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Xenobiotic Removal by *Trametes hirsuta* LE-BIN 072 Activated Carbon-Based Mycelial Pellets: Remazol Brilliant Blue R Case Study

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Abstract: As a toxic xenobiotic compound, the anthraquinone dye Remazol Brilliant Blue R (RBBR) poses a serious threat to aquatic ecosystems. In the present study, the ability of *Trametes hirsuta* to remove RBBR from the medium was investigated, and the role of adsorption by fungal mycelium and biodegradation by fungal enzymes was evaluated. It was shown that the whole fungal culture was able to remove up to 97% of the dye within the first four hours of incubation. Based on enzymatic activities in the culture broth, laccases were proposed to be the main enzymes contributing to RBBR degradation, and RT-qPCR measurements demonstrated an increase in transcription for the two laccase genes—*lacA* and *lacB*. Composite mycelial pellets of *T. hirsuta* with improved adsorption ability were prepared by adding activated carbon to the growth medium, and the induction of laccase activity by carbon was shown. For composite pellets, the RBBR decolorization degree was about 1.9 times higher at 1 h of incubation compared to carbon-free pellets. Hence, it was shown that using fungal mycelium pellets containing activated carbon can be an effective and economical method of dye removal.

Keywords: white-rot fungi; *Trametes hirsuta*; laccases; ligninolytic peroxidases; RBBR; dye biodegradation; biosorption; mycelial pellets; activated carbon; RT-qPCR

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1. Introduction

The textile industry is one of the most important sectors in the modern economy. At the same time, it produces an enormous volume of dye-containing wastewater, causing a serious impact on the environment. For example, about 10–50% of reactive dyes remain unbound after the dyeing process [1] and can be discharged into industrial effluents. After being released into water bodies, textile dyes reduce the penetration of solar light, which, in turn, inhibits the primary productivity of aquatic ecosystems and increases biological and chemical oxygen demand [2]. In addition, almost all dyes are typical xenobiotics that cannot be naturally formed in the environment and, therefore, can have a toxic, mutagenic, or carcinogenic effect on its inhabitants [3–5]. Therefore, the appropriate treatment of dye-containing wastewater is an issue of utmost importance.

Currently, many physical or chemical methods for dye effluent treatment are proposed. These methods include coagulation, ozonation, photocatalysis, absorption, etc. [6]. However, all these methods have several disadvantages, such as the use of additional chemicals and high operating costs for large remediation volumes. Moreover, commercial dyes are generally recalcitrant and light-resistant aromatic compounds, and their removal by the methods mentioned above is usually inefficient [6,7]. On the contrary, many studies have demonstrated the effectiveness of biological methods for dye removal. In addition, biological aquaremediation does not cause secondary pollution from the chemicals used and, as a result, is more environmentally friendly [8–10].

One of the promising environmentally friendly, effective, and inexpensive biological alternatives to physical and chemical methods of treating wastewater from synthetic dyes is the removal of pollutants using the mycelial biomass of various filamentous fungi [7]. Growing in liquid media, filamentous fungi can form pellets—dense spherical mycelial structures that can be regarded as ready-to-use granular biosorbents. This advantageously distinguishes fungi from bacteria, the immobilization of which requires additional materials. The fungal cell wall contains various functional groups that provide effective biosorption for a wide range of compounds [11].

Among all filamentous fungi, the fungi that cause white rot of wood (i.e., white-rot fungi) are of particular interest due to their unique ability to effectively metabolize many types of highly recalcitrant phenolic compounds, including aromatic dyes [12–14]. The first stages of the metabolic assimilation of aromatic compounds by white-rot fungi are their extracellular and intracellular oxidative degradation. Currently, the main extracellular oxidative enzymes of white rot fungi are considered to be laccases and class II (ligninolytic) peroxidases [15], both of which are capable of degrading many types of aromatic dyes through direct and indirect free radical oxidation processes [16–18]. The intracellular oxidative enzyme system of white-rot fungi is primarily composed of cytochrome P450 mixed-function oxidases that are capable of catalyzing a wide range of reactions, including hydroxylation, epoxidation, dealkylation, sulfoxidation, deamination, desulfurization, dehalogenation, and nitro reduction [19].

Trametes hirsuta (Wulfen) Lloyd 1924 is a white-rot fungus belonging to the order Polyporales. Its ligninolytic system has been extensively studied for the degradation of lignin, xenobiotics, and monolignol-related compounds [20–23]. It was shown that, depending on the structure of the compound being degraded, *T. hirsuta* may prefer either laccases or peroxidases for its degradation. In the present study, the process of xenobiotic removal by mycelial pellets of *T. hirsuta* was investigated using Remazol Brilliant Blue R (RBBR) synthetic dye as a model compound. The aim of this study was to evaluate the contribution of biosorption and enzymatic biodegradation to the overall dye removal process and to enhance the overall removal capacity of the fungal pellets by incorporating activated carbon into their structure. An additional goal of this study was to determine which oxidative enzymes are primarily responsible for RBBR degradation by the mycelial pellets of *T. hirsuta*.

2. Materials and Methods

2.1. Strain and Culture Conditions

The strain *T. hirsuta* LE-BIN 072 was obtained from the Komarov Botanical Institute Basidiomycetes Culture Collection (LE-BIN; St. Petersburg, Russia). It was stored on slant worth agar until further use. For all experiments, *T. hirsuta* was pre-cultured on glucose-peptone (GP) medium without agitation for 7 days at 28 °C. The composition of the GP medium was as follows: ($g \cdot L^{-1}$): glucose—10.0; peptone—3.0; KH_2PO_4—0.6; $ZnSO_4 \times 7H_2O$—0.001; $K_2HPO_4 \times 3H_2O$—0.4; $FeSO_4 \times 7H_2O$—0.0005; $MnSO_4 \times 5H_2O$—0.05; $MgSO_4 \times$ —0.5; $CaCl_2$—0.25.

For all decolorization experiments, *T. hirsuta* was grown on a liquid-agitated GP medium at 25 °C and 180 rpm. The dry weight of biomass was measured after reaching a constant weight at 70 °C.

2.2. RBBR Decolorization by the Whole Fungal Culture

For the whole culture (i.e., mycelial pellets and culture broth) decolorization experiment, *T. hirsuta* was grown for 5 days, and then RBBR was added to the final concentration of 240 $\mu M \cdot L^{-1}$. For residual RBBR content analysis and RT-qPCR analysis, samples of culture broth were taken at 1, 2, 4, and 24 h of cultivation. Heat-inactivated (65 °C for 3 days) samples were used as a control. For cytochrome P450 inhibition experiments, 500 µL of piperonyl butoxide (PBO) per 200 mL flask was added. All experiments were performed in three biological replicates.

2.3. RBBR Decolorization by Fungal Pellets

For RBBR decolorization by fungal pellets (FP), *T. hirsuta* was grown for 5, 7, and 10 days; pellets were collected using filtration through nylon mesh and washed twice with distilled water. Composite fungal pellets supplemented with activated carbon (FP-AC) were grown as previously described in Section 2.1. The only exception was the addition of activated carbon (1 g per 200 mL flask) ground into powder (<0.25 mm). Activated carbon was purchased from Sigma (Sigma, St. Louis, MO, USA), product number C-2889.

RBBR decolorization by fungal pellets was performed at 25 °C and 70 rpm. About 1.0 g (wet weight) of mycelial pellets was added to RBBR dissolved in 10 mL of citrate-phosphate buffer (pH 4.5). The RBBR concentrations were 50, 100, 200, or 300 μM. Heat-inactivated samples (65 °C for 3 days) were used as a control. All experiments were performed in three biological replicates.

2.4. RBBR Decolorization Assay

Prior to measurements, samples were centrifuged at 13,000 rpm for 3 min. Residual RBBR content in the samples was measured spectrophotometrically at 592 nm (absorbance maximum) using a Lambda 35 UV/VIS spectrophotometer (Perkin Elmer, Waltham, MA, USA). The decolorization degree was calculated as follows: $(OD_{592}{}^{initial} - OD_{592}{}^{final})/OD_{592}{}^{initial} \times 100$.

2.5. RT-qPCR Analysis

The RNA extraction, reverse transcription, and qPCR analysis for laccase genes were performed as described in Moiseenko et al. [21]. Briefly, collected fungal mycelium was ground in liquid nitrogen; RNA was extracted using Trizol reagent (Invitrogen, Waltham, MA, USA) and treated with DNase I (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The reverse transcription was performed with the MMLV RT kit (Evrogen, Moscow, Russia) using 100 ng of RNA and polyA-specific primers. All qPCR reactions were performed on the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using qPCRmix-HS SYBR+ROX (Evrogen, Moscow, Russia) according to the manufacturer's protocol. All primers are available in Moiseenko et al. [21].

The transcription levels of target genes relative to the transcription of the control gene, β-tubulin, were calculated as follows: $RQ = 2^{Ct(laccase)}/2^{Ct(\beta\text{-tubulin})}$.

2.6. Enzyme Activity Assays

Laccase activity was measured using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate [24]. The reaction mixture contained 50 μL of sample and 2 mL of 1 mM ABTS in sodium acetate buffer (0.1 M; pH 4.5). Absorbance increase at 436 nm (ε_{436} = 29,500 $M^{-1}cm^{-1}$) was monitored for 3 min using a Lambda 35 UV/Vis spectrophotometer (Perkin Elmer, Waltham, MA, USA).

Manganese-dependent peroxidase activity was measured using Mn^{2+} as a substrate. The method was adopted from work [25] and slightly modified. The reaction mixture contained 1760 μL of sodium tartrate buffer (0.1 M; pH 3.0), 200 μL of 1 mM $MnSO_4$, 40 μL of 5 mM H_2O_2, and 50 μL of sample. The formation of the Mn^{3+}-tartrate complex was monitored at 238 nm (ε_{238} = 6500 $M^{-1}cm^{-1}$) for 3 min using a Lambda 35 UV/Vis spectrophotometer (Perkin Elmer, Waltham, MA, USA).

Manganese-independent peroxidase activity was measured using veratryl alcohol as a substrate [26]. The reaction mixture contained 1760 μL of sodium tartrate buffer (0.1 M; pH 3.0 or 5.0), 200 μL of 100 mM veratryl alcohol, 40 μL of 5 mM H_2O_2, and 50 μL of sample. The formation of veratryl aldehyde was monitored at 310 nm (ε_{310} = 9300 $M^{-1}cm^{-1}$) for 3 min using a Lambda 35 UV/Vis spectrophotometer (Perkin Elmer, Waltham, MA, USA).

All enzymatic activities were expressed as units (U) per mL, where 1 U corresponds to the formation of 1 μmol of product per minute.

3. Results

3.1. Decolorization of RBBR by the Whole Fungal Culture

The role of sorption and enzymatic degradation in the process of RBBR (Figure 1B) decolorization by the whole culture of *T. hirsuta* (i.e., mycelial pellets and culture broth) was assessed during short-term incubation in the presence of the dye. To evaluate the role of extracellular enzymes, a culture broth without mycelium was used for RBBR decolorization. To evaluate the role of intracellular enzymes from the cytochrome P450 family, the inhibitor of cytochrome P450, PBO, was added to the whole culture prior to RBBR decolorization. To evaluate the role of sorption by fungal mycelium, the whole fungal culture was heat inactivated until extracellular enzymatic activities were totally inhibited.

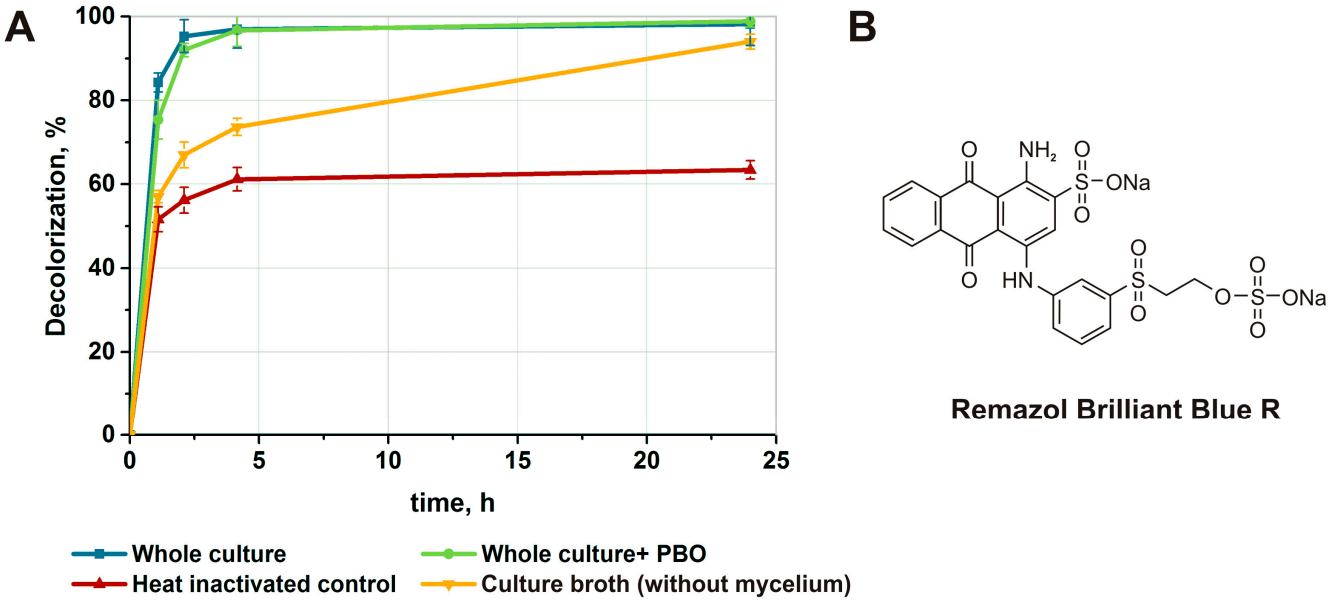

Figure 1. (**A**) The degree of RBBR decolorization by the whole culture, PBO-inhibited culture, culture broth, and heat-inactivated whole culture of *T. hirsuta*. (**B**) RBBR structure.

The whole culture of *T. hirsuta* decolorizes RBBR up to 97% within the first four hours of incubation (Figure 1A), and the addition of PBO to the whole culture did not significantly affect the decolorization degree. The latter suggests that cytochrome P450 enzymes were not largely responsible for RBBR decolorization in the chosen conditions. At the same time, extracellular enzymes contained in the culture broth were able to degrade up to 94% of RBBR in 24 h, but the decolorization rate was much lower than that of the whole culture. Heat-inactivated culture decolorizes about 60% of RBBR within the first 4 h with no significant changes afterwards. Thus, we can conclude that adsorption occurs mainly in the first 4 h of incubation.

Enzymatic activities of laccase, manganese-dependent peroxidase, and manganese-independent peroxidase were measured before the addition of RBBR and after 24 h of incubation with the dye. Laccase activity did not change significantly after the addition of RBBR; only a weak tendency ($0.05 < p < 0.1$, Student's *t*-test) for its increase was observed (26 ± 5 before RBBR addition vs. 31 ± 7 U·mL^{-1} after 24 h of incubation). The manganese-dependent peroxidase activity was 11 ± 3 U·mL^{-1} before the addition of RBBR and almost depleted after 24 h of incubation with the dye. Manganese-independent peroxidase activity was absent before and after RBBR addition.

3.2. Transcriptional Response of Laccases to RBBR

As demonstrated in Section 3.1, laccase activity was predominant in the culture broth during RBBR decolorization. It was previously demonstrated that the genome of *T. hirsuta* contains seven nonallelic copies of laccase genes (*lacA-lacG*) [21]. To find out which laccases

were induced by the presence of RBBR, a classical before-and-after experimental design with three biological replicates (i.e., three flasks) was adopted. From each flask, mycelium was collected before (0 h) the addition of RBBR and at certain time points (2, 4, and 24 h) after the introduction of the dye. The obtained values of gain scores (i.e., differences in $\log_2(RQ)$ and $\log_2(RQ_{0h})$) were averaged for all biological replicates and represented as a clustered heat-map in Figure 2.

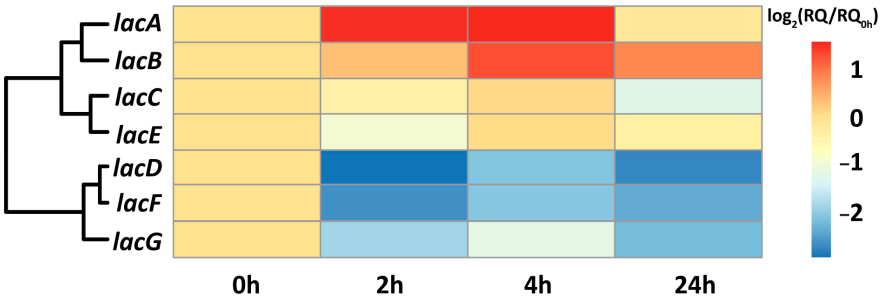

Figure 2. Clustered heat-map representing fold-changes (with a factor of two) in the transcriptional levels of laccase genes after RBBR introduction. The time point immediately before the RBBR introduction is designated as 0 h.

With respect to their transcriptional patterns, all laccase genes formed three clusters. The first cluster comprised *lacA* and *lacB*, whose transcription was induced by the presence of RBBR or its oxidation products in the culture broth. Transcription of *lacA* demonstrated almost three-fold induction at 2 and 4 h of cultivation, after which it returned to the same level as at the beginning of the experiment. In comparison with *lacA*, transcription of *lacB* was induced with a delay; it was unchanged at 2 h of cultivation and two-fold induced at 4 and 24 h. This suggests that the transcription of *lacB* may be more sensitive to the oxidation products of RBBR than to the dye itself. The second cluster comprised *lacC* and *lacE*, whose transcription levels were unaltered by the introduction of RBBR. The third cluster comprised *lacD*, *lacF*, and *lacG*, whose transcription levels were almost four-fold suppressed at all studied time points.

3.3. Formation of Composite Fungal Pellets Supplemented with Activated Carbon

In order to improve the adsorption properties of fungal pellets, activated carbon was added to the GP medium, and *T. hirsuta* was cultivated for 5, 7, and 10 days. The content of activated carbon in the composite FP+AC pellets was calculated based on the amount of carbon remaining after the cultivation. *T. hirsuta* pellets grown without the addition of activated carbon were used as a control.

The addition of activated carbon inhibited the growth of *T. hirsuta.* While FP achieved its maximal dry biomass weight on the 7th day of cultivation, FP+AC achieved the same dry biomass weight on the 10th day (Table 1). The activated carbon content was the highest on the 5th day of cultivation (64%), and it significantly decreased on the following days. Interestingly, activated carbon was mostly entrapped in fungal mycelium on the first days of cultivation. Fungal morphology also changed when activated carbon was added to the growth medium. While FP had developed hairy region, the hairy region was almost absent in FP+AC. Instead, FP+AC had a smooth black surface after 5 and 7 days of cultivation. However, on the 10th day of cultivation, a black core of FP+AC was totally covered by light and hairy fungal mycelium (Supplementary Figure S1), despite the fact that more than half of the initial amount of activated carbon remained in the cultural media at that moment.

Along with growth inhibition, strong laccase induction was observed during the cultivation of FP+AC. Compared to FP culture, laccase activity was induced more than 60 times in FP+AC, reaching 1518 ± 399 U·mL^{-1} on the 10th day of cultivation. In addition, during the cultivation of FP, laccase activity decreased from 5 to 10 days, but the opposite situation was observed during the cultivation of FP+AC. Manganese-dependent peroxidase

activity was slightly increased in FP+AC; in both FP and FP+AC, its growth was observed from day 5 to day 10. Manganese-independent peroxidase activity was observed at the trace level only in FP+AC on the 10th day of cultivation.

Table 1. Properties of fungal pellets (FP) and fungal pellets supplemented with activated carbon (FP+AC).

	FP			**FP+AC**		
Day	**5**	**7**	**10**	**5**	**7**	**10**
Dry weight, g	0.65 ± 0.11	1.23 ± 0.28	0.91 ± 0.20	0.34 ± 0.12	0.80 ± 0.19	1.52 ± 0.15
Activated carbon content, %	-	-	-	64	32	23
Laccase activity, $U \cdot mL^{-1}$	25 ± 3	16 ± 4	7 ± 1	936 ± 196	1254 ± 395	1518 ± 399
Mn-dependent peroxidase activity, $U \cdot mL^{-1}$	9 ± 2	12 ± 4	19 ± 3	0	15 ± 1	25 ± 3
Mn-independent peroxidase activity, $U \cdot mL^{-1}$	0	0	0	0	0	0.4 ± 0.1

3.4. Decolorization of RBBR by Composite Fungal Pellets Supplemented with Activated Carbon

The decolorization of RBBR by composite fungal pellets supplemented with activated carbon was studied in comparison with carbon-free fungal pellets. For both pellet types, heat-inactivated controls were used to estimate their adsorption properties in the absence of enzymatic activity. To avoid interference with the activity of already secreted enzymes, pellets were thoroughly washed prior to the RBBR decolorization measurements.

Both FP and FP+AC were tested for RBBR decolorization ability on different days of cultivation (Figure 3). The FP collected on the 5th and 7th days of cultivation were the most effective and demonstrated 84% and 87% of RBBR decolorization after 4 h of incubation, respectively. Both FP collected on the 5th and 7th days of cultivation were able to decolorize 93–95% of RBBR after 24 h of incubation; also, for these FP, the contribution of sorption and biodegradation to the overall RBBR removal was almost the same. The FP collected on the 10th day of cultivation were much less effective in RBBR decolorization. However, according to the data on heat-inactivated FP, FP collected on the 10th day of cultivation had more pronounced adsorption properties than FP collected on the 5th and 7th days. This could be explained by the increase in the adsorbing area of the mycelium due to its erosion, since at the late stages of cultivation (from 7 to 10 days of cultivation), the degradation of biomass was observed. Also, during the first 2 h of RBBR decolorization by FP collected on the 10th day of cultivation, only adsorption was observed, suggesting that oxidative enzymes were secreted by these FP only at the late stages of incubation with the dye. Thus, it was shown that the pellets collected somewhere between 5 and 8 days of cultivation are more effective for dye decolorization since, besides demonstrating good adsorption properties, they also actively secrete oxidative enzymes.

As with the decolorization of RBBR by the whole fungal culture, the dye was absorbed by FP within the first few hours of incubation, and the remaining dye was further degraded by the fungal extracellular enzymes. It is worth noting that the absorbed dye that was visible inside the pellets was degraded within the following 24 h (Supplementary Figure S1). Thus, after being absorbed by FP, RBBR was subjected to biodegradation, making the desorption of the dye from the pellets back into the environment impossible.

The overall tendency of RBBR decolorization by FP+AC was similar to that observed for FP (Figure 3); however, their adsorption efficiency was significantly increased. The most effective were FP+AC collected on the 7th day of cultivation, followed by FP+AC collected on the 5th and 10th days. Interestingly, the content of activated carbon in FP+AC collected on the 7th day of cultivation (32%) was lower than that in FP+AC collected on the 5th day (64%). This may be due to the different porosities and accessibilities of activated carbon entrapped in the pellets collected at different growth stages. The low adsorption efficiency of FP+AC collected on the 10th day of cultivation can be explained by the different pellet

morphology since access to activated carbon in the core of the pellets was hindered by the covering mycelium (see Section 3.2).

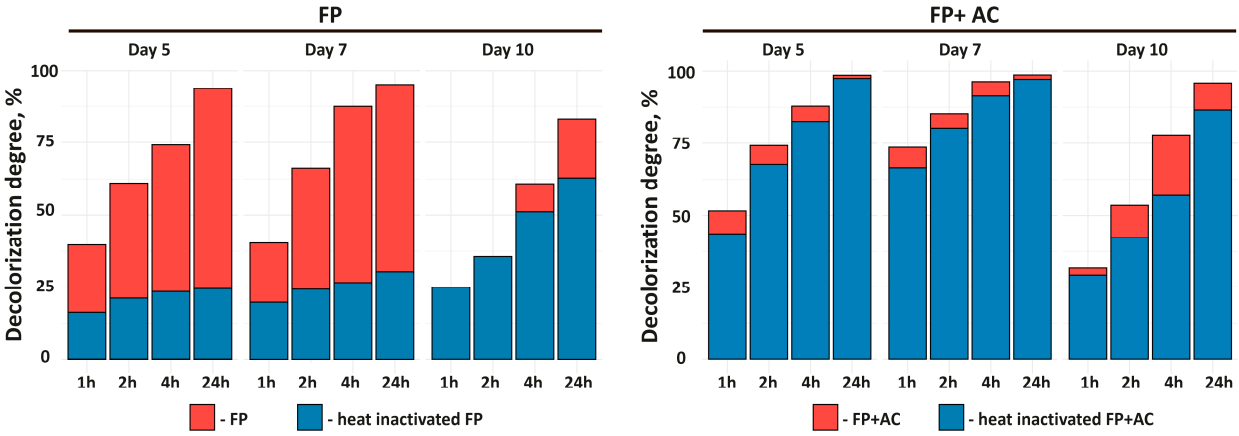

Figure 3. The decolorization of RBBR (with a starting concentration of 50 μM) by fungal pellets (FP) and fungal pellets supplemented with activated carbon (FP+AC) that were collected on the 5th, 7th, and 10th day of *T. hirsuta* cultivation.

The role of biodegradation by enzymes in FP+AC was not as significant as in the case of carbon-free pellets, since RBBR was readily adsorbed by pellets at the first hours of incubation with the dye. It should also be noted that the UV/Vis spectra of RBBR solutions decolorized by FP+AC showed significantly lower absorbance at ~500 nm (Supplementary Figure S2). This can be explained by the adsorption of not only RBBR but also its degradation products. Since some products of RBBR degradation can also be toxic to the environment [27], their adsorption is an additional advantage of FP+AC use.

Since at the initially tested concentration (50 μM) adsorption of RBBR to FP+AC prevailed over biodegradation in the overall dye decolorization process, the best-performing FP+AC, collected on the 5th and 7th day of cultivation, were tested at elevated RBBR concentrations. As can be seen from the presented data (Figure 4), with increasing concentrations of RBBR, the contribution of adsorption to the overall decolorization process was decreasing, and the contribution of biodegradation through the enzymatic process was increasing. At the same time, it can be noted that the efficiency of FP+AC collected on the 5th day of cultivation drops significantly, while FP+AC collected on the 7th day still decolorized about 90% of RBBR in 4 h of incubation, even at the highest concentration of RBBR (300 μM).

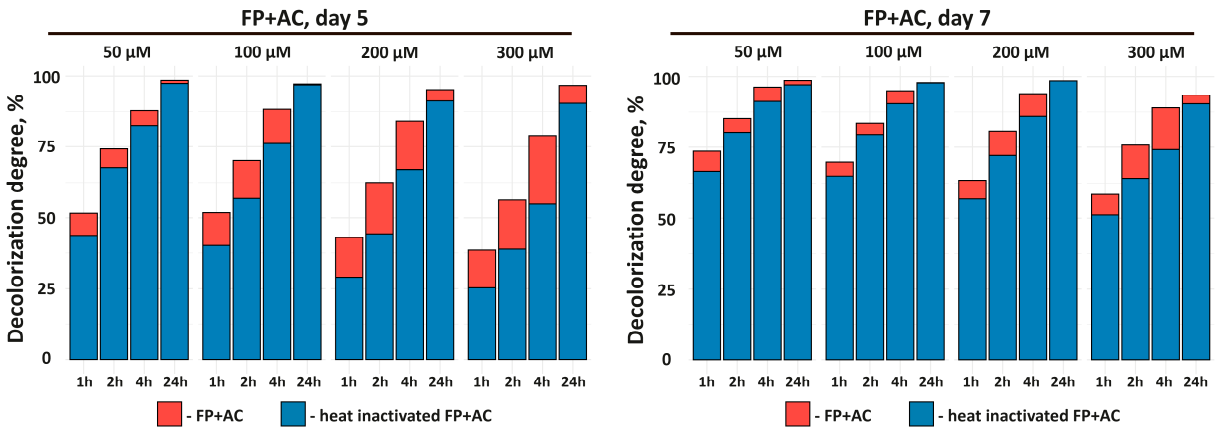

Figure 4. The decolorization of RBBR by FP+AC collected on the 5th and 7th days of cultivation at various RBBR concentrations.

4. Discussion

As a toxic xenobiotic compound, RBBR poses a serious threat to aquatic ecosystems. Belonging to anthraquinone dyes, RBBR has a complex aromatic structure and, therefore, is extremely recalcitrant to degradation [28]. Currently, there are two promising bioremediation methods suitable for removing complex, recalcitrant aromatic compounds from wastewater: biosorption and enzymatic biodegradation. It is noteworthy that mycelial pellets, which can be formed by white-rot fungi under certain cultivation conditions, can be both an efficient adsorbent of aromatic compounds and a source of oxidative enzymes that are able to degrade different xenobiotics of aromatic nature [7,9,29]. Moreover, the formation of mycelium-based composites by the inclusion of various materials into the fungal pellets can significantly improve their bioremediation potential. Fungal immobilization could be performed with lignocellulose agricultural residues (wheat straw, rice hull, corn cob, etc.), wood chips, polyurethane foam, alginate beads, biochar, activated carbon, and others [2,9,10,30]. Immobilized fungal cultures could have enhanced stability and enzymatic activity. Also, the use of fungal pellets and mycelium-based composites solves the very important problem in xenophobic adsorption—regeneration of adsorbents; since xenobiotics concentrated by fungal pellets or mycelium-based composites can be further locally degraded by fungal enzymes and even totally metabolized by fungal mycelium [2].

Despite their huge potential in bioremediation, there is a limited amount of information regarding the use of fungal pellets, especially fungal mycelium-based composites, for the removal of textile dyes. Most of the published studies are still concentrated on pure adsorption or pure enzymatic degradation processes. The current work showed that self-immobilized on activated carbon mycelial pellets of *T. hirsuta* can effectively decolorize RBBR in the concentration range of 50–300 μM in less than 24 h and showed the substantial contribution of both biosorption and biodegradation to this process.

From the perspective of enzymatic degradation, several previous works have investigated which enzymes may be involved in the degradation of RBBR by cultures of white-rot fungi. Based on enzymatic activities in a culture broth, it was shown that for the cultures of *Irpex lacteus* [31–33], *Ischnoderma resinosum*, and *Pleurotus calyptratus* [34], the main degrading enzymes were manganese-dependent peroxidases. At the same time, fungi such as *Pleurotus ostreatus* [35,36], *Trametes pubescens* [35], *Funalia trogii* [37], and *Trametes versicolor* [38] mainly relied on laccases in the process of dye degradation. Also, for *Dichomitus squalens*, the work of Šušla et al. [39] demonstrated the main role of laccases in the RBBR decolorization process, while the work of Eichlerová et al. reported cooperation between laccases and manganese-dependent peroxidases [34]. In our work, on the basis of enzymatic activities in the culture broth, it was shown that for *T. hirsuta*, the main RBBR-degrading enzymes were laccases. It should be noted that in the above-mentioned works, the maximum laccase activities, measured with ABTS as a substrate, varied from 0.05 to 2 U·mL^{-1} [34–36,39], while in our work, *T. hirsuta* demonstrated laccase activity of 31.7 U·mL^{-1}. This drastic difference may be due to different fungal cultivation conditions in those studies, specific features of *T. hirsuta* as a good laccase-producing strain, and individual characteristics of the major *T. hirsuta* laccase isozyme, LacA, which possesses high catalytic efficiency towards ABTS [40].

Since, on the basis of enzymatic activity in the culture broth, it is almost impossible to determine which of the seven laccase isozymes presented in the *T. hirsuta* genome was induced by RBBR, we used RT-qPCR to clarify this point. As a result, it was shown that for *T. hirsuta*, the presence of RBBR induced the transcription of *lacA* and *lacB*, while the transcription of the other laccase genes was either suppressed or unaffected. Previously, we have already shown that, as a universal "expressed by default" oxidative exoenzyme, LacA plays an important role in the detoxification of small aromatic compounds by *T. hirsuta* [21,23]. With respect to LacB, most probably its delayed induction was related to the accumulation of specific degradation products formed by the action of LacA. The sequential induction of several laccase genes by RBBR with simultaneous suppression of others was already reported for white-rot fungi. For *Ganoderma lucidum*, transcription of five laccase

genes increased immediately after RBBR introduction, and transcription of all laccase genes except one was suppressed after 10 h of cultivation [41]. For *P. ostreatus*, RBBR induced the transcription of four laccases [42]; however, it is worth noting that in this work, samples were taken within 120–552 h, and data on the depletion of the dye in the medium were not provided, so it is difficult to conclude whether the induction of laccases occurs as a result of RBBR addition or the appearance of its oxidation products.

From the perspective of biosorption, although some industrial dyes have already been tested for biosorption by the mycelia of white-rot fungi [43–46], there is very little information available on RBBR. In the studies of Erkurt et al., microscopic examinations of pellets formed by *P. ostreatus*, *T. versicolor*, and *F. trogii* did not show adsorption of RBBR to mycelium [47]. Similarly, several works in which adsorption was investigated via the extraction of residual dye from fungal mycelium did not report this mechanism as relevant to the decolorization process [34,48,49]. However, in later works, the action of intracellular or cell-wall-associated enzymes could be underestimated as they were not inactivated during the experiments. In our work, using the heat inactivation procedure, it was demonstrated that for *T. hirsuta*, the first rapid step in RBBR decolorization was the adsorption of the dye by fungal mycelium, and biodegradation followed thereafter. According to our data, adsorption of mycelium contributes a lot (up to 60%) to the RBBR decolorization by *T. hirsuta*. Importantly, similar conclusions were made for *F. trogii* when a heat-inactivated control was used [50]. Hence, heat-inactivated control seems to be extremely relevant for the evaluation of dye adsorption by fungal mycelium.

Although activated carbon is an effective adsorbent for many industrial dyes [51], the necessity of its regeneration seriously hinders its applications. Typically, pollutants adsorbed by activated carbon remain entrapped in its porous structure but are not degraded. This problem can be overcome by including activated carbon in mycelium-based composites, since these composites combine the advantages of physical adsorption on activated carbon, biosorption on fungal mycelium, and biodegradation by fungal enzymes. Previously, composite fungal pellets containing activated carbon or biochar were studied for the degradation of different dyes [44,52,53], phenanthrene [54], and heavy metal ions [55,56]. Remarkably, in our work, activated carbon not only significantly improved the adsorption capacity of fungal pellets but also drastically increased the laccase activity during the cultivation of *T. hirsuta*. To the best of our knowledge, this is the first report on the induction of laccase in white-rot fungus by activated carbon. In previous reports on the growth of *F. trogii* [57] and *Phlebia radiata* [58] in the presence of carbon, laccase induction was absent. There is only one report on laccase induction by carbon in *Physiporiopsis rivulosus* [59]; however, in addition to carbon, the cultivation medium was supplemented with sawdust.

A possible explanation for the induction of *T. hirsuta* laccases by activated carbon is the presence of polycyclic aromatic hydrocarbons or other aromatic compounds formed during pyrolysis in its composition [59]. As it was previously shown, these compounds could be laccase inducers [23,60–64]. Thus, at least in the case of *T. hirsuta*, the inclusion of activated carbon not only improves the adsorption properties of fungal pellets but also increases the production of laccase. However, to elucidate the exact mechanism of laccase induction by activated carbon, further investigations are needed.

5. Conclusions

Currently, bioremediation of synthetic dyes using pellets formed by filamentous fungi is a hot topic of research, and basidiomycete fungi causing white rot of the wood are extremely promising candidates for developing this technology. In this article, it was demonstrated that pellets of *T. hirsuta* were able to efficiently decolorize RBBR. The overall process of dye decolorization included two main mechanisms—biosorption onto fungal mycelium and biodegradation by the extracellularly secreted oxidative enzymes. While in the first few hours, biosorption was the predominant process, in the later stages of incubation, biodegradation became more and more pronounced. It was determined that

the main enzyme used by *T. hirsuta* for RBBR degradation was laccase, and it was proposed that the fungus mainly used two out of seven laccase isoenzymes (i.e., products of different nonallelic genes) encoded in its genome—LacA and LacB. The presence of activated carbon as a part of the mycelium-based composite not only increased the adsorption capability of the fungal pellets but also stimulated laccase secretion by the fungus. As a result of this synergistic effect, self-immobilized on activated carbon mycelial pellets of *T. hirsuta* were much more efficient than carbon-free mycelial pellets for RBBR decolorization. Therefore, fungal pellets of *T. hirsuta* supplemented with activated carbon could be a promising RBBR-removing agent that combines both adsorption by activated carbon, biosorption by fungal mycelium, and biodegradation by fungal enzymes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w16010133/s1. Figure S1: Mycelium appearance at different time points of RBBR degradation. Figure S2: UV/Vis spectra of RBBR before degradation (black line) and after degradation by fungal pellets (FP, red line) and fungal pellets supplemented with activated carbon (FP+AC, blue line) for 24 h.

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