J Chem Technol Biotechnol 80:669–676 (2005)
DOI: 10.1002/jctb.1246

Laccase improvement in submerged cultivation: induced production and kinetic modelling

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Abstract: Improvement of laccase production by *Trametes versicolor* was made by employing different operational strategies. In the cell growth medium, various glucose concentrations were compared for improving laccase production. A clear and significant stimulation of enzyme production under carbon limitation was obtained. Copper, 2,5-xylidine, and a phenolic mixture were also used as laccase inducers. A cooperative effect between the inducers on laccase production was identified. Mixtures of inducers produced higher laccase activities, reaching values of 5500 U dm^{-3}. Further productivity enhancement can be obtained using the inducers along with the carbon limitation strategy. It is shown that low laccase concentrations are obtained by a primary metabolism of *T versicolor*, and that phenolic compounds and carbon limitation induce a secondary metabolism, providing higher laccase concentrations. A mathematical model for laccase production based on a direct experimental measure of biomass, along with substrate consumption and enzymatic activity over time is proposed for non-homogeneous fermentations of *T versicolor*.

Keywords: carbon limitation; copper; inducers; laccase; *Trametes versicolor*; modelling

INTRODUCTION

Trametes (Coriolus) versicolor is a white-rot fungus that produces extracellular enzymes including laccase, manganese peroxidase (MnP), lignin peroxidase (LiP), cellulase and avicelase.[1–3] This fungus is a common habitant of dead woods, causing their decay due to the ability to degrade lignin (non-hydrolysable part of wood) using extracellular enzymes. *T versicolor* or its enzymes have a great potential as biocatalysts in oxidative processes: in the pulp and paper industry for delignification and bleaching of Kraft pulps,[4,5] in the textile industry for dye decolorization,[6–8] for wastewater treatments,[9] and for oxidizing lignin models with mediators in order to study mechanisms of biodegradation.[10,11]

Among the enzymes produced by this fungus, laccase (*p*-diphenoloxidase, EC 1.10.3.2) is of particular interest. It is a multi-copper oxidase for phenolic substrates, belonging to a family of enzymes, called the large blue copper proteins, with a copper content that varies between two and four atoms per laccase molecule.[12,13] The substrate range of this enzyme can be extended to non-phenolic subunits by inclusion of a mediator in the reaction mixture.[14]

Enzymatic induction of white-rot fungi is very important since their metabolic activity and growth are dependent on the environmental conditions. The addition of inducers such as xylidine,[15,16] ferulic acid[17] and pyrogallol[18] for *T versicolor*, and copper for the white-rot fungi *Marasmius quercophilus*[19] and *Pleurotus ostreatu*[20] have been found to increase laccase production. Veratryl alcohol and a number of other aromatic compounds have also been reported to enhance production of laccase in fungi.[21,22]

Fungal fermentation is a complex process with many kinetic parameters, most of them not available in the literature. To obtain the optimum conditions for process implementation it is necessary to develop new operational strategies. Mathematical models and optimization of culture conditions can be used as tools to improve enzyme production. Adequate models allow the estimation of kinetic parameters for both process optimization with enzyme production and substrate consumption and simultaneously cell growth prediction. Most of the reported models describe microbial growth in homogeneous culture suspensions, and computing procedures for kinetic parameters of such models are well-known. However, the estimation and the validation of filamentous fungi growth in submerged fermentations are difficult. Non-homogeneous cultures do not allow experimental determination of a representative cell concentration during operation of the batch.[23] There is no reported model for laccase production due to, among others, the difficulty in measuring the myceliar biomass of the fungus.

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(*Received 20 July 2004; revised version received 8 November 2004; accepted 15 November 2004*)
Published online 10 March 2005

The purpose of this work is to improve laccase production by *T versicolor*, employing different operational strategies such as culture medium supplementation, addition of laccase inducers and glucose suppression and also to present a kinetic model for carbon limited and non-limited situations. For the first time a laccase production model based on a direct experimental measure of biomass, along with substrate consumption and enzymatic activity over time, is proposed.

MATERIALS AND METHODS

Microorganism

The white-rot fungus *T versicolor* obtained from the National Institute of Industrial Engineering and Technology (INETI), Portugal, was employed. Its initial mycelial growth was held in Petri plates, on Tien and Kirk medium,[24] at 28 °C for 7 days, before utilization in the experiments.

Media

Experiments were carried on a slightly modified *Trametes* defined medium (TDM), described by Roy and Archibald.[25] The employed TDM contained: 5 mM glutamine, 5 mM NaCl, 5 mM KH_2PO_4, 1 mM $MgSO_4.7H_2O$, 0.1 mM $CaCl_2$, 10 mM dimethyl succinate, 2.4 μM thiamine and 1 $cm^3\,dm^{-3}$ of a trace metals solution containing: 20 μM $FeSO_4.7H_2O$; 2 μM $CuSO_4.5H_2O$; 5 μM $ZnCl_2$; 20 μM $MnSO_4.H_2O$; 6 μM $CoCl_2.6H_2O$; 0.1 μM $NiCl_2.6H_2O$ and 0.5 μM $(NH_4)_6MO_7O_{24}.4H_2O$. Tween 80 (0.5%) was added to stimulate the secretion of extracellular enzyme. The medium's pH was adjusted to 5.0 before sterilization at standard conditions of 121 °C for 21 min.

Inoculation procedure

Mycelium suspensions were obtained from the mycelia initially grown in Petri Plates. Ten cm^3 of modified TDM medium were added to each Petri dish. With a sterile loop the mycelium was collected and suspended on liquid medium from where it was transferred to a flask to obtain a concentrated suspension of cells, the inoculum. A fast determination of dry weight was carried with infra-red light in order to calculate the volume of inoculum to transfer to each of the 500 cm^3 Erlenmeyer flasks, with 250 cm^3 of culture medium. All shake flasks cultures were prepared with an initial cell concentration of 70 $mg\,dm^{-3}$. The flasks were maintained at 28 °C and 180 rpm for 12 days.

Culture conditions for enhancement of laccase production

Different sets of experiments were carried out:

(i) To evaluate the effect of copper on the nutrient media, concentrations up to 150 μM were added during media preparation, and before inoculation, 8 $g\,dm^{-3}$ of glucose were added:
(ii) To study the effect of glucose on laccase production, different glucose concentrations were employed (0–8 $g\,dm^{-3}$). In these last tests xylidine was added on the third day of culture.
(iii) To study laccase inducers, additions to the media were made at the third day of fermentation. Two classical inducers, copper (75 μM) and 2,5-xylidine (30 μM), were studied. Since one of the most promising applications of laccase is for dye decolorization in the textile industry, the potential as inducer of a phenolic mixture (made up of Procion Orange MX-2R, Remazol Red 3B, and Remazol Black GF 3, 150 $mg\,dm^{-3}$ each) was also studied in cooperation with the copper and xylidine. This study was carried (iiia) with and (iiib) without glucose. For organic carbon limitation conditions, glucose was suppressed on the third day by transferring the mycelium, through sterile filtration, to a medium without sugar.

The culture conditions for laccase production by *T versicolor* are summarized in Table 1.

ANALYTICAL DETERMINATIONS

Laccase activity

Laccase production was measured using 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate, at 40 °C. The reaction mixture contained 0.4 mM ABTS in 0.05 mM citrate/0.1mM phosphate buffer at pH 4.5 in a total volume of 2.0 cm^3. Oxidation of ABTS was monitored through absorbance increase at 420 nm ($\varepsilon = 36.000\,M^{-1}\,cm^{-1}$).[26] One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μM of ABTS per minute. The laccase activities were expressed in U dm^{-3}.

Glucose and pH determinations

Glucose quantification was made using 3,5-dinitrosalicylic acid (DNS).[27] The medium pH was monitored using a pHmeter (Crison, Barcelona, Spain, micropH 2000).

Biomass determination

The biomass concentration was determined by dry weight of fungal mycelium. The culture medium was vacuum-filtered through 0.45 μm glass microfibre filter (GF/C, Whatman, Oxon, UK). The biomass retained was washed with distilled water and dried at 100 °C to a constant weight.

BIOPROCESS MODELLING

Growth kinetics parameters of *T versicolor* fermentation were based on two phases of growth: a short period of exponential growth followed by a slow-deceleration profile, as described by Ikasari and Mitchell.[28] A Monod-type model was used for the exponential phase, described by eqn (1a). For the deceleration phase, two parameters were added, described by eqn (1b). The substrate consumption and the

Table 1. Culture conditions for laccase production by *Trametes versicolor*

Batch test	Carbon addition, glucose (g dm^{-3})	Media supplement, copper (μM)	Inducer addition		
			Copper (μM)	Xylidine (mM)	Phenolic mixture (mg dm^{-3})
Control	8.0	—	—	—	—
i	8.0	2.5			
i	8.0	16.0			
i	8.0	75.0			
i	8.0	150.0			
ii	—	—	—	30.0	
ii	1.5	—	—	30.0	—
ii	3.0	—	—	30.0	—
ii	5.0	—	—	30.0	—
ii	8.0	—	—	30.0	—
iiia	8.0	—	2.5	—	—
iiia	8.0	—	16.0	—	—
iiia	8.0	—	75.0	—	—
iiia	8.0	—	150.0	—	—
iiia	8.0	—	75.0	30.0	—
iiia	8.0	—	75.0	30.0	450.0
iiib	—	—	75.0	30.0	—
iiib	—	—	75.0	30.0	450.0

enzyme production were represented by eqns (2) and (3) respectively:

$$\frac{dX}{dt} = \left(\frac{\mu_{max} S}{(K_S + S)}\right) X, \text{ for } t < t_a \quad (1a)$$

$$\frac{dX}{dt} = (\mu_{max} L e^{-k(t-t_a)}) X, \text{ for } t \geqslant t_a \quad (1b)$$

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}\frac{dX}{dt}} \quad (2)$$

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X \quad (3)$$

where X is microbial biomass (g dm^{-3}), S is substrate (g dm^{-3}), P is enzyme (U dm^{-3}), t (day) is time, t_a is the time at the end of the exponential phase (day 3), μ_{max} is maximum specific growth rate (day^{-1}), K_s (g dm^{-3}) is saturation constant, L is survival factor and k (day^{-1}) is the exponential decay constant. Laccase production was considered to be associated with both growth and product by the Luedeking and Piret kinetic model,[29] where α is the growth-associated constant (U g^{-1}) and β the non-growth associated constant (U g^{-1} day^{-1}). This assumption is based on the experimental observation that concentration of the secreted laccase in the medium is not proportional to cell concentration. The differential equations were solved using MATLAB v.6.1 (The Mathworks Inc).

The parameters μ_{max}, K_S, $Y_{X/S}$, k, L, α and β were estimated with the MATLAB *Optimization Toolbox* by minimization of quadratic residuals between the experimental and model data, using the objective function shown in eqn (4). The Simplex—Nelder & Mead search method was employed in the optimization procedure. Experimental errors associated with cell and laccase activity were estimated to be 10% through replication performed in the shaker. Statistical significance of estimated parameters was determined by the t-test at a 95% confidence level.

$$\text{Minimize}_{\mu_{max},K_s,Y_{X/S},L,k,\alpha,\beta} \sum_{i}^{ne}\sum_{j}^{np}([X]_{exp} - [X]_{mod})^2 + ([S]_{exp} - [S]_{mod})^2 + ([P]_{exp} - [P]_{mod})^2 \quad (4)$$

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

RESULTS AND DISCUSSION

Effect of initial copper and glucose concentrations on laccase production

To improve laccase production by *T versicolor* in batch culture, different copper and glucose concentrations on the modified TDM medium were investigated. Copper addition in different culture media has been reported to enhance laccase production.[30,31] Low copper concentrations were chosen following the method of Giardina *et al*[32] who used 150 μM. In this study, copper concentrations up to 150 μM were tested to determine their effect on laccase production. The optimal concentration of copper was found to be 75 μM, leading to a four-fold increase of laccase production when compared with the control without copper. For higher copper concentrations, the laccase activity decreased (data not shown). This may be due to a deleterious effect of copper on the fungal metabolism. Thus, a concentration of 75 μM was selected for further studies.

The effect of the glucose concentration on the laccase production, with xylidine as inducer, is reported in Fig 1. These data show that laccase production

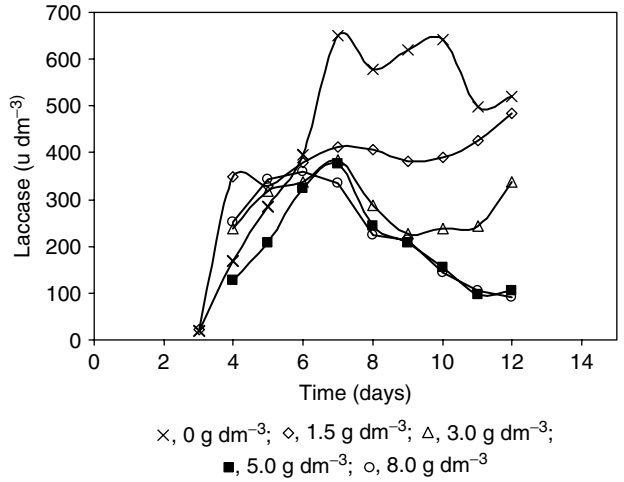

Figure 1. Laccase activity for *Trametes versicolor* batch cultures for different initial glucose concentrations.

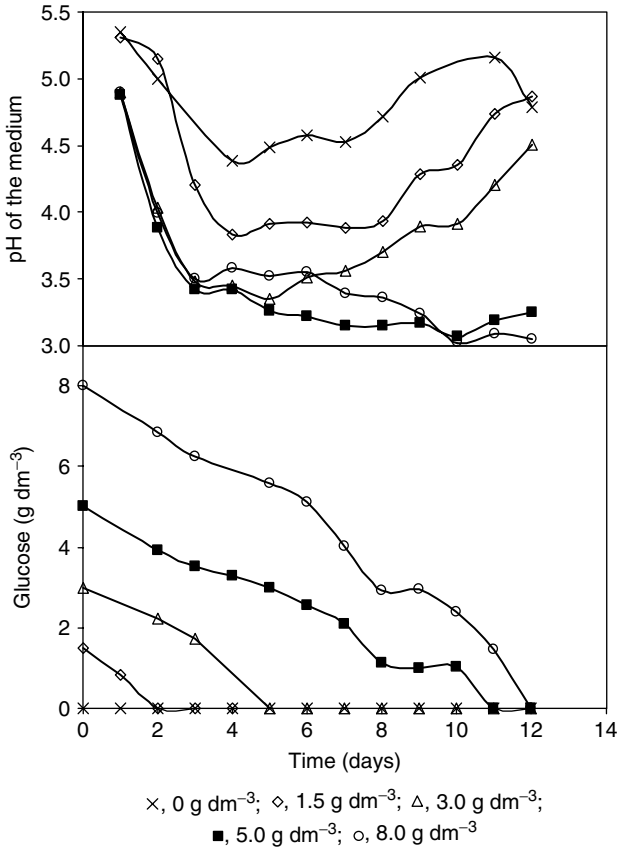

Figure 2. pH changes and glucose consumption during laccase production for *Trametes versicolor* batch cultures for different initial glucose concentrations.

is strongly dependent on glucose concentration. The fungus has a characteristic behaviour with a maximum laccase activity, attained on seventh day of fermentation, decreasing with increasing glucose concentrations and showing a maximum value of 650 U dm^{-3} for the carbon limitation condition

The pH evolution and glucose consumption during these experiments, are presented in Fig 2. The pH evolution shows that there are two distinct types of behaviour for the different experiments, strongly supporting the idea of a change in the *T versicolor's* metabolism. In all cases, the pH decreases while the fungus is using the glucose and stabilizes as soon as glucose exhaustion occurs. Accordingly, for the higher glucose concentration (8 g dm^{-3}), the sugar continues to decrease until the last day of fermentation, and the pH also continues to decrease, attaining values of 3 by the end of the process. These results indicate an association of the pH decrease with the glucose consumption by *T versicolor*. The pH decrease due to the synthesis of various organic acids, by the fungal primary metabolism,[25,33] and the pH increase that takes place afterwards indicates that an alternative secondary metabolism is taking place.

Figure 3 presents maximum laccase concentrations attained, respective pH values and also final biomass for each assay with different initial glucose concentrations. The final biomass concentration, a direct result of cell growth and primary metabolism, is dependent on the initial glucose concentration. This figure shows that laccase production is clearly associated with pH. In fact, there is a synchronism when comparing

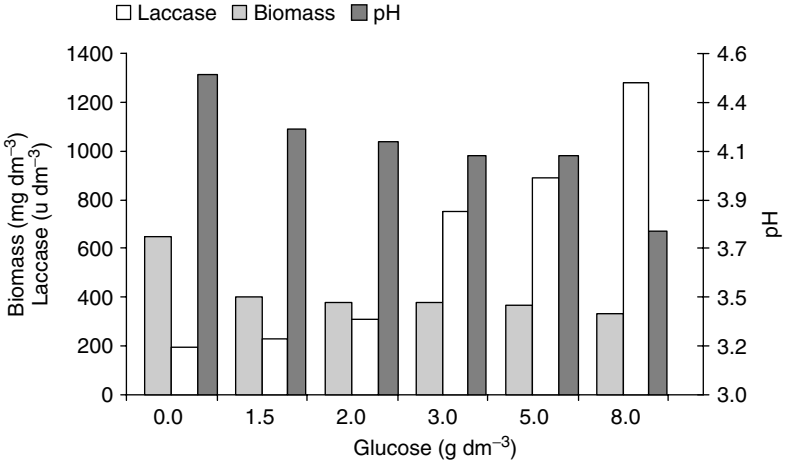

Figure 3. Final total biomass for *Trametes versicolor* batch cultures, maximum laccase activity and pH for different initial glucose concentrations.

maximum laccase concentration attained and respective pH. Assays with high glucose concentrations, where the fungus performs primary metabolism for a long time, decrease the pH and consequently inhibit laccase production secondary metabolism. Galhaup *et al*[31] also noticed that laccase production in *Trametes pubescens* took place only when glucose was depleted from the medium. This indicates that higher glucose concentrations inhibit laccase production. Ruel *et al*[34] suggested that the primary metabolism in the absence of inducer produces low laccase concentrations. The production of laccase in high concentrations may be a result of a secondary metabolism of *T versicolor,* not dependent on the fungal growth, but driven by an external stimulus.[35]

Effect of laccase inducers

Another factor that improves laccase production by white-rot fungi are inducers.[36] Using an adequate initial glucose concentration, different inducers were tested in TDM media with and without glucose. Three inducers for laccase production were studied: copper, xylidine and a phenolic mixture. The inducers were introduced in the medium on the third day of fermentation, when the fungus was already adapted to the media conditions, and exponential growth was starting. Those components included on the growth media are considered as micronutrients.

Considering that *T versicolor* laccase is a blue copper protein,[37] an increase in laccase activity by cultures employing copper as inducer was expected. The results herein described demonstrate that copper only induces an increase in laccase activity, from 66 to 190 U dm^{-3} over the control sample, without inducer, as shown in Fig 4. This is in accordance with the results of Collins and Dobson.[31] This does not correspond to the change of metabolism that, as suggested above, enhances the laccase productivity but only to an increase in laccase production by the primary metabolism. The results show that copper acts as a micronutrient on laccase production rather than as an inducer. Trupkin *et al*[38] have also studied the effect of copper as inducer and an enhancement on laccase production was reported with increased concentrations of copper. They state that copper is toxic for most fungi but that it can act for *T versicolor* as a laccase inducer with the laccase production possibly being a defence mechanism against oxidative stress.

Following Eggert *et al*[39] who used 10 μM of xylidine to induce laccase production on an other fungus, some experiments were carried out for concentrations between 5 and 50 μM. The concentration of 30 μM of xylidine was selected for *T versicolor* laccase induction since higher concentrations showed a toxic effect. The addition of xylidine in such a concentration produced laccase activities which were higher (from 190 to 360 U dm^{-3}) than those induced by copper, as shown in Fig 4. However, a cooperative effect between copper and xylidine was found. The simultaneous presence of both inducers further enhances the laccase production over its individual action (4.4 times over copper and 2.3 over xylidine). This is in agreement with results reported by Collins and Dobson.[31] The same cooperative effect of these two inducers in solid state cultures was also reported for *T versicolor* and *Trametes hirsuta*.[40]

Since laccase is an oxidizer acting on phenolic compounds, a complex phenolic mixture was employed to further enhance the effect of the inducers previously used. This phenolic mixture was previously tested by Amaral *et al*[8] for textile decolorization and it promoted a high production of laccase. A significant increase in

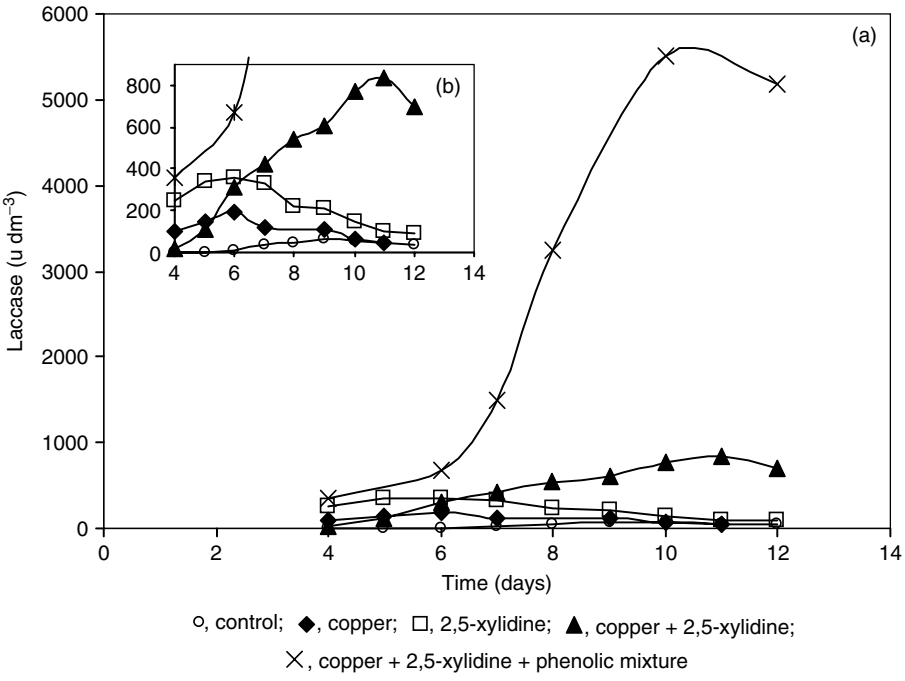

Figure 4. Laccase activity for *Trametes versicolor* batch cultures with different components on the medium (a). (b) Scale amplification of (a).

the enzymatic activity from 850 U dm^{-3}, in a medium with copper and xylidine, to 5500 U dm^{-3} (6.5 times) in the presence of this phenolic mixture was obtained.

Inducers with phenolic structures seem to allow this white-rot fungus to respond to a signal recognition, leading an intensive biological response that activates a secondary metabolism responsible for improving the lignolytic enzyme production mechanism.

Effect of carbon limitation in the laccase production using inducers

As reported above the organic carbon limitation stimulates laccase production by *T versicolor*. Results for the best induction conditions (copper + xylidine and copper + xylidine + phenolic mixture) are presented in Fig 5 for both presence and absence of sugar as a comparative study.

The enzyme activity obtained with copper and xylidine combined increases about three times (from 850 to 2700 U dm^{-3}) under carbon limitation conditions. A similar effect, although less pronounced, is also achieved when a complex phenolic mixture is employed. An increase of around 25% in laccase productivity (calculated on maximum of laccase activity) was obtained and an anticipation of 2 days on the maximum enzyme release was observed.

Table 2 shows both laccase productivity and its enhancement when different inducers are added alone or combined. The application of a glucose suppression strategy leads to an increase in enzyme productivity of 358% for copper and xylidine combined and an increase of 138% for a combination of all inducers. The result obtained for productivity enhancement in a carbon limitation strategy is better (104 times) for a combination of all inducers than just for copper and xylidine (37 times).

The addition of phenolic compounds stimulates the fungus to develop protection mechanisms to fight against their toxic effect. These results show the necessity of adding other components to *T versicolor* medium to improve laccase production and to permit the application of *T versicolor* in the reduction of the phenolic content during bioremediation or wastewater treatments as well as on valorization, of lignocellulosic compounds.

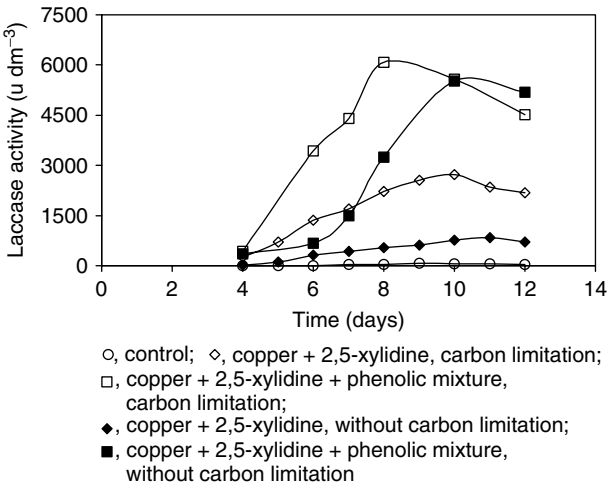

Figure 5. Laccase activity for cultures of *Trametes versicolor* with and without carbon limitation.

Table 2. Laccase productivity and laccase productivity enhancement for addition of inducers and carbon limitation in culture media of *Trametes versicolor*

Inducer	Productivity (U dm^3 day^{-1})	Productivity enhancement
Without carbon limitation		
Control	7.3	1.0
Copper	31.8	4.4
2,5-Xylidine	60.0	8.2
Copper + 2,5-xylidine	76.0	10.4
Copper + 2,5-xylidine + phenolic mixture	552.1	75.5
With carbon limitation		
Copper + 2,5-xylidine	272.3	37.3
Copper + 2,5-xylidine + phenolic mixture	759.8	103.9

Bioprocess modelling

The laccase production by *T versicolor* is not directly related to growth but instead is a complex process system. The results above show the importance of inducers and glucose concentration on laccase productivity. In order to study the fungal behaviour, namely cell growth, laccase production and glucose consumption on carbon limited and non-limited situations, with xylidine induction, mathematical modelling was carried to obtain the kinetic parameters of fermentation.

For the development of the model for cell growth, substrate consumption and enzyme accumulation it was necessary to consider the existence of two phases of mycelium growth: an exponential growth followed by a slow-deceleration profile. On the exponential growth phase there is a primary metabolism with biosynthesis associated with growth and biomass increase. The enzymatic production should be directed towards enzymes involved in growth pathways. In the slow-deceleration phase a different profile of enzymes is produced due to the conditions required to develop different metabolic pathways.

The model proposed by Ikasari and Mitchell,[28] for solid state fermentation, was adapted for this fermentation in submerged culture since the cell growth presented a similar profile. The model equations for these two phases of growth were developed separately.

The model was fitted to the experimental data using the approach described above. The kinetic parameters for laccase production by *T versicolor* with carbon and with carbon limitation are presented in Table 3 and the comparison of the model with the experimental data for the different media is presented in Figs 6 and 7. The results show that the maximum specific growth rate, μ_{max}, is very similar for both media: 0.87 day^{-1}

Table 3. Fermentation kinetics parameters of *Trametes versicolor* in different culture media

Kinetic parameter	Medium without carbon limitation	Medium with carbon limitation
μ_{max} (day^{-1})	0.87	0.82
K_s ($g\,dm^{-3}$)	4.21	1.30
$Y_{X/S}$ ($g\,g^{-1}$)[1]	0.17	0.38
L	0.37	0.41
k (day^{-1})	0.0921	0.253
α ($U\,g^{-1}$ cell)	0.059	0.147
β ($U\,g^{-1}$ cell day^{-1})	62.9	499

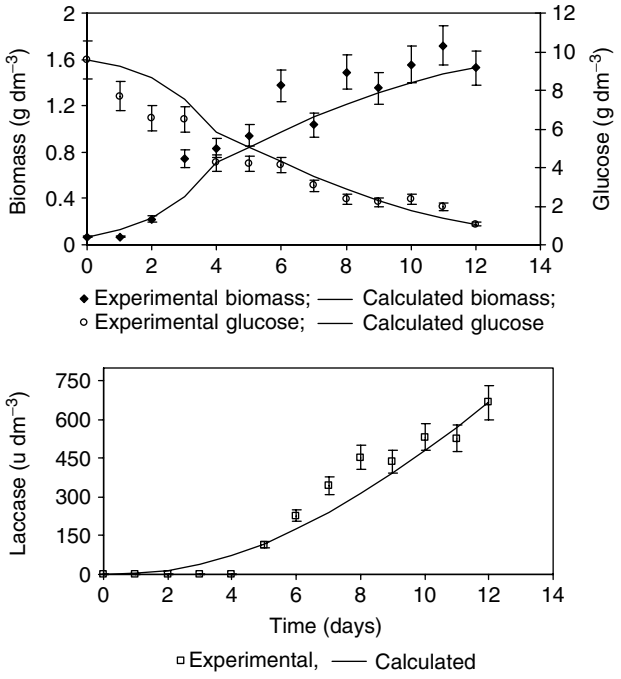

Figure 6. Experimental data and simulation of batch culture of *Trametes versicolor* on the medium with carbon.

for the medium with carbon and $0.82\,day^{-1}$ for the medium with carbon limitation. In fact, the survival mechanism just after inoculation must be very similar in both situations. On the other hand the substrate saturation constant (K_s) is three times higher for the medium with carbon since this saturation constant is related to the maximum of biomass attained, which show the same relationship. The medium affects the parameter values, L and k, during the deceleration phase. The value of k, constant of exponential decay, obtained on the medium with carbon limitation, is 2.7 times higher than for the medium with carbon, indicating that a higher deceleration in the cell growth occurred when carbon was present. Since during the exponential phase, the culture medium was the same, the parameter L (ratio of the specific growth rate at the start of deceleration phase to the specific growth rate during the previous exponential phase),[23] was practically the same for both media.

For laccase production, the Luedeking–Piret model (eqn (3)) was adopted because, as previously discussed, laccase is not a product from the primary

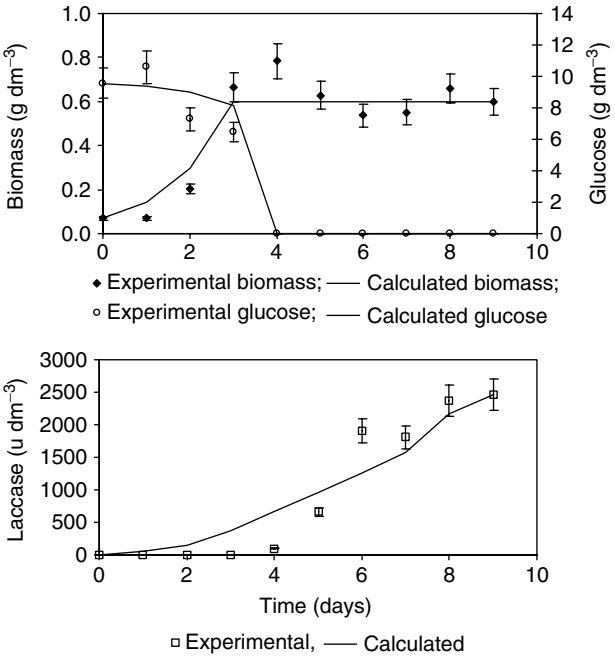

Figure 7. Experimental data and simulation of batch culture of *Trametes versicolor* on the medium with carbon limitation.

metabolism but from the secondary one. The results presented in Table 3 show that the α value is very low ($\alpha = 0.059$ or $\alpha = 0.147\,U\,g^{-1}$ cell) when compared with β ($\beta = 62,9$ or $\beta = 499\,U\,g^{-1}$ cell day^{-1}), meaning that for both media laccase production only started at the slow-deceleration phase of cell growth after the third day. This laccase production model is in accordance with the definition of the two distinct phases for biomass growth previously presented.

This kinetic model for *T versicolor* is here applied for the first time to laccase production. It accurately correlates microbial growth, substrate consumption and laccase synthesis in submerged non-homogeneous fermentations and certainly can be applied to different laccase-producing cultures.

ACKNOWLEDGEMENTS

The authors acknowledge the Instituto Nacional de Engenharia e Tecnologia Industrial (INETI) for *Trametes versicolor* cultures. APM Tavares acknowledges Fundação para Ciência e Tecnologia (FCT-Portugal) for a PhD grant (SFRH/DB/6606/2001) and MAZ Coelho thanks CNPq (Brazil).

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