

Full Length Research Paper

Comparison of synthetic dye decolorization by whole cells and a laccase enriched extract from *Trametes versicolor* DSM11269

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***Trametes versicolor* strain DSM 11269 was found to decolorize six out of seven different synthetic dyes when grown on dye-containing agar plates. Using a laccase enzyme extract, enriched from the fungal liquid culture supernatant, the anthraquinone derivative dyes (Alizarin Red S and Remazol Brilliant Blue R) were decolorized in three hours at 50°C by 55 and 70%, respectively. The four azo compounds (Amaranth, Cibacron Brilliant Red 3B-A, Direct Blue 71 and Reactive Black 5), and the indigo molecule (Indigo Carmine), showed a higher resistance to decolorization (<10% in 6 h), although of them (Amaranth, Reactive Black 5 and Indigo Carmine) were efficiently decolorized by *T. versicolor* in agar plate assays. This suggests that different oxidizing activities from laccase alone may be involved in the decolorization process.**

Key words: Synthetic dyes, *Trametes versicolor*, decolorization, white-rot fungus.

INTRODUCTION

Synthetic dyes are extensively used in the textile, dyeing, printing and leather industries, and their contamination in the water effluents from these industries often represents a major source of water pollution, affecting the water quality, transparency and oxygen content. The removal of such dyes from wastewater is of prime concern for environment protection but it remains a difficult task due to the structural complexity, toxicity and high stability

of these molecules. Physical and chemical methods, such as adsorption, coagulation-flocculation and filtration, are commonly used for the treatment of these effluents. However, these methods are expensive, not always efficient and sometimes produce hazardous by products.

As an alternative approach, microbial treatment by bacteria, or white rot fungi, is gaining in interest (Banat et al., 1996). In particular, the ability to biodegrade various types of dyes by whole cells of white rot fungi has proven to be effective, with their elimination being mediated through oxidation-reduction reactions catalyzed by the lignin degrading enzymes they produce, such as lignin peroxidase, manganese peroxidase (MnP) and laccase

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(Toh et al., 2003). Treatments with whole cells, either alive or dead, also show some drawbacks and in particular in the scale-up process and fungal biomass treatment (Zeng et al., 2011). Enzyme-based processes using crude or purified extracellular enzyme preparations constitute another alternative that has been less explored, although it may constitute an interesting route for dye bioremediation. In particular, decolorization by crude enzyme filtrates has many advantages. The production process is not expensive and allows a separate dye decolorization step from fungal growth and enzyme production, thus eliminating the problem of any fungal growth inhibition by the dye molecules (Papinutti et al., 2008; Zeng et al., 2011). In particular, laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) that catalyze the four-electron reduction of O₂ to water coupled with the oxidation of phenolic compounds have been shown to be efficient in degrading various dye molecules (Lu et al., 2007; Yang et al., 2009).

In this present study, we investigated the decolorization of seven dyes on solid plates using *Trametes versicolor* strain DSM11269. The dye molecules that were tested encompass four azo derivatives (Amaranth, Direct Blue 71, Reactive Black 5 and Cibacron Brilliant Red 3B-A), two anthraquinone derivatives (Alizarin Red S and Remazol Brilliant Blue R) and one indigo dye (Indigo Carmine). The decolorization results were then compared to those obtained when using an enzyme extract from the culture media that was enriched in laccase activity.

MATERIALS AND METHODS

Microorganism and chemicals

The fungal strain *T. versicolor* DSM11269 was purchased from the German collection of microorganisms and cell cultures (DSMZ), Germany. Guaiacol, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) and Amaranth were obtained from Fluka. Direct Blue 71, Reactive Black 5, Cibacron Brilliant Red 3B-A, Alizarin Red S, Remazol Brilliant Blue R (RBBR) and Indigo Carmine were obtained from Sigma-Aldrich.

Growth conditions and enzyme production

Five pieces (1 cm²) of *T. versicolor* DSM 11269, collected after seven days of growth on malt extract peptone agar plates (MEPA: 30 g malt extract l⁻¹, 3 g peptone l⁻¹ and 15 g agar l⁻¹) were used to inoculate 75 ml of basal fermentation medium (XH medium) (Xiao et al., 2006) in 250 ml Erlenmeyer flasks. The fungus was then grown at 28°C with continuous agitation at 100 rpm for 144 h before the addition of 2 mM guaiacol to induce laccase production and 0.5 mM CuSO₄. After five days, the culture broth was filtered and centrifuged at 10 000 rpm for 15 min. The supernatant was frozen and then defrosted to precipitate the remaining polysaccharides, which were removed by centrifugation. The resulting clear supernatant was used directly without additional purification for enzyme activity assays and dye decolorization experiments.

Enzyme activity assays

Laccase activity was determined by measuring the oxidation of 2, 2'-azino-bis (3-ethylthiazoline-6-sulfonate) (ABTS). The assay mixture contained 1.5 ml sodium acetate buffer (1 mM, pH 5.0), 1.5 ml ABTS (0.5 mM) and 1.5 ml culture medium. Oxidation of ABTS was monitored by measuring the absorbance at 420 nm ($\epsilon_{420} = 3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$), as described by Li et al. (2008). One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of ABTS per minute at 30°C.

Dye decolorization on solid plates

Dye agar plates were prepared using MEPA containing 0.2 mM of each individual dye. Plates were inoculated with a 1 cm² piece of *T. versicolor* fungus isolated from a seven day MEPA plate growth, cut out from the actively growing fungal culture. Dye agar plates that were not inoculated served as controls. All plates were incubated at 30°C. After seven days, the size of the decolorization halo was measured.

Dye decolorization by the crude enzyme preparation

Dye solutions were prepared by the method of Nyanhongo et al. (2002). The reaction mixture for decolorization experiments contained 0.5 ml of 250 mg dye l⁻¹, 0.5 ml of enzyme preparation at 1 U ml⁻¹ and 3 ml of 1 mM sodium acetate buffer (pH 5). The reaction mixture was incubated at 40, 50 or 60°C, to determine the effect of temperature on decolorization, with shaking at 90 rpm. The absorbance of the mixture at the appropriate maximum wavelength (Table 1) was recorded with a Zenyth 200-Anthos Microplate-Spectrophotometer. Decolorization was calculated by measuring the decrease in the absorbance according to the following expression:

$$\text{Decolorization (\%)} = (A_0 - A)/A_0 \times 100\%$$

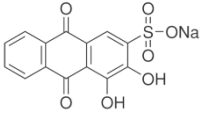

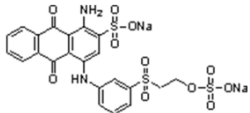
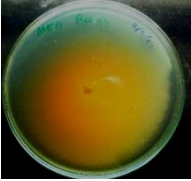
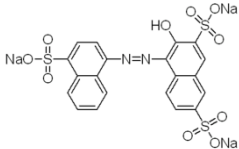

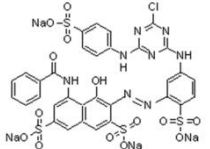
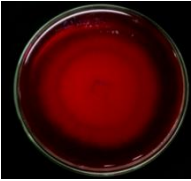
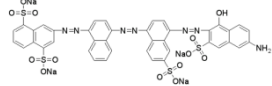
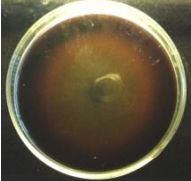
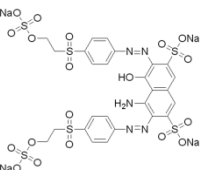

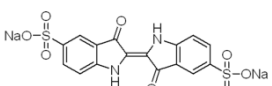

where, A₀ was the initial absorbance and A was the final absorbance. Enzymatic activity and decolorization assays were performed in triplicate.

RESULTS

Decolorization of dyes on solid plates

The ability of *T. versicolor* to decolorize seven selected dyes was first investigated using solid plate assays (Table 1). After seven days, Amaranth and Reactive Black 5 were completely decolorized, as revealed by the > 80 mm diameter of the decolorized zone. Decolorization of Remazol Brilliant Blue R (RBBR) was less extensive with a decolorized zone diameter of between 60 to 79 mm. The other four selected dyes (Alizarin Red S, Indigo Carmine, Direct Blue 71 and Cibacron Brilliant Red 3B-A) have never been tested before with *T. versicolor* on solid medium. Alizarin Red S and Indigo Carmine were completely decolorized whereas partial decolorization was obtained for Direct Blue 71. Finally, no decolorization was observed for Cibacron Brilliant red 3B-A.

Table 1. Characteristics, molecular structure and decolorization studies of the selected dyes during solid plate growth of *T. versicolor* strain DSM11269.

Dye (classification)	λ_{max} (nm)	Structure	Diameter of decolorized zone on solid media after seven days (mm)	Photograph
Alizarin Red S (anthraquinone)	423		>80	
Remazol Brilliant Blue R (RBBR) (anthraquinone)	595		60 - 79	
Amaranth (azo)	521		>80	
Cibacron Brilliant Red 3B-A (azo)	517		0 - 10	
Direct Blue 71 (azo)	594		60 - 79	
Reactive Black 5 (azo)	597		>80	
Indigo Carmine (indigo)	602		>80	

Laccase activity and dye decolorization by the crude enzyme preparation

The treatment of dyes by white rot fungi has been extensively studied, but the evaluation of the use of their

enzymes is more limited. The enzyme extract enriched in laccase activity was first prepared and tested for its ability to decolorize the seven selected dyes. Following induction with Guaiacol, the level of laccase production reached 3.23 U ml^{-1} of culture medium. The clear

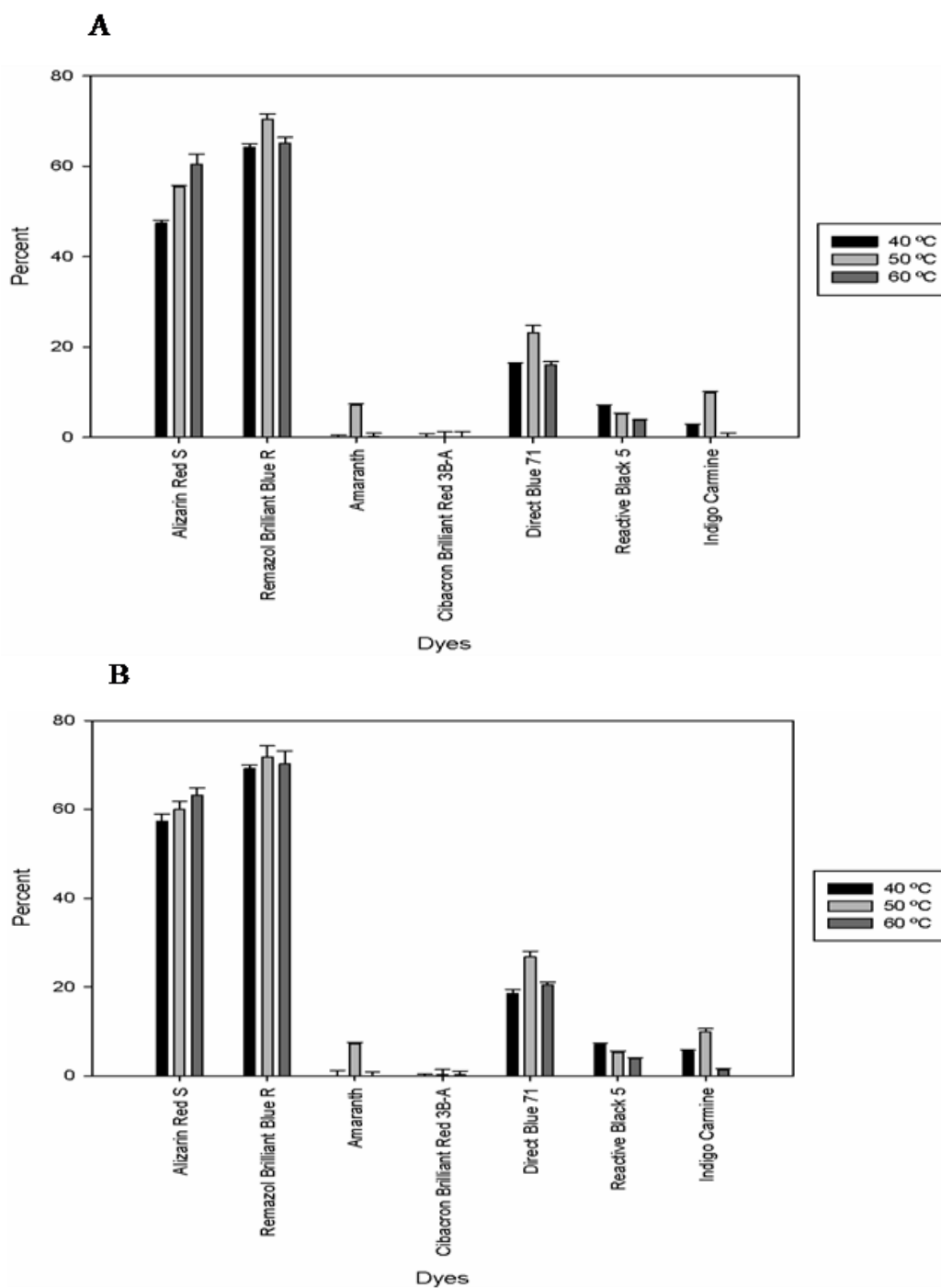


Figure 1. Effect of temperature on the decolorization of each dye by the crude laccase enzyme preparation from *Trametes versicolor* DSM 11269 after; A) three and B) six hours. Results are expressed as the percentage removal of dye (Percent) and are shown as the mean \pm 1 S.D. and are derived from triplicate experiments.

supernatant was diluted to 1 U ml^{-1} and incubated with the dyes at 40, 50 or 60°C. The decrease in the absorbance of the respective maximum absorption wavelength (λ_{max}) (Table 1) was determined after three and six hours of

incubation (Figure 1). The anthraquinone derivatives, Alizarin Red S and RBBR, were relatively strongly decolorized, reaching 60 and 70% decolorization, respectively, after three hours at 60 or 50°C (Figure 1).

Table 2. Percentage of dye decolorization obtained by the crude enzyme preparation from *T. versicolor* DSM 11269 after three and six hours at 50°C.

Time (h)	Dye						
	Alizarin Red S (anthraquinone)	Remazol Brilliant Blue R (RBBR)(anthraquinone)	Amaranth (azo)	Cibacron Brilliant Red 3B-A (azo)	Direct Blue 71 (azo)	Reactive Black 5 (azo)	Indigo Carmine (indigo)
3	55.4 ± 0.3	70.4 ± 1.2	7.1 ± 0.3	0.1 ± 1.1	23.2 ± 1.6	5.2 ± 0.3	9.9 ± 0.2
6	60.0 ± 1.9	71.8 ± 2.5	7.2 ± 0.3	0.3 ± 1.2	26.8 ± 1.3	5.2 ± 0.3	9.9 ± 0.7

Data are shown as the mean ± 1 S.D. and are derived from 3 independent repeats.

The azo and indigo dyes were more resistant to decolorization. The best azo dye decolorization was Direct Blue 71, where 23% decolorization was observed after three hours at 50 °C (Figure 1 and Table 2). By contrast, another two azo dyes (Amaranth and Reactive Black 5), and the indigo dye (Indigo Carmine), showed a very low level of decolorization at less than 10% during the same 3 to 6 h incubation time (Table 2).

DISCUSSION

T. versicolor showed different potentials to decolorize seven different dyes. The ability to decolorize Amaranth, Reactive Black 5 and Remazol Brilliant Blue R on solid plates are in agreement with the observations reported by Swamy and Ramsay (1999) and Junghanns et al. (2008) for *T. versicolor* strains ATCC 20869 and DSM 11269, respectively.

The strongly decolorized anthraquinone derivatives, Alizarin Red S and RBBR, by crude enzyme preparation were correlated with those obtained on solid medium. RBBR decolorization is efficient and comparable to that obtained with laccase enriched preparations from *Trametes hirsuta* and *Cerreña unicolor* (Moilanen et al., 2010) and this is the first report of Alizarin Red S

decolorization by laccase enriched enzyme extracts.

Regarding the indigo and three azo dyes (Direct Blue 71, Amaranth and Reactive Black 5), there is no correlation between the results obtained on solid medium and those obtained with the enzyme extract that is enriched in laccase. The decolorization observed on solid medium is, therefore, likely to be due to other oxidizing activities, that could be better excreted when growing the fungi on agar plates. Moreover, we suggest that this laccase may not be highly active on these substrates.

The azo dye Cibacron Brilliant Red 3B-A is highly recalcitrant to degradation. Indeed, no decolorization was observed either on solid medium or in liquid assay. This could be attributed to the complex structure of this dye. Indeed, similar results were reported by Wong and Yu (1999) with the laccase from *T. versicolor* ATCC 48424, where the azo (Acid Violet 7) and indigo (Indigo Carmine) dyes were not decolorized, whereas, in contrast, to the anthraquinone dye (Acid Green 27) was more easily decolorized. Additionally, Champagne and Ramsay (2005) reported that the purified laccase from *T. versicolor* ATCC 20869 did not efficiently degrade azo compounds compared to the MnP produced by the same strain. Our results are in agreement

with these observations. Regarding indigo, and by analogy with the results obtained with azo compounds, we can suggest that laccase is not responsible for the high level of degradation that is observed on the solid medium.

For all seven dyes tested, the decolorization rates were optimal at 50°C, except for Alizarin Red S and Reactive Black 5 that were slightly faster at 60 and 40°C, respectively. Previously, Nyanhongo et al. (2002) reported an optimal temperature of 50°C for the decolorization of Indigo Carmine using an enzyme extract from *Trametes modesta* that was enriched in laccase activity.

In addition, the decolorization was enhanced by the addition of redox mediators (Nyanhongo et al., 2002; Moilanen et al., 2010). In conclusion, we have shown here that the enzymatic extract enriched in laccase produced by *T. versicolor* strain DSM11269 can efficiently decolorize Alizarin Red S, RBBR and Direct Blue 71 without any addition of redox mediators, demonstrating the potential interest in such crude enzyme extracts for the removal of dyes issued from industrial effluents. Among the seven dyes tested, azo and indigo derivatives are resistant to the action of the enzymatic extract, indicating that the addition of redox mediators and/or engineering of laccase should be investigated to improve the decolorization process.

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