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Pleurotus ostreatus as a species with potentially high effectiveness in the removal of synthetic dyes belonging to different classes

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ABSTRACT

The widespread use of dyes causes a lot of problems in their elimination from wastewater, which is connected with their complex chemical structure. Nowadays, considerable attention is focused on decolourisation processes making use of the white rot fungi. The possibility of using two strains of *Pleurotus ostreatus* for decolourisation of azo, triphenylmethane, and anthraquinone dyes was investigated. In order to increase the efficiency of the decolourisation process, the mycelium was immobilised on a natural waste material (pistachio shell) and inexpensive artificial (sponge) supports. To assess the environmental safety of the process, ecotoxicity studies were conducted. In contrast to most of the previous reports about fungi, significant differences in the efficiency of the removal of each dye used were observed between both strains. The dyes were more effectively removed by the K4 strain than by the BWPH strain, especially if its mycelium was immobilised on a sponge. Immobilisation of the mycelium significantly improved both the efficiency and speed of the removal of the tested dyes. Complete decolourisation of Remazol brilliant blue R (RBBR) was obtained after 48 h in samples with immobilised mycelium. At the same time, the other two dyes (brilliant green [BG] and Evans blue [EB]) were removed in more than 90%. In samples with non-mmobilised biomass the efficiency of decolourisation was 10% and 11% of RBBR, 22% and 85% of BG, 11% and 42% of EB, respectively, for strains BWPH and K4. Pistachio nut shells may be used as a cheap and good material for mycelium immobilisation, which improves the process of dyes removal. Ecotoxicity tests have shown that decolourisation of dyes by *P. ostreatus* conducted in appropriate conditions may contribute to the reduction of the negative impact of dyes on aquatic organisms.

Keywords: Decolourisation; Ecotoxicity; Biomass immobilisation; White rot fungi

1. Introduction

The use of synthetic dyes in almost all branches of industry makes them one of the basic pollutants, introduced particularly into water reservoirs [1–2]. In spite of a number of studies conducted worldwide, the issue of limiting the penetration of dyes into the environment, as well as of their effective removal, has not found a suitable solution yet. One of the basic problems faced by industries using large amounts of various dyes in high concentrations is the improvement of the efficiency of wastewater treatment [1–3]. Even a slight presence of dyes in water causes changes in its physical properties, such as colour, taste, smell, which limits its usefulness and disturbs the biology of these ecosystems. Synthetic dyes can affect the life of water fauna and flora directly and indirectly. Their

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indirect influence occurs through the worsening of the light penetration and subsequently in the limitation of the photosynthesis processes. Their direct influence is associated with toxic properties of numerous dyes and their effect on living organisms [1–4]. Traditional methods of wastewater treatment, such as physicochemical (flocculation, sedimentation) and biological (e.g., activated sludge) methods, are commonly used to treat wastewater containing dyes, but with varying effectiveness. Methods such as ozonation, electrochemical and ultrasound techniques, membrane filtration, photocatalysis and adsorption are investigated. Among physical processes, filtration processes using membranes are popular, making use of nanofiltration, reverse osmosis and electrodialysis. The main disadvantages of these processes, however, are the occurrence of the fouling phenomenon and high costs of the membranes due to the necessity of their frequent replacement. Among the chemical methods of dye removal from wastewater, the main interest resides in coagulation and flocculation combined with flotation and filtration, precipitation–flocculation using Fe(II)/$Ca(OH)_2$, electroflowing, electrokinetic coagulation, conventional oxidation processes (using ozone), UV radiation or electrochemical processes. These techniques are often expensive and connected with the production and accumulation of a large amount of sludges, which must be stored or disposed of. Another problem is additional contamination associated with chemical agents used in the process, hence the interest in advanced oxidation processes, which are based on the production of hydroxyl radicals with a high oxidation ability. Unfortunately, despite high efficiency, these processes are quite expensive and, therefore, often unprofitable on an industrial scale [5,6].

Biological methods of elimination of dyes from wastewater are based on the use of different organisms: bacteria, fungi (including yeast), algae, and plants. There are different mechanisms of removing dyes by microorganisms. Biological decolourisation may be caused by the adsorption mechanism by living or dead biomass, as well as bioremediation based on biotransformation or biodegradation. Despite many limitations compared with physicochemical methods, these technologies are cheap, with a small amount of generated waste, and they generally have a much lower degree of negative impact on the environment, especially if the mechanism is based on biodegradation and the end products of microbiological changes are not toxic [1,2,7,8]. Until now, many studies have been carried out on the use of fungi in the decolourisation process, and the results have shown their varied effectiveness and process mechanisms. The interest in this group of organisms is related to their ability to degrade substances of the ring structure, to which lignin belongs, thanks to the production of non-specific exoenzymes with a significant redox potential [9–11].

Among various examined species of fungi, *Pleurotus ostreatus* (oyster mushroom) is recognised as one of the potential degrader of dyes. It is known as an edible, tasty and widely cultivated mushroom. Oyster mushrooms have numerous healing properties. In the fruiting bodies β-D-glucans are present, exhibiting anti-cancer properties, as well as substances lowering the cholesterol level. Oyster mushrooms contain B vitamins and significant amounts of potassium [12,13]. *Pleurotus ostreatus* is an important element of ecosystems. As a wood rot fungus, it contributes to the degradation of lignin. Fungi classified to *Pleurotus* genus produce Mn-depended peroxidase, which may degrade many aromatic substances [9,10]. Among others, the production of laccase by *Pleurotus ostreatus* was confirmed [11]. Although the fungus shows less variation within the species than the bacteria, it is assumed that the fact of belonging to a given species is not a guarantee of a high efficiency of the process. That is why the aim of the study was to determine the possibility of using an immobilised mycelium of two strains of *Pleurotus ostreatus* to remove dyes belonging to different classes. Another goal of the study was to assess the toxic impact of solutions of raw dyes and solutions after treatment on representatives of producers and consumers. A natural waste material, such as pistachio nut shells, was used as an inexpensive carrier for biomass immobilisation and a potential material to improve decolourisation.

2. Materials and methods

2.1. Pleurotus ostreatus strains

Two strains of *Pleurotus ostreatus* were used in the study (BWPH and K4). The sources of the material collection and the methods of isolation were different. The fungal strain BWPH was isolated by the tissue method with fruiting bodies of the fungi collected in the woods near Gliwice (Southern Poland, Upper Silesia), and strain K4 by the spore method (fruits collected in Ruda Śląska, Southern Poland, Upper Silesia). The strains were isolated, stored and cultivated on Petri plates with the MEA medium (Difco). Both strains used had been previously described (BWPH in Przystaś et al. [7,8,14] and K4 in Przystaś [15]).

2.2. Synthetic dyes

Three dyes belonging to the most commonly used classes of synthetic dyes were selected for the study: diazo Evans blue (Sigma-Aldrich) as azo dye, brilliant green (Sigma-Aldrich) as a triphenylmethane dye, and Remazol brilliant blue R ([RBBR] Acros Organics) as an anthraquinone dye. Short characteristics are provided in Table 1.

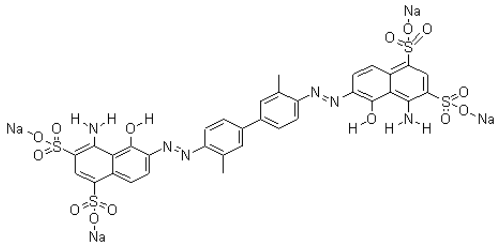
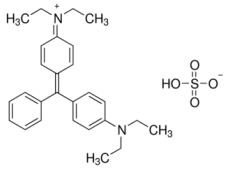
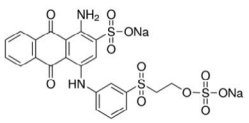
2.3. Solid supports

Two different materials were selected as a solid support for immobilisation of biomass of the fungi: a synthetic material (a sponge for washing dishes as a soft and porous material made of polyurethane) and an easily available and cheap natural material (pistachio nut shell). Before use, the sponge was washed four times in distilled water (each cycle – 15 min), dried, and cut into 1 cm pieces. The shells were not pre-treated. Both solid materials were sterilised by autoclaving before their use in the decolourisation processes (121°C, 30 min).

2.4. Media used for cultivation

Cultivation of biomass in all samples, including pregrowth before each experiment, was performed on a liquid PG medium (glucose 5 g L^{-1}, peptone 1 g L^{-1}, KH_2PO_4 0.1 g L^{-1}, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$; pH 5.6).

Table 1
Structure and short characteristic of dyes used in the study

Class of dye	Dye	Structure	Molecular structure and weight	Absorbance λ_{max} (nm)[a]
Azo	Evans blue (Direct Blue 53) C.I.: 23860 (EB)		$C_{34}H_{24}N_6Na_4O_{14}S_4$ 960.79 g mol^{-1}	606
Triphenyl methane	Brilliant green (Basic Green 1) C.I.: 42040 (BG)		$C_{27}H_{34}N_2O_4S$ 482.63 g mol^{-1}	624
Anthraquinone	Remazol brilliant blue R (Reactive Blue 19) C.I. 61200 (RBBR)		$C_{22}H_{16}N_2Na_2O_{11}S_3$ 626.54 g mol^{-1}	593

[a]Estimated on UV–VIS Hitachi 1900.

2.5. Preliminary tests of solid supports

Both solid supports were tested in two directions: their abilities to absorb the used dyes, and the possibilities of their colonisation by the fungal strains used. 1 g of each support and 10 mL of water solutions of dyes was added to 100 mL Erlenmeyer flasks. The final concentration of dye in each flask was 0.1 g L^{-1}. After 1 h of shaking in a rotary shaker (250 rpm), absorbance was measured at the wavelength appropriate for each dye (Table 1). The control samples were flasks with solutions of the dyes only.

The removal of the used dyes as a result of adsorption on solid supports was calculated according to the formula 1 (A – adsorption (%); C – concentration of dye in the control sample (g L^{-1}); S – concentration of the dye in the sample with a solid support (g L^{-1})):

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

In the second step of the test of solid supports, the possibilities of colonising them by fungi were estimated. To a 100 mL Erlenmeyer flask 1 g of dry sponge and 10 g of dried shells were weighted, the PG medium (10 mL) was added and autoclaved (121°C; 30 min). The mycelium of each strain used for inoculation was pregrown on the PG medium for 7 d and then homogenised (BagMixer 400P for 10 min). The mycelium suspension was introduced to each 0.1 mL tube of the strain. The biomass was cultured for 7 d in a rotary shaker (110 rpm). After the end of incubation, the samples were dried to a stable weight in temperature 55°C and the growth of the biomass was estimated. The intensity of the fungal colonisation was visually evaluated (***very intense, **medium, *weak, – no growth).

2.6. Influence of solid support on the efficiency of the removal of dyes and the growth of biomass

The main experiment was done with both the solid supports and the biomass of two strains of *Pleurotus ostreatus* suspended in the PG medium. Each sample contained a certain dye, one of the tested solid support and one of the fungi strain used. A few types of controls were prepared to evaluate the efficiency of the removal of dyes in different experiment conditions: medium, medium with an individual dye, medium with the biomass of the strains, medium with an individual solid support, medium with a solid support and an individual dye. All the controls as well as samples with dyes (medium with the biomass and dye, medium with solid supports, the biomass and dyes) were prepared in four replications. Solid supports were added to flasks with 80 mL of the medium: 1 g of the sponge and 10 g of the shells, respectively. The media with supports were sterilised for 30 min (121°C). Each strain was pregrown (7 d on liquid PG medium), homogenised (BagMixer) and 1 mL of inoculum was added to relevant samples. All samples were incubated in a rotary shaker for 7 d (26°C, 150 rpm). Filter sterilised dyes (pores diameter 0.2 μm) were added to each sample in order to reach the initial concentration of 0.1 g L^{-1}. After 1, 6, 24, 48, 72 and 96 h of incubation 2 mL were taken from the samples to evaluate the dye concentration on the UV–Vis Hitachi U-1900 spectrophotometer. The removal of individual dyes was

evaluated according to Eq. (2) (C – concentration in the control sample [g L^{-1}]; S – concentration in the sample with biomass [g L^{-1}]):

$$R\% = \frac{(C-S)}{C} \times 100\% \quad (2)$$

After the completion of the experiment, dry biomass (g $sample^{-1}$) was evaluated in the same way as in the preliminary test. Prior to the drying process, the samples were filtered through medium quality filters.

2.7. Ecotoxicity tests

At the end of the main experiment, ecotoxicity of each sample and of the control was evaluated. Tests were performed using two aquatic organisms: crustacea *Artemia salina* and plant *Lemna* sp. All the tests were performed in quadruple. Acute toxicity to *Artemia salina* was measured according to methods described by Hartl and Humpf [16]. Toxicity was estimated after 24 h of exposition. Phytotoxicity was evaluated according to the OECD *Lemna* sp. growth inhibition test no. 221. The EC50 value was estimated and the acute toxicity unit (TUa) was calculated to establish a toxicity class according to ACE 89/BE 2/D3 Final Report Commission EC (TUa < 0.4 – class I [non-toxic], 0.4 ≤ TUa < 1.0 – class II [low toxicity], 1.0 ≤ TUa < 10 – class III [toxic], 10 ≤ TUa ≤ 100 – class IV [high toxicity] and TUa > 100 corresponds to class V [extremely toxic]).

3. Results and discussion

3.1. Adsorption of dyes on solid supports

The main role of solid supports used for immobilisation of biomass is to increase the growth of fungal hyphae on it. Another advantage of immobilisation is the possibility of multiple use of the biomass. It has been proven that immobilisation has a positive effect on the growth of microorganisms and the activity of enzymes. It has been observed during different production and decomposition processes (including synthetic dyes decolourisation) [17,18].

Natural industrial waste as well as synthetic materials may be used as materials for immobilisation. When the biomass is immobilised, the removal of dyes may be reached through the adsorption of dyes on the support/biomass, as well as through biodegradation/biotransformation by the immobilised microorganisms [17,18]. Different solid supports have various absorption capacities, which is directly connected with their properties. Natural materials in particular, for example, natural waste from many processes, can be used in decolourisation processes because of their abilities to adsorb dyes [17,19,20]. In order to evaluate if the process of dyes removal occurs by biotransformation or by adsorption on supports, an initial test was carried out. The results (Fig. 1) showed that the used dyes may be adsorbed by both the supports used and the efficiency of the process is more dependent on the dye properties than on the properties of the supports. Triphenylmethane dye brilliant green was effectively removed by both supports (~72%), where both blue dyes (Evans blue and RBBR) were less adsorbed. Azo dye Evans blue was better removed by the natural support (27.5%) than by a synthetic sponge. Anthraquinone dye RBBR was almost not removed by both solid supports (<6%, which is within the limits of the measurement error).

Various natural and synthetic materials have been tested many times, mainly in terms of their use for the removal of dye by adsorption, and consequently in terms of the possibility of eliminating them from wastewater. In this respect, agricultural industry waste [17,19,20], chitosan [21], ashes [22], sawdust [23,24], peat [25], clay [26] have been tested, among other materials. Many factors influence the efficiency and mechanism of the process. Among them, there are the size of the sorbent surface, the size of the sorbent particles, interactions between the sorbent and the dye, the time of contact with the sorbent, the concentration of the dye, the temperature of the process, pH [23,27]. One of the most important factors is the structure of the dye, which was proven during our research and has been documented by other researchers [23,27]. Such waste as coconut mesocarp was used in the studies of Etim et al. [28] and Vieira et al. [29]. It was tested as a biosorbent for aqueous solutions of Blue Remazol R160 (BR 160), Rubi S2G (R S2G), Red Remazol 5R (RR5), Violet Remazol 5R (VR 5), and Indanthrene Olive Green (IOG). The dyes were adsorbed in the following order: R S2G > VR 5 > BR 160 > IOG > RR 5. Janaki et al. [30] used a composite made from polyaniline and chitosan to remove dyes from different classes (Congo red, Coomassie Brilliant Blue, Remazol Brilliant Blue R, methylene blue). The maximum removal was 95.4% for Congo Red (CR), 98.2% for Coomassie Brilliant Blue (CBB), and 99.8% for Remazol Brilliant Blue R (RBBR), which confirmed the effectiveness of this biopolymer as a sorbent. The reached results do not confirm the literature reports referred to above, especially in the case of azo Evans blue and anthraquinone RBBR blue, whose adsorption on the solid supports studied was insignificant (Fig. 1).

Pistachio shells used in the tests are considered as a waste material that may find potential use as a sorbent. Pistachio shells were used by Armagan and Toprak [31]. The researchers proved that the equilibrium between the azo dye Remazol Red and the adsorbent in the solution is reached within 10 min. and the maximum adsorption capacity of pistachio shells was 108 mg g^{-1} at 20°C.

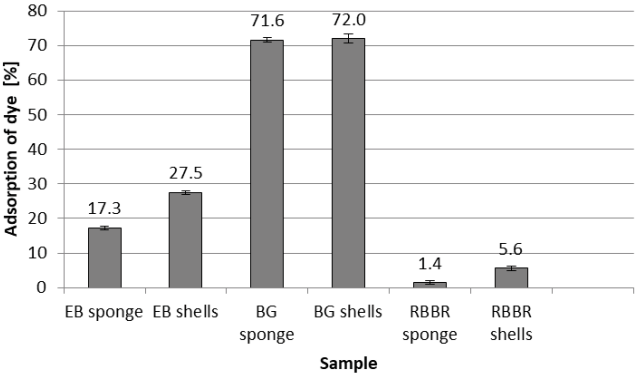


Fig. 1. Adsorption of dyes on the solid supports used.

Hashemian and Shayegan [32] studied agricultural cellulose materials as sorbents for the removal of Violet B. The maximum removal of the dye was obtained after 90 min. The highest adsorption capacity was presented by almond shells followed by orange peel, pistachio shells, tea waste, walnut shells. The level of adsorption was 96, 82, 71.4, 55.5 and 48.7 mg g^{-1}, respectively. Akl et al. [33] used them as a material to produce activated carbon. This material was identified to be a very effective adsorbent for the removal of methylene blue and brilliant green from an aqueous solution.

Results that show the intensity of the biomass growth on two different supports are presented in Table 2. There was no difference between individual strains in terms of the visual intensity of the growth on the sponge, but the concentration of dry biomass was a bit higher for the BWHP strain. Pistachio nut shells were also better covered by hyphae of that strain (BWPH), even if dry biomass was lower. For this natural solid support a decrease in the concentration of dry biomass was observed, which suggests that the solid support was used by the fungi as a source of nutrients. The intensity of the growth of the K4 strain was a bit lower than of the BWPH strain and the decrease in the concentration of dry biomass in the sample was four times lower (dry mass in samples with BWPH and K4 was –0.14 and –0.56 g $sample^{-1}$, respectively).

3.2. Influence of the solid support on the efficiency of the removal of the dyes and the growth of biomass

The influence of immobilisation of the fungal biomass on the removal of Evans blue is presented in Fig. 2. Both materials used for immobilisation took part in the removal of that azo dye. After 96 h of the process, the sponge adsorbed 12.2% and the nut shells even 32%. These results are comparable with the previous test described above (Fig. 1) and they indicate the possibility of using the shells in the dye removal process, but with low efficiency. Differences in the effectiveness of the dyes removal were noticed for both strains used as suspended biomass. Fluctuations in the colour of the samples with suspended biomass of the K4 strain during the process suggest that the process was mainly connected alternately with the adsorption on biomass and the partial desorption of the dye from hyphae. In comparison with the K4 strain, BWPH intensively removed that dye and eventually the loss of Evans blue was 61.7%. From the 4th hour of the experiment, the increase of the dye removal was linear in nature in the samples with

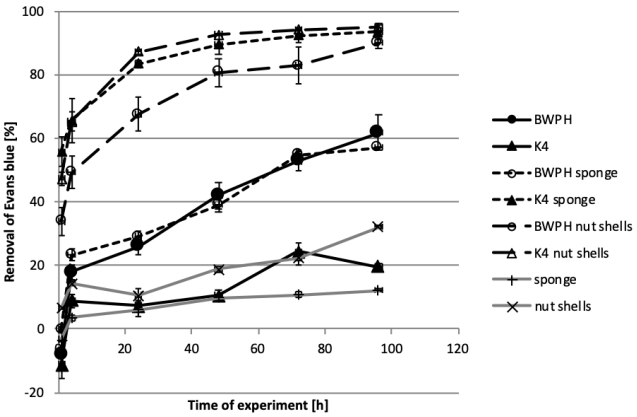


Fig. 2. Removal of Evans blue by two strains of *Pleurotus ostreatus* immobilized and not immobilized.

suspended biomass. Immobilisation of hyphae had a definitely positive influence on the decolourisation effectiveness. Even if the nut shells as well as suspended biomass of the strains did not adsorb high amounts of that dye, in the samples with the biomass of the strains immobilised on that material the removal of the dye increased. From the first hours of the experiment (4th h), the removal was higher than 30%, and eventually both strains used removed about 90% of the added dye. This may be connected with a few phenomena: a higher growth of the biomass of the strains which may remove this dye by sorption or biotransformation; changes in the properties of the material used for immobilisation (during the growth of the fungi) in such a way that it may bind the dye with higher efficiency than the material not partially degraded by hyphae. A confirmation of the impact of the higher growth of biomass may be provided by the results of the removal reached for biomass immobilised on a sponge – a material which is not used as a source of nitrogen and which is not degraded by the tested organisms. For the K4 strain immobilised on the sponge, the results of decolourisation observed during the experiment were about 10% worse that for K4 immobilised on the shells. The higher concentration of biomass pointed to the fact that the process is connected with the strain activity and could be the result of a higher production of enzymes involved in the process. As it is presented in Table 3, the biomass concentration in the sample with that strain immobilised on the sponge is more than four times higher than in the sample with suspended biomass. As a result, the

Table 2
Colonisation of the solid support by *Pleurotus ostreatus* strains

	Sponge		Pistachio nut shell	
	Visual evaluation of the intensity of the hyphae growth	Concentration of dry mass in the samples (g $sample^{-1}$)	Visual evaluation of the intensity of the hyphae growth	Concentration of dry mass in the samples (g $sample^{-1}$)
BWPH	***	0.13	***	–0.56[a]
K4	***	0.11	**	–0.14[a]

[a]Such a low value means that the shells were used as a substrate.

Table 3
Concentration of dry biomass (mg sample^{-1}) is samples (after 96 h of experiment)

Dye	Strain	Medium–dry biomass (mg sample^{-1})	Sponge–dry biomass (mg sample^{-1})	Pistachio nut shell–dry biomass (mg sample^{-1})
–	BWPH	110	140	160
–	K4	40	100	–780
EB	BWPH	100	180	0
EB	K4	30	130	–920
BG	BWPH	150	120	–120
BG	K4	40	90	–590
RBBR	BWPH	120	150	–350
RBBR	K4	40	110	–690

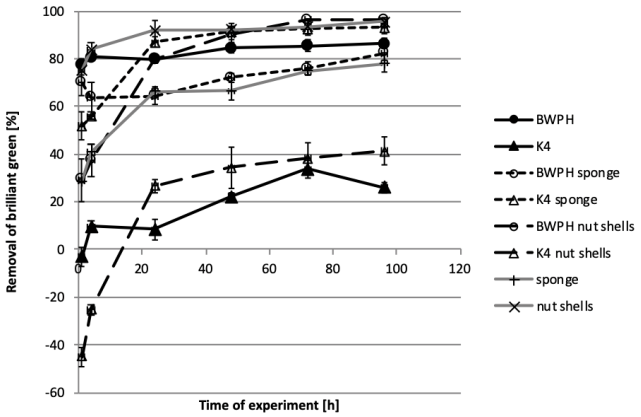


Fig. 3. Removal of Brilliant green by two strains of *Pleurotus ostreatus* immobilized and not immobilized.

mycelium immobilised on the washer removed almost five times more EB. Completely different results were reached for the BWPH strain. The concentration of biomass immobilised on the sponge was almost two times higher than in the sample with the suspended biomass (Table 3), but in spite of this the removal efficiency was the same for the suspended and immobilised mycelium (~60% after 96 h). Such results suggest that the process carried out by the BWPH strain is not inherently biochemical in nature and that immobilisation did not influence positively the production of enzymes involved in the process. The biomass of the BWPH strain immobilised on the same shells as for the strain K4 removed almost 90% of the dye after 96 h and the efficiency of the process was very high from the beginning of the experiment (more than 35%). As it has been mentioned above, the mechanism of the removal may be different. We cannot precisely evaluate the increase of the biomass growth for such a natural solid support because it is impossible to separate the biomass growing through the material. This makes the determination of the mechanism difficult, too. Such high effectiveness of the process may be a result of sorption on the fungal biomass, which grows intensely on this support, the increase in the activity of the fungal enzymes, as well as adsorption of the dye on the support, which may be physically and chemically modified by the mycelium to a significant extent. The NIR test showed statistically significant differences in the effectiveness of the decolourisation process between both strains. For suspended biomass, the value of p was 0.032, for the mycelium immobilised on the sponge p was 0.0001, and for the mycelium immobilised on the shells it was 0.0013. There were also statistically significant differences between the removal of Evans blue in the samples with the sponge and the mycelium of each strain, and in the samples with the shells and the mycelium ($p < 0.01$).

The influence of immobilisation on the efficiency of the dye removal was difficult to estimate in the samples with triphenylmethane brilliant green (Fig. 3). As it was visible in the preliminary test, this dye is easily adsorbed by both the materials used (~70% after 1 h). During the main experiment, sorption on the sponge was almost 80% after 96 h and on the pistachio shell – almost 96%. In the samples with the fungal biomass significant differences in the removal of brilliant green were observed. Suspended biomass of the BWPH strain removed about 77% of the dye after 1 h, which may be connected with its sorption on the biomass. After 96 h this strain removed ~86%. Completely different results were reached in the samples with the K4 strain, where the final removal (after 96 h) was only 26%. Differences in the biomass concentration in both samples (Table 3) were in the ratio BWPH:K4 = 3.75:1. Immobilisation of the biomass had a positive effect on the BG removal only in the case of the BWPH strain. Regardless of the material used for immobilisation, the decolourisation efficiency after 96 h was above 80% (82% for the sponge and 96% for the shell); therefore, the results are comparable with these observed in the samples with the supports only. An inexplicable phenomenon appeared in the samples with the biomass of the K4 strain immobilised on a shell proven as a material easily absorbing BG. After 1 h of the experiment, instead of the colour reduction, its growth was observed. An increase of colour instead of its reduction in the presence of different strains of *Pleurotus ostreatus* was observed also in the samples with RBBR (Fig. 4) and had been previously mentioned for other dyes [14]. After 96 h of the experiment in the sample with the K4 strain, only 20% more dye was removed in the sample with the biomass immobilised on the nut shells than in the samples with the suspended biomass (~46% and 26%, respectively). Eventually, the removal in the control with the nut shells was significantly higher than in the samples with the K4 strain and it reached almost 95%. The biggest influence on such a low efficiency of the BG removal by the K4 strain was connected with the degradation of the solid support by fungal hyphae. In the samples with the mycelium and shells, instead of an increase in the biomass, a huge decrease in its concentration was observed – 590 mg (Table 3). This means that during the degradation of the shells their adsorption capacity was significantly reduced. In the sample with K4 immobilised on the sponge, which is not susceptible to biological decomposition, the BG removal was eventually ~93%, which is 15% more than in the control with the sponge and 11% higher than in the sample with suspended hyphae of the BWPH strain. A higher efficiency of the BG removal in the samples with hyphae immobilised on the sponge was observed, though the biomass concentration was lower for the K4 strain (90 mg for K4 and 120 mg for the strain BWPH). Such results suggest that for this strain, the

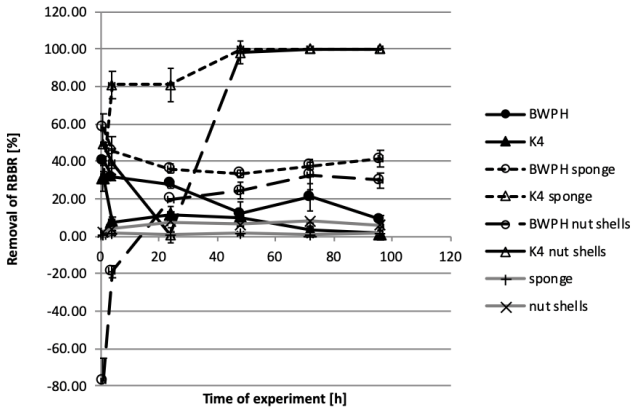


Fig. 4. Removal of RBBR by two strains of *Pleurotus ostreatus* using immobilised and non-immobilised biomass.

process might be connected both with the sorption and biochemical transformation of that dye. The same as for Evans blue, the NIR test showed statistically significant differences in the effectiveness of the decolourisation of brilliant green between both strains. For suspended biomass, the value of p was 0.005, for the mycelium immobilised on the shell p was 0.002. However, statistically insignificant differences in the efficiency of the process were observed when the mycelium was immobilised on the sponge (p = 0.46). Similarly, no statistically significant differences were observed for the strains immobilised on the sponge relative to the control (for BWPH p = 0.22, for K4 p = 0.06), and additionally for the BWPH strain immobilised on the shell (p = 0.30).

RBBR is a dye which was not adsorbed by any of the materials used in the study (<10% after 96 h). There was also a low decolourisation efficiency in the samples containing suspended biomass of both *Pleurotus ostreatus* strains. After a high result of the dye adsorption during the first hour of the experiment (up to about 32% for the K4 strain and 40% for BWPH), the dye desorption occurred, and eventually after 96 h the strains removed less than 2% and 10% of RBBR, respectively. Based on the results obtained for relevant controls and suspended mycelium, it can be concluded that in the case of using solid supports for immobilisation, a higher decolourisation efficiency (30%–100%) is mainly related to the biotransformation of this dye. Complete colour removal was observed in the samples with the K4 strain, regardless of the material used for the growth of hyphae. Different results were reached for the BWPH strain, which removed 30% and 41% on the shell and the sponge, respectively. There was also no correlation between the effectiveness of the process and the concentration of biomass of both strains (Table 3), because the ratio of biomass in the sample with the BWPH and K4 strain immobilised on the sponge was 1.4:1 and, as it has been mentioned above, the BWPH strain removed only 41% of the dye. Even more difficult is it to evaluate it for the samples with the shells as a support for the growth of biomass, where the decrease in the concentration of biomass was –350 and –690 mg sample^{-1} for strains BWPH and K4, respectively. The differences observed for both strains confirm that the decolourisation potential is not connected with the properties of the species, but of individual strains. An analysis by the NIR test demonstrated significant differences in the efficiency of the process carried out by both strains, both when the biomass was suspended in the substrate and when it was immobilised on a washer or shell (p-values of 0.034, 0.007, 0.007, respectively). Statistically significant differences in the process efficiency were observed in samples containing the sponge (p < 0.002). There were no statistically significant differences between samples containing an immobilised mycelium of the BWPH strain and the control containing shells (p = 0.45) and statistically significant differences for the second strain (K4) and the control (p = 0.034). The same test demonstrated statistically significant differences for both strains when the biomass was suspended in the medium and immobilised. For the mycelium immobilised on the sponge, the p-value is <0.001 for the BWPH strain and <0.03 for the K4 strain and when the biomass was immobilised on shell the p-value is <0.001 for the BWPH strain and 0.025 for the K4 strain. Statistically significant differences were observed for the strains immobilised on the sponge relative to the strains immobilised on the pistachio shell (p = 0.0021 and <0.0001, respectively).

The ability of fungi of the *Pleurotus* genus to decolourise various dyes has already been presented. Radhika et al. [34] in their decolourisation studies used strains classified as *Pleurotus* for the decolourisation of brilliant green, bromothymol blue, and methyl red in the concentration of 0.05% (w/v) in the PDA medium. The dyes were completely decolourised by the strains tested after 10–14 d. Particularly, fast decolourisation was achieved for brilliant green and methyl red. The *Pleurotus sajor-caju* strain decolourised those substances completely after 10 d of incubation. Eichlerova et al. [35] showed that when orange G and RBBR are added to the medium, they are decolourised during 12–28 d. Studies on strains of fungi belonging to the genus *Pleurotus* have been also conducted by Knapp et al. [36], Swamy and Ramsay [37] and Novotný et al. [38,39]. Knapp et al. [36] proved that *Pleurotus* sp. removes dyes from both the azo, anthraquinone, phthalocyanine and triphenylmethane groups. Novotný et al. [38] showed that *P. ostreatus* effectively decolourised Poly-R 478 and RBBR on all of the media used. The results presented in Figs. 2–4 indicate significant differences in the effectiveness of decolourisation between both strains of the same species. This confirms that the ability to decolourise dyes depends not only on the fungus species but also primarily on the strain. This is consistent only with the results of studies of Swamy and Ramsay [37], who found that *P. ostreatus* cannot decolourise azo dyes, which is in contrast to the research of Knapp et al. [36].

A positive effect of immobilisation on the effectiveness of the fungal decolourisation process was previously observed for other fungi. Similar to our study, Iqbal and Saeed [40] observed that biomass of a fungus belonging to the genus *Phanerochaete* immobilised on a natural loofah sponge removed dyes better than biomass suspended in a liquid medium. Nilsson et al. [41] in their studies on *Trametes versicolor* immobilised on a sponge, obtained a 70% reduction of the colour of azo reactive blues 4 and 2 after 3 d of the experiment. For comparison, Neelamegam et al. [42] obtained the removal of the dye even in 90%–95% using rice straw as a mycelium carrier. Domínguez et al. [43] used alginate beads for the immobilisation of the biomass of *Trametes hirsuta*. Already after 24 h, indigotine was decolourised in 95% and phenol red in 69%. These tests [40–43] are in line with the results presented earlier, because only

in the case of the RBBR dye did we manage to achieve full decolourisation in 48 h. The other two dyes were less efficiently removed. Studies by Tychanowicz et al. [44] were also based on the decolourisation of structurally different synthetic dyes (azo, triphenylmethane, heterocyclic, polymer) by the *Pleurotus pulmonarius* biomass immobilised on corn cobs. Within 6 d, a total decolourisation of Congo red, trypan blue, methyl green, RBBR, methyl violet, ethyl violet, and brilliant cresol blue was obtained. Methylene blue and Poly R-478 (heterocyclic dyes) were only partially discoloured. Rodriguez et al. [45] proved that the *P. ostreatus* strain immobilised on oat grains was characterised by higher activity of laccase and MnP than the biomass suspended in the substrate, which translated into a high degree of colour removal. Similar results for the mycelium of this species immobilised on polyurethane foam were also obtained by Casieri et al. [46]. The immobilised strain effectively removed RBBR and B49 with a high laccase activity. The importance of immobilisation was also emphasised by Franciscon et al. [47]. For the removal of colour, they used a non-ligninolytic fungus *Aspergillus ochraceus*, which – when grown on food waste – produced exoenzymes that degraded various dyes. At the same time, these researchers also observed that the carrier alone helped to remove the dyes by their adsorption. The enzymes released by the mycelium removed textile Violet dye completely, 57% of textile Green dye, 51.9% of blue methylene and 41.1% of Congo red.

3.3. Ecotoxicity test

No positive correlation was found between the effectiveness of removal of individual dyes of brilliant green and Evans blue and the changes in the toxicity of post-process samples (Tables 4 and 5). All controls containing only a medium with the dyes were classified as belonging to the 3rd class of toxicity. When the sponge was in the medium, a slight increase of toxicity was noticed in the controls with RBBR (from the 3rd to 4th class), which suggests that in contact with the dye some toxic substances could be released from this material. In contrast, in the case of shells, a decrease in the TUa value and maintenance of the toxicity class was observed. In the case of this material, this slight decrease probably resulted from the adsorption of the dye on the carrier (~6%). Interesting results were obtained in the case of brilliant green. The presence of a sponge that adsorbed almost 80% of the dye caused a significant decrease in the toxicity to *Artemia salina* (from the 3rd to 1st class). Surprisingly, in samples with shells, in which this dye was adsorbed in more than 95%, no decrease in toxicity was demonstrated.

There was no clear correlation between the removal of a given dye and a change in the toxicity to *Artemia salina* in the samples with the mycelium of both strains. In samples with Evans blue and the mycelium suspended in the medium and samples with biomass immobilised on sponge, a relationship was observed: the higher the degree of removal, the greater the reduction in the toxicity of this azo dye to the test organism. In samples with biomass immobilised on shells, the removal was almost similar for both strains, but a decrease of the toxicity was higher in the sample with the K4 strain (from the 3rd to first class of toxicity), which confirms our statement above that the process of removal using this strain is connected with biological transformation of this dye. An even smaller correlation between the removal of the dye and the toxicity of the samples was observed in

Table 4
TUa value and toxicity class and in test with *Artemia salina*

	Medium		Sponge		Pistachio shell	
	TUa	Class of toxicity	TUa	Class of toxicity	TUa	Class of toxicity
			Evans blue			
Control	2.77	3	2.43	3	1.30	3
BWPH	–	**1**	1.24	3	1.48	3
K4	1.47	3	1.00	**2**	**–**	**1**
			Brilliant Green			
Control	1.10	3	–	**1**	2.90	3
BWPH	2.71	3	1.40	3	2.27	3
K4	–	**1**	1.17	3	1.41	3
			RBBR			
Control	2.51	3	10.49	4	1.49	3
BWPH	1.82	3	–	**1**	1.54	3
K4	2.41	3	1.03	3	2.76	3
			Controls without dyes			
Medium	–	1	–	1	–	1
BWPH	1.13	3	–	1	–	1
K4	1.07	3	–	1	–	1

Table 5
TUa value and toxicity class and in test with *Lemna minor*

	Medium		Sponge		Pistachio shell	
	TUa	Class of toxicity	TUa	Class of toxicity	TUa	Class of toxicity
			Evans blue			
Control	3.49	3	1.72	3	1.87	3
BWPH	–	**1**	1.47	3	2.15	3
K4	2.53	3	2.03	3	2.18	3
			Brilliant Green			
Control	8.00	3	3.80	3	6.26	3
BWPH	16.00	4	16.18	4	1.43	3
K4	3.20	3	4.81	3	1.45	3
			RBBR			
Control	1.29	3	3.24	3	15.70	4
BWPH	–	**1**	–	**1**	2.28	**3**
K4	8.42	3	2.36	3	6.09	**3**
			Controls without dyes			
Medium	–	1	–	1	2.12	3
BWPH	–	1	–	1	1.09	3
K4	2.72	3	–	1	1.00	**2**

the case of brilliant green. The decrease in toxicity to *Artemia salina* was the highest in the sample with the suspended biomass of the K4 strain, where the removal was only 26%. An increase of toxicity from the 1st to 3rd class was noticed in the samples with biomass of both strains immobilised on the sponge. This phenomenon may be explained by the production of toxic dye metabolites by these strains, especially due to the fact that the biomass itself immobilised on this support does not release toxic compounds from this material, and it has been previously found that the decolourisation efficiency, especially for the K4 strain, was associated with biochemical transformation of brilliant green. There was also no correlation between the effectiveness of the RBBR removal and the toxicity of post-process samples. In the samples with an immobilised mycelium of the K4 strain, despite full decolourisation when the biomass carrier was a sponge, only one toxicity class drop was observed. At the same time, in the case of the BWPH strain, which removed only ~40%, the post-test samples were classified in the first toxicity class. When shells were used for the mycelium immobilisation, there was no decrease in the toxicity of the samples. For the K4 strain, a slight increase of TUa with the maintenance of the same toxicity class and full decolourisation suggests that the process was biochemical and caused the production of toxic metabolites.

Lack of correlation between the decolourisation efficiency and changes in ecotoxicity of the samples was also found in tests with *Lemna minor* (Table 5). A decrease of toxicity was noticed only in samples with suspended biomass of the BWPH strain (from 3rd to 1st class), where the removal reached 61%. However, in the samples with the immobilised mycelium in spite of the high degree of decolourisation (even over 90% for the K4 strain) no decrease in the toxicity class of the samples was observed. For brilliant green, even an opposite phenomenon was observed, that is, an increase in the toxicity class, especially in the samples with the BWPH mycelium suspended in the medium or immobilised on a sponge. While it seemed that in the case of this strain and brilliant green, the removal of the dye was the result of sorption, these toxicity test results suggest that the process could have been biochemical in nature, resulting in the production of toxic metabolites that had a negative effect on the growth of *Lemna minor*. Similar to the zootoxicity test, there was also no correlation between the effectiveness of the RBBR removal and the toxicity of the post-process samples. Irrespective of the degree of removal of this dye in the case of the K4 strain, no decrease in phytotoxicity was observed (the exception were the samples with the mycelium immobilised on pistachio shells). At the same time, despite the very low efficiency of the RBBR removal in the samples with the BWPH strain, a decrease in the toxicity class was observed. In the case of phytotoxicity tests, it should be emphasised that the presence of shells in the substrate adversely affected the growth of the examined plant. In this test, it was observed that the control samples, both with mycelia and non-treated, were mostly classified up to the 3rd class of toxicity. This makes it difficult to interpret the results of this test.

The results of ecotoxicological tests of samples after decolourisation have appeared only in recent years. The assessment of ecotoxicity is dominated by tests with mainly terrestrial plants. Casieri et al. [46] as one of the few used in their research a typically aquatic plant, which is *Lemna minor*. Acute toxicity tests using *Daphnia magna* were conducted by Franciscon et al. [47], Porri et al. [48], and Rizzo [49]. Ayed et al. [50] used *Artemia salina* and *Artemia nauplii* to assess zootoxicity.

The results presented above (Tables 4–5) are in accordance with the literature. Some information confirms that the used fungus not only removes colour, but also reduces the toxicity of wastewater containing dyes [51–53]. Casieri et al. [46] showed that both tested fungi (*Trametes pubescens* and *Pleurotus ostreatus*) not only efficiently decolourised the samples but also reduced phytotoxicity against *Lemna* sp. Particularly beneficial changes were found for *T. pubescens*. What is worth emphasising, as with both strains tested by us, these researchers also did not observe a clear correlation between the effectiveness of decolourisation and the decrease in toxicity.

4. Discussion

Both strains used in the study are able to remove almost 100% of the dyes from each of the analysed classes within 96 h, but the biomass must be immobilised on an appropriate solid support. Depending on the dye used in the studies, the influence of immobilisation on the efficiency of the decolourisation process was more or less significant. A statistical analysis with the NIR test proved that in the case of the K4 strain the immobilisation definitely positively affected the removal of each of the dyes tested. In the case of the BWPH strain, statistically significant differences for all the dyes were particularly evident when pistachio shells were used for immobilisation. This is important considering the fact that it is a waste matrix, and therefore a cheap and demanding development. It is necessary to emphasise that the effectiveness of decolourisation depends on the strain, the conditions of growth of the mycelium, but not on the biomass concentration.

Significant differences in the effectiveness of decolourisation between both strains of *Pleurotus ostreatus* were observed. This result confirms the necessity of conducting a series of tests on many strains and under different cultivation conditions before using them for the removal of pollutants. Literature data point to the possibilities of using various species of fungi in the decolourisation processes. The presented results proved that such knowledge is not sufficient and does not guarantee success because of significant differences in the decolourisation potential between various strains belonging to a given species.

A high efficiency of the dye removal, however, did not contribute to a significant decrease in the toxicity of post-test samples, which is mainly due to the fact that metabolites of these fungi, even on the substrate without dyes, have an impact on the producers and consumers used in the tests. However, especially in the case of the K4 strain, no increase in toxicity was observed, which allows to use these strains for the effective removal of dyes from various groups.

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Conflict of interest

The authors declare that they have no conflict of interest.

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