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ORIGINAL PAPER

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Optimization of a culture medium for ligninolytic enzyme production and synthetic dye decolorization using response surface methodology

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Abstract A Box-Wilson central composite design was applied to optimize copper, veratryl alcohol and L-asparagine concentrations for *Trametes trogii* (BAFC 212) ligninolytic enzyme production in submerged fermentation. Decolorization of different dyes (xylidine, malachite green, and anthraquinone blue) by the ligninolytic fluids from the cultures was compared. The addition of copper stimulated laccase and glyoxal oxidase production, but this response was influenced by the medium N-concentration, with improvement higher at low N-levels. The medium that supported the highest ligninolytic production (22.75 U/ml laccase, 0.34 U/ml manganese peroxidase, and 0.20 U/ml glyoxal oxidase) also showed the greatest ability to decolorize the dyes. Only glyoxal oxidase activity limited biodecoloration efficiency, suggesting the involvement of peroxidases in the process. The addition of 1-hydroxybenzotriazole (a known laccase mediator) to the ligninolytic fluids increased both their range and rate of decolorization. The cell-free supernatant did not decolorize xylidine, poly R-478, azure B, and malachite green as efficiently as the whole broth, but results were similar in the case of indigo carmine and remazol brilliant blue R. This indicates that the mycelial biomass may supply other intracellular or mycelial-bound enzymes, or factors necessary for the catalytic cycle of the enzymes. It also implies that this fungus implements different strategies to degrade dyes with diverse chemical structures.

Keywords Response surface methodology · Ligninolytic enzymes · White-rot fungi · Dye decolorization · *Trametes trogii*

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Introduction

Approximately 10,000 different dyes and pigments, mainly for use in the dye and printing industries, are produced annually worldwide. Synthetic dyes are chemically diverse, with those intended for industrial use divided into azo, triphenylmethane, and heterocyclic/polymeric structures. Product processing methods often lead to the loss of as much as 10–15% of dyes to wastewaters. Several of these dyes are very stable to light, temperature, and microbial attack, making them recalcitrant compounds; many of them also toxic [33].

White-rot fungi, a group of lignin-degrading basidiomycetes, have received considerable attention for their bioremediation potential. Their ability to degrade lignin is due to an extracellular nonspecific and nonstereoselective enzyme system consisting of laccases (EC 1.10.3.2), lignin peroxidases (LiP, EC 1.11.1.14), and manganese peroxidases (MnP, EC 1.11.1.13), which function together with H_2O_2-producing oxidases and secondary metabolites. White-rot fungi secrete one or more of the three enzymes essential for lignin degradation. The same unique nonspecific mechanisms that confer on these fungi the ability to degrade lignin also allow them to degrade a wide range of pollutants, e.g. polycyclic aromatic hydrocarbons, chlorinated phenols, polychlorinated biphenyls, dioxins, pesticides, explosives, and dyes. Purified laccases, LiPs, and MnPs are able to decolorize dyes of different chemical structure [29]. In addition, intracellular systems that are generally present in most fungi, such as cytochrome P-450 monooxygenase, may also be involved in organopollutant degradation [8].

Previous studies have focused on the lignin-degrading enzymes of *Phanerochaete chrysosporium* and *Trametes versicolor*. Recently, however, there has been growing interest in studying the lignin-modifying enzymes of a wider array of white-rot fungi, not only from the standpoint of comparative biology but also with the expectation of finding better lignin-degrading systems

for use in various biotechnological applications. The white-rot fungus *Trametes trogii* has received little research attention although it is an outstanding laccase producer [23], and its ability to degrade high-priority pollutants, such as polychlorinated biphenyls, polyaromatic dyes, and polycyclic aromatic compounds, has been demonstrated recently [24,40].

Enzyme overproduction can be achieved by media engineering. Traditional methods of optimization have involved changing one independent variable while fixing the others at a certain level. This single-dimensional search is laborious, time-consuming, and incapable of reaching a true optimum due to interactions among variables. Response surface methodology (RSM), first described by Box and Wilson [7], is an experimental strategy for seeking the optimum conditions for a multivariable system. Although this methodology has been widely applied in food science, e.g. in sensory analysis, and in pharmacology to assess drug interaction, it has not been well exploited to optimize fermentative enzyme production by microorganisms [38]. To the best of our knowledge there are only two reports regarding ligninolytic enzyme optimization using RSM, both of which concerned laccase production [28,38].

In this study we investigated the ability of a strain of *T. trogii* to decolorize a range of synthetic textile dyes, and the role of ligninolytic enzymes in the decolorization process. Response surface optimization techniques were applied to maximize enzyme production and decolorization efficiency.

Materials and methods

Microorganism

Strain 212 (BAFC: Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires) of *Trametes trogii (Funalia trogii)* (Berk. in Trog.) Bond. and Singer (Polyporaceae, Aphyllophorales, Basidiomycetes) was used in these experiments. Stock cultures were maintained on malt extract agar slants at 4 C.

Culture media

MEA medium (malt extract 12.5 g/l); GA medium (glucose, 10 g; L-asparagine monohydrate, 3 g; $MgSO_4{\cdot}7H_2O$,0.5 g; H_2KPO_4, 0.5 g; HK_2PO_4, 0.6 g; $CuSO_4{\cdot}5H_2O$, 0.4mg (1.6 μM); $MnCl_2{\cdot}4H_2O$, 0.09 mg; H_3BO_3, 0.07 mg; $Na_2MoO_4{\cdot}2H_2O$, 0.02 mg; $FeCl_3$, 1 mg; $ZnCl_2$, 3.5 mg; thiamin hydrochloride, 0.1 mg; biotin, 5 μg; distilled water up to 1 l). For the optimization studies, different asparagine, $CuSO_4{\cdot}5H_2O$ and veratryl (3,4-dimethoxybenzyl) alcohol (VA) concentrations were evaluated. Initial pH of the GA medium was adjusted to 6.0 with 1 N NaOH after autoclaving.

Culture conditions

Erlenmeyer flasks (250 ml) containing 25 ml medium were inoculated with two agar plugs (each 0.25 cm^2) cut out from a colony grown on 2% Bacto-agar. Incubation was carried out at 28 ± 1 C under stationary conditions. Cultures were harvested periodically (on day 26 for the optimization studies), filtered through filter paper using a Büchner funnel, and dried overnight at 70 C. Dry weight of mycelia was then determined. The culture supernatants were used as enzyme sources.To test the growth tolerance of the fungus towards the presence of different dyes, *T. trogii* was inoculated on plates containing malt extract (12.5 g/l) and agar (20 g/l), supplemented with different concentrations of anthraquinone blue, malachite green and xylidine (25–1000 mg/l). The inoculum consisted of a 0.25-cm^2 agar plug of a 5-day-old culture grown on MEA. A control plate with no dye added was also inoculated. Uninoculated plates served as controls for abiotic decoloration. The plates were incubated at 28 °C. Growth was followed by measuring the radial extension of the mycelium. A decolorized zone appeared when the fungus degraded the dye. Growth rates (mm/day) were calculated.

Experimental design and statistical analysis

A Box-Wilson central composite design [7] with five settings for each of three factors (copper, veratryl alcohol and asparagine concentrations) was run to optimize *T. trogii* ligninolytic enzyme production and decolorization ability in submerged fermentation, and to explore the interactions between those culture variables. The concentration of other media constituents was kept constant throughout the investigation. The 18 experiments carried out with different combinations of three independent variables had $n_c = 8{:}(\pm 1, \pm 1, \pm 1)$, $n_a = 6{:}(\pm \alpha, 0, 0)$, $n_o = 4{:}(0,0,0)$ where the value of $\alpha = n_c^{1/4} = 8^{1/4} = 1.682$ to make the design routable. The variable levels X_i were coded as x_i according to the following equation such that X_0 corresponded to the central value:

$$x_1 = \frac{X_i - X_0}{\Delta - X_i}, \quad i = 1, 2, 3, \ldots k$$

where x_i is the dimensionless value of an independent variable, X_i the real value of an independent variable, X_0 the real value of an independent variable at the center point, and ΔX_i the step change.

The experimental plan and levels of independent variables are shown in Table 1. X_1 (asparagine) had a lower limit of 0.51 g/l and an upper limit of 4.89 g/l, X_2 (VA) was varied between 0.056 and 2.5 mM. The lower and upper limits of X_3 ($CuSO_4{\cdot}5H_2O$) were 0.003 and 0.852 mM, respectively.

RSM was used to analyze the experimental design. We attempted to fit the response variable to a quadratic model in order to correlate the response variable to the independent variables. The general form of the second-degree polynomial equation is: $Y_i = \beta_o + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j$ (where Y_i is the predicted response, $x_i x_j$ are input variables that influence the response variable Y; β_o is the offset term; β_i is the ith linear coefficient; β_{ii} the ith quadratic coefficient and β_{ij} is the ijth interaction coefficient).

Experiments were replicated three times. The statistical analyses were carried out using multiple regressions and ANOVA with the programs Minitab v 13.1, InfoStat v 1.0 and Essential Regression v 2.2.

Analytical determinations

Extracellular proteins were estimated by the Bradford method [6] with bovine serum albumin as the standard. Reducing sugars remaining in the medium were measured by the glucose oxidase procedure [5] with glucose as standard. *Laccase activity* was measured with 2,2′-azino bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in 0.1 M sodium acetate buffer (pH 3.4) at 30 C. Oxidation of ABTS was determined by the increase in A_{420} ($\epsilon_{420} = 36$/mM cm) [26].

MnP was measured using phenol red as the substrate in 0.1 M sodium dimethylsuccinate buffer (pH 4.5) at 30 C ($\epsilon_{610} = 22$/mM cm) [20].

Table 1 Central composite design for media optimization, and measured response. *VA* Veratryl alcohol, *MnP* manganese peroxidase, *GLOX* glyoxal oxidase

Trial	Factor concentration						Volumetric enzyme activity (U/ml)			Decolorization ability (ΔA/min per l)		
	Asp (g/l)		VA (mM)		Cu^{2+} (mM)		Laccase	MnP	GLOX	Xylidine	Malachite green	Anthraquinone blue
1	(−1)[a]	1.4	(−1)	0.55	(−1)	0.18	3.57	0.08	0.01	0.44	1.3	2102
2	(+1)	4	(−1)	0.55	(−1)	0.18	7.84	0.19	0.07	0.75	1.31	3014
3	(- 1)	1.4	(+1)	2	(−1)	0.18	4.10	0.2	0.09	0.92	1.69	4461
4	(+1)	4	(+1)	2	(−1)	0.18	8.19	0.14	0.07	0.67	1.43	3189
5	(−1)	1.4	(−1)	0.55	(+1)	0.68	14.12	0.2	0.14	1.4	2.3	6367
6	(+1)	4	(−1)	0.55	(+1)	0.68	8.33	0.24	0.08	0.66	1.41	2803
7	(−1)	1.4	(+1)	2	(+1)	0.68	22.75	0.34	0.2	1.59	2.03	7889
8	(+1)	4	(+1)	2	(+1)	0.68	4.27	0.09	0.04	0.61	0.78	1487
9	(−α)	0.51	(0)	1.28	(0)	0.43	7.56	0.06	0.03	0.49	1.97	1623
10	(+α)	4.89	(0)	1.28	(0)	0.43	5.53	0.05	0.06	0.39	1.39	3286
11	(0)	2.7	(−α)	0.056	(0)	0.43	3.99	0.14	0.03	0.68	1.25	3175
12	(0)	2.7	(+α)	2.5	(0)	0.43	7.88	0.17	0.06	0.72	1.4	1269
13	(0)	2.7	(0)	1.28	(−α)	0.003	6.86	0.1	0.02	0.33	0.88	2486
14	(0)	2.7	(0)	1.28	(+α)	0.85	12.22	0.11	0.08	0.78	1.43	1401
15	(0)	2.7	(0)	1.28	(0)	0.43	6.44	0.1	0.06	0.81	1.15	2362
16	(0)	2.7	(0)	1.28	(0)	0.43	7.81	0.15	0.05	0.81	1.22	2361
17	(0)	2.7	(0)	1.28	(0)	0.43	9.87	0.09	0.11	1.06	1.96	3100
18	(0)	2.7	(0)	1.28	(0)	0.43	10.12	0.11	0.11	0.79	1.79	4796

[a]Numbers in parenthesis are the coded levels

Glyoxal oxidase activity (GLOX) was determined using a peroxidase-coupled assay with methylglyoxal as the GLOX substrate and phenol red as the peroxidase substrate ($\epsilon_{610} = 22$/mM cm) [18].

LiP was assayed by the azure B method [2]. International enzymatic units (U) were used (μmol/min) for all assays. Enzyme activities were expressed as U/ml culture filtrate.

In vitro decolorization of dyes

The reaction was carried out in test tubes at 30 C. The reaction mixture contained sodium acetate buffer (10 mM, pH 4.5), an aliquot of crude filtrate, and either xylidine (8.7 mg/l), malachite green (2.7 mg/l), or anthraquinone blue (60 mg/l); the total volume was 3 ml. Dye content was monitored at the maximum visible absorbance of each dye (respectively, 505, 615 and 620 nm), after 0.5, 1, 3 and 6 h. A control in which the extracellular liquid was replaced by distilled water was conducted in parallel. Decolorization activity was estimated as the decrease in absorbance at the maximum visible wavelength for each dye. Results are expressed as ΔA/min per l. The effect on the supernatant decolorization rate due to the addition of VA (2 mM), H_2O_2 (0.2 mM), and the laccase mediators HBT, phenol and tyrosine (0.1–0.5 mM) was also evaluated. Results are expressed as percent of remaining dye.

Decolorization rate of dyes in liquid cultures

Batch cultures of *T. trogii* were grown in 250-ml Erlenmeyer flasks containing 25 ml GA medium (with asparagine, 1.4 g/l; VA, 2.36 mM; $CuSO_4 \cdot 5H_2O$, 0.68 mM) at 28 C. The decolorization capacity of whole broth, cell-free supernatant and mycelium was evaluated. On day 26, the mycelium was collected by filtration under aseptic conditions, washed twice with distilled water and resuspended in sodium acetate buffer (0.1 M, pH 4.8). An aliquot of one of six different dyes (malachite green, remazol brilliant blue R (RBBR), indigo carmine, xylidine, poly R-478 and azure B) was added to each flask, containing either the whole culture, supernatant or mycelium. Dyes were added to a concentration that gave the same initial absorbance (1.0 absorbance unit at the maximum wavelength in the visible spectrum). Decolorization activity was determined by measuring the decrease of the dye absorbance at its maximum visible wavelength (respectively 615, 570, 609, 505, 520 and 650 nm).

Chemicals

Malt extract was purchased from Difco (Detroit, Mich., USA), anthraquinone blue was a gift from Dr. A. Vitale (Department of Organic Chemistry, Faculty of Exact and Natural Sciences, University of Buenos Aires), malachite green and Congo red were from Mallinckrodt (Phillipsburg, N.J., USA), indigo carmine, from ICN (Costa Mesa, Calif., USA), janus green from BDH (Poole, England). All other chemicals were from Sigma (St. Louis, Mo., USA).

Results and discussion

The kinetics of in vitro production of extracellular ligninolytic activities by *T. trogii* were studied in a synthetic medium (GA: glucose 10 g/l/ asparagine 3 g/l) and in a complex medium (malt extract 12.5 g/l). Although laccase, MnP and GLOX were detected in both media, the levels of all three enzymes were higher in the synthetic medium, with laccase activity being the highest. Figure 1 depicts the relationship between growth, glucose consumption, extracellular proteins and ligninolytic enzyme production by *T. trogii* in GA medium. All activities appeared before mycelial biomass peaked (i.e. they were present in the primary growth phase) but showed their maxima at the beginning of secondary metabolism (days 25–30, when the glucose in the culture medium was exhausted and the mycelial dry weight was decreasing). The greatest amount of extracellular proteins was recorded simultaneously with the highest enzyme production. Maximal levels detected were: 0.13 U

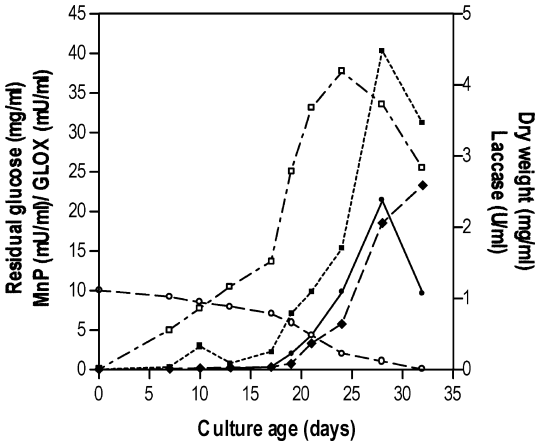

Fig. 1 Time course of growth and enzyme production by *Trametes trogii* in a medium with 10 g glucose/l and 3 g asparagine/l. □ Growth (mycelial dry weight (mg/25 ml)), ○ glucose remaining in medium (mg/ml), ♦ laccase activity (U/ml), (■) manganese peroxidase (MnP) activity (mU/ml), ● glyoxal oxidase (GLOX) activity (mU/ml)

laccase/ml, 0.015 U MnP/ml and 0.01 U GLOX/ml in malt extract medium; 2.7 U laccase/ml, 0.04 U MnP/ml and 0.02 U GLOX/ml in glucose/ asparagine. Attempts to detect LiP in the culture media assayed were unsuccessful. The negative LiP tests suggest that either the fungus produces no significant levels of this enzyme or that its production requires different growth conditions. LiP activity was detected previously in other strains of *T. trogii* [22,37].

GA medium was subsequently used for the optimization studies. The variables tested and their levels assayed were selected on the basis of preliminary studies [22,23]. Asparagine was chosen as the nitrogen source because it was the best of the different organic and inorganic sources tested with respect to growth of this fungus [21].

Data obtained with the aid of central composite design (Table 1) were analyzed by multiple regression. The results fit the following equation $Y_i = \beta_o + \Sigma\beta_i xi + \Sigma\beta_{ij} x_i x_j$ (where Y_i is the predicted response; $x_i x_j$ are input variables that influence the response variable Y; and β_o, β_i, β_{ij} represent the regression coefficients of the model). The quadratic terms were not statistically significant and were therefore rejected. The corresponding ANOVA is presented in Table 2. The coefficients of determination (R^2) were 0.799 for laccase production and 0.631 for GLOX production. In these models there was no lack of fit. No standardized residue was greater than 2 and all residues exhibited a normal distribution in both models, with $R > 0.97$, $p > 0.25$ in the Shapiro-Wilks test. In the case of MnP, it was not possible to fit the data obtained to either the lineal, lineal with interactions or quadratic models. MnP activity may be influenced by other variables not considered in this work.

Table 3 shows the estimates and *p*-values of the coefficients for both models (laccase and GLOX production).

Table 2 Analysis of variance (ANOVA) for the selected lineal with interactions model[a] for laccase (A) and glyoxal oxidase (B) production by *T. trogii*

Source	Sum of squares	Degrees of freedom	Mean squares	F-ratio	*p*-value
(A)					
Model	281.963	6	46.994	7.29	0.002
Linear	126.425	3	42.142	6.54	0.008
Interaction	155.538	3	51.846	8.05	0.004
Error (residual)	70.869	11	6.443		
Lack of fit	61.678	8	7.710	2.52	0.241
Pure error	9.191	3	3.064		
Total	352.832	17			
(B)					
Model	0.024	6	0.004	3.13	0.048
Linear	0.011	3	0.003	2.87	0.085
Interaction	0.013	3	0.004	3.34	0.058
Error (residual)	0.014	11	0.001		
Lack of fit	0.010	8	0.001	0.94	0.582
Pure error)	0.004	3	0.001		
Total	0.038	17			

[a] R^2 coefficient of determination = 0.799 for laccase and 0.631 for GLOX

Table 3 Parameter estimates and *p*-values for central composite design study of ligninolytic enzyme production

Factor	Laccase		GLOX	
	Estimate	*p*-value	Estimate	*p*-value
Constant	8.413	0.000	0.072	0.000
Asp	−1.415	0.064	−0.009	0.368
VA	0.877	0.228	0.013	0.219
Cu^{2+}	2.547	0.003	0.023	0.032
Asp–VA	−1.608	0.101	−0.021	0.116
Asp–Cu^{2+}	−4.080	0.001	−0.033	0.023
VA–Cu^{2+}	0.462	0.617	−0.006	0.648

Copper concentration positively affects enzyme synthesis (Cu^{2+} exhibited the largest positive value, $p < 0.05$). The initial asparagine concentration had a negative interactive effect with copper concentration on laccase and GLOX production (the interaction asparagine–Cu^{2+} had a negative value in both cases, $p < 0.05$). Other factors provided to be not statistically significant. Thus, it was evident from the results that although the linear effect of asparagine was not significant for enzyme production, at least in the range of 0.51–4.89 g/l, asparagine addition to the medium could not be totally overruled because of its interactive effect with copper. A single-parameter study would overlook this entity. The 3D response surface and the 2D contour plots are the graphic representations of the regression equation for laccase production (Fig. 2a, b). The effect of pairwise interaction of the parameters is better depicted in the three-dimensional graphs (Fig. 2a), in which the third parameter is kept constant. Similar results were obtained for GLOX production (not shown).

Aromatic alcohols such as VA (a secondary metabolite produced by ligninolytic cultures of white-rot

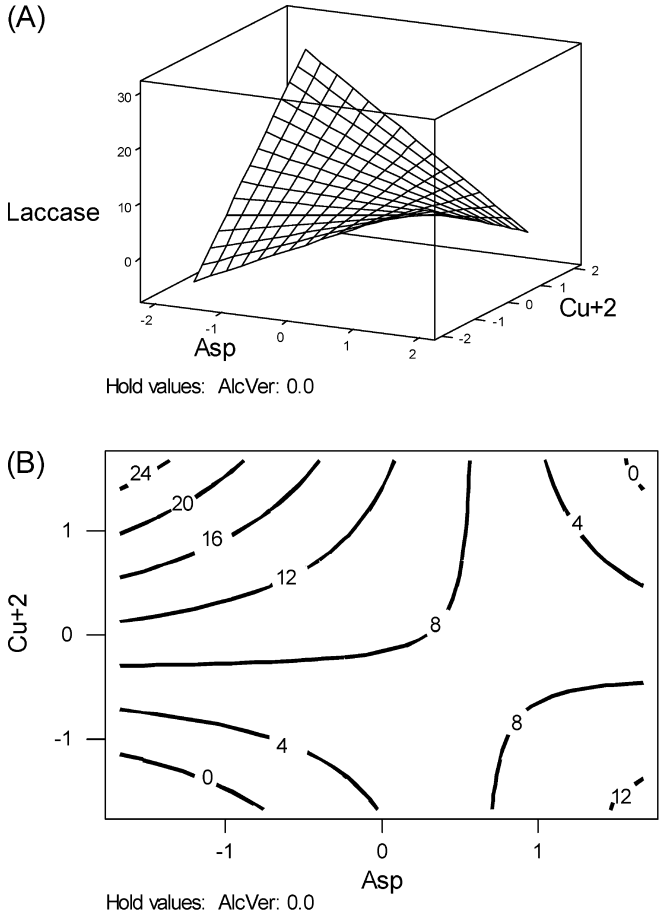

Fig. 2 **A** Surface response and **B** contour plots for the production of laccase by *T. trogii* as a function of asparagine and copper concentration, and with veratryl alcohol at its 0 level (1.275 mM), according to the experimental design and model in Table 1. Laccase activity is expressed in U/ml. Asparagine and $CuSO_4 \cdot 5H_2O$ values are shown in coded levels

fungi, and known to be a cofactor involved in the degradation of lignin and xenobiotic pollutants by LiP), which themselves are not substrates for laccases, have been reported to induce laccase production [11]. In a previous study using RSM to optimize laccase production by the ascomycete *Botryosphaeria* [38], the VA concentration was among the main factors increasing enzyme production; however, in the range assayed it did not significantly influence laccase and GLOX production by *T. trogii*. The addition of VA has been shown to induce laccase and GLOX production in other white-rot fungi and even in other strains of *T. trogii* [22,37].

The C:N ratio is another factor that usually influences ligninolytic enzyme production. The ligninolytic enzyme system of the best-studied model organism, *P. chrysosporium*, is triggered in response to N, C, or S limitation. Consequently, N-limited cultures are typically used to study the production of ligninases. However, many white-rot fungi produce higher titers of ligninolytic enzymes in N-sufficient media [16,19]. Although N-concentration in the range assayed did not significantly affect laccase and GLOX production by *T. trogii* (BAFC 212), low concentrations of asparagine in the medium enhanced the stimulatory effect of copper on enzyme production.

Copper has been reported to be a strong laccase inducer in several species, including *P. chrysosporium* [13] and *T. versicolor* [9]. It is known that copper induces both laccase transcription and activity [9], and the increase in activity is proportional to the amount of copper added. In the case of *T. trogii*, induction of MnP and GLOX activity has been observed as well [23]. But to the best of our knowledge the interactive effect of nitrogen and copper concentration on laccase production has not been described before. Laccase production may have been triggered by stress conditions, e.g. nitrogen starvation and toxicity due to high cooper concentration (in contrast to other essential metals, copper is toxic to most fungi even at very low concentrations [4]). One of the possible functions of fungal laccases is the polymerization of toxic aromatic compounds formed during the degradation of lignin. Therefore, laccases may function as a defense mechanism against oxidative stress [36].

Using the method of experimental factorial design and response surface analysis, the optimal conditions to obtain a high enzyme yield could be determined. The validity of the model was proved by fitting different values of the variables into the model equation and by carrying out the experiment at those values of the variables (Table 4).

The best conditions for the production of laccase, MnP, and GLOX were: 1.4 g asparagine/l, 2 mM VA and 0.68 mM copper (Table 1). This medium rendered the highest ligninolytic enzyme production (22.75 U laccase/ml, 0.34 U MnP/ml and 0.20 U GLOX/ml) and showed the greatest ability to decolorize the dyes.

Dye decolorization may be best predicted by the linear model ($Y_i = \beta_o + \Sigma \beta_i x_i$). The estimated coefficients yielded the following mathematical models:

- $y = 0.263 + 4.892$ [GLOX] (xylidine)
- $y = 1.123 + 7.256$ [GLOX] (malachite green)
- $y = 739.4 + 31.498$ [GLOX] (anthraquinone blue)

Table 4 Production of laccase and GLOX by *T. trogii*. Comparison of the experimental results with the predicted values computed from the mathematical model

Trial	Factor concentration			Laccase activity (U/ml)		Standardized residues	GLOX activity (U/ml)		Standardized residues
	Asp (g/l)	VA (mM)	Cu (mM)	Observed	Predicted		Observed	Predicted	
1	1.4	2.36	0.68	17.22	20.44	−1.58	0.2	0.18	0.87
2	3.35	1.28	0.55	10	8.19	0.88	0.06	0.07	−0.53
3	4	1.28	0.55	6	6.21	−0.10	0.04	0.06	−0.89

where y is dye degradation (ΔA/min per l) and [GLOX] is GLOX activity (U/ml).

The model determination coefficient R^2 (0.817 for xylidine decolorization, 0.552 and 0.727 for malachite green and anthraquinone blue degradation, respectively) suggested that the fitted models could explain only part of the total variation. The F-values (20.83, 12.45 and 5.74), and $p < 0.001$ for xylidine and anthraquinone blue, $p = 0.009$ for malachite green decolorization, respectively, indicate that the present models are able to well predict the experimental results.

The p-values suggest that the coefficient for the linear effect of GLOX activity (0.01 for xylidine and anthraquinone blue, and 0.04 for malachite green decolorization) is the most significant. By contrast, laccase and MnP activities, in the range assayed (laccase 3.57–22.75 U/ml and MnP 0.049–0.342 U/ml), did not significantly affect dye decolorization (probably because they were not in limiting quantities, allowing significant differences to be visualized by the analysis of variance). GLOX activity, however, seems to be rate limiting in the dye degradation process. Due to the participation of peroxidases in lignin breakdown, the extracellular production of H_2O_2 is essential. GLOX activity, which is produced extracellularly and expressed during secondary metabolism, when ligninases are also expressed, has been suggested to be the major enzyme responsible for the production of H_2O_2 in *P. chrysosporium* [18]. High levels of GLOX activity were produced by *T. trogii*. Only a few of the 67 strains analyzed by de Jong and coworkers [10] showed GLOX activity of up to 0.003 U/ml. Kersten [18], using an optimized liquid medium, obtained 0.032 U GLOX activity/ml in *P. chrysosporium*. Our results agree with those of previous reports demonstrating that xenobiotic compound oxidation by white-rot fungi cannot be improved by overproducing peroxidases without increasing the endogenous production of H_2O_2 [19].

The effect on the supernatant decolorization rate caused by the addition of compounds such as VA and H_2O_2, and the laccase mediators HBT, phenol and L-tyrosine [15] was also evaluated (results not shown). VA and H_2O_2 did not increase color removal, whilst phenol and tyrosine addition only slightly increased the percentage of decolorization. HBT was the most efficient mediator compound, suggesting the involvement of laccase activity in the decolorization process. Its addition not only increased the rate but also the range of dye decolorization (Fig. 3). *T. trogii* extracellular fluid in the presence of HBT decolorized eight synthetic textile dyes by more than 50% within 15 min. The respective rates for the azo dyes assayed were: xylidine (8.5 μg dye/ml per 15 min), Congo red (23.4), fast blue RR (54.6), janus green (12.5); the triphenylmethane dyes malachite green (5), bromophenol blue (20) and crystal violet (2.9); the anthraquinonic dyes anthraquinone blue (28) and RBBR (36.5); the polymeric dye poly R 478 (3); the heterocyclic azure B (0.5); and the indigoid indigo carmine (13.6). The different rates reflect the different

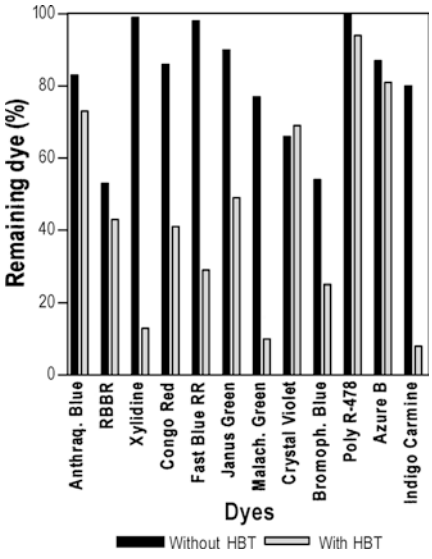

Fig. 3 Decolorization efficiency (as % dye remaining after 15 min incubation) of dyes with different chemical structures by the crude culture filtrate of *T. trogii*, with or without the addition of 1-hydroxybenzotriazole (HBT). The anthraquinonic dyes assayed were: anthraquinone blue and remazol brilliant blue R; the azo dyes: xylidine, Congo red, fast blue RR and janus green; the triphenylmethane dyes: malachite green, crystal violet and bromophenol blue; the polymeric dye poly R 478; the heterocyclic dyes: azure B; and the indigoid: indigo carmine

capacities of the crude culture filtrates of *T. trogii* to remove dyes with diverse chemical structures. Decolorization rates obtained compared favorably with those previously reported for other white-rot fungi. Decolorization rates for Congo red of 2.9 and 3.9 μg/ml per day were reported by Dey et al. [12] for *P. chrysosporium* and *Polyporus ostreiformis* grown on liquid media. Respective rates for Congo red, methyl red and the group of dyes including RBBR and bromophenol blue of about 10, 20 and 35 μg/ml per day, were described for cultures of *Irpex lacteus* [27]. Similar to *T. trogii,* the laccase of *Coriolopsis gallica* decolorized 13 of 38 industrial dyes tested but, in the presence of 1 mM HBT, the enzyme decolorized 26 of 38 dyes, increasing both the range and rate of decolorization [32].

Commercial dyes are not uniformly susceptible to microbial attack in conventional aerobic treatment because of their unique and stable chemical structures. Anthraquinone, azo and indigo dyes were decolorized by the laccase of *T. versicolor*; however, the mechanism of laccase-catalyzed decomposition, was different depending on the dye structure. While anthraquinone was oxidized directly by the laccase, azo and indigo dyes were not substrates of laccase, and small-molecule metabolites mediated the interaction between the dyes and the enzyme. The decolorization rate of the non-substrate dyes was limited by the concentration of mediating compounds rather than by laccase activity in the solutions [39]. Our results confirmed these findings: while HBT addition did not remarkably increase decolorization of anthraquinonic dyes, decolorization of

azoic and indigoid dyes was much less efficient in the absence of HBT. Poly R and azure B degradation have been correlated mainly with peroxidase production [14,41]; therefore, we did not expected a significant increase in their decolorization rate upon addition of HBT. Complete decolorization of two triphenylmethane dyes (bromophenol blue and malachite green) was achieved by cultures of *Pycnoporus sanguineus* producing laccase as the sole phenoloxidase [30]. The faster decolorization of those dyes by *T. trogii* supernatants with HBT addition suggests that laccase may play an important role in this process. Nevertheless, other enzymes, which were not assayed for, or hitherto unknown enzymes may contribute to decolorization by *T. trogii* extracellular fluids. A RBRR peroxidase is involved in decolorization of RBBR and several other dyes, including triphenylmethane, heterocyclic, azo and polymeric dyes [35].

The decolorization capacities of whole broth, cell-free supernatant and mycelium from *T. trogii* were evaluated. The results are depicted in Table 5. All of the dyes tested (xylidine, poly R-478, RBBR, malachite green, azure B and indigo carmine) were decolorized to some extent by whole broth, with varying percentages of decolorization (1–76% in 15 min). Adsorption of the dyes to the heat-killed mycelium was often observed by eye in *T. trogii* cultures, suggesting that the mycelial mass might be used as an adsorption system for removal of pollutant dyes. Heat-killed pellets of *Funalia trogii* adsorbed 55% of Astrazon red in 24 h [40]. Nevertheless, when considering the living microorganism, sorption in dye decolorization seems to be the first step. The dyes were rapidly removed from the medium by physical adsorption, but, in a second step, they were eliminated from both the solution and the surface of the mats, as a consequence of the enzymatic degradation.

In a recent work, *F. trogii* pellets showed a rapid dye decolorization activity. Spectrophotometry and microscopy also showed that the process involved initial adsorption of the dye compounds, which was followed by decolorization through microbial metabolism [40]. The crude extracellular extracts were able to decolorize the dyes xylidine, malachite green, poly R-478 and azure B, but not as efficiently as the cultures. Similarly, although *P. ostreatus* decolorized 12 of 23 industrial dyes when grown on solid media, the crude extracellular extracts were able to decolorize only five dyes, showing that other enzymatic mechanisms could be involved in dye decolorization in in-vivo experiments [34]. Cytochrome P-450 monooxygenases (as suggested for malachite green degradation by the non-ligninolytic fungus *Cunninghamella elegans* [8]) or other hitherto uncharacterized enzyme(s) may be involved in this process. Nevertheless, cell-free supernatants and whole cultures of *T. trogii* degraded comparable amounts of the dyes RBBR and indigo carmine, suggesting that this fungus implements different strategies to degrade dyes with diverse chemical structures. The best decolorization was obtained for RBBR (79%). Purified laccase proved to be able to decolorize RBBR [1], whereas MnP played a major role in the decolorization of RBBR by *I. lacteus* [17]. However, a RBBR decolorizing peroxidase was purified from culture filtrates of *P. ostreatus* [35], and might also be present in culture supernatants of *T. trogii*. Indigo carmine is a dye that is easily decolorized by different wood-rotting fungi. It can be degraded either by purified laccase, LiP or MnP [1,31]. Assistance in decolorization of the dyes may be credited to these or other extracellular or mycelia-bound enzymes, which were not assayed. Although laccase (17.22 U/ml), MnP (0.2 U/ml) and GLOX (0.2 U/ml) (but not LiP) activities were detected in the extracellular fluid of *T. trogii*, H_2O_2 or other necessary factors involved in the catalytic cycle of the enzymes are probably lacking. While these enzymes and their associated components, such as H_2O_2, are present extracellularly, in biobleaching studies with *T. versicolor* Archibald [3] showed that they must be constantly replenished from active biomass. Mycelial presence becomes significant if decolorization requires such biomass-associated factors. *T. trogii* showed considerable tolerance to dyes such as anthraquinone blue and xylidine, and concentrations of up to 1,000 mg/l did not significantly affect its growth. Indeed, colony sizes were similar to those of the control (rates of growth: among 9.3 and 7.8 mm/day). Only malachite green, widely used as the most efficacious antifungal agent in the fish farming industry [8], partially (25–50 mg/l, 65–80% decrease) or totally (100 mg/l) inhibited growth of the fungus. The fungus was able to decolorize the dyes at the concentrations assayed, but the increase in dye concentration delayed the appearance of the decolorized zones.

Two strategies for degrading recalcitrant compounds have been pursued: (1) direct transformation of pollutants by active cultures of white-rot fungi, and (2) the use of enzymes extracted from the culture media. The advantage of the second strategy is greater independence from influences of wastewater composition, e.g. high salinity, that may affect the activity of the fungi. A disadvantage is the higher costs resulting from the need for extraction and cleaning steps. By contrast, crude culture filtrates offer distinct advantages: they are not as

Table 5 Decolorization (% per 15 min) of xylidine, malachite green, indigo carmine, RBBR, poly R-478 and azure B at their maximum absorbance (505, 615, 609, 570, 520 and 650 nm respectively) by *T. trogii* on the 26th day of growth in GA medium (with 1.4 g L-asparagine/l, 2.4 mM veratryl alcohol, 0.68 mM $CuSO_4 \cdot 5H_2O$)

	Xylidine	Malachite green	Indigo carmine	RBBR	Poly R-478	Azure B
Extracellular fluid	3.88	12.34	44.71	79.05	0.61	0.48
Mycelium	5.21	5.48	22.89	57.76	0.71	1.48
Whole culture	34.26	21.16	40.37	76.08	1.13	2.17

expensive to obtain, and proteins or other factors present in the medium may stabilize crude enzymes, as evidenced by the finding that lower levels of poly R-478 decolorization were obtained with pure MnP of *P. chrysosporium* than with the nonpurified extracellular liquid [25], and that the highest poly R-478 decolorization rate by crude MnP of *P. chrysosporium* occurred in parallel with the maximum H_2O_2 decomposition rate. When H_2O_2 was depleted, no further decolorization was observed but when H_2O_2 was supplied semi-continuously, the decolorization rate was much higher [25]. Thus, a crude culture filtrate with high ligninolytic and H_2O_2-producing activities, such as those produced by *T. trogii* (BAFC 212), capable of decolorizing a wide range of industrially important textile dyes may be a good candidate for the bioremediation of textile processing effluents.

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