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Comparison of Alkali-Tolerant Fungus *Myrothecium* Sp. IMER1 and White-Rot Fungi for Decolorization of Textile Dyes and Dye Effluents

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Abstract

A new isolated nonligninolytic fungus, strain *Myrothecium* sp. IMER1, was found to decolorize five different synthetic dyes when grown on dye-containing agar plates. The capability of *Myrothecium* sp. IMER1 for decolorization of Remazol Brilliant Blue R (RBBR) and dye effluents was compared with that of five white-rot fungi. More than 65% RBBR removal by *Myrothecium* sp. IMER1was observed at various pHs (5-10). About 60-95% of decolorization was observed with these white-rot fungi in the acidic pH range of 5.0-6.0, whereas color removal rate was less than 30% in the basic pH range of 8.0-10.0. *Myrothecium* sp. IMER1 had a more efficient decolorization of the dye in a broad pH range than white-rot fungi tested. In comparison with color removal performance, *Myrothecium* sp. IMER1 was approximately 2-5-fold better than white-rot fungi tested in the basic pH range. Additionally, the visual observation and Ultraviolet-Visible (UV-VIS) spectral analysis demonstrated that decolorization of dye by *Myrothecium* sp. IMER1 was due to biodegradation and biosorption. Biomass production was not affected by changes in the pH range of 7-10, indicated that *Myrothecium* sp. IMER1 is alkali-tolerant fungus. Decolorization of dye effluents by *Myrothecium* sp. IMER1 at pH 7 and 9 was 73 and 70%, respectively, while less than 25% of decolorization was observed in the case of white-rot fungi tested. Our results showed that *Myrothecium* sp. IMER1 exhibited efficient decolorization of dye effluents compared to white-rot fungi, indicating that the alkali-tolerant strain *Myrothecium* sp. IMER1 will be potential candidates for wastewater treatment of dye effluents, especially alkaline dye effluents.

Keywords: *Myrothecium* sp. IMER1; White-rot fungi; Alkali-tolerant; Decolorization

Introduction

Worldwide, over 10,000 different dyes and pigments are used in textile, cosmetic, printing, drug, and food-processing industries [1]. Most of synthetic textile dyes are mutagenic and/or carcinogenic and belong to the most dangerous pollutants [2-4]. Although conventional chemical and physical techniques such as precipitation, adsorption, and ozonation have been employed for the decolorization of dye effluents, they possess inherent limitations such as high cost, formation of hazardous by-products, and intensive energy requirements [4-6]. As a feasible alternative, dye decolorization using microorganisms has recently received much attention owing to their cost effectiveness [7-11]. Currently, a lot of studies have focused on white rot fungi that seem to be more prospective organisms [12]. These fungi are efficient ligninolytic organisms capable of degrading many xenobiotic compounds including various types of dye such as azo, anthraquinone, reactive, and triphenylmethane dyes [13-17].

Due to the complexity of the biodegradation mechanism of ligninolytic system, requirements for some redox mediators and low pH for the optimum activity of the enzymes will be present in a wastewater, which are major disadvantages of bioremediation by white rot fungi [14,18]. On the other hand, studies of nonligninolytic fungi degrading dyes are few [19]. Such studies are very interesting not only from the standpoint of comparative biology but also with the expectation of finding better fungi for use in the treatment of dye effluents. Our previous studies showed that the nonligninolytic fungus, *Myrothecium* sp. IMER1, can decolorize dyes and dye effluents. Furthermore, unlike white rot fungi, this strain can grow at high pH [20-22].

However, decolorization efficiency of dye/dye effluents by *Myrothecium* sp. IMER1 and white-rot fungi were not systematically compared. Therefore, the major objective of this study is to investigate efficiencies of different white rot fungi strains and *Myrothecium* sp. IMER1 for decolorization of the dye (RBBR) and dye effluents at various pHs. The results would help to evaluate decolorizing performance of these strains tested and select the right strains for an effective decolorization process.

Materials and Methods

Materials

2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Sigma Chemical Company (St. Louis, MO, USA). RBBR (C.I. 61200) was purchased from Sigma (St. Louis, MO, USA), Congo Red (C.I. 22120) and Indigo Carmine (C.I. 73015) from Tianjin Damao Chemical Reagent Factory (Tianjin, China). All other chemicals used were analytically pure. Highly colored effluents (dark red) were collected from a textile dye-producing plant situated in Foshan, Guanzhou, China. The dye effluents contained both acid and direct dyes. The main components were azo dyes. Apart from azo phenyl and cyclohexyl substituted anthraquinonic dyes were coloring

the effluent. It was an alkaline wastewater with a pH value of 10-10.5. Prior to laboratory decolorization treatment, the effluent was centrifuged at 10,000×g for 15 min to remove large suspended particles and then was sterilized. Sample absorbances were measured using a Varian CARY50 UV-Vis (St. California, USA) spectrophotometer; pH values were measured with a pHS-3A pH-meter (Hangzhou Wanda Instrument Factory, China).

Organism and culture conditions

Fungal strains used in this study were: SDK (*Polyporus* sp. SDK), AX3 (*Leutinus* sp. AX3), DS1 (*Schizophyllum* sp. DS1), CD3 (*Irpex* sp. CD3), BP2 (*Pleurotus* sp. BP2), IMER1 (*Myrothecium* sp. IMER1). The strain *Myrothecium* sp. IMER1 was isolated from soil from the suburb of Wuhan, P.R.China. On the basis of the comparison of sequences of the ITS regions and 5.8SrRNA gene with those found in databanks, its morphology, and microscopy observations, it was identified as *Myrothecium* sp. (GenBank accession no. EF458487). These strains were isolated and identified in our laboratory. They were grown in potato dextrose broth medium (PDB), or on potato dextrose agar medium (PDA). Every strain was maintained on PDA medium slant. The slant was inoculated and incubated at 28°C for 5-6 days, and then stored at 4°C and periodically sub-cultured.

Dye decolorization on solid plates

Dye agar plates were prepared using PDA containing 60mg L^{-1} of each individual dye. Plates were inoculated with a 1 cm^2 piece of the fungus isolated from a six day PDA plate growth, cut out from the actively growing fungal culture. Dye agar plates that were not inoculated served as controls. All plates were incubated at 28°C. After six days, the size of the decolorization halo was measured.

Decolorization experiments in PDB and determination of biomass production

For the decolorization experiments, five agar plugs (6 mm diameter) of active mycelium from PDA plate was transferred aseptically into 500 ml Erlenmeyer flasks containing 100 ml of autoclaved PDB medium and RBBR (60mg L^{-1}) or the dye effluent at different concentration of 30% (v/v). The pH of PDB medium was adjusted using 1.0 M NaOH or 1.0 M HCl. The cultures were incubated at 28°C in rotary shakers running at 150 rpm. Biotic (sterilized medium, without dye addition) and abiotic (sterilized medium containing the effluent but not inoculated with the fungus) control experimental sets were also prepared and maintained in parallel with the decolorization experiments. The cultivations were carried out in triplicate. Culture samples were withdrawn at defined intervals, and after centrifugation at 10,000×g for 20 min at 10°C, the supernatants were used as samples for decolorization assay. Biomass production was evaluated by determining the dry mass of mycelia. Mycelia were harvested from the cultivation liquid by filtration using a piece of filter paper, dried at 105°C for 24 h and weighed.

Enzyme assays

Bilirubin Oxidase (BOX) or laccase activity was quantified using an assay based on the oxidation of ABTS. The assay reaction mixture consisted of 1.5 mM ABTS, sodium acetate buffer (0.2 M, pH 4.0) and a suitable amount of enzyme to create a total reaction volume of 3.0 ml that was incubated at 25°C. The unit activity was expressed as 1μM of product formed per min under the assay conditions [23,24].

Decolorization analysis

Decolorization of RBBR was analyzed in culture filtrates and monitored spectrophotometrically at the maximum wavelength of absorbance (595 nm). The intensity of effluent color was measured at its maximum absorbance wavelength 510 nm. Color removal rate (P) was calculated according to the following formula: $P=(A_1-A_2)/A_1\times100\%$. Where A_1 represented the absorbance of the control, A_2 represented the absorbance of the corresponding untreated sample, and P was the dye/effluent color removal rate.

Statistical analysis

All decolorization experiments were performed in three sets. The results reported are an average of the three data points with standard deviations calculated. The data from phytotoxicity studies were analyzed by a one-way analysis of variance (ANOVA) using a Tukey-Kramer multiple comparison test.

Results and discussion

Decolorization of textile dyes by *Myrothecium* sp. IMER1 on PDA plates

The selected dyes (Indigo Carmine, RBBR, Congo red, Malachite green and Bromophenol Blue) have never been tested before with *Myrothecium* sp. IMER1 on solid medium (Figure S1). First, Indigo Carmine was completely decolorized, as revealed by the >78 mm diameter of the decolorized zone after six days. Decolorization of RBBR was less extensive with a decolorized zone diameter of between 63 to 70 mm. Second, partial decolorization was obtained for Congo red and Malachite green. Finally, little decolorization was observed for Bromophenol Blue. *Myrothecium* sp. IMER1 showed different potentials to decolorize five different dyes which belong to four classifications. The class of the dye which defines its structure is influential in deciding the extent to which dye is decolorized. Wong and Yu observed that anthraquinone dye was *Trametes versicolor* laccase substrate while azo and indigo dyes were not the substrates of laccase [25].

Thus it is seen that that dye decolorization by fungus was dependent on dye structures. Spadaro et al reported that aromatic rings with substituents such as hydroxyl, amino, acetamido, or nitro functions were mineralized to a greater extent than unsubstituted rings in dye decolorization by *Phanerochaete chrysosporium* [26]. Knapp et al reported that relatively small structural differences could markedly affect decolorization and this might be due to electron distribution and charge density as well as steric factors [27].

Comparison of *Myrothecium* sp. IMER1 and five white-rot fungi for decolorization of RBBR at various pHs

RBBR is a typical anthraquinone dye used in the textile industry, represents an important class of toxic and recalcitrant organopollutants [28]. It was reported that the dye can be decolorized by many white-rot fungi and *Myrothecium* sp. IMER1 [20,28]. Therefore we compared decolorization capability of *Myrothecium* sp. IMER1 for Remazol Brilliant Blue R (RBBR) with five different white-rot fungi over a pH range of 5.0 to 10.0. The decolorization rate of RBBR was measured in culture filtrates after 6 d of incubation. As shown in Figure 1a, more than 65% of decolorization was observed with these white-rot fungi in the acidic pH range of 5.0-6.0, whereas

color removal rate was less than 30% in the basic pH range of 8.0-10.0. By contrast, 60-70% color removal was obtained with *Myrothecium* sp. IMER1 in the acidic pH range of 5.0-6.0, and more than 65% of decolorization was observed in the basic pH range of 8.0-10.0. These results showed that *Myrothecium* sp. IMER1 exhibited excellent decolorizing performance at all the pH value tested (5.0 to 10.0). Kapdan *et al.* reported the optimum growth pH of *Coriolus versicolor* as 4.5 [29]. Decolourization of Solar golden yellow R by *Schizophyllum commune* indicated that maximum decolourization efficiency (73%) was observed at pH 4.5 after 6 days [30]. It has been widely reported that for majority of the fungi the optimum pH for dye decolorization is in the acidic range. Unfortunately, such a low pH is not suitable for the wastewater treatment. Therefore, fungal strain showed an appreciable decolorization of dye over a wide range of pH, which is desirable for industrial applications since it can be used for wastewater treatment without a previous pH adjustment stage [31].

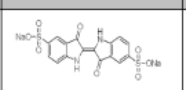
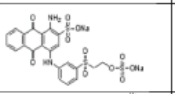
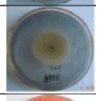
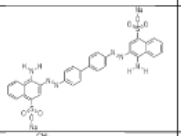
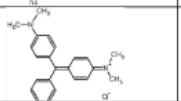
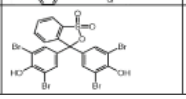
Dye (classification)	λ max (nm)	Structure	Diameter of decolorized zone (mm)	Photograph
Indigo Carmine (indigo)	602		>78	
Remazol Brilliant Blue R (RBBR) (anthraquinone)	595		63-70	
Congo red (azo)	488		46-52	
Malachite green (triarylmethane)	618		20-23	
Bromophenol Blue (triarylmethane)	422		2-9	

Figure S1: Characteristics, molecular structure and decolorization studies of the selected dyes during solid plate growth of *Myrothecium* sp. IMER1

Decolorization of dyes by most fungi could be due to adsorption to microbial cells or to biodegradation [32,33]. If the dye removal were attributed to biodegradation, either the major UV-VIS light absorbance peak would completely disappear or a new peak would appear. In adsorption, cells of fungus become deeply colored because of adsorbing dyes [34,35]. The absorbance spectra of the dye were scanned before and after decolorization and changes in its absorption spectrum (350-800 nm) were recorded. The absorption peaks of RBBR in the visible region disappeared, suggesting that removal of dye by *Myrothecium* sp. IMER1 should be partly attributed to biodegradation (Figure 1b). In addition, in the decolorization of RBBR by *Myrothecium* sp. IMER1, the cells were stained deeply colored at pHs (5.0-10.0), indicating that the dye was adsorbed on the cell surface, which was partly due to biosorption [Figure 1c]. It suggested that decolorization by *Myrothecium* sp. IMER1 involved biosorption and biodegradation. This result was in agreement with our previous studies [20,21].

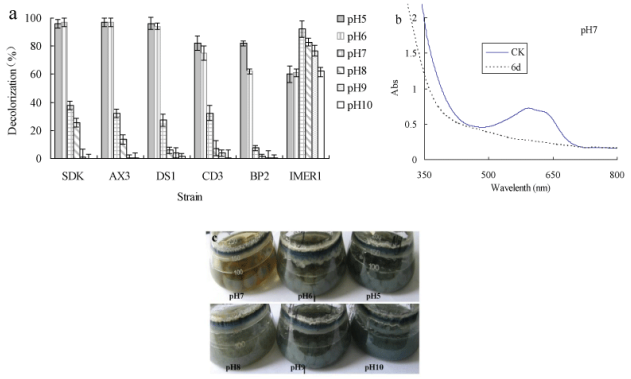


Figure 1: Effect of pH on decolorization of RBBR by *Myrothecium* sp.IMER1/five white-rot fungi (pH5-10). a) Decolorization of RBBR by fungi at various pHs, b) UV-VIS absorbance spectra of decolorization for RBBR by IMER1 and c) Photographs of decolorization for RBBR by IMER1at different pH

To investigate the effect of pH on growth of *Myrothecium* sp. IMER1, biomass production was evaluated by determining the dry mass of mycelia. No decrease or increase in biomass production was observed throughout the study in the pH range of 5.0-8.0 (Figure 2a). Interestingly, cells of *Myrothecium* sp. IMER1 grew almost as well as the control at pH 9.0 and 10.0 (Figure 2b). Although biomass production of *Myrothecium* sp. IMER1 decreased slightly with increase pH value (9.0-10.0), data statistically analyzed showed that there were no significantly difference in biomass production between control group and experimental group. In comparison with *Myrothecium* sp. IMER1, growth of white-rot fungi tested such as *Pleurotus* sp. BP2 was strongly inhibited and low biomass production was observed in the basic pH range of 9.0-10.0 (Figure 2c). The results indicated that biomass production of *Myrothecium* sp. IMER1 might not be affected by changes in the pH range of 7-10, which also confirmed that *Myrothecium* sp. IMER1 is alkali-tolerant fungus. Previously, Sulistyaningdyah et al. had reported that *Myrothecium verrucaria* 24G-4 is an alkali-tolerant fungus and can grow at pH10.0 [36].

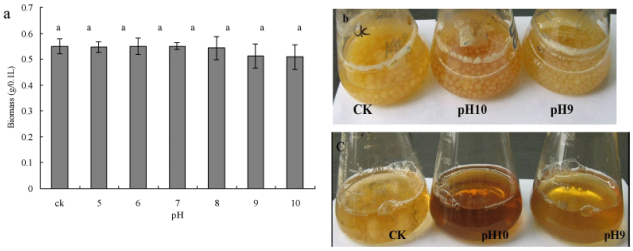


Figure 2: Effect of pH on the biomass production of *Myrothecium* sp.IMER1 (a), photographs of IMER1 during growth at pH 9 and 10 (b), and photographs of BP2 during growth at pH 9 and 10 (c).

Comparison of *Myrothecium* sp. IMER1 and five white-rot fungi for decolorization of dye effluents

Although a large number of lab-scale studies have been conducted on decolorization of single synthetic dye solutions and simulated dye wastewaters by fungal biosorption/biodegradation, there is a need to generate relative performance data on real dye effluents [37,38]. Decolorization of industrial effluent from dyeing industry is shown in Figure 3. At pH 7.0 and 9.0, decolorization by *Myrothecium* sp. IMER1 at the effluent concentrations of 30% (v/v) was 73 and 70%, respectively, whereas in the case of white-rot fungi tested less than 25% of decolorization was observed. The result showed that *Myrothecium* sp. IMER1 had higher color removal efficiency than these white-rot fungi tested under neutral and alkaline conditions. On the one hand, when UV-VIS spectra of dye effluent-containing culture fluid for defined intervals were scanned from 350-800 nm, a marked decrease in absorbance maximum at 510 nm was observed at 6-days incubation, which was related to the breakdown of the chromophoric group in dyes of the effluent (Figure 3b and 3c). On the other hand, the mycelia became deeply colored at the beginning of decolorization, and then became faint, finally almost disappeared (Figure 3d). Spectra analyses of culture supernatants and color changes in fungal cells suggested that dyes in the effluent were removed by *Myrothecium* sp. IMER1 mainly due to enzymatic reaction.

It was reported that most white-rot fungi only exhibit good decolorization ability in the acidic pH range of 3-6, which due to low pH for the optimum activity of extracellular ligninolytic enzymes [39,40]. The decolorization capability of *Myrothecium* sp.IMER1 under alkaline conditions will be an advantage in the case of practical applications to dye effluents, because pulping, bleaching and dye-producing are mainly performed under highly alkaline conditions and their effluents generated are also alkaline.

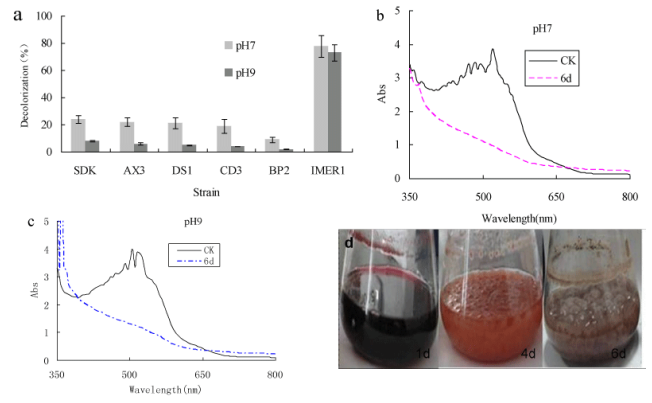


Figure 3: Decolorization of dye effluents (30% , v/v) by *Myrothecium* sp. IMER1/five white-rot fungi at pH 7 and 9. (a) Decolorization of dye effluents by fungi, (b)UV-VIS absorbance spectra of decolorization for dye effluents by IMER1 at pH7, (c)UV-VIS absorbance spectra of decolorization for dye effluents by IMER1 at pH9 and (d)Photographs of decolorization for dye effluents by IMER1at different time interval

It has previously been reported that BOX as a main extracellular oxidoreductase of *Myrothecium* sp. IMER1 plays a major role in the dye decolorization, while in the case of many white-rot fungi; main decolorization enzyme is laccase [20-22]. Therefore, BOX/laccase activity and the biomass production of *Myrothecium* sp. IMER1/ white-rot fungi tested were evaluated during decolorization. Both enzyme activity and biomass production of *Myrothecium* sp. IMER1 were higher than that of all white-rot fungi tested, which attributed to the fact that *Myrothecium* sp. IMER1 is alkali-tolerant fungus and BOX activity keeps good under neutral and alkaline pHs (Figure 4).

Conclusions

The non-ligninolytic fungus *Myrothecium* sp. IMER1 was capable of decolorizing five different synthetic dyes. Congo red, Malachite green and Bromophenol Blue were decolorized to some extent with varying percentages of decolorization, whereas, to Indigo Carmine, RBBR were more easily decolorized. Decolorization efficiencies of RBBR and dye effluents with *Myrothecium* sp. IMER1 were compared to five white-rot fungi. *Myrothecium* sp. IMER1 exhibited better decolorizing performance than white-rot fungi tested at the pH value tested (7 to 10). Biomass production of *Myrothecium* sp. IMER1 was not affected by changes in the pH range of 7-10, which confirmed that *Myrothecium* sp. IMER1 is alkali-tolerant fungus. Moreover, *Myrothecium* sp. IMER1 had higher color removal efficiency of dye effluents than these white-rot fungi tested under neutral and alkaline conditions. The alkali-tolerant fungal strain *Myrothecium* sp. IMER1 exhibited high decolorization activity in the basic pH range, which would be suitable for the treatment of dye effluents with alkaline pH but also for other industrial applications, such as bio-bleaching.

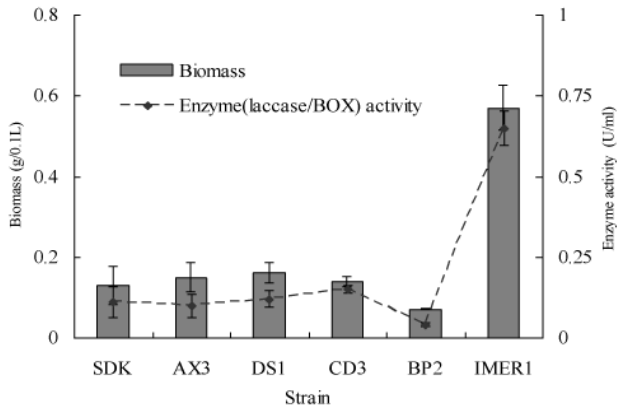


Figure 4: Production of biomass and enzyme (BOX/laccase) by *Myrothecium* sp. IMER1/five white-rot fungi at pH 9.

Acknowledgments

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