

Synthetic dyes decolorization and bioremediation by indigenous fungi isolated from dye-effluent contaminated environments

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Biodegradation and detoxification of dyes, Methylene blue (MB), Bromophenol blue (BB), Crystal violet (CV) and Orange G (OG) have been carried out using two fungal isolates *Aspergillus niger*, and *Phanerochaete chrysosporium*, isolated from dye contaminated soils. Different approaches were utilized to study biodegradation, viz. agar overlay and broth media means; static and shake conditions at 25°C. *Aspergillus niger* recorded maximum decolorization of the BB dye under shake/static (68.12 / 71.39 %) followed by MB (60.21 / 75.34 %), CV (49.12 / 53.31 %) and OG (35.23 / 46.16 %). Whereas, *P. chrysosporium* displayed decolorization under shake/static conditions with MB (73.72 / 86.98 %) followed by BB (70.23 / 88.24 %), CV (69.56 / 75.62 %) and OG (68.47 / 72.27 %). Under stationary conditions, the fungal isolates performed better, compared to shaking approach. Fungal inoculation raised the pH of the dye solutions to neutral from acidic. Seed germination bioassay showed seed germination, when soaked with bioremediated dye solutions, on the other hand, seeds soaked in non-inoculated dyes, no germination was observed even after five days. Similarly, bacterial growth was also inhibited by uninoculated dyes, which shows their toxic effect against bacteria. The novelty of this work is to assess the biodegradation efficiency of filamentous fungi *Aspergillus niger* and *Phanerochaete chrysosporium* for remediation of synthetic dyes, methylene blue, bromophenol blue, crystal violet, and orange G. The results show the viability of these indigenous fungal species as an efficient bioremediation organisms.

Keywords: *Aspergillus niger*, Bioassay, Bioremediation, *Phanerochaete chrysosporium*, Synthetic dyes

INTRODUCTION

Synthetic dyes prevalent application in different industrial sectors, such as textiles, paints, papers, cosmetics, tanneries, pharmaceutical, and food leads to huge amounts discharge of colored and noxious effluents into water bodies^{1,2}. Annually, about 280,000 tons of synthetic dyes are being discharged through industrial effluent³. Directly discharging the synthetic dyes pollutants into aqueous systems can unfavourably influence both human beings and the environment⁴. Synthetic dye pollutants intervene with oxygen solubility and penetration of light in water,

thus restraining aquatic ecosystems photosynthesis process, devastating food chains, disturbing aquatic biota growth, and also creating health risks owing to their carcinogenic and mutagenic features^{5,6}.

Owing to the complicated chemical structure, the synthetic dyes showed significant resistance against naturally biodegradation mechanism. Generally conventional treatment approaches removing color from chromatic wastewater consists of procedures encompassing combination of physical, chemical, and biological decolorization approaches^{7,8}. Physical and

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chemical approaches for dye wastewater treatment are not generally applied to textile industries owing to not being cost effective, generation of sludge, requirement of large areas, and disposal issues⁹. Green approaches to solve these issues include sorption of dyestuffs microbial biomass^{10,11} or low-priced non-conventional dyestuffs using microbial adsorbents could be another choice^{12,13}.

An alternative approach could be mycoremediation, which can be applied for decolorising synthetic dyes and is known for its cost-efficacy and eco-friendly characteristics in removal of colored pollutants from effluent^{14,15}. Fungi possess a unique ability to metabolize diverse pollutants and exploit them as source of energy and carbon by breaking them into lesser toxic forms^{16,17}. Additionally, fungi exhibit the capability to synthesize degradative extracellular enzymes, such as manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase (Lac), which play an important role in bioremediation¹⁸. The fungi (*Aspergillus niger*, and *Phanerochaete chrysosporium*) used in this study, have also been studied in our earlier work (Rani *et al.*¹⁴), for remediation of synthetic dyes (Basic fuchsin, Nigrosin, and Malachite green), where the fungal isolates exhibited encouraging bioremediation potential. To the best of our understanding, there is no previous report in one specific article, showing remediation of MB, BB, CV, and OG using *Aspergillus niger*, and *Phanerochaete chrysosporium*). Keeping the above points in view, the aim of the work was to employ selected potential textile dye effluent soil fungal sp. capable to decolorize and detoxify the synthetic dyes using solid and liquid media under shaking and stationary conditions.

METHODS AND MATERIALS

Chemicals and media

All the chemicals, dyes and media such as Potato Dextrose Agar (PDA), Potato Dextrose broth (PDB) and Nutrient agar (NA) were procured from Himedia, Mumbai, India.

Dyes used

Methylene blue (3,7-bis(dimethylamino)phenothiazin-5-ium chloride) Molecular formula: C₁₆H₁₈ClN₃S; Bromophenol blue: 3',3'',5',5''-tetrabromophenolsulfonphthalein, Molecular formula: C₁₉H₁₀Br₄O₅S; Crystal violet: Tris(4-(dimethylamino)phenyl)methyl cation chloride Molecular formula: C₂₅H₃₀ClN₃; Orange G: Disodium 7-hydroxy-8-[(E)-phenyldiazenyl] naphthalene-1,3-disulfonate Molecular formula: C₁₆H₁₀N₂O₇S₂Na₂. Structures of synthetic dyes are shown in Fig. 1.

Source of fungal strains

Both of these fungi have already shown their potential in bioremediation of dyes, malachite green, Nigrosin, and basic fuchsin¹⁴, and therefore, selected in present study

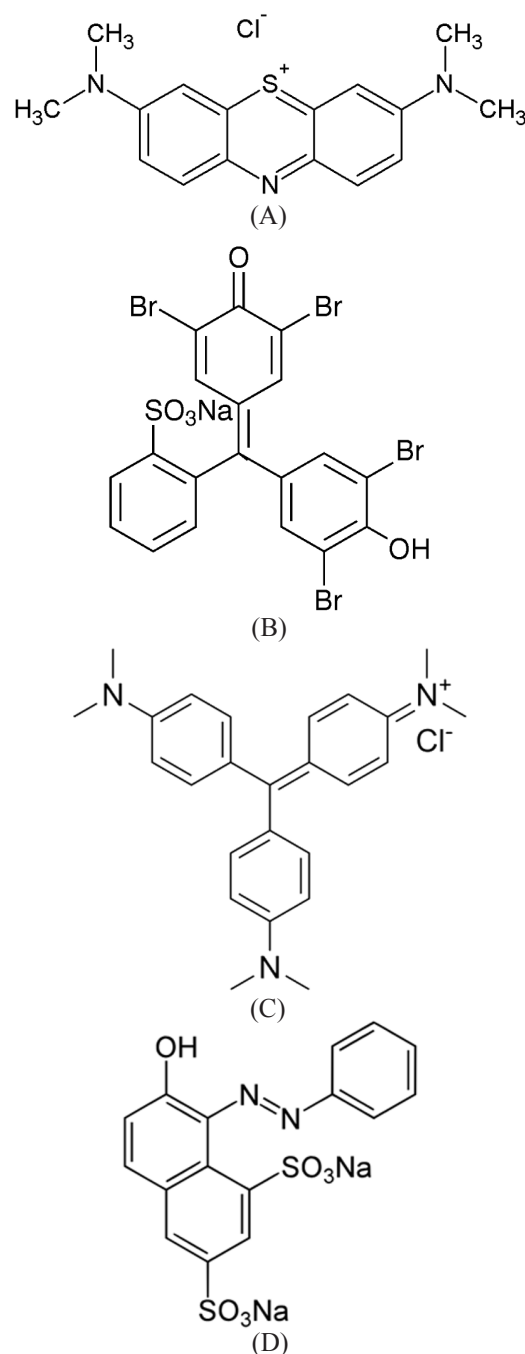


Figure 1. Structures of (A) methylene blue, (B) bromophenol blue, (C) crystal violet and (D) orange G

for methylene blue, bromophenol blue, crystal violet, and orange G dyes decolorization.

Dye Decolorization in solid medium

The two selected fungal strains were tested for their ability to decolorize on potato dextrose agar (PDA) medium, Himedia, Mumbai, India. Following incubation, fungal mycelial agar plugs (~5 mm²) were cut approximately 5 mm from the colony margin and inoculated on test tubes (in

triplicates) each pre-filled with 2 ml of the Potato Dextrose Agar (PDA) medium, supplemented separately with either with following dye 0.01 % (w/v) methylene blue, bromophenol blue, crystal violet, orange G¹⁴. The culture tubes were then incubated at room temperature (~ 25°C). The growth of the fungi and its ability to decolorize the dye were observed weekly up to four weeks. The depth of dye decolorization (in mm) indicated by clearing of the dye was then measured.

Dye decolorization assay in broth

The spores and mycelia were then dislodged using a flame-sterilized inoculating loop. Then, 10 µL of the inoculum were added on culture vials (in triplicates) pre-filled with 25 mL Potato Dextrose Broth (PDB) supplemented with 0.01% of either one of the following dyes: methylene blue, bromophenol blue, crystal violet, and orange G. Three sets were prepared and were incubated either under constant agitation/shaking (120 rpm, Narang Calton Shaker Incubator) or under stationary/without shaking condition¹⁹. All culture vials were incubated at room temperature (~25°C) for 10 days and all assays were performed in triplicate. Growth and dye decolorization were noted every day. Following culture for 10 days, the culture filtrates were decanted and subjected to spectrophotometric analysis (Shimadzu UV-1800). Absorbance maxima of the tested dyes were read as methylene blue-665 nm, bromophenol blue -592 nm and orange G-474 nm wavelength. The extent of dye decolorization by the soil fungal strains on liquid media was calculated using the formula below:

Percent dye decolorization (%) = (Absorbance control–Absorbance inoculated) / Abs control × 100

Later, the mycelial biomass was harvested on clean Petri dishes and observed. Fungal hyphae were also mounted on clean glass slides and observed under a compound light microscope for the biosorption of dyes.

Enzyme assay

Laccase activity was measured by using syringaldazine as a substrate as per the method of (Dias *et al.*²⁰). The activity was assayed using using 1.0 ml of 0.2 M sodium phosphate buffer (pH 5.7) and 0.2 ml syringaldazine (1.6 mg/ml) in absolute ethanol, (4.47 Mm). Reactions were initiated by the addition of syringaldazine and after mixing; incubations were conducted at 30°C for 1h, because after 1h highest enzyme laccase activity was observed. The absorbance was measured in a spectrophotometer (Shimadzu UV-1800) before (0 time) and after incubation (60 min) at 526 nm and the increase in absorbance was calculated. One unit activity was defined as the enzyme producing one absorption unit/ min at 526 nm.

Seed germination bioassay

Effect of bioremediated and untreated dye solution was

observed on mustard seed germination. Mustard seeds were sterilized using 0.1 % HgCl₂ solution for 60 sec, washed with 70 % alcohol for 2 minutes, and finally washed with sterile distilled water 5-6 times to remove traces of sterilizing agents. In sterile Petri dishes sterile filter paper was kept soaked in treated and untreated dye solution, while in other Petri dishes, sterile distilled water-soaked filter paper was kept as control. Ten mustard seeds were kept in each Petri dish, and the experiment was conducted in triplicate. Observation on seed germination was taken for five days.

Bacterial toxicity

Effect of treated and untreated dye solution was observed on bacterial growth by measuring zone of inhibition. 0.1 ml of twenty-four h grown *E. coli*, cells (10⁻⁸ cells / ml) were plated evenly on Petri dishes and sterile filter paper discs impregnated with treated and untreated dye solution were kept on the seeded bacterial cells at equal distance and pressed lightly, so that discs firmly adhere on to the surface. The Petri dishes were incubated at 30°C for 48 h, zone of inhibition was observed around the discs.

Statistical analysis

All the experiments were carried out in triplicate; The data were analyzed statistically by using analysis of variance (ANOVA). In figures, error bars indicate standard error of the mean, where error bars are not visible; they are smaller than the marker.

RESULTS

In tube overlay method, highest decolorization was observed in methylene blue and bromophenol blue (92.17 and 91.68 % respectively), followed by crystal violet (68.45 %), and orange G (60.30%) by *A. niger*. In case of *P. chrysosporium* highest decolorization was observed in methylene blue and bromophenol blue 88.64, and 86.75%, followed by crystal violet (65.85%) and least was orange G (60.3%) (Fig. 2).

Dye decolorization in liquid medium exhibited different trend as that of tube overlay method. In this approach, highest decolorization by *A. niger* was observed in bromophenol blue (68.12%), followed by methylene blue (60.21%), crystal violet (49.12%) and least by orange G (35.23%) under shaking conditions. While, under static conditions, highest decolorization was observed in methylene blue (75.34%), followed by bromophenol blue (71.39%) and crystal violet (53.31%), least by orange G (46.16%) (Fig. 3).

Dye decolorization in liquid medium by *P. chrysosporium* also showed interesting trend, highest decolorization was observed in methylene blue (75.73%), followed by bromophenol blue (72.19%), orange G

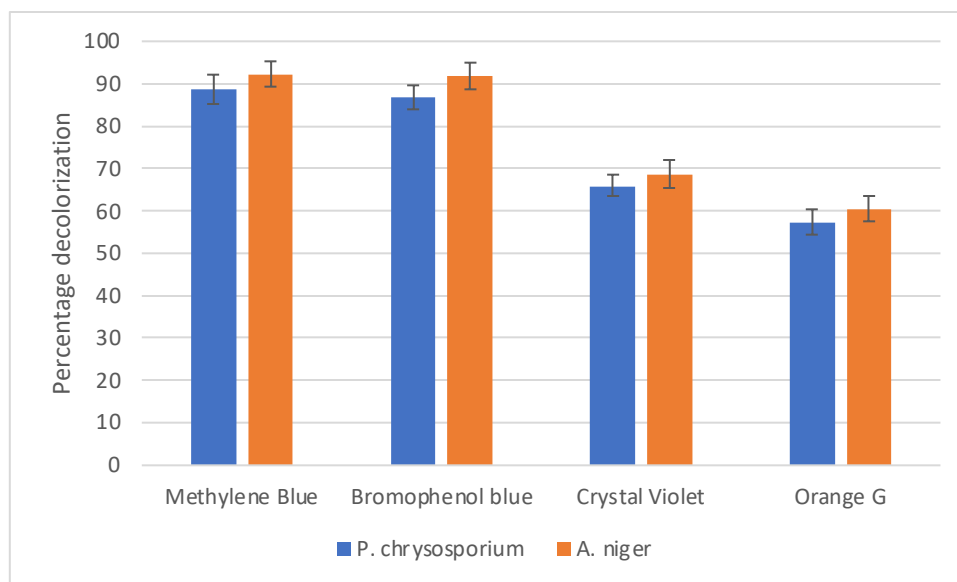


Figure 2. Dye decolorization by *P. chrysosporium*, and *A. niger* using tube overlay method

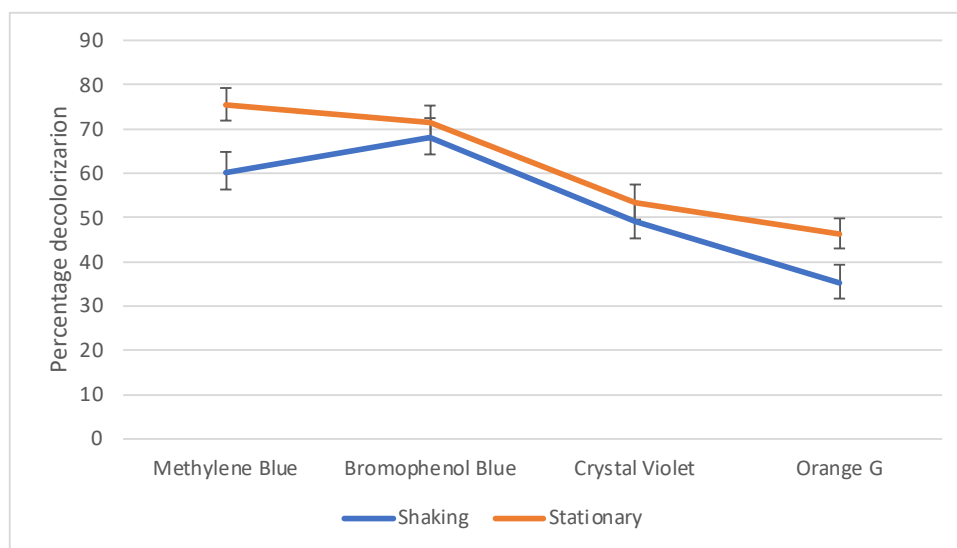


Figure 3. Percentage dye decolorization by *A. niger* under shaking and stationary conditions

(71.56%) and least by crystal violet (68.47%) under shaking conditions (Fig. 3). While under stationary conditions, maximum decolorization was observed in bromophenol blue (88.87%), followed by methylene blue (87.67%) and crystal violet (77.75%) least by orange G (73.89%) (Fig. 4).

Inoculation of fungal strains also exhibited pH change in the dyes samples, as observed in *A. niger* and *P. chrysosporium* inoculated treatments under shaking and stationary conditions. After inoculation, the pH of all the dye samples was increased to neutral or near neutral (Fig. 5, 6).

Microbial bioassay study showed that untreated dye (control) inhibited the growth of *E. coli* on Petri dishes by forming a higher zone of inhibition, while the treated dye showed very little zone of inhibition (Fig. 7).

Moreover, effect of untreated and treated dye on mustard seed germination was also examined. It was observed that germination percentage was higher by treated dye, while the untreated (control) dye inhibited the germination of mustard seeds (Fig. 8).

DISCUSSION

Bioremediation is an efficient, cost-effective and suitable treatment method for contaminating materials²¹. Using this approach, the waste and noxious chemicals are transformed into harm-less or less toxic components by means of using microbes²². Fungi are well known for their several applications in diverse biotechnological fields^{23,24}, and are the admirable resources of diverse extracellular enzymes such as peroxidases and laccases which have remarkable ability of dye's biodegradation²⁵. Fungi are the widely

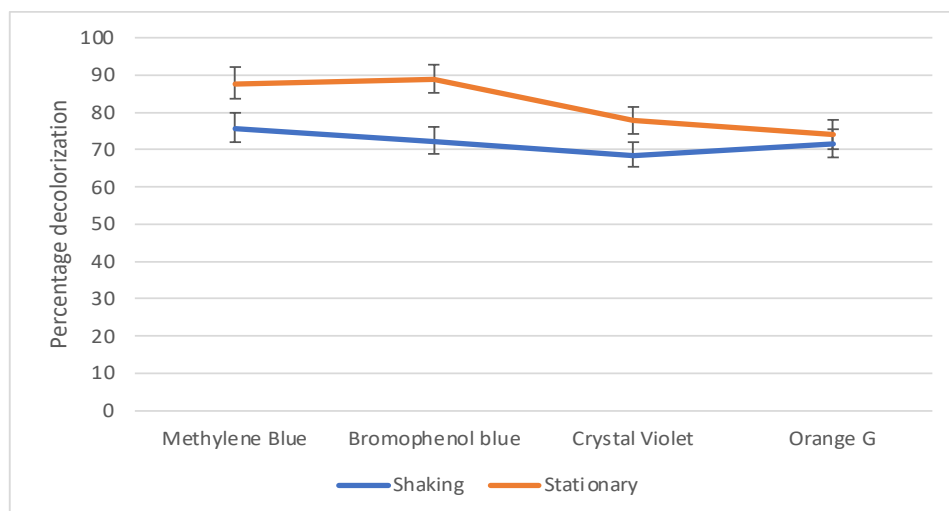


Figure 4. Percentage dye decolorization by *P. chrysosporium* under shaking and stationary conditions

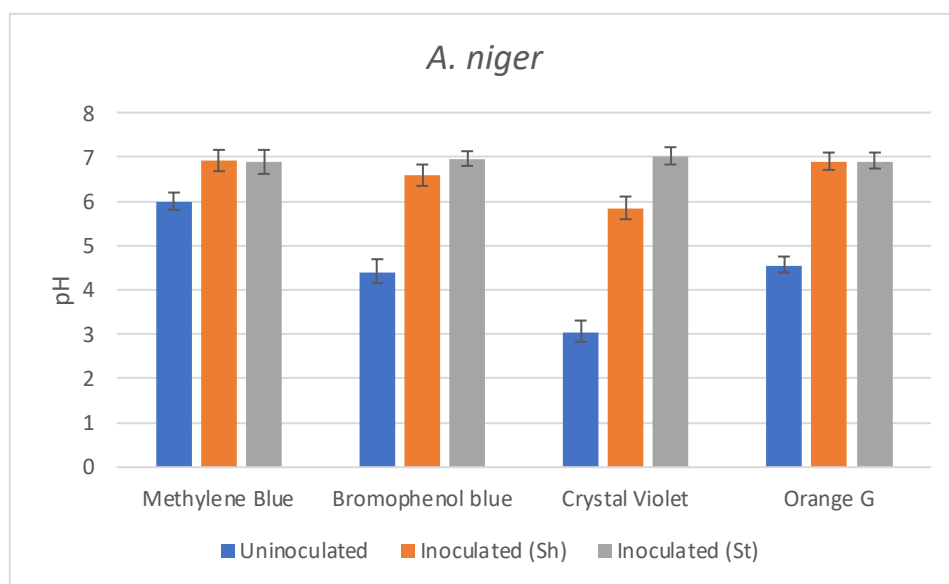


Figure 5. Effect of *A. niger* inoculation on change in pH value in dye liquid medium

distributed microorganisms and their natural ability to work as great decomposer makes them also the effective bio-degrading agents of diverse organic molecules²⁶.

The removal of the dye color is vital in the potential application of effluent soil fungal organisms as bioremediation agents in wastewater treatment plants and in runoff waters. Thus, it is essential to test dye contaminated soil fungal strains for dye decolorization in liquid medium. *Aspergillus niger* and *P. chrysosporium* bioremediated methylene blue, bromophenol blue, crystal violet, and orange G within 10 days up to 88%. Al-Jawhari (2015)²⁷ reported decolorization of methylene blue and crystal violet by *Aspergillus fumigatus*, and *A. niger*, by production of ligninolytic enzymes.

Use of *A. niger* and *P. chrysosporium* as dye biodegrader or decolorizer has been reviewed in this report and the efficient decolorization may be attributed to either through the action

of extracellular enzymes such as laccase and/or biosorption by the fungal biomass. Fig. 9 shows the degradation of MB by *A. niger*: adsorption, transformation, and detoxification process. This diagram represents the stepwise scheme of the degradation of the synthetic dye MB by *A. niger*. In the first step, the dye is adsorbed on the cell wall of the fungus because of the interaction with the functional groups in the cell wall of the fungus. Next, the extracellular oxidative enzymes facilitate the cleavage of the dye into intermediate forms. These intermediates are then transferred inside the cell where they undergo further enzymatic reaction for their degradation via oxidation, reduction, demethylation, and ring cleavage. The intermediates thus formed get converted into simpler and less toxic metabolites and finally get mineralized to form harmless end products like CO₂ and water.

Laccase production by the soil fungal species have

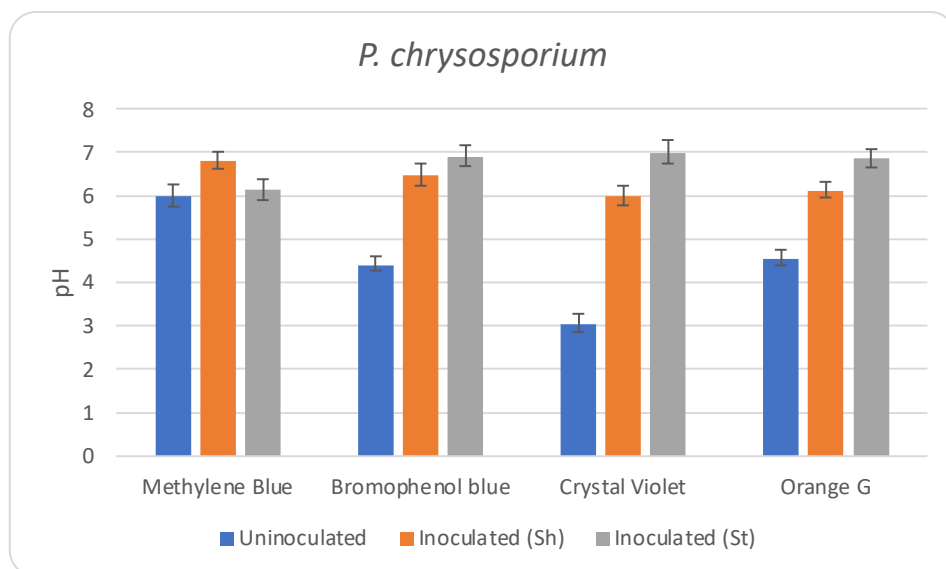


Figure 6. Effect of *P. chrysosporium* inoculation on change in pH value in dye liquid medium

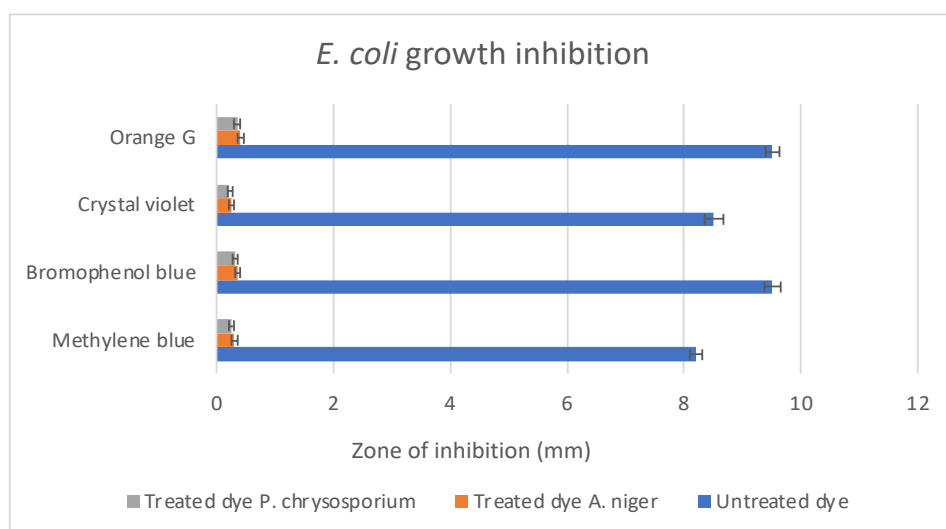


Figure 7. Effect of treated and untreated dyes on growth of *E. coli*

been studied, this test was performed to know, whether laccase enzyme plays any role in biodegradation of dyes. Furthermore, the soil fungal strains also showed promising decolorization activities against tested dyes (Fig. 4, 5). Decolorization of high-molecular weight azo dye, viz., Orange G by *Podoscypha elegans* has been reported by (Pramanik and Chaudhuri, 2018)²⁸. The maximum utilization of orange G by fungal isolate *P. elegans* occurred within 14 to 21 days of incubation, the results indicated the fungus utilized the dye at different rates depending on their concentration in the culture medium.

Soil fungi possess ligninolytic enzymes and play an important role in the degradation of lignocellulose in soil ecosystems²⁹. These lignin-degrading enzymes are directly involved not only in the degradation of lignin in their natural lignocellulosic substrates but also in the degradation of various xenobiotic compounds, including

dyes. Moreover, ligninolytic enzymes have been reported to oxidize many recalcitrant substances such as chlorophenols, polycyclic aromatic hydrocarbons (PAHs), organophosphorus compounds, and phenols^{30,31,32}.

Biosorption of dyes occur essentially either through complexation, adsorption by physical forces, precipitation, entrapment in inner spaces of fungal mycelium, ion exchange due to surface ionization, and by formation of hydrogen bonds³³. Due to an increased cell-to-surface ratio, fungi have a greater physical contact with the environment. The efficient decolorization of crystal violet, a triphenylmethane dye, indicates the ability of fungi to break down complex aromatic rings. Crystal violet is known to be toxic and resistant to degradation due to its stable molecular structure³⁴. The observed reduction in color intensity suggests that fungal strains produce ligninolytic enzymes such as laccase, lignin peroxidase (LiP), and

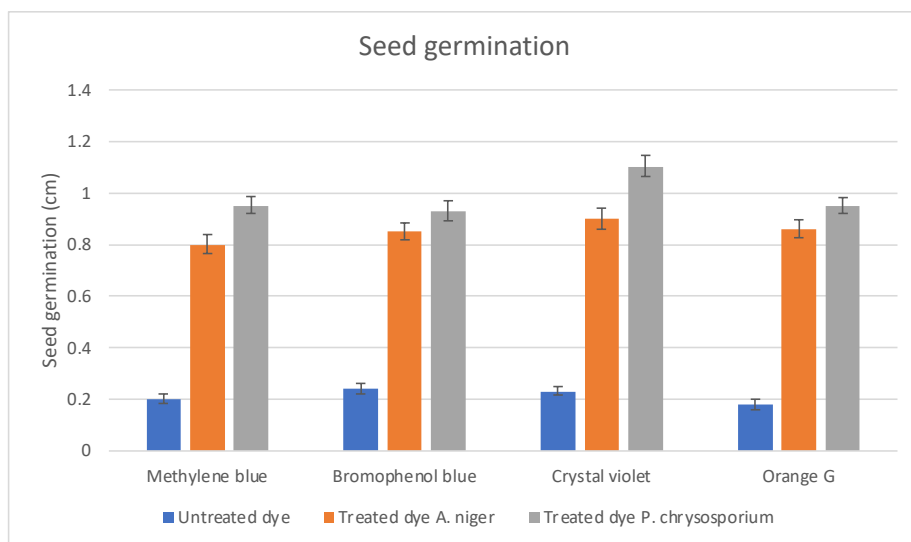


Figure 8. Effect of treated and untreated dyes on the germination of mustard seeds

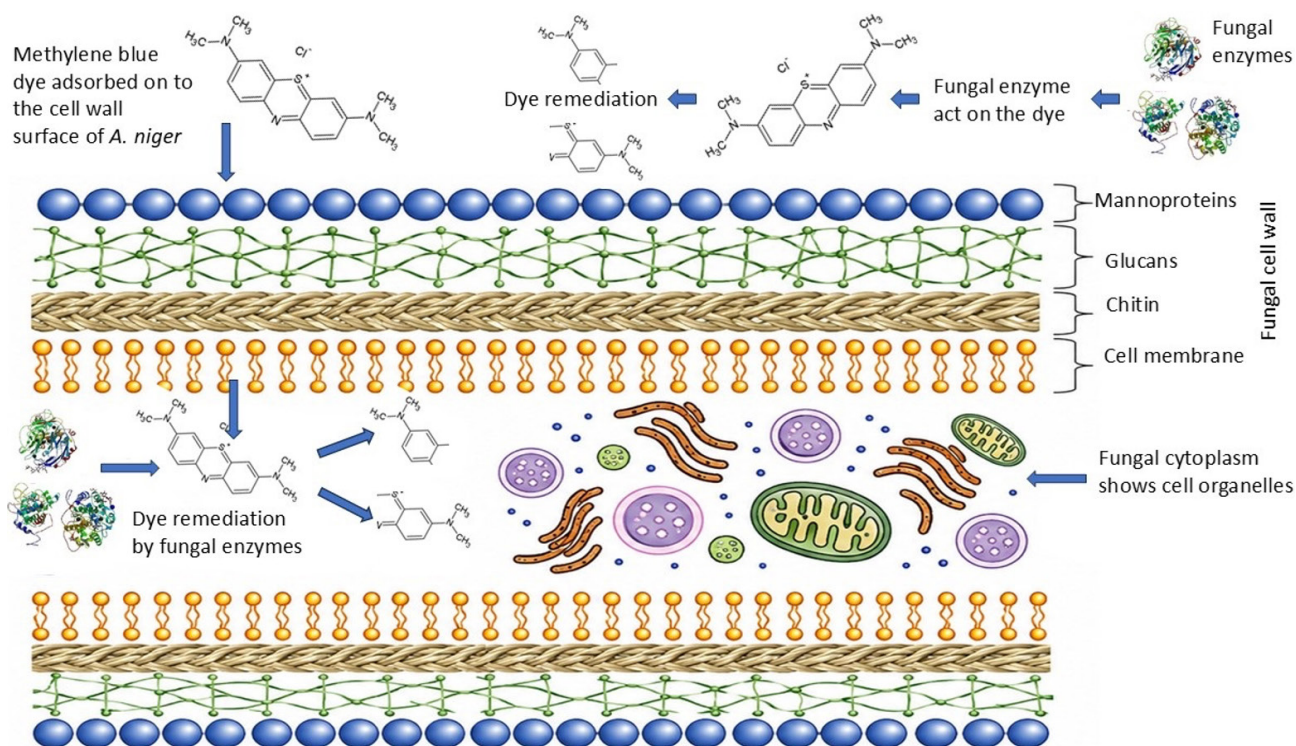


Figure 9. The image shows the simple pathway of synthetic dye (methylene blue) absorption by *A. niger*. The fungal extracellular and intracellular enzymes act on the dye to remediate it (adapted from Modi *et al.* 2025)

manganese peroxidase (MnP), which oxidatively cleave the chromophoric groups of the dye. Similar findings have been reported where white-rot fungi effectively degraded triphenylmethane dyes through enzymatic oxidation and subsequent mineralization³⁵.

The decolorization of bromophenol blue, a sulfonated triphenylmethane dye, further demonstrates the metabolic versatility of fungi in handling dyes with sulfonic groups. The removal of bromophenol blue may involve enzymatic

reduction or oxidative cleavage of the dye structure, leading to breakdown of the chromophore and formation of less complex intermediates³⁶. Fungi possess adaptive metabolic pathways that allow them to utilize dye molecules as secondary carbon sources or transform them through co-metabolic processes³⁷.

Similarly, methylene blue, a heterocyclic thiazine dye commonly used in textile and medical applications, showed considerable decolorization by fungal isolates³⁸.

Methylene blue degradation is often associated with both biosorption and biodegradation mechanisms. Initially, dye molecules may adsorb onto fungal biomass due to electrostatic interactions with cell wall components such as chitin, glucans, and proteins. Subsequently, enzymatic reactions catalyzed by oxidoreductases lead to structural modification and breakdown of the dye molecule³⁹.

The azo dye Orange G exhibited comparatively variable decolorization efficiency among fungal strains, which can be attributed to the presence of azo bonds (-N=N-) that confer high stability to the dye⁴⁰. Fungal degradation of azo dyes generally involves enzymatic reduction of the azo bond by azoreductases, followed by further oxidative degradation of the resulting aromatic amines by ligninolytic enzymes⁴¹. This sequential enzymatic process leads to cleavage of the chromophore and loss of color from the medium⁴².

Recently, Modi et al.⁴³, reported the decolorization efficiencies of 95.26, 97.51, and 98.23 % for Reactive Orange 16 (200 mg/L), Reactive Green 19 (200 mg/L), and Remazol Brilliant Blue R (200 mg/L), respectively using *Aspergillus aculeatus* (KCHW-1) and *Cladosporium tenuissimum* (MNDS-3). In another interesting work by Mustafa et al.⁴⁴, potential of *Penicillium chrysogenum* for degradation of four types of dyes: Everzol Black B (EBB), Basic Yellow (BY), Reactive Red (RR), and Acid Blue 62 (AB62) were analyzed. The results indicated that each dyes exhibited maximum removal efficiency on different days. For example, 94.6% removal efficiency was obtained from AB62 on the sixth day. In the case of RR and BY, the maximum removal efficiencies were observed after the eighth day, amounting to 88.7% and 89.4%, respectively. HPLC spectra showed that the dyes had been broken down through the absence of their respective peaks and the presence of new peaks. For instance, the disappearance of the peak at 472 nm and appearance of a new peak at 136 nm implied changes in the molecule of EBB.

Effect of treated dyes on seed germination compared to the untreated dyes showed that treated dyes favoured the mustard seed germination, while the seeds cultured in untreated dyes showed poor germination (radicle and plumule) even after five days of treatment. Our results are in corroboration with Ghanaim et al.⁴⁵, which used four fungal strains-*Aspergillus flavus*, *Aspergillus terreus*, *A. niger*, and *Fusarium oxysporium* to screen for the degradation and detoxification of azo dyes-congo red, crystal violet, bromophenol blue, and malachite green. After eight days, *A. flavus* had degraded azo dyes at the maximum proportion. Phytotoxicity assay results exhibited that the degraded products were less harmful to maize and common bean plant's growth and germination rates, as compared to untreated dyes. The enzymes, laccases, lignin peroxidase, and manganese peroxidase production by *A. flavus* showed that enzymes play an important part in discolorizing of the detrimental azo dyes. Similarly, Marwein and Ramamoorthy (2025) reported that synthetic

dyes remediation by *Penicillium singorense*, and *Aspergillus ochraceus* resulted in reduced toxicity on *Triticum aestivum* growth parameters compared to untreated dyes.

Similarly, filter paper discs impregnated with untreated dyes exhibited *E. coli* inhibition on solid agar medium in Petri dish, while the treated dye solution showed very little zone of inhibition. This showed that fungi treatment detoxified the dyes, which slightly inhibited the tested bacterial growth. Our observations are in substantiation with Rani et al.¹⁴, who also reported that treated dyes favoured wheat seed germination, compared to the untreated dyes. Also, the treated dyes exhibited little or no inhibition against the tested bacterium. Increase in pH from acidic towards neutral or near neutral in treated dyes solution by fungi, shows that fungal inoculation remediated the acidic aspects of dyes, which is also a positive signal towards dyes bioremediation^{46,47}.

Overall, the differential decolorization patterns observed among the tested dyes may be attributed to variations in dye structure, molecular weight, functional groups, and solubility. Fungal strains capable of producing a broad spectrum of extracellular enzymes are particularly effective in degrading structurally diverse dyes. Environmental factors such as pH, temperature, dye concentration, and nutrient availability may also influence the rate and extent of fungal decolorization^{46,48}.

The findings support the growing evidence that fungi, particularly ligninolytic and filamentous species, play an important role in the decolorization and bioremediation of dye-contaminated wastewater. Their ability to produce non-specific oxidative enzymes allows them to degrade a wide range of xenobiotic compounds, making them promising candidates for sustainable wastewater treatment strategies. Further studies focusing on enzyme characterization, metabolic pathways, and toxicity assessment of degradation products will help in optimizing fungal systems for large-scale bioremediation applications.

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