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Optimization and Modeling of Laccase Production by *Trametes versicolor* in a Bioreactor Using Statistical Experimental Design

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Abstract

Experimental design and response surface methodologies were applied to optimize laccase production by *Trametes versicolor* in a bioreactor. The effects of three factors, initial glucose concentration (0 and 9 g/L), agitation (100 and 180 rpm), and pH (3.0 and 5.0), were evaluated to identify the significant effects and its interactions in the laccase production. The pH of the medium was found to be the most important factor, followed by initial glucose concentration and the interaction of both factors. Agitation did not seem to play an important role in laccase production, nor did the interaction agitation × medium pH and agitation × initial glucose concentration. Response surface analysis showed that an initial glucose concentration of 11 g/L and pH controlled at 5.2 were the optimal conditions for laccase production by *T. versicolor*. Under these conditions, the predicted value for laccase activity was >10,000 U/L, which is in good agreement with the laccase activity obtained experimentally (11,403 U/L). In addition, a mathematical model for the bioprocess was developed. It is shown that it provides a good description of the experimental profile observed, and that it is capable of predicting biomass growth based on secondary process variables.

Index Entries: Bioreactor; enzyme production; kinetic parameters; modeling; optimization; *Trametes versicolor.*

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Introduction

Trametes (Coriolus) versicolor is one of the most common white-rot fungi. It is highly valued in biotechnology because of its abilities to degrade wood (especially lignin) and decompose phenolic compounds. This fungus has a great potential as a biocatalyst in oxidative biologic processes owing to the production of extracellular oxidative enzymes, such as lignin and manganese peroxidases and laccase, that initiate decomposition of the lignin *(1–4)*, the complex aromatic polymer that constitutes the nonhydrolyzable part of wood. Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a multicopper enzyme that oxidizes various aromatic substrates such as polyphenols, aromatic amines, and methoxy-substituted phenols *(5)*. Oxidation by laccase is a one-electron reaction with the reduction of oxygen to water *(6)*. Fungal laccase has been intensively studied owing to its applications in wood delignification *(7)*, bioremediation *(8,9)*, wine clarification *(10)*, and decolorization of dyes *(11–14)*, among others.

For laccase application in industrial processes, large amounts of enzyme are required. Laccase production could be improved using new strategies of enzymatic production such as the use of inducers and substrate limitation conditions studied by our group in a previous work *(15)*. Fermentative process optimization is required to maximize productivity and minimize costs. Statistical experimental design can be used in biological processes to evaluate the effects and interactions of the different parameters that rule a biochemical system *(16)*. Such designs have been successfully employed to optimize medium composition and environmental factors *(17–20)*.

Recent studies have used statistical methodologies to improve laccase production. Prasad et al. *(21)* used the methodological application of Taguchi DOE for the optimization of submerged culture conditions for laccase production by *Pleurotus ostreatus* 1804. To evaluate the relative importance of various nutrients for laccase production by *Trametes trogii* in submerged fermentation, the Plackett-Burman experimental design was used to maximize enzyme production *(22)*.

In the present work, a 2^k factorial design was employed to determine the effects of initial glucose concentration, pH of the medium, and agitation at two different levels on laccase production by *T. versicolor*. The kinetic parameters of the fungal fermentation and enzyme production were determined through a modeling approach.

Materials and Methods

Experimental Design

To maximize laccase production by *T. versicolor* in bioreactor submerged fermentation, a two-level factorial design with three factors was applied. The selected culture factors were initial glucose concentration, pH of the medium, and agitation, in order to determine the interactions

Table 1
Factor Levels for a 2^3 Factorial Design

		Coded level	
Variable	Parameter	+1	–1
G	Glucose (g/L)	9	0
pH	pH of medium	5.0	3.0
A	Agitation (rpm)	180	100

between these culture variables. A two-level full factorial design consists of 2^k experiments, in which k is the number of factors, each with a high and a low value (level). In this context, a factor is an experimental variable, and the response is the quantitative measure of the parameter of interest, i.e., laccase activity. A total of eight experiments were performed with different combinations of three independent variables, in order to find the optimal conditions. Table 1 presents the factor levels, upper and lower limits, of independent variables. A pH of 3.0 ± 0.1 was obtained without pH control in culture medium and a pH of 5.0 ± 0.1 was obtained with pH control, as described in Fermentation Conditions. The values of each variable were coded as minus (–) for lower and plus (+) for upper limits. The response was the maximum laccase activity attained during the production.

The experimental design based on the analysis of the software *Statistica* v.5.1 (Statsoft) was used to estimate the responses from dependent variables. Equation 1 describes the regression model of the present system, which includes the interaction terms:

$$\hat{Y}\hat{y} = \beta_0 + \beta_1 x_G + \beta_2 x_{pH} + \beta_3 x_A + \beta_{12} x_{GpH} + \beta_{13} x_{GA} + \beta_{23} x_{pHA} \quad (1)$$

in which $\hat{Y}\hat{y}$ is the predicted response, i.e., laccase production; x_G, x_{pH}, and x_A are the independent variables; and the regression coefficients are as follows: β_0 is the intercept term; β_1, β_2, and β_3 are the coefficients for linear effects; and β_{12}, β_{13}, and β_{23} are the coefficients for interaction effects.

The experimental details of bioreactor fermentations with *T. versicolor* are described in the following sections. The experiments were produced in random order, and to identify optimum levels of variables, the response surface methodology was applied. The condition estimated to give maximum laccase activity was confirmed experimentally.

To obtain the kinetic parameters of the fungal fermentation and laccase production, a modeling approach, described in Mathematical Modeling, was followed.

Microorganism

T. versicolor was obtained from the National Institute of Industrial Engineering and Technology Portugal. The culture was maintained on Tien and Kirk *(23)* agar Petri plates at 4°C.

Culture Medium

Experiments were carried on a *Trametes* defined medium (TDM), described by Roy and Archibald *(24)*. The composition of the medium was 9 g/L of glucose, 5 m*M* glutamine, 5 m*M* NaCl, 5 m*M* KH_2PO_4, 1 m*M* $MgSO_4 \cdot 7H_2O$, 0.1 m*M* $CaCl_2$, 10 m*M* dimethyl succinate, 2.4 µ*M* thiamine, and 1 mL/L of a trace metals solution containing the following: 20 µ*M* $FeSO_4 \cdot 7H_2O$, 2 µ*M* $CuSO_4 \cdot 5H_2O$, 5 µ*M* $ZnCl_2$, 20 µ*M* $MnSO_4 \cdot H_2O$, 6 µ*M* $CoCl_2 \cdot 6H_2O$, 0.1 µ*M* $NiCl_2 \cdot 6H_2O$, and 0.5 µ*M* $(NH_4)_6MO_7O_{24} \cdot 4H_2O$. Sterilization was performed under autoclave at 121°C for 20 min. Tween-80 (0.5% [v/v]) was added to the culture medium to stimulate the secretion of extracellular enzyme *(25)*.

Inoculum

T. versicolor was grown in Petri dishes for 7 d at 28°C with Tien and Kirk *(23)* medium. To obtain the inoculum, a mycelium suspension was prepared from Petri dish plates. Ten milliliters of specific liquid culture medium was added to each Petri dish. With a sterilized wire loop, the mycelium was collected and suspended on liquid medium, and it was then transferred to a flask to obtain a concentrated suspension of cells, the inoculum. A given volume of suspension was filtered through 0.45-µm fiberglass filters (GF/C; Whatman), and biomass concentration by dry weight was quickly determined using an infrared light for 2 h to dry the sample. This allows calculation of the volume of inoculum necessary to obtain an initial cell concentration of 70 mg/L. The inoculum was transferred to each 500-mL Erlenmeyer flask containing 250 mL of culture medium and maintained at 28°C and 180 rpm for 3 d.

Fermentation Conditions

After 3 d in a rotary shaker, the flasks were transferred to a sterile 1-L bioreactor (Braun) equipped with automatic controls for pH, agitation, and temperature and operated in a batch mode for 9 d. The fermentations were performed under the following conditions: working volume of 1 L and temperature of 28°C, with compressed air continuously supplied to the bioreactor, maintaining oxygen saturation in the fermentation medium. In some experiments, the pH was controlled at 5.0 ± 0.1 by automatic addition of an aqueous NaOH (1 *M*) or H_3PO_4 (1 *M*). For studies of glucose suppression (0 g/L), the cultivated biomass in the rotary shaker for 3 d was filtered and transferred to a new TDM culture medium without glucose.

Following Eggert et al. *(26)*, who used xylidine to induce laccase production on other fungi, some experiments were carried out for concentrations between 5 and 50 µ*M* (data not shown). For *T. versicolor* laccase induction, a concentration of 30 µ*M* xylidine was selected, because higher concentrations showed a toxic effect. Such concentration of xylidine was added soon after the culture had been transferred to the bioreactor.

One sample (5 mL) of the medium was collected from the reactor once a day.

Laccase Stability Tests

The effect of pH on laccase stability was investigated at pH 3.0 and 4.5, because laccase production is much reduced when pH decreases to 3.0. A sample of culture medium at the end of fermentation with an initial laccase activity of 2500 U/L was colleted. It was incubated at 28°C for 3 d in 0.05 m*M* citrate/0.1 m*M* phosphate buffer at pH 3.0 and 4.5. The remaining laccase activities were measured each day under standard conditions. A control was incubated at the same conditions without any buffer, but with water.

Analytical Methods

Laccase activity was evaluated spectrophotometrically by the method of Ander and Messner *(27)* using 2,2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate. The reaction mixture contained 0.4 m*M* ABTS in 0.05 m*M* citrate/0.1 m*M* phosphate at pH 4.5 and 40°C in a total volume of 2.0 mL. Oxidation of ABTS was monitored through an increase in absorbance at 420 nm ($\varepsilon = 36.000\ M^{-1}cm^{-1}$). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 mmol of ABTS/min. Laccase activities were expressed in units per liter.

Glucose quantification was measured by the 3,5-dinitrosalicylic acid method *(28)* using D-glucose as standard.

The final biomass concentration was determined by dry weight of fungal mycelium. The culture medium was vacuum filtered through a 0.45-µm fiberglass filter (GF/C; Whatman). The biomass retained was washed with distilled water and dried at 100°C until reaching constant weight.

Mathematical Modeling

To identify the kinetic parameters of fermentations performed with the factorial design by *T. versicolor* in the bioreactor, a mathematical model was applied. A modeling approach was also herein adopted owing to the impossibility of quantifying the biomass along the batch studies. This impossibility derives from the morphology of the fungus, which does not allow a representative sample. Regarding the optimization of laccase, biomass quantification was mathematically performed with the aid of process secondary variables such as substrate concentration and product formation.

Equations 2–5 describe the model for laccase and biomass production, and glucose consumption, by *T. versicolor* in the bioreactor. To estimate the kinetic parameters, the model proposed by Mitchell et al. *(29)* for microbial growth was applied as described by Eqs. 2 and 3. This model considers two growth phases: the first, an exponential phase, is described by a Monod-

type model that is presented in Eq. 2; the second describes the growth deceleration, for which two more parameters (L and k) were required (Eq. 3).

Biomass concentration cannot be directly assessed during the experiments because *T. versicolor* grows in pellets, presenting heterogeneous liquid cultures. To estimate the biomass, it was assumed that the batch growth in the bioreactor was related to the growth observed in similar batch tests carried out in Erlenmeyer flasks in which total biomass was analyzed each day by dry weight. A parameter, A, was added to the model to represent the environmental changes from the Erlenmeyer flask to the bioreactor. Equations 4 and 5 describe the substrate consumption and enzyme production, respectively:

$$\frac{dX}{dt} = A\left[\frac{\mu_{max} S}{(K_S + S)}\right] X \quad \text{for } t < t_a \tag{2}$$

$$\frac{dX}{dt} = A\mu_{max}\left(\frac{S}{K_S + S}\right)[Le^{-k(t - t_a)}]\, X \quad \text{for } t \geq t_a \tag{3}$$

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}}\frac{dX}{dt} \tag{4}$$

$$\frac{dP}{dt} = K_1 e^X - K_2 P \tag{5}$$

in which X (g/L) is the microbial biomass; S (g/L) is the substrate; P (U/L) is the enzyme product; t (d) is the process time; t_a (d) is the time at the end of exponential phase (d 3); μ_{max} (d^{-1}) corresponds to the maximum specific growth rate; K_S (g/L) is the saturation constant; L is the survival factor; k (d^{-1}) is the exponential decay constant; $Y_{X/S}$ (g cell/g substrate) is the substrate yield coefficient to biomass; A is the environmental conditions adjustment; K_1 (U/[g cell·d]) is the enzyme synthesis rate constant; and K_2 (d^{-1}) is the enzyme decay rate constant.

The kinetic parameters used on the bioreactor modeling were estimated from Erlenmeyer cultures grown on media with glucose and without glucose as previously reported *(15)*. The kinetic parameters obtained have the following values: $\mu_{max} = 0.87\ d^{-1}$, $K_S = 4.21$ g/L, $Y_{X/S} = 0.17$ (g cell/g substrate), $k = 0.0921\ d^{-1}$, and $L = 0.37$ for a medium with 9 g/L of glucose; and $\mu_{max} = 0.82\ d^{-1}$, $K_S = 1.30$ g/L, $Y_{X/S} = 0.38$ (g cell/g substrate), $k = 0.2534\ d^{-1}$, and $L = 0.41$ for a medium without glucose.

The differential equations were solved, and the remaining parameters, A, K_1, and K_2, were estimated using the software MATLAB v.6.1 (Mathworks) by minimizing quadratic residuals between the experimental and model data using the objective function shown in Eq. 6. The Simplex-Nelder & Mead search method was used in the optimization procedure. Experimental errors associated with cell and laccase activity were estimated to be 10% through replication performed in the shaker studies. Statistical significance of estimated parameters was determined by the t-test at a 95% confidence level.

$$\text{Minimize} \sum_{i}^{ne} \sum_{j}^{np} ([X]_{exp} - [X]_{mod})^2 + ([S]_{exp} - [S]_{mod})^2 + ([P]_{exp} - [P]_{mod})^2 \tag{6}$$

Table 2
2^3 Factorial Design and Responses for Laccase Production by *T. versicolor*

Run	Agitation (rpm)	pH	Glucose (g/L)	Final biomass (g/L)	Maximum laccase activity (U/L)	Laccase productivity (U/[L·d])
F1	180	3.0	0	0.37	1410	117
F2	100	5.0	0	0.60	1780	130
F3	180	3.0	9	1.51	771	37
F4	180	5.0	9	1.59	7217	902
F5	100	3.0	0	0.29	2034	169
F6	180	5.0	0	0.27	2178	242
F7	100	3.0	9	1.28	444	49
F8	100	5.0	9	1.54	7113	889

in which *np* is the number of parameters; *ne* is the number of experiments; and *exp* and *mod* correspond to experimental and model data, respectively.

Results and Discussion

Experimental Design

Because enzyme production depends on the culture conditions, three factors, pH of the medium, agitation, and initial glucose concentration, were evaluated for maximization of laccase production. To select the optimal experimental conditions, a factorial design with two levels and three variables was adopted. Table 2 presents the results of *T. versicolor* fermentations used in the factorial design. The analysis showed that the media with glucose and a pH of 3.0 (without pH control) did not stimulate laccase formation (runs F3 and F7) and resulted in activities less than 1000 U/L, accompanied by high biomass concentrations.

The best results (F4 and F8) were obtained when glucose was present (9 g/L) and at a pH of 5.0 (controlled pH). A 16-fold increase in laccase activity for an agitation of 100 rpm (F7 and F8) and 9-fold for 180 rpm (F3 and F4) was obtained when pH was controlled (5.0). For all experiments, laccase productivity followed the same trend as maximum laccase activity.

Table 3 provides the individually calculated effects and their interactions for the 2^3 factorial design. The responses show that the linear terms of pH of the medium, initial glucose concentration, and their interactions have remarkable effects on maximum laccase activity. The interaction of agitation × pH of the medium and agitation × initial glucose concentration did not represent any effect on maximum laccase activity. The significance of the estimated effects was tested by analysis of variance (ANOVA). The ANOVA test indicates that the model adequately describes the yield of laccase synthesis. The significance of each coefficient was determined through a p value test ($p < 0.1$) considering 90% of confidence in which low

Table 3
Effects Estimate and ANOVA for Laccase Activity by *T. versicolor* Using 2^3 Factorial Design[a]

Effects	Estimate	*p* value	*t* value	SS	df	MS	*F*
Main effects							
pH	*3452.7*	*0.0579*	*10.9515*	*232,214 E²*	*1*	*232,214 E²*	*119.93*
G	*2170.8*	*0.0965*	*6.5420*	*8,286,928*	*1*	*8,286,928*	*42.80*
A	186.4	0.8962	0.1643	5233	1	5233	*0.0270*
Two factors interaction							
A × pH	64.4	0.6368	0.6416	79720	1	79720	*0.4117*
A × *G*	29.1	0.6906	0.5282	54022	1	54022	*0.2790*
G × pH	*3014.8*	*0.0626*	*10.1239*	*19,845,630*	*1*	*19,845,630*	*102.49*
Error				193,629	1	193,629	
Total SS				516,866 E^2	7		

[a]SS = sum of squares; df = degrees of freedom; MS = square means; $R^2 = 0.99625$; *A* = agitation; *G* = glucose; pH = pH of the medium.

The numbers in italic represent the response of the factors that have a significant effect on laccase production.

p values indicate high significance of the corresponding coefficient. The variable with the largest effect was the pH, and the glucose H pH interaction term is similar in significance. The Pareto chart presented in Fig. 1 shows the statistically relevant effects. These are sorted from the largest to the smallest, and the effects to the right of the divisor line are significant. The Pareto chart clearly shows that the variable pH is the most pronounced factor that affects laccase production, followed by the interaction glucose H pH and by glucose and that agitation was not a relevant factor. Additionally, Fig. 2 shows the correlation between the model predicted values of maximum laccase activity and experimental data (observed values).

Factorial design results may be represented through a surface, called response surface. The response surface was used to optimize laccase production. The resulting response surface on laccase production (Fig. 3A–C) shows the effect of two variables, while the third was kept constant. Figure 3 represents a three-dimensional plot of the following variables: glucose concentration and pH (at an agitation of 180 rpm) (Fig. 3A), agitation and pH (at a glucose concentration of 9 g/L) (Fig. 3B), and agitation and glucose concentration (at pH 3.0) (Fig. 3C). The darker color means higher laccase activity. The optimal conditions were found at a glucose concentration and a pH of 11 g/L and 5.2, respectively. Under these conditions laccase activity >10,000 U/L was predicted (Fig. 3A). After obtaining the optimized conditions, it is appropriate to validate the same by running one experiment. Fermentation at the optimal conditions (initial glucose concentration of 11 g/L and pH 5.2) was performed to verify the predicted optimum. The result was a maximum laccase activity of 11,403 U/L, which was in good agreement with the predicted value, showing the adequacy of

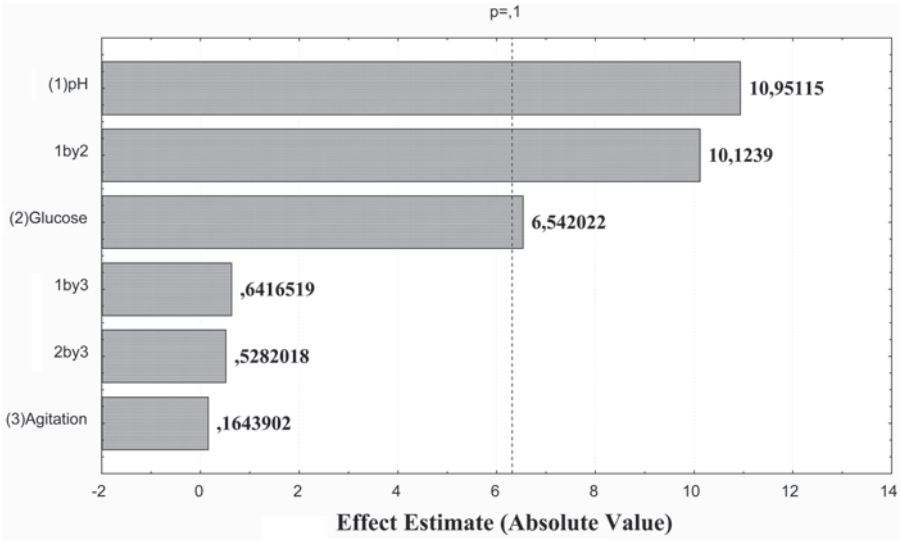

Fig. 1. Pareto chart for 2^3 factorial design.

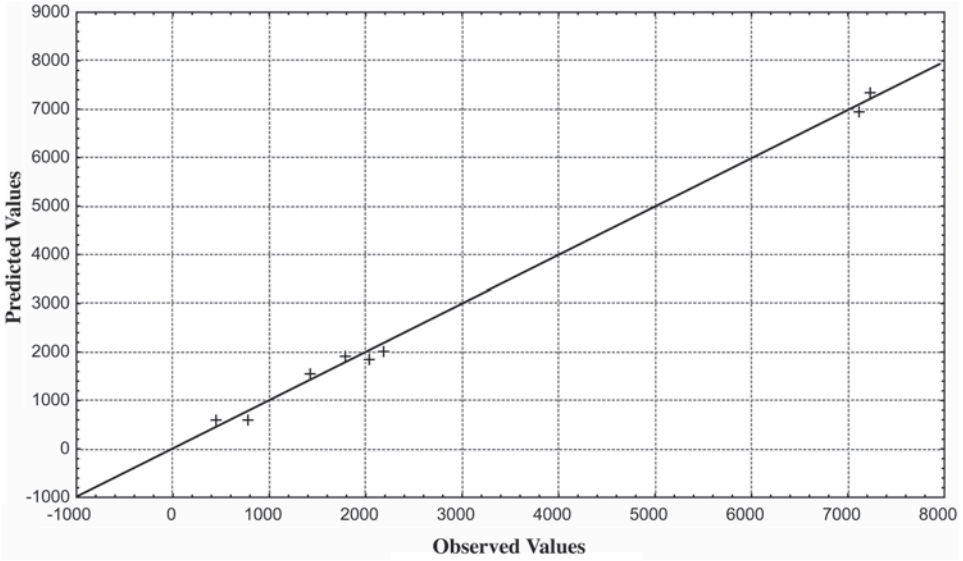

Fig. 2. Correlation between observed and predicted values of laccase activity. +, experimental results.

the experimental design used. Figure 3B,C once more demonstrates that agitation had no individual effect and neither interaction effect on laccase activity in the range studied. The respective fitted function that represents Fig. 3A–C is given by Eq. 7:

$$\text{Laccase activity (U/L)}: 2931.55 - 220.69\ \text{pH} - 1237.77\ G - 11.40\ A + 350.01\ \text{pH} \times G + 2.46\ \text{pH} \times A + 0.46\ G \times A \quad (7)$$

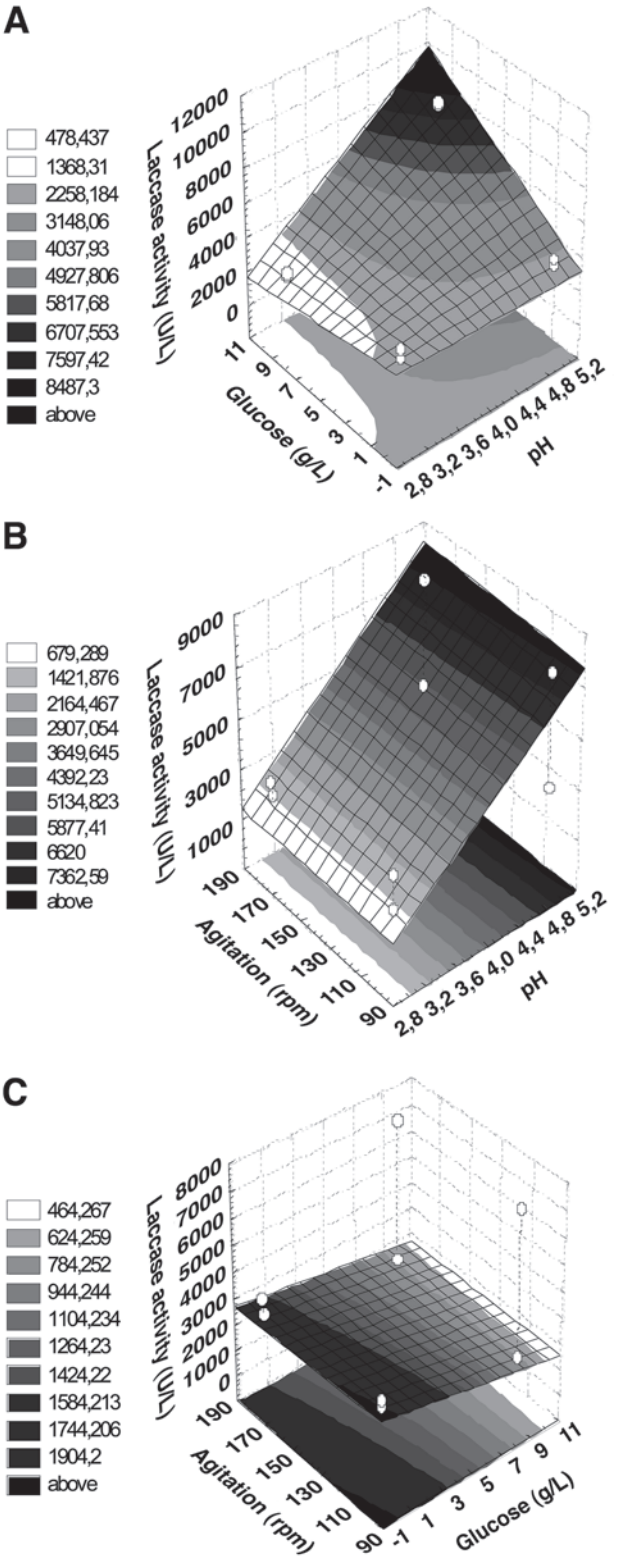

Fig. 3. Response surface plot for laccase production by *T. versicolor* as function of **(A)** glucose and pH, **(B)** agitation and pH, **(C)** glucose and agitation and respective fitted function.

These results indicate that control of the pH of the medium (5.0) is required for attaining high laccase activities. During the fermentations without pH control and with a glucose concentration of 9 g/L, it was observed that the pH decreased rapidly, reaching a minimum of 3.0, which coincided with glucose depletion (data not shown), after which pH increased again. The literature shows that organic acids such as malate, oxalate, fumarate, and glyoxalate are produced during the fermentation of *T. versicolor* as well as of other fungi *(30–33)*. These acids are responsible for the drop in pH. Low pH values of the culture medium are not favorable for laccase production by *T. versicolor*. There could be two explanations for this: (1) either the metabolism of laccase synthesis is repressed at low pH values; or (2) conformational changes in the enzyme's three-dimensional structure are promoted by low pH values, affecting the active site, not allowing biocatalytic reactions.

To identify the reason for the reduction in laccase activity with pH, enzyme stability was studied at pH 3.0 and 4.5. A loss of 50% of laccase activity was observed for pH 3.0 during the first day of incubation, attaining 80% after 3 d, whereas for pH 4.5 after 3 d a loss of only 11% was observed. The control did not present any reduction in activity for 3 dat pH 5.0. When comparing the results for fermentations with glucose at the same agitation (100 or 180 rpm), for pH 3.0 and 5.0 (*see* F3 and F4, and F7 and F8 in Table 2), a very significant loss in activity is observed, corresponding to a decrease of 90–93% in laccase activity for fermentations with a pH of 3.0 (without pH control). The studies of laccase stability at low pH values showed that enzyme degradation is the main cause for the reduction in activity observed but do not exclude that any metabolic inhibition of the laccase production pathway by this fungus at pH 3.0 may take place.

Several studies also show that laccase stability is very dependent on pH and temperature *(34–36)*. Nyanhongo et al. *(37)* reported that laccase stability from *Trametes modesta* is significantly affected by pH values <4.5. Another study by Jönsson et al. *(38)* showed that pH less than 4.0 is detrimental for laccase production and suggests that a possible explanation for this is laccase's susceptibility to acidic proteases.

Galhaup et al. *(39)* reported that for *Trametes pubescens*, when glucose concentration is present in culture medium above a critical value (0.25 g/L), laccase synthesis is repressed and laccase productivity increases considerably when glucose is depleted from the medium. In the present study with *T. versicolor*, when working without pH control and with 9 g/L of glucose maximum laccase production was indeed attained when glucose was exhausted. However, for controlled pH, even with 9 g/L of glucose in the culture medium, a larger laccase biosynthesis was achieved. Contrary to the results reported by Galhaup et al. *(39)* for *T. pubescens*, laccase synthesis was not inhibited by the presence of glucose in high concentrations but by low pH values of the culture media. The drop in laccase activity was a direct result of the lowering of the pH, rather than of a glucose repression mechanism on laccase biosynthesis.

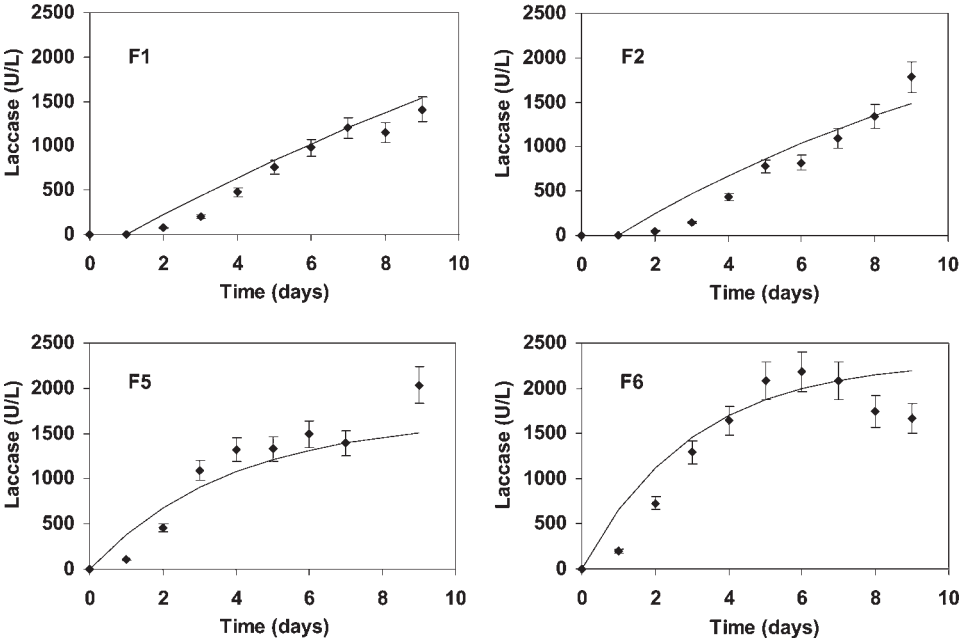

Fig. 4. Experimental and modeled data for laccase production performed in bioreactor by *T. versicolor* at different conditions F1, F2, F5, and F6 (media without glucose). Symbols represent the experimental data and lines represent modeling results.

Bioreactor Modeling

A description of the cell growth, glucose consumption, and laccase synthesis in TDM medium by *T. versicolor* in the bioreactor was achieved using the mathematical model previously described.

Figures 4–6 provide the experimental data for glucose concentration, laccase production, and biomass concentration for the eight conditions studied along with the model results. As shown, a good description of the experimental data was obtained. Figures 4 and 5 show laccase production in medium without glucose (F1, F2, F5, and F6) and with glucose (F3, F4, F7, and F8), respectively. Figure 6 shows the glucose consumption and cell growth. The model used can accurately describe the value of the experimental final biomass, indicating that the model results may be a good description of the inaccessible growth curve. In the best conditions of fermentations, with high glucose concentration and pH control (F4 and F8), laccase production attained maximum activity at d 5, and the culture medium still presented glucose and biomass growth, whereas for a medium with glucose but at pH 3.0 (F3 and F7), laccase activity displayed a continuous increase without attaining the high values presented under higher pH values.

According to the model and final biomass concentration, for the experiments with glucose, the biomass growth of *T. versicolor* was not influ-

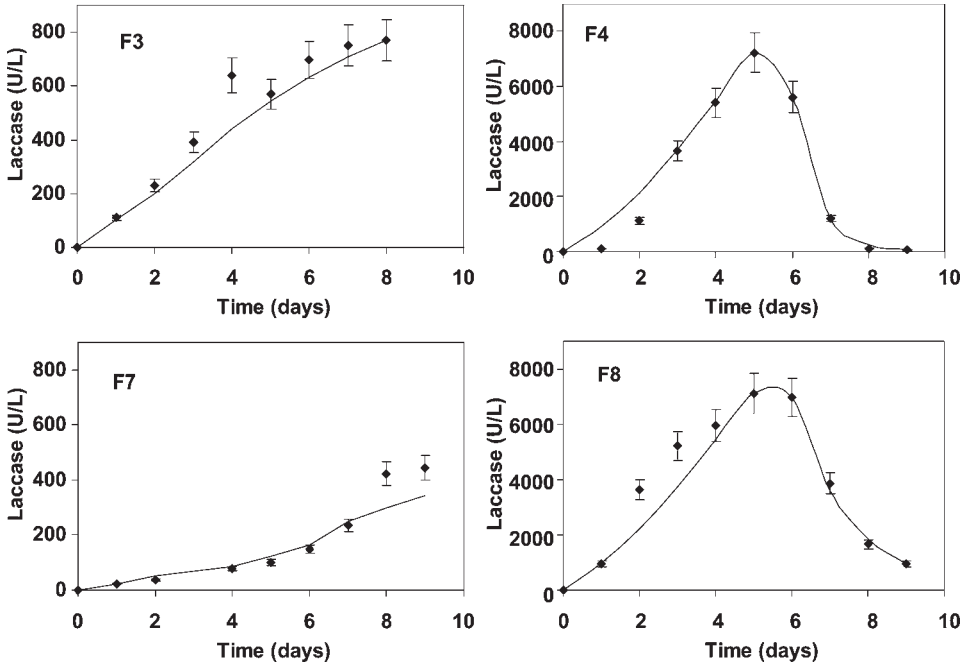

Fig. 5. Experimental and modeled data for laccase production performed in bioreactor by *T. versicolor* at different conditions F3, F4, F7, and F8 (media with glucose). Symbols represent the experimental data and lines represent modeling results.

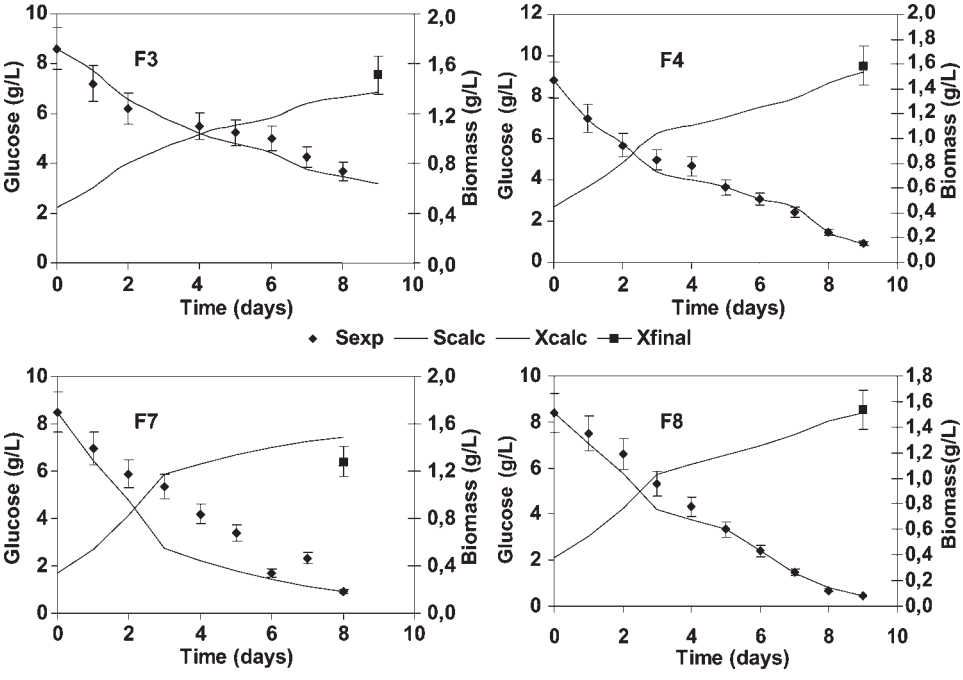

Fig. 6. Experimental and modeled data for glucose consumption (Sexp and Scalc, respectively) and modeled data for biomass growth (Xcalc) performed in bioreactor by *T. versicolor* at different conditions F3, F4, F7, and F8 (media with glucose). Symbols represent the experimental data and lines represent modeling results.

Table 4
Values of Model Parameters
for Laccase Production in Bioreactor by *T. versicolor*

Run	A	K_1 (U/[g cell·d])	K_2 (d^{-1})
F1	1	1443	0.263
F2	1	1293	0.335
F3	0.51	14.3	0.026
F4	0.54	1932	0.667
F5	1	771.3	0.039
F6	1	564.4	0.084
F7	0.85	78.9	0.377
F8	0.64	2088	1.55

enced by the conditions studied, attaining a final concentration of 1.5 g/L, indicating that the increase in laccase activity is not owing to the increase in biomass, but to the different culture conditions. The growth profile was the same for all conditions studied and started the stationary phase at the end of fermentation.

Table 4 provides the kinetic parameters for laccase synthesis (K_1), laccase decay (K_2), and the parameter A. For the fermentations without glucose, the parameter A was set as the unity (1), because the change from the Erlenmeyer flask to the bioreactor environment may affect only the fungal growth, and not the enzymatic synthesis, and under these conditions no cell growth was observed. The data presented in Table 4 demonstrate that *T. versicolor* laccase synthesis was determined essentially by the relation between the presence of glucose and pH control, because for those experiments higher K_1 values were estimated. Once more it can be verified that the influence of the system's agitation did not determine the enzyme production in the range analyzed. K_2 values presented a profile achieved similar to that of K_1; that is, higher K_1 values indicate higher K_2 values, as expected. This behavior can be considered because higher levels of enzyme inactivation were expected with increasing enzyme synthesis.

Conclusion

The experimental design and response surface methodology were successfully used to determine the effect of the main parameters initial glucose concentration, agitation, and pH on laccase production by *T. versicolor* in a bioreactor. These factors led to a substantial increase in laccase yield. The obtained surface methodology optimal culture conditions for laccase production were validated by performing an experiment with such obtained conditions.

The pH of the medium and its interaction with initial glucose concentration were the main factors that affected laccase production, whereas agitation and the interactions agitation × pH and agitation × glucose did not

promote any change in the range studied. pH control seems to be the key factor in improving laccase production.

Using mathematical modeling, it was possible to determine the kinetic parameters of *T. versicolor* fermentation for laccase production. The model used provided a good description of glucose consumption and laccase production, as well as good estimation for biomass growth.

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