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Decolorization of heterocycle dye Neutral Red by white-rot fungus *Perenniporia subacida*

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ABSTRACT

A white-rot fungus *Perenniporia subacida* was developed for decolorizing twelve structurally various dyes from anthraquinone, azo, heterocycle, thiazine, and triphenylmethane groups. Among them, heterocycle dye Neutral Red with preferable decolorization performance was selected for further experiments and this strain could be utilized sequentially for three consecutive cycles with declining decolorization (%). After a 10-day incubation period, higher dye uptake (96.56%) was obtained at constant inoculum and agitation speed with the optimum physicochemical parameters like initial pH at 4.0, temperature at 35°C, initial dye concentration at 100 mg/L, and ionic strength at 0.1 mol/L. Noteworthy induction of various dye decolorizing enzymes viz. lignin peroxidase, laccase, manganese peroxidase, tyrosinase, and nicotinamide-adenine dinucleotide hydrogen-2,6-dichlorophenol indophenol reductase compared to control, point out toward their involvement in overall decolorization and degradation process. Analytical studies like fourier transform infrared spectroscopy and gas chromatography–mass spectroscopy were used to identify the degraded metabolites and scrutinize the degradation process. Phytotoxicity studies indicated that the fungal treatment favors detoxification of dye Neutral Red. It is suggested that *P. subacida* has great potential for decolorizing heterocycle dyes.

Keywords: *Perenniporia subacida*; Heterocycle dye; Dye decolorization; Reusability; Metabolites characterization; Detoxification

1. Introduction

Synthetic dyes are extensively used in textile dyeing, paper printing, color photography, pharmaceutical, food, cosmetic, leather, and carpet industries [1]. It is estimated that between 10 and 15% of the total dyes used in the dyeing processes can be found in wastewaters [2]. The effluents from textile and dyeing industries have high biochemical oxygen demand, chemical oxygen demand, color, pH, and also, it contains salts [3]. The presence of even trace concentrations of dyes in effluents is highly visible and undesirable. Such effluents lead to a reduction in sunlight penetration, which in turn decrease photosynthetic activity, dissolved oxygen concentration, and water quality, and have acute toxic effects in aquatic flora and fauna, causing severe environmental problems worldwide [4]. Additionally, recent studies indicated that some synthetic dyes and their degraded metabolites might be mutagenic and/or carcinogenic to lives [5]. Heterocycle dyes represent a major group

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of dyes that cause environmental concern because of their color and potential toxicity to animals and humans. They are constituted with –C–N– functional groups and are characterized by basic groups that form covalent bonds with OH–, NH–, or SH– [6]. Considering the appearance and toxic nature, removal of this kind of dyes from water is of major scientific interest.

As the dyes are relatively recalcitrant to biodegradation, the elimination of colored effluents in wastewater treatment systems is mainly based on physical or chemical methods such as adsorption, photocatalytic, chemical transformation, filtration, and coagulation [7,8]. They always involve high costs and therefore their use is restricted in scale of operation and pollution profile of the effluent and they are practically ineffective [9]. Instead, bioremediation is definitely an attractive tool which is currently of interest to overcome the problems arising from the industrial wastewater as this is an environment-friendly and cost-competitive alternative [10]. Many biological agents that are capable of decolorizing textile dyes by biosorption or degradation using different types of enzymes have been reported, including bacteria, fungi, yeast, actinomycetes, algae, and plants [11–16].

To date, white-rot fungi are the most common micro-organisms for this approach. Some extracellular ligninolytic enzymes produced by white-rot fungi, such as lignin peroxidase (LiP), laccase, manganese peroxidase (MnP), and tyrosinase, are responsible for degrading dyes [17,18]. In addition, white-rot fungi have been found to possess high adsorption capacities due to the presence of polysaccharides, proteins, or lipids on the cell wall surface containing various functional groups such as amino, hydroxyl, carboxyl, phosphate, and sulfate, which can act as binding sites for dye molecules [19]. Until now, many studies have been developed on dyes decolorization by a variety of fungal species, such as *Aspergillus niger*, *Bjerkandera adusta*, *Cerrena unicolor*, *Daedalea quercina*, *Funalia trogii*, *Ganoderma lucidum*, *Irpex lacteus*, *Lentinus edodes*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Pycnoporus cinnabarinus*, *Scyzophyllum commune*, *Trametes villosa*, and *T. versicolor* [20–23]. Nevertheless, few reports regarding the use of white-rot fungus *Perenniporia subacida* to decolorize heterocycle dyes are available.

Hence, the goal of the present study was to evaluate the potential of white-rot fungus *P. subacida* for the decolorization of heterocycle dye Neutral Red. Influences of initial pH, temperature, initial dye concentration, and ionic strength on the decolorization capacity were investigated. This work also included characterization and identification of Neutral Red degraded metabolites using analytical tools viz. Fourier transform infrared spectroscopy (FTIR) and gas chromatography–mass spectroscopy (GC–MS), involvement of extracellular enzymes responsible for degradation, and toxic effect on germination of plant seed. Finally, the suggested fungus was successfully used for the decolorization of heterocycle dye Neutral Red.

2. Materials and methods

2.1. Dyes and chemicals

The dyes used in this study were prepared by being filtered through a 0.22-μm membrane to remove bacteria before use. Their characteristics are listed in Table 1. 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), nicotinamide-adenine dinucleotide hydrogen (NADH), and 2,6-dichlorophenol indophenol (DCIP)

Table 1
Decolorization of structurally various dyes by *Perenniporia subacida*

Dye	Color index number	Chemical class	Wavelength (nm)	Percentage Decolorization
Reactive Brilliant Blue X-BR	61,205	Anthraquinone	603	42.57
Disperse Blue B	61,500	Anthraquinone	640	45.67
Congo Red	22,120	Azo	497	76.62
Methyl Red	13,020	Azo	523	75.34
Sudan Black B	26,150	Azo	498	70.58
Trypan Blue	23,850	Azo	607	72.23
Neutral Red	50,040	Heterocycle	553	80.34
Methylene Blue	52,015	Thiazine	661	74.41
Crystal Violet	42,555	Triphenylmethane	595	33.57
Ethyl Violet	42,600	Triphenylmethane	596	35.78
Victoria Blue B	44,045	Triphenylmethane	599	33.67
Brilliant Green	42,040	Triphenylmethane	623	41.83

were all Sigma-Aldrich products (St. Louis, Mo, USA). Other chemicals used were of analytical reagent grade.

2.2. Fungus and dye decolorization

P. subacida was collected on fallen trunk of *Pinus koraiensis* from Changbai Mountain of Jilin Province in China. This strain was maintained through periodic (monthly) transfer on yeast extract glucose agar (YGA) at 4˚C. The YGA medium used for the experiment contained (g/L of distilled water): yeast extract 5.0, glucose 20.0, agar 20.0, KH_2PO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, $ZnSO_4{\cdot}7H_2O$ 0.05, and vitamin B1 0.01, and the pH was adjusted to 5.0 before sterilization. Prior to use, the stored strain from slant was transferred to newly prepared YGA plates and grown at 30˚C. Five mycelial disks (1 cm diameter) were removed from the peripheral region of the 8-day-old YGA plate and were used to inoculate into a 250-mL Erlenmeyer flask containing 100 mL of yeast extract glucose medium (YG, identical to YGA without agar). The cultivation was carried out in a dark chamber under 150 rpm shaking speed at 30˚C. After 6 days, mycelia were homogenized using an Ace Homogenizer (Hengao Co., Tianjin, China) at 5,000 rpm for 30 s, and the suspensions were later prepared as inocula for the next experiments.

The decolorization capacity of *P. subacida* for structurally various dyes was monitored by the decrease in absorbance at the wavelength of each dye listed in Table 1. An aliquot of 5.0 mL of the inocula (0.023 g, dry weight) was inoculated into a 250-mL Erlenmeyer flask containing 100 mL of YG medium supplemented 50 mg/L of each dye. In all the cases, the mixtures were incubated in a dark chamber under 150 rpm shaking speed at 30˚C. In parallel, the negative control contained all components expect inocula and experiments were all performed in triplicate. Aliquot of samples was collected after a 10-day incubation period and was centrifuged at 12,000 rpm for 20 min and the supernatant was used for percentage decolorization determination. Decolorization is defined as a percent color variation and was expressed in terms of percentage and calculated as follows:

$$\%\ \text{Decolorization} = \frac{A_i - A_o}{A_i} \times 100$$

where A_i refers to the initial absorbance of the dye and A_o refers to the absorbance measured after decolorization. Thereafter, the dye with the highest decolorization was selected as the target dye for the next experiments.

2.3. Reusability of fungal biomass for target dye decolorization

An aliquot of 5.0 mL of inocula was inoculated into a 250-mL Erlenmeyer flask containing 100 mL of YG medium spiked with 50 mg/L of target dye solution. For the sequential experiments, flask was repeatedly supplemented after every 10 days with new 50 mg/L of the target dye solution. In order to determine dye decolorization, samples were analyzed prior to addition of dye solution and after every 10 days. Uninoculated (without inocula) flasks were selected as controls under identical conditions and experiments were all performed in triplicate. Percentage decolorization determination and expression were mentioned in section 2.2.

2.4. Enzyme activities during the time course of decolorization

The broth was harvested at regular intervals and was centrifuged at 12,000 rpm for 20 min and the cell-free supernatant was used for extracellular enzymes measurement during the time course of decolorization. In parallel, the supernatant obtained from the cultivation without addition of target dye was selected as control under identical conditions and experiments were all performed in triplicate. Activities of LiP, laccase, MnP, tyrosinase, and NADH-DCIP reductase were determined spectrophotometrically at room temperature. All enzyme assays were run in triplicates and average rates were calculated, where one unit is defined as the amount of enzyme that oxidizes 1 μmol or 1 mg of substrate per minute.

Laccase was determined in a reaction mixture of 2.0 mL containing 50.0 μL of enzyme solution and 1.0 mmol/L of ABTS in 0.1 mol/L of sodium citrate buffer (pH 5.0), and the optical density was measured at 420 nm [24]. LiP was determined by monitoring the formation of propanaldehyde at 300 nm in a reaction mixture of 2.5 mL containing 100.0 mmol/L of *n*-propanol, 250.0 mmol/L of tartaric acid, 10.0 mmol/L of H_2O_2, and 50.0 μL of enzyme solution [24]. Tyrosinase activity was determined by the formation of *o*-benzoquinone and dehydro-ascorbic acid in a reaction mixture of 3.0 mL containing 50.0 mmol/L of catechol, 2.1 mmol/L of ascorbic acid, and 50.0 μL of enzyme solution in 50.0 mmol/L of potassium phosphate buffer (pH 6.5), and the decrease in absorbance was measured at 265 nm [25]. NADH-DCIP reductase activity was determined using the procedure reported by Salokhe and Govindwar [26]. The assay mixture contained 50.0 μmol/L of DCIP, 50.0 μmol/L of NADH in 50.0 mmol/L of potassium phosphate buffer (pH 7.4),

and 0.1 mL of enzyme solution in a total volume of 5.0 mL. The DCIP reduction was monitored at 595 nm. MnP was determined by the modified method of Hatvani and Mecs [27]. The assay mixture of 2.5 mL contained 1.0 mmol/L of $MnSO_4$ and 50.0 μL of enzyme solution in 50.0 mmol/L of sodium tartarate buffer (pH 4.5). The reaction was started by adding 10.0 mmol/L of H_2O_2 and was monitored at 238 nm.

2.5. Influences of physicochemical parameters on target dye decolorization

The batch dye decolorization experiment was conducted in a 250-mL Erlenmeyer flask containing 100 mL of YG medium spiked with 50 mg/L of the target dye solution. A weighed amount of 5.0 mL of fungal inocula was added to the solution. The reaction mixture was shaken with agitation speed of 150 rpm for 10 days in the dark to avoid dye polymerization by light. The influences of initial pH (2.0–10.0), temperature (10°C–80°C), initial dye concentration (20–300 mg/L), and ionic strength (sodium chloride, 0.05–1.0 mol/L) were evaluated to optimize the physicochemical parameters for the target dye decolorization by varying the factor under study and keeping other factors constant. Uninoculated (without inocula) flasks were selected as controls under identical conditions and experiments were all performed in triplicate. Percentage decolorization determination and expression were mentioned in section 2.2.

2.6. Identification of target dye degraded metabolites

Once complete dye decolorization was achieved, the culture broth was collected by vacuum filtration and was centrifuged at 12,000 rpm for 20 min. An equal volume of ethyl acetate was used to extract metabolites from the supernatant and the extracts were evaporated after removal of aqueous content with anhydrous $CaCl_2$ in rotary evaporator, dried at 40°C and used for further analysis.

The FTIR analysis was done on Perkin Elmer, spectrum one instrument in the mid IR region of 400–4,000 cm^{-1} with a scan speed of 16 (Spectrum One, Perkin Elmer, USA). Disks were prepared by first mixing 2 mg dried samples with 200 mg KBr in an agate mortar. The resulting mixture was pressed at 10 MPa for 3 min using a YP-2 tablet press (Shanghai, China). GC–MS analysis was carried out using a QP2010 gas chromatography coupled with mass spectroscopy (Shimadzu). Ionization voltage was 70 eV and temperature of the injection port was 280°C. Gas chromatography was conducted in the temperature programming mode with a Restek column (0.25 mm × 30 mm, XTI-5). Initial column temperature was 80°C for 2 min, which was later increased linearly at 10°C per minute up to 280°C and held for 7 min. GC–MS interface was maintained at 290°C and helium was used as the carrier gas at a flow rate of 1.0 mL/min with a 30 min run time.

2.7. Phytotoxicity study

Phytotoxicity of the target dye and its metabolites were evaluated on the plant seeds of *Sorghum vulgare*, *Phaseolus mungo*, and *Triticum aestivum*. Target dye degraded metabolites extracted in ethyl acetate were dried and dissolved in 50 mL of distilled water to a final concentration of 2.0 g/L. The experiments were carried out at room temperature by placing 50 seeds in separate 5.0 mL of solutions containing either distilled water, control dye solution, or its degraded metabolites per day. Germination (%) and lengths of plumule (cm) and radicle (cm) were recorded after 7 days.

2.8. Statistical data analysis

The results obtained during experimentation were expressed in terms of means and standard error means. Data were subjected to statistical analysis of one-way ANOVA, *t*-test, and LSD test by using Microsoft excel and MSTAT software. Correlation between different parameters was calculated by SPSS 18.0 software. Probability (*P* value) less than 0.05 or 0.01 was considered significant or highly significant, respectively.

3. Results and discussion

3.1. Dye decolorization

In this study, twelve representatives of structurally various dyes were used to evaluate the potential of *P. subacida* for dye decolorization. When the dyes were subjected to the fungal treatment, the batch cultures turned from an initial deep colorization to a lighter color, eventually becoming colorless, indicating that dye adsorption occurred before degradation during the dye decolorization process. As shown in Table 1, an apparent increase of heterocycle dye Neutral Red decolorization by *P. subacida* occurred, and a peak of 80.34% decolorization was observed, which was 2.39 times higher than that of triphenylmethane dye Crystal Violet at the same level after 10 days of incubation. Therefore, based on the decolorization capacities of *P. subacida*, the heterocycle dye Neutral Red was selected as the target dye for the next experiments.

It is well-known that the industrial effluents generally contain a mixture of dyes and so, non-specific method of decolorization is a prerequisite. Since the dyes with various chromophores demonstrated higher decolorization performances, it seemed to be that there exist some other special factors explaining for these. One factor is that the non-specific property of the fungal strain causes an increase in cell permeability and mass transfer speed, thus leading to an increase in dye decolorization [28]. The other factor is that fungal physiological characteristics and structural differences can affect dye decolorization process [29].

P. subacida has been found to be a common wood-decaying and pathogenic fungus [30]. However, reports on the biodegradation and adsorption of heterocycle dye by *P. subacida* are very limited [31]. Hence, the ability of *P. subacida* to decolorize the heterocycle dye Neutral Red was investigated and the influences of initial pH, temperature, initial dye concentration, and ionic strength on the decolorization process were explored. Additionally, the degradation pathway of Neutral Red treated by *P. subacida* was also elucidated. The results of this study may facilitate a better understanding of the degradation mechanism of the heterocycle dye Neutral Red by *P. subacida* and support the use of fungal applications in bioremediation.

3.2. Reusability of fungal biomass for decolorization of heterocycle dye Neutral Red

To explore the reusability of *P. subacida* biomass for decolorization of heterocycle dye Neutral Red, the reaction system was repeatedly spiked with new 50 mg/L of Neutral Red dye solution. In Table 2 it is shown that the biomass sequentially decolorized 50 mg/L of Neutral Red dye solution up to three cycles and demonstrated better decolorization performance; while during the fourth cycle, the biomass could not survive. In the second cycle, the decolorization of Neutral Red was 56.48%, and for the third cycle decolorization was 37.14% after a 10-day incubation period. The observed decline in decolorization with re-use of the biomass may be due to the poisonous effect of the dye on the fungus, obstruction of the active sites of the involved enzymes by complex dye structure and/or insufficient production of biomass for decolorization of dye at higher concentrations [32]. The results indicated that the *P. subacida* biomass exhibited excellent reusability and persistence in sequential decolorization operations.

3.3. Enzyme activities during the time course of decolorization

The biotransformation process or mechanism of toxic pollutant is only possible because of the catalytic species present in the micro-organisms, which is nothing but the oxido-reductive enzymes [9]. Oxidative degradation takes place by the action of enzymes such as peroxidases and laccase. To study the effect of dye Neutral Red on enzymes production, liquid cultures with or without Neutral Red were assayed for enzyme activities. Screening of various oxido-reductive enzymes which were responsible for decolorization of Neutral Red is presented in Table 3. Within 10 days of cultivation, LiP and NADH-DCIP reductase production in fungal treatment was remarkably higher than other enzymes. Additionally, during the decolorization process the activities of LiP, laccase, MnP, tyrosinase, and NADH-DCIP reductase were found to be reduced by 21.28, 25.53, 17.03, 40.18, and 21.64% as compared to the culture without dye. Although, the presence of heterocycle dye Neutral Red resulted in reduced levels of all the enzymes, the collective role of all these enzymes might be the key of efficient decolorization of heterocycle dye Neutral Red by *P. subacida* within short period [33]. Contrary to the results of this study, the addition of dye Bromphenol Blue significantly improved MnP production of *I. lacteus* [34]. It is inferred that synthetic dyes influence the production of ligninolytic enzymes, depending on not only the dye structure but also the enzyme producer [35]. However, the mechanism that influences enzyme activity remains unknown.

Table 2
Reusability of *Perenniporia subacida* biomass for decolorization of heterocycle dye Neutral Red[a]

Repeated cycle	Percentage decolorization				
	2th day	4th day	6th day	8th day	10th day
1	35.47 ± 2.55	50.87 ± 1.41**	77.45 ± 2.15**	79.23 ± 2.55	80.34 ± 2.57**
2	28.86 ± 2.65	41.87 ± 1.10**	53.22 ± 2.32	54.47 ± 0.26*	56.48 ± 2.65**
3	12.05 ± 3.26	20.41 ± 4.54	34.41 ± 2.70**	35.81 ± 2.53	37.14 ± 1.55**

Note: [a]Each value is the mean value ± standard error mean of triplicate.

Table 3
Enzymes activities monitored during decolorization process of heterocycle dye Neutral Red by *Perenniporia subacida* after a 10-day incubation period[a]

Enzyme	Time	LiP[b]	Laccase[b]	MnP[b]	Tyrosinase[b]	NADH-DCIP reductase[c]
Control (Cultivation without dye Neutral Red)	2th day	0.48 ± 0.05**	0.21 ± 0.02	0.38 ± 0.05	0.51 ± 0.03*	16.35 ± 0.96*
	4th day	0.92 ± 0.14*	0.74 ± 0.11	0.81 ± 0.12**	0.60 ± 0.13	24.57 ± 0.84**
	6th day	1.19 ± 0.21	1.21 ± 0.21**	1.24 ± 0.32	0.77 ± 0.14**	17.25 ± 0.95
	8th day	2.35 ± 0.12**	1.41 ± 0.15*	1.82 ± 0.24**	1.12 ± 0.21	15.57 ± 1.05**
	10th day	1.84 ± 0.05*	1.05 ± 0.20	1.54 ± 0.17*	1.43 ± 0.16	11.62 ± 1.21
Cultivation with dye Neutral Red	2th day	0.56 ± 0.19**	0.17 ± 0.16	0.47 ± 0.19*	0.37 ± 0.12	15.54 ± 0.82**
	4th day	0.87 ± 0.10	1.02 ± 0.21**	0.57 ± 0.11	0.45 ± 0.20*	23.18 ± 0.96**
	6th day	1.07 ± 0.18**	0.86 ± 0.14	0.98 ± 0.13**	0.64 ± 0.21**	15.19 ± 1.01
	8th day	1.85 ± 0.11**	1.05 ± 0.20	1.51 ± 0.09**	0.67 ± 0.18	12.20 ± 0.97*
	10th day	1.67 ± 0.22**	0.53 ± 0.13**	1.43 ± 0.10	1.37 ± 0.17*	9.61 ± 1.07**

Notes: [a]Each value is the mean value ± standard error mean of triplicate. [b]Enzyme unit U/mL.[c]μg DCIP reduced/min mg/protein.

3.4. Influences of physicochemical parameters on decolorization of Neutral Red

3.4.1. Initial pH

Initial pH of solution plays an important role in the whole decolorization process. It affects not only the surface charge, the degree of ionization and the dissociation of functional groups on active sites of the fungal biomass, but also the structure of the dye molecules [36]. In this study, the influence of initial pH on the decolorization of heterocycle dye Neutral Red by *P. subacida* was studied by varying pH from 2.0 to 10.0, while the temperature and initial dye concentration were kept constant at 30˚C and 50 mg/L, respectively. The plot of dye uptake vs. initial pH is shown in Fig. 1(a). The maximum decolorization of Neutral Red was obtained at initial pH 4.0, indicating that the optimum decolorization of Neutral Red by *P. subacida* occurred under acidic conditions. Thereafter, the decolorization of Neutral Red started to decrease and was found to be minimum at the initial pH 10.0. When the initial pH was 4.0, the percentage decolorization was 85.51%, which was 3.70-fold higher than that with initial pH at 10.0. Higher dye uptake obtained at relatively lower pH might be due to the electrostatic interaction between negatively charged dye molecules and positively charged fungal cell surface [37]. At high pH, a high concentration of OH^- could neutralize the positively charged surface of biomass and form a negatively charged surface. Thus, it would increase repulsion between colored dye ions and biomass and cause a decrease in the decolorization capacity. As maximum decolorization of dye Neutral Red was observed at initial pH 4.0, therefore, further studies were carried out at initial pH 4.0.

3.4.2. Temperature

Temperature is well-known to play an important role in decolorization process. The fungal growth is supported in a limited temperature range, but its related metabolizing property fairly differs from the other micro-organisms at different temperatures [38]. Thus, in order to determine the optimum temperature for decolorization of heterocycle dye Neutral Red, a weighed amount of 5.0 mL of *P. subacida* inocula was treated with 100 mL of YG medium spiked with 50 mg/L of Neutral Red dye solution at initial pH 4.0. As can be seen from Fig. 1(b), decolorization of dye Neutral Red increased with varying the temperature from 10 to 35˚C and maximum dye removal was obtained with temperature at 35˚C. Further increase in the temperature, resulted in a marginal reduction in the decolorization capacity.

3.4.3. Initial dye concentration

Fig. 1(c) depicts the influence of different initial concentrations of heterocycle dye Neutral Red on the decolorization capacity of *P. subacida* at the ranges of 20–300 mg/L. It was noted that the decolorization of the dye at different concentrations was rapid in the initial stages and gradually decreased with the progress of decolorization. The initial concentration provides an important driving force to overcome all mass transfer resistances of the dye between the aqueous and solid phases, which suggested that increasing the initial concentration of the dye increases the probability of contact between dye molecules and fungal biomass [39]. Hence a higher initial concentration of the dye will enhance the fungal

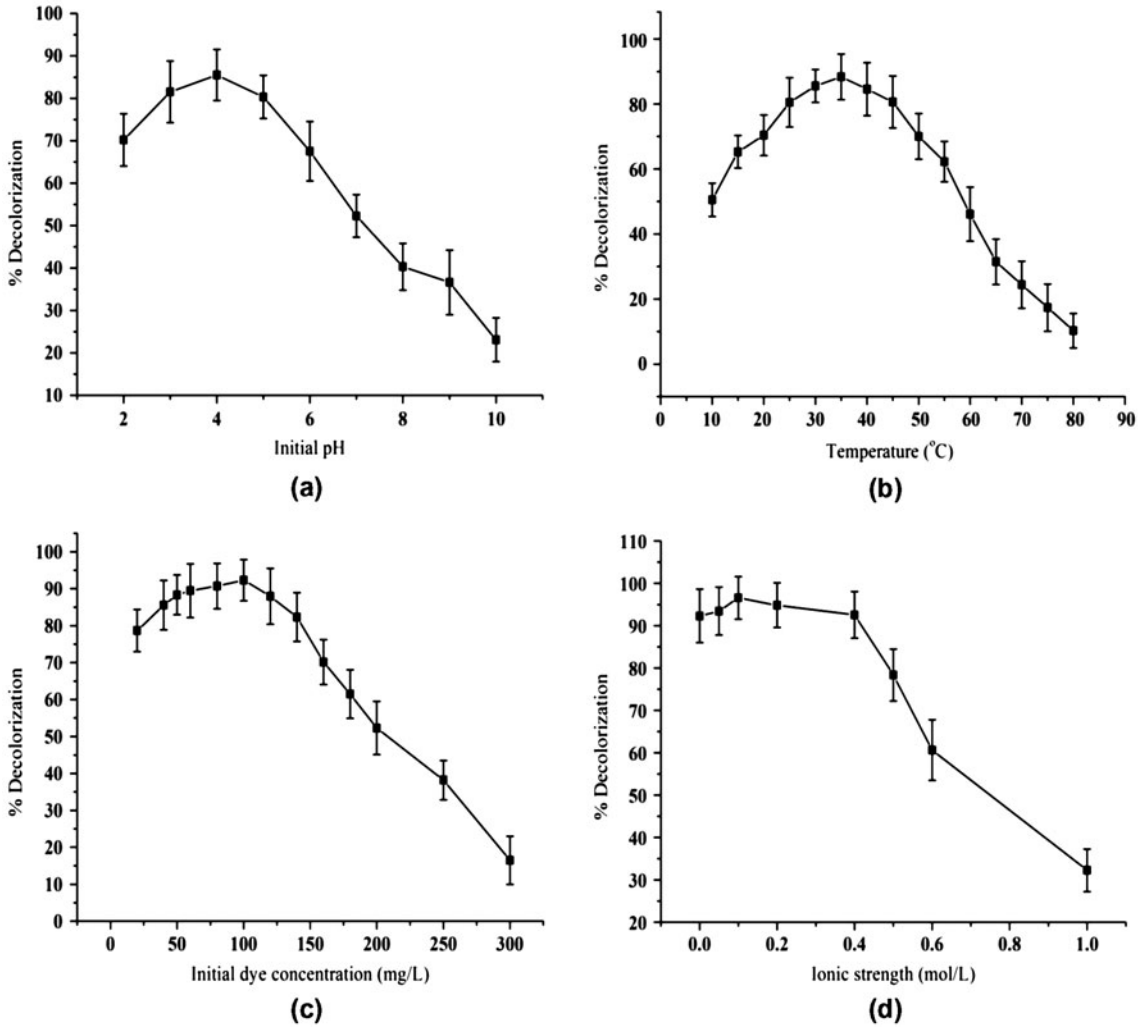

Fig. 1. Influences of physicochemical parameters on the decolorization of heterocycle dye Neutral Red by *Perenniporia subacida* after a 10-day incubation period. (a) Initial pH; (b) Temperature; (c) Initial dye concentration; and (d) Ionic strength. Each value is the mean value ± standard error mean of triplicate.

decolorization capacity. A peak of 92.31% decolorization was observed with the initial dye concentration at 100 mg/L, while higher dye concentrations such as 250 and 300 mg/L showed 38.20 and 16.47% decolorization, respectively, after a 10-day incubation period. Decline in decolorization at relatively higher dye concentrations may be due to the toxicity of the dye to the biomass and/or inadequate biomass concentration for the uptake of higher concentrations of dye [40].

3.4.4. Ionic strength

High amounts of salts are generally used in dyebath to ensure the maximum fixation of dye to the cellulose fiber [41]. The presence of these salts leads to high ionic strength affecting the performance of the decolorization process. The influence of ionic strength on decolorization of heterocycle dye Neutral Red by *P. subacida* was analyzed in the sodium chloride solutions with concentrations ranging from 0.05–1.0 mol/L, while the initial pH, temperature, and initial dye concentration were kept constant at 4.0, 35˚C and 100 mg/L, respectively. As demonstrated in Fig. 1 (d), the decolorization of dye Neutral Red was enhanced by approximately 1.05 times (96.56%) in the presence of sodium chloride at 0.1 mol/L and returned to its original level at 0.4 mol/L, thus stimulating the applicability of *P. subacida* for environmental applications. Thereafter, an increase in ionic strength exhibited an adverse effect on dye decolorization. As salt concentration increased from 0.4 to 1.0 mol/L, the

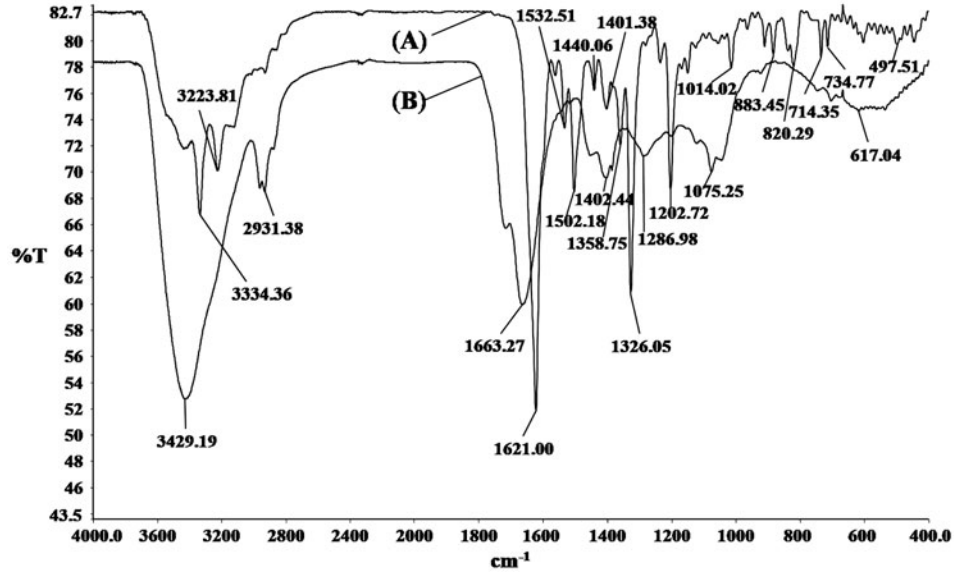

Fig. 2. FTIR spectra of (A) heterocycle dye Neutral Red and (B) its metabolites degraded by *Perenniporia subacida*.

Table 4
GC–MS spectral data of metabolites formed after degradation of heterocycle dye Neutral Red by *Perenniporia subacida*

S. no.	Metabolites	CAS. no.	Rt. time (min)	Mw (m/z)	Area (%)
1	Methylbenzene	108-88-3	21.687	92	37.39
2	N,N-Dimethylphenylenediamine	99-98-9	27.866	136	36.18

decolorization of dye reduced from 92.57 to 32.27%. Thus, the presence of lower concentrations of salt may stimulate the growth of fungal biomass and the production of oxido-reductive enzymes; whereas, higher salt concentrations have deleterious effects on their growth [3]. Moreover, chloride anions existing in the solution may also form complexes with the dye and, therefore, affect the adsorption process adversely and limit transfer of the dye to the fungal surface [42].

3.5. Identification of heterocycle dye Neutral Red degraded metabolites

A noticeable difference was observed between the FTIR spectra of heterocycle dye Neutral Red and its metabolites. Comparison of FTIR spectrum of control dye with extracted metabolites after complete decolorization clearly indicated the degradation of dye Neutral Red by *P. subacida* [28]. As can be seen from Fig. 2, the FTIR spectrum of control dye displayed a peak at 3223.81 cm^{-1} for asymmetric $-CH_3$ stretching vibration; peaks at 1440.06, 1326.05, and 883.45 cm^{-1} for the −C=C−H in plane −C−H bend; a peak at 1014.02 cm^{-1} for $=CH_2$ out of plane twist; a peak at 734.77 cm^{-1} for ring vibrations; and a peak at 1502.18 cm^{-1} for the −C=C− stretching of the benzene ring—this confirmed the aromatic nature of the dye. The peak at 1202.72 cm^{-1} for −C−N confirmed the heterocycle nature of Neutral Red, with peaks at 3334.36, 1532.51, and 1621.00 cm^{-1} representing −N−H stretching in amide. The FTIR spectrum of extracted metabolites demonstrated significant changes in positions of peaks when compared to control dye spectrum. A new peak at 1075.25 cm^{-1} represented −C−N−, deformation of aniline, where a peak at 1663.27 cm^{-1} was observed for −N−H deformation. It was obtained that −C−H stretching at 2931.38 cm^{-1} for alkane and −N−H stretching at 3429.19 cm^{-1} for amines.

GC–MS analysis was carried out to investigate the metabolites formed during the degradation process. The structures of the detected compounds were assigned from the fragmentation pattern and (m/z) values, and the GC–MS spectral data are concluded in Table 4. According to the identified metabolites, a pathway has been proposed for the degradation of dye Neutral Red in Fig. 3. During the time course of decolorization, the degraded enzymes initially

catalyzed the cleavage of the bond, giving rise to methylbenzene with mass peak (m/z) 92 and N,N-dimethylphenylenediamine with mass peak (m/z) 136. The conversion of complex dye molecules to simpler metabolites by oxidative and reductive mechanism with the aid of various oxido-reductive enzymes is well documented [43].

3.6. Phytotoxicity study

Assessment of the toxic nature of original dye and its metabolites formed after degradation on plant populations is of great importance as plants are important commercial products and are consumed by people. Table 5 depicts the relative sensitivities toward the original dye Neutral Red and its degraded metabolites in relation to plant seeds of *P. mungo*, *S. vulgare* and *T. aestivum*. Percentage germination, plumule length, and radicle length for all the plant seeds were less with control dye Neutral Red treatment as compared to its metabolites obtained after degradation. The original dye Neutral Red significantly reduced the lengths of plumule and radicle than its degraded metabolites, which indicated the less toxicity of the metabolites formed after its degradation. Therefore,

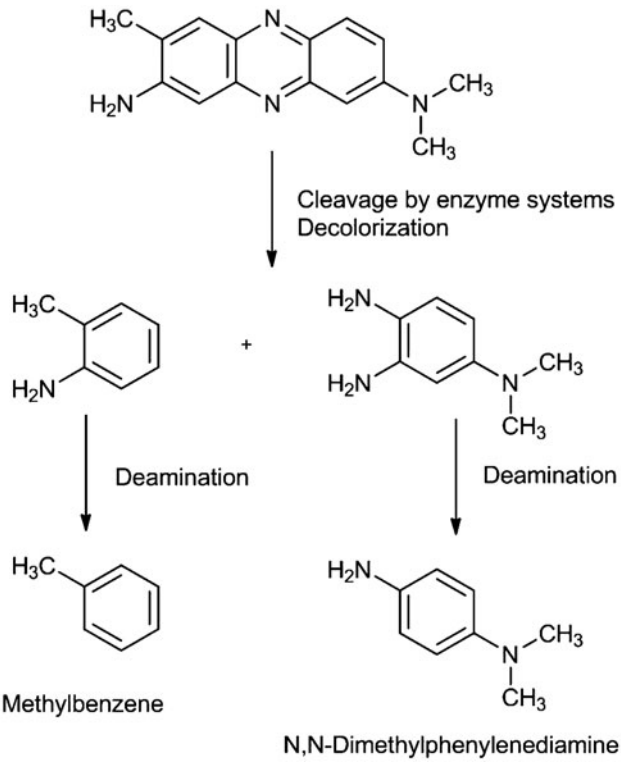

Fig. 3. Proposed pathway of degradation of heterocycle dye Neutral Red by *Perenniporia subacida*.

Table 5
Phytotoxicity of heterocycle dye Neutral Red and its metabolites formed after degradation by *Perenniporia subacida*[a]

Parameters studied	*Phaseolus mungo*			*Sorghum vulgare*			*Triticum aestivum*		
	Distilled water	Neutral Red	Metabolites	Distilled water	Neutral Red	Metabolites	Distilled water	Neutral Red	Metabolites
Germination (%)	100	65	100	100	70	95	100	60	90
Plumule (cm)	18.50 ± 0.64*	6.83 ± 0.21**	13.34 ± 045*	15.16 ± 0.32*	6.44 ± 0.36**	11.34 ± 0.46	17.20 ± 0.61**	7.64 ± 0.71**	12.87 ± 0.08*
Radicle (cm)	2.30 ± 0.08**	0.75 ± 0.08**	1.84 ± 0.14*	8.17 ± 0.11	2.20 ± 0.13**	4.57 ± 0.57*	3.60 ± 0.13**	1.10 ± 0.08**	2.40 ± 0.18*

Note: [a]Each value is the mean value ± standard error mean of triplicate.

phytotoxicity studies revealed that the degradation of the heterocycle dye Neutral Red by *P. subacida* led to detoxification of the pollutant.

4. Conclusion

Results of this work showed that the white-rot fungus *P. subacida* possessed high decolorization capability and reusability. Dye removal efficiency was dependent on various physicochemical parameters such as initial pH, temperature, initial dye concentration, and ionic strength. Degradation of heterocycle dye Neutral Red by *P. subacida* was proved and the related metabolites were identified using FTIR and GC–MS. Phytotoxicity studies indicated that *P. subacida* transformed the original dye Neutral Red into less toxic metabolites. It is apparently suggested that *P. subacida* can be a promising candidate for heterocycle dye effluents treatment.

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