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Decolorization of azo and triphenylmethane dyes by MW113 *Beauveria bassiana* strain

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ABSTRACT

Dyes are used extensively to add color to various substrates in the manufacturing process and they are difficult to remove from wastewater. There is constant need for good biological alternatives to wastewater treatment. Fungi have a great potential to degrade and biotransform color dyes because of their broad enzymatic abilities. We performed the toxicity tests of the processed products to prove the possibility of using fungal processes to degrade synthetic dyes. In the experiments, two dyes (brilliant green and Evans blue) belonging to different classes – triphenylmethane and azo, respectively, were used (as well as their 1:1 w/w mixture). The MW113 fungal strain of *Beauveria bassiana* was chosen as a potential organism to degrade the dyes in a bioreactor. The experimental conditions included dyes concentration, static/shaken culture, mycelium immobilization, and medium composition and were established during earlier studies. The changes in zootoxicity (against *Daphnia magna*) and phytotoxicity (against *Lemna minor*) were estimated at the end of the experiments. The investigated MW113 fungal strain effectively removed dye mixture in the bioreactor. The highest removal reached 99.4% after 168 h at third week of experiment. The toxicity tests showed that *B. bassiana* metabolites were not toxic to *L. minor* and highly toxic (Bioreactor I) and toxic (Bioreactor II) to *D. magna*.

Keywords: Dye decolorization; Zootoxicity; Phytotoxicity; *Beauveria bassiana*; Triphenylmethane dye; Azo dye

1. Introduction

Dyes are chemical compounds that are widely used in various industries (e.g., leather, textile, and paper) and, therefore, they are found in wastewater, which leads to the pollution of surface water deposits. These kinds of pollution may be toxic to organisms (mutagenic, carcinogenic, and allergenic), cause colorization of water, and reduce light penetration and photosynthetic activity [1]. The dyes can be grouped based on their chemical structures (e.g., the presence of chromophoric group), and azo and triphenylmethane dyes are the largest classes of commercially produced colorants [2]. Triphenylmethane dyes are used for coloring acryl fibers and paper during the production of *n*-type photoconductors and the production of CDs and diskettes (as an IR-absorbing dye to read the information on a storage medium). Currently they are less frequently used in textile industry due to fast fading. Azo dyes occupy the first place among all classes of dyes in terms of the number of representatives and diversity in chemical structure. They account for about 70% of the total

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discharge of dyes from the textile industry to surface waters. Over 2000 of azo dyes are used to color leather, cosmetics, and food [3,4].

Aromatic structure of dyes makes them more resistant to biodegradation [5]. Therefore, intensive research on decolorization and detoxification of dyes using physical, chemical, and physicochemical processes (include membranes) has been widely initiated. Combining these methods can result in higher efficiency of the removal of dyes. However, this technology is energy-consuming and expensive [4]. Therefore, cheaper and more effective methods of wastewater treatment are needed, and biological treatment may be one among them. Thus, intensive research is being continued to identify the organisms which are most effective in biological removal of dyes. Decolorization of synthetic dyes by microorganisms can be very effective, less expensive, and friendlier method for the environment [6]. Biodegradation is the most efficient process of organic substance removal, flowing with wastewaters into the soil or water ecosystems [7]. Decolorization of synthetic dyes by white and brown rot fungi used as sorbents and/or producers of non-specific enzymes as laccase or manganese peroxidase and lignin peroxidase is well-known in scientific literature [8]. Moreover, studies on removal of synthetic dyes are conducted on fungi which do not cause typical rot of wood, for example, *Aspergillus* spp., *Fusarium* spp., and *Beauveria* spp., whereas the biological degradation depends on their enzyme production abilities, and on the fact that each species has a different potential of biotransformation/biodegradation and may lead to complete mineralization of the dyes or transformation them to less toxic products [9,10].

Beauveria bassiana is a broad spectrum enthomopatogenic fungus used also as an alternative to pesticides [11]. Entomopathogenic fungi produce chitinases, proteases, and lipases to penetrate the cuticle of the host. Hydrolytic role of the enzymes could have additional important application. Besides the degradation of the cuticle, they can also biotransform other chemical compounds, such as synthetic dyes. The goal of the research was to determine the efficiency of the biotransformation of single dyes and dye mixture by *B. bassiana.* Ecotoxicity tests with *Daphnia magna* and *Lemna minor* were carried out to determine if the decolorization may lead to the production of toxic by-products.

2. Materials and methods

2.1. Strain used in the experiment

In this study *B. bassiana* strain MW113 was used. Isolation method and results of previous decolorization tests were shown in earlier paper by Przystaś et al. [12]. The isolate was grown on Malt Extract Agar medium (Difco/Fluka Analytical Sigma-Aldrich, India) with chloramphenicol (concentration 0.1 g/L) to eliminate bacterial contaminants. Then, growing mycelium of MW113 strain was transferred on the liquid yeast-extract peptone glucose (YEPG) medium [13] and maintained in pure culture for future studies on decolorization. In both cases strain MW113 was cultivated for 7 d at 20°C–25°C. Isolate of *B. bassiana* (strain MW113) was deposited in the collection of the Environmental Biotechnology Department, Silesian University of Technology, Gliwice, Poland.

2.2. Genomic DNA isolation and polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region

The culture of *B. bassiana* was grown stationary in Lindeberg and Holm [14] medium at room temperature (25°C) for 7 d. Mycelia were harvested through Miracloth (Merck, Whitehouse Station, NJ, USA), washed twice with TE buffer, and frozen in liquid nitrogen. DNA was isolated according to Borges et al. [15]. The purity and quantity of the DNA samples were evaluated using an ND-1000 spectrophotometer (Thermo Scientific, West Palm Beach, FL, USA). PCRs were performed using Sigma REDTaq in a T-personal thermal cycler (Biometra, Goettingen, Germany). To confirm the fungus identity, the ITS region in the nuclear ribosomal repeat unit was determined by direct sequencing of the PCR products amplified with ITS1-ITS4 primers as described previously [16]. Automatic sequencing was performed using a BigDye™ Terminator Cycle Sequencing Kit and an ABI PRISM 310 or ABI PRISM 3730 XL sequencer (Applied Biosystems, Carlsband, CA, USA).

2.3. Bioinformatic tools

Data from sequencing were analyzed with Lasergene v.8.0 software (DNASTAR, Inc.). Database searches were performed with the BLASTn algorhitm using nucleotide databases at the National Centre for Biotechnology Information (Bethesda, MD, USA) and the European Bioinformatic Institute (Hinxton, UK).

2.4. Dyes used in the experiments

Triphenylmethane dye brilliant green (POCh, Gliwice, Poland) and azo dye Evans blue (Sigma-Aldrich, St. Louis, MO) water solutions sterilized by Millipore cellulose filters 0.20 μm were used during the study. The dye mixture was prepared with an equal proportion (1:1 w/w) of both dyes. The characteristics of dyes were presented in previous paper [17]. The wavelength of maximum absorbance for brilliant green (624 nm) and for Evans blue (591 nm) was determined experimentally. The wavelengths for dye mixture were 606, 624, and 591 nm. Dyes concentrations were measured in spectrophotometer UV-VIS (Hitachi U1900).

Percentage of dye removal was calculated according to the following formula:

$$R(\%) = \frac{(C - S)}{C} \times 100\% \quad (1)$$

where C is the current concentration of dye in a control sample (sterile medium with the dye or dye mixture) (mg/L), and S is the current residue concentration of dye in samples with fungal biomass (mg/L).

2.5. Influence of single dye concentration on the efficiency of decolorization by strain MW113

We analyzed different concentrations of the dye to select those which are not toxic to the mycelium and the process of decolorization occurs with maximum efficiency. These solutions were used in the next step.

After 7 d of MW113 strain incubation in tubes containing 10 mL of YEPG medium, water solutions of brilliant green and Evans blue were loaded. Solutions were added to tubes with fungal biomass with adequate concentrations. For brilliant green they were 1.25, 0.6, 0.3, 0.15, 0.08, 0.04, and 0.02 g/L and for Evans blue 0.6, 0.3, 0.15, 0.08, 0.04, 0.02, and 0.009 g/L. Each modification—brilliant green Z1, Z2, Z3, Z4, Z5, Z6, and Z7, and Evans blue N1, N2, N3, N4, N5, N6, and N7—was prepared in four replicates, at the same time control samples were prepared (medium, medium + dye [brilliant green or Evans blue]).

After 8 d of incubation at 25°C, absorbance of samples was measured, and percentage of removal was calculated based on Eq. (1).

2.6. Influence of shaking condition on efficiency of dyes decolorization

Mycelium of MW113 strain was added to flasks containing 150 mL of YEPG medium. Water solutions of brilliant green and Evans blue were added to 7-d-old fungal samples. According to results of the previous test, final dye concentrations in samples were 0.15 g/L for both brilliant green and Evans blue and 0.08 g/L (0.04 g/L brilliant green and 0.04 g/L Evans blue) for the mixture of both dyes. The same concentrations of dyes and their mixture were added to flask with dead biomass for the estimation of bio-sorption and were obtained by samples autoclaving (15 min., 121°C, 1.5 atm). Three biological replicates were prepared: living biomass with single synthetic dye or their mixture incubated in shaken condition—100 rpm (MW113 LB-D); living biomass with single synthetic dye or their mixture incubated in static conditions (MW113 LB-S); and dead biomass with single synthetic dye or their mixture incubated in static conditions (MW113 DB-S).

Absorbance was measured after 1, 3, 6, 24, 48, 72, and 96 h, and percentage of the removal was calculated based on Eq. (1).

2.7. Influence of immobilization of B. bassiana strain on decolorization efficiency

Plastic washer was used for immobilization of strain MW113. Mycelium was added to flasks containing 250 mL of YEPG medium. Inoculated samples were incubated for 7 d in shaken conditions (100 rpm) to ensure the growth of mycelium on the material.

After 7 d of incubation, the solutions of brilliant green and Evans blue and the mixture were prepared. Dyes were added to flasks with fungal biomass with adequate concentrations: brilliant green and Evans blue—0.1 g/L, and dye mixture 0.08 g/L (brilliant green and Evans blue in proportion 1:1 w/w). At the same time, control samples were prepared. Three biological replicates were prepared for each experimental option, and the values were averaged.

Absorbance was measured after 1, 6, 12, 24, 48, 72, 96, 120, and 144 h, and percentage of the removal was calculated based on Eq. (1).

2.8. Optimization of medium composition

To increase the effectiveness of the decolorization of synthetic dyes, liquid YEPG medium was modified using different concentrations of three components: glucose, peptone, and yeast extract. The concentration of those components per liter was as follows: Medium 1—10 g glucose, 2 g yeast extract, and 5 g peptone; Medium 2—10 g glucose, 2 g yeast extract, and 1 g peptone; Medium 3—10 g glucose, 0 g yeast extract, and 5 g peptone; Medium 4—10 g glucose, 0 g yeast extract, and 1 g peptone; Medium 5—5 g glucose, 2 g yeast extract, and 5 g peptone; Medium 6—5g glucose, 2g yeast extract, and 1 g peptone; Medium 7—5 g glucose, 0 g yeast extract, and 5 g peptone; Medium 8—5 g glucose, 0 g yeast extract, and 1 g peptone; Medium 9—2.5 g glucose, 2 g yeast extract, and 5 g peptone; Medium 10—2.5 g glucose, 2 g yeast extract, and 1 g peptone; Medium 11—2.5 g glucose, 0 g yeast extract, and 5 g peptone; and Medium 12—2.5 g glucose, 0 g yeast extract, and 1 g peptone.

Mycelium was added to the samples with modified medium (250 mL) and was incubated for 7 d, then water solutions of Evans blue and brilliant green mixture were prepared and added to the flasks. Final mixture concentration in the samples was 0.08 g/L. After 7 d of incubation, the absorbance was measured, and percentage of the removal was calculated based on Eq. (1). Change in the amount of biomass after 7 d of incubation was examined in addition to the determination of the optimal composition of the substrates for the decolorization of dyes.

2.9. Bioreactors with immobilized biomass

Experiments were carried out on a modified YEPG medium (10 g/L glucose and 1 g/L of peptone). Two different reactors were prepared.

Plastic washer was used for the immobilization of the mycelium, and the capacity of the bioreactor was 2,000 mL. Samples were incubated for 7 d at room temperature (24°C) on rotary shaker (100 rpm). Samples were aerated—air volume flowing through the bioreactor was 1.16×10^{-3} cm^3/s. In the second reactor, a modification was introduced which included glucose reduction by a half in comparison with the 1st bioreactor (5 g/L glucose and 1 g/L of peptone) in order to compare the effectiveness of decolorization of the dye mixture by *B. bassiana* on substrates with different nutrient concentrations. Bioreactor was aerated (injection of 20 mL of air at every day) and shaken at 100 rpm. After 7 d of incubation, the dye mixture was prepared and added to bioreactor (final concentration was 0.08 g/L). Bioreactors were prepared in two biological and three technical replicates. Samples were collected after direct addition of dye mixture to the bioreactors—0 and after 0.5, 24, 48, 72, 96, 120, 144, and 168 h. Absorbance was measured as well as the pH (pH changes were corrected with 0.1 M NaOH). The study was carried out for 4 weeks (new portion of the fresh medium and the dye mixture was added weekly, the same amount each time). The mycelium was grown on the liquid medium (150 mL) having the same composition as in the bioreactor (pH 5.6–5.8).

2.10. Toxicity tests

The zootoxicity was evaluated using *D. magna* (OECD 202), and phytotoxicity was tested using OECD *Lemna* sp. growth inhibition test no. 221. Tests were performed in quadruple. EC50 value was estimated. Based on these data, acute toxicity unit (TU_a) was calculated {Eq. (2)) and toxicity classes were established.

$$TU_a = \frac{100}{EC50} \quad (2)$$

where EC50 is the effective concentration of a wastewater sample that causes 50% inhibition of tested organisms. Samples were classified according to the ACE 89/BE 2/D3 Final Report Commission EC: $TU_a < 0.4$—not toxic (I class); $0.4 \le TU_a < 1.0$—low toxicity (II class); $1.0 \le TU_a < 10$—toxic (III class); $10 \le TU_a \le 100$—high toxicity (IV class); and $TU_a > 100$—extremely toxic (V class).

3. Results and discussion

3.1. PCR amplification of the ITS region

The strain of *B. bassiana* used in this study was genetically identified by determination of the ITS sequences. One product of 569 bp was obtained by PCR with ITS1-ITS4 primers and subjected to direct sequencing. The complete sequence of this product indicated over 99% identity to the *B. bassiana* ITS sequences and was deposited in GenBank under accession number GU109336.

3.2. Influence of dyes concentration on the efficiency of decolorization by strain MW113

Efficiency of dyes' degradation depends on many factors such as the type of media, temperature, pH, complexity of chemical structures of the dyes, and their concentration [18]. Selvam and Shanmuga Priya [19] in their study confirmed this hypothesis. Dyes having complex structures were more susceptible to enzymatic degradation. The tested MW113 strain tolerated all dye concentrations used in experiment, which was confirmed by the visible growth of the mycelium. However, the removal of Evans blue (Fig. 1) and brilliant green (Fig. 2) showed various degrees of intensity related with concentrations. The highest efficiency of decolorization of Evans blue by MW113 strain was noticed in samples with concentration 0.08 g/L—94.4%. For brilliant green, the highest noticed decolorization was 78.76% also in samples with 0.08 g/L of this dye. Further increasing of dyes' concentration resulted in decrease in dyes removal. Evans blue was more toxic to fungal biomass, because in concentration of ~0.6 g/L the removal was only about 15% where in case of brilliant green it was almost 30%. Similar research was carried out by Radhika et al. [20]. Among three tested dyes bromophenol blue was degraded with the highest efficiency, whereas brilliant green was the one with the lowest score. Efficiency of decolorization depended on dyes' concentration and fungal species used in the experiment. Increasing of brilliant green concentration in medium from 10 to 20 mg/L caused the decrease of decolorization effectiveness of *Pleurotus florida* strain from

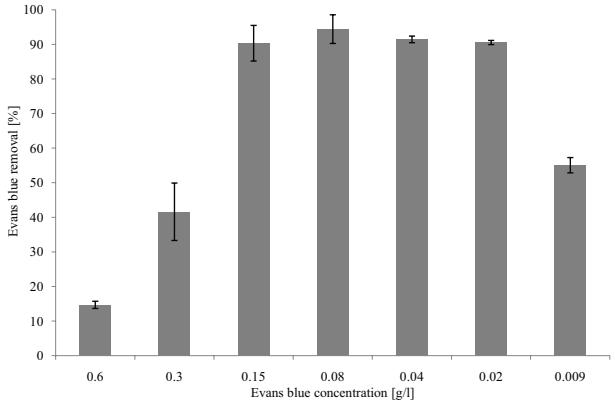

Fig. 1. Removal of different Evans blue concentrations by MW113 strain.

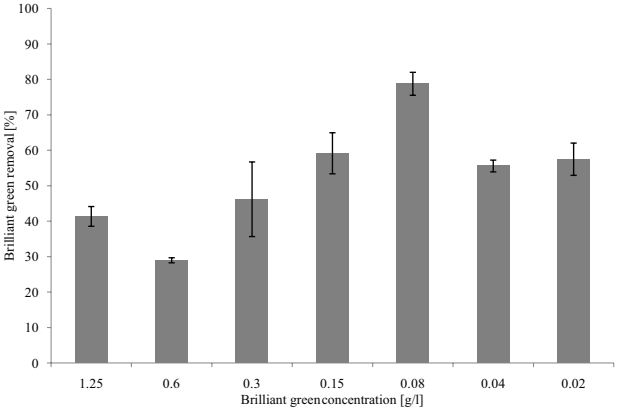

Fig. 2. Removal of different brilliant green concentrations by MW113 strain.

16% to 11%. Yang et al. [21] investigated the efficiency of Reactive Black 5 decolorization by *Debaryomyces polymorphus*. In samples with 200 mg/L of dye, the effectiveness of decolorization reached 100%, in samples with 400 mg/L of dye the effectiveness of decolorization dropped to 80% and in samples with 1,000 mg/L of dye the effectiveness of decolorization was only 30%. Also Ali and Mohamedy [22] noticed that increasing dye concentration caused the decrease of dyes degradation efficiency.

According to the results of presented tests, 0.15 g/L of Evans blue as well as brilliant green was selected for further study. This concentration was chosen because higher concentrations of the dye had negative influence on the mycelium growth. At concentration up to 0.15 g/L, the decolorization was observed and an increase in the biomass of the tested strain.

3.3. Influence of shaking on efficiency of dyes decolorization

The results obtained in the early tests provided the evidence for the ability of the MW113 strain to decolorize dyes. Here, the decolorization of brilliant green, Evans blue, and their mixture was investigated. The results of dye decolorization process and their adsorption on the dead biomass are shown in Fig. 3. Tested strain absorbed both

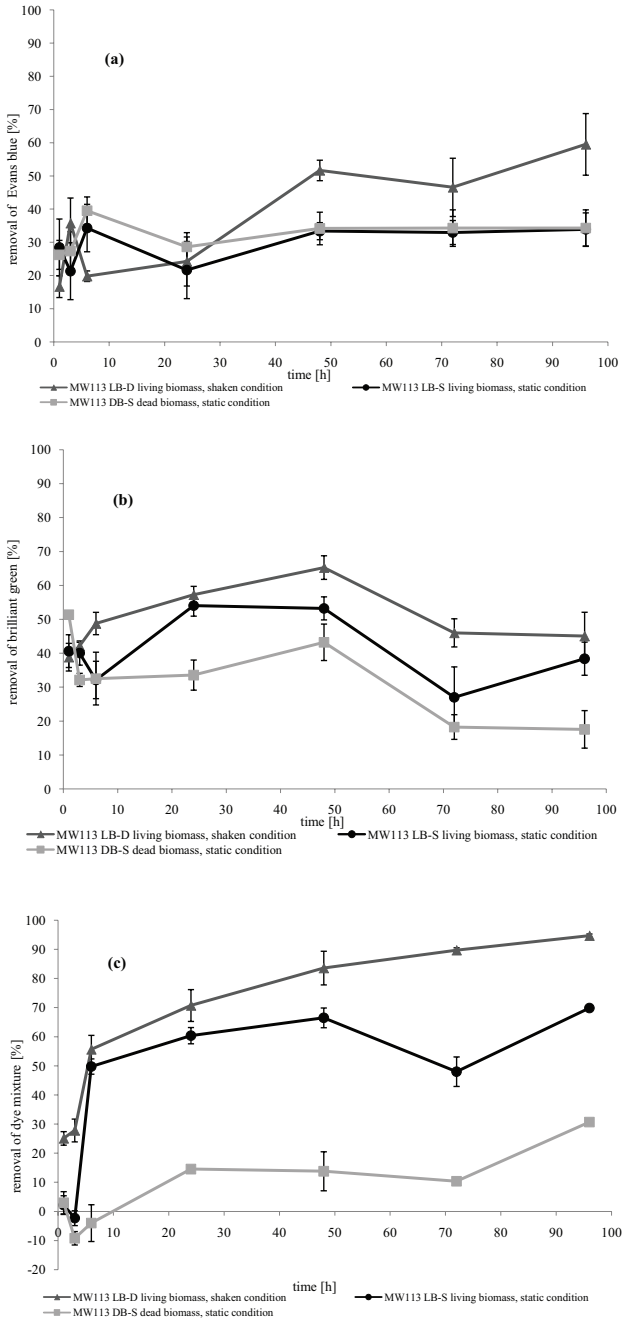

Fig. 3. Removal of (a) Evans blue, (b) brilliant green, and (c) dye mixture by living and dead biomass of MW113 strain.

dyes and their mixture, which was confirmed by intense staining of biomass, both living and dead. Removal of color may be the result of the only physical process of sorption of dye through the cell wall of the fungus, or it may be a preliminary stage of its biodegradation [23]. Generally, decolorization by living biomass is higher in comparison with the dead biomass, because both the adsorption effect and the enzymatic transformation occur simultaneously [9]; however, sometimes the intensity of the sorption of dead biomass is similar to the degradation of dyes by living biomass [24]. After the first hour of experiment, the sorption on dead biomass for Evans blue was observed (26.2%) (Fig. 3(a)). Changes in adsorption were not significant during the study, and after 96 h it remained at a similar level as at the beginning—34.3%.

After the first hour of experiment, sorption of brilliant green on dead biomass was 51.4%, but desorption was noticed during next hour, and on the last day the sorption level was only 17.6% (Fig. 3(b)). Polman and Breckenridge [25] found that the biomass has still a higher sorption capacity than the living biomass, due to the cracking of dead cells when they were killed at high temperature. Moreover, Selvam et al. [24] found that sorption of the dye by the cells of the microorganisms is the most important mechanism in the decolorization of wastewater. After the first hour, sorption of dye mixture on the dead biomass was 3.01% (Fig. 3(c)) and increased to 30.71% on the last day of experiment. Sorption value varied with alternatively overlapping processes of sorption and desorption of dye from the dead biomass. It is important to notice that mixture of dyes behaved differently than individual dyes. Such results were explained previously [13].

Seyis and Subasioglu [9] reported that each fungal strain removed azo dye—methyl orange with varying intensity. For example, *Trichoderma harzianum* removed only 5% of the dye from the medium, the sorption was lower—3%; *Trichoderma viride* was characterized by a higher efficiency of dye decolorization—50% of dye was degraded by living biomass and 40% by dead biomass. Top degrader of azo dye was the species *Fusarium acuminatum*, the effectiveness of dye decolorization by the living biomass was 70%, and by dead biomass was 39%. The effectiveness of the decolorization of dyes by living biomass basically depends on conditions and time. Strain MW113 removed Evans blue in static condition in 21.6%, in dynamic condition 24.2% in the first 20 h. However, on the last day of decolorization for the static condition it was 33.9% and for the dynamic it was 59.5% (Fig. 3(a)). In the first hour MW113 strain removed 40.6% of brilliant green in the static condition, in dynamic condition 38.4%. On the last day of research effectiveness of decolorization was 38.4% in static conditions and 45.1 % in the dynamic conditions (Fig. 3(b)). Verma and Madamwar [6] showed that the dye decolorization occurred with better efficiency (70%) in static conditions than in the dynamic ones (35%).

Strain MW113 removed the mixture of dyes with the highest efficiency in dynamic conditions. In the first hour, the decolorization of the dye mixture was 2.21% in static conditions and 25.11% in dynamic conditions. After 96 h of the experiment, the effectiveness of decolorization in the dynamic conditions was 94.74% compared with less than 70% in static samples (Fig. 3(c)).

3.4. Influence of immobilization of B. bassiana strain on decolorization effectiveness

Immobilization of biomass is known to increase decolorization process efficiency. Brilliant green and Evans blue were used in concentration of 0.1 g/L, and their mixture's (1:1) concentration was 0.08 g/L. Boehmer et al. [26] applied a natural material in the form of pine shavings and olive palm fiber in their studies on immobilization. The problem with this material was the adsorption of dyes on the surface. However, the material went back to its natural color

after 2–3 d, indicating the degradation of azo dye—remazol red diamond by *Trametes versicolor*. The result was a 52% removal for pine chips and 51% on the fibers of the olive palm after 4 d. In addition to natural materials, an artificial material can be used, as well as alginate beads [27]. Evans blue decolorization by living biomass in the first hour of the experiment was 98% (0.08 g/l), and then reached 100% (0.1 g/l) at the sixth hour. Sorption on dead biomass in the first hour was 77% (0.06 g/L) and increased to 91% after 96 h. The Fig. 4(a) shows the efficiency of Evans blue decolorization by *B. bassiana*, which increased at lower dye concentrations and biomass immobilization. The removal of brilliant green by living biomass and dead biomass ranged at similar range of 88% (0.08 g/L) to 95% (0.11 g/L), respectively (Fig. 4(b)). Removal of the dye mixture by living biomass was also high. After 6 h of experiment, sorption on dead biomass was 81% (0.09 g/L), and decolorization by living biomass was 75% (0.09 g/L). In the following hours, desorption of dyes from the biomass was observed. Slight increase of dye removal by the dead biomass was observed during next 3 d, and finally after 96 h dead biomass adsorbed 75% (0.09 g/L) of a mixture of dyes (Fig. 4(c)).

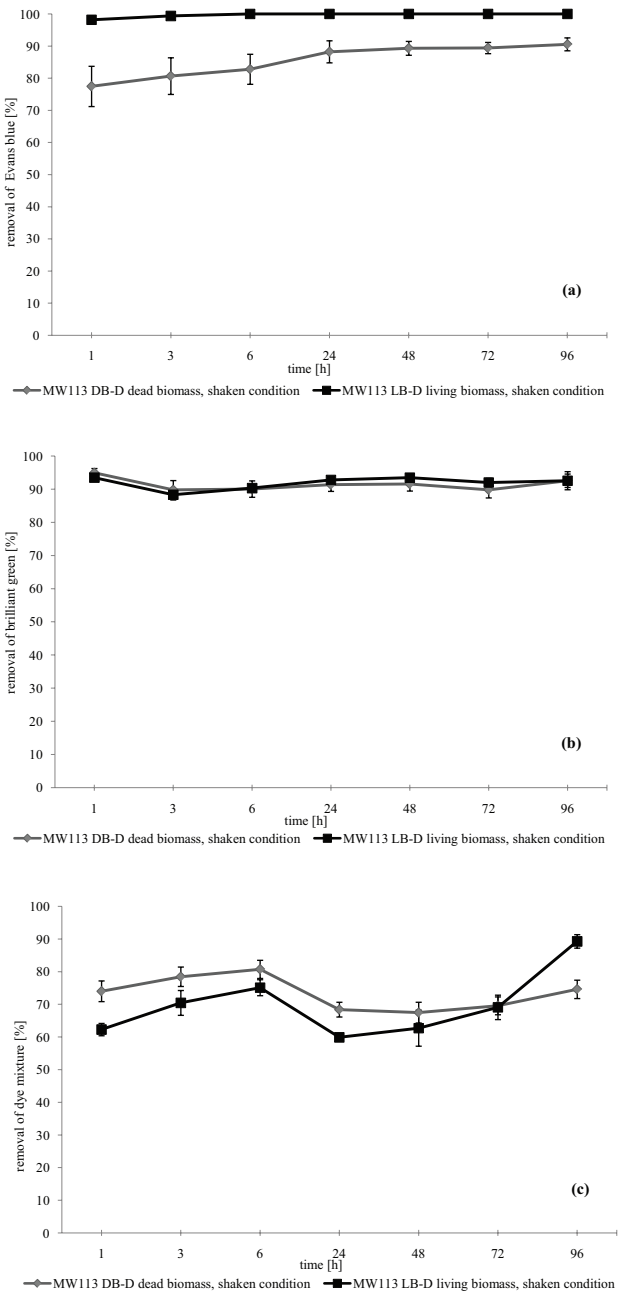

Fig. 4. Removal of (a) Evans blue, (b) brilliant green, and (c) dye mixture by immobilized living and dead biomass of MW113 strain.

Eichlerova et al. [7] concluded that the dyes differing structurally are not removed in the same way. Triphenylmethane dyes (malachite green, crystal violet at concentrations of 2 g/L) were poorly decolorized. Orange G and amaranth (azo dyes) were effectively removed from the substrate, but other azo dye Orange I was removed at low levels. Differences in the decolorization may be caused by varying rate of decomposition of chromophoric groups. Even small differences in structures may cause difficulties in removing the dye from the substrate.

3.5. Optimization of medium

Biodegradation of dyes depends on many factors including composition of the medium and culture conditions [28]. To select the optimal substrate tests were performed for 12 modifications consisting of different concentrations of glucose, peptone, and yeast extract (Table 1). The removal of dye mixture (mg/mg) by the gram of dry biomass was also examined. Decolorization of a mixture proceeded with various degrees of intensity for each substrate (Table 1). The most effective decolorization occurred on the medium 6 (5 g glucose, 2 g yeast extract, and 1 g peptone), where the loss was 88% and medium 4—81% (10 g of glucose, 0 g of yeast extract, and 1 g peptone). Medium 4 was selected for the tests on decolorization with strain MW113 in the bioreactor due to the most effective distribution of the dye mixture with 1 mg of dry biomass (Table 1) which was 11.8 ($mg/mg_{dry\ biomass}$), so 4.6 ($mg/mg_{dry\ biomass}$) higher than in sample with Medium 6. During the experiment, efficiency of dye mixture decolorization was not correlated with the concentration of biomass ($mg/mg_{dry\ biomass}$). Seyis and Subasioglu [28] had the same observation that the effectiveness of decolorization is independent from the growth of biomass and that with a small increase in mycelium the removal of dye may be associated with higher synthesis and release of enzymes to the substrate.

3.6. Bioreactor with immobilized biomass

Dyes are among the most enduring groups of compounds polluting surface waters. Modern and alternative environmental-friendly technologies are being sought for wastewater treatment. One of them may be the use of bioreactors [10]. *B. bassiana* has not been tested previously for biodegradability of dyes, apart from the work of Seyis and Subasioglu [28], where satisfactory results in decolorization were not achieved. Studies in bioreactors were conducted in four cycles (648 h) with sample collection after 0.5, 24, 48, 72, 144, and 168 h after adding a mixture of dyes. The concentration of the dye mixture was 0.08 g/L in 1:1 ratio of

Table 1
The loss of Evans blue and brilliant green dye mixture (g/L and mg/$mg_{dry\ biomass}$) in modified YEPG media

Medium	Loss of dye mixture (g/L) (wavelength 591 nm)	Loss of dye mixture (mg/$mg_{dry\ biomass}$) (wavelength 591 nm)
Medium 1	0.025 ± 0.0036	2.3
Medium 2	0.020 ± 0.0016	3.7
Medium 3	0.016 ± 0.0022	1.7
Medium 4	0.060 ± 0.007	11.8
Medium 5	0.008 ± 0.0061	1.2
Medium 6	0.053 ± 0.0043	7.2
Medium 7	0.024 ± 0.0005	6.5
Medium 8	0.010 ± 0.0009	2.7
Medium 9	0.012 ± 0.001	3.2
Medium 10	0.021 ± 0.0012	9.6
Medium 11	0.021 ± 0.0011	2.8
Medium 12	0.016 ± 0.0001	6.2

brilliant green and blue Evans, respectively. For Bioreactor I, Medium 4 was selected (10 g glucose, 0 g yeast extract, and 1 g peptone), where the mixture of dyes was most effectively degraded. For Bioreactor II, we selected Medium 8 (5 g of glucose, 0 g of yeast extract, and 1 g peptone) in order to compare the efficiency of removal of dye mixture with two substrates. The results of dye mixture decolorization are shown in Fig. 5.

For Bioreactor I, the loss of mixture of dyes ranged from 20% to 95.6% depending on the sampling date and cycle (Fig. 5). During the first cycle, the removal of dye mixture grew from 50.6% (dye concentration in the bioreactor—0.052 g/L; 0.5h) to 92.7% (concentration of the mixture in bioreactor—0.004 g/L, 168 h). In the second cycle, removal was growing and reached 93.8% at about 72nd h and maintained for 168 h of the second cycle on the same level. In the third cycle, after 0.5 h by adding a mixture of dyes the loss was 57.9% and grew to 48 h—92.5%. At 72nd hour when NaOH was added, a mixture of dyes removal dropped to 20% (the concentration of dye mixture in the bioreactor—0.088 g/L). After 168 h the decolorization efficiency increased to 95.6%. In the fourth cycle in 48th h removal increased to 92.7% and until 168 h of experiment remained at a similar level.

For Bioreactor II in first cycle the tendency was the same. Mostly after 30 min the efficiency of decolorization was among 30%–77.8% and after each cycle removal during the first few minutes was lower. During next hours decolorization efficiency was growing. At 168 h of experiment concentration in the bioreactors was between 81.1% and 90.8% (Fig. 5).

It is known that chitin, chitosan, and lignocellulose are used as adsorbents. Because fungi have a cell wall built from these sugar polymers, they can efficiently adsorb dyes. The use of live biomass as an adsorbent may be a very advantageous alternative method of removing wastewater, because the effectiveness of decolorization depends on the efficiency of adsorption on fungal biomass. The availability and cost of use in the industry also support the use of living biomass as an adsorbent [9]. It was observed that in all bioreactors results

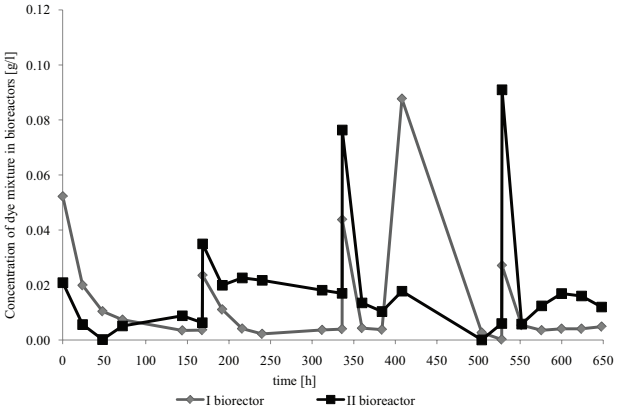

Fig. 5. Removal of dye mixture in bioreactors with immobilized biomass of MW113 strain.

of removal after 0.5 h of each cycle were lower from cycle to cycle, which may be connected with decreasing sorption capacity of fungal biomass. High levels of decolorization reached after 168 h of each cycle suggests that the removal of dye mixture is a result of adsorption as well as biochemical transformation of them.

Decreasing the medium glucose concentration from 10 to 5 g/L substrate had no significant effect on the efficiency of decolorization. It was found that the loss of the mixture of dyes in bioreactors I and II remained at a similar level. Eichlerova et al. [7] studied the removal of dyes Orange G and remazol brilliant blue by *Dichomitus squalens* on two different substrates with various composition of glucose and obtained similar results. The concentrations of glucose in malt extract (ME) medium and Kirk media had no effect on the efficiency of decolorization of remazol brilliant blue, final dye content in the samples was between 90% and 100%. Similarly for Orange G, the concentration of glucose in ME media had no effect on efficiency of decolorization (80%–90%), whereas in Kirk media changes in effectiveness of Orange G degradation were observed (increasing glucose concentration from 0.1% to 1% resulted in decrease in dye loss from 90% to 60%).

3.7. Toxicity tests

3.7.1. Biomass growth in static and shaking conditions

Zootoxicity and phytotoxicity of media after decolorization process were evaluated for all variants (Table 2). The samples containing dyes were toxic and extremely toxic to the tested organisms. The only sample classified as low toxic (II class) was medium with dead biomass before addition of dyes. Controls with medium and brilliant green dye and medium with dye mixture were extremely toxic (V class). Control with medium and Evans blue was toxic (III class).

The decolorization of Evans blue had no effect on the toxicity. MW113 removed the dye with 59.5% efficiency under dynamic conditions, but the toxicity was at the same level as the control with the same dye (III class). It suggests that the removal of Evans blue was not related to the production of large amounts of highly toxic metabolites, such as aromatic amines. The same results were achieved in the test with brilliant green. Even high removal of this dye did not lead

to the decreased zootoxicity. All samples with brilliant green dye were extremely toxic. To conclude, even high efficiency of decolorization did not contribute sufficiently to the reduction of zootoxicity. The same relation was observed in phytotoxicity tests. Samples after decolorization were classified to the same class as controls. Changes in growth of *L. minor* were observed on the third day of the experiment. Yellowing of the leaves was observed on individual lashes at the highest concentrations (Table 2). In toxicity studies carried out by Ramsay and Nguyen [29], the components of the substrate turned out to be more toxic than the dye, which may be due to the number and weight of the medium, as in the case presented in our research.

3.7.2. Biomass immobilized on washer

Ecotoxicity tests were conducted for samples with immobilized fungal biomass, where the efficiency of decolorization was higher than in samples with suspended biomass. Immobilization improved the decolorization efficiency and led to the decrease of zootoxicity. Samples with dead biomass and plastic washer were extremely toxic similarly to control samples with brilliant green dye and controls with mixture of dyes (V class). Only controls with Evans blue were classified as highly toxic to *D. magna* (IV class). In comparison with the results presented in Table 3, the increase in toxicity was visible. It may result from the substances that could be released from washer to the medium.

Table 2
Influence of *Beauveria bassiana* MW113 strain cultivation in shaking condition on zootoxicity and phytotoxicity tests

Samples	Samples with strain MW113						Controls	
	Shaken condition		Static condition		Dead biomass			
	TU_a	Interpretation/class	TU_a	Interpretation/class	TU_a	Interpretation/class	TU_a	Interpretation/class
Zootoxicity								
Medium			5.6	Toxic/III class	0.6	Low toxicity/II class	1.97	Toxic/III class
Medium + brilliant green	>100	Extremely toxic/V class	>100	Extremely toxic/V class	>100	Extremely toxic/V class	>100	Extremely toxic/V class
Medium + Evans blue	1.62	Toxic/III class	4.19	Toxic/III class	2.26	Toxic/III class	2.03	Toxic/III class
Medium + dye mixture	3.22	Toxic/III class	2.62	Toxic/III class	2.14	Toxic/III class	>100	Extremely toxic/V class
Phytotoxicity								
Medium			1.07	Toxic/III class	1.51	Toxic/III class	1.05	Toxic/III class
Medium + brilliant green	>100	Extremely toxic/V class	>100	Extremely toxic/V class	>100	Extremely toxic/V class	>100	Extremely toxic/V class
Medium + Evans blue	1.2	Toxic/III class	1.27	Toxic/III class	3.42	Toxic/III class	2.77	Toxic/III class
Medium + dye mixture	1.43	Toxic/III class	2	Toxic/III class	2.47	Toxic/III class	>100	Extremely toxic/V class

Table 3
Influence of immobilization of *Beauveria bassiana* MW113 strain on zootoxicity tests against *Daphnia magna*

Samples	Samples with strain MW113				Controls	
	Living biomass		Dead biomass			
	TU_a	Interpretation/class	TU_a	Interpretation/class	TU_a	Interpretation/class
Zootoxicity						
Medium + plastic washer	82	High toxicity/IV class	>100	Extremely toxic/V class	72	High toxicity/IV class
Medium + brilliant green + plastic washer	>100	Extremely toxic/V class	66	High toxicity/IV class	>100	Extremely toxic/V class
Medium + Evans blue + plastic washer	34	High toxicity/IV class	27	High toxicity/IV class	30	High toxicity/IV class
Medium + dye mixture + plastic washer	15	High toxicity/IV class	28	High toxicity/IV class	>100	Extremely toxic/V class

The zootoxicity test of samples with immobilized mycelium showed that the toxicity did not decrease in the case of Evans blue (IV class), although the decolorization reached 100% what suggests that some toxic metabolites were released by the strain to the medium. The toxicity of samples with brilliant green decreased from extremely toxic to highly toxic but only in the samples with dead biomass. Samples with living biomass, which were classified as extremely toxic (although the loss of dye was 93%), suggesting that this strain produces some toxic metabolites. Only in the samples containing a mixture of dyes the toxicity class decreased from V to IV (the loss of dye mixture in samples with living biomass was 89% and in samples with dead biomass 75%). It can, therefore, be concluded that even the high efficiency of decolorization process by the strain MW113 did not contribute sufficiently to the reduction of zootoxicity.

3.7.3. Bioreactors

Differences were observed between toxicity of medium used in bioreactors I and II. Medium in Bioreactor I was more toxic to *D. magna* than medium in Bioreactor II but it could be related with the concentrations of compounds used in both reactors (2× more glucose in Bioreactor I). The results of zootoxicity and phytotoxicity tests are shown in Table 4. After 30 min and after 24 h, the samples were extremely toxic or toxic to *D. magna* throughout the four cycles while after 168 h the toxicity decreased, which suggested that decolorization is effective and leads to detoxification of the dye mixture. After 168 h of first cycle, the toxicity decreased to Class III in Bioreactor I and to Class IV in Bioreactor II. In the second week at the end of the process, both reactors were again classified to III and IV class. During the third cycle 30 min after adding the dye mixture, the toxicity increased again to the extremely toxic for all bioreactors and then was reduced to the toxic after 24 h for Bioreactor I, and after 168 h for all bioreactors. After the fourth cycle of work, the Bioreactor II reached II class of toxicity, but the samples from Bioreactor I were highly toxic to tested *D. magna*. Efficiency of decolorization by strain MW113 immobilized on washer in batch reactor reached satisfactory level as well as did the decrease of zootoxicity measured.

The results of the phytotoxicity test and their interpretation are presented in Table 4. Samples after 0.5 h of the experiment were mostly toxic but the process of dyes removal by immobilized biomass led to the decrease in phytotoxicity. All samples after each cycle were classified as not toxic. It is noteworthy that only in Bioreactor I experiment the decrease of toxicity was correlated with decolorization of dye mixture, but in Bioreactor II no correlations have been observed.

Table 4
Results of zootoxicity tests against *Daphnia magna* and phytotoxicity tests against *Lemna minor* in bioreactor experiment

		Zootoxicity				Phytotoxicity			
		Bioreactor I		Bioreactor II		Bioreactor I		Bioreactor II	
		TU_a	Interpretation/ class	TU_a	Interpretation/ class	TU_a	Interpretation/ class	TU_a	Interpretation/ class
1st week	Before addition	1.75	Toxic/III class	0.47	Low toxicity/II class	<0.4	Not toxic/I class	<0.4	Not toxic/I class
	After 0.5 h	>100	Extremely toxic/V class	>100	Extremely toxic/V class	1.49	Toxic/III class	<0.4	Not toxic/I class
	After 24 h	>100	Extremely toxic/V class			1.49	Toxic/III class		
	After 168 h	8	Toxic/III class	19.79	High toxicity/IV class	<0.4	Not toxic/I class	<0.4	Not toxic/I class
2nd week	After 0.5 h	3	Toxic/III class	>100	Extremely toxic/V class	<0.4	Not toxic/I class	1.5	Toxic/III class
	After 24 h	1.49	Toxic/III class			<0.4	Not toxic/I class		
	After 168 h	1.98	Toxic/III class	12.56	High toxicity/IV class	<0.4	Not toxic/I class	<0.4	Not toxic/I class
3 rd week	After 0.5 h	>100	Extremely toxic/V class	>100	Extremely toxic/V class	1.67	Toxic/III class	<0.4	Not toxic/I class
	After 24 h	3	Toxic/III class			<0.4	Not toxic/I class		
	After 168 h	3	Toxic/III class	2.37	Toxic/III class	<0.4	Not toxic/I class	<0.4	Not toxic/I class
4th week	After 0.5 h	>100	Extremely toxic/V class	>100	Extremely toxic/V class	<0.4	Not toxic/I class	<0.4	Not toxic/I class
	After 24 h	3	Toxic/III class			<0.4	Not toxic/I class		
	After 168 h	13	High toxicity/IV class	6.36	Toxic/III class	<0.4	Not toxic/I class	<0.4	Not toxic/I class

4. Conclusion

The isolated strain of the fungus *B. bassiana* (MW113) has a high potential to remove azo and triphenylmethane dyes and mixture of them in laboratory conditions, which had a decisive influence on the effectiveness of dyes removal. Decolorization of dyes was the highest under dynamic conditions, particularly for Evans blue dye and dye mixture. Degradation of dyes in static conditions was less efficient than in dynamic conditions, however, was more effective than the sorption on dead biomass.

High efficiency of decolorization did not change the toxicity. Zootoxicity of samples with brilliant green did not decrease after the process, similarly to the samples with Evans blue. Only for the mixture of dyes, toxicity decreased from the V class of toxicity to the III class of toxicity, nevertheless, phytotoxicity remained unchanged. It was found that metabolites of mycelium and the substrate itself are also toxic *to D. magna* and *L. minor*.

The degree of decolorization of dyes by immobilized biomass depended on the type of dye. The highest removal of dyes was found in the samples with Evans blue. There was no significant influence of the effectiveness of decolorization on the change in toxicity. For Evans blue dye zootoxicity was not impaired. For brilliant green the zootoxicity dropped from V to IV class of toxicity in samples with dead biomass, and for mixture of dyes the toxicity dropped from V to IV class of toxicity in samples with dead and living biomass.

It was found that the increase in biomass often had no effect on the efficiency of the removal of dye mixture. The *B. bassiana* strain MW113 effectively removed the mixture of dyes in the bioreactor, reaching 99.4% of dye mixture added to the bioreactor.

In each cycle of the experiment, the efficiency of the removal of dye mixture was at least 80%. However, there was a fluctuation in the removal of a dye mixture resulting from the adsorption of dyes on fungal biomass. *B. bassiana* metabolites were not toxic to *L. minor*, and highly toxic (Bioreactor I) and toxic (Bioreactor II) to *D. magna*. It was noted that the ecotoxicity was associated with the loss of dye mixture in bioreactors. When the effectiveness of decolorization grew, the medium was less toxic to *D. magna*. In the future, MW113 strain cultured in a bioreactor can be used as an environmental-friendly alternative for the treatment of industrial wastewater containing toxic dyes. Continued research should be conducted on dye decolorization involving this strain and the optimization of the process.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

[1] A. Alinsafi, M. Motta, S. Le Bonte, M.N. Pons, A. Benhammou, Effect of variability on the treatment of textile dyeing wastewater by activated sludge, Dyes Pigm., 69 (2006) 31–39.

[2] Y. Liu, Y. Jiang, M. Hu, S. Li, Q. Zhai, Removal of triphenylmethane dyes by calcium carbonate–lentinan hierarchical mesoporous hybrid materials, Chem. Eng. J., 273 (2015) 371–380.

[3] S. Agarwal, V.K. Gupta, M. Ghasemi, J. Azimi-Amin, *Peganum harmala*-L Seeds adsorbent for the rapid removal of noxious brilliant green dyes from aqueous phase, J. Mol. Liq., 231 (2017) 296–305.

[4] W. Somasiri, W. Ruan, L. Xiufen, C. Jian, Decolourization of textile wastewater containing acid dyes in UASB reactor system under mixed anaerobic granular sludge, Electron. J. Environ. Agric. Food Chem., 5 (2006) 1224–1234.

[5] W.M. El-Rahim, H. Moawad, A.Z.A. Azeiz, M.J. Sadowsky, Optimization of conditions for decolorization of azo-based textile dyes by multiple fungal species, J. Biotechnol., 260 (2017) 11–17.

[6] P. Verma, D. Madamwar, Decolourization of azo dyes using Basidiomycete strain PV002, World J. Microbiol. Biotechnol., 21 (2005) 481–485.

[7] I. Eichlerova, L. Homolka, F. Nerud, Synthetic dye decolorization capacity of white rot fungus *Dichomitus squalens*, Bioresour. Technol., 97 (2006) 2153–2159.

[8] N. Wang, Y. Chu, F. Wu, Z. Zho, X. Xu, Decolorization and degradation of Congo red by a newly isolated white rot fungus, *Ceriporia lacerata*, from decayed mulberry branches, Int. Biodeterior. Biodegrad., 117 (2017) 236–244.

[9] I. Seyis, T. Subasioglu, Comparison of live and dead biomass of fungi on decolorization of methyl orange, Afr. J. Biotechnol., 7 (2008) 2212–2216.

[10] D. Wesenberg, I. Kyriakides, S.N. Agathos, White-rot fungi and their enzymes for the treatment of industrial dye effluents, Biotechnol. Adv., 22 (2003) 161–187.

[11] W. Fang, J. Feng, Y. Fan, Y. Zhang, M.J. Bidochka, R.J. St. Leger, Y. Pei, Expressing a fusion protein with protease and chitinase activities increases the virulence of the insect pathogen *Beauveria bassiana*, J. Invertebr. Pathol., 102 (2009) 155–159.

[12] W. Przystaś, E. Zabłocka-Godlewska, E. Grabińska-Sota, M. Urbaniak, Potential Ability of Some Ligninolytical Fungal Strains to Decolorize Synthetic Dyes, Ochrona Środowiska, 32 (2010) 15–20.

[13] W. Przystaś, E. Zabłocka-Godlewska, E. Grabińska-Sota, Efficacy of fungal decolorization of a mixture of dyes belonging to different classes, Braz. J. Microbiol., 46 (2015) 415–424.

[14] G. Lindeberg, G. Holm, Occurrence of tyrosinase and laccase in fruit bodies and mycelia of some Hymenomycetes, Physiol. Plant, 5 (1952) 100–114.

[15] M.J. Borges, M.O. Azevedo, J.R. Bonatelli, M.S.S. Felipe, S. Astolfi-Filho, A practical method for the preparation of total DNA from filamentous fungi, Fungal Genet. Rep., 37 (1990) 1.

[16] T. White, T. Bruns, S. Lee, J. Taylor, Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics, Vol. 315–322, M. Innis, D. Gelfand, J. Shinsky, T. White, Eds., PCR Protocols: A Guide to Methods and Applications, Academic Press, 1990. doi:citeulike-article-id:671166.

[17] W. Przystaś, E. Zabłocka-Godlewska, E. Grabińska-Sota, Effectiveness of dyes removal by mixed fungal cultures and toxicity of their metabolites, Water Air Soil Pollut., 224 (2013) 1534–1543.

[18] R.D. Koyani, G.V. Sanghvi, R.K. Sharma, K.S. Rajput, Contribution of lignin degrading enzymes in decolourisation and degradation of reactive textile dyes, Int. Biodeterior. Biodegrad., 77 (2013) 1–9.

[19] K. Selvam, M. Shanmuga Priya, Biological treatment of Azo dyes and textile industry effluent by newly isolated White rot fungi *Schizophyllum commune* and *Lenzites eximia*, Int. J. Environ. Sci., 2 (2012) 1938–1947.

[20] R. Radhika, G.R. Jebapriya, J.J. Gnanadoss, Decolorization of synthetic textile dyes using the edible mushroom fungi Pleurotus, Pakistan J. Biol. Sci., 17 (2014) 248–253.

[21] Q. Yang, A. Yediler, M. Yang, A. Kettrup, Decolorization of an azo dye, *Reactive Black* 5 and MnP production by yeast isolate: *Debaryomyces polymorphus*, Biochem. Eng. J., 24 (2005) 249–253.

[22] N.F. Ali, R.S.R. El-Mohamedy, Microbial decolorization of textile waste water, J. Saudi Chem. Soc., 16 (2012) 117–123.
[23] L. Growther, M. Meenakshi, Biotechnological approaches to combat textile effluents, Internet J. Microbiol., 7 (2008) 1–7.
[24] K. Selvam, K. Swaminathan, K.S. Chae, Decolourization of azo dyes and a dye industry effluent by a white rot fungus *Thelephora* sp., Bioresour. Technol., 88 (2003) 115–119.
[25] J.K. Polman, C.R. Breckenridge, Biomass-mediated binding and recovery of textile dyes from waste effluents, Text. Chem. Color., 28 (1996) 31–35.
[26] U. Boehmer, S.H. Suhardi, T. Bley, Decolorizing reactive textile dyes with white-rot fungi by temporary immersion cultivation, Eng. Life Sci., 6 (2006) 417–420.
[27] A. Domınguez, R.S. Couto, M. Sanroman, Dye decolorization by *Trametes hirsuta* immobilized into alginate beads, World J. Microbiol. Biotechnol., 21 (2005) 405–409.
[28] I. Seyis, T. Subasioglu, Screening of different fungi for decolorization of molasses, Braz. J. Microbiol., 40 (2009) 61–65.
[29] J.A. Ramsay, T. Nguyen, Decoloration of textile dyes by *Trametes versicolor* and its effect on dye toxicity, Biotechnol. Lett., 24 (2002) 1757–1761.