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DECOLORIZATION OF INDUSTRIAL DYES BY IMMOBILIZED MYCELIA OF *TRAMETES VERSICOLOR*

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ABSTRACT

The aim of this study was to compare the decolorization of several of the most utilized synthetic dyes in textile applications by immobilized white-rot fungus Trametes versicolor. Different immobilization methods for achieving maximum decolorization were explored. The initial dye concentration was 125 mg/L and the immobilized preparation concentration was 10 % (w/v). Different degrees of decolorization were observed. Different operational stability of the immobilized preparations was accomplished. Increase of the immobilized preparation concentration up to 30 % (w/v) was investigated. The study was performed in two stages: simulated incubation in a 'batch' reactor and trickle-bed continuous flow reactor. During the decolorization process, laccase activity was detected. The dye-decolorizing activity of the immobilized culture was found to be associated with the processes of biodegradation, bio-oxidation and biosorption.

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Keywords: decolorization, immobilization, laccase, synthetic dyes, *Trametes versicolor*

Introduction

About one million tons of synthetic dyes are produced per year in the world (3, 5). They are the main pollutant in textile wastewater. It is well-known that they are difficult to be processed. These dyes represent a group of compounds with different chemical structure and are classified either by their chromophore group or by their application (15). Azo dyes are the most utilized dyes in textile applications. Their resistance to biological treatment is due to their specific structures: azolink and aromatic sulfonic groups (16). It is established that dyes are never 100 %fixed in textiles. This leads to the emission of up to 50 % of the used coloring agents in the wastewaters (18, 20). Due to the toxicity of their decomposition products to aquatic organisms, azo dyes have to be removed from effluents before release (5).

The limitations of the conventional physical, chemical and biological techniques for wastewater treatment are well-known, including high cost, high sludge formation, low efficiency, etc (27). Furthermore, traditional methods for wastewater treatment, such as aerobic biodegradation and adsorption, have proven to be ineffective in the removal of textile synthetic dyes (14, 17). These problems have led to increased interest in the application of microbes in wastewater treatment. Moreover, wastewater always contains more than one pollutant. That is why, the strains used need to be not only highly active, but also stable enough to other toxic mixture components.

Bioremediation remains the favored approach for domestic and industrial wastewater treatment, and for removal of heavy metals, toxic chemicals and radioactive contaminants. The term bioremediation (12) covers a wide variety of processes that use natural resources to control pollution problems caused by xenobiotics. Xenobiotics do not decompose naturally in nature. They are highly toxic and have mutagenic, carcinogenic, teratogenic and allergenic properties. They have high persistence and can accumulate in the surroundings. They have electrophilic groups like halogen, sulfo, azo, or nitro groups (13).

Fungi are effective bioremediators because of their dye adsorption and pollutant degradation capabilities (22). Hence, the biodegradation abilities of basidiomycete fungi as well as of other fungi are subject of intensive investigations. The main focus of these studies is the ability of fungi to remove synthetic dyes in industrial wastewater by enzymatic biodegradation or mineralization (4, 19, 21, 23, 24, 28, 29, 33, 34). Mycelia of *Botrytis cinerea*, *Endothiella aggregata*, *Geotrichum fici*, *Tremella fuciformis*, *Xeromyces bisporus*, *Inonotus hispidus*, *Phlebia tremellosa*, *Coriolus versicolor, Trametes hirsuta*are investigated for their capabilities to decolorize textile wastewaters. Native biomass and mixed cultures decolorization provides insufficiently satisfactory results (8, 18) so there is increased interest in the application of immobilized filamentous fungi in different processes of biodegradation of pollutants. Researchers have focused on the immobilization of basidiomycetes, which are known as efficient producers of laccase and peroxidase (1, 7, 9, 10, 26) in order to develop effective systems for detoxification and decolorization of wastewater.

Laccase (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) has a specific structure that makes it an ideal candidate for the treatment of wastewater from industrial effluents such as those from the textile industry (6, 25, 30).

The aim of the present work was to investigate the possibility for biological decolorization of seven of the most commonly used synthetic dyes (Phenol Red, Orange II, Congo Red, Reactive Violet 12, Brilliant Blue R, Reactive Blue 4 and Indigo Carmine) with immobilized mycelia of *Trametes versicolor* in different modes of operation.

Materials and Methods

Dyes

The model solutions of dyes used in the study are listed in **Table 1**.

TABLE 1

Used synthetic dyes

Dye	Class	Absorbance maximum (nm)
Phenol Red	Triarylmethane	475
Orange II	Azo	483
Congo Red	Secondary diazo	492
Reactive Violet 12	Antraquinondichlorotriazine	492
Brilliant Blue R	Reactive	587
Reactive Blue 4	Anthraquinone-basechlorotriazine	595
Indigo Carmine	Azo (food color)	680

Microorganism and inoculums

A fungal strain of *T. versicolor* 1 collected from hardwood trees in the city of Plovdiv (Bulgaria) was used in this work. The culture belongs to the collection of the Department of Biotechnology at the University of Food Technologies in Plovdiv (Bulgaria). The culture was maintained on 2 % lima bean agar plates and slants at 4 °C. Mycelial inoculum was prepared by inoculating 300 mL shake flasks containing 50 mL of Wort medium (7.5°B) with 10^7 spores of the fungus from an agar-slant culture. The inoculated flasks were incubated at 30 °C, pH 6.5 and 220 r/min for 72 h.

Immobilization of *T. versicolor*

In the present work three different methods for immobilization were used:

Immobilization by self-aggregation. Biomass in the form of pellets was filtered and washed out with saline and was then added to 10 % glutaraldehyde solution in 1:1 ratio (to a final concentration of 5 %). The suspension was placed in a thermostatic shaker bath at 40 °C for 5 h. The obtained immobilized preparation was washed out and stored under a layer of saline at 4 °C.

Immobilization by calcium alginate gel entrapment. Fungi or spores and Na-alginate solution (1 g of alginate in 15 mL of saline) were mixed in a 0.5:1 ratio and the homogenized mixture was dispensed into a chilled 0.5 mol/L $CaCl_2$ solution as droplets. After filtration and washing out with saline, the formed beads were allowed to cure at 4 °C for 2 h.

Immobilization by chitosan gel entrapment. A 2 % chitosan solution (2 g of chitosan in 87 mL of water and 13 mL of CH_3COOH) was prepared at 80 °C by stirring. Fungi, chitosan solution and 25 % glutaraldehyde solution to a final concentration of 2 % were mixed by intensive stirring. The resulting gel was crushed through a sieve to particles of 1 mm. The immobilized preparation was washed out with phosphate buffer (pH 6.0) and was kept for 12 h at 4 °C.

Decolorization

The immobilized biomass and the model dye solution (0.125 g/L) in a 1:10 ratio (w/v) were added in flasks to a final volume of 50 mL, simulating a 'batch' type reactor for the decolorization process. Decolorization was carried out at 30 °C in a thermostatic shaker bath. The model dye solution was periodically replaced with a fresh one (once a day and once an hour for 10 % (w/v) and 30 % (w/v) immobilized biomass, respectively). One operational stability cycle is considered as one replacement of the dye solution during usage of the same immobilized biomass. Samples were taken periodically from dye solution already taken for HPLC analyses, laccase activity and absorbance measurement (for each dye at a specific wavelength).

Glass columns, 13 mm in diameter with a 15 cm packed bed layer of self-aggregated pellets and glutaraldehyde cross-linked pellets from *T. versicolor* 1, were used for the continuous process of synthetic dye decolorization. The dye solution (0.125 g/mL) was constantly fed through the column. The flow rate was not controlled and the liquid droped gravitationally, simulating a 'trickle-bed' bioreactor. Samples were taken periodically for HPLC analyses, laccase activity and absorbance measurement (for each dye at a specific wavelength).

Laccase activity

Laccase activity was measured spectophotometrically using syringaldazine as a substrate. A 0.008 % ethanol solution of syringaldazine (0.5 mL), 1.5 mL of acetate buffer (pH 4.5) and 1 mL of sample were mixed at 30 °C. Absorbance was measured at 530 nm against a control containing distilled water instead of a sample. Samples were kept at 30 °C and after 10 min the absorbance was measured again. One unit of laccase activity (*LA*) was defined as 0.001 absorbance change for 1 min at pH 4.5 and 30 °C: $LA = \Delta A.R/10$, U/mL, where ΔA is the absorbance change; R is the dilution factor, 10 is the hydrolysis time [min].

HPLC analyses

HPLC analyses were carried out with a C18 10 μm Bondapac column (3.9 mm × 300 mm) and a 'Waters' 484 UV detector. The mobile phase was a methanol–water mixture in a ratio of 70:30. The flow rate was 0.2 mL/min, the temperature was 22 °C. The sample volume was 20 μL and the effluence time of the sample was 15 min.

All decolorization capability and enzyme activity experiments were performed in triplicates. The data shown in the figures and tables are average values.

Results and Discussion

T. versicolor 1 was chosen for this study because it is known as an efficient laccase producer and for its previously established capability of synthetic dye degradation (1, 2, 21, 28, 29, 31).

Decolorization by immobilized cells is more perspective than traditional dye-removing processes (18). Immobilized preparations contain a higher biomass concentration per volume unit and have higher operational stability. This leads to process intensification and acceleration of decolorization rates. Four different immobilized preparations were obtained: 1) self-aggregated pellets cross-linked with glutaraldehyde; 2) biomass immobilized in chitosan gel; 3) biomass immobilized in alginate gel; 4) spores of *T. versicolor* 1 immobilized in alginate gel.

Fig. 1 shows decolorization of Phenol Red by the immobilized preparations. An initial dye concentration of 0.125 g/L for 144 h was decolorized up to 89 % by preparation **1**, up to 78 % by preparation **2**, up to 43 % by preparation **4**, and up to 35 % by preparation **3** of *T. versicolor* 1. Based on these results, self-aggregated pellets cross-linked with glutaraldehyde (preparation **1**) were chosen for all further experiments. The significance of the present work lies in the utilization of this specific kind of immobilized biomass, the so-called immobilization without carrier, meaning that the immobilized preparation contains only biomass.

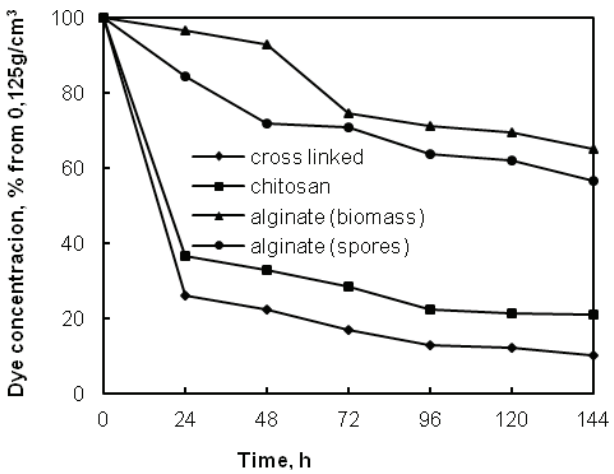

Fig. 1. Phenol Red decolorization by different immobilized preparations of *T. versicolor* 1 (10 % (w/v) immobilized biomass concentration).

To determine the decolorization rates (10 % immobilized preparation), laccase activity and the correlation between them, experiments were carried out in flasks simulating a 'batch' reactor. The initial concentration of all model dye solutions was 0.125 g/L. The obtained laccase activity values are presented in **Table 2**. **Fig. 2** shows that the immobilized preparation was able to decolorize Orange II, Congo Red, Reactive Violet 12, Brilliant Blue R, Reactive Blue 4 and Indigo Carmine for 48 h. The decolorization rates reached 88 %, 99.8 %, 100 %, 97.2 %, 89.8 % and 95 %, respectively.

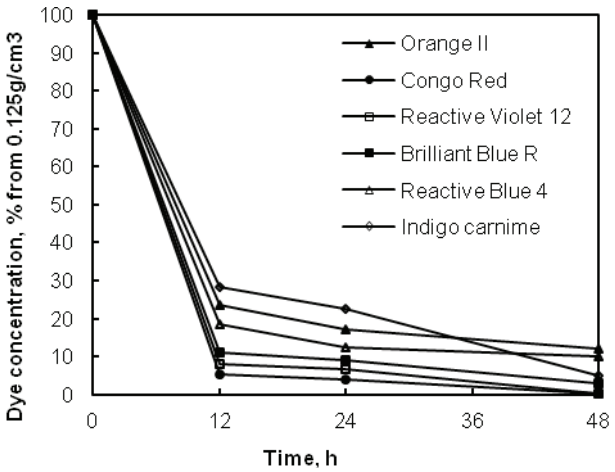

Fig. 2. Periodic decolorization process of different synthetic dye by self-aggregated glutaraldehyde cross-linked *T. versicolor* 1 pellets (10 % (w/v) immobilized biomass concentration).

TABLE 2

Laccase activity in the liquid phase during periodic synthetic dye decolorization (10% (w/v) immobilized biomass concentration)

Dye	Laccase activity, U/L		
	0 h	24 h	48 h
Orange II	0	47	43
Congo Red	0	13	13
Reactive Violet 12	0	41	18
Brilliant Blue R	0	41	30
Reactive Blue 4	0	47	24
Indigo Carmine	0	16	11

HPLC analyses also proved complete dye degradation and absence of color. This study, however, did not consider the toxicity of the degradation compounds some of which could be toxic to aquatic life. The focus was on dye removal because these kinds of effluents increase the water turbidity and temperature which significantly reduces the amount of dissolved oxygen in the effluent and changes the composition of the aquatic life.

The results obtained by us indicate that the self-aggregated cross-linked pellets are suitable for synthetic dye removal. The applied method was found to be fast (48 h) and highly efficient (100 %). As reported by Faraco et al. (9) and Moreira et al. (18), the variation of laccase activity (13.5 U/L to 47.5 U/L) and decolorization are not necessarily closely interconnected as it could be expected in principle. The *T. versicolor* 1 strain was shown in our previous experiments (32) to have enzymes able to oxidize, transform and decompose different aromatic compounds. HPLC analyses demonstrated presence of degradation compounds (data not shown). However, the decolorization process could not be attributed only to laccase

activity. Other factors that could also affect the process are the utilization of dyes as a sole carbon source and adsorption on the immobilized preparation. The synthetic dye decolorization process was found to be complex and very complicated and to result from the simultaneous action of dye degradation, bio-oxidation and biosorption processes.

To study the effect of immobilized biomass concentration on the process of decolorization experiments were carried out in flasks simulating a 'batch' reactor with 30 % (w/v) of immobilized biomass concentration. The obtained results clearly showed that the increase in the concentration of immobilized preparation led to higher decolorization rates (**Fig. 3**). At 2 h of the dye removal process 97 % of Reactive Blue 4 and 98 % of Indigo Carmine were decolorized and 100 % removal efficiency was reached at 4 h. The immobilized preparation led to decolorization of 83 % of Orange II, 93 % of Reactive Violet 12, 96 % of Brilliant Blue R and 98% of Congo Red within 4 hours. Neither the first, nor the second experiment confirmed the results of Hu (11) who reported that the color removal rate was faster in the case of monoazo dyes compared to diazo and triazo ones. The higher immobilized preparation content allowed higher laccase production from the very beginning of the process (**Table 3**). Laccase activity was observed to have an important role in the decolorization processes.

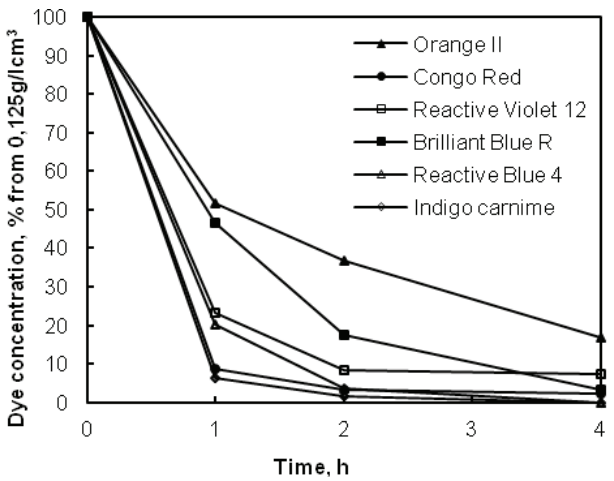

Fig. 3. Periodic decolorization process of different synthetic dye by self-aggregated glutaraldehyde cross-linked *T. versicolor* 1 pellets (30 % (w/v) immobilized biomass concentration).

Operational stability (half-life) is one of the most significant characteristics of immobilized preparations. It defines the time for 50 % activity loss during usage of the same immobilized biomass. **Fig. 4** shows operational stability of the used immobilized preparations. The operational stability of repeatedly used self-aggregated cross-linked *T. versicolor* 1 pellets was five cycles in the case of Orange II and Reactive Blue 4 decolorization. There was rapid loss of dye decolorization activity shortly after the third cycle. The operational stability of the immobilized preparation for Indigo Carmine decolorization was six cycles. Rapid loss of activity of about 30 % was observed after the fifth cycle. Seven cycles was the operational stability of the immobilized preparation for Reactive Violet 12 decolorization. Gradual activity loss was detected after the third implementation cycle. The achieved operational stability for Brilliant Blue R decolorization was nine cycles, with a gradual loss of enzyme activity after the fifth cycle. After 36 cycles of Congo Red decolorization, 90 % dye removal was achieved, which is close to the results obtained by Enayatzamir et al. (7), who reported almost total decolorization of different azo dyes by *Phanerochaete chrysosporium* micelle immobilized in alginate pellets.

TABLE 3

Laccase activity in the liquid phase during periodic synthetic dye decolorization (30% (w/v) immobilized biomass concentration)

Dye	Laccase activity, U/L			
	0 h	1 h	2 h	4 h
Orange II	0	60	63	3
Congo Red	0	54	11	2
Reactive Violet 12	0	40	21	6
Brilliant Blue R	0	36	11	2
Reactive Blue 4	0	62	20	2
Indigo Carmine	0	63	18	6

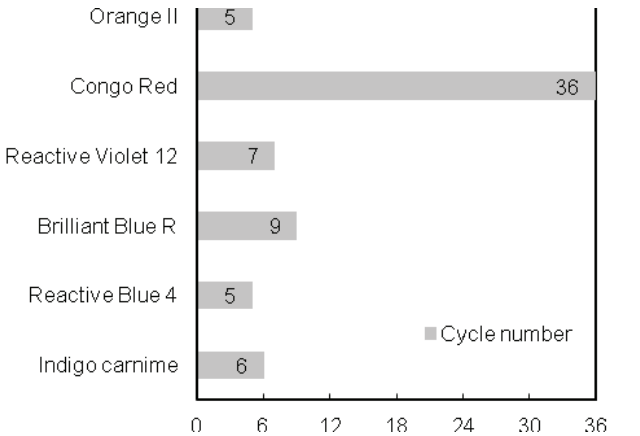

Fig. 4. Operational stability of repeatedly used self-aggregated glutaraldehyde cross-linked *T. versicolor* 1 pellets for dye decolorization.

The moment of dye addition is also an important factor. The dyes were added when about 50 U/L enzyme activity was detected. To be efficiently reused, the immobilized preparation had to be kept in sterilized calcium chloride (2 g/L) for 3 weeks at 4 °C. The present experiment was carried out with simultaneous addition of dye and immobilized preparation. Our immobilized preparation did not need any nutrient supplementation between the cycles.

The most significant advantage of immobilized preparations is the possibility to design continuous processes. For continuous decolorization a 'trickle-bed' column bioreactor was simulated. Self-aggregated glutaraldehyde cross-linked *T. versicolor* 1 pellets were used as the column filling. Complete

decolorization was detected for all synthetic dyes. Hundred percent removal of all synthetic dyes was achieved in the first 24 h to 26 h of the continuous process (**Fig. 5**). The immobilized preparation was capable of removing Orange II, Brilliant Blue R and Indigo Carmine with a removal efficiency of about 50 % within 48 h, 72 h and 84 h, respectively. The operational stability for Reactive Violet 12 decolorization was 100 h, the time for more than 50 % decolorization (**Fig. 6**). The immobilized preparation was found to be extremely efficient for decolorization of Reactive Blue 4 and Congo Red . The operational stability was 300 h and 1050 h, respectively. Complete Congo Red decolorization was observed after more than 600 h. About 80 U/L laccase activity was detected in the decolorized outflow. After 30 h, gradual enzyme activity loss was observed. However, the decolorization processes lasted much more than 30 h. This suggests that the decolorization process was due not only to laccase activity (18), but also to dye degradation and especially to biosorption processes.

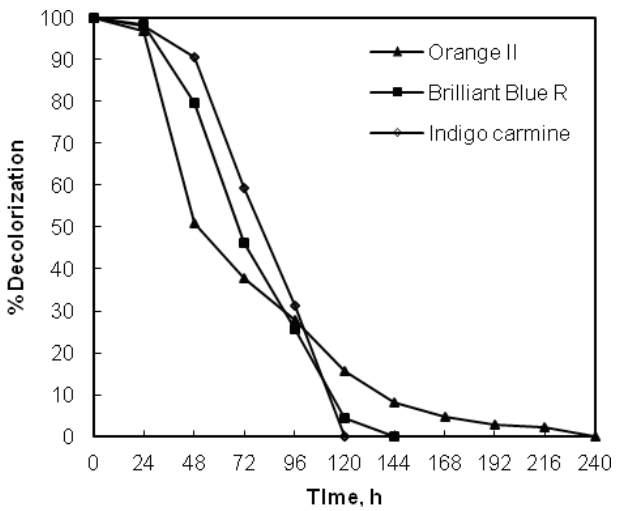

Fig. 5. Continuous decolorization process of Orange II, Brilliant Blue R and Indigo Carmine by self-aggregated glutaraldehyde cross linked *T. versicolor* 1 pellets in 'trickle-bed' bioreactor.

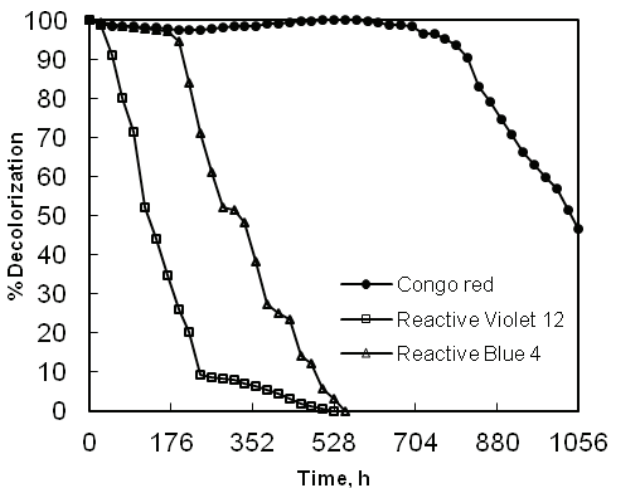

Fig. 6. Continuous decolorization process of Congo red, Reactive Violet 12 and Reactive Blue 4 by self-aggregated glutaraldehyde cross-linked *T. versicolor* 1 pellets in 'trickle-bed' bioreactor.

The investigated processes and interrelations between them are the focus of a large body of research. The mechanism of decolorization and degradation of synthetic dyes with aromatic structure is poorly studied. This problem remains a subject of great importance and is open for new research. Hence, the main contribution of the present work lies in the possibility for complete decolorization of synthetic dyes. Moreover, the immobilized biomass showed high operational stability and was obtained by an extremely easy and simple method.

Conclusions

Various immobilization methods and approaches for decolorization of synthetic dyes were explored. Although the methods and experiments presented in this work are not comparable to other studies because of the different synthetic dyes, strains and preparations (native and immobilized) used, the obtained data demonstrate the high decolorization capability of the fungus *T. versicolor* 1. Complete or near complete decolorization of all investigated dyes was achieved. The advantages of immobilized preparations usage for the treatment of highly polluted wastewaters were demonstrated as well as their multiple-cycle processing capability without the necessity of nutrient addition.

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