Biosorptive and Biodegradative Mechanistic Approach for the Decolorization of Congo Red Dye by *Aspergillus* Species

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Abstract

In this work, *Aspergillus terreus* GS28 and *Aspergillus flavus* CR500 isolated from industrial waste sludge examined for the decolorization of Congo red (CR) dye. The rate of CR decolorization raised due to optimum pH, temperature, carbon, nitrogen, and heavy metals. In the comparative study, *A. terreus* has the maximum ability (95%) to decolorize CR ($\approx$ 100 mg L^{-1}) as compared with *A. flavus* (92.96%) under optimized condition after 120 h. GC–MS and FTIR analysis of the fungal-metabolite and fungal-biomass shows bio-degradation and biosorption processes respectively. The degraded products were benzenepropanic (Rt-26.147), 3, 4-diaminonapthelene-1-sulfonic acid, and benzenedicarboxylic acid (Rt-26.660) by *A. terreus*, and benzenedicarboxylic acid (Rt-41.467) by *A. flavus*. The phytotoxicity assay revealed that a decrease in toxicity of the degraded product towards the growth and germination rate of two plant seeds compared to CR. Thus, the finding suggests that both the fungi act promising CR remediation candidates, induces restoration of CR polluted wastewater and save soil-land.

Keywords Biodegradation · Adsorption · Laccase · Manganese peroxidase · GC–MS analysis · Phytotoxicity

The indiscriminate disposal of chemical compounds in water bodies and soil adversely affects the whole biosphere (Bankole et al. 2018; Syafiuddin and Fulazzaky 2021). Synthetic dyes increased their attention because of their broad range of use in different sectors for coloring purposes (Tan et al. 2019). Among synthetic dyes, azo dyes have taken great attention due to their frequent use in the industrial sector because of their stability in nature and less cost production as other groups like anthraquinone dyes (Sen et al. 2016). Subsequently, azo dye contains an azo group of a bond (–N=N–) is joint with benzene or naphthalene rings (Mnif et al. 2016; He et al. 2018). About 10%–15% of dyes can be discharged from coloring industries in the aquatic environment which affects the light penetration, reduces photosynthetic activities, causes eutrophication and interference in the aquatic-ecosystem (Asses et al. 2018). It has an offensive impact on life-forms like allergy and their nature is carcinogens, toxic and mutagenic (Sen et al. 2016; Tan

et al. 2019). Benzidine-based anionic dye, CR (Congo red) is the most common and frequently used in textile industries (Mnif et al. 2016; Singh et al. 2021).

Several physicochemical techniques are available for the removal of dye but all the methods are so costly and also release a vast quantity of sludge by which their use is not beneficial at the economic level and not ecofriendly at the industrial level (Sen et al. 2016). The microbial bioremediation processes are ecofriendly suitable method for reducing CR azo dye in wastewater through its degradation in less toxic components (Syafiuddin and Fulazzaky 2021). In the decolorization of dye, fungi contain exclusive features as their biomass (live or dead biomass) is used as adsorption and their supernatant may use as the degradation process (Singh and Dwivedi 2020). Several researchers have investigated and revealed that the fungus have ability to degrade various type of azo dye including Congo red (CR) from waste water (Levin et al. 2010; Almida and Corso 2014; Zhang et al. 2020). Wang et al. (2017) illustrate that *Ceriporia lacerates* decolorize CR up to 90% at 100 mgL^{-1}. The fungal cell wall/ biomass can remove contaminants from environments through the adsorption process (Tan et al. 2019). The extracellular enzymes of fungi, such as laccase (lac), manganese peroxidase (MnP) and lignin peroxidase

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(LiP) etc. are also involved in the degradation of the azo dye (Wang et al. 2017; Asses et al. 2018; Iark et al. 2019).

Fungi can achieve maximum dye decolorization or degradation on different parameters such as pH, temperature, time, nutrient sources, dye concentration and carbon–nitrogen sources etc. (Singh and Dwivedi 2020). Keeping this fact, the present study aimed with (i) comparative study of CR decolorization by both fungi at optimized condition (ii) identify the mechanisms of decolorization (degradation/adsorption) process of CR by both native fungal isolates and (iii) phytotoxicity test to confirm the eco-toxicological effect of fungal dye degraded products.

Materials and Methods

Congo red (azo dye) was obtained from Sigma-Aldrich while further chemicals were procured from HiMedia, Pvt. Ltd., Mumbai, India. CR solution of 1000 mg L^{-1} was prepared, preserved at 4°C and used as a stock solution.

A. terreus GS28 was previously isolated from the mud of carpet industrial area (Singh and Dwivedi 2020) and *A. flavus* CR500 was isolated from wastewater of electroplating industries (Kumar et al. 2019). Both fungi were molecularly characterized and ITS region sequencing was submitted to genebank NCBI database under the accession number (isolate GS28-MK574833 and isolate CR500-MK450338).

Decolorization experiments at several concentration (100–1000 mg L^{-1}) and time interval (24-168 h) were conducted as illustrated by Singh and Dwivedi (2020) and by taking the absorbance of supernatants at $\lambda_{max} = 497$ nm using a spectrophotometer (SYSTRONICS Double beam spectrophotometer 2203). The final dye concentration was calculated (Chakraborty et al. 2013). However, after 168 h, the biomass was collected by filtering culture through Whatman filter paper No. 1 (Singh and Dwivedi 2020) and dried at 60 °C (6 h) followed by weighing on Weighing balance (Sartorius BT 224S). Percent decolorization concentration after treatment was determined by the following formula (Singh et al. 2021).

$$\text{Decolorization rate (\%)} = \frac{Ai - Af}{Ai} \times 100 \qquad (1)$$

where Ai = Initial dye concentration before treatment, Af = Final dye concentration after treatment.

To optimize the pH for better removal, the experiment was conducted at various pH (4.0, 6.0, 8.0, 10.0 and 12.0) at 30°C, 120 rpm, for 120 h and 800 mg L^{-1} of CR containing solution. pH was maintained by 0.1 M NaCl and HCl solution (Singh and Dwivedi 2020).

To examine the role of various temperature (25, 30, 35, 40, 45°C) on removal of CR was analyzed by incubating the both fungi in different conical flask containing CR (800 mg L^{-1}) amended in 50 mL of PDB medium. Temperature was maintained in thermostat incubator shaker (pH 7.0 and 120 rpm in shaking condition) for 120 h (Kumar and Dwivedi 2019).

The consequences of carbon and nitrogen sources on CR decolorization by fungi were analyzed using CR (800 mg L^{-1}) containing 50 mL of PDB medium supplemented with 0.25 g of different carbon (Glucose, Dextrose) nitrogen sources (Peptone, Yeast extract and Beef extract) (Singh et al. 2021). All the treatments were conducted in triplicates in the experimental condition at 30°C, pH 7.0, and 120 rpm for 120 h. Carbon sources were added in a concentration of 0.5% w/v each (Basak et al. 2013).

To analyze the effect of heavy metal on the percent decolorization (degradation and adsorption) of CR were performed. These metal salts ($MnSO_4$, $ZnSO_4$, $Pb(NO_3)_2$, $CdCl_2$ and $NiSO_4$) were used with single concentration of 100 mg L^{-1} supplemented with PDB medium added with single concentration of CR (800 mg L^{-1}) at 30°C, pH 7.0,120 rpm for 120 h (Chakraborty et al. 2013; Singh et al. 2021).

Among these heavy metals Mn (II) act as enhancer for the CR decolorization activity, so the various concentration of Mn (50, 100, 150 and 200 mg L^{-1}) were also tested to check the decolorization rate of CR. All experiments were conducted in triplicate and the percentage decolorization was calculated by Eq. (1).

The decolorization efficiency of CR by both fungi was investigated under optimum conditions. The optimized condition was selected from the above experiments. The decolorization assay with *A. flavus* was performed at pH 6.0 and 800 mg L^{-1} of CR using glucose (5 gL^{-1}) and Mn (100 mgL^{-1}) amended PDB and incubated at 30°C for 120 h in an incubator shaker at 120 rpm. The same conditions followed for decolorization with *A. terreus* except for temperature (35 °C) and pH (8.0) and the further process was performed as above discussed and decolorization percent was calculated by Eq. (1) (Singh et al. 2015). After 168 h treatment, biomass obtained from culture broth and dip into 50 ml of distilled water followed by autoclaving at 121°C and 18 psi for 15 min (Singh and Dwivedi 2020). OD of the obtained solution was taken at 497 nm. The adsorption amount was calculated as follows

$$Decolorization = Degradation + Adsorption \qquad (2)$$

To assess the reusability of obtained biomass, the biomass obtained at the treatment of only 100 mgL^{-1} of CR was rinsed with different types of solution (0.1 M HCl and NaOH solution) at room temperature for 1 h separately and subsequently rinsed by distilled water. Different solution rinsed biomass were used of decolorization of 100 mg L^{-1} of CR prepared in distilled water (30°C, pH 7.0, 48 h and

120 rpm in shaking condition) (Gautam et al. 2018). The adsorption intensity of extracted metabolites was taken at the wavelength 497 nm and decolorization percent was calculated using Eq. (1). Similar work was also performed with the HCl solution. After each chemical treatment process, the biomass was washed multiple times with distilled water until pH value become neutral (7.0) for the filtrate (Sen et al. 2016; Gautam et al. 2018). Then retreated fungal mycelia used for removal of CR dye with the same concentration (100 mg L^{-1}) in 50 ml of an aqueous solution for 48 h. The fungal mycelia were recycled up to two times under the same environmental condition at shaking condition with 120 rpm.

To investigate the role of enzyme on dye decolorization, fungi (GS28 and CR500) were grown in different concentrations of CR (100, 500 and 1000 mg L^{-1}) containing PDB medium. After 120 h, treated and untreated culture were filtered and kept at 4°C for further analysis. After decolorization, the extracted metabolites were used as crude enzyme extract to examine the extracellular enzyme and obtained biomass used for H$_2$O$_2$ and protein content estimation which involved during dye decolorization by fungi (*A. terreus* and *A. flavus*). For the determination of laccase activity, guaiacol was used as a substrate. The laccase activity was determined as described by Srinu et al. (2017) and the value presented in Umin^{-1} mg protein^{-1}(Adnan et al. 2014).

The manganese peroxidase (Mnp) activity examined on the basis of Srinu et al. (2017). By using, a spectrophotometer (Systronic, 2203), the absorbance was taken at the wavelength of 465 nm and the activity of MnP was computed. Bovine serum albumin used as a standard for Protein content (Lowry et al. 1951) and H$_2$O$_2$ content was determined by Velikova et al. (2000).

For FTIR analysis, CR (200 mg L^{-1}) treated and untreated fungal biomass (*A. terreus* and *A. flavus*) was filtered by filter paper (Whatman No.1) and oven-dried for 6 h at 60°C. Obtained dried fungal biomass was crushed with the help of mortal-pestle and mixed with a 1:100 (w/w) ratio of the KBr (potassium bromide). In the chamber of the sample cabin, the obtained pellets were fixed and examined by the FTIR spectrometer (Thermo Scientific Nicole 6700, USA). The FTIR spectra of the sample were scanned in IR wavelength ranged from 400 to 4000 cm^{-1} (Bankole et al. 2018).

To extract the metabolites for GC–MS analysis, sample were prepared as described He et al. (2018). The extracted product was further dried using a rotatory evaporator. The obtained residues were passed in GC- vial and dried by nitrogen gas as well as suspend in a little amount of methanol which is related to HPLC grade. GC–MS analysis was performed by Gas chromatography-mass spectroscopy [Shimadzu QP-2010 plus with Thermal Desorption System TD 20] (Pandi et al 2018; Singh and Dwivedi 2020).

A phytotoxicity test was conducted to determine the toxicity of fungal treated and untreated CR (100 mg L^{-1}) dye.

The seeds of *Solanum lycopersicum* (tomato) and *Triticum aestivum* (wheat) were used to analyze the phytotoxicity test. Seeds were surface sterilized using 10% H$_2$O$_2$ content for ten minutes and rinsed with autoclaved distilled water (Srinu et al. 2017). Twenty seeds of each plant were dispersed on Petri-plate at a similar distance and poured with the same volume (10 mL) of autoclave distilled water, untreated CR (100 mg L^{-1}) solution and fungal treated (100 mg L^{-1}) solution separately. The plates were watered on alternate days with 10 mL of sterilized water and fungal treated as well as untreated CR solution and incubated at room temperature. After eight days, the root and shoot length of seedlings was measured and the percent germination rate of seed was calculated by Eq. (3) (Kannan and Upreti 2008).

$$Germination\ rate = \frac{Number\ of\ seeds\ germinated}{Total\ number\ of\ seeds} \times 100$$

(3)

Each experiment was performed in three-replicates to enhance the analytical accuracy of data. Therefore One Way Analysis of Variance (ANOVA) was used to analyze the obtained data followed by Duncan Multiple Range Test (DMRT; $p \leq 0.05$) (post–hoc test) by SPSS software (version 20.0).

Results and Discussion

In this work, Congo red (CR) decolorization efficiency by *A. terreus* and *A. flavus* were investigated. Both the fungi play a crucial role in CR decolorization from lower concentration to higher concentration (100–1000 mg L^{-1}) within 168 h at shaking condition of 120 rpm and results are shown in Fig. 1a. Maximum decolorization activities were recorded at 100 mg L^{-1} of CR (95% – *A. terreus* and 92.96% – *A. flavus*) by both the fungi. The decolorization rate was decreased drastically ($p < 0.05$; DMRT) with an increase in CR concentration up to 1000 mg L^{-1} and was reduced to 78.18 and 73.81% by *A. terreus* and *A. flavus* respectively. The fungal biomass and dye concentration was opposite to each other as dye concentrations were increased the fungal biomass was decreased from 100 to 1000 mg L^{-1} of CR (Fig. 1a). Result indicated that both fungi might feel stress condition at higher concentration by which their growth was inhibited and also released few extracellular enzymes (such as manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase) which involved in the CR decolorization via degradation (Table 1) (Srinu et al. 2017). Both fungi were used for the study but *A. terreus* was more efficient as compare to *A. flavus* for decolorization of CR. For the decolorization of dye, both (adsorption and degradation) mechanism were involved (Fig. 1f). The growth of fungal strain was reduced as dye concentration was increased because of increasing
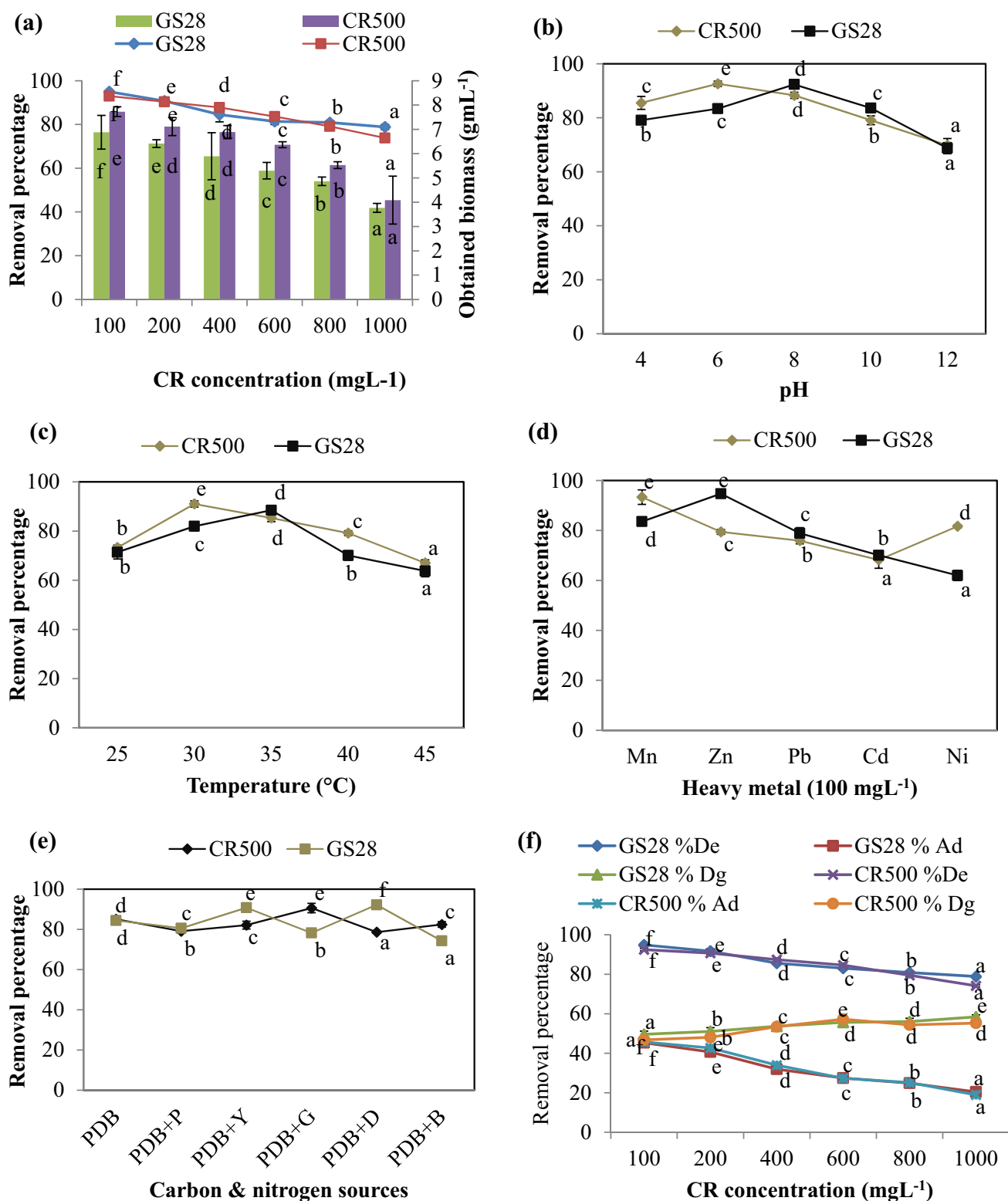
Fig. 1 **a** Percent removal of CR (100–1000 mgL^{-1}) and Obtained biomass (gmL^{-1}) after decolorization by *Aspergillus flavus*-CR500 and *Aspergillus terreus*-GS28 after 168 h **b** Percent removal of CR (800 mgL^{-1}) at different pH **c** at several temperature **d** different heavy metal with single concentration (100 mgL^{-1}) **e** Percent decolorization of CR (800 mgL^{-1}) under the various concentration of Carbon and nitrogen sources (PDB, PDB+P, PDB+Y, PDB+G, PDB+D and PDB+B) with the concentration of 5 gmL^{-1} **f** *De* decolorization, *Ad* adsorption and *Dg* degradation percent of CR at several concentration by *A. flavus* and *A. terreus*. Bar above the graph denotes standard deviation (n=3). Same letters above the graph denotes no significant differences using DMRT ($p \leq 0.05$)

Table 1 Effect of different dye concentration on the activity of Laccase and Manganese peroxidase and the level of protein and H_2O_2 content (Different letter above the values (n=3) denotes significant differences within the variables using DMRT ($p \leq 0.005$))

Dye concentration (mgL^{-1})	Laccase (U min^{-1} mg protein^{-1})	Manganese peroxidase (Umin^{-1} mg protein^{-1})	Protein (mg g^{-1} FW)	H_2O_2 content (nM g^{-1} FW)
A. terreus GS28				
0	0.924 ± 0.011[a]	0.454 ± 0.033[d]	0.444 ± 0.017[c]	0.796 ± 0.037[a]
100	1.118 ± 0.016[b]	0.380 ± 0.013[c]	0.415 ± 0.023[c]	1.304 ± 0.173[b]
500	1.265 ± 0.028[c]	0.267 ± 0.029[b]	0.35 ± 0.041[b]	1.512 ± 0.130[c]
1000	1.514 ± 0.022[d]	0.163 ± 0.019[a]	0.268 ± 0.021[a]	2.049 ± 0.057[d]
A. flavus CR500				
0	1.161 ± 0.053[c]	0.448 ± 0.032[a]	0.298 ± 0.013[b]	0.817 ± 0.016[a]
100	1.263 ± 0.047[d]	0.524 ± 0.018[b]	0.365 ± 0.017[c]	1.160 ± 0.026[b]
500	1.095 ± 0.018[b]	0.625 ± 0.023[c]	0.266 ± 0.038[b]	1.599 ± 0.038[c]
1000	1.05 ± 0.022a	0.757 ± 0.025[d]	0.165 ± 0.028[a]	2.064 ± 0.059[d]

stress and the degradation process become slower due to a reduction in the number of enzymes (Wang et al. 2017; Tan et al. 2019). Similarly, the dye decolorization in a liquid medium with different fungi has also been reported in several works (Sen et al. 2016; Bankole et al. 2018; Syafiuddin and Fulazzaky 2021). The decolorization capacity of GS28 and CR500 was higher than *Alternaria alternata* CMERI F6 for which the decolorization efficiency was 78% at 800 mg L^{-1} (Chakraborty et al. 2013). Similar work has been done in the report of Bosco et al. (2016) but their decolorization efficiency of CR was less than *A. terreus*. Both fungi have enormous differences for decolorization of CR by their enzymatic activity and adsorption rate.

The removal rate of CR was influenced by various pH (4.0, 6.0, 8.0, 10.0 and 12.0) observed in the present study. The rate of CR decolorization and obtained biomass was affected by the pH of the CR-containing solution (Chakraborty et al. 2013). The highest decolorization of CR 800 mg L^{-1} by *A. terreus* was showed at the pH of 8.0 was recorded which corresponds to 92.35% and *A. flavus* treated showed maximum decolorization (92.60%) at 6.0 pH at the temperature of 30°C, 120 rpm within 120 h (Fig. 1b). An interesting observation was found in this study that maximum CR (800 mg L^{-1}) decolorization was observed at slightly acidic (6.0) for *A. flavus* and slightly alkaline (8.0) condition for *A. terreus* and this could happen due to copping behavior of fungi. The pH of the medium is a critical environmental factor to affect the azo biodegradation (Asses et al. 2018; Singh et al. 2021) and the degradation of dyes depends on [H$^+$] ions (Basak et al. 2013). During the adsorption process, the structure of the dye molecule and mycelial surface charge is also affected by pH value. The pH value and isoelectric point of mycelia interacted with each other. As mycelia surface showed negative charges which reflect the pH value is more than the isoelectric point of mycelia and when mycelia surface shows positive charges which reflect the pH value is less than isoelectric point of mycelia (Basak et al. 2013). A strong acidic or alkaline condition,

decolorization rate was slow due to the existence of the same charge on mycelia and dye molecule that cause repulsion between them (Asses et al. 2018). The pH values from 6.0 to 8.0 were more suitable for better decolorization (Wang et al. 2017).

Temperature is a most influencing factor that affects the growth of fungi and the rate of degradation/adsorption mechanism (Adnan et al. 2014). The effects of various temperatures (25 to 45°C) were examined for CR decolorization at 800 mg L^{-1}. Both fungi can decolorize CR up to 35°C while maximum CR decolorizes were 88.48% at 35°C by *A. terreus* and 91.02% by *A. flavus* at 30°C (Fig. 1c). An increase in temperature above 35°C, the decolorization ability of both fungi was reduced due to a decrease in their growth, biomass as well as metallic activity. The result showed that optimum temperature (25–35°C) was favorable for dye decolorization by fungi. However, optimum temperature plays an important role to enhance the microbial metabolic activity and their growth by which metabolic activity constantly depends on the enzymatic activity of microbes (Sen et al. 2016). Thus in the adsorption process, dye molecule surface and kinetic energy are enhanced by optimum temperature and in the degradation mechanism, optimum temperature involved in the increasing production of enzymes that participate in dye decolorization (Zhang et al. 2020). It showed that a high temperature reduces cell viability and inactivation of enzyme which is responsible for CR decolorization (Singh and Dwivedi 2020).

The CR decolorization activity in 50 mL of PDB medium supplemented with different carbon and nitrogen sources (Yeast extract, Peptone, Beef extract, Dextrose, and Glucose) was studied with a single concentration of (800 mg L^{-1}) CR at the temperature of 30 °C, pH 7.0 and 120 rpm for 168 h. PDB supplemented with glucose medium showed maximum (90.59%) decolorization while PDB supplemented dextrose showed minimum (78.61%) decolorization by *A. flavus* and PDB supplemented with dextrose showed maximum decolorization (92.19%) and beef extract showed

less (74.28%) decolorization by *A. terreus* (Fig. 1e). The result of the present work showed that among all carbon and nitrogen sources, glucose is an excellent carbon source for the removal of CR by both fungi. Usually, carbon sources increase the growth of microbe and relocate the reducing equivalents to the dye for the cleavage of –N=N– (azo bond) (Kumar et al. 2009; Zhang et al. 2020) while less amount of carbon–nitrogen sources in PDB medium allow the slow rate of biomass production and decrease fungal metabolic activity which is also essential for the dye degradation (Kumar et al. 2009). Degradation/decolorization process of azo dye by microbe depends on the types and presence of carbon–nitrogen sources due to their activities which play the role of electron donor in the azo dye decolorization process (Singh et al. 2021).

The existence of heavy metals in the PDB medium affects the rate of CR decolorization extensively at the level of $p \leq 0.05$ followed by DMRT (Fig. 1d). However, the chosen metals were Mn(II); $MnSO_4$, Zn(II); $ZnSO_4$, Pb(II); $Pb(NO_3)_2$, Cd(II); $CdCl_2$ and Ni(II); $NiSO_4$ used to investigate the percent decolorization ability with 100 mgL^{-1} single concentration. *A. flavus* decolorized maximum CR (93.33%) with the presence of Mn and less decolorization showed with Cd- 68.21% and Ni- while *A. terreus* decolorized maximum under the presence of Zn (94.71%) and least decolorization (61.79%) occurred with the presence of Ni (Fig. 1d). Results exposed that the decolorization via degradation of CR azo dye was enhanced in the presence of manganese which might be due to Mn(II) induced MnP activity (Gao et al. 2010; Singh and Dwivedi 2020). In general, the toxicity of heavy metal present in wastewater can influence the fungal growth and decolorization ability (Kumar et al. 2019). Cd and Ni also have been reported to cause toxicity to the fungi by inducing reactive oxygen species production that possibly reduces the growth of *A. terreus* and *A. flavus* which led to a decrease in CR decolorization (Kumar et al. 2019). While in the present study, isolate *A. terreus* and *A. flavus* have a proficient ability to decolorize CR with the presence of some specific heavy metal (Mn(II) and Zn(II)). Results demonstrated that both fungi can be used for the handling of dye-containing wastewater co-contaminated with heavy metal.

In the present study, different concentration (50, 100, 150 and 200 mg L^{-1}) of Mn(II) was also used to check the maximum decolorization rate of CR (Fig. S2a). Maximum decolorization (93.66% and 90.52%) showed in PDB supplemented with 100 mg L^{-1} of Mn by *A. flavus* and 150 mg L^{-1} by *A. terreus*. While as increasing concentration of Mn decrease the decolorization rate of CR (76.96% by *A. flavus* and 71.76% by *A. terreus* at 200 mg L^{-1} of Mn). Increasing concentration of heavy metal inhibits the growth and enzymatic activity of microbe by which decolorization rate is also affected (Chakraborty et al. 2013; Singh et al. 2021).

Under optimum conditions, both fungi (*A. terreus* and *A. flavus*) illustrated better results within 120 h (Fig. S2b). The decolorization of CR (800 mgL^{-1}) by *A. terreus* was at 35°C, pH 8.0, 5 gm L^{-1} of dextrose and 150 mg L^{-1} of Mn in shaking condition for 120 h and the decolorization rate of CR was 96.40%. However the optimum condition for luxurious growth and highest decolorization rate of CR by *A. flavus* was at 30°C, pH 6.0, 5 gm L^{-1} of glucose and 100 mg L^{-1} of Mn in shaking condition for 120 h and the decolorization rate was 95.24%. Same work has been reported by which showed that maximum decolorization (> 90%) and 99.9% decolorization by *Alternaria alternata* under optimum condition (Chakraborty et al. 2013) Optimum condition is better for the growth of microbes, enzymatic activity and it increases both adsorption and degradation process for excellent decolorization activity (He et al. 2018).

Incubation time is the most influencing factor for decolorization (adsorption and degradation) of dye and biomass production (Asses et al. 2018). Results revealed that as contact time of CR with both (*A. terreus* and *A. flavus*) fungi increase than their rate of decolorization increase due to their increasing biomass and enzymes involvement (Fig. S2b). After 24 h, 800 mg L^{-1} of CR decolorized 40.62% by *A. terreus* and 34.66% by *A. flavus* under optimized condition while after 120 h, CR decolorize up to 96.40% by *A. terreus* and 95.24% by *A. flavus*. Both fungi play a unique role in CR decolorization with regard to incubation time. Incubation time and shaking conditions amplify the mixing of oxygen which exists in the PDB medium thus helps to promote the growth of fungi for decolorization (Kumar et al. 2009; Tan et al. 2019). Time course is also important for the increase in the enzymatic activity, metabolic rate, (laccase, MnP and liP) and biomass content for degradation and adsorption of CR.

The finding of the present work examined that adsorption via degradation process involves dye decolorization by both (*A. terreus* and *A. flavus*) fungi. Adsorption of CR by *A. flavus* was 45.70% at lower concentration (100 mg L^{-1}) and 18.91% at higher concentration (1000 mg L^{-1}) of CR (Fig. 1f) and almost similar results were recorded for CR adsorption by *A. terreus* (Fig. 1f). In the report of Sen et al. (2016), Biosorption activity depends on the functional group and cell surface area of fungi which have a great correlation with their adsorption mechanism (Zhang et al. 2020). Several reports highlighted that fungal biomass is involved as an adsorption process in the removal of dye (Almida and Corso 2014; Wang et al. 2017). In microbes, fungal cell walls has charged functional groups (active sites) of different varieties such as carboxyl (–COOH), hydroxyl (–OH), phosphate (PO_4^{3-}) and others. Heteropolysaccharide releases by fungi (Mahmud et al. 2021), which play important role in the adsorption process for dye decolorization (Vitor and Corso 2008). The amount of fungal biomass decreased

with increasing concentration of dye which reflects that the adsorption rate might be decreased due to less availability of surface functional group in fungi and reduces CR adsorption (Cheng et al. 2012; Syafiuddin and Fulazzaky 2021).

In the present degradation process also involved in CR decolorization as concentration increases degradation process increased (100–1000 mg L^{-1} = 46.76–55.23% by A. flavus and 49.55–58.42% by A. terreus) compared to adsorption process (Fig. 1f). The changes found after the decolorization process specified the transformation of new products which is formed after the degradation process by enzymes. The increasing concentration of dye increase laccase activity and decrease MnP activity (Table 1) and which indicates that degradation was also involved in decolorization of CR by A. terreus and A. flavus. During the biodegradation process, fungi released enzymes (azo-reductases, peroxidases and laccases) which have great concern for the breakdown of azo bond and aromatic ring which is present in azo dye (Singh and Dwivedi 2020). The biodegradation process involves the change of additional organic molecules without a change in the main structure of the dye compound as well as a breakdown of the complex dye molecules in mineral forms (Asses et al. 2018).

For the reusability assay, a block of 5 mm diameter of fungal culture was used to inoculate the CR (100 mg L^{-1}) amended PDB medium and incubated for 168 h at 30°C, pH 6.0. The filtered biomass was again used for decolorization at the concentration of 100 mg L^{-1} of CR. The regeneration study of fungal biomass (A. terreus and A. flavus) demonstrated good results by desorption with 0.1 M of NaOH in comparison to 0.1 M HCl. It might be occurred due to the acidic nature of CR dye which makes that it highly soluble in alkaline solution. CR Adsorption was to be 71.40% and 46.86% by CR500 and 68.03% and 44.13% by A. terreus in 2nd and 3rd reusability cycle respectively by treating with 0.01 M NaOH solution as a desorption agent. However, 67.62% and 42.59% decolorization was recorded by A. flavus and 65.33% and 45.72% by A. terreus in the 2nd and 3rd cycle with the treatment of 0.1 M HCl solution (Table S1). A similar reusability essay has been done in the previous report (Wang et al. 2017) where only II cycle of regenerative ability of mycelia had been done for dye treatment while in this study III cycle of reusability of fungal biomass for CR decolorization has been done. The nature of azo dye reflects the desorption ability which adheres to the adsorbent surface (Tan et al. 2019). Formation of the weak bond (such as hydrogen bond and ionic bond) among dye and adsorbent may conduct to simply and higher desorption (Syafiuddin and Fulazzaky 2021).

High laccase and low MnP activity were detected at the end of degradation of the CR after 168 h treated with A. terreus while low laccase and high MnP activity were found after degradation of CR treated with A. flavus. In this study,

laccase enzyme increased (1.118 ± 0.016, 1.265 ± 0.028 and 1.514 ± 0.022 U min^{-1} mg protein $^{-1}$) with increasing concentration of CR (100, 500 and 1000 mg L^{-1}) treated by A. terreus and decrease with increasing concentration of CR treated with A. flavus showed in Table 1. Results revealed that both fungi degrade CR dye but their enzymatic activities changed because of dye nature and behavior of fungi. Synthetic dye degradation by releasing oxido-reductases like laccase, MnP and LiP enzymatic activity through fungi has been highlighted in various research papers (Pandi et al. 2018; He et al. 2018). Literature supported that fungi have extensive reaction potential as well as wide substrate specificity together with no conditions for co-factor by which they degrade azo dye due to their enzymatic activity (Cheng et al. 2012).In this investigation, extracellular (laccase) enzyme effectively involved in the degradation of CR by GS28 and Mnp by CR500 which demonstrated that Parent CR dye compound change in the less toxic compound by oxido-reductase enzymes (Singh and Dwivedi 2020). If the degradation of CR done by laccase enzyme, it directly cleavage the azo groups by non-specific free radical mechanism and no aromatic amines formation occurred after cleavage of azo bond (Zhang et al. 2020). Similarly, MnP enzymes occurred in the family of ligninolytic peroxidase which also able to mineralize azo dye by redox reaction and oxidize phenolic compound due to alteration of Mn^{2+} in Mn^{3+} (Sen et al. 2016). Whereas there are few reports found that under in vitro decolorization studies, two or more ligninolytic enzymes are involved in which laccase possibly collaborate with MnP for dye decolorization (Levin et al. 2010; Srivastava et al. 2019).

In the present investigation, the secretion of extracellular protein was decreased with increasing concentration of CR by both fungi (Table 1). The decreasing concentration of protein may be due to the stress condition in fungi with increasing concentration of dye (Adnan et al. 2014). The production of H_2O_2 indirectly interlinked with the enzymatic activity which is involved in the decolorization process of CR. H_2O_2 content (1.304 ± 0.173, 1.512 ± 0.130 and 2.049 ± 0.057 nM g^{-1} FW by GS28 and 1.160 ± 0.026, 1.599 ± 0.038 and 2.064 ± 0.059 nM g^{-1} FW by CR500) increased with increasing concentration of CR (100,500 and 1000 mg L^{-1}) treated with both fungi (Table 1). Production of enzymes (laccase and MnP) which is involved in the decolorization process was too high or low due to fluctuation in the concentration of H_2O_2 in the PDB medium (Kumar et al. 2009; Srivastava et al. 2019). It plays a unique role in the redical generation process which involves in the lignocellulose degradation process (Singh and Dwivedi 2020). A high quantity of H_2O_2 required as a co-factor for peroxidase action and however, sometimes it can also inhibit the enzymatic activities and a low quantity of H_2O_2 support to dye decolorization by the enzyme (Pandi et al. 2018).

Table 2 FTIR spectral band of CR treated and untreated fungal biomass with both fungus (GS28 and CR500) and their assigned functional group

A. Terreus GS28		A. flavus CR500		Assigned functional group
Untreated fungal biomass (cm^{-1})	CR Treated fungal biomass (cm^{-1})	Untreated fungal biomass (cm^{-1})	CR Treated fungal biomass (cm^{-1})	
3442.5	3441.4	3376.9	**3283.6**	–O–H Stretching vibration and –N–H stretching of protein or acetamide groups of chitin fraction
3009.9	**2924.2**	2927.8	2926.3	=C–H stretch
–	–	2362.2	–	P–H stretching
2654.2	**2853.7**	–	–	C–H stretching
2270.7	–	–	–	C≡C stretching of unsaturated fatty acid
1745.8	1742.3	–	–	Stretching of C=O group, enol form
1650.5	1647.6	1649.4	1653.1	C=O stretching
1556.3	1552.8	1550.7	1541.8	Ring stretching
1459.5	–	–	**1449.0**	C–H bend at stretching
1413.6	1412.6	1408.9	–	C–N stretching (Amide III band)
1374.2	1374.3	–	**1377.0**	CH_3 sym deformation
1314.8	1312.5	–	–	CF_3 antisym stretching
1235.2	-	1236.7	1221.1	C–N stretching
1158.0	1155.3	1149.8	–	C=S stretching
1081.6	1077.1	1077.4	–	C–N stretching vibration of amine groups
1033.7	1038.2	1038.2	1036.4	C–N stretching vibration of the chitin-chitosan and protein
–	–	885.3	–	C–H out of plane deformation
721.3	720.0	–	–	CH_2 rocking in methylene chains
602.6	**636.9**	605.2	**537.6**	SO_2 deformation

Bold values denotes change in spectral band as compared to untreated biomass and

"–" represents the spectral bands were not detected

To encounter the role of the functional group of fungal (*A. terreus* and *A. flavus*) mycelia surface in decolorization of CR, FTIR analysis was done. The results of the FTIR investigation of CR treated and without treated biomass of fungi (control) are shown in supplementary Fig.S3 and the detected peaks and their assigned functional group are listed in Table 2. FTIR result demonstrated the association of surface-active site (functional groups) in the biosorption of CR on the fungal cell surface. The major peaks were found at wave number 2270.7 cm^{-1}, 1459.5 cm^{-1}, 1235.2 cm^{-1} before CR biosorption in mycelia biomass corresponds to the C≡C stretching of unsaturated fatty acid, C–H stretching and C–N stretching respectively (Kumar and Dwivedi 2019) which completely disappeared from CR treated mycelia biomass of *A. terreus*. Azo dye biotransformation into several metabolites was confirmed by the disappearance of above mentioned major peaks (Singh and Dwivedi 2020). The peaks 3009.9 cm^{-1} (=C–H stretch), 2654.2 cm^{-1} (C–H stretching modes), 602.6 cm^{-1} (SO_2 deformation) were shifted to 2924.2 cm^{-1}, 2853.7 cm^{-1} and 636.9 cm^{-1}. Some minor shifts in the bands were also observed at 1745.8 cm^{-1}, 1650.5 cm^{-1}, 1081.6 cm^{-1} and 1033.7 cm^{-1} which were shifted to 1742.3 cm^{-1}, 1647.6 cm^{-1}, 1077.1 cm^{-1} and

1038.2 cm^{-1} respectively for CR loaded biomass of *A. terreus*. No new peak was observed on CR treated mycelia of *A. terreus* biomass. However, few major IR peaks 2362.2 cm^{-1} (P–H stretching), 1408.9 cm^{-1} (C–N stretching of Amide III band), 1149.8 cm^{-1} (C=S stretching), 1077.4 cm^{-1} (C–N stretching vibration of amine groups) were not detected on CR treated mycelia of CR500 while high-intensity peak was detected on CR untreated biomass. Two new peaks (at 1449.0 cm^{-1} and 1377.0 cm^{-1}) also occurred in CR treated biomass of CR500 which correspond to CH_3 and CH stretching. These peaks may belong to the adsorb CR molecules. New peaks formation and vanishing of major IR peaks may be acknowledged the mineralization of parent (dye molecules) compound (Wang et al. 2017). The obtained results revealed that *A. flavus* biomass was more effective for surface sorption of CR as compare to *A. terreus* because *A. flavus* biomass treated new peaks that affect the parent compound of CR. The disappearance of the peaks also might indicate the C-N bond breaking and deformation of ammonium ion (NH_4^+) (Bankole et al. 2018).

To examine the degradation pattern and metabolites produced during the degradation pathway of CR by *A. flavus* and *A. terreus* were investigated by GC–MS analysis.
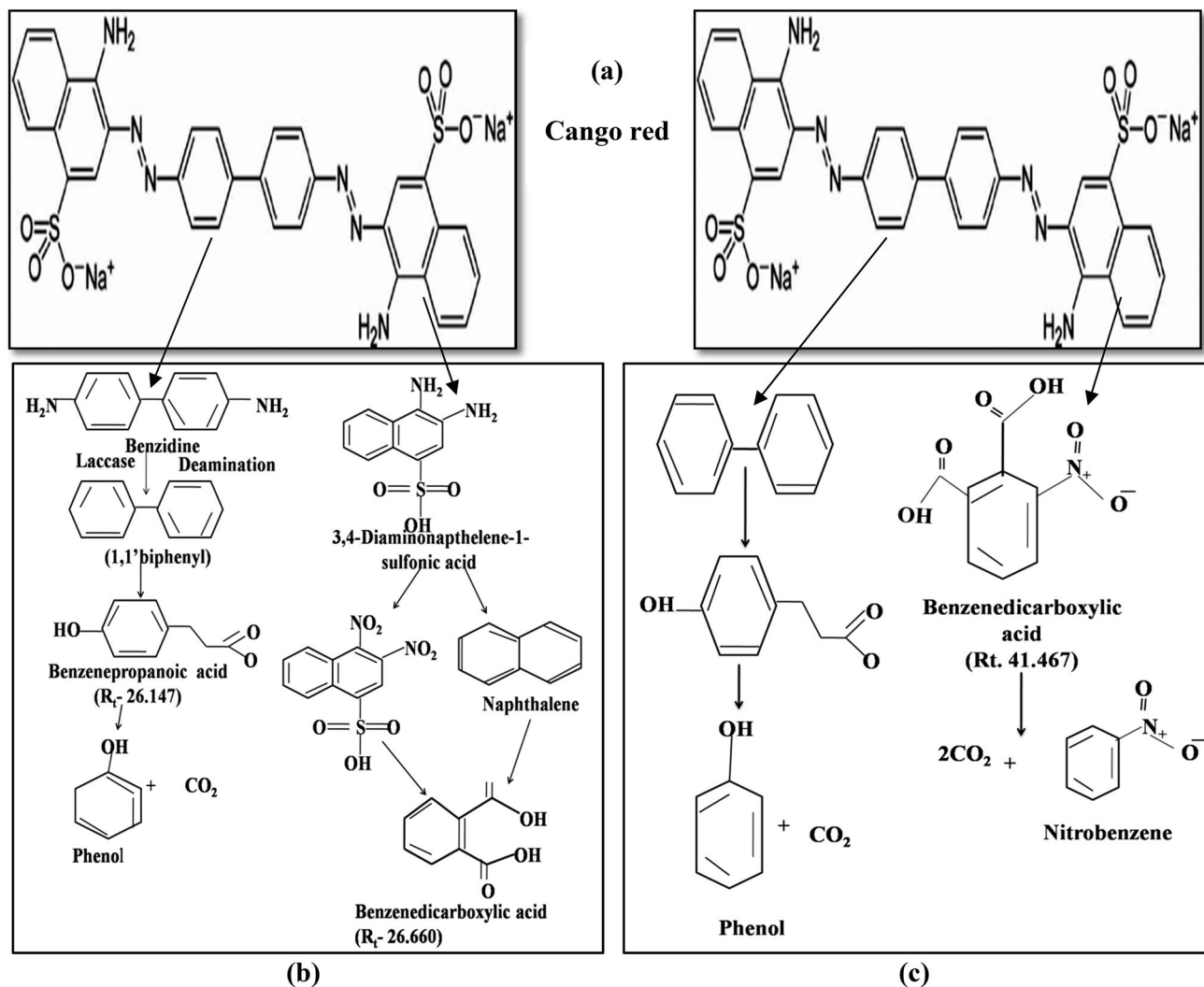
Fig. 2 **A** Congo red dye structure **B** Proposed biodegradation mechanism of CR by *A. terreus*-GS28 as analyzed by GC–MS investigation **C** Proposed biodegradation mechanism of CR by *A. flavus*-CR500 as analyzed by GC–MS investigation

The dye degraded metabolites were analyzed by GC–MS investigation based on m/z values. The chromatogram of GC–MS analysis has demonstrated the existence of several peaks, fragmentation patterns in the degraded metabolites (Fig. S4). The intermediate products benzidine and 3,4-diaminonapthelene-1-sulfonic acid were found in the degradation pathway of CR by *A. terreus* and similarly, several intermediate products were found in the degradation of CR by *A. flavus* (Fig. 2b, c). The end products were phenol and benzenedicarboxylic acid (Rt-41.467) by *A. terreus* and phenol and nitrobenzene by *A. flavus*. In the degradation of CR dye, the first step is the cleavage of the azo bond by releasing enzymes such as laccase and MnP. CR has a maximum functional group by which the biodegradation process is so tough and several intermediates may be released(He et al. 2018). Benzidine and Napthylamine both are extremely toxic for the organism by which they further degraded in harmless

products which are beneficial for aquatic life (Zhang et al. 2020). Two types of the pathway (Symmetrical and asymmetrical) are involved in the cleavage of azo bond based on peroxidase degradation of sulfonated azo dyes (Bosco et al. 2016). Amino groups are not found at any step in asymmetrical cleavage while in symmetrical cleavage amino groups exists at one or multiple-step (He et al. 2018). In the present investigation, asymmetrical cleavage of CR happened to exists with the treatment of *A. flavus* however, symmetrical cleavage occurred with the treatment of *A. terreus*. Several researchers have reported the CR degradation pathway by GC–MS (Bankole et al. 2018).

For the assessment of the toxicity of dye degraded product, the Phytotoxicity test was conducted using used to evaluate the toxicity of fungal treated dye solution and pure dye solution using *S. lycopersicum* and *T. aestivium* seeds. The result of this work is given surety that uneven

Table 3 Phytotoxicity test (in terms of germination rate and growth) of fungal treated and without treated 100 mgL^{-1} of CR solution

Parameters	Solanum leucopersicum				Triticum aestivum			
	DW	CR (100 mgL^{-1})	CR500 treated 100 mgL^{-1} of CR solution	GS28 treated 100 mgL^{-1} of CR solution	DW	CR	CR500 treated 100 mgL^{-1} of CR solution	GS28 treated 100 mgL^{-1} of CR solution
Germination rate (%)	90	50	80	75	95	45	75	84
Root length (cm)	2.36 ± 0.13	1.8 ± 0.11	2.15 ± 0.22	2.28 ± 0.18	6.73 ± 1.42	2.18 ± 0.43	3.18 ± 0.27	4.13 ± 0.97
Shoot length (cm)	3.56 ± 0.31	2.31 ± 1.11	1.16 ± 0.21	2.06 ± 0.25	4.58 ± 1.13	1.80 ± 0.24	2.38 ± 0.60	3.44 ± 1.26

discharge of these substances may not give a negative impact on the aquatic system due to check their phytotoxicity test (Table 3). Similar work has been done in the previous report (Almida and Corso 2014; Singh et al. 2021) with the same fungi for degradation of Procion Red MX- 5B which gave a negative impact on the aquatic ecosystem due to their highly toxic degraded product as compared to pure dye. In the present study, in the control treatment and fungal treated dye solution the germination rate (90%, 80%, 75% for *S. leucopersicum* and 95, 75, 84% for *T. aestivum*), root length and shoot length was higher as compared with pure dye treatment (Table 3). According to Nouren and Bhatti (2015) GI values the toxicity of fungal degraded metabolites was less as compared to parent dye after the treatment with *A. terreus* and *A. flavus*.

Two fungi (*A. terreus* and *A. flavus*) were investigated in this study for decolorization (adsorption and degradation) of Congo red dye which was isolated from wastewater. Both fungi have efficient decolorization ability for CR up to the higher concentration (1000 mg L^{-1}) under optimized condition (pH 8.0, 35°C, PDB + Glucose and Mn(II) for *A. terreus* and pH 6.0, 30°C, PDB + glucose and Mn(II) for *A. flavus*). Extracellular enzymes (laccase and MnP) are engaged in the decolorization via degradation of CR by both fungi. The role of adsorption of CR was confirmed by FTIR analysis and degradation of CR has been investigated by GC–MS analysis. The degraded product was benzenepropanic (Rt-26.147), 3, 4-diaminonapthelene-1-sulfonic acid and benzenedicarboxylic acid (Rt-26.660) by *A. terreus*, and benzenedicarboxylic acid (Rt-41.467) by *A. flavus*. In the phytotoxicity study, good germination rate and growth of the seedlings confirm those degraded dye products showed less toxicity as compared to crude dye (CR). Therefore, both fungi have high CR decolorization capability via degradation and adsorption mechanism and can be potentially applied in the removal of CR contaminated industrial effluent in a safe and environmentally pleasant way.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Approval This manuscript does not contain any studies with animals performed by any of the authors.

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