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Removal and degradation of the fungicide dye malachite green from aqueous solution using the system wheat bran–*Fomes sclerodermeus*

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Abstract

The triphenylmethane dye malachite green (MG), commonly used as fungicide, was adsorbed onto wheat bran (WB) by using a batch technique. The effects of contact time, dye concentration and pH were investigated. The equilibrium was attained after 40 min of contact time irrespective of MG concentration. The pH of MG aqueous solution greatly influenced the adsorption capacity and intensity, it was found that maximum adsorption of dye occurred at pH range 7–9, where the amount of dye removed was nearly 90%. Data obtained on adsorption at different dye concentrations and pH range 4–7 were used to plot the Freundlich isotherms. WB with MG adsorbed at pH range 4–7 was used as substrate for the growth of the white rot fungi *Fomes sclerodermeus* and *Phanerochaete chrysosporium*. The presence of MG (nearly 24 mg g^{-1} dry WB) delayed the fungal growth. MG was completely degraded by *F. sclerodermeus* cultures at pH 5, in concordance with the highest ligninases production. Thus, pH values not only influenced the adsorption capacity of WB but they were also important for growth, enzyme production and finally, dye degradation. This technique should have broad applications in bioremediation processes of water and wastewater.

Keywords: Adsorption; Bioremediation; Dyes; Ligninases; White rot fungi

1. Introduction

Synthetic dyes are common contaminants of water, it is estimated that the production of these compounds is around of 10,000 tonnes per year and it is assumed that the quantity of dyes discharged in the environment is about 1–10% [1]. Malachite green (MG) is a triphenylmethane dye used as a fungicide and antiseptic in fish cultures (The Merck Index, 12th edition), its toxicity to bacteria and mammalian cells was also demonstrated [2]. MG also is used as a direct dye for silk, wool, jute and leather. Because of the toxicity to major microorganisms, its presence in wastewaters makes difficult the biodegradation. In a previous report it was demonstrated that supernatants from *Fomes sclerodermeus* with laccase activity were able to degrade and detoxify MG [3], although a primary problem is the establishment of the organism to aqueous contaminated environments. Thus, wastewater has the problem that the toxic compounds have to be removed and immobilized. Different adsorption materials had been studied for removal of these compounds from water and wastewater in order to decrease their impact on the environment [1]. Traditionally, activated carbon adsorption and solvent extraction are the most widely used methods for the removal of contaminant compounds [4,5]. But there is a low cost alternative to this process called biosorption that began to be used recently, where biomass (usually lignocellulosic or microbial residues) is the adsorbent material. Biosorption is the non-specific term used to denote the complex process whereby biomass is utilized to remove solutes or colloidal material from wastewater or other aqueous solutions. Brown marine macroalgae, which are an important source of biomass, were used for biosorption of nickel and cadmium showing a high capacity for the uptake of these heavy metals, in this case the biosorption could be mostly a surface process in which the metal cation is adsorbed due to charges on the surface of the adsorbent [6]. Wheat and rice bran were used as adsorbents for chloroform, organochlorine compounds and benzene, which, due to their lipophillic character were taken up into intracellular oil containing particles present in lignocellulosic materials and called spherosomes [7,8]. Biosorption probed also to be a good method to remove dyes from wastewater [9,10]. A second step should be the degradation of

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the organic recalcitrant compound adsorbed. There are a wide range of microorganisms capable of degrade these compounds, being white rot fungi included in this group.

White rot fungi are the only organisms able to mineralize the lignin, these fungi possess a ligninase system composed at least by three unspecific enzymes: lignin peroxidase (LiP), manganese dependent peroxidase (MnP) and laccase; some fungi are capable to produce all three classes of such enzymes while others produce only one or two of them [11]. These enzymes not only attack lignin but it was also demonstrated that their substrates include a wide range of pollutants, such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and synthetic dyes [12].

F. sclerodermeus is a white rot basidiomycete. In previous reports, the capacities of this fungus to produce large amounts of ligninases (laccase and MnP) growing on wheat bran (WB) as well as to degrade and detoxify the fungicide malachite green were demonstrated [3,13]. The aims of this work were to study the adsorptive capacity of wheat bran for the removal of malachite green from aqueous solution at different pH and to study the degradation of the dye adsorbed to wheat bran by *F. sclerodermeus*. *Phanerochaete chrysosporium*, widely known for its ligninases production and xenobiotic degradation capability was used for comparison.

2. Materials and methods

2.1. Organisms

Pure cultures of *F. sclerodermeus* BAFC 2752 and *P. chrysosporium* BAFC 246 were obtained from the culture collection of the Facultad de Ciencias Exactas y Naturales (Universidad de Buenos Aires). Stock cultures were maintained on malt extract (1.2%) agar (2%) slants at 4 °C.

2.2. Adsorption of malachite green: Freundlich equation

WB with a particle size of 8–20 mesh, was purchased at a local market, it was dried at 60 °C overnight and stored dry in a vacuum desiccator until required. MG was obtained from Sigma (St. Louis, USA), and used without further purification. Stock solution of dye was prepared using MG salt in double distilled water. Adsorption of MG was investigated in a batch system to obtain rate and equilibrium data. Batch technique is very simple allowing the scale up of the process. Effects of initial concentrations, contact time and pH of the medium on the adsorption rate and capacity were studied. The pH of the media was adjusted over a range of 2–9 using citrate–phosphate buffer (50 mM final concentration). The pH of the solutions was measured with a pH meter (HI 9321, Hanna Instruments). The adsorption of MG to wheat bran was monitored spectrophotometrically at 618 nm in the aqueous solution [14] and the values were expressed as A_t 100/A_0 where: A_t = absorbance at time t and A_0 = absorbance at time 0. Dye concentrations (mg l^{-1}) were correlated with the absorbance values. A control flask containing only distilled water and WB was also used to determine the zero level absorbance.

In the experiments to estimate the capacity and affinity of adsorption, 500 mg of WB were placed in each 100-ml conical flask with 10 ml of MG solutions of varying concentration. Citrate–phosphate buffer 50 mM (final concentration) was used to adjust the pH of solutions. Samples from the aqueous phase were taken at proper time intervals to study the adsorption kinetics.

Due to its empirical character and applicability on heterogeneous systems, Freundlich model equation was used to describe adsorption isotherms of MG to WB. Freundlich equation was adopted to describe the adsorption isotherm.

Freundlich equation:

$$q_e = aC_e^{1/b}$$

where q_e is the dye uptake capacity (mg dye/g dry WB); C_e is the equilibrium concentration of dye in solution after the adsorption assay (mg l^{-1}); a and b are the Freundlich constants to be estimated, being a the maximum dye uptake capacity and b the adsorption affinity constant.

For plotting the isotherms q_e versus C_{eq}, the contact time and pH values were selected on the basis of the above experiment. Due to the fact that color intensities of MG depends on pH, calibration curves at each pH were calculated. Experimental data points were obtained by keeping constant the amount of WB at 1 g and varying the concentration of the dye from 12 to 348 mg g^{-1}.

Dye uptake capacity was calculated from the initial concentration in the aqueous medium. The dye concentration in the aqueous medium was calculated from calibration curves at different pH values. The constants were calculated by non-linear regression using STATISTICA 5.1 (StatSoft, Tulsa, OK).

2.3. Cultures

WB with 24 mg of MG adsorbed per gram of dry matter at a pH range 4–7, and 75% moisture content, was used as a substrate for the growth of *F. sclerodermeus* and *P. chrysosporium*. Inoculation was carried out using cubes of agar-malt extract colonized by mycelium.

All experiments were run in triplicate at 28 ± 1 °C.

2.4. Growth estimation

Because of the structure and porosity of the solid substrate the mycelium penetrates into and binds to it. Thus, the fungal growth cannot be estimated as dry weight, therefore this variable was estimated from the dry weight loss of the complex substrate-mycelia. Fungal biomass production correlated very well with the dry weight loss of this system, therefore, higher losses of dry weight (%) are assumed as higher biomass production [15].

2.5. Degradation of malachite green

To calculate the degraded dye, the substrate colonized by the fungus was placed in a desorption medium that consisted of ethanol:water (1:1) and stirred for 60 min, the concentration of MG in the desorption medium was calculated spectrophotometrically as described above. Absorption spectra were carried out for MG at time 0 and after 50 days of incubation.

2.6. Enzyme activities and proteins

For the extraction of extracellular proteins and enzymes solid cultures were shaken 20 min with 5 vol. of water and centrifuged. Supernatants were used for assays.

Extracellular protein was determined by the Bradford method [16]. Bovine serum albumin served as the reference. Laccase activity was determined by oxidation of 2,6-dimetoxyphenol (2,6-DMP) to coerulignone (ε_{469} = 27500 M^{-1} cm^{-1}). The reaction mixture contained 0.1 M sodium tartrate buffer, pH 5, 10 mM 2,6-DMP and the appropriate amount of enzyme in a total volume of 1 ml [17]. MnP activity was determined by oxidation of phenol red (0.01%). The reaction product was measured at 610 nm (ε_{610} = 22000 M^{-1} cm^{-1}). The reaction mixture contained 50 mM succinate buffer pH 4.8, 0.1 mM $MnSO_4$, 0.1 mM H_2O_2 and the appropriate volume of enzyme in a total volume of 1 ml [18]. LiP was determined by the H_2O_2-dependent veratraldehyde formation from 2 mM veratryl alcohol in 100 mM sodium tartrate buffer (pH 3), reactions were started by the addition of H_2O_2 0.4 mM [19]. One unit of enzymatic activity was defined as the amount of the enzyme needed to oxidize 1 μmol of substrate per minute.

3. Results

3.1. Adsorption of malachite green to wheat bran

The MG adsorption kinetics to WB performed with different dye concentrations at pH 7 are shown in Fig. 1. The equilibrium

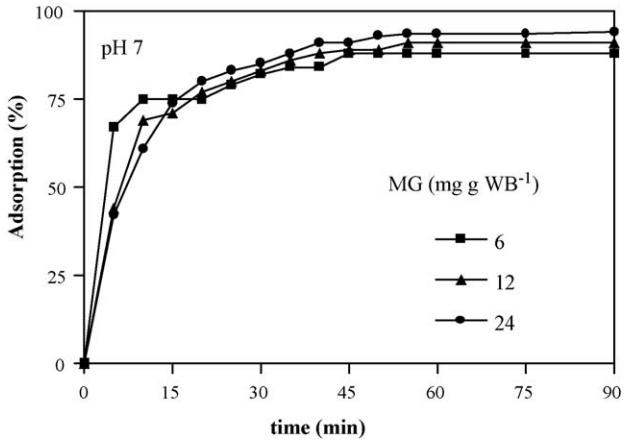

Fig. 1. Effect of MG concentration on the adsorption onto WB. Each flask contained 1 g WB and 40 ml buffer citrate–phosphate (50 mM) at pH 7, the amount of MG per gram WB was variable as is observed in the figure. Each point represents the mean of three replicate measurements, standard error of the mean was less than 5%.

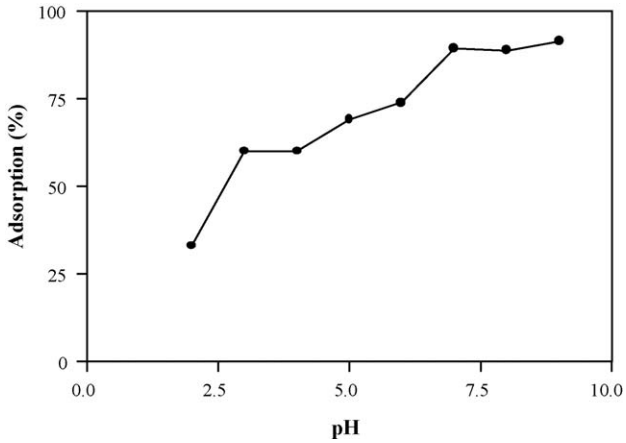

Fig. 2. Effect of pH on the adsorption of MG onto WB. Each flask contained 1 g WB and 12 mg MG and 40 ml buffer citrate–phosphate (50 mM) in the pH range 2–9. Each point represents the mean of three replicate measurements, standard error of the mean was less than 5%.

was attained after 40 min of contact time with the three dye concentrations tested. The system reached around 70% adsorption within 15 min of contact time irrespective of concentration. Therefore, amount (mg) of MG removed by adsorption as well as the rate (mg g^{-1} min^{-1}) increased with the increase in MG concentration.

It is well known that pH greatly influences the adsorption capacity and intensity. To study this effect, experiments were performed using citrate–phosphate buffer at pH range 2–9. Since the color intensities of MG are influenced by the pH values, concentrations of the dye were estimated from calibration curves performed at each pH assayed. The amount of MG adsorbed onto WB after 2 h of contact time is shown in Fig. 2. From these results, it is evident that the maximum adsorption of dye was attained at pH range 7–9, where the amount of dye removed was nearly 90%.

Data obtained of q_e and C_{eq} at different dye concentrations and pH range 4–7, were used without any prior transformation to fit the Freundlich equation. Plots of C_{eq} versus q_e, estimated parameters and R^2 values are shown in Fig. 3. The two coefficients were estimated by non-linear regression. The high values of R^2 provide strong evidence that the model accurately reflects the process. The R^2 values were >0.9; this means that more than 90% of the variability in MG adsorption observed can be accounted for by the Freundlich equation prediction. It was taken as evidence of the applicability of the model within the range of variables included in this work. With respect to Freundlich constants, it is evident that pH affects the adsorption capacity of WB. At pH 7, this constant showed its maximum value (4.55), while the adsorption capacity decreased at lower pH values. The affinity constant showed similar values at the pH range assayed.

3.2. Fungal growth, ligninases production and degradation of MG

Growth, enzyme production and MG decolorization in *F. sclerodermeus* and *P. chrysosporium* were greatly influenced by pH values (Table 1). LiP was not detected in any media tested. The growth of *F. sclerodermeus*, as estimated by dry weight loss, at pH 4 and 5 was twice that at higher pH values. On the other hand, *P. chrysosporium* was significantly more affected, reaching maximum dry weight loss at pH 6. Control cultures (without MG) showed their maximal growth after 18 and 12 days postinoculation with dry weight losses of 55 and 65% for *F. sclerodermeus* and *P. chrysosporium*, respectively. In the presence of MG at 50 days postinoculation only *F. sclerodermeus* attained maximal growth, comparable to control cultures with-

Table 1
Growth, extractable protein, enzyme production and MG decolorization with increasing pH values at 50 day postinoculation of *F. sclerodermeus* and *P. chrysosporium*. Adsorption conditions: WB, 4 g; MG, 96 mg; buffer citrate–phosphate, 50 mM; contact time, 1 h

pH	*F. sclerodermeus*					*P. chrysosporium*				
	Dry weight loss (%)	Protein (mg g^{-1})	Laccase (U g^{-1})	MnP (U g^{-1})	MG decol. (%)	Dry weight loss (%)	Protein (mg g^{-1})	Laccase (U g^{-1})	MnP (U g^{-1})	MG decol. (%)
4	53.25	31.16	37.42	0.48	88.03	27.50	0.58	0	0.59	34.56
5	54.50	32.04	110.8	5.28	100	33.25	0.83	0	0.71	45.47
6	26.75	22.01	14.42	0.04	96.23	52.00	3.94	0	0.18	25.30
7	23.42	20.42	3.62	0	74.84	20.50	8.26	0	0.27	32.51

Values shown are the means of triplicate cultures, standard error of values was less than 10%.

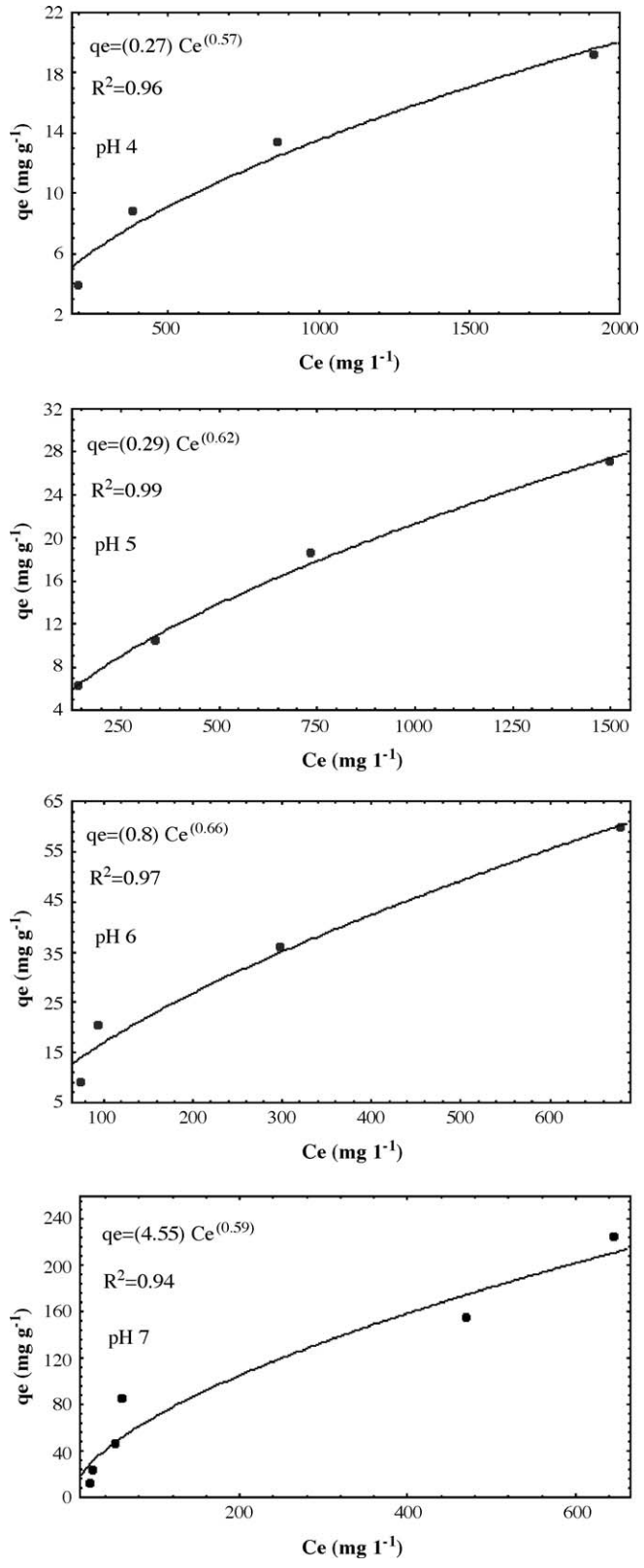

Fig. 3. Estimated Freundlich (—) isotherms fitting to experimental data (●) at the four pH values tested. Concentrations of MG were determined spectrophotometrically as described in Section 2.

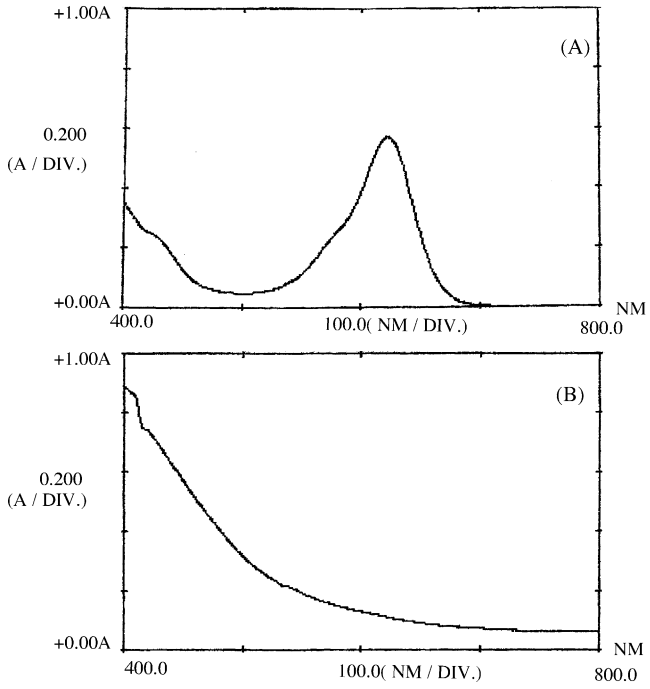

Fig. 4. Absorption spectra of desorption media from *F. sclerodermeus* cultures at pH 5 on WB with MG adsorbed. Time zero (A) and 50-day-old cultures (B).

out MG, at pH 4 and 5. Absorption spectra of desorption medium from *F. sclerodermeus* growing at pH 5 are shown in Fig. 4. The absorbance peak at 618 nm in the absorption spectrum obtained at day 0 showed the presence of MG in the desorption medium (Fig. 4A). This peak disappeared at 50 day postinoculation (Fig. 4B).

Regarding ligninases, in *F. sclerodermeus* cultures at pH 5 laccase and MnP activities increased 3- and 10-fold, respectively, compared to the titres obtained at pH 4. This fungus attained the highest enzyme production and MG decolorization at pH 5 where it showed up to 100% of dye degradation. Further increase of pH values strongly decreased ligninolytic activities. On the other hand, *P. chrysosporium* did not produce laccase activity and the highest MnP titre was obtained at pH 5 along with the highest MG decolorization (45%).

4. Discussion

Bioremediation of aquatic media polluted with dyes or other xenobiotics, presents two problems: removal and degradation. In this work, we deal with both of them. Commonly, these compounds are recalcitrant to the degradation and toxic to major microorganisms, its presence in water further increases the problem of its degradation. Thus, the removal of these compounds from water and wastewater by means of an efficient adsorbent is the first step of the treatment processes. Second one is the degradation using proper microorganisms able to produce the enzymatic machinery involved in the degradation of the xenobiotic compounds adsorbed.

WB is an inexpensive agronomic residue. Its content of polysaccharides such as cellulose and starch is useful as carbon source for fungal growth. This is an advantage compared to

other lignocellulosic materials used as adsorbents like pine sawdust which, due to its higher lignin content, is a poor substrate for fungal growth. In addition, degradation of MG occurred naturally without the addition of exogenous compounds such as laccase mediators, H_2O_2 or ligninase inducers to promote enzyme action.

Effect of pH on adsorption capacities in many different systems has been extensively studied. The pH affects surface binding sites of the adsorbent as well as the availability of the adsorbate compound, MG was completely soluble in the pH range tested in this work. It was described previously that at basic pHs the surface of the adsorbent acquires negatively charged species, while the adsorbate compounds are still positively charged or vice versa [20–22]. Therefore, the significant effect of pH on the adsorption of MG onto WB suggests that an interaction based on the charged groups of the dye and WB surface probably occurred.

The relatively fast capacity of adsorption is similar to values reported for other systems [10]. The adsorption velocity has a significant importance from the standpoint of the applicability of the process to bioremediate water and wastewater. Faster adsorption velocities require smaller reactor volumes, thus decreasing the cost and increasing the efficiency. The time required to attain the equilibrium is a very important parameter to know, if the contact time is not enough, only a partial removal of the solute is achieved. Obviously, it would be expected that adsorption parameters (a and b) as well as adsorption velocities are different from system to system. For example, the time required to attain equilibrium (85–90%) for the system Methylene Blue/Red mud or fly ash was 70–80 h [22], the dye attained faster equilibrium (1 h) when the adsorbent was *Azadirachta indica* leaf powder [9].

pH values not only influenced the adsorption capacity of WB but this factor was important for growth, enzyme production and finally, dye degradation. At pH 5, the highest values of MG degradation were detected in both fungi, the pattern of growth observed, as function of pH cannot be related to the pattern of degradation. By contrast, both fungi showed that the production of ligninases was strongly related to MG decolorization. This finding shows that the process is consistent with the unspecific activities of ligninases towards MG rather than biomass dependent. Moreover, *F. sclerodermeus* degraded over 100% more than *P. chrysosporium* in concordance with its high enzymatic activities. Absorption spectra showed that MG was degraded, although we cannot assert it was completely mineralized.

White rot fungi have long been used for ligninases production but reports dealing with the cultivation of these fungi on contaminated substrates are scanty. Ligninases can be used to develop bioremediation processes which, may imply either whole fungal cultures or enzymatic extracts. A problem facing white rot fungi use is survival and colonization of the polluted substrate, thus it is necessary to test for the sensitivity of the fungus to be used.

In conclusion, (1) due to its low cost and high adsorption capacity (up to 240 mg g^{-1}), WB was found to be a promising material for the removal of MG from wastewater; (2) *F. sclerodermeus* and *P. chrysosporium* were able to grow on WB/MG and to degrade the dye adsorbed, being *F. sclerodermeus* the most efficient; and (3) degradation of dye adsorbed could be strongly attributable to the extracellular ligninolytic enzymes (laccase and MnP) secreted by the fungi.

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