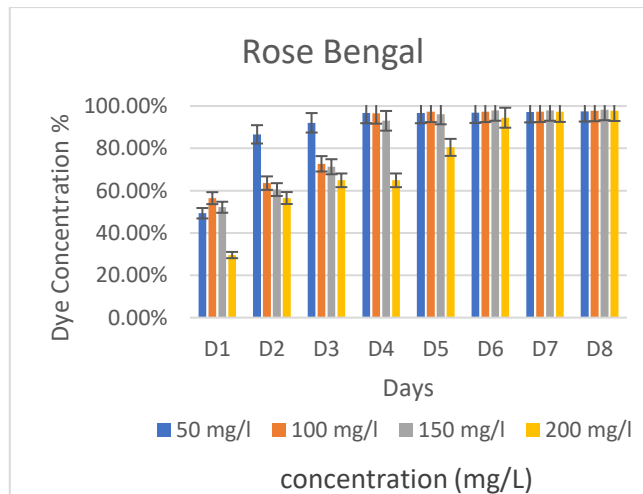
Comparatively, in figure we observed that agitated condition was more effective in decolorizing both Congo red and Rose Bengal dyes treated by the two fungal isolate, than in static condition (Fig. 4). Under stationary conditions, Congo red treated by *Penicillium singorense* show highest decolorization in 50 ppm concentration (50.20%) followed by 100ppm (49.89%), 150 ppm (44.30%), 200 ppm (36.04%) and Rose Bengal treated by penicillium sp, it was also observed in 50ppm concentration (61.71%), 100ppm (59.74%), 150 ppm (56.69%), 200 ppm (46.57%). Whereas, in Congo red treated by *Aspergillus ochraceus* how highest decolorization in 100 ppm concentration (47.34%) followed by 50ppm (47.07%), 150 ppm (42.95%), 200 ppm (20.22%) and Rose Bengal treated by *Aspergillus* sp, it was also observed in 50ppm concentration (41.48%), 100ppm (31.10%), 150 ppm (26.97%), 200 ppm (15.08%).

The authors state that the higher decolorization observed under agitated conditions compared to static ones primarily hinges on oxidative reactions facilitated by essential enzymes like LiP and MnP (Shedbalkar et al., 2008; Zhuo et al., 2011). Additionally, it has been noted that agitation enhances for faster and complete adsorption and decolorization of Crystal Violet (CV) and Methyl Violet (MV) by *Corioloopsis* sp., as well as Cotton Blue (CB) by *Penicillium simplicissimum* KP713758 or *Corioloopsis* sp. in contrast to static conditions (Chen et al., 2019; Ting et al., 2016). It has also been indicated that this might be attributed to improved oxygenation of the fungus and continuous interaction of released enzymes with dye molecules for decolorization; furthermore, agitation promotes fungal growth (Rani et al., 2014). Nevertheless, in a different scenario, the decolorization process may not need oxygen and is likely facilitated by reductive reactions involving a distinct group of enzymes. We hypothesized that the difference in decolourisation between stationary and agitation conditions appeared to

be linked to the fungal isolates. In summary, our results indicated that decolourisation could be more efficient when maintained under agitated conditions.

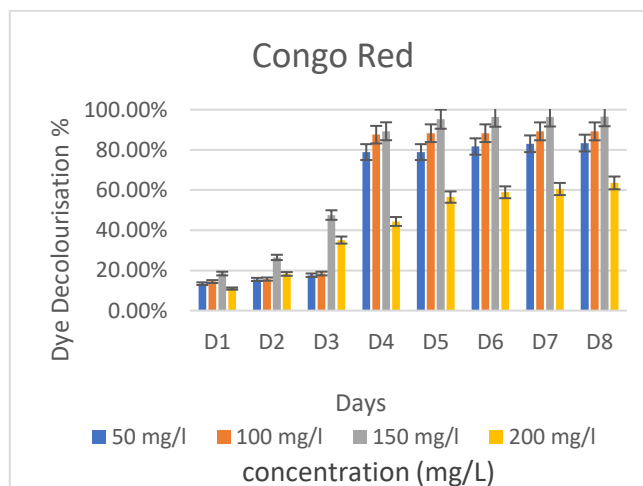


(a)



(b)

Fig. 2. Dye Decolourisation % over eight days of different dye concentration for (a)Congo Red and (b)RoseBengal Dye treated by *Penicillium singorense*



(a)

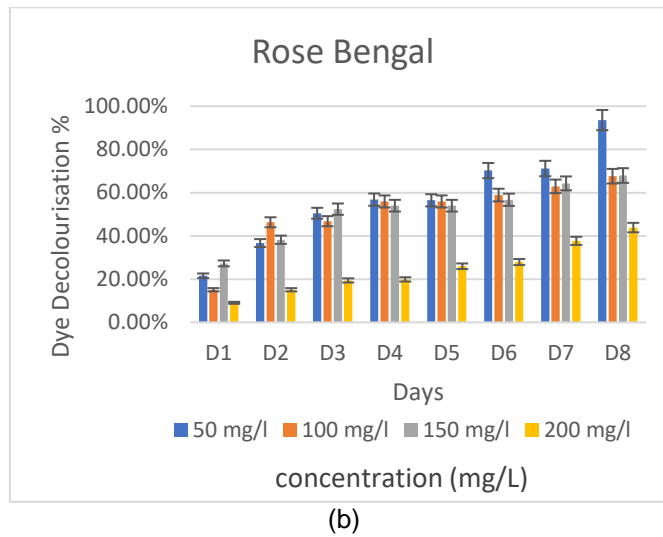


Fig. 3. Dye Decolourisation % over eight days of different dye concentration for(a)Congo Red and (b)RoseBengal Dye treated by *Aspergillus ochraceus*

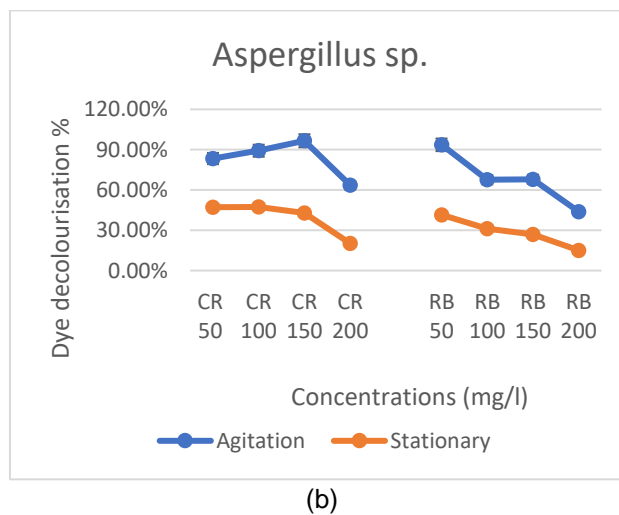
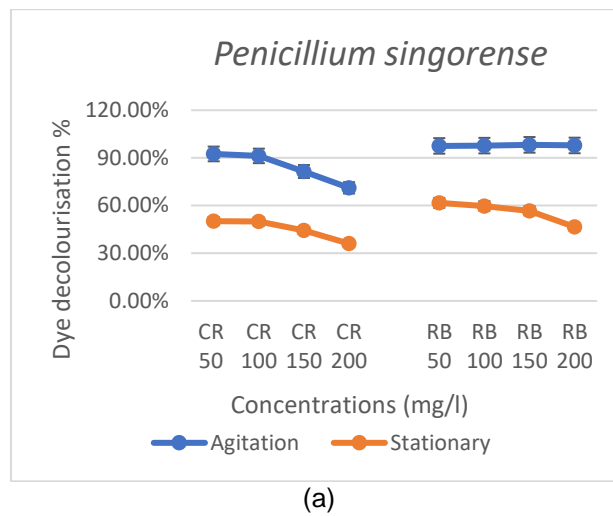
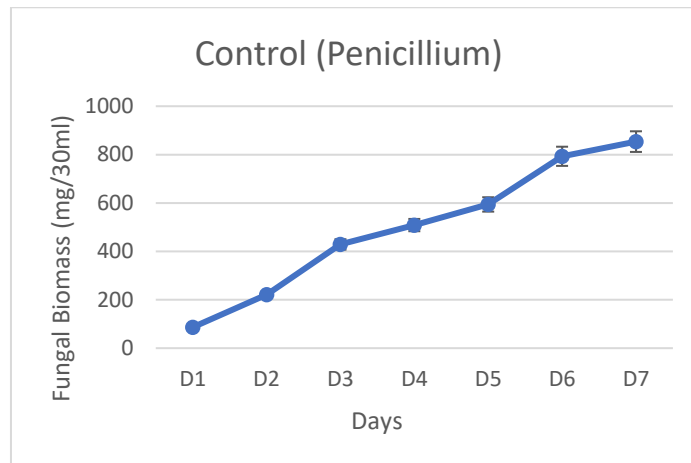
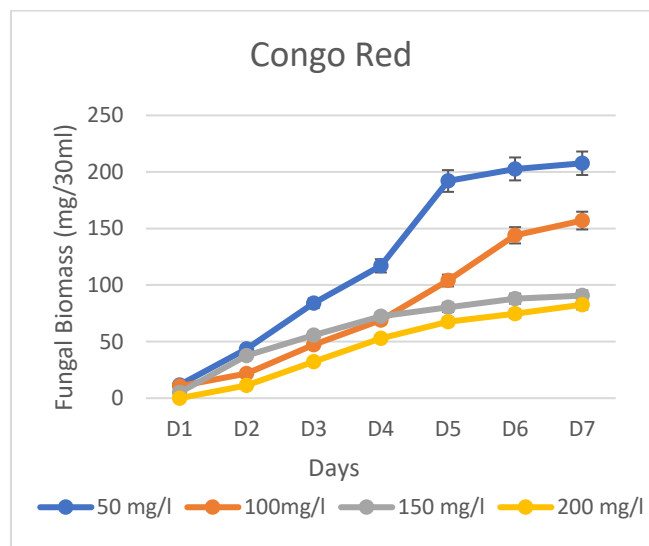


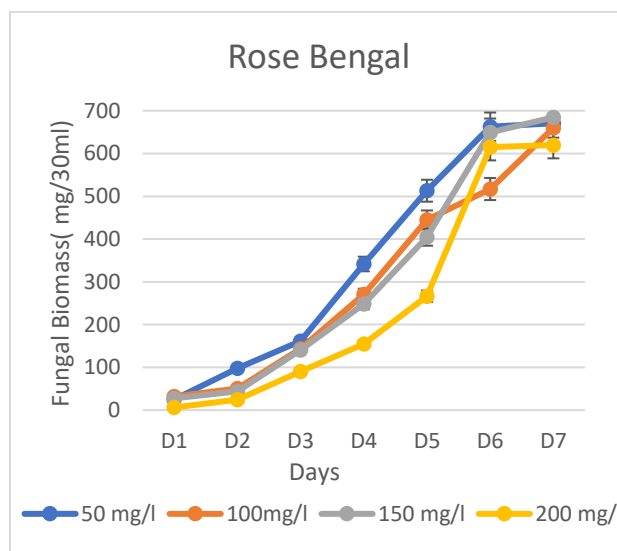
Fig. 4. Comparison of Dye Decolourisation % for Agitation and Stationary of different dye concentration for(a) Congo Red and Rose Bengal Dye treated by *Penicillium singorense* (b) Congo Red and Rose Bengal Dye treated by *Aspergillus ochraceus*



(a)

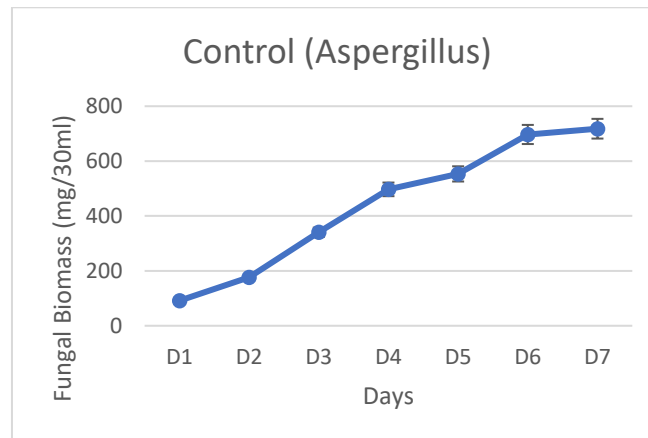


(b)

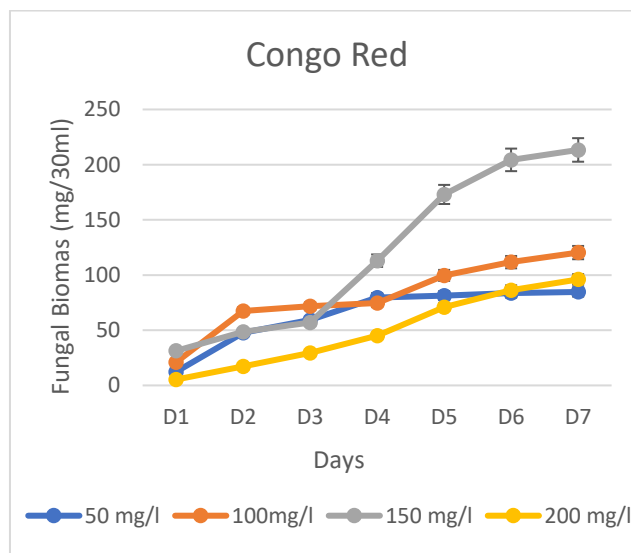


(c)

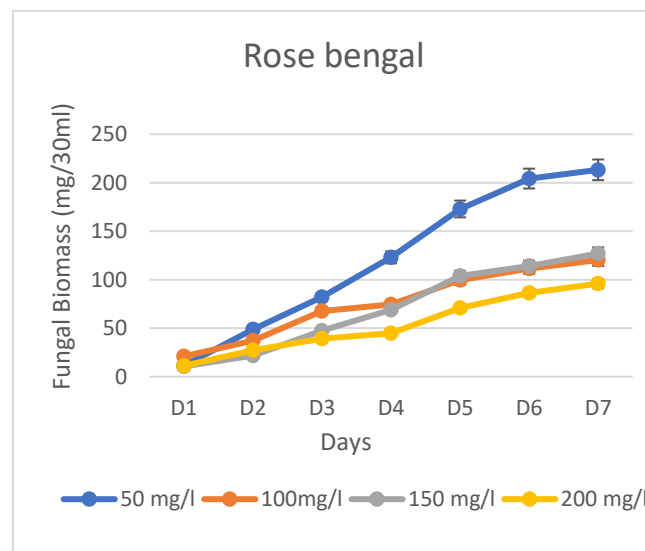
Fig. 5. Fungal biomass growth over seven days (a)Control, with increasing dye concentration for both (b)Congo Red and (c) Rose Bengal dye treated by *Penicillium singorensis*



(a)



(b)



(c)

Fig. 6. Fungal biomass growth over seven days (a)Control with increasing dye concentration for both (b) Congo Red and (c) Rose Bengal dye treated by *Aspergillus ochraceus*

3.5 Fungal Biomass Analysis

Microorganisms can serve as delicate systems for evaluating the toxicity of specific pollutants. Biomass significantly contributes to the decolorization of dyes by augmenting the decolourisation process through enhanced enzyme activity. Cell biomass has been proposed as an efficient approach for colour elimination via biosorption. The toxicity of the dyes (CR and RB) incorporated into the decolorization media was assessed by their suppressive impacts on isolates biomass growth. After 7 days, the dry weights of the isolates in a medium without dye (control) were higher compared to those in dye-containing media, indicating that the dyes inhibit isolate's growth (Fig. 5a and 6a) In the present study (Fig. 5b and 6b) Congo red was tested for 7 days to determine the ability of *Penicillium singorense* and *Aspergillus ochraceus* to decolorise the dye. One ml of fungal inoculum was taken and incubated simultaneously in an aqueous solution containing varying concentrations of Congo red (50-250 mg/l). Weight analysis for Congo red treated by *Penicillium singorense* showed that fungal growth was inhibited based on the dye concentration, with higher concentrations (rising from 50 to 250 mg/l) leading to greater toxicity against fungal biomass (Fig. 5a).

Previously, research conducted by (Young & Yu, 1997) indicated that the overall pattern within the fungal system shows that increased dye concentrations lead to reduced growth rates and a prolonged duration for decolourisation. Similar findings were noted when *Penicillium singorense* was tested for Rose Bengal decolorization at different concentrations starting from 200 mg/l, which achieved over 95% decolorization after 6 days of incubation. In contrast, the dyes at concentrations between 50 and 150 mg/l were decolorized after 3 to 4 days of incubation, and this process stabilizes (Fig. 5b). Similarly, in work done by (Tehrani et al., 2014) their study shows the decolorization rate stayed roughly stable while the growth of the fungus steadily rose.

In case of Congo red treated by *Aspergillus ochraceus*, a peculiar pattern of fungal growth was observed where the fungal growth increases with the dye concentration (50-150mg/l) and is drastically reduced at 200 mg/l (Fig. 6a). Where as in Rose Bengal dye treated with *Aspergillus ochraceus*, high fungal growth was observed in lower concentration (50 mg/l) in contrast to a high dye concentration (200 mg/l), indicate the dye's structural instability or toxicity, leading to

growth inhibition, as seen in the current study (Fig. 6b) While the dyes restricted the growth of the fungal isolates, they did not fully prevent growth or decolorization (Apoohan & Yesilada, 2005) also demonstrated that textile dyes Astrazon Blue and Red suppress the growth of *Funaliatrogii* and *Staphylococcus aureus* on solid media in a concentration-dependent manner. The effectiveness of the decolorization methods relies on the fungus's ability to adjust to the textile dyes present in the effluent (Assadia & Jahangirib, 2001). The screening experiment found that the best decolorization occurred with fungal isolates that exhibited significant growth in the effluent. This discovery indicates that growth and mycelia formation significantly influence decolorization and suggests that the decolorization process in these fungi relies more on absorption than on enzyme production. However, (Fu & Viraraghavan, 2001) proposed that a biosorption mechanism could also significantly contribute to the decolorization of dyes by living fungi, alongside biodegradation. Also, in previous study reported by (Tehrani et al., 2014) demonstrated that the decolorization mechanism of the fungus tested occurs through both surface and internal cell absorption.

3.6 Phytotoxicity Studies

A study on phytotoxicity was carried out to assess the toxicity of Rose Bengal and Congo Red (at 50, 100, 150, and 200 mg/l) to *Triticum aestivum* seeds. As demonstrated in Table 3, seeds treated with distilled water and treated dyes by *Penicillium singorense* exhibited a greater germination rate than those using the original dye. Seeds that were treated with Congo red exhibited an average shoot length of 2.9 ± 0.45 cm and an average root length of 0.35 ± 0.2 cm. The shoot and root lengths of dyed seeds were, ranges from 6- 4.08 cm and 4.14 -2.1 cm as we increase the concentration. (50 – 200 mg/l) respectively.

Seeds treated with Rose Bengal showed an average shoot and root length of 3.13 ± 0.3 cm and 0.75 ± 0.28 . Seeds that were treated with dye had shoot and root lengths ranges from 6.22 - 4.27 cm, and 2.79 - 1.31 cm as we increase the concentration. (50 – 200 mg/l) respectively.

Moreover, the germination index (GI) of Congo red was 7.14%, and after treatment at different concentrations, it was 84.4%, 63.26%, 55%, and 42.85%. The germination index (GI) of Rose Bengal was 15.3%, and after treatment at different concentrations, they were 56.93%, 46.93%, 38.77%, and 26.73%.

Table 3. Phytotoxicity of the congo red and rose bengal treated by *Penicillium singorense*

Parameters	Distilled Water	Congo Red dye	Concentration				Rose Bengal Dye	Concentration			
			50	100	150	200		50	100	150	200
GR%	100	20	80	60	33	20	13.3	83	80	56	40
Shoot length (cm)	6.3 ± 0.06	2.9 ± 0.45	6.00±0.24	5.87 ± 0.17	4.67 ± 0.2	4.08 ± 0.69	3.13 ± 0.3	6.22 ±0.16	6.89 ± 0.1	4.38 ± 0.24	4.27 ± 0.03
Root length(cm)	4.9 ± 0.9	0.35 ± 0.2	4.14 ± 0.06	3.1 ± 0.2	2.7 ± 0.01	2.1± 0.04	0.75 ± 0.28	2.79± 0.06	2.3 ± 0.5	1.9 ± 0.27	1.31 ± 0.03
GI%		7.14	84.40	63.26	55	42.85	15.3	56.93	46.93	38.77	26.73

Table 4. Phytotoxicity of the congo red and rose bengal treated by *Aspergillus ochraceus*.

Parameters	Distilled Water	Congo Red dye	50	100	150	200	Rose Bengal Dye	50	100	150	200
GR%	100	20	70	40	30	30	13.3	80	70	53	40
shoot length (cm)	6.3 ± 0.06	2.9 ± 0.45	5.19 ± 0.07	4.57 ± 0.13	4.48 ± 0.14	3.87 ± 0.11	3.13 ± 0.3	4.90 ±0.20	5.01 ± 0.17	4.35 ± 0.1	3.9 ± 0.19
Root length(cm)	4.9 ± 0.9	0.35 ± 0.2	0.9 ± 0.07	1.21 ± 0.07	1.37 ± 0.01	0.11 ± 0.06	0.75 ± 0.28	3.1 ± 0.07	2.26 ± 0.06	2.18 ± 0.03	1.25 ± 0.07
GI%		7.14	18.36	24.69	27.90	2.24	15.3	63.26	46.12	44.48	25.50

As indicated in Table 4, seeds treated with distilled water and treated dyes by *Aspergillus ochraceus* exhibit a greater germination rate than those with the untreated dye. Seeds exposed to Congo red dye exhibited an average shoot and root length of 2.9 ± 0.45 cm and 0.35 ± 0.2 . Nevertheless, the average lengths of shoots and roots for seeds subjected to treated dye ranges from 5.19 - 3.87 cm, and 0.9 - 0.11 cm as we increase the concentration. (50 – 200 mg/l) respectively.

Seeds treated with Rose Bengal showed an average shoot and root length of 3.13 ± 0.3 cm and 0.75 ± 0.28 . Nonetheless, the average lengths of shoots and roots of seeds subjected to the treated dye ranges from 4.90- 3.9 cm, and 3.1 - 1.25 cm as we increase the concentration. (50 – 200 mg/l) respectively.

Moreover, the germination index (GI) of Congo red was 7.14%, and after treatment at different concentrations, they were 18.36%, 24.69%, 27.90%, and 2.24%. The germination index (GI) of Rose Bengal was 15.3%, and after treatment at different concentrations, it was 63.26%, 46.12%, 44.48%, and 25.50%. Shazia Nouren indicates that GI values under 50% reflect high phytotoxicity, values from 50–80% suggest moderate phytotoxicity and values beyond 80% denote nontoxicity (Nouren & Bhatti, 2015b).

The incredibly low GI percentages in the results show that untreated dyes (CR and RB) significantly inhibit root elongation and seed germination. This suggests that the original dye solutions are highly toxic to plant development. However, after treatment by *Penicillium singorense* (CR and RB), there is a noticeable improvement in germination, especially at lower concentrations (50 and 100 mg/l). This implies that partial decolorisation reduces toxicity, allowing better seed development. After treatment with fungal *Aspergillus ochraceus* in Rose Bengal, it shows a similar trend, but in Congo red, the germination index rises with higher concentration, peaking at 150 mg/l, and then sharply declines at 200 mg/l. While the dyes significantly hampered the development of the shoots from the seeds, the seeds treated with fungal isolate displayed a growth pattern similar to the control. A similar study by (Thakor et al., 2022) illustrates that the filtrate obtained following the degradation process by *Penicillium oxalicum* and *Aspergillus tubingensis* was less harmful to plant growth than the Congo red dye.

4. CONCLUSION

The removal of dyes was investigated under particular conditions; positive results emerged after the second or third day, but the highest level of decolorization for all dyes occurred after a week. In this research, we saw that both *Penicillium singorense*. and *Aspergillus ochraceus*. were better at decolorizing at lower concentrations (50 mg/l) compared to high concentrations (200 mg/l). This might be because the fungus was getting more oxygen and its enzymes were coming into regular contact with the dye molecules to degrade them down, or it could be because the fungus was growing better. The colour may have disappeared because the chromophore in the dye molecule is being broken down by enzymes made outside of cells by fungi, as well as absorption and adsorption. The dye molecules adsorption onto the cell surface occurs rapidly. Dyes with varying structures are decolorized at distinct intrinsic enzymatic rates, and as we elevate the concentration, typically leads to a reduced rate of decolourisation. The results suggest that operational parameters such as agitation and fungal growth can affect decolouration rates. Many studies focus only on well-known fungal species like *Trametes versicolor*, *Pleurotus ostreatus*, *Aspergillus niger* and *Phanerochaete chrysosporium*, while underexplored strains from the regional areas have not been explored and is needed for novelty. Mainly, dye decolourisation focuses on azo dyes like Congo red; in this study along with an azo dye, a xanthene dyes has been studied with a more complex structure. While dye decolorization is reported, many studies fail to assess the toxicity of intermediate metabolites formed during degradation. Some degradation products may be more toxic than the parent dye, yet few studies conduct ecotoxicological evaluations.

Bioremediation has been described as a soft technology because of the eco-friendly methods it employs. Its cost-effectiveness and minimal environmental disruption make this technology a highly appealing alternative method. Molecular techniques will help scientists find and study new fungal isolates, which will make the practical uses of fungi even better. Fungal remediation is poised to become a dependable and competitive method for dye remediation.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models

(ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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