

## DECOLORIZATION OF TWO DYES USING WHITE ROT FUNGUS *P.OSTREATUS* (BWPH) STRAIN AND EVALUATION OF ZOOTOXICITY OF POST PROCESS SAMPLES

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Received: 30.03.2022; Revised: 19.07.2022; Accepted: 1.08.2022

### Abstract

Synthetic dyes are extensively used in various industries like textile and food industry and are responsible for generation of colored and toxic wastewater. The aim of study was to evaluate decolorization efficiency of White Rot Fungi *Postreatus* (strain BWPH) for dyes belonging to different classes: Anthanthrone Red (anthraquinone dye) and Disazo Red (azo dye) having concentration of 0.08g/l. The *Daphnia magna* immobilization test was performed to check zoo toxicity of samples during and after treatment. The result show that maximum decolorization efficiency achieved for Anthanthrone Red and Disazo Red dye after 168h was 94.31% and 73.13% respectively. The zoo toxicity test reflects that the pure dyes were much less toxic to *D.magna* at higher concentration. In contrast, the post process samples were more toxic to organism. It reflected production of toxic metabolites because of enzymatic degradation/biotransformation of dye. For anthraquinone dye, post process sample of 0.25 h was less toxic as compared to 168 h sample. Toxicity Unit was 23.52(class IV), and 2.61(class III) respectively for Anthanthrone Red and Disazo Red post process sample. The conducted research showed high potential of BWPH strain for decolorization dyes belonging to different classes. But the mycelium produces toxic substances during the decolorization process. It may be related to the biodegradation of these substances to toxic metabolites. Further studies have indicated to optimize the process of decolorization.

Keywords: Anthraquinone dye; Azo dye; Decolorization; *Pleurotus ostreatus*; Zoo toxicity.

## 1. INTRODUCTION

The maintenance of environment quality has become a global concern as the environment is highly vulnerable to various kinds of toxic pollutants which are emerging as a result of industrialization and urbanization and may have adverse effect on ecosystem by creating disturbance at various levels [1]. The continuous usages of different kinds of resources coupled with lack of proper treatment of effluents are majorly responsible for polluting natural environment like water, soil or air [2]. The water pollution is also one of

the serious environmental concerns. The major contributors of the wastewater are domestic sewage and industrial effluents. The latter being the most concerned issue as it is responsible for generation of gallons of wastewater throughout the year which is harmful to aquatic plants, animals and even humans directly or indirectly [3]. As a result, the ecosystem gets imbalanced by entry and accumulation of carcinogenic and teratogenic compounds. The concern has been raised globally and many groups of scientists are focusing on priority basis to find advanced technology for pollutant removal [4].

The synthetic dyes are one of the popular substances being utilized in textile, leather, pharmaceutical industry. The large amounts of industrial effluents contain dyes which are released during the dyeing process and results in generation of colorful, baleful, toxic wastewater. Their chemical properties are also harmful showing high chemical oxygen demand (COD), high pH and temperature, strong coloration [5- 6]. The synthetic dyes belong to different classes like azo dye, triarylmethane dye and anthraquinone dye and based on the classes, they have different properties, degradability and effect on ecosystem. The vat dye like Anthanthrone Red is a synthetic anthraquinone dye and it's derivative 4,10-DiBromo Anthanthrone is being utilized in preparation of industrial paints and decorative ink etc. It is insoluble dye in water and shows higher persistence in environment as it is containing aromatic compound with halogen substituents. In terms of toxicity, the more toxic synthetic dyes include azo dyes and more particularly disazo dyes. It shows carcinogenic effect on humans and animals as the electron withdrawing nature of azo group creates electron deficiencies [7]. The dye wastewater treatment has been accomplished by various means of conventional physical and chemical process including adsorption, flocculation, precipitation, photodegradation [8]. But these processes are not much cost effective and on top of that, it also ended up in land filling.

As an alternative option, the dye removal by using bacteria, algae, fungi as biological remediation processes, have been proven to be more advantageous. It is more beneficial because of cost effectiveness, easy maintenance and it results in complete mineralization of dye containing wastewater [9]. Among bioremediation agents, Filamentous fungi shows its unique properties for degradation of broad spectrum of pollutants. It is reported that White Rot Fungi is able to degrade range of pollutants like synthetic dyes, pesticides, Polyaromatic Hydrocarbons (PAHs), Polychlorinated biphenyls (PCBs), Pharmaceutically active compounds (PhACs) [11–14]. The White Rot Fungi (WRF) demonstrates stable colonization at different kinds of locations like soil, marine water, fresh water and even it can survive in effluent treatment plants (ETPs) for treatment of wastewater [10]. The utilization of WRF in bioremediation of various pollutants is gaining attention. The morphology and chemical nature of WRF provide an added advantage by enormous biosorption capacity and diverse metabolic capacity. WRF have potential to transform or even mineralize pollutants. It has

nonspecific extracellular as well intra cellular enzymatic system. Extracellular degradation is majorly carried out by ligninolytic enzymes (mostly laccases and manganese or versatile peroxidases) and intracellular oxidation is performed by cytochrome P450 system associated enzyme complex [11].

As mentioned previously, the synthetic dyes have negative ecotoxicological effect. It is important to conduct toxicity analysis of metabolites which are produced at the end of decolorization of dye. Several studies have been available on the chemical properties of metabolites being produced but very few papers are available on toxic effect of pure dye and biologically treated water on environment and organisms residing in the water bodies where ultimately the water is going to be released after treatment or may be recycled and utilize for farming. It is highly important to evaluate the toxicity effect of post process samples on all three levels like producers, consumers and decomposers [16].

The aim of the study was to perform a small scale decolorization experiment study before applying to field. In this study, the removal of Anthanthrone Red and Disazo Red dye was targeted to achieve degradation of dyes by providing WRF *Pleurotus ostreatus* (strain BWPH) as a biological agent. The decolorization was measured spectrophotometrically at regular time intervals. The zoo toxicity of pure dye and post process sample was also tested on crustacean *Daphnia magna* in order to get an idea about the hazardous nature of intermediates and/or final metabolites being produced as a result of biodegradation. It gives better idea about efficiency of remediation technique.

## 2. METHODOLOGY:

### 2.1. Fungal species and Culture condition

*Pleurotus ostreatus* (strain BWPH) was chosen for this study, which were collected from the Fungal Strain Collection of Environmental Biotechnology Department, The Silesian University of Technology, Gliwice, Poland. For the multiplication of *Pleurotus ostreatus* mycelium, 4 cubes of mycelium from MEA plates were transferred to the flask containing the medium Glucose – 5g/L, Peptone – 1g/L, MgSO<sub>4</sub> x 7H<sub>2</sub>O – 0.5g/L and KH<sub>2</sub>PO<sub>4</sub> – 0.1g/L (pH 5.6). The media sterilization was performed at 121°C for 20 minutes in autoclave.

### 2.2. Test Compound and Solution Preparation

Two dyes belonging to anthraquinone and azo group were selected: Anthanthrone Red (C.I 59300; CAS 4378-61-4) and Disazo Red (C.I 20730; CAS 3905-19-9) respectively. The dyes were prepared in deionized water in concentrated solution form, autoclaved at 121°C and then added to the media containing 5 days old fungal biomass at the final concentration 0.08 g/L.

### 2.3. Decolorization Experiment

The BWPH strain's capacity to decolorize dyes belongs to different groups was checked by performing decolorization experiment. After approximately 5 days of growing fungal biomass on the media described above (100 ml of medium with 7 days cultured biomass), the biomass was broken up using a BagMixer and added to the reactor containing liquid organic medium having volume of 1500 ml. The reactors were left for 7 days on a shaker (150 rpm) in order to multiply the biomass. After growth of fungal biomass, Anthanthrone Red and Disazo Red were added separately to bioreactor with a final concentration of 0.08 g/L. After addition of dye, the reactor was operated in the static version. For 5 days a week, 200 ml of air was pumped through the filter using a syringe. The absorbance of the dye containing samples was measured spectrophotometrically by using Hitachi U-1900 UV-VIS spectrophotometer. The  $\lambda$  values were determined by measuring the absorbance at different wavelengths and determined that the Anthanthrone Red dye gave maximum absorbance at three wavelengths 538 nm, 510 nm and 462 nm and for Disazo Red dye the value of maximum absorbance was at 556 nm and 510 nm. The samples were collected, and absorbance was measured at mentioned time interval from the reactor after addition of dye; 0.25 h, 24 h, 48 h, 120 h, 144 h and 168 h. Changes in pH value were measured during the process by pH-meter. The percentage of decolorization (DP) of dye at fixed time interval was calculated using the below mentioned equation 1:

$$DP[\%]=\frac{C_i-C_t}{C_i} * 100 \tag{1}$$

Where,  $C_i$  is the initial concentration of dye (in milligram per Liter), and  $C_t$  is the concentration of dye in samples at time  $t$  (in milligram per Liter).

The total removal percentage was measured using equation 2:

$$Removal[\%]=\frac{C_i-C_f}{C_i} * 100 \tag{2}$$

Where,  $C_i$  is the initial concentration of dye (in milligram per Liter), and  $C_f$  is the final concentration of dye in samples (in milligram per Liter).

### 2.4. Toxicity Assessment

For the assessment of toxicity, the acute immobilization test was conducted on crustacean *Daphnia magna* in accordance with OECD guideline 202. The test was performed in special 24-well plates. The range of the concentrations of the 5 tests sample arranged in a geometric series with a factor 2 for both dyes (3.125% – 100%) and the post process sample were collected from reactor. The same test was carried out for dyes solution in water. In each well, suitable concentration of test solution and post process sample were added followed by addition of five *Daphnia magna* organisms. After 24 hours of incubation at room temperature, the number of mobile and immobile test subjects was noted.

The concentration of the sample causing any change in 50% of the organisms in the population is considered as a measure of toxicity –  $EC_{50}$ . The  $EC_{50}$  value was determined by the method of graphical interpolation of the obtained results. In order to classify the toxic substances, this value was converted into toxicity units according to the below mentioned Equation 2:

$$TUa=100/EC_{50} \tag{3}$$

Based on the above calculations, the pure dye and its metabolites found in the post process sample were classified to appropriate toxicity group. Persoone toxicity classes has been described in the table below (Persoone et al., 2003).

**Table 1.**  
Persoone Toxicity Classes

Toxicity Unit (TUa)	Class	Toxicity
<0.4	Class I	no acute toxicity
0.4 < TU < 1	Class II	low acute toxicity
1 < TU < 10	Class III	acute toxicity
10 < TU < 100	Class IV	high acute toxicity
TU > 100	Class V	very high acute toxicity

## 3. RESULT AND DISCUSSION

The biological removal of different kinds of synthetic dyes from wastewater could be achieved by using

White Rot Fungi. The WRF shows enormous ability to degrade and decolorize different dyes belonging to various classes [16–17]. The WRF *P. ostreatus* (strain BWPH)'s ability to decolorize the two recalcitrant dye – Anthanthrone Red and Disazo Red was evaluated by measuring decolorization at different wavelength. Also, the zoo-toxicity of post process sample was evaluated in order to decide its effect on animals when the wastewater is released into water stream.

### 3.1 Effectiveness of White Rot Fungi mediated decolorization of Dye

The decolorization effectiveness of the dyes Anthanthrone Red and Disazo Red were measured spectrophotometrically at specific mentioned time interval. As soon as the dye was added to the reactor, the gradual decrease in the absorbance of dye was observed at particular wavelength which correlates with the commencement of dye degradation process by fungi. With reference to Fig.1, the decrease in concentration of dye started just within 0.25 h after addition of dye. Starting decolorization may correspond to great biosorption capacity of WRF. As the homogenized biomass is utilized for the experiment, it serves for larger surface area for dye adsorption. The sorption of dye depends on the chemical nature of dye, interaction between functional group of dye and cell wall of fungi, pH of medium. The Anthanthrone dye contains halogen chromophore which could be absorbed by negative charges on fungal cell wall.

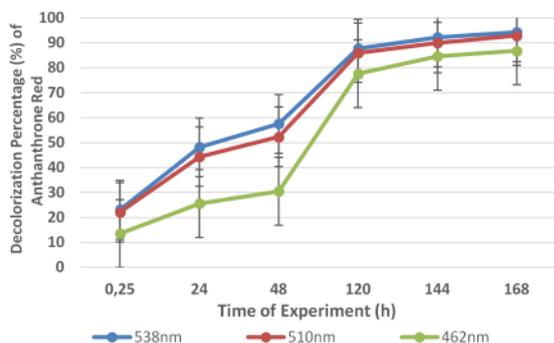
As mentioned, the DP was calculated for three absorbance maxima for Anthanthrone Red dye at 538 nm, 510 nm and 462 nm. The UV-VIS wavelength scan was utilized to monitor the changes in absorbance peaks at each wavelength which helps in estimation of changes in chemical structure of dye. The final decolorization measured at 168 h, was highest (93.4%) at 538 nm, followed by 92.9% at 510 nm and was lowest (86.8%) at 462 nm. It suggests that enzymatic degradation showed maximal effect at 538nm wavelength. In case of Disazo Red Dye, almost similar percentage of decolorization was observed at both wavelengths 556 nm (73.1%) and 510 nm (70.3%). The process of Anthanthrone Red decolorization was faster and more efficient than Disazo Red, as after 0.25 h, the decolorization percentage were observed 23.09% and 11.99% respectively (Fig. 1, Fig. 2). The results of decolorization experiment were as expected and it is found to be correlating with published literature as well [20].

The differential changes in absorbance maxima

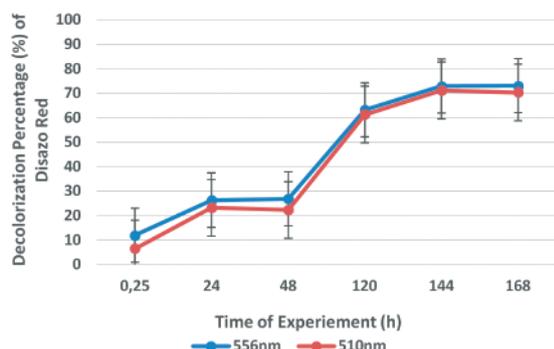
attributed to the possibility of removal of both dyes via biochemical transformation [21]. Because when the dye is removed via biosorption or bioaccumulation route, mostly it does not correspond to changes in wavelength peaks. Lu et al., 2016 [22] proposed removal of RBBR, an anthraquinone dye via Lignin modifying enzyme whereas Kunjadia et al., 2016 [23] mentioned enzymatic removal of recalcitrant azo dye with help of laccase as a major extracellular enzyme responsible for degradation of dye. The Lignin modifying enzymes and oxidases are responsible for degradation of pollutants. The enzyme activity is influenced by various parameters like pH, temperature, fungal species etc. Gahlout et al., 2013 has observed that the presence of glucose as carbon source and peptone as nitrogen source have major effect on laccase and Manganese Peroxidase MnP enzyme activity and apparently resulted in highest decolorization percentage [19]. So, it is evident that the medium composition utilized here for degradation studies had suitable components for efficient enzyme production. Jureczko et al., 2020 has measured laccase enzyme activity for *P.ostreatus* (BWPH) strain. It suggested that BWPH strain is potent producer of laccase enzyme which is responsible for degradation of recalcitrant pollutants [14]. The mechanism of microbial degradation of azo dyes involves the reductive cleavage of azo bonds (-N=N-) with help of azoreductase and results in formation of degraded products. The degradation pathway of anthraquinone dye was proposed and first reaction was hydrolysis of C-N bond in anthraquinone dye degradation [24]. And it is well documented that hydroxyl radicals play important role in degradation process by fungi.

In addition to the biochemical removal, the white rot fungi showcase great sorption capacity due to cell wall structure and remove dye efficiently via biosorption in living and dead biomass [25–26]. A regular increase in DP was found in case of the Anthanthrone Red dye whereas the Disazo Red showed almost a halt in decolorization between 24h and 48h. Here, shaking condition was utilized for growth of biomass and after addition of dye, the static condition was maintained. The proper aeration was provided after time intervals. It also corresponds to enhanced dye decolorization as it helped in oxygen transfer reaction. The parameters like pH and temperature also play important role in dye degradation mechanism. The pH of culture medium is critical for growth of fungi, metabolic activity, surface structure, enzyme production and hence, efficiency of recalci-

trant compound degradation [10]. The charge present on surface of fungal biomass and ionic forms of dye pollutant can be controlled by pH of solution [27]. Here, pH of medium was set at 5.6 which generally imparts in good range of pH for effective dye degradation. But the pH of medium was dropped to 3.6 as compared to initial pH of medium. With time passed, a gradually the medium turned acidic. Overall, the set parameters for this experiment supported combined dye degradation mechanism.



**Figure 1.**  
Effectiveness of Anthranthrene Red Decolorization (%) by *Postreatus* (BWPH)  
(Parameters:  $T=22\pm 2^{\circ}\text{C}$ ,  $t = 0.25\text{-}168$  h, final concentration of dye – 0.08 g/L)



**Figure 2.**  
Effectiveness of Disazo Red Decolorization (%) by *Postreatus* (BWPH)  
(Parameters:  $T=22\pm 2^{\circ}\text{C}$ ,  $t = 0.25\text{-}168$  h, final concentration of dye – 0.08 g/L)

### 3.2. Evaluation of Zoo-toxicity of dye and samples treated with fungi

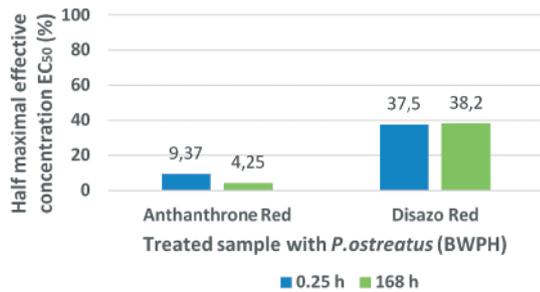
The result of dye degradation is not always complete mineralization of dye, but it may produce toxic intermediates or final products of degradation which are sometime even more harmful than pure dye [28]. The ultimate goal of fungal bioremediation is not just

decolorization but also toxicity of post process samples should be decreased as compared to start sample in order to utilize fungal bioremediation as a prime tool for cleaning wastewater effluent. The Ecological and Toxicological Association of Dyestuffs Manufacturing Industry (ETAD) was established with the aim of mitigating environmental damage, safeguard consumers and coordinating with government and public issues in relation to toxicological effect of their products [29–30].

The results of the toxicity test were obtained in form of percentage of immobilized *D.magna*. Based on the observation of immobile organism, the samples were distributed to toxicity class. In case of pure dye toxicity assessment, the Anthranthrene Red dye showed no toxicity but in contrast, Disazo Red Dye exhibited almost 20% immobilized organism at 100% concentration (0.08 g/L) of dye. At lower concentration of disazo dye (up to 25%), no mortality of organism was observed but as the concentration increases (50–100%), there was not harmful effect on the crustacean (data not shown here).

The post process samples were collected from two-time interval: in the beginning of dye decolorization process (0.25 h) and at the end of experiment (168 h). The purpose of evaluation of these samples was to compare the toxicity of metabolites in the starting and at the end stage. When the post processed sample of time period 0.25 h and 168 h were assessed for toxicity, they belonged to higher toxicity classes of Persoone as compared to pure dye. The Anthranthrene Red dye executed higher percentage of immobilization of test organism at all ranging concentration, starting from 100% immobilization at highest concentration to 25% inhibition at lowest (3.125%). Whereas Disazo Red showed harmful effect up to 50% concentration of dye in 168 h post process sample. The  $EC_{50}$  value was determined using extrapolation. In case of Anthranthrene Red dye the  $EC_{50}$  value was 9.37 and 4.25 respectively at 0.25h and 168 h whereas it was 37.5 and 38.2 for Disazo Red dye (Fig. 3). The difference between  $EC_{50}$  value for post process samples with increasing time period showed the gradual accumulation of toxic metabolites which results in higher number of test organism mortality. As in case of Anthranthrene Red dye, the toxicity increased as the time passed (168 h). It corresponds to that fact that as enzymatic degradation proceeds, it resulted in conversion of chemical property of dye. Overall, Anthranthrene Red dye showed much higher toxicity to crustacean *D.magna* than Disazo Red. The aromatic nature of

Anthranthone Red dye proved to be more toxic. The data reported here is in contrast with existing literature as it resulted in increase in zoo toxicity as compared to pure dye [31]. Such results can confirm formation of toxic metabolites of dyes as well as production of toxic metabolites by fungi. Mostly post process samples obtained as a result of decolorization were classified as toxic by-products [32].



**Figure 3.** maximal effective concentration EC<sub>50</sub> (%) of post-process samples treated with *P.ostreatus* (BWPH) strain containing Anthranthone Red and Disazo Red dye

**Table 2.** Zoo-toxicity of Pure dye and Post process sample classified according to Persone Toxicity Classes

Fungal Culture	Dye	Sample Description	Toxicity class (TU <sub>a</sub> )
<i>P.ostreatus</i>	Anthranthone Red	Pure Dye	Non-Toxic
		0.25 h post process sample	IV (10.67)
		168 h post process sample	IV (23.52)
<i>P.ostreatus</i>	Disazo Red	Pure Dye	Non-Toxic
		0.25 h post process sample	III (2.67)
		168 h post process sample	III (2.61)

## 4. CONCLUSIONS

The ability of White Rot Fungi *Pleurotus ostreatus* for removal of recalcitrant dye belonging to major two classes, anthraquinone and azo dye, were analysed during this study. *Postreatus* was capable of decolorization of Anthranthone Red and Disazo Red dye containing solution at concentration of 80 mg/L. Although it was concluded that *Postreatus* was more efficient in decolorization of Anthranthone Red as compared to Disazo Red after 168 h incubation. But when the zootoxicity analysis was performed on *D.magna*, it was observed that the metabolites giving absorbance at different wavelengths were much more toxic in case of Anthranthone Red as compared to Disazo Red. The results suggests that the fungal treatment is promising tool for dye con-

taining wastewater for their efficient decolorization as fungi robustly degrade complex organic pollutants.

## ACKNOWLEDGEMENT

The authors would like to acknowledge the funding body for this research. The Faculty of Energy and Environmental Engineering, Silesian University of Technology provided grant (BKM-668/RIE7/2021) to support young scientist research activity.

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