Decolourisation of synthetic textile dyes by *Phlebia tremellosa*

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Abstract

*Phlebia tremellosa* decolourised eight synthetic textile dyes (200 mg l⁻¹) by greater than 96% within 14 days under stationary incubation conditions. High performance liquid chromatography analysis of culture supernatants indicated that Remazol Black B was degraded by the fungus, however, complete mineralisation did not occur as a colourless organic breakdown product accumulated. Laccase activity was detectable in culture supernatants after 5 days when the fungus was grown in the presence of an artificial textile effluent, with activity reaching a maximum of 15 U l⁻¹ on day 14. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Wastewater from the textile industry is a complex mixture of many polluting substances ranging from organochlorine-based pesticides to heavy metals associated with dyes or the dying process [1]. During textile processing inefficiencies in the dyeing process result in 10–15% of all dyestuff being lost directly to wastewater which ultimately finds its way into the environment [2]. Once in the environment, certain dyes, containing azo linkages, have the potential to form carcinogenic breakdown products [3]; whilst concerns have been raised over the toxicity of certain metal complex dyes [4]. Due to increasingly stringent environmental legislation the textile industry in the UK and elsewhere is seeking to develop effective wastewater remediation technologies. Despite the existence of a variety of chemical and physical treatment processes, bioremediation of textile industry effluent is still seen as an attractive solution due to its reputation as a low cost, environmentally friendly and publicly-acceptable treatment technology [4].

A number of biological processes, such as sequenced anaerobic/aerobic digestion, have been proposed as having potential in the treatment of textile wastewater [4]. The use of white-rot fungi has attracted increasing attention as these organisms have the ability to metabolise a diverse range of polluting compounds [5]. *Phanerochaete chrysosporium*, the most extensively studied white-rot fungus, has been shown to metabolise compounds such as PCB’s and organophosphorus insecticides but is also capable of decolourising and metabolising synthetic textile dyes with the organism’s ligninolytic enzymes being implicated in the degradation process [4]. To date, studies have largely concentrated on the microbial decolourisation of azo dyes which are currently of wide use in the textile industry, although the biodegradation of other commercially important classes of textile dye, such as the phthalocyanines, have recently been addressed [6].

The white-rot fungus *Phlebia tremellosa* has received little research attention although its laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) have been purified [7] and its ability to metabolise lignin and decolourise Kraft bleach plant effluent are described in the literature [8,9,10]. In this paper we investigate the ability of a strain of *Ph. tremellosa* to decolourise a range of synthetic textile dyes and investigate the role of ligninolytic enzymes in the decolourisation process.

2. Materials and methods

2.1. Chemicals

All chemicals were of reagent grade unless otherwise
stated and obtained from Sigma (Poole, UK) with the exception of the synthetic textile dyes (Table 1) which were supplied courtesy of Fruit of the Loom International (Buncrana, Republic of Ireland). All textile dyes used were azo-based synthetic dyes, except Remazol Turquoise Blue G133, which is a copper–phthalocyanine dye and whose structure we have previously described [6]. The structure of the azo dyes is not in the public domain with the exception of Remazol Black B which is a tetrasulfonated diazo dye whose structure is described by Oxspring et al. [11].

2.2. Microorganism and culture conditions

Ph. tremellosa was provided by Dr. R.T. Moore (University of Ulster, Coleraine, UK) and maintained on potato dextrose agar at 4°C. The organism was routinely grown on a modified basal culture medium described by Reid [9] containing glucose at 5.0 g l⁻¹. The organism was routinely incubated at 28°C under stationary conditions unless stated otherwise. Dye decolourisation was measured photo- metrically at the visible wavelength maximum of each dye using a Shimadzu UV-2101 PC scanning spectrophotometer.

2.3. Enzyme assays

The method of Paszczynski et al. [12] was used to measure manganese peroxidase activity at 30°C over 5 min. The method of Tien and Kirk [13] was used to measure lignin peroxidase activity at 26°C over 200 s. Laccase was measured by the method of Szklarz et al. [14] at 30°C over 4 min. All assays were carried out in triplicate and with suitable controls.

2.4. High performance liquid chromatography (HPLC) analysis

HPLC separation was performed as previously described by Oxspring et al. [11] using a Phenomenex (4.6 mm i.d. × 250 mm) (Macclesfield, UK) reverse phase Luna C18 column, a ThermoSeparation Products (San Jose, CA, USA) spectraSYSTEM P4000 pump, a spectraSYSTEM AS3000 autosampler, a spectraSYSTEM UV2000 detector and a Rhodyne model A4169-030 loop injector with a 100-μl loop. The column was equilibrated with acetonitrile:water (60:40) containing 4.5 mol dm⁻³ CTAB at a flow rate of 0.6 ml min⁻¹. Culture supernatants were filtered through 0.45-μm filters prior to analysis and were monitored at 254 nm.

3. Results and discussion

The ability of white-rot fungi to decolourise synthetic textile dyes has been extensively studied, particularly with P. chrysosporium and Trametes versicolor [4]. The aim of the current study was to investigate the ability of a little studied white-rot fungus, Ph. tremellosa, to decolourise industrially-important synthetic dyes and if possible to identify the role, if any, of ligninolytic enzymes in the decolourisation process. Our motivation in studying a wider range of white-rot fungi stems from the relative unreliability of enzyme production under many experimental situations.

Ph. tremellosa was capable of decolourising eight commercially used textile dyes and an artificial textile effluent, consisting of a mixture of each of these dyes, by greater than 96% within 14 days under stationary incubation conditions (Table 1). The time taken for Ph. tremellosa to decolourise these synthetic dyes compares favourably with reports for other white-rot fungi which indicate periods of between 7 and 20 days to achieve greater than 90% decolourisation of diverse synthetic dyes [6, 15, 16]. However when cultures where agitated (250 rpm) the ability of Ph. tremellosa to decolourise Cibacron Red and Remazol Golden Yellow significantly decreased with 27 and 43% colour remaining, respectively, after 14 days, although the data were subject to a high level of standard deviation under these experimental conditions (Table 1). A number of factors are likely to have contributed to this observation of reduced dye decolourisation. For example in P. chrysosporium agitation has been reported to suppress the expression of the ligninolytic system [17], something perhaps not unexpected considering the normal growth environment of these fungi; whilst Young and Yu [18] proposed, not surprisingly, that the chemical structure of the dye molecules themselves could be responsible for their incomplete decolourisation under certain conditions.

Flushing cultures of Ph. tremellosa with oxygen also had a major effect on the organism’s decolourising ability.

<table>
<thead>
<tr>
<th>Textile dye (λmax)</th>
<th>Colour remaining (%) ± S.D.</th>
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<tbody>
<tr>
<td></td>
<td>agitation</td>
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<tr>
<td>Cibacron Red (515 nm)</td>
<td>27.4 ± 17.4</td>
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<tr>
<td>Remazol Navy Blue (620 nm)</td>
<td>6.7 ± 6.7</td>
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<tr>
<td>Remazol Red (520 nm)</td>
<td>0</td>
</tr>
<tr>
<td>Cibacron Orange (487 nm)</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>Remazol Golden Yellow (410 nm)</td>
<td>42.6 ± 29.9</td>
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<tr>
<td>Remazol Blue (590 nm)</td>
<td>3.8 ± 1.5</td>
</tr>
<tr>
<td>Remazol Turquoise Blue (675 nm)</td>
<td>6.4 ± 6.1</td>
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<tr>
<td>Remazol Black B (600 nm)</td>
<td>0.8 ± 0.5</td>
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<tr>
<td>Mixture (602 nm)</td>
<td>3.8 ± 1.5</td>
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Cultures were incubated at 28°C and 250 rpm where indicated and readings taken after 14 days incubation. All experiments were set up in triplicate and values represent the percentage colour remaining ± S.D.
After 14 days the fungus was capable of decolourising an artificial textile effluent by 98% when flushed daily with oxygen compared to only 25% colour removal when sterile air was used. There are numerous reports in the literature describing the beneficial effect that oxygen has on the ligninolytic activity of *P. chrysosporium* [19] whilst Reid and Seifert [20] described enhanced lignin degradation by *Ph. tremellosa* under an oxygen atmosphere rather than air.

HPLC analysis was used to monitor decolourisation of Remazol Black B by *Ph. tremellosa* under optimal culture conditions. This dye was chosen because of its extensive decolourisation by our isolate and for its well characterised structure. Metabolism of the parent dye molecule (retention time of 4.57 min, peak area $4.6 \times 10^5$ U s$^{-1}$) did not commence until day 4 with 97% reduction in peak area by day 6 and complete removal from culture supernatants by day 10. One other major metabolite was detectable by HPLC analysis, this metabolite (retention time 3.8 min) was first detectable on day 6 rising to a maximum peak area of $1.4 \times 10^6$ U s$^{-1}$ by the end of the study. The metabolite had no visible optical density maximum and at present HPLC-mass spectroscopy techniques are being used to try and identify its structure as a first step towards elucidating the degradation pathway of Remazol Black B by this fungus. Our data suggest that *Ph. tremellosa* is capable of the metabolism and decolourisation of the Remazol Black B parent dye but that complete mineralisation does not occur with a major colourless metabolite accumulating in culture supernatants.

An investigation of the ligninolytic enzymes involved in dye decolourisation by cultures of *Ph. tremellosa* failed to detect any lignin or manganese peroxidase activity. Our findings also concur with those of a number of groups who failed to detect lignin peroxidase activity in culture supernatants of *Ph. tremellosa* [9,10]. Manganese peroxidase activity (10 U l$^{-1}$) could however be detected if the culture medium was modified by a supplementation of MnCl$_2$ (12 mg l$^{-1}$). As seen in Fig. 1, laccase activity first occurred in culture supernatants on day 5, coinciding with the start of dye decolourisation, with a maximum of 15 U l$^{-1}$ detectable on day 14. Similar patterns of biomass production and laccase activity were, however, observed in control flasks in which the textile dyes were omitted. Laccase has been reported to be responsible for the decolourisation of Remazol Brilliant Blue R by *Pycnoporus cinnabarinus* [21] whilst Lankinen et al. [22] demonstrated that laccase was important in the decolourisation of Kraft bleaching effluent by *Ph. tremellosa*. Indeed, recently, Leontievsky et al. [7] purified two distinct types of laccase from a strain of *Ph. tremellosa*.

In an attempt to investigate the role of laccase in dye decolourisation by *Ph. tremellosa*, sodium azide (2.5 mM final concentration) was added to culture supernatants on day 5 of incubation, the time at which laccase activity was first detectable. Previously it had been reported that sodium azide inhibited laccase activity in *Coriolus versicolor* [22]. Colour removal in these flasks was only 50% compared to 99% in control flasks with no sodium azide addition and no laccase activity was detectable in culture supernatants over a 14 day period. Biomass production in the presence of sodium azide was identical to control flasks. The findings demonstrate that laccase is involved in the decolourisation of textile dyes by *Ph. tremellosa*, however, another process must account for the remaining colour removal observed in the absence of detectable levels of this enzyme.

In conclusion, it is clear that the white-rot fungus *Ph. tremellosa* is capable of decolourising a range of industrially-important textile dyes. Only laccase of the three enzymes

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**Fig. 1.** Decolourisation of artificial textile effluent by *Ph. tremellosa*. Symbols: ▲, percentage colour remaining was measured at 602 nm with a $T_0$ optical density of 2.1; ●, cell dry weight; ●, laccase activity. Experiments were carried out in triplicate with error bars representing ± S.D.
major ligninolytic enzymes was detected in culture supernatants, in both the presence and absence of textile dye, however activity did coincide with the dye decolourisation period. Future work with this isolate should concentrate upon finding the breadth of pollutants that it can metabolise and also the development of suitable bioreactor configurations is required if the isolate is to be exploited for bioremediation purposes.

References