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Differential Approach of Bioremediation by *Sclerotium rolfsii* Towards Textile Dye

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Abstract

Synthetic dyes are extensively used in various industries and are one of the major contaminants of industrial effluents. Dyes being xenobiotic, carcinogenic, and toxic there is need for their effective removal and detoxification to conserve water resources. Tremendous research has been carried out to identify potent microorganisms that facilitate bioremediation of these harmful dyes. A static batch culture has proved white rot fungi *Sclerotium.rolfsii* as an efficient catalyst in bioremediation of textile dyes and to compare their efficiency in decolourisation of two different azo dyes. Studies revealed the organism employ different remedial approach to cationic dye (Malachite green) and anionic dyes (Rose Bengal). Decolourisation of malachite green was a gradual with degradation and biotransformation to colourless, non-toxic by products while Decolourisation of rose Bengal was quick process of biosorption. *S.rolfsii* exhibited 89% of decolourisation of malachite green dyes at higher concentration of 900mg/L while 96% for rose Bengal at 900mg/L. The mechanism of dye decolourisation was proposed using the UV Vis spectrophotometry, FTIR, XRD, HPLC and SEM. Microbial toxicity studies confirmed the dye metabolites of degraded malachite green was less toxic compared to original dye. Comprehensively studies illustrate the sustained application of *S. rolfsii* as model organism for bioremediation of complex industrial effluents due to its differential bio remedial approach can potentially decolourise or remove various dyes.

Keywords: Biodegradation, Bioadsorption, *Sclerotium rolfsii*, Malachite green, Rose Bengal.

Introduction

Dyes are extensively used in various sectors of industries like paper, printing, textile, pharmaceuticals, and food industries. The industrial effluents of these industries carry huge amounts of dyes and are mostly released into water bodies [27, 32]. Dyes present in waterbodies affect aquatic plants and animals. They hinder in the penetration of sunlight thereby affecting photosynthesis in plants. Ecosystems are interconnected and disturbance in aquatic ecosystem will affect overall integrity of biosphere. Dyes being recalcitrant in nature they tent to persist in environment. They are carcinogenic and teratogenic affecting human health. Across worldwide nearly 5-20% of dyes are released as part of industrial effluent to waterbodies leading to environmental issues.

Discharges of these dyes alters the phys-

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io-chemical properties of water like pH, increases BOD and COD [23]. They intensely colour the water and imparts unpleasant odour. Removal of these dyes can be achieved by physio-chemical method like flocculation, adsorption, ionisation, oxidation, irradiation etc. These methods have certain limitations like they are not effective, expensive, produces secondary sludge whose disposal is a problem and produces toxic by-products [1]. A variety of microorganisms like bacteria, actinomycetes, fungi and yeast can be used in biodegradation and complete mineralisation of dyes [29, 33]. Bioremediation have received wide acceptance by public mainly because this technique eliminates waste completely and permanently, cost effective, less impact on environment and can be coupled with physical and chemical methods [17, 28].

White rot Fungi have proved to be most potent in bioremediation of dye compared to bacteria mainly due to its biomass and secretion of ligninolytic enzymes such as laccase, manganese peroxidase, and lignin peroxidase [4, 17, 30]. These enzymes play a vital role in detoxification of dyes and bleaching of woods [16, 36]. White-rot fungi, belonging to the basidiomycetes, are the only organisms capable of mineralizing lignin efficiently and thus can enzymatically degrade dyes [14]. In recent years many researches are carried out to exploit the use of white rot fungi in degradation of complex aromatic compounds. *Rhizopus arrhizus, Rhizopus oryzae, Penicillium sp., Trichoderma harzianum and Haematonectria haematococca*, which have been used to remove dyes [7, 22, 35, 36].

Biosorption is another alternative method of bioremediation of dyes where dyes can be effectively removed from aqueous medium. Biosorption is a physiochemical process which occurs on the surface of microbial cell wall. This process includes electrostatic interaction, ion exchange, complexation, chelation and microprecipitation [35]. The process is cheap, rapid and effective and microbial biomass obtained from industries like yeast slurry from brewing industry or agricultural waste like barley husk, wood chips, fruit peel can be used as absorbents [3, 5, 19, 18, 34, 35, 37].

In recent years, there has been a growing interest in the field of screening of fungal strains that can produce these enzymes that can help in degradation of xenobiotics substances like textile dyes and petrochemicals [25]. *S. rolfsii* is a facultative parasite that survives in the soil primarily in the form of sclerotia, which are the source of inoculum and can remain viable for several years together [6, 10, 13, 32]. Of the major extracellular enzymes produced by this pathogenic fungus, laccase and lignin peroxidase are known to have a functional role in degrading toxic xenobiotic substances.

Literature reviews reveals unexplored potential of *Sclerotium rolfsii* in removal of Malachite green and Rose Bengal dyes. The objective of current research was to understand the differential mechanism employed by fungi to decolourize and detoxify the widely used textile dyes.

Materials and Methods

Source of culture and chemicals

The fungal culture of *Sclerotium rolfsii* was obtained from Indian Institute of Horticulture Research, Bangalore. The acquired fungal sample was subcultured on Potato Dextrose Agar (PDA) medium and incubated at 26°C for 7~10 days till sclerotia were formed on mycelial mat, and served as the master culture. All chemicals like dyes and growth media components were purchased from Sigma Aldrich and Himedia.

Dye decolourisation on solid media

To screening the ability of fungi to decolourise different dyes was performed as per Park's work with certain modifications [25]. Extensively grown mycelia of *S. rolfsii* were divided into 1 cm2 pieces along with sclerotia and were placed on PDA media with 100 mgL-1 concentration of selected dyes. Control and Test

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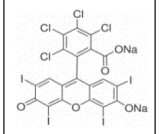
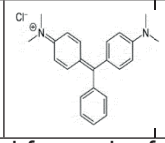
Table 1: Dyes used in the studies

Names of Dyes	Structure	λ max
Rose Bengal (Acid Red)		595 nm
Malachite Green (Basic Green)		680 nm

were maintained for each of the selected dyes. Control was not inoculated with fungi while the test media was inoculated. Both control and test plates were incubated at 26°C. This method was used to examine dye decolourization on agar media.

Dye decolourisation in liquid media

Malachite Green (Basic Green 4) and Rose Bengal (Acid Red 94) of concentration ranging from 100-900 mgL-1 were prepared. Protocol for decolourisation in liquid media was analysed as per Lefebvre's work. Control samples were not inoculated while test samples were inoculated [15]. Cultures were incubated at static conditions at 26˚C. Colour intensity of culture fluids were determined using UV-Vis Spectroscopy at 620 nm for Malachite Green (MG) and 595 nm for Rose Bengal (RB) at regular interval from fourth day to sixteenth day. The percentage of dye decolourisation was estimated.

Extraction of dye metabolites for analysis

Culture broth with malachite green after incubation period was subjected to solvent extraction using equal volume of ethyl acetate. Broth and ethyl acetate were mixed for 30 mins followed by the separation of organic layer which was subjected to rotatory evaporated to concentrate the dye intermediate metabolites. Which was used for HPLC analysis and toxicity studies.

Spectroscopic analysis of degradation and biosorption studies

The dye metabolites of malachite green after fungal Dye degradation were isolated for FTIR, HPLC analysis and for toxicity studies. To study the surface morphology, change on the fungal mycelium after adsorption of rose Bengal was subjected to XRD and SEM analysis

Results and Discussion

Dye decolourisation on solid media

S. rolfsii was grown on Potato Dextrose Agar (PDA) with 100 mgL-1 of selected dyes, fungi grew as a smooth mycelial mat that spreads radially from its origin. It proceeded to grow on media in a radial manner up to the edge of the plate uniformly. Complete growth covering the media as uniform mycelial mat was observed on 5th day of inoculation. Test plate showed prominent dye decolourisation with Malachite Green and Rose Bengal on comparison with control.

Dye degradation in liquid medium and effect of dye concentration

To understand the effect of dye concentration on rate of decolourization Potato Dextrose Broth (PDB) medium with dye concentration ranging from 100-900 mgL-1 was incubated at 26°C for a period of 16 days in a static mode. On day 4 Mycelia growth reached maximum forming a uniform mat on surface of the medium. Absorbance was read at 680 nm for MG and 595 nm for RB at interval of 4 days for 16 days. There was a progressive decolourization observed for MG, while abrupt fall and its persistence during incubation was observed for RB (Fig.4). This clearly indicated the mechanism of decolourization adopted by *S.rolfii* was for basic dye (MG) and acidic dye (RB) were different.

Studies of degradation of rose bengal

In case of RB culture, the media had completely lost the colour and dye had adsorbed on mycelial mat. Absorbance studies of culture

media from day 4 to 16 showed negative values indicating complete absence of dye in medium. This explains that adsorption of dye is quick on mycelium and had removed the dye from culture media significantly on day 4 itself and thus absorbance readings value had dropped and persisted till end of incubation period as indicated in (fig.1).

Initially when *S. rolfsii* culture was set for decolourisation of RB the pH of the medium was maintained at 7 but as the fungal mycelium grew the pH of the medium dropped to 4.8 and had become acidic. The change in pH of the medium is due to release of chemicals like oxalic acid due to the metabolism of fungi [24]. This leads to protonation of the amino group on chitin cell wall of fungi [5]. Which led to enhanced biosorption of anionic dye (RB) on the net positively charged mycelial mat.

The functional groups associated with fungal biomass also influence rate of dye adsorption. After 5 days of inoculation mycelial mat grew to maximum in static liquid culture with RB concentration ranging from 100-900 mgL-1. High biomass density and surface area increased the number of amino group availability for electrostatic attraction of anionic dye [2, 20, 34].

Absorbance spectrum obtained for RB decolourization for 700 mgL-1 from day 5 to 16 showed big drop in the color intensity (Fig.1) proving *S.rofii* as excellent organism for absorption of dye. Hence is promising microorganism for bioremediation of RB.

Studies of degradation of malachite green

The rate of decolourization of MG was studied using UV-Vis Spectroscopy. A gradual decrease in the absorbance values over incubation period for dye concentration ranging from 100 mgL-1 to 900 mgL-1 was recorded from day 5 to 16 days.

As per the (fig.1) *S. rolfii* exhibited efficient degradation of Malachite Green with initial concentration of 100 mgL-1 reduced to 11.12 mgL-1. Similarly with the other concentrations: 300 mgL-1 to 43.26 mgL-1, 500 mgL-1 to 74.4 mgL-1 , 700 mgL-1 to 92.15 mgL-1 and 900 mgL-1 to 121.68 mgL-1 on 16th day of inoculation. Gradual decrease in concentration of dye with time proves the ability of fungi to metabolise the dye at higher concentrations as well.

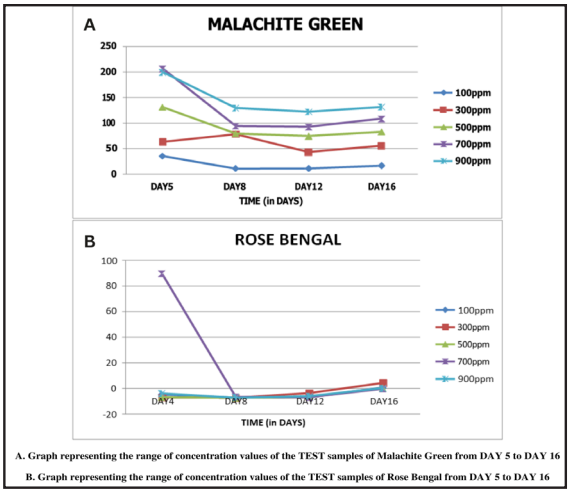


Figure 1. Degradation of Malachite green and Rose Bengal by S. rolfsii

Dye degradation ability of *S. rolfsii* is attributed to lignolytic enzymes like laccase (lac) and Manganese Peroxidase (MnP) which are extracellular and catalyse dye degradation process [17, 19]. Increase in biomass during the incubation period signifies increasing amounts of these extracellular enzymes production that facilitates effective dye degradation. *S.rolfii* produces acid stable extracellular laccase that catalyses the degradation of dye at low pH. The presence of dyes also acts as inducer for enzyme produced that catalysis dye degradation or transformation of dye. Additionally, the Production of MnP increased the dye degradation ability. *S.rolfii* exhibited tolerance and decolourisation at higher concentration of dye. This study shows that as the fungi was exposed to increasing concentration of dye they get acclimatized and degrade MG. The characteristic Absorbance spectrum of 700 mgL-1 obtained

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during the incubation period exhibited direct correlation between growth, incubation time and decolorization. As growth and incubation period increased the dye decolorization increased. The decrease in intensity of dye absorbance peak over time is attributed to enzymatic degradation of dye.

Percentage of dye degradation with increasing concentration of MG exhibited high potential of fungi to degrade upto 86% of 300 mgL-1, 700 mgL-1 and 900 mgL-1 respectively while 100 mgL-1 was degraded upto 89% by end of the incubation period. This proves *S.rolfsii* as promising organism to degrade MG at higher concentration.

Fourier-transform infrared spectroscopy studies

FTIR were used to confirm dye degradation and presence of intermediate metabolites. Parental dye malachite green and its dye metabolite extracted from media were subjected to FTIR studies.

Interpretation of the FTIR spectrum of control indicated as Control in (Fig 2) for Malachite Green dye, we observed sharp peak at 1592 cm-1, corresponding to the C=N bond stretching. The sharp peak at 1509 cm-1 relates to C-C stretching in aromatic ring. The sharp peak at 1229 cm-1 agrees with the C-N aliphatic stretching. Peak at 1190 cm-1 corresponds to C-N aromatic stretching. The sharp peak at 826 cm-1 matches to C-H out of plane bending in aromatic ring. The weak peaks at 3036 cm-1 and 2883 cm-1 gives the C-H stretching in aromatic ring and alkanes respectively.

The IR spectrum of the test or the dye metabolites of malachite green after degradation represented in (Fig.2) and comparing it with control there was much difference giving us the knowledge that Malachite Green dye has degraded. There are new peaks formed at 3300 cm^{-1} corresponding to O-H stretching indicating the formation of alcohols or carboxylic acids during degradation. The sharp peak observed at 2921 cm-1 relates to the N-H stretching of aromatic amines and strong peak at 1714 cm^{-1} corresponds to C=O functional group due to aromatic ketones or aldehydes formed in the course of degradation. Peaks at 1235 cm^{-1} corresponds to the C-N aromatic stretching and peak at 1097 cm-1 corresponds to C-O stretching of alcohols. Peak at 800 cm-1 gives information about the disubstituted benzene ring derivatives formed. This change in spectrum of test suggest dye degradation by *S. rolfii.*

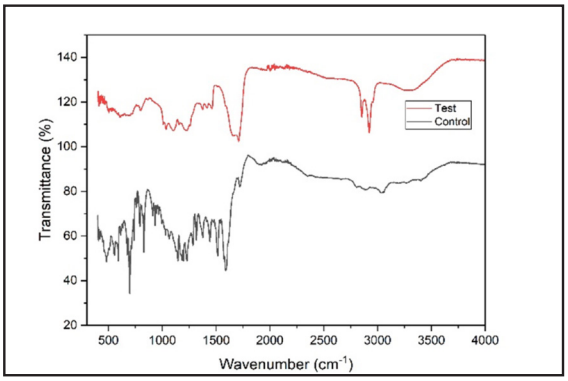

Figure 2. FTIR spectrum of Malachite Green dye after degradation (Test), Parental Dye (control)

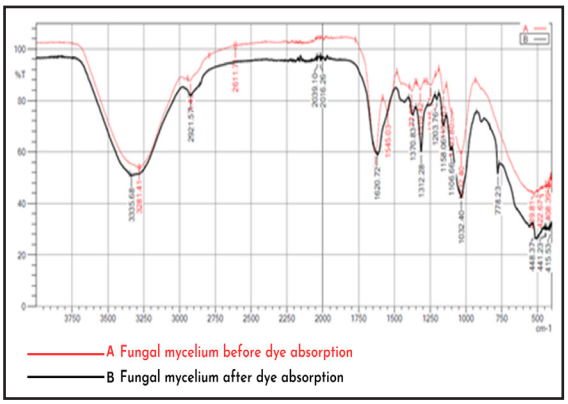

Figure 3. FTIR spectra of Sclerotium.rolfsii biomass before (A) and after dye sorption (B) of Rose Bengal dye

The spectrum of the *Sclerotium.rolfsii* biomass with and without dye loading were compared. The study revealed slight shifting of peaks (3281.41, 1633.57, 1545.03, 1319.42 and

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1032.40 cm-1) from their position with alteration in intensity and appearance of new peaks indicating the involvement of some functional group of the fungi in the biosorption of Rose Bengal dye on the surface of the fungal biomass.

XRD analysis of fungal mycelia

XRD pattern was analysed for Rose Bengal dye absorption on the mycelia of *S.rolfii*. Both adsorbed and un-adsorbed mycelia was subjected to XRD analysis. Significant variation in the pattern of peaks was observed. The un-adsorbed fungal mycelial XRD pattern showed hollow peak indicating the non-crystalline and amorphous nature of fungal mycelia. After the adsorption of the dye to the fungal biomass showed few crystalline peaks at around 25.9, 29.2, and 35.8o of 2 theta values indicating that there was transformation in the nature of the fungal biomass due to adsorption of Rose Bengal dye. This change in fungal mycelial morphology from amorphous to crystalline structure confirms dye biosorption on mycelia.

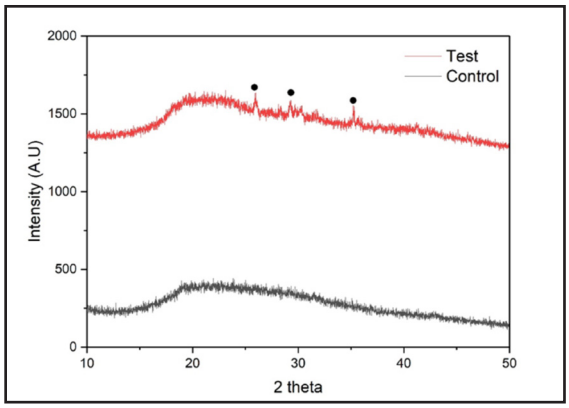

Figure 4**:** XRD pattern of S. rolfsii mycelium before and after adsorption of Rose Bengal

Scanning electron micrograph studies

Surface morphology change on the fungi after adsorption of rose Bengal was subjected to SEM analysis. Fungal mycelium acting as adsorbent clearly showed a distinct change in its morphological change. Mycelium surface was uniform and smooth with deep pore and pockets as shown in (Figure 5A) before the adsorption of dye while after adsorption its surface is rough no pores and with fine particles on surface (**Figure 5B**). Similar results were reported when fungal biomass was used for the adsorption of Acid blue 161 [38] [39] [40]

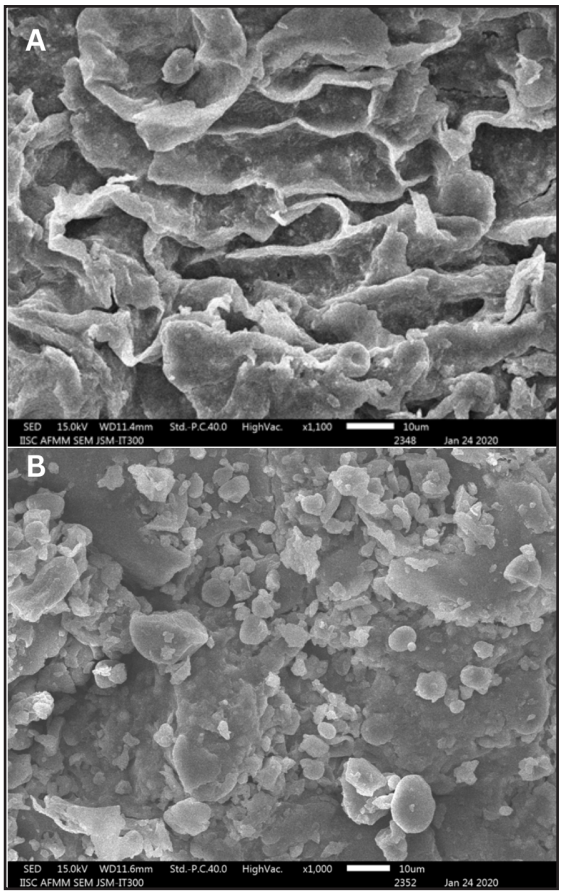

Figure 5**:** Micrograph of scanning electron 1000X (A- Fungal Biomass before dye adsorption, B- Fungal Biomass after dye adsorption)

HPLC analysis for malachite green degradation

The metabolites obtained before and after the malachite green dye degradation by *S. rolfsii* was concentrated and subjected to HPLC analysis at two different absorbance wavelengths of 680 nm and 280 nm. The absorption

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spectra of the samples (control and test) are presented in (fig.6). The HPLC elution profile of the dye malachite green (Control) showed single sharp peaks at 680nm with retention time (RT) of 4.5minutes (Figure 6B). The elution profile obtained for the fungal treated samples significantly differed from the control in terms of height of the peak obtained and retention time at 680nm. The HPLC profile of sample treated with fungi *S. rolfsii* showed one peaks with retention time 4.3 with reduced height of peak and shift in retention time clearly indicating the degradation of dye (Fig. 6A). The UV absorption spectrum at 280 nm for dye metabolite obtained after 16 days of incubation showed no peak at 680 nm

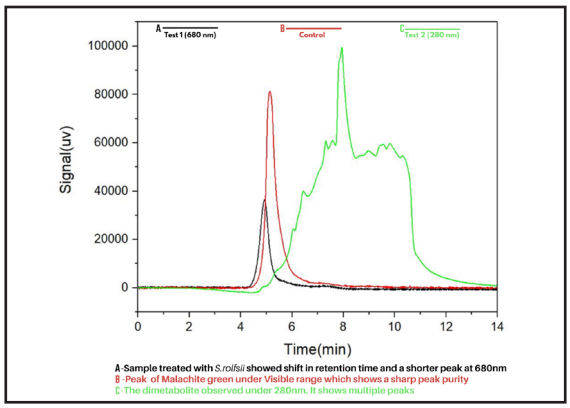

Figure 6**:** HPLC chromatogram of Malachite green and dye metabolites at 680nm and 280nm

but with multiple peaks at 280nm as shown in (Figure 6C). From these observations' microbial degradation and transformation of malachite green was confirmed.

Microbial toxicity studies of malachite green and metabolic intermediates

Toxicological effect of parental dye and their metabolites obtained after microbial degradation was studied. Bacillus subtilis was incubated with malachite green dye before and after dye degradation to ensure the degraded products are environmentally safe. A clear zone of inhibition was observed with parental Malachite Green indicating the toxic effect of azo dye while the dye metabolites showed no zone of inhibition. This proves the *S. rolfsii* had degraded the toxic dye to nontoxic metabolic intermediates. The products obtained after fungal dye degradation did not show any growth inhibition.

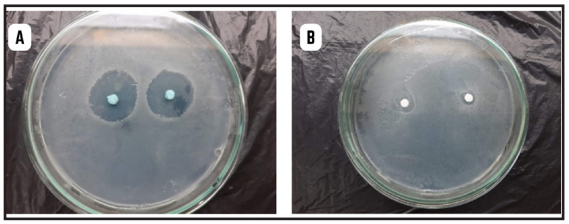

Figure 7. Toxicity study of dye before (A) and after (B) MG degradation

Conclusion

This study was done to explore the potential of *S. rolfii* as promising microorganism for bioremediation of Malachite Green and Rose Bengal the most extensively used textile dyes which has not been studied. Static batch culture system with higher concentration of dyes ranging from 100-900 mgL^{-1} were established to test degradation ability of the organism. Fungi exhibited excellent potential to degrade the dye at high concentration of 900 mgL-1 with 89% of degradation. *S. rolfsii* follows two different mechanisms for removal of dye like bioadsorption for Rose Bengal (anionic dye) and biodegradation for Malachite Green (Cationic dye). UV-Vis spectroscopy, XRD, FTIR and HPLC were the tools used to analyse the bioremediation of dyes. The degraded intermediate or transformed products were reported. Toxicity studies of degraded products exhibits no toxic effect on test microorganism. In this mycoremediation fungi employs two main mechanism of like biodegradation and biosorption of dyes and hence identifying such microorganisms with differential bioremediation approach will facilitate the dye decolourisation and detoxification of complex industrial effluents with various dyes. Fungi can be considered as best and reliable mediators of dye remediation.

Conflict of interest

Authors declare no conflict of interest

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