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Mechanism of textile metal dye biotransformation by Trametes versicolor

P. Blánquez[a], N. Casas[a], X. Font[c], X. Gabarrell[a], M. Sarrà[a], G. Caminal[b], T. Vicent[a,*]

[a] Departament d'Enginyeria Química, i Unitat d'Enginyeria Bioquímica, del CeRBA, Escola Técnica Superior d'Enginyeria, Universitat Autònoma de Barcelona, Edifici C.C. Nord, Bellaterra 08193, Spain

[b] Unitat de Biocatàlisis aplicada Asociada al IIQAB (CSIC-UAB), Universitat Autònoma de Barcelona, Bellaterra 08193, Spain

[c] Escola Universitaria Politècnica del Medi Ambient, Mollet del Vallès, Spain

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Abstract

The biodegradation of Grey Lanaset G, which consists of a mixture of metal complexed dye, was studied. Experiments were carried out in a bioreactor with retained pellets of the fungus *Trametes versicolor* that was operated under conditions of laccase production. Although decolorization was highly efficient (90%), no direct relationship to extracellular enzyme was apparent. Moreover, the extracellular enzyme was found to be unable to degrade the dye in vitro. The process involves several steps. Thus, the initial adsorption of the dye and its transfer into cells is followed by breaking of the metal complex bond in the cells release of the components. The metal (Cr and Co) contents of the biomass and treated solutions, and their closer relationship to intracellular enzyme and degradation of the dye, confirm the initial hypothesis.

Keywords: *Trametes versicolor*; Textile dye; Fluidized bioreactor; Intracellular and extracellular laccase; Decolorization steps

1. Introduction

The colour of textile effluents is due to the dyes. Dyes vary in chemical composition, but share a common feature: they are highly stable to external agents such as chemical compounds or light. This makes it difficult to remove colour from the wastewater and low concentrations of dye are visible [1]. Most synthetic industrial dyes possess an azo bond connected to various aromatic structures; some, however, are polymeric structures containing metals.

Conventional methods for the removal of colour from textile effluents are physical or chemical methods (coagulation–flocculation, adsorption,…) [2,3]. The coagulation–flocculation process has a major operational problem: the production of abundant sludge. On the other hand, adsorption is quite expensive as it usually involves the use of powdered activated carbon as adsorbent. The dye is transferred from the liquid to solid phase undegraded and removing it from the adsorbent is difficult, which hinders the reuse of powdered activated carbon. Recently, some authors used biomass as low cost adsorbents [4–6]. In the activated sludge process, decolorization is generally accomplished by adsorption of the dyes on bacteria rather by oxidation though aerobic metabolism.

The ability of white-rot fungi to degrade a wide range of synthetic chemicals including dyes is widely documented [7–17] and is a result of the non specificity of their extracellular ligninolytic enzyme system [7–13], which produces enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. Although early studies focused on the fungus *Phanerochaete*, in recent years fungi such as *Trametes versicolor*,

*Corresponding author. Tel.: +34-93581-2142; fax: +34-93581-2013.

E-mail address: teresa.vicent@uab.es (T. Vicent).

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Bjerkandera, *Clitocybula dusenii* and *Pleurotus eryngii*, have been tested for the decolorization of wastewaters [7–12,14–17].

Most biological decolorization work involves azo or diazo dyes, or a combination thereof [8–10,12,14, 15,18,19]. Azo dyes constitute the largest class of water-soluble synthetic dyes and exhibit the greatest variety of colours; also, most are resistant to conventional aerobic biodegradation processes. Some authors have examined various microorganisms (generally fungi) with a view to identifying most efficient ones for decolorizing dyes [8,12,15,16]. Other have shown the ability of new microorganisms to decolorize various dyes [7,20,21]. In some studies, the type of dye or its chemical composition was altered by changing the substituent on the phenol ring or between two azo bonds, and the mineralization or transformation of the dye was compared depending on the type of substituent used [14]; the chemical structure of both the phenol ring and that distal to the phenolic moiety were found to affect the mineralization kinetics of the dye.

Reported papers on this topic can be classified into two groups. In one, the authors provide no information about enzyme activity during the decolorization process [15,21] or the information is not related to the effect [14,17,19]. Some authors explain the process in terms of bioadsorption mainly [4,5,22,23]. The other group of papers show the occurrence of degradation or biotransformation in the dye and relate the decolorization with most of the ligninolytic enzymes produced [8,12,14,20]. In some cases, a purified enzyme (usually laccase or manganese peroxidase) was used and the rate of decolorization was also found to depend on the structure and substituents of the dye molecule [9,10,18].

However, the biodegradation of dyes containing a metal bond does not seem to have been studied to date. Grey Lanaset G (Ciba) is a mixture of metal complex dyes containing chromium and cobalt. In this paper, we evaluate dye degradation from the results of spectrophotometric colour analysis and metal atomic absorption analysis. A simple mechanism for the decolorization process is proposed and the optimisation of these stages would allow to design the dye biodegradation process.

2. Materials and methods

2.1. Dye

Grey Lanaset G, which is a commercial mixture of several metal complex dyes, was complimentarily supplied by Ciba (ref. 080173.5).

2.2. Strain

Trametes versicolor was obtained from the American Type Culture Collection (ATCC # 42530). The fungus was maintained on 2% malt agar slants at 25°C until use. Subcultures were routinely prepared as required from the mother culture.

2.3. Media and culture conditions

A mycelial suspension of *Trametes versicolor* was obtained by inoculating four 1 cm^2 plugs from the growing zone of fungi on malt agar (2%) to a 500 ml Erlenmeyer flask containing 150 ml of malt extract medium (2%). Flasks were placed in an orbital shaker (135 rpm, $r = 25$ mm) at 25°C. After 4–5 days, a thick mycelial mass was formed, that was ground with a X10/20 (Ystral GmbH) homogenizer. The resulting mycelial suspension was stored in sterilized saline solution (0.85% NaCl) at 4°C. This suspension was used to obtain pellets by inoculating 1 ml of the suspension in 250 ml malt extract medium (2%) (adjusted to pH 4.5) in a 1 l Erlenmeyer flask. The flask was incubated in an orbital shaker (135 rpm, $r = 25$ mm) at 25°C for 5–6 days. The pellets thus obtained can be stored in sterilized saline solution (0.85% NaCl) at 4°C where they will remain active for up to 2 months without loosing their morphology.

2.4. Synthetic dye wastewater

The batch reactor medium contained per litre: 8 g glucose, 1.9 g NH_4Cl, 11 ml of a supplemented medium [24], 100 ml of 2,2-dimethylsuccinate buffer (80 mM) and 0.15 g dye. The pH was adjusted to 4.5 with 0.5 M NaOH and the solution was sterilized at 120°C for 30 min. The culture medium was inoculated with an amount of pellets equivalent to 3.2 g/l dry weight. For continuous operation, the wastewater feed consisted only of dye (0.15 g/l), glucose (2 g/l) and supplemented medium (11 ml/l).

2.5. Equipment and operating conditions

A glass fluidized bioreactor with a useful volume of 1500 ml was furnished with a pH controller in order to maintain pH 4.5. Fluidized conditions in the reactor were maintained by using air pulses [25]. The aeration rate was 0.8 l/min. The temperature was maintained at 25°C. For the continuous process, a hydraulic residence time of 120 h was used and the biomass, in pellet form, was retained in the bioreactor throughout the experiment with no loss in the efluent.

2.6. In vitro dye biodegradation

The reactor broth was centrifuged at 10 000 × g at 4°C for 10 min. The supernatant was passed through filters of 0.45 μm pore size and the pH adjusted to 4.5. The laccase capacity to degrade the dye was evaluated in three different assays. In one, a sample of the filtered solution was used directly. In the second, the filtered solution was concentrated 3.7 times by ultrafiltration (Stainless Steel Minitan II). In the third, the ultrafiltrated solution was diafiltrated against 250 mM sodium malonate buffer (volume ratio $\frac{1}{10}$). Finally, the biodegradation capacity was determined by adding a 150 mg/l concentration of dye to 100 ml of each solution obtained. The dye concentration and enzyme activity were measured after a 24 h period.

2.7. Analytical methods

Colour determination: Spectrophotometric measurements were carried out at the visible maximum absorbance, 590 nm on a PV 8620 Philips spectrophotometer.

Glucose determination: Glucose concentrations were measured with an YSI 2000 enzymatic analyser from Yellow Springs Instruments and Co.

Laccase activity: Enzymatic activity was determined using a modified version of the method of Paszczynski [26] for the determination of manganese peroxidase. The reaction mixture used consisted of 200 μl of 250 mM sodium malonate at pH 4.5, 50 μl of 20 mM 2,6-dimethoxyphenol (DMP) and 600 μl of sample. DMP is oxidized by laccase even in the absence of a cofactor. Changes in the absorbance at 468 nm were monitored for 2 min on a Varian Cary 3 UV/Vis spectrophotometer at 30°C. One activity unit (AU) was defined as the number of micromoles of DMP oxidized per minute. The DMP extinction coefficient is 10,000 $M^{-1}\,cm^{-1}$.

Intracellular laccase activity: The biomass used was filtered, washed with water and resuspended in 250 mM sodium malonate buffer at pH 4.5. Samples of 3 ml were taken and disrupted in a Constant Cell Disruption System (Constant Systems LTD) using one shot at 2.86 atm. Finally, the mixture was centrifuged at 20 000 × g at 4°C for 30 min. The laccase assay was conducted on the clear liquid.

Metals (*chromium and cobalt*): Metal concentrations were measured by flame atomic absorption spectroscopy on a Perkin-Elmer 2100 spectrophotometer, using a nitro-acetylene flame for Cr and an air–acetylene one for Co.

3. Results and discussion

Two types of experiments were carried out. In one, the batch operation mode was used. In the other, after a short period of batch operation and once the glucose concentration was about 2 g/l, the system was switched to continuous operation. The wastewater feed was pumped at a flow rate of 0.30 l day^{-1}. The biomass was retained in the bioreactor while the treated solution was continuously withdrawn from it.

The variables analyzed in both types of experiments were the glucose concentration, dye concentration and laccase activity in the solution. The percent decolorization was calculated as the difference in colour between the inlet and outlet concentration divided by the inlet concentration in the continuous process; or that between the initial and final dye concentrations in the batch process.

Fig. 1 shows the results of a five days batch process, during which the glucose was depleted. Although the maximum laccase activity (1685 AU/l) was reached on day four, the decolorization occurred largely within the first 24 h when the extracellular laccase concentration in the broth was very low. The final colour reduction was 90%. No MnP activity was detected.

Fig. 2 shows the results obtained in a continuous process. After a 5-day batch stage, continuous operation was maintained for 40 days. The maximum enzyme activity was 2028 AU/l. It was reached after 21 days remained at high levels for a further 10 days, after, which extracellular enzyme activity decreased through the end of the run. By contrast, the percent decolorization achieved under continuous operation conditions remained virtually constant at 90%. Therefore, no direct relationship between extracellular enzyme activity and decolourization rate exists. However, low enzyme activity may be required to catalyze the initial colour reduction process.

In both processes, the most substantial colour reduction in the medium occurred within the first 24 h,

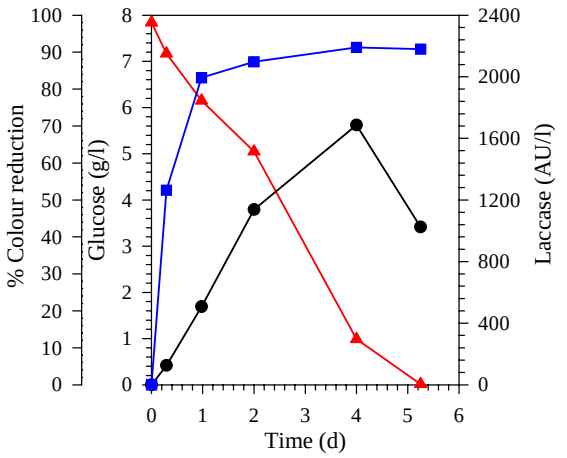

Fig. 1. Time course of glucose concentration (▲), laccase activity (●) and percentage of colour removal (■) during the batch process.

during which the pellets became completely dark. This was clearly the result of the dye being adsorbed on the biomass. The dye adsorption process had previously been studied [17]. The adsorption equilibrium of Grey Lanaset G on the pellets was reached within 24 h and conformed to a Langmuir isotherm. These tests were carried out on dead biomass where the dye changed phase from the solution to become adsorbed to the surface of the fungus. The dye was desorbed by less than 5%, so, its adsorption was virtually irreversible. On the other hand our microorganisms were alive and colour reduction of the biomass was observed after 24 h of treatment with no increase in colour of the liquid phase.

Pellets became quite discoloured during the continuous process, even when the dye was continuously fed to the reactor, pellets did not darken as in the firsts few hours of treatment, rather they remained quite discoloured. This can be ascribed to the reactor being the continuous flow type and fully homogenized, so the dye concentration in the effluent was the same as inside the reactor and hence very low. Fig. 3 shows the significant difference between the colour of the solution and those of the pellets obtained after being in contact for 1 h and for 42 days. Both the solution and the biomass were darker after 1 h than after 42 days of treatment, which reflects that the dye disappeared from both phases during the process.

Grey Lanaset G is a commercial mixture of several metal complex dyes. The chemical formula is unavailable, because it is a patented dye; based on its specifications, however, the dye is known to contain cobalt (0.79%) as an organo-metal complex and

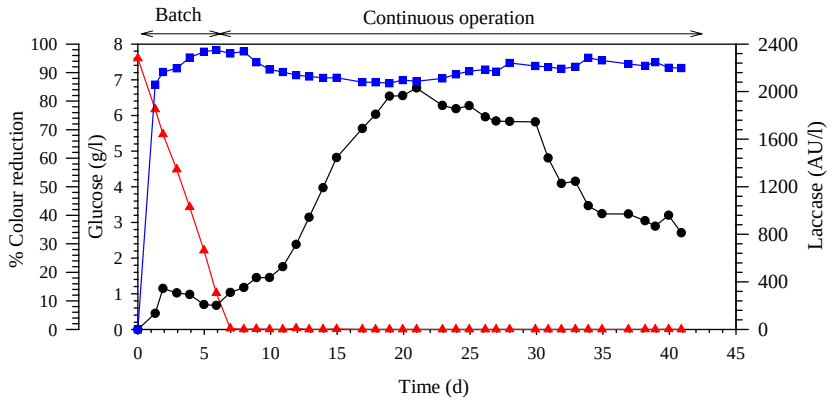

Fig. 2. Glucose concentration (▲), laccase activity (●) and percentage of colour removal (■) during the continuous biodegradation of the dye Grey Lanaset G by *Trametes versicolor*.

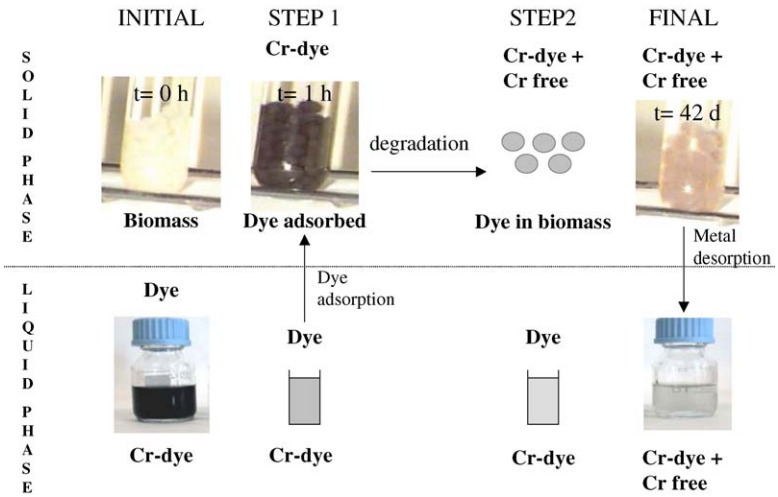

Fig. 3. Scheme of the proposed mechanism for the dye biodegradation and how the chromium is, (Cr-dye means chrome bound to colorant whereas Cr-free means chrome non bound). Pictures show the initial, 1 h and 42 days biomass (on the top); and initial and final wastewater (on the bottom).

chromium (2.5%) as a Cr III organo-metal complex. The contents in both metals were determined to facilitate a better understanding of the decolorization mechanism.

The hypothesis behind the dye degradation mechanism relies on experimental findings. Fig. 3 shows the scheme of the proposed mechanism. The dye is first adsorbed by the microorganism (step 1) and then biodegraded within it (step 2), and being finally the metals are released into the medium, where they are separated on the basis of the chromophoric group or discoloured though their alteration.

The initial and final metal concentrations in the solution and biomass were determined in the batch processes; in the continuous process, analyses were performed in the feed flow efluent, the average in the flow out and the biomass at the end of the process. Similar results were obtained for both Cr and Co (see Table 1). From these results, the percent contents of the different forms of the metals were calculated. Table 2 shows the metals contents in each phase as percentages of the total metal contents in the outlet. At the end of the process, metals can be either bound to the dye molecule in the culture medium (Cr-dye in Fig. 3), free in the medium (Cr-free in Fig. 3) or in the biomass. The last form cannot be determined if the metals in the biomass are free or dye-bound as they change over time. Free Cr was determined as the difference between as total Cr measured and calculated dye-bound Cr in the liquid phase. The two metals exhibited nearly identical proportions in the batch process. In the continuous processes they exhibited a slight difference that might be due to an analytical error or to the different adsorption rates of the metals. Only Cr is considered in the discussion of the results because it presents a higher concentration than Co in the dye, even though the arguments applies to both metals.

A general balance of Cr between the inlet and the outlet values reveals a loss of about 10%. This is acceptable because the metal concentrations were very low. In both the batch and continuous process, between 7.5% and 8% of Cr in the effluent was bound to the dye molecule. In the batch process, 37% of metal at the end of the process was free in the medium, as was 54% in the continuous process. These differences are related to the metal bond to the biomass. In the batch process, 55% of metal was in the biomass, while only 37% was in the continuous process. This difference can be ascribed to the batch process being allowed to develop for only five days, during which the desorption equilibrium of the metals probably could not be reached.

Based on the proposed mechanism, the degradation of the dye may be related to intracellular enzyme activity, the variation of which may be consistent with the degree of decolorization obtained. These hypothesis was tested in a new batch experiment where we examined intracellular enzyme activity. Fig. 4 shows the results obtained in the second batch process. Decolorization amounted to about 98%. The figure shows the intra and extracellular activity profiles, as well as colour development in the liquid medium. Intracellular activity units were determined per gram of biomass, and extracellular ones per millilitre of solution, in the figure, however activities are expressed in total AU to facilitate the discussion.

Table 1
Concentration of chromium, cobalt and dye in each phase for the experiments corresponding to Fig. 1 (batch process) and Fig. 2 (continuous process)

	Batch process			Continuous process		
	Liquid phase (mg/l)		Solid phase (mg/g DCW)	Liquid phase (mg/l)		Solid phase (mg/g DCW)
	Initial	Final	Final	In	Out[a]	Final
Chromium	3.0	1.3	0.432	3.0	1.96	1.376
Cobalt	0.98	0.4	0.134	0.98	0.80	0.303
Dye	150.0	10.7	nd	150.0	12.68	nd

[a] Means average value and nd means not determined. DCW: dry cell weight.

Table 2
Metal content in each phase of the system at the end of the biodegradation processes

		Continuous process		Batch process	
		Cr	Co	Cr	Co
Solid phase (biomass) (%)		37.2	24.3	55.0	55.1
Liquid phase	Dye bounded (%)	8.1	7.5	7.4	7.5
	Free (%)	54.7	68.1	37.7	37.2

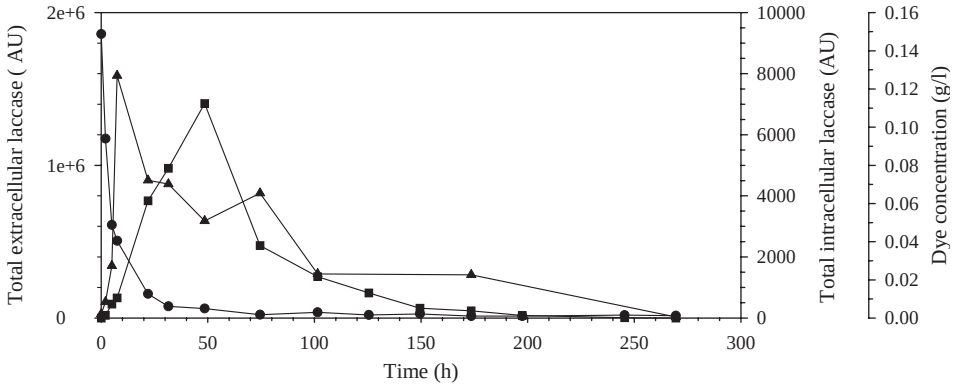

Fig. 4. Evolution over time of the total extracellular laccase (■), dye concentration (●) and total intracellular laccase (▲) for a batch process.

A large amount of biomass was required in each sample to be able to determine intracellular enzyme, so the Cr balance could not be established in this test. However, a percent decolorization of 90.6% after 22 h of treatment was determined, and 23% of the Cr was found not to be bound and hence at least a similar proportion of dye was degraded by the fungus. As it can be seen in Fig. 4, intracellular laccase activity peaked at 7936 AU at 32 h. However, the highest extracellular activity was detected at 48 h, time by which no dye remained in the medium.

In order to ascertain whether the extracellular enzyme laccase was responsible for the decolorization, a new experiment was carried out. After 48 h of batch conditions (when the extracellular enzyme activity was 692 AU/l), the in vitro enzyme capacity to degrade the dye was tested as described under Materials and methods. This experiment showed that, although the enzyme retained its activity after 24 h no decolorization was observed in any of the three fractions [culture broth (692 AU/l), broth by ultrafiltration concentrated (2560 AU/l) and dialysed concentrated broth (2092 AU/l)]. Although laccase (intra or extracellular) was invariably detected whenever some decolorization was observed, the results of this experiment clearly show that the presence of laccase, without the microorganism, does not suffice to degrade Gris Lanaset G, which contradicts results obtained by several authors [9,18].

4. Conclusions

The mixture of metals containing dyes Grey Lanaset G was successfully biodegraded in a bioreactor filled with pellets of the fungus *Trametes versicolor*. The reactor was operated in the batch and continuous modes. In the latter case, the process proceed for more than 40 days with an acceptable decolorization efficiency and no operational problems. Measurements of the extracellular enzyme activity ruled out a direct relationship with dye degradation. In addition, no decolorization was detected in vitro, when the biomass was withdrawn from the broth. Even when concentrated and dialyzed, the enzyme was unable to degrade the dye. Moreover, visual observations of the liquid and solid phases revealed that the colour had almost disappeared from both phases by the end of the process. Therefore, the microorganism is capable of degrading the dye. On the other hand, the presence of a high proportion of chromium and cobalt released from the dye is consistent with the breakage of the metal complex. All these results reveals that the degradation occurs in several steps including the initial adsorption of the dye onto the biomass, followed by its transfer into cells. Degradation occurs within cells and the resulting products are finally released. In the absence of the microorganism, not even a high concentration of extracellular enzyme is capable of degrading Grey Lanaset G.

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