Decolorization of methylene blue by new fungus: *Trichaptum biforme* and decolorization of three synthetic dyes by *Trametes hirsuta* and *Trametes gibbosa*

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**ABSTRACT**

In order to screen dye decolorization by *Trametes hirsuta*, *Trametes gibbosa* and new fungus *Trichaptum biforme*, Methylene Blue (MB), Methyl Green (MG) and Reactive Black 5 (RBS) were studied in Broth culture at concentrations of 25, 50 and 70 ppm. Decolorization of these three kind dyes by *T. hirsuta* decreased by increasing of concentration. Decolorization of RBS by *T. hirsuta* was examined in two different medium contains of Mn²⁺ or veratryl alcohol (VA), as activators. At the presence of laccase activator (VA), RBS was decolorized 91% at concentration of 70 ppm, and in the medium contains of MnP activator (Mn²⁺), dye was decolorized less than 50% at same concentration. Decolorization of RBS by *T. hirsuta* was more than decolorization of dye at the presence of MnP activator (Mn²⁺). *T. gibbosa* decolorized MB, MG and RBS in more than 80% in the presence of MnP activator. Decolorization of the three kind dyes by *T. gibbosa*, was independent of concentration. *T. gibbosa* decolorized different dyes during 20 days and *T. hirsuta* did it during 32 days, this is due higher potential of decolorization of synthetic dyes for *T. gibbosa* than *T. hirsuta*. Decolorization of MB by new fungus *T. biforme* was studied in two medium contains of MnP activator and laccase activator. Rate of decolorization in the presence of MnP activator was more than decolorization in the presence of laccase activator. Decolorization of MB by new fungus *T. biforme* was higher than decolorization of MB by two other fungi.

1. Introduction

Environmental pollution containing recalcitrant xenobiotic materials has become one of the major ecological problems. Many of these compounds are major environmental pollutants such as nitrotoluenes, dioxin, organic acid, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), pesticides, synthetic dyes, wood preservatives, synthetic polymers and olive mill wastewater [1-5]. Most of these compounds are toxic, carcinogenic and highly resistant to degradation [6-8]. Synthetic dyes are extensively used in a number of industries, such as textile dyeing or paper printing. Synthetic dyes represent a large group of chemically different compounds, which are classified by their chromophore as azo, anthraquinone, triphenylmethane, heterocyclic or phthalocyanin dyes [9,10]. The total world colorant production is estimated to be about 800000 ton/year. More than 10000 dyes are commercially available and at least 10% of the used dyestuff enters the environment through wastes [9,11-16]. The elimination of such dye-containing effluents is mostly based on physical and chemical procedures, e.g. adsorption, concentration, chemical transformation and incineration. These methods are rather costly and sometimes produce hazardous products [3,10,17-19]. Therefore, several investigations have been carried out several investigations to identify a cost effective and environmentally acceptable technology that could be applied to remediate the contamination. Bioremediation is one of these techniques that involve almost exclusively microbial processes [20-23]. White rot fungi have been studied as one of the possible agents of biodegradation since their extracellular degradation systems are basically non-specific. This fact that allows the degradation of mixture refractory substances and can be applied to environmental pollutants [24,25].

The primary source of carbon for the white rot fungi is trees and plant cellulose, which is protected by a complex polymer known as lignin. The lignin degrading enzymes such as manganese peroxidase (MnP) (EC 1.11.1.13), lignin peroxidase (LiP) (EC 1.11.1.14) and Laccase (Lac) (EC 1.10.3.2 ) degrade lignin [26,27]. Taxonomically, white rot fungi are mostly basidiomycetes, and a few ascomycetes are also capable of white rot decay [28]. Fungal strains have different ligninolytic systems which distinguish them from each other [16,23,29-30]. Laccase requires oxygen and peroxidase need oxygen peroxide for reactions. *Phanerochaete chrysosporium* was the first fungus that was examined its ability to decolorization [31,32]. A fungus species can degrade pollutants. In contrast of fungi a consortium of bacteria may be needed to completely degrade the same mixture. Also bacteria which rely on various enzymes must first adsorb the chemicals. Then these chemicals induce the production of enzymes needed for degradation [23]. Hitherto has been studied on ability of different fungus species in dye decolorization [28]. Kling and Neto (1991) [33], studied oxidation of MB by crude lignin peroxidase from *Phanerochaete*
chrysosporium. This reaction depended on peroxide concentration. Fungi Coniophora versicolor decolorized Methylene Blue at concentrations 5 and 10 mg/L. At this study various glucose and NH₄H₂OPO₄ were effective in rate of decolorization [34].

2. Experimental

2.1. Effect of temperature on growth of fungi

Trametes hirsuta (Fr.) Pilit., Trametes gibbosa (Pers.) Fr. and Trichaptum biforme (Fr.) Ryvarden were collected in April 2008 from Abbas Abad Forest, in North of Iran. The fungi were growing on dead wood of hornbeam (Carpinus betulus L.) from Betulaceae. Specimens were identified based on morphological features according to Ryvarden and Gilbertson [35]. T. hirsuta based on following features was identified: Basidiocarp annual, effused-reflexed, upper surface hirsute, gray, with brownish margin, cap 8 cm wide, 5 cm long and 2 cm thick. Context trimitic with clamp connection. Hyphal system trimitic. Pores white, 3 per mm. Spores 5*2 µm, smooth, cylindrical. Indehible. T. gibbosa according to these features was identified: Basidiocarp annual, applanate, semicircular, grayish white, upper surface colored green due to algal growth. Pores creamy-white, elongated and stollike, 2 per mm. Hyphal system trimitic. Spores hyaline, cylindric, 4.5*2.5 µm. T. biforme according to following characters was identified: Basidiocarp annual, imbricate, dimidiate, hirsute to glabrous with age, surface imbricate, dimidiate, hirsute to glabrous with age, surface imbricate, dimidiate, hirsute to glabrous with age, surface imbricate, dimidiate, hirsute to glabrous with age, surface imbricate, dimidiate, hirsute to glabrous with age, surface imbricate, dimidiate, hirsute to glabrous with age.

2.2. Study of optimum pH for growth of mycelium

To study the effect of pH on growing fungi, mycelia were incubated on Malt Extract Broth at different pH = 4, 6, 7 and 9 during 15 days and dry weight of mycelium was measured every 3 days.

2.3. Dye decolorization experiments

In order to study of decolorization of three structurally different dyes (Scheme 1) MB (Heterocyclic dye), MG (Triphenylmethane dye) and RB5 (Diazo dye), by T. hirsuta, T. gibbosa and MB by T. biforme were selected. These fungi were grown on broth medium. At first two different medium containing MnP activator including Mn²⁺, according to Mohorčič et al. [37] procedure and laccase activator including veratryl alcohol, according to Minussi et al. [36] procedure were used.

The composition of the culture medium to induce laccase production and activation was prepared according to Minussi et al. procedure [36]. The basal medium containing per litre (g/L) malt extract (5.0); peptone (10.0); glucose (20.0); and CuSO₄·5H₂O (0.005), (0.5) mM pyrogallol; veratryl alcohol (laccase activator) (0.07); pH was 4. The medium for screening Mn Peroxidase production by fungus, prepared according to Mohorčič et al. procedure [37] per litre (g/L) contain of: Glucose (10.0); Yeast Extract (0.2), tartaric acid (3.0); Tween 80 (1.0); KH₄PO₄ (0.2); CaCl₂ 2H₂O (0.146); (NH₄)₂HPO₄ (0.157); MgSO₄ 7H₂O (50.0 mg); ZnSO₄ 7H₂O (42.5 mg); MnSO₄·H₂O (MnP activator) (33.8 mg); CoCl₂·6H₂O (7.0 mg); CuCl₂·2H₂O (0.7 mg); FeCl₃ (0.54 mg); NaCl (0.9 mg), pH was 4.5. All chemicals were obtained from Sigma.

The following synthetic dyes were obtained from Sigma: Methylene Blue (MB), Methyl Green (MG), Reactive Black 5 (RBS) was obtained from a national dyeing factory.

To test the ability of fungi to decolorize synthetic dyes in vivo, each dye was membrane-filtered with a 0.25 µm cellulose nitrate filter and was concentrated to final concentrations of 25, 50 and 70 ppm. The dye disappearance was determined spectrophotometrically (Agilent 8453 UK). Line equation of tested dyes was obtained by Sigma Plot 8.0. Afterward amount of dye absorptions were placed in equation and were obtained dye concentrations. To obtain percentage of dye removal was used Removal Efficiency formula:

\[
\text{RE} = \frac{\text{C}_0 - C}{\text{C}_0} \times 100
\]

\(\text{C}_0\) = Dye concentration in broth medium; \(\text{C}\) = Dye concentration in medium of mycelium; \(\text{X}\) = 100

Dye concentration in control medium

Scheme 1
Table 1. Dry weight of mycelium (g) from three fungal species at different pH.

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3. Results and discussion

3.1. Effect of temperature on growth of fungi

Optimum temperature for T. hirsuta, T. gibus and T. biforme was 35, 30 and 25 °C respectively. Optimum temperature for pure laccase and MnP activity was 40 to 60 °C [28], but at this experiment decolorization was determined in vivo and at these temperatures growing of both T. hirsuta, T. gibus and T. biforme stopped. Therefore the experiments were followed at 35, 30 and 25 °C, respectively.

3.2. Study of optimum pH for growth of mycelium

Three fungal mycelium at different studied pH = 4, 6, 7, and 9 were compared at Table 1. Dry weight of mycelium (g) from three fungal species at different pH and times were measured. Therefore optimum pH for highest growth of T. hirsuta, T. gibus and T. biforme was 4 (Table 1).

3.3. Dye decolorization experiments

3.3.1. Decolorization by Trametes hirsuta

Three dyes (MB, MG and RB5) were examined at three concentrations of 25, 50 and 70 ppm in medium culture containing the Mn²⁺ (Mn peroxidase activator). MB at concentrations of 25, 50 and 70 ppm was degraded more than 80% at 16th day. It is agree to Novotný et al. [3] that reported Irpex lacteus (Fr.) Fr. Decolorized MB by 80% within two weeks. This experiment was continued until 32 day. Rate of decolorization was reduced by increasing concentration, so that at concentration of 25 ppm, MB was degraded completely (100%) and at 50 and 70 ppm, MB degraded 90% and 80% respectively (Figure 1).

Rate of decolorization of MG at concentration 25 ppm was less than decolorization at concentrations 50 and 70 ppm until 22nd day, but in following days, rate of decolorization was achieved more than 50 and 70 ppm. Dye was decolorized completely at 24th day. MG was decolorized at concentrations 50 and 70 ppm at 32nd day (Figure 2). Results showed decolorization of MB was more than MG by T. hirsuta, it is agree with this report that highly substituted triphenylmethane dye such as MG required longer time to be decolorized or could only be decolorized to a certain extent [38].

RB5 was decolorized 91% at concentration of 70 ppm in medium contains of laccase activator (VA), but in the medium contains of MnP activator (Mn²⁺), dye was decolorized less than 50%. This dye was decolorized completely at concentrations 25 ppm and 50 ppm in the presence of VA while in the presence of Mn²⁺ rate of decolorization was achieved 65% (Figure 3 and 4). RB5 was decolorized slower than MB and MG, because of its complex structure. It is notable that some compounds that were rapidly decolorized by MnP were resistant to other enzymes and the other compounds which are resistant to MnP [39]. Moreover, λmax of RB5 was changed during study. λmax of RB5 changed from 602 at day one to 610 at day 8 and to 616 and 620 at days 16 and 32, respectively. Therefore RB5 may be converted to other dyes products and this delay the decolorization. Spectrum of MB and MG showed complete decolorization.

Rodriguez Couto et al. [40] studied the effect of redox mediators (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1-hydroxybenzotriazole (HBT) Remazol Brilliant Blue R (RBBR) on synthetic dyes; Sella Solid Red and Luganì Green by laccase from Trametes hirsuta. Result showed higher activities of enzyme in the presence of mediators than those obtained without mediators addition. HBT showed a decolorization percentage of 88% in 10 min for Sella Solid Red and of 49% in 20 min for Luganì Green.

3.3.2. Decolorization by T. gibus

Decolorization of MB, MG and RB5 by T. gibus was studied during 20 days at three concentrations (25, 50 and 70 ppm) in medium contains of Mn²⁺. MB was degraded completely at concentration of 25 ppm at 20th day. Decolorization of MG, MB and RB5 was independent of concentration. Decolorization rate of MB at 70 ppm, was less than 50 ppm in the first 8 days, but in following days the rate of decolorization at 70 ppm was more than 50 ppm (Figure 5).
3.3. Decolorization by Trichaptum biforme

Decolorization of MB was studied in two medium contains of MnP activator and laccase activator by new fungus *T. biforme* during 20 days. Maximum decolorization of MB in medium culture contains of Mn²⁺ was achieved 40% and in medium contains of VA was achieved 98% at 20th day (Figure 8 and 9). According to cures, can be claimed MnP is the main enzyme of *T. biforme* in dye decolorization. The most important result obtained is that in medium contain of MnP activator, new fungus *T. biforme*, decolorized MB higher than two other species.
4.3. Effect of dye on dry weight of mycelium

To obtain the amount of fungal growth in the presence of dye, dry weight of mycelium which was grown at different concentrations of dyes, was determined (Table 2). Dry weight of mycelium of *T. hirsuta* in medium contains of MB was more than MG and RB5. These results showed less toxicity of MB in comparison with other dyes. Also different concentrations of MB did not show any significant effect on fungal growth. In medium containing the MG, mycelium growth decreased with increasing concentration. In medium containing RB5 and laccase activator, dry weight increased surprisingly with increasing dye concentration. In MnP medium, the most important result is new fungus *T. gibbsosa* obtained more than 50 ppm (Table 2).

4. Conclusion

We studied dye decolorization of three different synthetic dyes (Remazol Black 5, Diazo dye; Methylene Blue, Heterocyclic dye; Methyl Green, Triphenylmethane dye) by *Trametes hirsuta*, *Trametes gibbsosa* and *Trichaptum biforme* from white rot fungi. At this study was determined kind of fungus is effective on decolorization as *T. gibbsosa* decolorized MG (has highly substituted and need more time to decolorization) faster than MB while *T. hirsuta* could not do it. Decolorization of MB by new fungus *T. biforme* in medium contains of Mn²⁺ was more than decolorization of this dye in medium contains of VA that showed Mn²⁺ is main enzyme of *T. biforme* in dye decolorization. The most important result is new fungus *T. biforme* decolorized MB higher than two other species and can more testing be done on it and could be used in future.

Acknowledgements

The authors are grateful to Dr. Jamil Vaezi and Samira Hozhabr for their helpful comments and collaborations.
Figure 7. Removal Efficiency (%) of RB5 by T. gibbosa within 20 days.

Figure 8. Comparison of removal efficiency of MB by T. biforme at concentration of 25 ppm in medium contain of laccase activator and MnP activator.

Figure 9. Removal efficiency of MB by T. biforme at concentrations of 25 ppm and 50 ppm, in medium contain of MnP activator.

References