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Production of lignocellulolytic enzymes from three white-rot fungi by solid-state fermentation and mathematical modeling

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Three species of white rot-fungi (*Pleurotus ostreatus*, *Coriolus versicolor*, and *Lentinula edodes*) were grown on 12 solid media based on several lignocellulosic materials (oak sawdust, coconut husks, coffee husks and corn bran) during 49 days. The media had varied carbon/nitrogen ratios and $CuSO_4$ content. The objective of the work was to evaluate the effect of the media formulation on the production of lignocellulolytic enzymes and degradation of lignocellulosic components by the three fungal species. *C. versicolor* exhibited the highest ability to degrade the three main polymers of the lignocellulosic waste materials employed and to produce ligninases with titers as high as 107 U/g solid substrate in the case of laccase. In addition, a mathematical model describing the fermentation kinetics of the cell biomass growth, degradation of lignocellulosic components, and lignocellulolytic enzyme production for the fungal species/medium combination exhibiting the best performance under solid-state fermentation conditions was proposed and validated in the case of *C. versicolor*. The mathematical model could be used to provide valuable information on the process itself as well as to contribute to the development of a future commercial process for lignocellulolytic enzyme production.

Key words: White-rot fungi, cellulases, ligninases, degradation of lignocellulosic materials, fermentation kinetics.

INTRODUCTION

The lignocellulolytic enzymes have a wide spectrum of applications in several industrial sectors. These enzymes are being used for breakdown of lignocellulosic materials (particularly, agricultural and agro-industrial wastes) into fermentable sugars, which can be converted into valuable products like ethanol, lactic acid and butanol. In particular, cellulases are currently used at pilot and semi-industrial plants during the hydrolysis of cellulose derived from lignocellulosic biomass for fuel ethanol production (Sánchez and Montoya, 2012). Cellulolytic enzymes are also

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employed for improvement of paper texture, biodeinking during paper recycling, biopolishing of the fabrics, and detergent production. In the same way, ligninases are used for delignification of forage grass and agricultural wastes for animal feed (Ruqayyah et al., 2013). Ligninolytic enzymes are also used to decolorize synthetic dyes contained in wastewater from textile industry (Grassi et al., 2011). In fact, ligninases are being involved in the transformation and mineralization of organopollutants with lignin-like structures, so they have been proposed to treat and degrade polychlorinated biphenyl pesticides, polycyclic aromatic hydrocarbons, effluents from bleaching mills and synthetic polymers, among others (Pointing, 2001).

The so-called white-rot fungi synthesize and secrete into the media based on lignocellulosic materials (liquid or solid) an important amount of cellulases, xylanases, and ligninases. For this reason, the cultivation of these fungi under conditions of submerged or solid-state fermentation is being intensively studied. Basidiomycetes like *Pleurotus ostreatus* (Carabajal et al., 2012), *Coriolus versicolor*, and *Lentinula edodes* (Elisashvili et al., 2008) produce the above-mentioned enzymes using different lignocellulosic feedstocks, especially agro-industrial wastes. The enzymes released by these fungi synergistically act on the lignocellulosic complex in order to degrade it and generate the sugars and other compounds needed for their development. The cellulose biodegradation implies the initial action of the endoglucanases (ENG) and exo-cellobiohydrolases (also called exoglucanases, EXG), which attack the amorphous and crystalline structure of the cellulose forming cellotrioses and cellobiose. These oligosaccharides are later converted into glucose by the β-glucosidase. The hemicellulose (xylan) hydrolysis requires the action of the endoxylanase (ENX) and β-xylosidase, although other enzymes that hydrolyze the substituted xylans are needed to completely degrade this polysaccharide. The lignin is broken down by a non-hydrolytic mechanism through the interaction of different extracellular enzymes, mainly phenoloxidases like laccase (LAC), and peroxidases like manganese peroxidase (MnP) and lignin peroxidase.

The production of lignocellulolytic enzymes by white-rot fungi depends on several different factors. In particular, the micro-environmental and nutritional conditions in the substrate (that is, lignocellulosic materials) directly affect the growth rate of each one of the fungal species employed. Thus, the composition of the media on which these fungi are cultivated plays a crucial role during the synthesis of lignocellulolytic enzymes. In particular, the titers of oxidoreductases such laccase and manganese peroxidase are influenced by the concentration of metals like copper in the culture medium. Although it is not a common fact that the manganese peroxidase is induced with a metal other than manganese, Mouso et al. (2003) already has shown that copper and cadmium induce the synthesis of this enzyme in *Stereum hirsutum*, and Levin et al. (2002) have demonstrated that laccase and manganese peroxidase production is enhanced by adding $CuSO_4$ to the medium in the case of *Trametes trogii*. On the other hand, the carbon/nitrogen (C/N) ratio is an important factor when white-rot fungi are cultivated to produce lignocellulolytic enzymes, especially under solid-state fermentation (SSF) conditions. For instance, Bento et al. (2014) pointed out that an appropriate C/N ratio during solid-state cultivation of macromycetes like *L. edodes* is critical to achieve the complete colonization of the substrate and suitable levels of ligninolytic activity. Therefore, the selection of the solid materials (substrates) and the determination of the medium composition are crucial to attain a significant amount of active lingo-cellulolytic enzymes.

The mathematical modeling of the cultivation process has a paramount importance in order to develop an efficient fermentation process at pilot scale or industrial level. Several mathematical expressions have been proposed to describe the fungal growth. These models involve linear or logistic expressions for growth rate. However, these models do not include the effect of the nutrient concentration on the fungal development (Ikasari and Mitchell, 2000; van de Lagemaat and Pyle, 2005). Likewise, Monod-type and modified logistic expressions have been employed to describe the biomass growth for different fermentation types (Mitchell et al., 2004; Tavares et al., 2005). The mathematical expressions used to model the product formation depend on the type of metabolite synthesized by the fungus. For primary metabolites, the models are simple since a directly proportional relationship between growth and product formation rates is generally assumed. The biosynthesis of intermediary and secondary metabolites is much more complex to describe because there exist no well-defined simple expressions covering the formation of these compounds. Currently, there are not available mathematical models allowing the description not only of the growth of macromycetes fungi, but also of the biosynthesis of enzymes obtained from them during SSF. This difficulty is related to the reduced knowledge on the combined influence of factors like the physical characteristics and chemical composition of the solid media employed, environmental conditions, features of the metabolic pathways involved, and presence of inhibitors, among others.

The objective of this work was to evaluate the effect of the carbon/nitrogen ratio and copper sulfate (II) concentration on production of lignocellulolytic enzymes by three white-rot fungi species (*P. ostreatus*. *C. versicolor* and *L. edodes*) grown on 12 different solid media based on lignocellulosic substrates. In addition, this work was aimed at proposing and validating a mathematical model describing the fermentation kinetics of the cell biomass growth, degradation of lignocellulosic components, and lignocellulolytic enzyme production for the fungus/medium combination exhibiting the best

Table 1. Carbon/nitrogen (C/N) ratios and copper sulfate (II) concentrations employed for formulations of twelve solid media.

Formulation	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
$CuSO_4$ (wt.%)	0.16	0.08	0.16	0.08	0.16	0.08	0.16	0.08	0.16	0.08	0.16	0.08
C/N ratio	142.6	89.7	114.6	93.6	100.9	91.8	85.3	84.9	55.1	51.6	117.1	111.0

The solid media contained (dry basis) 40% oak sawdust, 20% coconut husks, and 2% soybean oil. In addition, the media contained coffee husks and corn bran, which were used to adjust the C/N ratio.

performance under SSF conditions.

MATERIALS AND METHODS

Microorganisms

Three species of basidiomycetes were employed for production of lignocellulolytic enzymes by SSF. These species are deposited at Culture Collection of Macrofungi at Universidad de Caldas (Manizales, Colombia). *L. edodes* CICL54 strain was donated to this collection by the National Coffee Research Center, Cenicafé, located in Chinchiná (Colombia). *C. versicolor* PSUWC430 strain was acquired to the Pennsylvania State University (USA). *P. ostreatus* UCC001 strain belongs to the collection. The species were maintained on potato dextrose agar (PDA) at 4°C with periodic transfer.

Spawn production

The three fungal species were adapted by inoculating wet wheat grains with five pieces of 1.0 cm^2 of the mycelium extended on PDA until complete colonization. Spawn of all the species evaluated was prepared on wheat grains previously cleaned and hydrated until 40% moisture content. The wheat grains were packed in bi-oriented polypropylene bags. Each bag of 20 cm diameter and 30 cm height contained 1 kg of solid substrate. One square hole with 2.54 cm side was made at the top of each bag and covered with a microporous breather strip to allow for gas exchange. The hydrated grains contained in the bags were tyndallized at 121°C for 30 min. Then the grains were aseptically inoculated with 4% (wet basis) of each fungal species previously adapted as described above and incubated for 12-15 days at 25°C until complete colonization.

Culture media

Twelve (12) different formulations of the solid media for SSF named F1, F2,..., F12 were employed. These media were formulated on dry basis with 40% oak sawdust, 20% coconut husks and 2% soybean oil as fixed components. The concentration of these fixed components was defined in a previous work (Montoya, 2012). The remaining components (coffee husks and corn bran) were varied in order to modify the C/N ratio as shown in Table 1. The concentration of copper sulfate (II) was varied on two levels: 0.16 wt.% for odd formulations and 0.08 wt.% for even formulations. All media were formulated at 60% moisture. The media were packed in bi-oriented polypropylene bags with 200 g solid material and tyndallized at 121°C for 30 min. Then the media were inoculated with 4% (wet basis) spawn related to the solid mass in laminar flow chamber, and incubated at 25°C under dim light for 49 days. Fifteen (15) samples were taken (two per week); for each sample, three replicas from different bags cultivated under the same conditions were performed.

Carbon/nitrogen ratio was determined based on the measurement of the organic matter in the 12 formulations using the method reported by Walkley and Black (1934), which employs concentrated sulfuric acid and a potassium dichromate solution. The sample is read at 585 nm. The total carbon content corresponds to the 58% organic matter. The total organic nitrogen was determined by Kjeldahl method (Kjeldahl, 1883).

Quantitative determination of enzymatic activities

Extracts for the determination of enzyme activities were obtained from 1 g fresh substrate in 12 mL sterile neutral distilled water. Then the resulting suspension was sonicated for 5 min and stirred for 10 min, with subsequent filtration and centrifugation.

Cellulolytic and xylanolytic activities

Endo-1,4-β-*D*-glucanase (EC 3.2.1.4): A reaction between 0.5% carboxymethyl cellulose (CMC) in sodium acetate buffer solution with a pH of 4.8 and 100 µL enzymatic extract is performed for 30 min at 50°C. The enzymatic reaction was stopped by adding 3,5-dinitrosalicilic acid (DNS). Immediately after this, the same DNS added is used for determination of reducing sugars according to the method of Miller (1959) for which the absorbance is read at 540 nm. One unit of enzyme activity (U) was defined as the amount of enzyme that produces 1 µmol reducing sugars per minute. A calibration curve of reducing sugars constructed from different glucose concentrations was used to quantify the enzyme activity.
Exo-1,4-β-*D*-glucanase (EC 3.2.1.91): A reaction between 1% crystalline cellulose in sodium acetate buffer solution with a pH of 4.8 and 100 µL enzymatic extract is performed for 60 min at 50°C. The enzymatic reaction was finished by adding DNS. Immediately after this, the same DNS added is used for determination of reducing sugars according to the method of Miller (1959). The samples were centrifuged before reading the absorbance at 540 nm. One unit of enzyme activity (U) was defined as the amount of enzyme that releases 1 µmol reducing sugars per minute.
β-glucosidase (EC 3.2.1.21): A reaction between 0.02% *p*-nitrophenyl β-*D*-glucopyranoside (the substrate) in sodium acetate buffer solution with a pH of 4.8 with 100 µL enzymatic extract is performed for 30 min at 50°C. The enzymatic reaction was finished by adding Clark and Lubs buffer solution with pH of 9.8 and reading the absorbance at 430 nm (Wood and Bhat, 1988). One unit of enzyme activity (U) was defined as the amount of enzyme that produces 1 µmol *p*-nitrophenol per minute. A calibration curve was constructed using *p*-nitrophenol in order to quantify the enzyme activity.
Endo-1,4-β-*D*-xylanase (EC 3.2.1.8): A reaction between 0.2% xylan in sodium acetate buffer solution with pH of 4.8 and 100 µL enzymatic extract is performed for 30 min at 50°C. The enzymatic reaction was stopped by adding DNS. Immediately after this, the same DNS added is used for determination of reducing sugars according to the method of Miller (1959) for which the absorbance is read at 540 nm. One unit of enzyme activity (U) was defined as

the amount of enzyme that produces 1 μmol reducing sugars per minute. In this case, the calibration curve was constructed using xylose.

Ligninolytic activities

Laccase (EC 1.10.3.2): A reaction between 0.5 mM ABTS (2.2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate)) solution in 0.1 M sodium acetate buffer with a pH of 3.6 and the enzymatic extract was performed according to the method of Paszczynski and Crawford (1991). The absorbance was read at 420 nm after 3 min of reaction at 30°C, time needed for color change from transparent to turquoise green. One unit of enzyme activity (U) was defined as the amount of enzyme required to oxidize 1 μmol ABTS in 1 min.

Manganese peroxidase (EC 1.11.1.13): The substrates of the enzymatic reaction were 0.01% phenol red in 0.1 M sodium succinate buffer solution with a pH of 4.5 and manganese sulfate (0.22 g/L) with 0.2 mM hydrogen peroxide as initiator. After 10 min of reaction of these substrates with the enzymatic extract, the process is finished by adding 5 N NaOH in order to read the absorbance increase at 610 nm (Paszczynski et al., 1988). One unit of enzyme activity (U) was defined as the amount of enzyme needed to oxidize 1 μmol red phenol in 1 min.

Chemical analyses

During the incubation of each solid medium (substrate), 15 samples were collected, two per week, until the 49th day for each one of the three fungal species (*C. versicolor*, *P. ostreatus* and *L. edodes*). The samples were dried at 101°C until constant weight, ground and stored for determination of chitin content. The content of fungal biomass in the solid media was indirectly estimated based on the determination of the structural component of chitin, the N-acetyl-*D*-glucosamine (NAGA), after hydrolysis with 6 N HCl according to the method of Plassard et al. (1982). The NAGA content per gram of mycelium for each one of the three species grown in liquid medium was determined in a parallel way; for this, 250 mL flasks containing 100 mL liquid medium were used. The liquid medium employed for these purposes had the following composition (in g/L): glucose, 30; yeast extract, 6; $MgSO_4.5H_2O$, 0.5 and $CaCl_2$, 0.1. The NAGA content for the mycelium grown in liquid medium was determined according to the method of Plassard et al. (1982) as well.

The fiber components (cellulose, hemicellulose and lignin) for each one of the formulations of the solid media and for all the 15 samples collected during the cultivation of each fungal species, as well as the soluble fraction, were determined by using the results of the determination of neutral detergent fiber, acid detergent fiber, and acid detergent lignin. To determine these fiber types, each dried and ground solid sample underwent three hydrolysis in series during 70 min each: i) hydrolysis with sodium lauryl sulfate and others; ii) hydrolysis in ammonium bromide in 1 N sulfuric acid solution; and iii) hydrolysis with 72% (w/v) sulfuric acid. At the end of each hydrolysis, samples were washed and dried at 105°C until constant weight (Leterme, 2010). The content of reducing sugars as glucose was determined by the DNS method (Miller, 1959).

Experimental design

In order to find the most appropriate combination of the three fungal species and 12 medium formulations having the best performance in terms of lignocellulolytic enzyme production and degradation of lignocellulosic components of the substrates, a randomized bi-factorial experimental design was used with three levels for the first factor (fungal species) and 12 levels for the second factor (formulation of the solid medium). The formulations (F1 to F12) varied their C/N ratio and the copper sulfate (II) concentration as shown in Table 1. The response variables were six enzyme activities (ENG, EXG, BG, ENX, LAC, and MnP), and the fiber components (content of cellulose, hemicellulose, and lignin in the samples). The response variables for each fungus/formulation combination were measured for 15 incubation times during the fermentation process. Thus, three different experimental series were performed (one series for each fungal species) by varying the medium formulation. Each sample was taken by triplicate from different bags cultivated under the same conditions. All the statistical analysis for this work were performed by using the software Matlab® 2010b (MathWorks, USA). The analyses were carried out in three steps. Firstly, an analysis of variance (ANOVA) was done for all the data obtained during the experimental design considering all the fungus/formulation combinations with 5% significance level. For this, the anovan Matlab function was applied. Then a comparative Kruskal Wallis analysis for degradation of cellulose, hemicellulose and lignin was performed for each one of the three fungal species studied by using the kruskalwallis and multcompare functions. Once the degradation maximum was determined for each species, a new comparative analysis to find the combination with the best performance in terms of fiber degradation at the end of fermentation (49th day) was carried out.

Mathematical modeling of fungal growth and enzyme production

Matlab® was also used to solve the mathematical models proposed in this work. For this, the ode45 function based on an explicit Runge-Kutta (4,5) formula using a Dormand-Prince pair as well as the ode15s function based on a variable-order formula employing numerical differentiation were applied. The model parameters were determined by non-linear regression from the experimental data obtained by using the nlincon and Fmincon Matlab functions.

RESULTS

Effect of C/N ratio and cupric sulfate on degradation of lignocellulosic wastes and enzyme production

The interval of C/N ratio used for the 12 medium formulations was from 50 to 140 with a variation in the nitrogen content ranging from 0.29 to 0.8% in dry basis. All the three fungal species were able to grow on all the media formulations and to produce significant enzyme activities for all the C/N ratios during 49 incubation days. All the nitrogen contents in the formulations stimulated the macromycetes growth during the vegetative phase increasing the biomass growth and cellulose consumption as observed in Figure 1 (all formulations are not presented due to space constraints). It is worthy to point out that the variation of nitrogen content and carbon source was ensured employing the same materials (oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil) and not modifying the nitrogen and carbon sources. The ANOVA performed considering the lingo-cellulolytic enzymes titers exhibited during the SSF (Figure 2) demonstrated the variability in the seven enzyme activities measured depending on the species, formulation and incubation time. The first ANOVA indicated that there exist significant differences within each factor

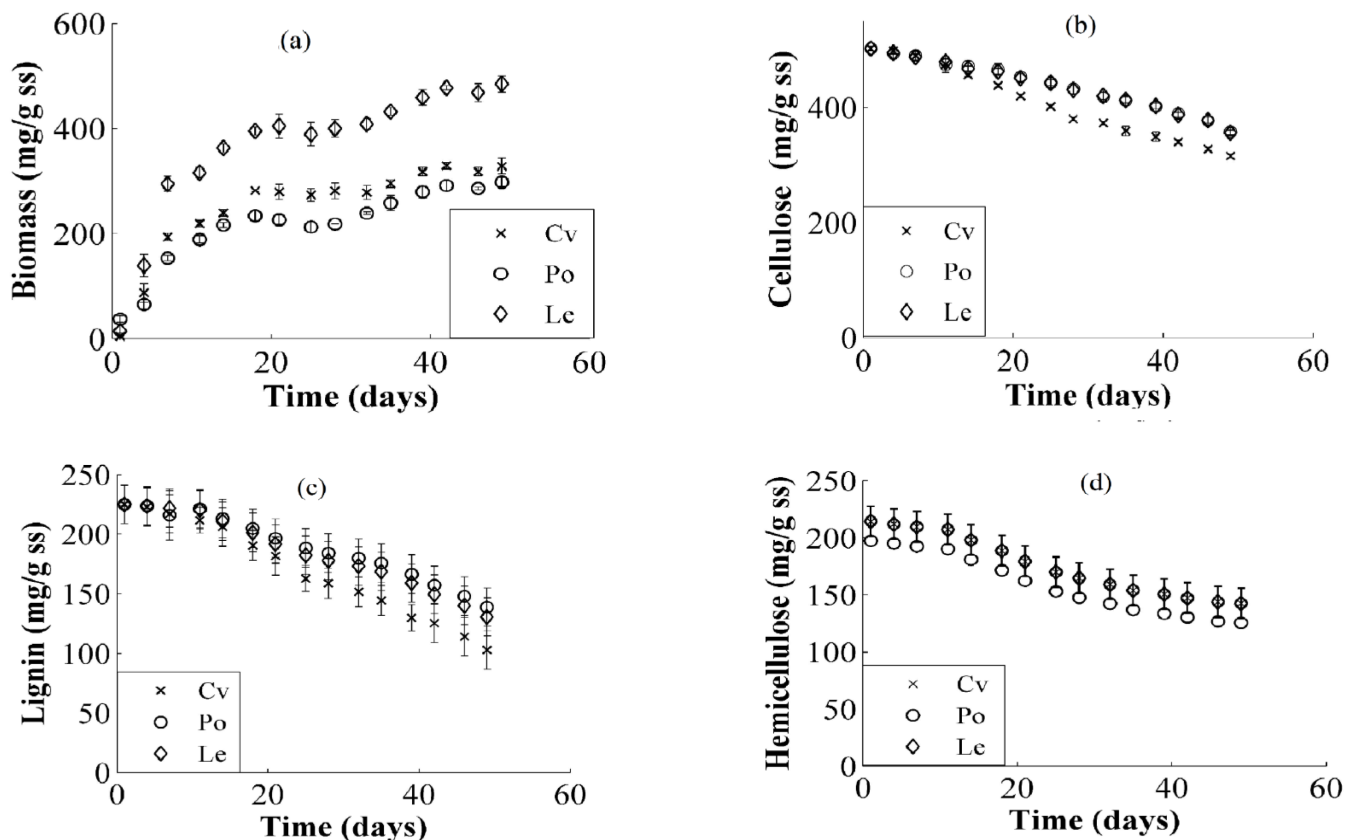

Figure 1. Biomass production and cellulose consumption for *C. versicolor* (Cv), *P. ostreatus* (Po), and *L. edodes* (Le) grown on different solid media based on oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil during 49 cultivation days under SSF conditions. **(a)** Biomass growth on F1 medium. **(b)** Cellulose consumption for F12 medium. **(c)** Lignin degradation for F3 medium. **(d)** Hemicellulose consumption for F7 medium. The formulation for each medium is deciphered in Table 1.

(species and formulation) and between them (5% significance level). Then the maximum lignocellulolytic enzyme activities for each fungal species were determined graphically considering the 15 incubation times sampled as presented in Table 2. For instance, the highest EXG levels were obtained for *L. edodes* grown on F6 medium at 21st day (106.44 U/g solid substrate), while the lowest EXG corresponded to *P. ostreatus* grown on F12 medium at 35th day (44.05 U/g solid substrate).

As observed in Figure 1, fungal biomass increased for all the formulations during 49 days of fermentation. In the case of the hydrolases (ENG, EXG, BG and EXN), the variability of the enzymatic activities found is as wide among the three fungal species and 12 media formulations, that there exists no clear evidence that the cupric sulfate amounts added to the substrates (0.08% and 0.16%) cause neither inhibition nor direct activation of the fungal ability to produce these enzymes.

Time course of enzyme production during SSF

The time course of enzyme production measured during 49-day SSF did not present a defined tendency for any fungus or medium formulation. Nevertheless, from all the experimental data obtained in this work, an overall trend consisting in the decrease of enzymes production after a specific incubation time can be observed. ENG and EXG tend to decrease after 20 days of incubation, in coincidence with the moment when the three species have completed the colonization of the solid medium (substrate). However, some combinations showed a different behavior, as in the case of *P. ostreatus* grown on F3 medium for which the ENG presented a stable trend until the 35th day with a decrease until the end of the process. In the case of EXG for all the fungi, the F7–F10 formulations presented an increase in enzyme activities until the 30th day with a decrease until the end of the measurements (Figure 2b). On the other hand, EXN activity was more disperse than in the case of the two foregoing hydrolases although values as high as 90 U/g ss (solid substrate) for F5 medium were achieved for *C. versicolor*. Regarding the BG activity, there is no clear trend of the data, but a decrease in the activity with the time is noticeable for all fungus/formulation combinations. The two oxidoreductases studied in this work exhibited different behaviors. The LAC activity considerably varies

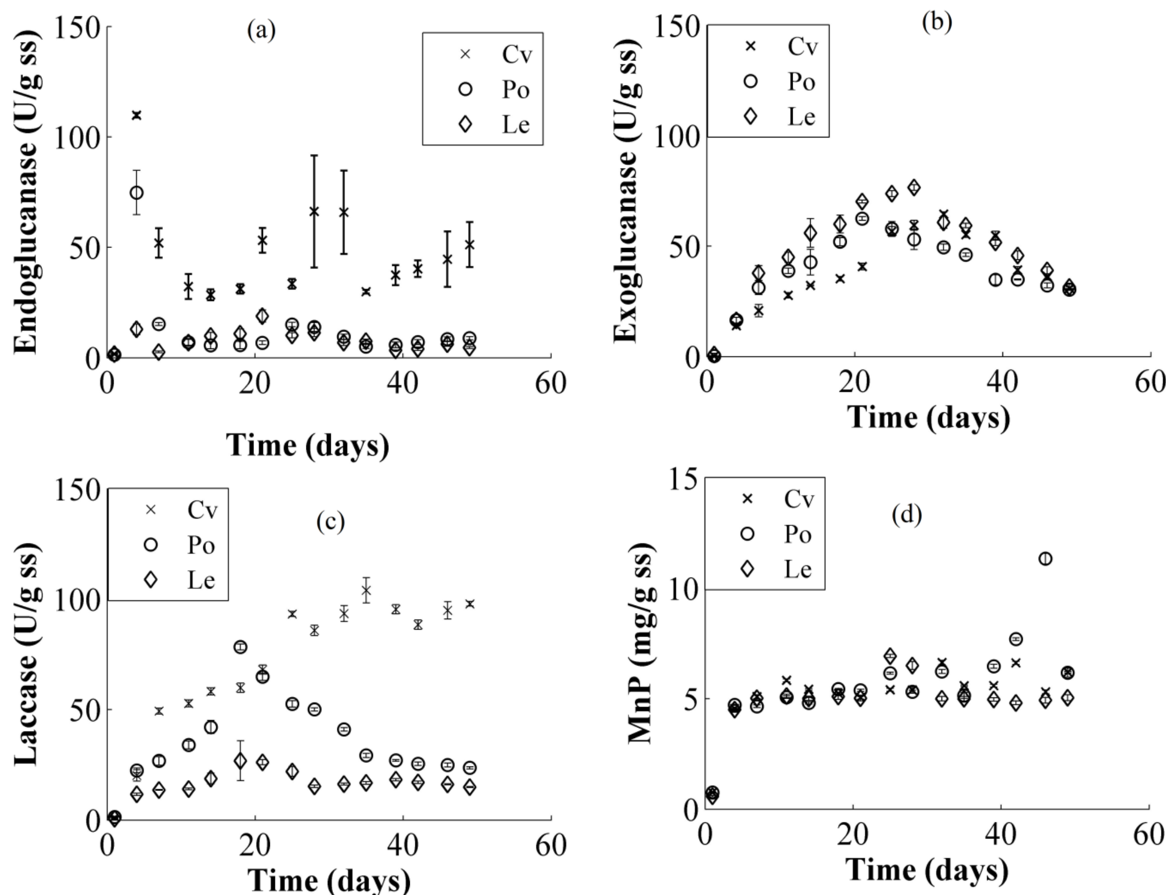

Figure 2. Time course of lignocellulolytic enzyme production by *C. versicolor* (Cv), *P. ostreatus* (Po), and *L. edodes* (Le) grown on different solid media based on oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil during 49 cultivation days under SSF conditions. **(a)** Endoglucanase acitvity (ENG) for F6 medium. **(b)** Exoglucanase activity (EXG) for F10 medium. **(c)** Laccase activity (LAC) for F9 medium. **(d)** Manganese peroxidase activity (MnP) for F4 medium. The formulation for each medium is deciphered in Table 1.

among the combinations, being *C. versicolor* the species with highest titers of this enzyme, which were in the range 80-100 U/g ss for some formulations (Figure 2c). In contrast, the MnP activity was generally low for the time interval of the measurements (Figure 2d).

Degradation of lignocellulosic components

In order to choose the fungus/formulation combination with the best performance regarding the degradation of the main components of the lignocellulosic wastes (cellulose, hemicellulose and lignin) caused by the release of an important amount of lignocellulolytic enzymes, several ANOVAs were performed. The results of the first ANOVA showed that there exist significant differences among the treatments for each fungal species as mentioned above. For each fungus, the maximum of each enzyme activity was determined. Then a comparative analysis of these maxima was carried out using the multcompare Matlab function and applying a Kruskal-Wallis test for each activity. In this way, significant differences were found among the maxima of enzyme activity.

To choose the best fungus/formulation combination, a new comparative analysis of the degradation percentages of the fiber components was done. For this, the results of degradation percentages for each fungus and formulation at the end of SFF (49th day) were analyzed by a Kruskal-Wallis test. Then, selected combinations were compared by using the multcompare function. These analyses showed that *C. versicolor* exhibited the highest ability to degrade the main components of the lignocellulosic biomass (cellulose, hemicellulose and lignin) for all formulations as presented in Table 3. In particular, this fungus grown on F8 and F9 formulations showed the best degradations of cellulose. The highest lignin degradation corresponded to the F1 and F9 formulations again for *C. versicolor*. In the case of hemicellulose, *C. versicolor* exhibited the highest degradation when grown on F1, F9 and F12 media. An ANOVA was performed between F1

Table 2. Maximum lignocellulolytic enzyme activities of three white-rot fungi species for different carbon/nitrogen ratios and two levels of copper sulfate (II).

Enzyme activity	ENG	EXG	ENX	BG	LAC	MnP
P. ostreatus						
Maximum activity (U/g ss)	47.32	91.34	80.88	47.32	90.42	9.57
Day of max. activity	46	39	21	46	28	28
Medium formulation [a]	F3	F3	F6	F3	F3	F5
C. versicolor						
Maximum activity (U/g ss)	78.75	74.50	124.18	71.05	106.76	7.36
Day of max. activity	11	7	21	32	28	28
Medium formulation	F9	F5	F9	F5	F7	F3
L. edodes						
Maximum activity(U/g ss)	66.85	99.29	107.53	84.01	47.25	6.92
Day of max. activity	11	32	21	14	18	25
Medium formulation	F3	F5	F10	F2	F11	F4

[a]According to the formulations presented in Table 1. ENG, Endoglucanase activity; EXG, exoglucanase activity; ENX , endoxylanase activity; BG, β-glucosidase activity; LAC, laccase activity; MnP, manganese peroxidase activity; U, enzyme unit; g ss, gram of solid substrate.

and F9 formulations for *C. versicolor*, but the results indicated that there are no significant differences. Therefore, the combination *C. versicolor* – F9 medium was selected for further modeling considering that it presented high degradation values for these three biopolymers. In fact, this combination showed the highest average of the three mean degradation values.

Mathematical modeling

For the fungus/formulation combination exhibiting the highest average degradation of the main components of the lignocellulosic wastes used (*C. versicolor* – F9 medium), a non-structured non-segregated deterministic mathematical model was proposed in order to describe the behavior of the SSF process, particularly, the fungal growth, substrate degradation and lignocelullolytic enzyme production. In addition, the behavior of the reducing sugars (mostly glucose, cellobiose and xylose) was also included in the model considering that these sugars are intermediary products released and consumed during the utilization of the lignocellulosic biomass.

The proposed model is composed of 11 differential equations that are shown in Table 4. The values of the model parameters are as follows: μ_m = 6.7416 day^{-1}; C_{bm} = 358.0071 mg/g ss; n = 0.0279; q_p = 0.2636 mg×mg^{-1}×day^{-1}; k_L = 8.238x10^{-3} mg×g ss×day^{-1}×U^{-2}; k_{lac} = 1.341x10^{-3} U×g ss×mg^{-1}×mg^{-1}; k_{MnP} = 1.035x10^{-4} U×g ss×mg^{-1}×mg^{-1}; μ_{LAC} = 0 U×mg^{-1}×day^{-1}; μ_{MnP} = 5.659x10^{-4} U×mg^{-1}×day^{-1}; k_{HM} = 0.0245 mg×day^{-1}×U^{-1}; k_{ENX} = 2.353x10^{-3} U×g ss×mg^{-1}×mg^{-1}; μ_{ENX} = 0.2563 U×mg^{-1}×day^{-1}; k_C = 0.0101 mg×g ss×day^{-1}×U^{-2}; k_{ENG} = 2.511x10^{-3} U×g ss×mg^{-1}×mg^{-1}; μ_{ENG} = 0,08 U×mg^{-1}×day^{-1}; k_{EXG} = 5.064x10^{-4} U×g ss×mg^{-1}×mg^{-1}; μ_{EXG} = 0.0810 U×mg^{-1}×day^{-1}; k_{BG} = 0.0742 U ×mg^{-1}; μ_{BG} = 0.0256 U×mg^{-1}×day^{-1}. For *C. versicolor* grown on F9 medium, the following initial conditions were established: C_{b0} = 25.2333 mg/g ss; C_{AR0} = 12.8 mg/g ss; C_{L0} = 205.45 mg/g ss; C_{lac0} = 1.7374 U/g ss; C_{MnP0} = 0.5335 U/g ss; C_{HM0} = 225.36 mg/g ss; C_{ENX0} = 1 U/g ss; C_{C0} = 490 mg/g ss; C_{ENG0} = 1.888 U/g ss; C_{EXG0} = 0.4793 U/g ss; C_{BG0} = 3,999 U/g ss. The names of the 11 variables are indicated in the last column of Table 4.

The Equation 1 proposed for cell biomass description corresponded to a logistic model modified by Mitchell et al. (1999a). To describe the variation of reducing sugars, the Equation 2 including a constant production factor that affects growth rate was proposed; in this case, the change of these sugars had a better fit to the growth rate than to the cell biomass itself. The Equations 3, 6, and 8 for consumption of lignin, hemicellulose, and cellulose, respectively, were expressed as a function of the specific activities of the enzymes responsible for degradation of the corresponding substrates. The changes of cellulolytic (ENG and EXG), xylanolytic (ENX) and ligninolytic (LAC and MnP) specific enzyme activities (the equivalent of the concentration for enzymes) were considered to be dependent on the concentration of the corresponding substrate and on growth rate with an inhibition factor. This factor is related to the concentration of reducing sugars for the two cellulases and the xylanase as indicated in Equations 9, 10, and 7. In the case of ligninases, the inhibition factor was related to the lignin concentration in the medium as can be observed in Equations 4 and 5. Some authors (Tengerdy and Szakacs, 2003) state that the inhibition

Table 3. Degradation percentages of cellulose, hemicellulose, and lignin during the SSF using three white-rot fungi for different media formulations.

Formulation[a]	Cellulose			Hemicellulose			Lignin		
	C. versicolor	*P. ostreatus*	*L. edodes*	*C. versicolor*	*P. ostreatus*	*L. edodes*	*C. versicolor*	*P. ostreatus*	*L. edodes*
F1	37.83	29.49	27.83	37.86	35.65	34.45	65.22	46.01	50.39
F2	38.17	25.55	30.90	29.94	29.28	27.91	53.31	41.97	41.18
F3	39.18	29.84	29.79	35.92	32.68	33.96	58.96	41.60	45.55
F4	39.15	32.27	26.04	28.77	25.74	27.88	60.44	42.64	46.69
F5	42.88	30.28	28.49	29.41	25.42	26.55	57.67	40.68	44.55
F6	40.67	32.92	23.27	32.47	29.11	30.56	58.18	41.04	44.95
F7	37.35	31.13	27.04	33.02	32.90	31.33	55.93	44.94	43.21
F8	43.64	29.55	30.79	32.88	28.80	30.11	54.47	42.88	42.08
F9	43.21	29.68	28.26	36.54	26.50	29.54	63.69	44.93	49.21
F10	38.50	26.87	26.59	28.12	25.15	27.12	53.28	37.58	41.16
F11	34.71	26.89	24.82	32.32	26.88	30.28	53.94	38.05	41.67
F12	37.32	28.76	27.93	37.53	27.39	29.39	47.40	33.44	36.62

[a]According to the formulations presented in Table 1.

of these enzymes could be caused by intermediary compounds formed during the fermentation or, probably, by high lignin concentrations. Although there is still no certainty of the causes of this inhibition, the experimental data obtained in this work suggest a possible inhibition of ligninases; as no intermediary compounds were determined during the SSF, a substrate inhibition relationship was proposed, in this case, by the lignin. Finally, for description of BG production, Equation 11 was formulated as a dependence on the growth rate with an inhibition term affected by the reducing sugars concentration.

The follow-up of cell biomass was accomplished by determining the chitin contained in the cell wall of fungi through the quantification of NAGA at different times during the cultivation process. From parallel experimental runs under conditions of submerged fermentation, the NAGA content in the dry mycelium of *P. ostreatus*, *C. versicolor*, and *L. edodes* were determined. Thus, the NAGA content for these fungi were 15.83, 14.15, and 10.08 wt.%, respectively. These percentages were used to calculate the amount of cell biomass attached to the solid media. The behavior of the cell biomass during the fermentation is depicted in Figure 3 along with the time profile of the reducing sugars. As can be seen, the data fit was quite adequate. It should be noted that the model appropriately described the appearance/disappearance of the reducing sugars as key intermediary substances playing a crucial role during this type of SSF. The data fitting for lignin degradation and production of the corresponding ligninolytic enzymes (LAC and MnP) was satisfactory as well (Figure 4). The model was able to capture the decrease in the MnP activity after 20 h of cultivation. Finally, the data fitting was quite appropriate to describe the behavior of the hydrolytic enzymes released during the SSF using *C. versicolor* as can be observed in Figure 5 for hemicellulose consumption and ENX production, and in Figure 6 for cellulose consumption and production of cellulases measured (ENG, EXG, and BG). The suitability of the model was proven through 1-tailed *F*-test comparing the variance of the model residuals (deviation of the values calculated by the model related to the experimental data) and the variance of the experimental series for each variable considered in the model. The model was tested for all the 12 media formulations on which *C. versicolor* grew. This fact enabled to provide enough confidence about the validity of the representation of the experimental data by the model proposed.

DISCUSSION

Effect of C/N ratio and cupric sulfate on degradation of lignocellulosic wastes and enzyme production

The results presented in the present work regarding the C/N ratios used for formulation of the 12 solid media indicate that there exists no apparent direct

Table 4. Mathematical model describing the production of fungal biomass and lignocellulolytic enzymes, and consumption of lignocellulosic matrix.

Equation	Number	Description	Parameters and variables
$\frac{dC_b}{dt} = \mu_m.C_b\left(1-\left(\frac{C_b}{C_{bm}}\right)^n\right)$	1	Biomass	μ_m: Specific growth rate (day^{-1}) C_{bm}: Maximum biomass concentration (mg/g ss) n: $n < 1$ The organism is relatively sensitive to the auto-inhibition and it occurs for very low values of C_b $n = 1$ Logistic equation $n > 1$ The organism is relatively resistant to the auto-inhibition and it occurs only when $C_b \approx C_{bm}$
$\frac{dC_{AR}}{dt} = q_P.\mu_m.\frac{dC_b}{dt}\left[1-(n+1)\left(\frac{C_b}{C_{bm}}\right)^n\right]$	2	Reducing sugars	q_p: Production coefficient for reducing sugars ($mg \times mg^{-1} \times day^{-1}$) C_{AR}: Reducing sugars concentration ($mg \times g\ ss^{-1}$)
$\frac{dC_L}{dt} = -k_L.C_{LAC}.C_{MnP}$	3	Lignin	k_L: Lignin degradation coefficient ($mg \times g\ ss \times day^{-1} \times U^{-2}$) k_{LAC}: Laccase production coefficient ($U \times g\ ss \times mg^{-1} \times mg^{-1}$) k_{MnP}: Mn peroxidase production coeff. ($U \times g\ ss \times mg^{-1} \times mg^{-1}$) μ_{LAC}: Inhibition coefficient for laccase ($U \times mg^{-1} \times day^{-1}$) μ_{MnP}: Inhibition coefficient for Mn peroxidase ($U \times mg^{-1} \times day^{-1}$) C_L: Lignin concentration ($mg \times g\ ss^{-1}$) C_{LAC}: Laccase activity ($U \times g\ ss^{-1}$) C_{MnP}: Manganese peroxidase activity ($U \times g\ ss^{-1}$)
$\frac{dC_{LAC}}{dt} = k_{LAC}.\frac{dC_b}{dt}.C_L - \mu_{LAC}.C_L$	4	Laccase	
$\frac{dC_{MnP}}{dt} = k_{MnP}.\frac{dC_b}{dt}.C_L - \mu_{MnP}.C_L$	5	Manganese peroxidase (MnP)	
$\frac{dC_{HM}}{dt} = -k_{HM}.C_{ENX}$	6	Hemicellulose	k_{HM}: Hemicellulose consumption coefficient ($mg \times day^{-1} \times U^{-1}$) C_{HM}: Hemicellulose concentration ($mg \times g\ ss^{-1}$)
$\frac{dC_{ENX}}{dt} = k_{ENX}.C_{HM}.\frac{dC_b}{dt} - \mu_{ENX}.C_{AR}$	7	Endoxylanase	k_{ENX}: Endoxylanase production coefficient ($U \times g\ ss \times mg^{-1} \times mg^{-1}$) μ_{ENX} : Inhibition coefficient for endoxylanase ($U \times mg^{-1} \times day^{-1}$) C_{ENX}: Endoxylanase activity ($U \times g\ ss^{-1}$)
$\frac{dC_C}{dt} = -k_C.C_{ENG}.C_{EXG}$	8	Cellulose	k_C: Cellulose consumption coefficient ($mg \times g\ ss \times day^{-1} \times U^{-2}$) C_C: Cellulose concentration ($mg \times g\ ss^{-1}$)
$\frac{dC_{ENG}}{dt} = k_{ENG}.\frac{dC_b}{dt}.C_C - \mu_{ENG}.C_{AR}$	9	Endoglucanase	k_{ENG}: Endoglucanase production coeff. ($U \times g\ ss \times mg^{-1} \times mg^{-1}$) μ_{ENG} : Inhibition coefficient for endoglucanase ($U \times mg^{-1} \times day^{-1}$) C_{ENG}: Endoglucanase activity ($U \times g\ ss^{-1}$)
$\frac{dC_{EXG}}{dt} = k_{EXG}.\frac{dC_b}{dt}.C_C - \mu_{EXG}.C_{AR}$	10	Exoglucanase	k_{EXG}: Exoglucanase production coeff. ($U \times g\ ss \times mg^{-1} \times mg^{-1}$) μ_{EXG} : Inhibition coefficient for exoglucanase ($U \times mg^{-1} \times day^{-1}$) C_{EXG}: Exoglucanase activity ($U \times g\ ss^{-1}$)
$\frac{dC_{BG}}{dt} = k_{BG}.\frac{dC_b}{dt} - \mu_{BG}.C_{AR}$	11	β-glucosidase	k_{BG}: β-glucosidase production coeff. ($U \times mg^{-1}$) μ_{BG} : Inhibition coefficient for β-glucosidase ($U \times mg^{-1} \times day^{-1}$) C_{BG}: β-glucosidase activity ($U \times g\ ss^{-1}$)

Remarks: g ss – gram of solid substrate, U – unit of enzyme activity.

effect of this parameter on the variation of the six enzyme activities. This could be explained taking into account that the solid materials are not changed during the media formulation and that the levels of the C/N ratio evaluated (from 50 to 140) could not be different enough to cause significant variation in the enzyme activities measured. In this work, the data of ENG and EXG obtained were much lower than those ones obtained by other researchers like Kachlishvili et al. (2006) with different C/N ratios and for *L. edodes* and two *Pleurotus* species different to *P. ostreatus*. However, the EXG, BG, LAC, and MnP titers were higher related to those ones obtained with different C/N ratios for white-rot basidiomycetes reported by other authors (Baldrian and Gabriel, 2002; Reddy et al., 2003). Nevertheless, the enzyme titers can vary according to the efficiency of the method used to extract the enzymes from the solid substrate. In fact, the methodology employed could not necessarily ensure the extraction of all the enzymes absorbed to the substrate.

The presence of heavy metals affects the growth of

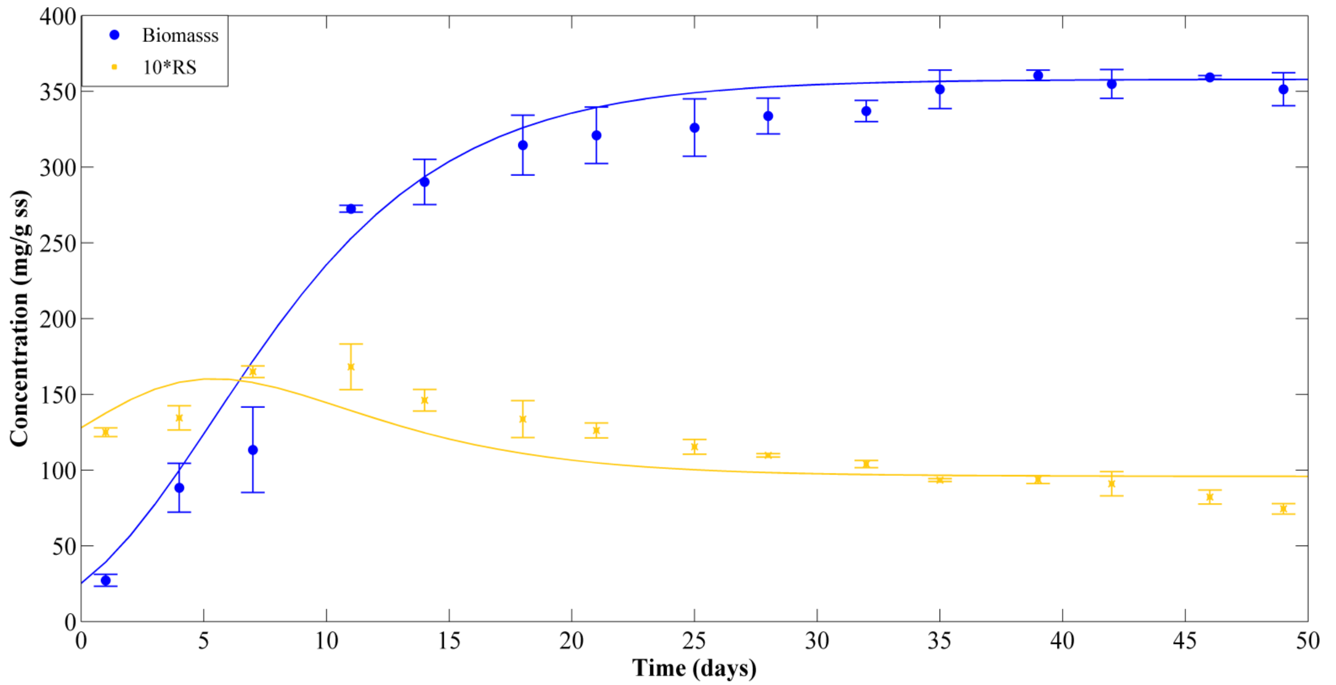

Figure 3. Time profile of cell biomass and reducing sugars for *C. versicolor* grown on the solid medium F9 based on oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil under SSF conditions. The continuous lines were calculated by the model proposed. The values for reducing sugars are multiplied by 10.

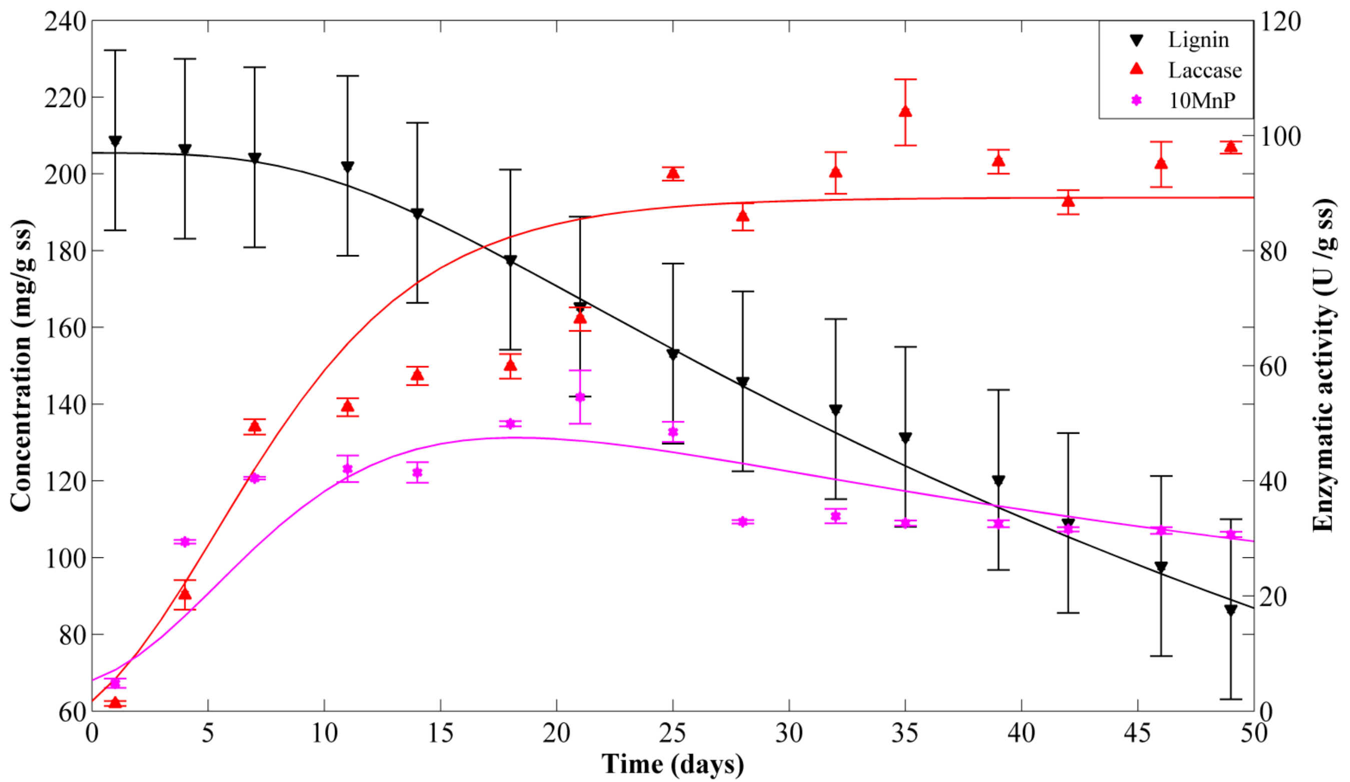

Figure 4. Time profile of lignin, laccase (LAC), and manganese peroxidase (MnP) for *C. versicolor* grown on the solid medium F9 based on oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil under SSF conditions. The continuous lines were calculated by the model proposed. The values for MnP are multiplied by 10.

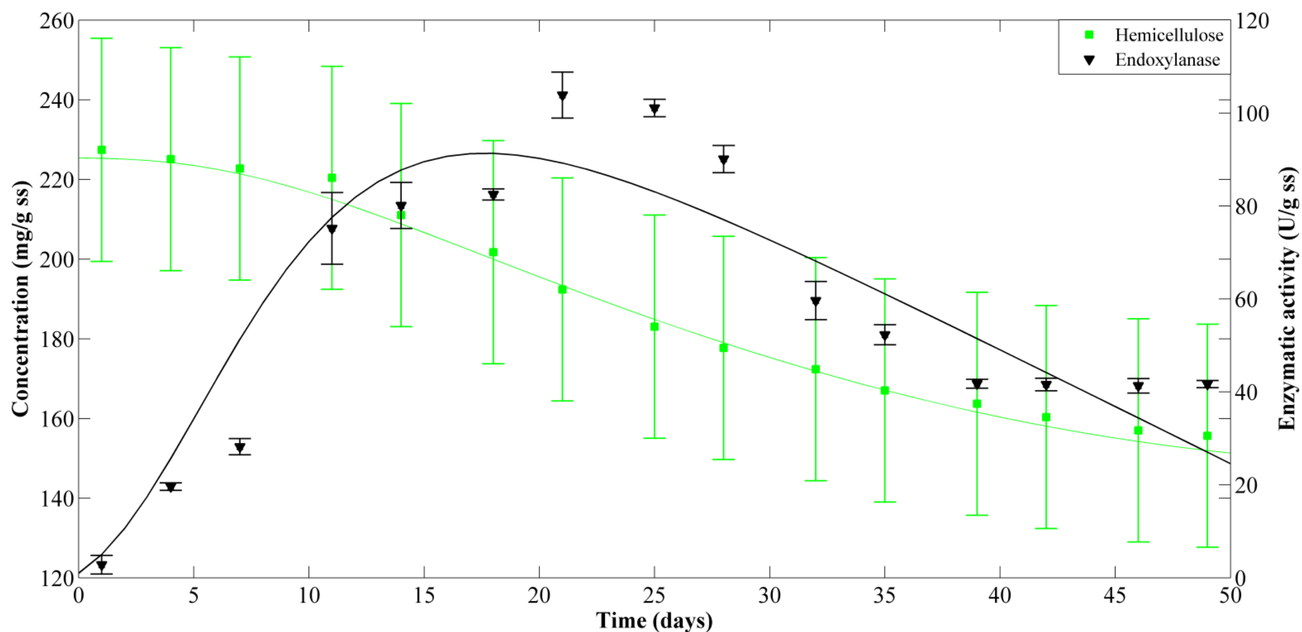

Figure 5. Time profile of hemicellulose and endoxylanase (ENX) for *C. versicolor* grown on the solid medium F9 based on oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil under SSF conditions. The continuous lines were calculated by the model proposed.

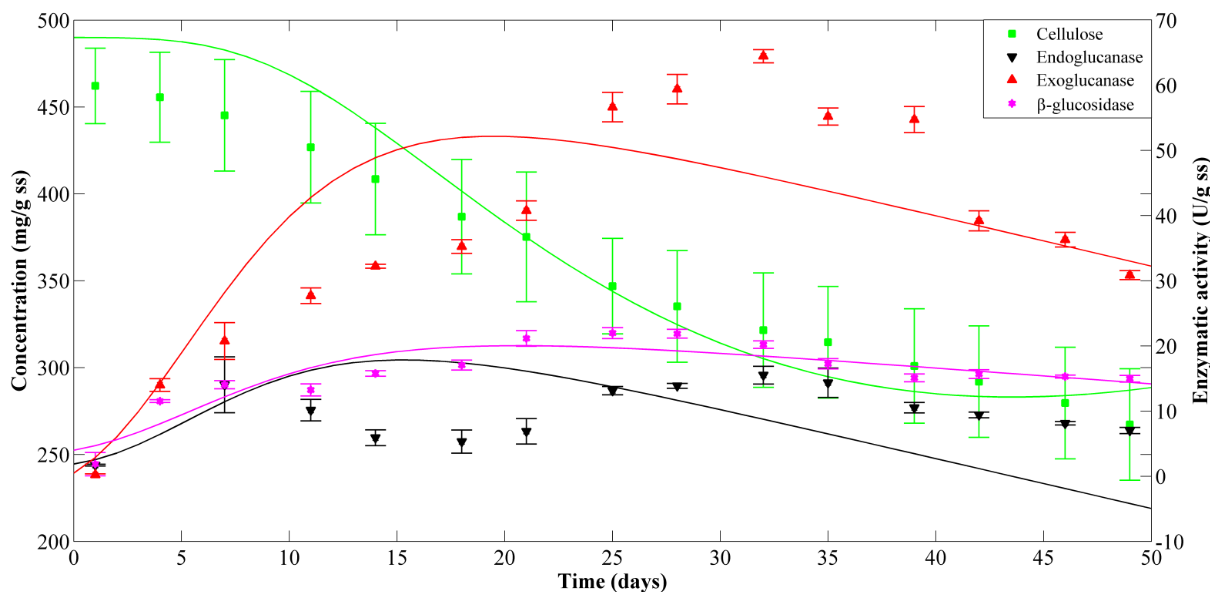

Figure 6. Time profile of cellulose, endoglucanase (ENG), exoglucanase (EXG), and β-glucosidase (BG) for *C. versicolor* grown on the solid medium F9 based on oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil under SSF conditions. The continuous lines were calculated by the model proposed.

white-rot fungi. This effect can cause the decrease of fungal growth rate, which is sometimes accompanied with a prolonged lag phase. Thus, copper inhibited the growth of *Ganoderma lucidum* at concentrations less than 1 mM, while 150 ppm copper decreased the growth rate of *Phanerochaete chrysosporium* (Baldrian, 2003), but concentrations used in this work did not generate apparent inhibition on the growth of the three organisms analyzed.

Extracellular cellulolytic and ligninolytic enzymes are

regulated by heavy metals at the transcription level as well as during their catalytic action (Baldrian, 2003). 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* (Baldrian, 2003); but, in the range assayed in this work, copper had no influence on the production of cellulolytic and xylanolytic enzymes by the three fungi evaluated.

In the case of ligninolytic enzymes, several researchers have studied the effect of $CuSO_4$ addition as an inductor of laccase and peroxidases (Levin et al., 2002; Niladevi and Prema, 2008). Copper has been reported to be a strong laccase inducer in several species, among them *Ganoderma applanatum*, *Peniophora* sp., *Pycnoporus sanguineus*, and *Coriolus versicolor* f. *antarcticus* (Fonseca et al., 2010). It is known that copper induces both laccase transcription and activity (Collins and Dobson, 1997), and the increase in activity is proportional to the amount of copper added. Laccase production for all the three fungal species and 12 formulations could have been stimulated by $CuSO_4$ addition since LAC activities reached values higher than those ones reported in other works. For instance, in a previous report (Montoya et al., 2012), LAC activities of the white-rot fungus *Grifola frondosa* were not higher than 15 U/g ss (solid substrate) while activities as high as 90.42 U/g ss for *P. ostreatus* and 106.76 U/g ss for *C. versicolor* at 28 days were achieved in the present work. These activities are comparable to those obtained by Gassara et al. (2010) for one of the most studied basidiomycetes, *P. chrysosporium*, in presence of $CuSO_4$ on different lignocellulosic materials. These authors did not achieve any LAC activity when inducers were not used. Rosale et al. (2007) reported LAC activities comparable to this work as well. These authors showed an increase in LAC activity for *Trametes hirsuta* grown on ground orange peelings due to the addition of 1 mM cupric sulfate. Therefore, the presence of copper in cultivation media plays a crucial role for their induction and production. On the other hand, MnP activities did not show any considerable variation among the species and formulations. The activities obtained were comparable to those ones previously reported for *G. frondosa* (Montoya et al., 2012) and *P. chrysosporium* (Gassara et al. (2010) grown on lignocellulosic wastes.

Time course of enzyme production during SSF

The time course of the biosynthesis of the lignocellulolytic enzymes studied in this work showed interesting patterns as presented in the results section. Many basidiomycetes have the ability to simultaneously synthesize hydrolytic and oxidative enzymes, which are needed to degrade the substrates contained in the lignocellulosic complex. These enzymes are extracellular and inducible. In particular, the white-rot fungi show higher titers for ligninases than for cellulases. This implies an advantage for these fungi since they should start the lignin degradation in order to access the cellulose. In fact, the higher ability to degrade lignin by *C. versicolor* (Table 3) allowed a faster access to the cellulose leading to an enhanced cellulose consumption and, consequently, to an improved utilization of the carbon source.

The enzyme activities measured in this study presented decreases at different times during the SSF process. This fact could be explained by several issues like the efficiency of the extraction method employed; changes in the composition of the substrate during the fermentation since new compounds inhibiting the enzymes can be formed; repression exerted by the products generated during the enzymatic reactions; and synthesis of several isoenzymatic forms of the same enzymes at different times under different substrate conditions leading to different activities. This reduction in the enzyme activities has also been observed by other authors who have cited some of the above-mentioned causes for such decline (Mata et al., 2005).

Mathematical modeling

The SSF is a complex process that is strongly influenced by the cultivation conditions. The mathematical description of such process is very difficult considering several issues related to the determination of cell biomass (that is attached to the solid matrix), complex consumption or degradation of substrates, heterogeneity of the solid materials employed, and the impossibility to determine all the intermediary compounds generated from the biochemical reactions occurring during the different fermentation phases. For these reasons, the model proposed in this work should be considered as an attempt to describe the complex behavior of white-rot fungi grown on residual lignocellulosic materials. In fact, the modeling of the lignocellulolytic enzyme production by SSF has not been disclosed in the available literature for the case of macromycetes. The models proposed by other authors (Mitchell et al., 1999b; Mitchell et al., 2004; Viccini et al., 2001) have emphasized the fungal growth rate for micromycetes, and the biosynthesis of simple metabolites. From those works, the logistic equation was successfully applied in this paper to predict the behavior of the fungal biomass. However, the degradation of the fiber components (cellulose, hemicellulose, and lignin) and the synthesis of lignocellulolytic enzymes required mathematical expressions not reported before in order to fit the experimental data obtained as was done in the present work.

Although there exists an important amount of previous

works dealing with the production of lignocellulolytic enzymes by different white-rot fungi, degradation of lignocellulosic materials, and approximations to the determination of the intermediary compounds formed during SSF processes, a greater insight is required in order to generate the corresponding kinetic curves for growth rate, nutrient consumption, biopolymer degradation, and lignocellulolytic enzyme production. Undoubtedly, these kinetic relationships formulated within a comprehensive mathematical model are crucial to conceptually design SSF processes for enzyme or biomass production as well as for their scale-up and operation. As far as we know, this is the first attempt to model these complex processes in the case of *C. versicolor* using such heterogeneous materials as oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil. The formulation of the model equations was made considering the biological sense of the phenomena studied. In fact, the expressions proposed acceptably described each one of the variables measured. For future works, it is necessary to take into account the key intermediary compounds formed during the SSF using white-rot fungi, or other enzymes not evaluated in this work along with their products. Furthermore, it is necessary to consider the intraparticle constraints to the heat and mass transfer during solid cultivation as studied by Mitchell et al. (2004). This will contribute to a deeper understanding of the complex phenomena occurring during the growth of macromycetes on lignocellulosic materials. The model proposed representing the kinetic relationships of the SSF process studied in this work is a powerful tool that could be used not only to provide valuable information on the process itself, but also to develop a commercial process for production of lignocellulolytic enzymes in the future. In addition, the model offers the possibility to reduce the number of experimental runs as well as to optimize the process.

Conclusions

In this work, three species of white-rot fungi were grown on 12 media formulations during 49 days of SSF varying their carbon/nitrogen ratios and their content of cupric sulfate. Some fungus/formulation combinations showed a promising performance regarding enzyme production. The fungus *C. versicolor* exhibited the highest ability to degrade the three main polymers of the lignocellulosic residual materials employed. The mathematical model proposed to describe the process studied could be used to provide valuable information on the process itself as well as to contribute to the development of a future commercial process for lignocellulolytic enzyme production.

Conflict of interest

The authors did not declare any conflict of interest.

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