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TEXTILE DYES DECOLOURISATION BY WHITE-ROT FUNGUS *Fomes fomentarius*

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Abstract. This paper summarises experimental results obtained during textile dyes decolorisation (Fast Black HS-02 and Derma 2RN) by white-rot fungus *Fomes fomentarius*. White-rot fungi produce extracellular ligninolytic enzymes for degradation of the complex substrates, such as wood polymers, laccases playing an important role in these processes. Thus, prior to decolorisation tests, laccase activity was assayed in different culture media, highest value of 51.3 U/l being obtained using medium supplied with cellulose as a source of organic carbon and KNO_3 as a source of nitrogen. After 168 h of reaction time, decolorisation efficiencies of Black HS-02 and Derma 2RN dyes were ranged between 54.9 and 67.7%, and 57.6 and 61.1%, respectively, for the following initial dyes concentrations: 20, 40, 60, 80 and 100 mg/l. No inhibitory effect was noticed on the decolorisation efficiency by initial dyes concentrations. Dyes decolorisation efficiency was also analysed using crude enzymatic extract produced under solid-state fermentation conditions. In the case of Black HS-02 dye, maximum decolorisation efficiency was registered for 40 mg/l (1.75%), the lowest value being recorded for 100 mg/l (1.14%). Regarding Derma 2RN dye, decolorisation efficiency decreased continuously from 1.13 to 0.09% per unit of laccase activity simultaneously with the increase of the initial dye concentration.

Keywords: white-rot fungi, textile dye, laccase activity, decolorisation.

AIMS AND BACKGROUND

Dyes degradation is of high concern nowadays as due their complex molecular structure, low biodegradability[1] and wide chemical diversity, almost 100 000 of dyes being identified so far[2]. The discharge of these pollutants in the environment is followed by negative effects generated on the ecosystems health, colour pollution being one of the major problems[3]. Another effect is caused as a result of bioaccumulation and bioconcentration of dyes or intermediary degradation products, which can lead to the toxic, mutagenic and carcinogenic[4] effects on the trophic networks.

Textile industry is one of the most developed industries worldwide and also an important source of dyes-containing effluents, about 50% of globally produced dyes being involved in textile dyeing processes[5] and about 10–15% of them remain

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unused, being discharged through industrial effluents[6]. As a result, many efforts are made in order to ensure high efficiency removal of these pollutants, several physical, chemical and biological methods being applied so far[7,8]. From economic and ecologic point of view, application of the biological methods is preferential[9]. However, the treatment of textile industry wastewater using activated sludge processes is not always efficient, being reported foaming and bulking problems[10] and low treatment performances[11]. A lot of research was conducted on optimisation of bacteria and fungi strains for dyes removal. The interest is determined by the properties of the intra- and extracellular enzyme systems of the tested species[12].

Among tested microorganisms, white-rot fungi are recognised as potential candidates for dyes decolour isation and degradation. Extracellular ligninolytic enzymes produced by these species are characterised by low specificity for substrate, being able to catalyse degradation reactions of the complex, low-biodegradable chemical structures. One of the most important fungal enzymes involved in dyes decolorisation and degradation processes are laccases, which use molecular oxygen as a final electron acceptor[13].

Therefore, this study investigated the efficiency of using popular, medicinal white-rot fungus *Fomes fomentarius* for decolorisation of two textile dyes: Fast Black HS-02 and Derma 2RN, at different initial concentrations. The experiments were performed using fungi biomass and crude extract of extracellular enzymes obtained after fungi cultivation in solid-state fermentation conditions. The decolorisation efficiencies were evaluated based on spectrophotometric analysis. The suitable culture medium which led to higher laccase production by the tested species was also determined.

EXPERIMENTAL

Microorganism isolation. The fungal species were isolated from wild fruit body provided by the Department of Botany and Microbiology, University of Bucharest, Romania. Several plugs of the fungi biomass were inoculated in 100 ml of autoclaved basal medium distributed in 250 ml Erlenmeyer flasks. Taken into consideration the results obtained by Chen and collaborators[14], the basal medium included: 20 g/l glucose, 3 g/l peptone, 3 g/l yeast extract, 10 g/l cellulose, 1 g/l KH_2PO_4, 0.126 g/l KNO_3, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l $CaCl_2 \cdot 2H_2O$, 0.04 g/l $FeSO_4 \cdot 7H_2O$, 0.005 g/l $MnSO_4 \cdot H_2O$, 0.01 g/l $CuSO_4 \cdot 5H_2O$, 0.03 g/l NaCl and 0.005 g/l $ZnSO_4 \cdot 7H_2O$. The pH value was adjusted to 5.5 with 5% KOH. All flasks were incubated for 7 days at 25°C, under stirring condition (120 rpm), in an incubator shaker (Model Innova®44R, New Brunswick Scientific, USA). The cultures pH was checked daily and adjusted to 5.5 with KOH (5%) or H_2SO_4 (1 N). After incubation, a fragment of fungal mycelia was sampled, added in 5 ml distilled water and homogenised (1 min, 2000 rpm) with a vortex shaker (Genius 3,

IKA®, Germany). 1 ml of mixed solution was used for fungi isolation on glucose agar plates applying dilution technique. Solid medium for isolation procedure was prepared following the recipe of the basal medium, but without cellulose and with agar addition. Inoculated agar plates were incubated at 25°C for 7 days. Pure fungi culture was maintained at 4°C and subcultured every 3 months.

Laccase activity assay. Laccase activity of fungus *Fomes fomentarius* was evaluated comparatively in 7 experimental variants differentiated by source and concentration of carbon and nitrogen as presented in Table 1. Each culture medium contained also the following nutrients: 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l $CaCl_2 \cdot 2H_2O$, 0.1 g/l $FeSO_4 \cdot 7H_2O$, 0.005 g/l $MnSO_4 \cdot H_2O$, 0.25 g/l $CuSO_4 \cdot 5H_2O$, 0.036 g/l NaCl, and 0.005 g/l $ZnSO_4 \cdot 7H_2O$. The pH of all solutions was adjusted to 5.5 with 5% KOH. Fungus cultivation was performed in cotton-plugged Erlenmeyer flasks (250 ml) containing 50 ml of proper culture medium. Fungal inoculum was obtained as follows: several agar plugs, with developed fungal biomass, were added in 10 l of distilled water, mixed for 1 min (2000 rpm) and resulted suspension was serially diluted up to 10^6 times; from the last dilution 1 ml was used as inoculum for each flask. Flasks were maintained at 25°C in dark, being continuously mixed (83 rpm). Laccase activity in each experimental variant was assayed after 48 and 168 h of incubation when 1.5 ml of solution were sampled, centrifuged for 20 min at 4500 rpm (using centrifuge model U-320, Boeco, Germany) and the resulted supernatant was tested. Laccase activity was calculated based on spectrophotometric determinations (using UV/vis. spectrophotometer DR/5000™, Hach Lange, Germany) at 530 nm, reaction mixture containing: 1.6 ml acetate buffer (pH 5), 0.6 ml syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine) (60 mg/l) (Sigma-Aldrich, Steinheim, Germany) as substrate ($\varepsilon_{525} = 65\ mM^{-1}\ cm^{-1}$), and 0.6 ml of supernatant containing laccases. Laccase activity was expressed in units (U/l), one unit being defined as the amount of enzyme required to oxidise 1 μmol of syringaldazine per min. Black HS-02 and Derma 2RN dyes were obtained from National Research and Development Institute for Textile and Leather, Bucharest, Romania.

Table 1. Composition of the culture medium for laccase activity assay

Experimental variant	Glucose 20 g/l	Glucose 30 g/l	Peptone 3 g/l	KNO_3 2.5 g/l	Cellulose 10 g/l
1	+		+		
2		+	+		
3	+			+	
4	+		+		+
5	+			+	+
6			+		+
7				+	+

Decolorisation assays using fungi biomass. Decolorisation tests were performed in two steps. In the first step, the fungi biomass was cultured in an incubator shaker for 7 days, at 25°C applying 83 rpm. Cultivation was performed in 10 Erlenmeyer flasks (250 ml), in each being added 50 ml of the culture medium which included: 20 g/l glucose, 1 g/l peptone, 3 g/l yeast extract, 1 g/l KH_2PO_4, 1.5 g/l KNO_3, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.05 g/l $CaCl_2 \cdot 2H_2O$, 0.1 g/l $FeSO_4 \cdot 7H_2O$, 0.001 g/l $MnSO_4 \cdot H_2O$, 0.125 g/l $CuSO_4 \cdot 5H_2O$, 0.036 g/l NaCl and 0.001 g/l $ZnSO_4 \cdot 7H_2O$. The pH was adjusted to 5.5 using KOH (5%). Each flask was inoculated with 1 ml of inoculum solution obtained as follows: several agar plugs with developed fungi biomass were added in 10 ml of distilled water, mixed for 1 min (2000 rpm) and resulted culture was serially diluted up to 10^4 times.

In the second step, after 7 days of fungi incubation, Black HS-02 and Derma 2RN dyes were added individually in the flasks to reach the final concentrations of 20, 40, 60, 80 and 100 mg/l. Samples were taken from each flask after 2, 4, 48 and 168 h after dyes addition. Prior to sampling, shaking was stopped and the fungal mycelia were allowed to settle for 30 min. From the surface, 1.5 ml of liquor were sampled, centrifuged at 4000 rpm for 10 min and the resulted supernatant was analysed spectrophotometrically. The decolorisation efficiency was calculated using equation (1) based on the spectrophotometric measurements performed at maximum absorbance wavelength for each dye: at 468 nm for Black HS-02 dye and at 470 nm for Derma 2-RN dye.

$$\mathrm{DE}\ (\%) = \frac{\mathrm{MA_I} - \mathrm{MA_F}}{\mathrm{MA_I}} \times 100, \tag{1}$$

where DE (%) is the decolorisation efficiency; MA_I – initial absorbance, and MA_F – the final absorbance.

During experiments, it was emphasised the importance to know the trend between the decolorised quantity of the two consecutively tested concentrations. The indicator was calculated by applying the following equation:

$$\mathrm{DET} = \frac{\mathrm{DE}_x\, x}{\mathrm{DE}_y\, y}, \tag{2}$$

where DET indicates the trend of the decolorised substrate quantity between two consecutively tested dyes concentrations (x, y); DE_x (%) – the decolorisation efficiency recorded for concentration x (mg/l) testing, and DE_y (%) – the decolorisation efficiency obtained for the previous tested dye concentration – y (mg/l).

Decolorisation assays using crude enzyme extract. Crude enzyme solution was produced under solid-state fermentation conditions taking into consideration the experimental operation described by Neifar and collaborators[15]. Fungi cultivation was performed in cotton-plugged Erlenmeyer flasks (250 ml) in which was added

sterilised wheat bran (2.5 g) as a substrate, obtained from a local market, and 15 ml as a culture medium containing: 5 g/l glucose, 1 g/l peptone, 2 g/l yeast extract, 1 g/l KH_2PO_4, 1.5 g/l KNO_3, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.05 g/l $CaCl_2 \cdot 2H_2O$, 0.1 g/l $FeSO_4 \cdot 7H_2O$, 0.001 g/l $MnSO_4 \cdot H_2O$, 0.125 g/l $CuSO_4 \cdot 5H_2O$, 0.036 g/l NaCl and 0.001 g/l $ZnSO_4 \cdot 7H_2O$. The pH value was adjusted to 5.5 with KOH (5%). Each flask was inoculated with 1 ml of fungal mycelium solution obtained as it was described previously. Flasks were incubated statically at 30°C in darkness for 14 days. After the cultivation period, 25 ml acetate buffer (pH 5) were added in each flask, resulted culture being mixed for 3 h at 100 rpm and filtered through prefiltration paper. The resulted extract was sequentially filtered through mixed cellulose ester membrane filter (0.45 µm) (Frisenette, Denmark) and regenerated cellulose membrane filter (0.20 µm) (Sartorius, Germany), frozen, defrosted, in order to remove the polysaccharides, and ultrafiltrated through 10 kDa membrane of regenerated cellulose (Vivaspin®15, Sartorius, Germany). Resulted concentrated extract was used for decolorisation tests which were carried out directly in the spectrophotometric cuvette by preparing the following reaction mixture: 100 µl of enzymatic stock solution, 150 µl dye solution, and 1250 µl acetate buffer (pH = 5). The following dyes concentrations were tested: 20, 40, 60, 80 and 100 mg/l. Dye decolorisation efficiency was calculated according to the following equation:

$$\mathrm{DE} = \frac{A_\mathrm{i} - A_\mathrm{f}}{A_\mathrm{i}} \times 100\%, \tag{3}$$

where DE (%) represents decolorisation efficiency; A_i – the area of the absorption spectrum from 400 to 700 nm recorded at the initial time, and A_f – the area of the absorption spectrum from 400 to 700 nm recorded after 30 min. Final results were defined as decolorisation efficiency (DE_U) obtained by using 1 U of laccase activity for decolorisation of 1 l of dye solution and was calculated applying the following formula:

$$\mathrm{DE_U} = \frac{1.5\ \mathrm{DE_C}}{0.1\ \mathrm{La}}, \tag{4}$$

where DE_U (%) represents decolorisation efficiency obtained by using 1 U of laccase activity for decolorisation of 1 l of dye solution; DE_C (%) – the decolorisation efficiency obtained for the tested concentration and La (U/l) is the laccase activity of the crude extract. As in the previous case, it was emphasised the necessity to determine if the decolorised quantity differs between two consecutively tested dyes concentrations. Therefore, the following formula was applied:

$$\mathrm{DET_U} = \frac{\mathrm{DE_{Ux}}\, x}{\mathrm{DE_{Uy}}\, y}, \tag{5}$$

where DET_U indicates the trend of the decolorised substrate quantity between two consecutively tested dyes concentrations; DE_{Ux} (%) – the decolorisation recorded during concentration *x* (mg/l) testing, and DE_{Uy} (%) – the decolorisation efficiency obtained for the previous tested dye concentration – *y* (mg/l).

All presented results represent the means of two replicates, the experimental error being below 10%.

RESULTS AND DISCUSSION

Laccase activity of fungus Fomes fomentarius in different culture media. Laccase activity recorded in different culture media is represented in Fig. 1*a*. As it can be noticed, the increase of the glucose concentration from 20 (experimental variant No 1) to 30 g/l (No 2) had a positive effect on the laccase activity, which increased from 8.9 to 15.4 U/l after 48 h, and from 25.6 to 31.2 U/l at the end of the experiment. No significant differences were noticed between the presence of organic and inorganic nitrogen supply in the cultures enriched with glucose substrate (Nos 1 and 3). However, in the presence of both glucose and cellulose as substrates (Nos 4 and 5), after 48 h of incubation, a higher laccase activity was recorded in the culture medium supplyed with KNO_3. A different situation was noticed at the end of the experiment, when laccase activity in the culture medium supplyed with peptone (No 4) increased with about two times than that registered in the culture including KNO_3 (No 5), and was close to that recorded in the case of the cultures which were not enriched with cellulose substrate (Nos 1, 2 and 3). However, after 168 h of incubation, maximum laccase activity (52 U/l) was registered in the culture supplyed with cellulose substrate and KNO_3 (No 7). The effect on the laccase activity in the culture supplyed with cellulose and peptone (No 6) was pronounced only in the first 48 h, at the end of the experiment laccase activity being with about three times lower than maximum registered level. It is worth to point that in experimental variants of Nos 2, 3 and 5, pellets of the fungal biomass were developed, as it is represented in Fig. 1*b*.

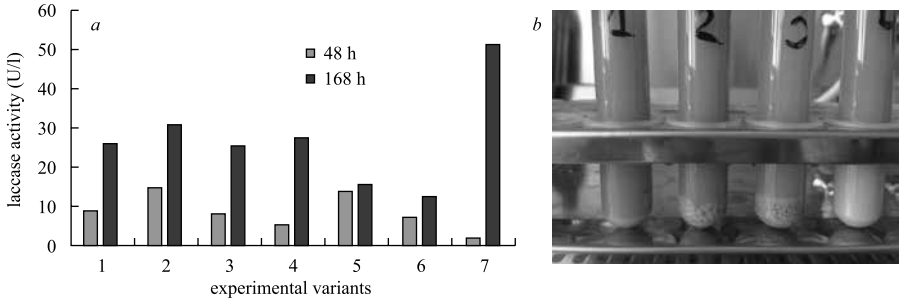

Fig. 1. Variation of the laccase activity after 48 and 168 h of cultivation in different culture liquors (*a*) and image of the pelletised fungi biomass developed during cultivation in experimental variants Nos 2, 3 and 5 (*b*)

Dyes decolorisation using fungal biomass. Results recorded during 168 h of fungi cultivation in contaminated-dye culture emphasised the increase of the decolorisation efficiency in time for both tested dyes, indicator value ranging between 57.6 and 61.1% in the case of Black HS-02 dye (Fig. 2*a*), close results being registered in the case of Derma 2RN dye 54.9–67.7% (Fig. 2*b*). Differences between dyes decolorisation efficiencies were noticed mainly after 2 h of incubation, when decolorisation efficiency recorded in the case of Derma 2RN dye (35.4–45.1%) was with about two times higher than that registered in the case of Black HS-02 (17.6–27.7%), for the same initial concentrations. Moreover, close results registered in the case of Derma 2RN after 2 and 4 h and lower decolorisation efficiency increase until 48 h, as in comparison with Black HS-02 dye, could indicate higher availability degree of the Derma 2RN dye for fungus enzymatic activity. However, it must be pointed that decolorisation efficiency could be also influenced by adsorbtion processes.

Another fact noticed during the experiments was the positive correlation established between initial dye concentration and quantity of the decolour ated substrate in the case of both dyes (Fig. 2*c*), as DET indicator did not decrease below 1.

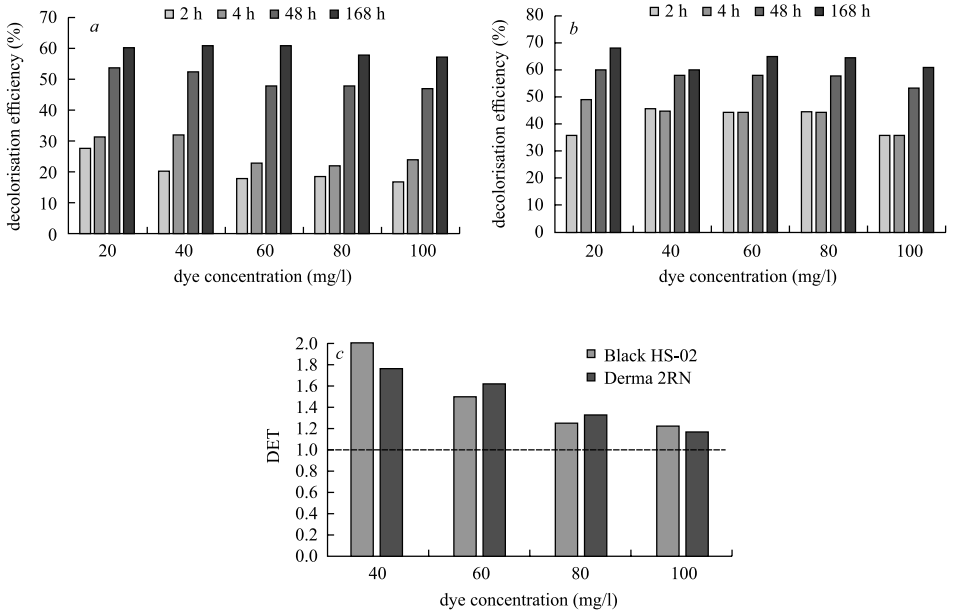

Fig. 2. Variation of the decolour isation efficiency during 168 h of reaction for Black HS-02 (*a*) and Derma 2RN dye (*b*) and variation of the decolorised substrate quantity between two consecutively dye concentrations (DET) (*c*)

Enzymatic dyes decolorisation. *Fomes fomentarius* was tested in several experiments being described as a good producer of laccases on wheat bran medium[13].

Laccase activity of the concentrated extract, obtained after fungus cultivation in solid-state fermentation conditions, was 33 U/l. Laccase activity and the initial dye concentration represents the most important factors that govern the decolourisation efficiency[16]. Therefore, the obtained results clearly indicated that, with simultaneous increase of the Derma 2RN concentration from 20 to 100 mg/l, decolour isation efficiency ensured by one unit of laccase activity decreased continously from 1.13 to 0.09% (Fig. 3*a*). In the case of Black HS-02 dye, decolorisation efficiency increased from 1.18 to 1.75% along with the increase of dye concentration from 20 to 40 mg/l. However, as in the case of Derma 2RN dye, the increase of the dye concentration conducted to the continous decrease of the decolorisation efficiency to 1.14% (Fig. 3*a*). Therefore, using crude laccase extract, higher decolorisation efficiency was obtained in the case of Black HS-02 even if previously mentioned results showed similar decolorisation efficiencies between tested dyes, after 168 h of incubation, by using fungi biomass. As a result, it could be concluded that adsorbtion processes could influence significantly the dye decolourisation performance, further research being needed in this way.

Analysing DET_U value variation, it was noticed that by increasing the concentration of Black HS-02 dye, the amount of decoloured substrate also increased as it is represented in Fig 3*b*. However, not the same effect was registered in the case of Derma 2RN, dye concentration above 40 mg/l lead to the decrease of the amount of decoloured substrate, DET_U value decreasing below 1. Therefore, concentration higher than 40 mg/l of Derma 2 RN dye influenced negatively laccase activity.

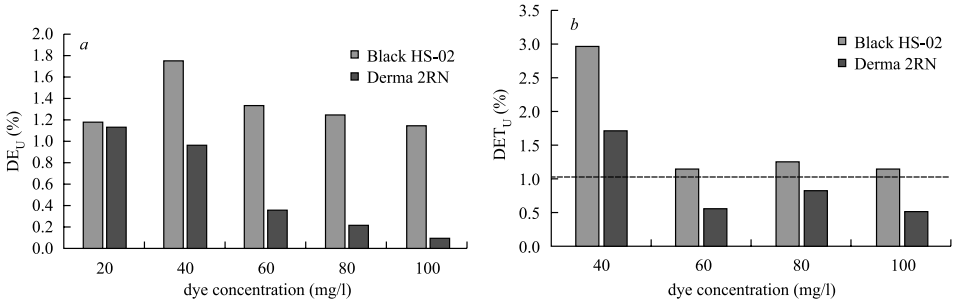

Fig. 3. Dyes decolorisation efficiencies recorded after 30 min of reaction (*a*) and variation of the amount of decolorised substrate between two consecutively dye concentrations (DET_U) (*b*)

CONCLUSIONS

The study shown that white-rot fungus *Fomes fomentarius*, which is widely used in traditional medicines, represents also a potential candidate for textile dyes decolorisation. Recorded results underlined the correlation between laccase activity and substrate concentration, the inhibitory effect of dye (at relative high concentration)

on fungus enzymatic activity being recorded in the case of Derma 2RN dye at a concentration higher than 40 mg/l. However, using fungal biomass in decolorisation process, no inhibitory effect was registered during testing. Also, it was shown that the presence of low-biodegradable substrate in the culture medium (such as cellulose) promoted the increase of the laccase activity, result that could be taken into consideration in order to promote cost-efficient technologies for increasing laccases production.

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