

Optimization of lignocellulolytic enzyme production by the white-rot fungus *Trametes trogii* in solid-state fermentation using response surface methodology

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Abstract

The white-rot basidiomycete *Trametes trogii* (MYA 28-11) is an outstanding producer of laccase. A Doehlert experimental design was applied to optimize its lignocellulolytic enzyme production in solid-state fermentation. The impact on enzyme production of three quantitative variables, namely pH, copper and nitrogen concentrations, was investigated by using a wood-based solid medium supplemented with malt extract. Optimization was aimed at simultaneously minimizing cellulase activity and maximizing ligninolytic enzyme production. Such conditions were: pH 4.5, peptone 12.5 g l⁻¹, and CuSO₄ 11 mM. Highest activities achieved were: laccase 901 and Mn-peroxidase 20 U g⁻¹. There was not a direct correlation between the levels of enzymatic activities and the extent of losses of wood weight or components. The factorial approach also allowed us to quantify the enzyme activity in any part of the experimental domain, consequently, it was also possible to determine a culture medium to obtain crude extracellular extracts with high laccase (510 U g⁻¹) and endoxylanase (780 U g⁻¹) yields. These enzymes have gained renewed interest mainly due to their applications in paper industries for pulp treatment, improving the effectiveness of conventional bleaching. Considering the results obtained, *T. trogii* could be an attractive source of both enzymes.

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1. Introduction

White-rot fungi (WRF) are so far unique in their ability to completely degrade all components of lignocellulosic materials. Considering that WRF are able to degrade lignin selectively or simultaneously with cell wall polysaccharides, two white-rot patterns have been described, namely selective delignification and simultaneous rot [1]. The fungal isolate, solid substrate and ecophysiological factors affect the fungal growth and wood degradation pattern [1]. Cellulose biodegradation is a synergistic process involving endo- β -1,4-glucanase, cellobiohydrolase and β -glucosidase. The internal glucosidic bonds in the β 1-4 chain are first hydrolyzed by endoglucanases to generate free non-reducing ends, from which the exoglucanases then remove cellobiose units that are converted to glucose by β -glucosidase. Hydrolysis of xylans mainly requires the action of endo- β -

1,4-xylanase and β -xylosidase. However, the presence of other accessory enzymes are needed to hydrolyse substituted xylans. The capability to degrade lignin is due to their extracellular non-specific and nonstereoselective enzyme system composed by laccases, lignin peroxidases and Mn-peroxidases, which function together with H₂O₂-producing oxidases and secondary metabolites. WRF secrete one or more of the three enzymes essential for lignin degradation [2,3]. The same unique nonspecific mechanisms that give these fungi the ability to degrade lignin also allow them to degrade a wide range of pollutants, including: polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), explosives, pesticides and dyes [3].

The majority of information so far available concerns the lignin-degrading enzyme systems of *Phanerochaete chrysosporium* and *Trametes versicolor*. Recently however, there has been a growing interest in studying the lignin-modifying enzymes of a wider array of WRF, from both a basic and applied viewpoint. *Trametes trogii* (MYA 28-11), an Argentinean white-rot strain, besides efficiently degrading lignin in wood [4], has been tested successfully in biomechanical pulping experiments [5] and also

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proved to be a good producer of xylanases and ligninases (laccase and Mn-peroxidase) [6,7]. Its ability to almost completely remove high priority pollutants such as PCBs, PAHs, nitrobenzene, anthracene, and a wide range of textile dyes, has been recently demonstrated [8–10].

The pulp and paper industry is emerging as one of the potential large markets for enzyme application. Microbial enzymes: cellulases, xylanases and ligninases, create new technologies for pulp and paper processing. Xylanases reduce the amount of chemicals required for bleaching; cellulases smooth fibers, enhance drainage and promote ink removal. Lignin-degrading enzymes remove lignin from pulps. Currently the most important application of enzymes in the pulp and paper industry is in the prebleaching of Kraft pulp. Pulp bleaching with a laccase mediator system has reached pilot plant stage and is expected to be commercialized soon. Xylanase prebleaching technology is now in use at several mills worldwide [2,11,12].

Reducing the cost for enzyme production is still needed in order to develop enzymatic treatment processes for different industrial and environmental applications, which might be more competitive than conventional and other novel treatment technologies. In alternative to recombinant DNA techniques, this might also be achieved by means of both process optimization using statistical experimental designs and the use of cheap growth substrates [13]. The fungal production of lignin-modifying enzymes through the bioconversion of lignocellulosic residues has been widely investigated in recent years. This approach is attractive because of foreseeable effects on cost reduction, waste reuse and enhanced enzyme production. Solid-state fermentation (SSF) processes have shown to be particularly suitable for the production of enzymes by filamentous fungi, since they reproduce the natural habitats of such fungi [14]. Enzyme overproduction can be achieved by media engineering. Traditional methods of optimization 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 since it does not allow to estimate interactions among experimental variables. The use of different statistical designs for medium optimization has been recently employed for xylanase and laccase production by fungal cultures [15–19]. Response surface optimization techniques were applied in submerged fermentation to maximize laccase and Mn-peroxidase production by *T. trogii* [10,20].

In this work, a surface response methodology based on a Doehlert experimental design [21] was applied to optimize *T. trogii* lignocellulolytic enzyme production in SSF. The factors analyzed were: initial pH value, copper (as CuSO_4) and nitrogen concentration (as peptone), using a cheap natural medium, containing Poplar wood (as sawdust and shavings) and malt extract as substrates. In order to reduce the effects on the cellulose fraction of lignocellulose, of the crude extracellular extracts, with the expectation of applying them in biopulping, optimization of the process was based on minimum cellulase production and maximal ligninolytic enzyme production. Some relevant hydrolytic enzymes (endo- and exo-glucanase, β -glucosidase, endoxylanase and β -xylosidase) and oxidative enzymes (lac-

case, lignin- and Mn-peroxidases) present in the culture extracts were studied. Consequently, it was also possible to determine optimal operating conditions to obtain extracts with high laccase and endoxylanase yields for biobleaching purposes. The extent of polysaccharides and lignin removal in the solid substrate was also determined in order to assess whether the degradation pattern was correlated with activity levels of the aforementioned enzymes.

2. Materials and methods

2.1. Fungal strain and culture conditions

Strain MYA 28-11 (BAFC 463) of *Trametes trogii* (*Funalia trogii*) (Berk. in Trog.) Bond and Singer (Aphyllophorales, Basidiomycetes), was maintained at 4 °C on malt agar slants. Incubation was carried out at 28 ± 1 °C under stationary conditions in 100-ml Erlenmeyer flasks for up to 43 days. Poplar wood (in the form of sawdust 5 g and shavings 0.89 g per Erlenmeyer (dry basis)) was used as solid substrate for cultures, moistened with 16 ml of distilled water and 1 ml mycelial suspension (inoculum) (initial moisture content 77%). The medium for maximum ligninolytic production was first optimized by a 'one-variable-at-a-time' approach. The effect of additives was evaluated by replacement of the distilled water with an aqueous solution of the additive to be tested at the following concentrations: malt-extract (30 g l^{-1}), peptone from meat (20 g l^{-1}), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (3 mM). Initial pH of the medium was adjusted to 5.0 with either NaOH 1N or HCl 1N. The medium was then autoclaved for 20 min at 121 °C, and inoculated aseptically with the mycelial suspension. To prepare the inoculum, one 0.25 cm^2 agar plug cut out from the margin of a 5-day-old colony grown on malt-extract/agar medium was transferred to 100-ml Erlenmeyer flasks containing 50 ml of malt-extract (12.5 g l^{-1}) medium and incubated for 5 days, at 28 °C and 125 rpm, the mycelium obtained was blended in three cycles of 15 s. The medium composition that resulted in the highest enzyme titers of laccase and Mn-peroxidase, was considered as the basal medium, and used for optimization by response surface methodology (RSM) using the Doehlert design [21].

2.2. Sample preparation

Weight losses were determined on the basis of the initial and final dry weights, drying the content of each flask to constant weight at 80 °C. Dried samples were ground in a mortar and stored until they were used for chitin, cellulose and lignin determinations. Enzyme extraction: crude extracts were obtained by adding $5 \text{ ml (g wet solid)}^{-1}$ of 50 mM sodium acetate buffer, pH 4.8, to the contents of each flask, stirring for 20 min, followed by filtration and centrifugation. The supernatants were stored at -20 °C until needed.

2.3. Enzyme assays

Laccase activity (E.C.:1.10.3.2) was measured with 2,2'-azino bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in

Table 1
Production of laccase, MnP, endoglucanase and β -glucosidase activities, by *T. trogii* in different media

Medium	Laccase (U g ⁻¹)	MnP (U g ⁻¹)	Endoglucanase (U g ⁻¹)	β -Glucosidase (U g ⁻¹)
Wood ^a	2.12 ⁽⁴³⁾	0	167.25 ⁽²⁹⁾	0.35 ⁽⁴³⁾
Wood + malt extract ^b	11.58 ⁽⁴³⁾	1.17 ⁽⁴³⁾	253.49 ⁽³⁶⁾	0.66 ⁽⁴³⁾
Wood + malt extract + peptone (20 g l ⁻¹)	10.75 ⁽⁷⁾	0.55 ⁽⁷⁾	503.81 ⁽⁷⁾	0.89 ⁽¹⁴⁾
Wood + malt extract + peptone (20 g l ⁻¹) + Cu ²⁺ (3 mM)	62.80 ⁽¹⁴⁾	2.88 ⁽¹⁴⁾	512.21 ⁽¹⁷⁾	0.87 ⁽¹⁴⁾

The numbers in parenthesis indicate the day of maximum activity.

^a Poplar wood (sawdust 5 g and shavings 0.89 g per Erlenmeyer (dry basis)).

^b Malt extract (30 g/l).

0.1 M sodium acetate buffer (pH 3.4). Oxidation of ABTS was determined by the increase in A_{420} ($\epsilon_{420} = 36 \text{ (mM cm)}^{-1}$) [22]. Mn-peroxidase activity (MnP) (E.C:1.11.1.13) was measured using phenol red as the substrate in 0.1 M sodium dimethylsuccinate buffer (pH 4.5) ($\epsilon_{610} = 22 \text{ (mM cm)}^{-1}$) [23]. Lignin peroxidase activity (LiP) (E.C:1.11.1.14) was assayed by the Azure B method [24]. Endo- β -D-1,4-glucanase (E.C:3.2.1.4), exo- β -D-1,4-glucanase (E.C:3.2.1.91) and endo- β -D-1,4-xylanase (E.C:3.2.1.8) activities were determined measuring the reducing sugars released from carboxymethyl cellulose, cellulose crystalline or oat xylan, respectively, as substrates, in 50 mM sodium acetate buffer, pH 4.8. Liberated reducing sugars were quantified by the Somogyi–Nelson method [25], using either glucose or xylose as standards. β -glucosidase (E.C:3.2.1.21) and β -xylosidase (E.C:3.2.1.37) were determined in 50 mM sodium acetate buffer, pH 4.8, by measuring *p*-nitrophenol released from *p*-nitrophenyl- β -D-glucopyranoside and *p*-nitrophenyl- β -D-xylopyranoside, respectively [26]. All enzyme activities were determined at 50 °C. International enzymatic units (U) were used ($\mu\text{mol min}^{-1}$). Enzymatic activities in the extracts recovered from the solid-state cultures were reported in U ml⁻¹. In terms of production, the activity was defined as U per g dry residue (substrate plus mycelium) (U g⁻¹). Analysis of chitin, cellulose, and lignin: the chitin content of dried samples and mycelium from malt-extract liquid cultures

was determined by measuring *N*-acetylglucosamine (NAGA) released from chitin after hydrolysis with 6N HCl [27]. Cellulose and lignin in the dried samples were determined by the TAPPI method [28].

2.4. Experimental design and statistical analysis

A Doehlert uniform shell design was applied to study the effects of initial pH, Cu²⁺ and peptone (N). The levels and sampling days were selected according to preliminary studies (Table 1). Results were analyzed using the software STATISTICA 5.1 (StatSoft, Tulsa, Okla.). To study the effect of three independent variables, Doehlert proposed an experimental design based on 13 combinations of the three variables studied. The replicates of the central point allowed the calculation of the pure error of the method. The equally spaced values of each independent variable and the combination between them were adopted and coded following the Doehlert design [21]. The coded (in brackets) and real values are shown in Table 2. The responses measured at 14 days of growth were: laccase, MnP, endoglucanase, exoglucanase, β -glucosidase, endoxylanase and β -xylosidase activities, as well as biomass (chitin), and the changes in the polysaccharides and lignin contents in the substrate. The values are the mean of triplicate cultures. The processing of data was performed by direct curvilinear regres-

Table 2
Doehlert experimental design for media optimization and measured responses

Trial	Factor concentration (coded and real levels)						Enzyme activity (U g ⁻¹)						
	Cu ²⁺ (mM)		Peptone (g l ⁻¹)		pH		Laccase	MnP	Endoglu	Exoglu	β -Glu	Endoxil	β -Xil
1	(0) ^a	6.5	(0)	15	(0)	4.5	330.25	15.10	448.92	123.19	1.15	826.31	0.11
2	(0)	6.5	(1)	20	(0)	4.5	69.05	5.69	496.18	246.39	0.80	762.75	0.13
3	(0.866)	11	(0.5)	17.5	(0)	4.5	510.39	16.70	531.62	161.10	1.89	778.64	0.15
4	(0.289)	8	(0.5)	17.5	(0.816)	6	210.16	8.25	779.71	331.68	3.50	1144.13	0.23
5	(0)	6.5	(-1)	10	(0)	4.5	750.58	21.60	307.16	151.63	1.79	540.28	0.08
6	(-0.866)	2	(-0.5)	12.5	(0)	4.5	105.08	12.38	248.09	142.15	0.58	508.50	0.09
7	(0.866)	11	(-0.5)	12.5	(0)	4.5	900.71	19.65	271.72	113.72	1.54	413.15	0.09
8	(-0.866)	2	(0.5)	17.5	(0)	4.5	81.06	6.87	378.04	265.35	2.26	587.95	0.15
9	(-0.289)	5	(-0.5)	12.5	(-0.816)	3	195.15	8.84	425.29	217.96	2.06	746.86	0.09
10	(0.289)	8	(-0.5)	12.5	(0.816)	6	90.07	5.70	567.06	398.02	2.31	1112.35	0.14
11	(-0.289)	5	(0.5)	17.5	(-0.816)	3	162.12	8.64	448.93	132.67	1.40	842.21	0.09
12	(-0.577)	3.5	(0)	15	(0.816)	6	66.05	5.89	779.71	369.59	2.80	1175.91	0.22
13	(0.577)	9.5	(0)	15	(-0.816)	3	300.23	12.97	307.16	142.15	2.10	556.17	0.08
14	(0)	6.5	(0)	15	(0)	4.5	252.19	11.00	378.04	161.11	1.03	810.42	0.11

The numbers in columns 2 and 3, 4 and 5, and 6 and 7 correspond to the coded (in brackets) and real values (in bold) of the variables assayed. For example for copper the real value is the concentration of copper in the medium and is expressed in mM.

^a The numbers in parenthesis are the coded levels [21].

sion, without any prior transformation. A full quadratic model containing ten coefficients was used to describe the responses observed to fit Eq. (1), where Y represents the experimental response, pH, Cu^{2+} , N (peptone) the independently evaluated factors (in coded variables), b_0 the intercept, and b_i the parametric coefficients of the model obtained by multiple regression (which represent the linear, quadratic and cross-product effects of the factors on the response, respectively).

$$Y = b_0 + b_1 \times \text{pH} + b_2 \times \text{Cu}^{2+} + b_3 \times N + b_4 \times \text{pH}^2 + b_5 \times (\text{Cu}^{2+})^2 + b_6 \times N^2 + b_7 \times \text{pH} \times \text{Cu}^{2+} + b_8 \times \text{pH} \times N + b_9 \times \text{Cu}^{2+} \times N \quad (1)$$

3. Results

The time course of lignocellulolytic enzyme production by *T. trogii* in SSF using a cheap natural medium, containing poplar wood (as sawdust and wood shavings), was studied. The effect of different additives was evaluated: malt-extract (30 g l^{-1}), peptone from meat (20 g l^{-1}) and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (3 mM). Results are shown in Table 1. Enzymes exhibited different responses in the diverse media assayed. When using wood, as unique substrate, MnP was not detected and the titers of laccase were very low, but *T. trogii* produced appreciable levels of endoglucanase and β -glucosidase activities. When malt-extract was added to the medium, laccase and MnP titers increased. Results were improved by the addition of peptone combined with copper, considering the shorter incubation period needed for high ligninolytic enzyme production. Regardless of culture conditions, LiP activity was not detected.

The final optimization was aimed at simultaneously minimizing cellulase activity while maximizing ligninolytic enzyme production, to this end the Doehlert uniform design was employed. The effects of initial pH, Cu^{2+} , and peptone, were studied. Those factors hold the most promising for optimizing the fermentation on the basis of the preliminary research, along with previous studies in this strain [6,10,29,30]. Cultures were harvested on day 14, to guarantee appreciable levels of laccase and MnP. The values of the variables analyzed and the responses measured are shown in Table 2. Biomass (chitin) and the changes in the polysaccharides and lignin contents in the substrate were also determined in each trial (data not shown).

Activity data were fitted by a polynomial quadratic equation [31] (Table 3). Linear, quadratic and interaction coefficients of variables under study that were found to be significant at $P < 0.1$ were retained in reduced models. The quality of the model fit was evaluated by the coefficient R^2 and its statistical significance was determined by an F -test. R^2 represents the proportion of variation in the response data that can be explained by the fitted model. The lack-of-fit term in the residual indicates the variation due to model inadequacy. Although for β -glucosidase, endoxylanase and β -xylosidase activities the lack-of-fit test was significant, it has been pointed out that when a large amount of data is included in the analysis, a model with a significant lack of fit could still be used [32]. We considered the high R^2 as an evidence for the applicability of the model in the range of variables included. The iso-response curves are shown for each factor couple (Fig. 1). Shaded zones determine the conditions that maximized the production of laccase and MnP (in black) and minimized the production of the other enzymes (in gray). Contour plots indicate an optimum enzyme activity with initial pH 3, in the range of low peptone (10 g l^{-1}) and high

Table 3
Model coefficients estimated by multiple linear regression and best selected prediction models

Coefficient	Laccase	MnP	Endoglucanase	Exoglucanase	β -Glucosidase	Endoxylanase	β -Xylosidase
Constant	291.22	13.05	413.48	142.15	1.091	818.37	0.113
Linear							
Cu^{2+}	294.90**	4.47*	4.263	-34.20	0.198	-20.64	-0.006
PeP	-211.29*	-4.74*	125.52*	26.06	0.074	127.13*	0.038**
pH	-56.60	-2.17	193.03**	123.89**	0.622	262.91**	0.066**
Interactions							
$\text{Cu}^{2+} \times \text{PeP}$	-211.49	1.48	75.03	-43.78	-0.773	165.15	-0.003
$\text{Cu}^{2+} \times \text{pH}$	-31.60	-2.11	-4.12	6.69	-0.060	97.67	-0.005
$\text{PeP} \times \text{pH}$	168.73	1.16	89.24	27.11	1.409	-97.44	0.052*
Quadratic							
$(\text{Cu}^{2+})^2$	104.59	0.94	-70.88	18.96	0.570	-272.81*	0.010
PeP^2	118.60	0.60	-11.81	56.86	0.204	-166.85	-0.005
pH^2	-236.96	-7.39	227.70*	166.04*	1.713**	277.07**	0.041*
R^2	0.838	0.810	0.902	0.869	0.806	0.945	0.953
Lack of fit	NS	NS	NS	NS	S	S	S

Reduced equations for enzyme production: best selected models: laccase = $291.22 + 294.90\text{Cu}^{2+} - 211.29\text{PeP}$; MnP = $13.05 + 4.47\text{Cu}^{2+} - 4.74\text{PeP}$; endoglucanase = $413.48 + 125.52\text{PeP} + 193.03\text{pH} + 227.70\text{pH}^2$; exoglucanase = $142.15 + 123.89\text{pH} + 166.04\text{pH}^2$; β -glucosidase = $1.091 + 1.713\text{pH}^2$; endoxylanase = $818.37 + 127.13\text{PeP} + 262.91\text{pH} - 272.81(\text{Cu}^{2+})^2 + 277.07\text{pH}^2$; β -xylosidase = $0.113 + 0.038\text{PeP} + 0.066\text{pH} + 0.041\text{pH}^2 + 0.052\text{PeP} \times \text{pH}$. NS: not significant.

* Significant ($p < 0.1$).

** Significant ($p < 0.05$).

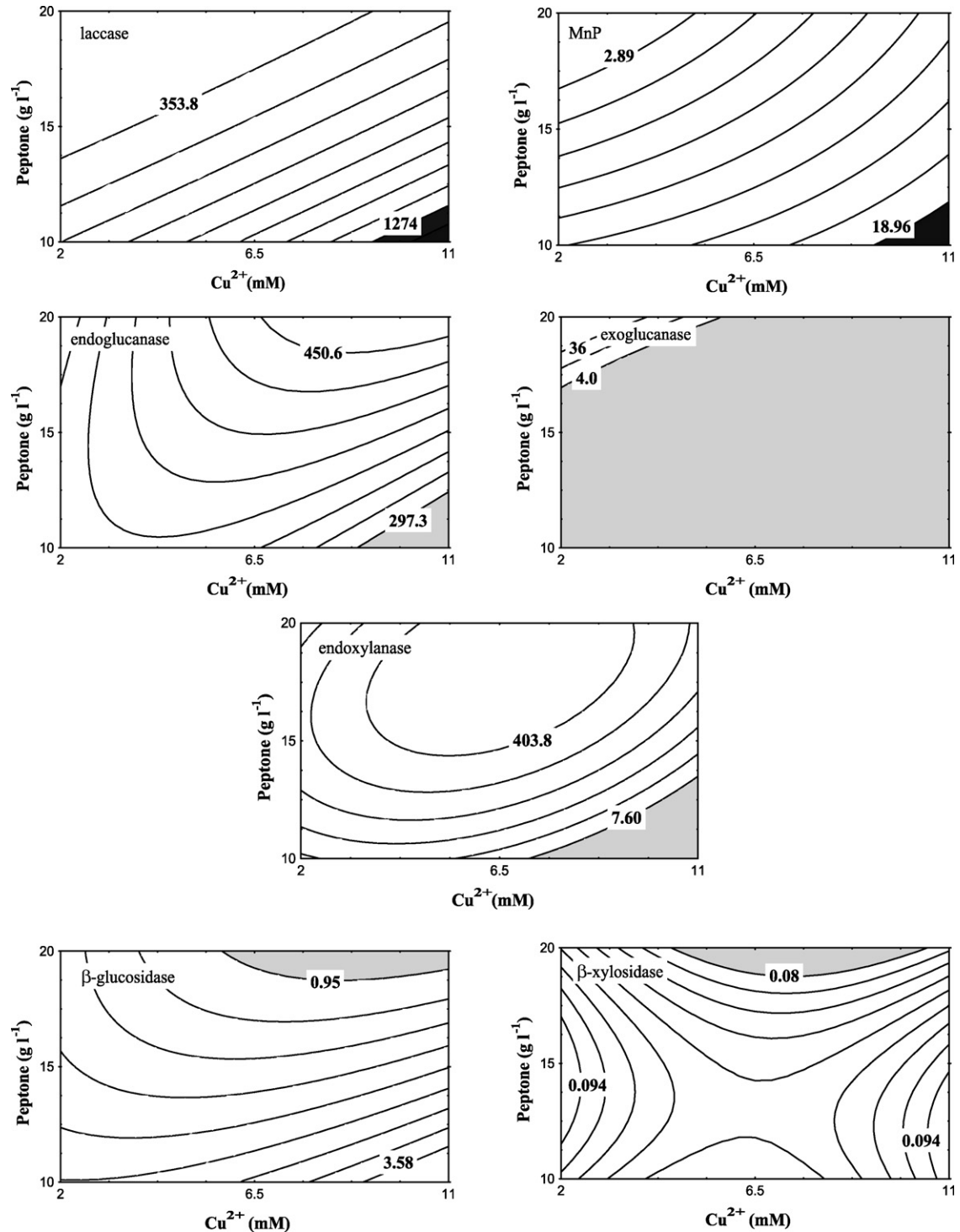


Fig. 1. Contour plots based on the equations shown in Table 3, for the effects of Cu²⁺ and nitrogen (as peptone) concentrations on lignocellolytic activity of *T. troglia* (at pH 3.0). Shaded zones determine the conditions that maximized the production of laccase and MnP (in black) and minimized the production of the other enzymes (in gray).

Cu²⁺ concentrations (11 mM). Analysis of the iso-response surfaces at pHs 4.5 and 6.0 displayed similar results, but cellulase production was higher (not shown).

The validity of the model was confirmed by fitting different values of the variables in the model equation and by actually carrying out the experiment at those values of the variables. The

differences between actual and predicted responses were always <10%, thus proving its validity.

The present model and data analysis allowed us not only to define the optimal media composition for lignocellolytic enzyme production, but it also showed the combined effects among the three factors studied. The large value for Cu²⁺ con-

centration in the linear term (Table 3) illustrates the significant, positive effect of this metal on laccase and MnP production. This positive linear coefficient indicates that production of laccase and MnP increased with increasing concentration of Cu^{2+} , whereas the negative linear coefficient for peptone suggests its inhibitory effect on the production of both enzymes. Conversely, peptone had a positive linear effect on endoglucanase, endoxyylanase and β -xylosidase production indicating an increase in activity with N concentrations. The positive linear effect of pH on cellulolytic and xylanolytic enzymes demonstrates an increase in their production when initial pH rises, in the range assayed; while the positive quadratic term indicates the existence of a minimum for these activities. Additionally, a positive or synergistic interaction between peptone and pH for β -xylosidase production was observed. The Cu^{2+} negative quadratic coefficient indicates the existence of a maximum of endoxyylanase activity as a function of Cu^{2+} concentration. Beyond this point Cu^{2+} had an inhibitory effect. From these coefficient values, it can be seen that Cu^{2+} is the factor with the most positive influence on ligninolytic secretion, while pH exerts a positive effect on cellulase and xylanase production. On the other hand while an increase in peptone decreases ligninolytic enzyme production it increases the production of endoglucanase and endoxyylanase activities. Although it was not possible to find experimental conditions leading to an almost complete suppression of cellulase production; conditions of low pH, limited peptone, and high Cu^{2+} concentrations, are suitable when searching for crude extracellular extracts with high ligninolytic but reduced cellulolytic activities.

The choice of culture conditions had a great impact on the final amount of enzymes produced. The optimal conditions to obtain minimum cellulase production and maximal ligninolytic enzyme production, were initial pH 4.5, peptone 12.5 g l^{-1} , and CuSO_4 11 mM. The highest ligninolytic activities achieved under these conditions were: laccase 901 U g^{-1} solid (42 U ml^{-1}) and MnP 20 U g^{-1} solid (0.91 U ml^{-1}). Lowest cellulolytic activities determined were: endoglucanase 272 and exoglucanase 113 U g^{-1} solid. There was not a direct relation, either between the titers of enzymatic activities and biomass of the fungus, or with the extent of losses of wood weight or components. Growth was monitored by measuring the chitin content in the dried substrate. As mycelium of *T. troglia* contains $27 \mu\text{g NAGA mg}^{-1}$, actual biomass could be estimated, attaining a maximum of $530 \text{ mg (g dry solid)}^{-1}$ in treatment 6 and a minimum of 350 mg in treatment 1. Although in different treatments biomass measured was similar (i.e. treatments 7 and 11: 397 and 400 mg, respectively), remarkable differences in enzyme production were observed (Table 2). On the other hand, there were no appreciable differences in the percentages of remaining lignin and cellulose when comparing treatments (data not shown).

Of the lignocellulolytic enzymes assayed, endoxyylanase was detected in the highest quantity, in terms of total units per culture, attaining a maximum value of 1176 U g^{-1} solid. This technique allowed us to quantify the enzyme activity in any part of the experimental domain. Therefore, it was also possible to determine operating conditions to obtain crude extracellular extracts with high laccase (510 U g^{-1} solid) and endoxyylanase

(780 U g^{-1} solid) yields (Cu^{2+} 11 mM, peptone 17.5 g l^{-1} , pH 4.5).

4. Discussion

Lignocellulolytic enzyme production by *T. troglia*, using Poplar wood as substrate, was investigated. In the absence of supplements, MnP activity was not detected while laccase activity levels were very low. Production of laccase and MnP by *T. troglia* was increased by the addition of easily available carbon and nitrogen sources to the culture medium, such as malt-extract and peptone (Table 1). Similar results were obtained in solid-state cultures of *Ceriporiopsis subvermispora* on *Pinus taeda* wood chips [33]. LiP activity was detected previously in this strain of *T. troglia* only when sawdust was added to a synthetic medium containing glucose and asparagine as carbon and nitrogen sources, respectively [29]. However, in the present work attempts to detect LiP were unsuccessful. The presence of Mn^{2+} is known to induce the production of MnP in many WRF, but Mn^{2+} lowers LiP titers [34]. Likewise, high Cu^{2+} concentration could inhibit LiP production by *T. troglia*, or LiP levels produced could be too low to be detected. On the other hand, the extraction procedure may have not been appropriate to guarantee the recovery of the enzymes adsorbed on the wood substrate [33].

The addition of malt-extract increased the secretion of *T. troglia* ligninolytic enzymes, but maximal production was registered only after 43 days of growth. When adding peptone to this medium, the peaks were detected remarkably earlier (at day 7). With the addition of Cu^{2+} to the medium a boost in laccase and MnP production was registered, although enzyme activity peaks were detected one week later. Malt-extract broth also increased the production of laccase in *Phlebia radiata* and *Phlebia fascicularia* [35]. Malt-extract is rich in the aromatic amino acids tryptophan and tyrosine. Collins et al. [36] reported a large increase in LiP production when adding tryptophan to the cultures of *T. versicolor*, *P. chrysosporium* and *Chrysosporium lignorum*, thus indicating that tryptophan, as provided by malt extract, might be responsible for better enzyme yields. *T. troglia* laccase production was stimulated by the addition of tryptophan to a synthetic medium containing glucose and asparagine as carbon and nitrogen sources, respectively [29]. Among different, mainly complex organic N sources, peptone from meat was the best for laccase production by *Trametes pubescens* [37]. MnP production in *Bjerkandera* sp. strain BOS 55 was also greatly stimulated in media containing peptone [38].

Extracellular ligninolytic and cellulolytic enzymes are regulated by heavy metals at the transcription level as well as during their catalytic action [39]. Cu^{2+} has been reported to be a strong laccase inducer in several species, among them *T. pubescens* [37] and *T. versicolor* [22]. Production of additional laccase isoenzymes, not present under natural conditions, was observed after Cu^{2+} addition in *P. chrysosporium* [40] and *Marasmius quercophilus* [41], but not in *T. troglia* [7]. In *T. troglia*, addition of copper increased the activities of MnP and glyoxal oxidase, as well as the decolorization of the polymeric dye Poly R-

478. Highest enzyme activities and decolorization rates were obtained with 1.6 mM Cu^{2+} , the highest concentration tested in submerged fermentation [7]. The presence of heavy metals affect the growth of WRF, the decrease of fungal growth rate is sometimes accompanied with a prolonged lag phase. Cu^{2+} inhibited the growth of *Ganoderma lucidum* at concentrations less than 1 mM, while 150 ppm of Cu^{2+} decreased the growth rate of *P. chrysosporium* [39]. 1.6 mM Cu^{2+} reduced the growth of *T. trogii* in submerged fermentation [7], but concentrations of up to 11 mM did not affect the final biomass obtained under the conditions of the present work. Yet, they may have increased the lag phase, considering that the peaks of enzyme activity were detected later when adding copper to the medium (Table 1). The presence of heavy metals can also interfere with the carbon and energy supplying system of cellulases and hemicellulases. Cellulase of *P. chrysosporium* in liquid media was inhibited in the presence of 50–150 ppm cadmium, copper, lead, manganese, nickel and cobalt. Mercury, iron and copper strongly inhibited the activity of β -glucosidase from *Trametes gibbosa* [39]. But, in the range assayed in this work, copper had no influence on the production of cellulolytic enzymes by *T. trogii*.

The experimental design employed indicated that ligninolytic enzyme production by *T. trogii* is not much affected by pH variation in the range 3–6, but an increase in pH stimulates cellulase and xylanase production. In a synthetic medium with xylan as carbon source, pH 4.0 was optimal for endoxylanase production by *T. trogii*, but enzyme activity was also detected when initial pH values of culture media ranged from 3.1 to 6.5 [6]. On the contrary in the same synthetic medium at pH 3.6 but with crystalline cellulose *T. trogii* did not produce endoglucanase nor β -glucosidase activities and exoglucanase production was minimal [30].

The choice of culture conditions had a great impact on the final amount of enzymes produced, but there was not a direct relation, either between the titers of enzymatic activities and biomass of the fungus, or with the extent of losses of wood weight or components. This indicates that *T. trogii* grew and produced enzymes probably at the expense of the nutrients supplied to the solid substrate, but did not degrade the wood components effectively. Several reasons may account for this effect, among them, their low permeation into the wood cell walls. When analyzing wood biodegradation and enzyme production by *C. subvermispora* during SSF of *Eucalyptus grandis*, Ferraz et al. [42] also found that the high levels of enzymatic activities detected after 15 days of decay did not correlate with the extent of initial wood weight or component losses. The capacity of WRF for wood degradation seemed to be correlated with the levels of oxidative activities only after longer biodegradation periods [42].

By proper selection of the initial pH, copper and peptone concentrations it was possible to obtain fermentation extracts with desire lignocellulolytic composition. To this end, the Doehrl design proved to be an excellent tool. From the coefficient values obtained, it can be seen that Cu^{2+} is the factor with the most positive influence on ligninolytic secretion, while pH exerts a positive effect on cellulase and xylanase production. On the other hand while an increase in peptone decreases ligninolytic

production it increases the production of the endoglucanase and endoxylanase activities. Although it was not possible to find experimental conditions where cellulolytic activity is completely suppressed; conditions of low pH, limited peptone, and high Cu^{2+} concentrations, are suitable when searching for crude extracellular extracts with high ligninolytic but reduced cellulolytic activities.

In the present study laccase production by the white-rot basidiomycete *T. trogii* MYA 28-11, which only recently had been identified as an excellent source of this enzyme [7], was successfully optimized using a mathematical model. Laccase has been purified in other strain of *T. trogii* [43,44], but it produced much lower titers (maximal production 0.5 U ml^{-1}) than those secreted by this isolate (93.8 U ml^{-1}) [7]. Comparison of the sequence of laccase encoding gene *lcc1* of *T. trogii* 201 with known laccases showed that this enzyme was most closely related to Lac1 from basidiomycete PM1 and *Trametes* C30 (98% similarity). The laccases from these fungi differ from laccases of other *Trametes* species in their higher thermal stability [43]. Laccase from *T. trogii* MYA 28-11 was stable up to 5 d at 30°C , and for 24 h at 50°C [7].

Productivity of laccase in SSF of selected agro-residues by *Pycnoporus sanguineus* reached a maximum of 48.7 U g^{-1} [45], while *Fomes sclerodermeus* grown on wheat straw produced 270 U g^{-1} of laccase [46]. In this work, very high yields of laccase (901 U g^{-1}), accompanied also but MnP activity (20 U g^{-1}), were obtained on a simple medium that does not contain aromatic toxic inducers, routinely used for stimulating laccase synthesis. No addition of expensive media is required and the use of inexpensive agro-industrial wastes will have important economic advantages. Thus, because of its low cost and the resulting high levels of enzyme production, this medium could be used to scale up the production of both laccase and MnP.

The application of cheap crude enzyme mixtures can be more efficient than isolated enzymes, in cases where high substrate specificity is not required [2]. Crude culture filtrates offer additional advantages over the use of purified enzymes: their obtention process is not as expensive, and in addition, proteins or other factors present in the medium may stabilize crude enzymes. The fact that *T. trogii* produces high levels of both laccase and MnP is a further advantage with respect to their possible synergism in bioremediation processes. Furthermore, this technique also allowed us to estimate the enzyme activity in any part of the experimental domain. Consequently, it was also possible to determine optimal operating conditions to obtain extracts with high laccase and xylanase yields. Therefore, this strain could be an attractive and alternative source of these enzymes, which have gained renewed interest in recent years, mainly due to their applications in paper industries for pulp treatment, improving the effectiveness of conventional bleaching.

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