Bioprocess Biosyst Eng (2014) 37:2463–2473
DOI 10.1007/s00449-014-1224-3

ORIGINAL PAPER

Interactions between fungal growth, substrate utilization, and enzyme production during solid substrate cultivation of *Phanerochaete chrysosporium* on cotton stalks

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Received: 24 January 2014 / Accepted: 13 May 2014 / Published online: 8 June 2014

Abstract Fungal pretreatment, using lignin-degrading microorganisms to improve lignocellulosic feedstocks with minimal energy input, is a potential alternative to physiochemical pretreatment methods. Identifying the kinetics for fungal pretreatment during solid substrate cultivation is needed to help establish the processing conditions for effective scale up of this technology. In this study, a set of mathematical models were proposed for describing the interactions between holocellulose consumption, lignin degradation, cellulase, ligninolytic enzyme, and the growth of *Phanerochaete chrysosporium* during a 14 day fungal pretreatment process. Model parameters were estimated and validated by the System Biology Toolbox in MatLab. Developed models provided sufficiently accurate predictions for fungal growth ($R^2 = 0.97$), holocellulose consumption ($R^2 = 0.97$), lignin degradation ($R^2 = 0.93$) and ligninolytic enzyme production ($R^2 = 0.92$), and fair prediction for cellulase production ($R^2 = 0.61$). The models provide valuable information for understanding the interactive mechanisms in biological systems as well as for fungal pretreatment process scale up and improvement.

Keywords Cotton stalk · Pretreatment · Bioethanol · *Phanerochaete chrysosporium* · Kinetics · Solid substrate cultivation

List of symbols

α_C	Growth-associated constant for cellulase formation, (g enzyme protein) (g DM)$^{-1}$
α_L	Growth-associated constant for ligninolytic enzyme formation, (g enzyme protein) (g DM)$^{-1}$
β_C	Non-growth-associated constant for cellulase formation, (g enzyme protein) (g DM)$^{-1}$
β_L	Non-growth-associated constant for ligninolytic enzyme formation, (g enzyme protein) (g DM)$^{-1}$
μ_{max}	The maximum specific growth rate, day^{-1}
C_{FPU}	Cellulase activity per ml of enzyme extract, IU ml^{-1}
$C_{TKN,E}$	Amount of TKN per ml of enzyme extract, (g TKN) ml^{-1}
$C_{TKN,base}$	Base TKN content in autoclaved cotton stalk extract, (g TKN) ml^{-1}
$C_{TKN,S}$	Amount of TKN in pretreated solids, (g TKN) (g DM)$^{-1}$
CCP	Cumulative CO_2 production (mmol)
COU	Cumulative oxygen uptake (mmol)
CPR	CO_2 production rate (mmol day^{-1})
DM	Dry matter
DML	Dry matter loss
$k_E = 1/1070$	Conversion coefficient from cellulase activity to cellulase proteins, (g cellulase proteins) IU^{-1}
k_{LD}	Coefficient of lignin degradation
k_{LL}	Coefficient of ligninolytic enzyme production, (g enzyme protein) (g fungal biomass)$^{-1}$ day^{-1}
$k_T = 10.3$	Conversion coefficient from TKN to fungal biomass, based on the amount of TKN in pure *P. chrysosporium* fungal biomass, (g fungal biomass) (g TKN)$^{-1}$
m	Maintenance coefficient, (g cellulose and hemicellulose) (g fungal biomass)$^{-1}$ day^{-1}
m_O	Maintenance coefficient, (mmol O_2) (g fungal biomass)$^{-1}$ day^{-1}

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OU	Oxygen uptake, mmol
OU_0	Initial oxygen uptake, mmol
OUR	Oxygen uptake rate, (mmol O_2) day^{-1}
P_C	Cellulase concentration, (g enzyme protein) $(g\ DM)^{-1}$
$P_{C,0}$	Initial cellulase concentration at time 0, (g enzyme protein) $(g\ DM)^{-1}$
P_L	Ligninolytic enzymes concentration, (g enzyme protein) $(g\ DM)^{-1}$
$P_{L,0}$	Initial ligninolytic enzymes concentration, (g enzyme protein) $(g\ DM)^{-1}$
S_C	Cellulose and hemicellulose content, g $(g\ DM)^{-1}$
$S_{C,0}$	Initial cellulose and hemicellulose content, g $(g\ DM)^{-1}$
S_L	Lignin content, g $(g\ DM)^{-1}$
$S_{L,0}$	Initial lignin content, g $(g\ DM)^{-1}$
t	Pretreatment time, day
TKN	Total Kjeldahl Nitrogen, %
$V = 20$	Volume of enzyme extracts, ml
w	Dry weight of pretreated sample, g DM
w_0	The initial sample dry weight before pretreatment, g DM
X	The fungal biomass content, g $(g\ DM)^{-1}$
X_0	Initial fungal biomass content, g $(g\ DM)^{-1}$
X_{max}	Maximum biomass content, g $(g\ DM)^{-1}$
$Y_{X/O}$	The oxygen to fungal biomass yield coefficient $(g{-}X\ mmol^{-1}\ O_2)$
$Y_{X/S}$	The substrate to fungal biomass yield coefficient, (g fungal biomass) (g cellulose and hemicellulose DM)$^{-1}$

Introduction

The conversion of agricultural residues, energy crops, and forest biomass to biofuels has become a research priority in the recent years. Although numerous challenges exist in the conversion of these lignocellulosic materials to biofuels, the pretreatment step, accounting for roughly one fifth of the total production costs, remains as one of the main barriers preventing commercial success [1, 2]. Existing pretreatment methods have largely been developed on the basis of physiochemical technologies [3]. Microbial pretreatment, an alternative to physiochemical pretreatment, employs lignin-degrading microorganisms (usually white rot fungi) to break down lignocellulosic biomass and makes it suitable for enzymatic or acid hydrolysis. This approach has received considerable emphasis in the recent years [4–10] due to its potential advantages such as energy-savings, low cost, relatively simple processes and equipment, and environmental friendliness when compared with prevailing physiochemical pretreatment technologies [11].

One of the most investigated white-rot fungi, *Phanerochaete chrysosporium*, is well suited for microbial pretreatment because of its fast growth rate and exceptional lignin biodegradation effectiveness compared with many other basidiomycetes [12]. Although a number of studies have been reported on pure *P. chrysosporium* cultures [13–15], an understanding of the fungal behavior and cultivation conditions on heterogeneous substrates especially lignocellulosic materials still remains inconclusive and requires further investigation [16]. In the previous study, the influence of moisture content, nutrient supplements, and oxygen flushing on lignin degradation, solids recovery, and carbohydrate availability during solid substrate cultivation (SSC) of *P. chrysosporium* on cotton stalk wastes was studied [8]. Additional investigation through the modeling of fungal kinetics can provide critical information for further development of the fungal pretreatment process and its scale up. Until now, limited effort has been made to model the growth kinetics of *P. chrysosporium* on lignocellulosic biomass. Mathematical equations for modeling fungal growth and substrate utilization by *P. chrysosporium* during static submerged cultivations have been developed previously [15]. In another study, respiration kinetics for *P. chrysosporium* mycelia pellets under agitated submerged conditions was studied [17]. However, these studies were conducted on submerged cultures of *P. chrysosporium* using chemically defined media, which limited their applicability to heterogeneous lignocellulosic biomass. In the recent study, we reported mathematical models for describing the interactions between fungal growth, substrate utilization, and enzyme production during submerged stationary cultivation of *P. chrysosporium* on cotton stalks [18]. However, limited effort has been made to model SSC systems due to several factors including the structural and nutritional heterogeneity of lignocellulosic substrates, the difficulties with parameter measurement, and the complexity of mechanistic equations [19]. The other challenges associated with modeling SSC growth kinetics are the estimation of fungal biomass and difficulties in separating it from the substrate [20]. Several approaches have been developed to estimate biomass indirectly by correlating with specific cell constituents such as glucosamine, ergosterol, nucleic acids, and protein or microbial respiration activities [oxygen uptake rate (OUR) and carbon dioxide production rate (CPR)] or dry matter loss [21–24]. However, it is necessary to thoroughly examine and select a specific method that may be suitable for the substrate and fungus used. A study on the fungus' behavior and its interaction with substrate utilization, enzyme formation, and culture conditions will provide

important knowledge for processing development of an effective fungal pretreatment technology [11, 16, 25].

The purpose of this research was to examine the interactions between fungal growth, substrate utilization, and enzyme production during the pretreatment of cotton stalks by SSC of *P. chrysosporium*. In order to understand the growth kinetics, mathematical relationships were established and key growth-dependent parameters were estimated for determination of holocellulose (cellulose and hemicellulose) reduction and lignin degradation (substrate consumption), cellulase and ligninolytic enzyme production (enzyme production), and generation of *P. chrysosporium* biomass (fungal growth). In addition, dry matter loss, OUR, CPR, and organic nitrogen content [through Total Kjeldahl Nitrogen (TKN) method] were investigated as indirect methods for fungal biomass estimations.

Materials and methods

Strain and inoculation

The fungal strain, *P. chrysosporium* (ATCC 24725), was obtained from the Forest Products Laboratory of USDA Forest Service (Madison, WI) and maintained as a frozen culture (−80 °C) in 30 % glycerol. The microorganism was propagated on Potato Dextrose Agar (PDA) plates for 2 days at 39 °C. Spore suspensions were prepared by washing the agar surface with 10 ml of sodium acetate buffer (50 mM, pH 4.5). Spore counts were determined with a hemacytometer (Hausser Scientific, Horsham, PA) and an inoculum with a final spore concentration of 5×10^6 spores ml^{-1} was prepared.

Cotton stalk preparation

Cotton stalks (shredded and baled in the field) were harvested at the Cunningham Research Station (Kinston, NC; 35.298°N, 77.575°W). The stalk material with an initial air dry moisture content of 7 % was ground to 1 mm particle size for compositional analysis, while the stalk material destined for pretreatment was ground to 3 mm by a Thomas Wiley Laboratory Mill (Model No. 4, Thomas Scientific, Philadelphia, PA) and stored in air tight containers at room temperature until use.

Solid substrate cultivation pretreatment

Pretreatments were performed in 250 ml Erlenmeyer flasks sealed with a customized silicon stopper supporting gas inlet and exhaust ports and inline 0.33 μm sterile gas filters. Each treatment was prepared in triplicate with 3 g of air dry, autoclaved (20 min, 121 °C, 15 psi) cotton stalks. Based on the results of the previous study [8], the initial moisture content was adjusted to 75 % (wet-basis) through the addition of sterile 50 mM acetate buffer (pH 4.5) and the moist substrate was inoculated with the inoculum (spore suspension) in a sterile biological safety cabinet. Controls (no fungal inoculum) were performed to account for culture changes in the absence of microbial and enzymatic activity. Pretreatments were carried out in an air convection incubator at 39 °C and destructively sampled at predetermined time intervals (0, 2, 4, 5, 6, 7, 8, 10, 12, and 14 days). Flasks were flushed with oxygen every day starting on day 0 for 10 min at a flow rate of 125 ml min^{-1}. In between flushing events, the flasks were closed by clamping off inlet and exhaust tubing lines.

Analysis methods

Solid substrate cultivation samples were removed at each time interval and sodium acetate buffer (30 ml, pH 4.5, 0.05 M) was added to the cultures and mixed thoroughly prior to incubation at 39 °C for 30 min. The pretreated cotton stalk suspensions were then filtered through preweighed and ignited fritted glass crucibles (Kimax 30 M, Kimble Glass Inc, Vineland, NJ). The filtrates (enzyme extract) were further filtered through a 0.7 μm glass microfiber filter (Type GF/F, Whatman Inc., Florham Park, NJ) and stored at −80 °C for future enzyme activity and TKN analysis.

The solid fractions were washed with 100 ml DI water and dried in a convection oven at 105 °C for 12 h or until constant weight was achieved. The total solids and lignin content (acid soluble and acid insoluble) of untreated and pretreated cotton stalk samples were determined by a modified NREL Laboratory Analytical Procedures [26]. The filtrate from lignin analysis was used for quantification of reducing sugars by a modified DNS assay and then used for estimating holocellulose (cellulose and hemicellulose) content [27].

Fungal biomass was estimated by TKN [28] based on the assumption that the fungus consists of a stable percentage of organic nitrogen (∼9.7 %) which is much higher than that of cotton stalks (∼0.9 %, previously measured). The conversion from TKN to fungal biomass was completed using Eq. 1.

$$X = \frac{k_T \cdot C_{\mathrm{TKN},S} \cdot w}{w_0} \quad (1)$$

Besides TKN, oxygen uptake, CO_2 production, and dry matter loss (DML, as a percentage of initial sample, dry weight) were also determined to predict fungal biomass. The O_2 and CO_2 concentrations (%, v/v) in the headspace of cultivation flasks were measured every 12 h during days 0–10 and every 24 h during days 11–14 with a calibrated gas

analyzer (PAC CHECK 650, Minneapolis, MN). The oxygen uptake rate (OUR, mmol day^{-1}), CO_2 production rate (CPR, mmol day^{-1}), cumulative oxygen uptake (COU, mmol), cumulative CO_2 production (CCP, mmol), and microbial respiratory quotient (RQ, moles of carbon dioxide produced per mole of oxygen consumed) were calculated based on changes in O_2 and CO_2 concentrations over time [29].

Holocellulose (substrate, S_C) and lignin content (substrate, S_L) remaining after pretreatment were defined as gram of holocellulose or lignin left per gram of initial substrate (dry basis). Cellulase activities (C_{FPU}, IU ml^{-1}) were tested using a modified DNS assay [27]. To estimate the amount of enzyme produced, a published value of protein coefficient ($k_E = 9.35 \times 10^{-4}$ g IU^{-1}) was used to convert the cellulase activities into gram cellulase protein per gram initial dry cotton stalks, namely cellulolytic enzyme (P_C) as shown in Eq. 2 [18]. Based on challenges in determining lignin peroxidase (LiP) and manganese peroxidase (MnP) activities derived on cotton stalks using spectrophotometric methods [30], ligninolytic enzymes (LiP and MnP $\{P_L\}$) were estimated by measuring TKN (organic nitrogen) in the enzyme extracts and adjusting for the base TKN in autoclaved cotton stalk extract ($C_{TKN,\,base}$) and the amount of cellulase (P_C) as shown in Eq. 3.

$$P_C = \frac{k_E \cdot C_{FPU} \cdot V}{w_0} \tag{2}$$

$$P_L = \frac{k_T \cdot (C_{TKN,E} - C_{TKN,base}) \cdot V}{w_0} - P_C \tag{3}$$

Model assumptions and setup

Several kinetic equations of varying complexity, such as linear, exponential, and logistic equations, as well as the Monod equation have been suggested to describe microbial biomass growth in SSC [31]. Among these, the logistic equation is commonly used due to its relative simple form [32]. The model describes the growth of a microbial population as a function of maximum population density, specific growth rate, and time [33]. It has been proven that the logistic equation can be successfully applied to many, but not all, cases of fungal growth on solid substrates [34]. One limitation of the logistic equation is its non-association with substrate consumption, making it unable to represent the effects of substrate variation, especially in the case of fungal growth on heterogeneous or multiple substrates. In this study, the consumption of both lignin and holocellulose as substrates had significant effect on the optimization of the fungal pretreatment process. Thus, the kinetic equations were designed to model the interaction between fungal growth and consumption of both substrates. The Monod equation, relating limiting nutrient concentration to microbial biomass growth rate, is capable of representing both aspects. Hence, the generic Monod equation [35] can be written as

$$\frac{dX}{dt} = \frac{\mu_{max} \cdot S}{Ks + S} \cdot X \tag{4}$$

In case of growth on mixed substrates, an extended Monod equation that contains parameters with multiple substrates (1 to k) has been proposed as shown in Eq. 5 [36].

$$\frac{dX}{dt} = \left(\frac{\mu_{max1} \cdot S_1}{Ks_1 + S_1} + \frac{\mu_{max2} \cdot S_2}{Ks_2 + S_2} + \cdots \frac{\mu_{maxk} \cdot S_k}{Ks_k + S_k}\right) \cdot X \tag{5}$$

The substrate is consumed by the fungus to support the metabolism for growth, product synthesis, and maintenance of microbial cells [37]. A commonly used equation for change in substrate concentration (soluble and insoluble) over time can be written as [38, 39]

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \cdot \frac{dX}{dt} - \frac{1}{Y_{P/S}} \cdot \frac{dP}{dt} - m \cdot X \tag{6}$$

Enzymes play a major role during the breakdown of complex lignocellulosic materials by fungi. Enzyme production associated with fungal growth can be described by the Luedeking–Piret model [40] as follows:

$$\frac{dP}{dt} = \alpha \cdot \frac{dX}{dt} + \beta \cdot X \tag{7}$$

In this study, by analyzing the microbial biomass growth, substrate consumption and enzyme production curves during the 14 day period can be inferred that microbial biomass growth follows a multi-phase behavior (Fig. 1). As supported by data shown in Fig. 1a, holocellulose content decreased significantly from 0.494 to 0.377 g g^{-1} during 0–4 days, which coincides with the first exponential phase of fungal growth (Fig. 1a) and the cellulase peak (Fig. 1b). Lignin content remained constant at 0.308 g g^{-1} DW and no ligninolytic enzyme content was detected until day 4. From days 4–7, lignin content decreased significantly ($P < 0.05$), while the holocellulose content became stagnant. Ligninolytic enzyme production was stimulated during this period because of carbon starvation (limited availability of easily accessible holocellulose) [14]. Meanwhile, cellulase activity depressed and dropped below 0.0003 g g^{-1} DW. As lignin was removed due to the action of ligninolytic enzymes, the fungus *P. chrysosporium* gained access to the inner cellulose and hemicellulose, therefore using both lignin and holocellulose simultaneously after day 7. As shown in Figs. 1a, b, during 7–14 days, both lignin and holocellulose were degraded and both ligninolytic and cellulolytic enzymes were present. The fungal biomass also showed an overall increasing trend from 0.0132 to 0.0366 g g^{-1} DW, yet fell into three distinct phases as described above.

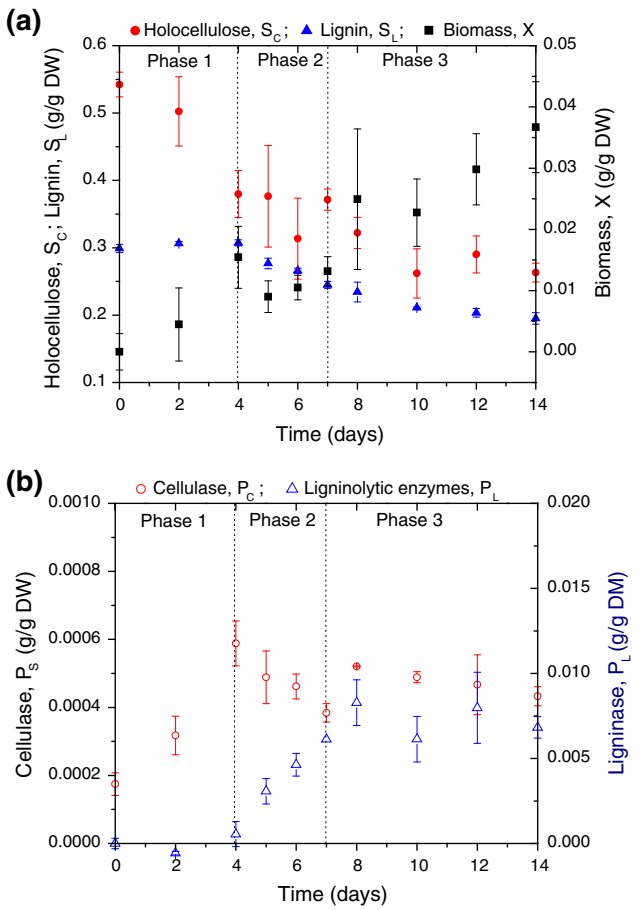

Fig. 1 **a** Holocellulose, lignin, fungal biomass; **b** cellulase and ligninase during 14 days treatment by *Phanerochaete chrysosporium*

Based on these analyses, the following assumptions were made to set up kinetic models derived from Eqs. 4 to 7:

(1) Phase 1—days 0–4: fungus prefers holocellulose (S_C) rather than lignin (S_L) as its primary carbon source; Lignin content remains constant ($dS_L/dt = 0$); and no ligninolytic enzyme is produced ($dP_L/dt = 0$).

$$\frac{dX}{dt} = \frac{\mu_{max} \cdot S_C}{K_S + S_C} \cdot X \tag{8}$$

$$\frac{dS_C}{dt} = -\frac{1}{Y_{X/S}} \cdot \frac{dX}{dt} - \frac{1}{Y_{P/S}} \cdot \frac{dP_C}{dt} - m \cdot X \tag{9}$$

$$\frac{dP_C}{dt} = \alpha \cdot \frac{dX}{dt} + \beta \cdot X \tag{10}$$

(2) Phase 2—days 4–7: easily accessible holocellulose is depleted and fungus switches to lignin (S_L) and ligninolytic enzyme production (P_L) is stimulated, while holocellulose (S_C) consumption and cellulase (P_C) production cease ($dS_C/dt = 0$, $dP_C/dt = 0$).

$$\frac{dX}{dt} = \frac{\mu_{max} \cdot S_L}{K_S + S_L} \cdot X \tag{11}$$

$$\frac{dS_L}{dt} = -\frac{1}{Y_{X/S}} \cdot \frac{dX}{dt} - \frac{1}{Y_{P/S}} \cdot \frac{dP_L}{dt} - m \cdot X \tag{12}$$

$$\frac{dP_L}{dt} = \alpha \cdot \frac{dX}{dt} + \beta \cdot X \tag{13}$$

(3) Phase 3—days 7–14: fungus regains access to the inner holocellulose and starts utilizing both holocellulose (S_C) and lignin (S_L) simultaneously and ligninolytic enzyme production (dP_L/dt) and cellulase production (dP_C/dt) are correlated with the fungus' growth (dX/dt) and the accumulation of fungal biomass (X).

$$\frac{dX}{dt} = \left(\frac{\mu_{max,C} \cdot S_C}{K_{S,C} + S_C} + \frac{\mu_{max,L} \cdot S_L}{K_{S,L} + S_L}\right) \cdot X \tag{14}$$

$$\frac{dS_C}{dt} = -\frac{1}{Y_{X/S_C}} \cdot \frac{dX}{dt} - \frac{1}{Y_{P_C/S_C}} \cdot \frac{dP_C}{dt} - m_C \cdot X \tag{15}$$

$$\frac{dS_L}{dt} = -\frac{1}{Y_{X/S_L}} \cdot \frac{dX}{dt} - \frac{1}{Y_{P_L/S_L}} \cdot \frac{dP_L}{dt} - m_L \cdot X \tag{16}$$

$$\frac{dP_C}{dt} = \alpha_C \cdot \frac{dX}{dt} + \beta_C \cdot X \tag{17}$$

$$\frac{dP_L}{dt} = \alpha_L \cdot \frac{dX}{dt} + \beta_L \cdot X \tag{18}$$

Data analysis and parameter estimation

All treatments were conducted in triplicate. Primary models for fungal growth, lignin and holocellulose consumption, and the production of ligninolytic and cellulolytic enzymes were developed using an average of two randomly selected experimental replicates. The third data set was used to validate the developed models. Data were analyzed using PROC GLM in SAS 9.1 software (SAS Inc., Cary, NC) for means and variances. Model parameters were estimated and validated by the System Biology Toolbox applying a global optimization algorithm [41] under MatLab 7.1 (The MathWorks, Inc., Natick, MA).

Results and discussion

Fungal biomass estimation

Direct measurement of fungal biomass in SSC is tedious and essentially impossible due to the difficulties in quantitatively separating intimately bound microorganisms from the solid particles as well as the extremely heterogeneous solid substrate and fungal biomass distribution

[20]. Indirect methods measuring specific cell constituents such as glucosamine, ergosterol, nucleic acids, and the widely used protein have been developed [21, 22] and shown to be effective indicators for microbial biomass concentration. It is reported that microbial respiration activities (OUR and CPR) or dry matter loss can also be correlated to fungal growth, with results affected minimally by fungal type [23, 24]. Based on the information available, it is necessary to select one or several indicators that are well adapted to the specific microorganism and the substrate. Factors such as presence and stability of the selected indicator under fungal development and cultivation conditions, presence of background indicators in the substrate itself, and the accuracy and feasibility of methods should be considered for selection of an indicator [42].

In this study, fungal biomass was estimated by quantifying the organic nitrogen content (through TKN method) in solids (Fig. 2a). Raw cotton stalk substrate (untreated samples) showed a TKN level at 9.0 mg g^{-1} (0.9 %, w w^{-1}) dry cotton stalks, while the TKN content increased with fungal growth during pretreatment and reached 15.9 mg g^{-1} dry pretreated sample. The changes in TKN content were correlated to the growth of *P. chrysosporium* that utilizes nitrogen in cotton stalk to support its metabolic activities especially protein synthesis. By doing a nitrogen balance, it was seen that the fungus in fact accumulated nitrogen in its cells when grown on cotton stalks. A linear correlation (by a factor of $k_T = 10.3$, as shown in Eq. (1) between the fungal biomass and presence of TKN was established by cultivating pure *P. chrysosporium* on PDA medium (data not shown). Therefore, organic nitrogen content was used as an indicator of fungal growth on cotton stalks, and has also been successfully applied for fungal biomass estimation in the previous studies [23, 43].

Two alternative approaches for indirect fungal biomass measurement, (1) dry matter loss and (2) oxygen uptake and carbon dioxide production, were also investigated (Fig. 2a). These measurements are simple, convenient, and can be done continuously using a weighing device or O_2/CO_2 analyzer, which has been widely reported as being used for fungal growth estimation in SSC [44, 45]. The measured values were tested for correlation with TKN-derived fungal biomass data (Fig. 2b). COU and CCP correlated well with the TKN-derived fungal biomass values measured, with coefficients of determination (R^2) of 0.8495 and 0.8476, respectively. DML also showed good correlations of 0.9505 and 0.9504 with COU and CCP, respectively. However, correlation between DML and TKN-derived fungal biomass was relatively low ($R^2 = 0.8708$). A possible explanation is that dry matter loss was mainly due to CO_2 evolution, formation of volatile products, and evaporation/consumption of water during polysaccharide metabolism, which implicates a direct

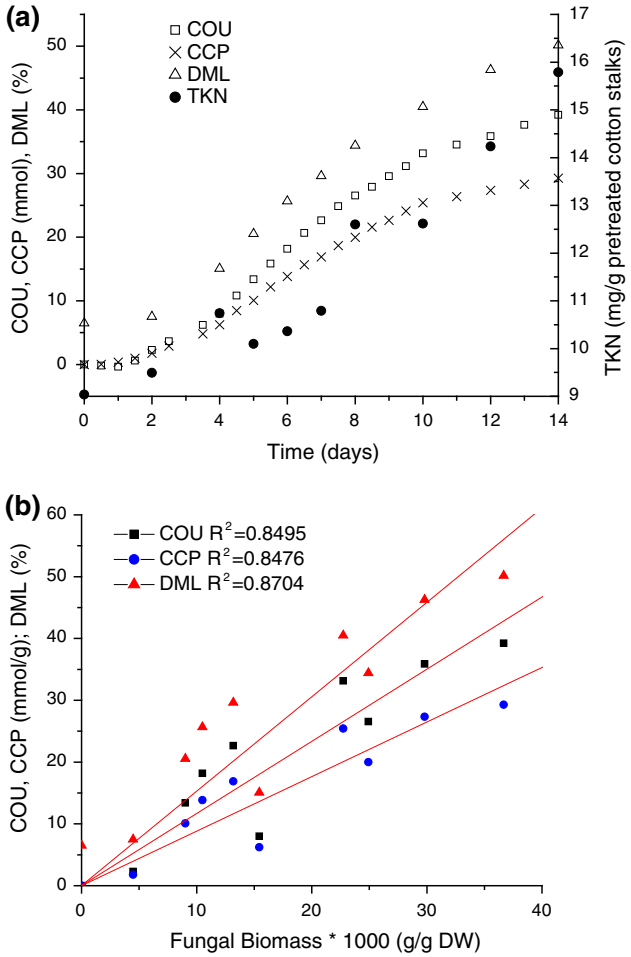

Fig. 2 Comparison of fungal biomass estimation methods **a** time course of COU, CCP, DML and TKN during 14 days SSC pretreatment by *Phanerochaete chrysosporium*; **b** correlations between COU, CCP, DML and TKN derived fungal biomass

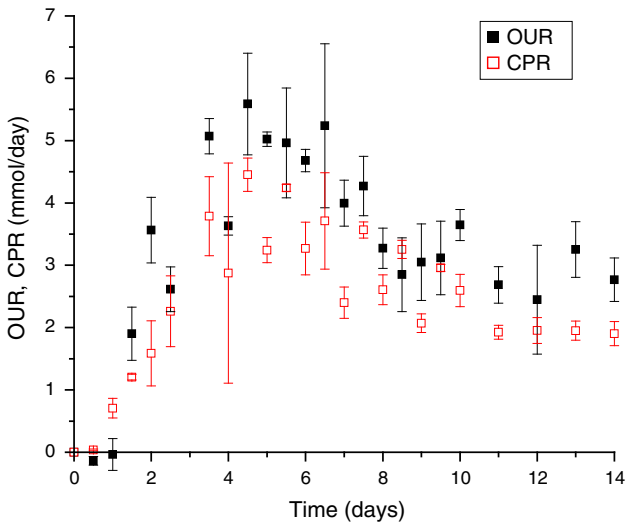

Fig. 3 Changes of OCR/CPR during time course of 14 days cultivation of *Phanerochaete chrysosporium*

correlation to COU/CCP in the SSC system [24, 46]. DML and COU/CCP present good information about metabolic activities, nevertheless, they may not be consistent (linear) indicators of fungal biomass since they can vary during the different stages of fungal growth, as shown in Fig. 3 and reported elsewhere [20, 47, 48].

Fungal respiratory metabolism

The fungal respiratory activities such as OUR, CPR, and RQ not only serve as indicators of fungal growth but also reflect metabolic activities especially lignin consumption. It was noted that both OUR and CPR peaked during days 4–8, which coincided with the period when lignin degradation occurred (Figs. 1a, 3). This may be attributed to the oxidative nature of delignification by white-rot fungi, as lignin molecules were oxidized in the presence of peroxidases and oxygen [49]. The RQ, calculated from the ratio of CO_2 produced/O_2 consumed, has been reported to vary when different organic components are degraded during various growth phases under aerobic conditions [50]. RQ less than 1 indicates oxidation of lignin combined with metabolism of polysaccharides, where lower RQs would suggest strong delignification activities [20, 49]. In the previous study, an RQ of 0.83 was observed along with 32 % lignin degradation during a 14 day solid state fermentation of oat straw by *P. chrysosporium* under 1 day intermittent oxygen flushing at 30 °C [49]. On average, the RQ value in this study was 0.74, indicating substantial lignin degradation (34 %) observed during the 14 day microbial pretreatment. These results also suggested that oxygen supplementation was a critical factor for effective delignification.

Model parameter estimation

Mathematical models provide important information for understanding interrelations between substrate consumption, enzyme production, and fungal growth as well as serve as a tool for optimizing the design, operation, and scale-up of SSC systems. In this study, empirical kinetic models in forms of differential equations were used to describe the interaction between substrates (S_C and S_L), fungal biomass (X), and enzymes (P_C and P_L). Experimental data were used to determine the model parameters. Parameter estimation not only allowed fitting of the experimental profiles but also helped provide useful information on understanding and analyzing the microbial pretreatment process. The parameters estimated by the System Biology Toolbox are tabulated in Table 1.

In general, when two or more substrates are present in the medium initial metabolism will take place on the substrate that is preferred, followed by potential use of the remaining substrates. This phenomenon of utilization of one substrate followed by the second is defined as diauxic growth on mixed substrates [35]. Cotton stalk is a heterogeneous and chemically complex lignocellulosic feedstock, which consists of holocellulose (49.4 %), lignin (30.8 %), and other components (19.8 %) such as extractives and ash [8]. There is a noticeable amount of easily accessible cellulose, which consists of cotton fiber residues (mostly pure cellulose) in untreated cotton stalk, hence preferential metabolism of holocellulose was observed during phase 1 (day 0–4). After depleting the easily accessible holocellulose, the fungus switched to lignin substrate by adjusting its enzyme systems to ligninolytic enzymes (phase 2, day 4–7). During 7–14 days, the fungus utilized both holocellulose and lignin as its substrates. This could be a simultaneous process or an alternative process that impacts shifting from one substrate to another.

Table 1 Summative parameter estimation results for SSC 1d oxygen flushing pretreatment models

Parameters	Day 1–4	Day 4–7	Day 7–14	
			S_C	S_L
μ_{max}	0.8765	2.10E−08	0.4105	0.0192
Ks	1.8378	0.9581	0.1504	0.3609
$Y_{X/S}$	0.0949	0.0102	0.4371	0.0098
$Y_{P/S}$	0.0026	0.0896	0.0039	0.0018
m	0.0053	0.0255	0.0365	0.0477
α	0.0060	0.0048	0.0029	1.00E−08
β	4.15E−09	0.0175	0.0034	1.00E−08

Maximum specific growth rate (μ_{max}) and the half-saturation coefficient (K_S) are the two parameters in the Monod equation. μ_{max} corresponds to the maximum specific growth rate that the substrate could support while K_S is the substrate concentration at which μ is one-half of its maximum. It was noted that holocellulose as a substrate supported a higher maximum specific growth rate (μ_{max}) than lignin. During phase 1 (day 0–4), when holocellulose was consumed as the major substrate, a greater μ_{max} of 0.8765 day^{-1} was observed than a μ_{max} of 2.10E−08 day^{-1} when the fungus switched to lignin as its main substrate (day 4–7). A similar phenomenon occurred during 7–14 days, when *P. chrysosporium* utilized both lignin and holocellulose as its substrates. Holocellulose supported a higher maximum specific growth rate (μ_{max}) of 0.4105 day^{-1} relative to lignin (0.0192 day^{-1}). The maximum specific growth rate (μ_{max}) of 0.4105–0.8765 day^{-1} observed for holocellulose as a substrate is comparable to an average μ_{max} of 0.489 reported in the previous study that modeled the growth kinetics of *P. chrysosporium* in submerged static cultivation using glycerol-based media [15].

The glycerol-based substrate, however, supported a higher $Y_{X/S}$ of 0.4405 than observed in this study [15]. Yields (Y_{X/S_c}) of fungal biomass from holocellulose as substrate (0.0949 and 0.4371 for phase 1 and phase 3, respectively) were higher than those of lignin as a substrate (Y_{X/S_L}) (0.0102 and 0.0098 for phase 2 and phase 3, respectively) (Table 1). It has been inferred that yield of new fungal biomass from the lignin metabolic process is extremely low [51]. As a complex polymer of phenylpropane units, lignin has been proven to be resistive to microbial degradation. As one of the best candidates capable of effectively breaking down lignin, *P. chrysosporium* requires much higher levels of metabolic energy and nutrients to degrade these polymers [52], supporting the lower fungal biomass yield values observed. Previous studies have suggested that lignin degradation requires a more readily degradable substrate to support the energy gain of white-rot fungi [51]. At a stage during the pretreatment of cotton stalks when lignin was the primary carbon source (phase 2), the fungal biomass growth rate remained at a very low level as no significant increase in X was observed. Energy spent for maintaining the metabolic activities of fungal biomass increased as the fungal biomass increased. As shown in Table 1, the maintenance coefficients during days 4–7 ($m = 0.0255$) and 7–14 ($m = 0.0365$ and 0.0477 for S_C and S_L, respectively) were greater when compared with phase 1 ($m = 0.0053$). The maintenance coefficients derived from this study were lower than the values reported in the previous studies [53], where researchers observed a higher growth rate constant of 0.0348 h^{-1} and a substrate-based maintenance coefficient of 0.028 h^{-1} on the growth of *P. chrysosporium* biofilm on polysulphone capillary membrane in an air-sparged reactor with glucose as the carbon source. It is possible that the growth rate and maintenance energy were higher because glucose (rather than a complex carbohydrate) was used as the carbon source for *P. chrysosporium* [54].

Interestingly, the growth/non-growth associated constants (Eq. 7) obtained ($\alpha = 0.0060$ g-cellulase/g-fungal biomass and $\beta = 4.15E{-}09$ g-cellulase/g-fungal biomass/d) through the kinetics study inferred that a growth-associated factor played a more important role in cellulase production during day 0–4 as shown by the magnitude difference of α over β. However, it seemed that ligninolytic enzyme production during 4–7 days was more likely a process not associated with fungal growth as indicated by the greatness of β over α ($\alpha = 0.0048$ g-ligninolytic enzymes/g-fungal biomass and $\beta = 0.0175$ g-ligninolytic enzymes/g-fungal biomass/d). This suggests that ligninolytic enzymes are produced in the presence of lignin only when necessary (e.g., easily accessible carbon gone). Since lignin cannot support high fungal biomass formation, as indicated by the low Y_{X/S_L}, it appears that these enzymes are produced under low or non-growth-associated conditions and are not necessarily a secondary product [52]. The determined alpha and beta values support this hypothesis.

Model validation

Model curves were generated by substituting the estimated kinetic parameters (Table 1) into the respective phase equations and compared by plotting both experimental (independent data set) and the model predicted values (Fig. 4). Further comparison was achieved via a linear regression on experimental data and predicted values. The resulting R^2 (coefficient of determination) values assess how well the model described the data, with higher

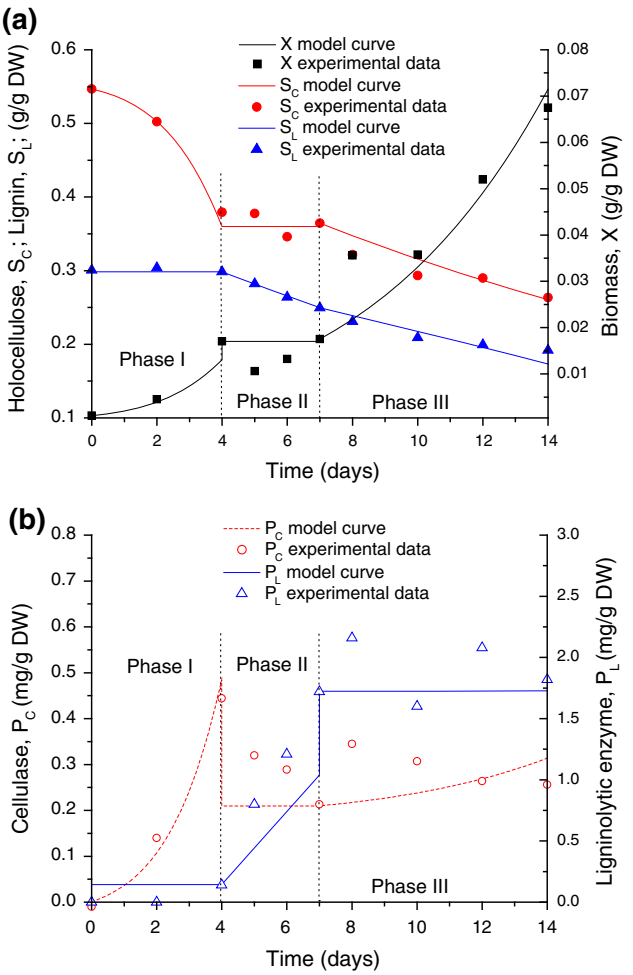

Fig. 4 Experimental and predicted fungal biomass, holocellulose, lignin, cellulase and ligninolytic enzyme during 14 days treatment by *Phanerochaete chrysosporium*; **a** fungal biomass, X, holocellulose, S_C and lignin, S_L; **b** cellulase production, P_C and ligninolytic enzyme production, P_L

Table 2 Summative model validation results for SSC 1d oxygen flushing pretreatment models

Model variables	R^2				Slope
	Phase 1	Phase 2	Phase 3	Overall	
Fungal biomass, X	0.9973	N/A	0.8492	0.9309	1.0122
Holocellulose, S_C	0.9798	N/A	0.8505	0.9724	0.9967
Lignin, S_L	N/A	0.9986	0.7769	0.9686	1.0033
Cellulase, P_C	0.9710	N/A	N/A	0.6138	1.0639
Ligninolytic enzymes, P_L	N/A	N/A	0.7898	0.9203	1.1129

coefficients and slopes near unity indicating a better fit (Table 2).

Overall, the models proposed in this research predicted the experimental data well. Estimated parameters yielded sufficiently accurate predictions (Table 2) for fungal growth (TKN, slope = 0.9967; $R^2 = 0.9724$), holocellulose consumption (slope = 1.0033; $R^2 = 0.9686$), and lignin degradation (slope = 1.0122; $R^2 = 0.9309$). Although, generally the models gave a good estimation of ligninolytic enzyme production over time ($R^2 = 0.9203$), there was under estimation of the predicted values as represented by a slope of 1.1129. The predictability of cellulase production ($R^2 = 0.6138$) by the models established in this study was relatively low although a good linear correlation was obtained (slope = 1.0639). This may be associated with the bias introduced in cellulase activity measurements due to the high affinity of cellulase to substrates [55]. Another possible explanation for lower predictive ability is that the model may have been oversimplified by assuming that no change occurs in holocellulose and cellulase content during day 4–7.

Implications of modeling on improving fungal pretreatment efficiency

Mathematical modeling has important implications on the design and optimization of fungal pretreatment processes. The purpose of pretreatment is to enhance the enzymatic digestibility of cellulose through breakdown of the lignin while preserving the cellulose content [56]. An effective fungal pretreatment process requires high selectivity for lignin degradation over cellulose consumption. Previous biological pretreatment studies have shown that moisture content, nutrients, aeration, and time are critical for fungal growth and metabolism to achieve good selectivity [57]. As indicated in this study, majority of the lignin degradation occurred during 4–7 days, since the fungus first depleted the easily accessible holocellulose until it experienced preferential nutrient starvation. In chemically defined media, ligninolytic peroxidases (ligninolytic enzymes) are secreted only in response to nutrient depletion, especially nitrogen limitation and carbon limitation [14]. However, in this study where cotton stalks served as a heterogeneous feedstock, nitrogen may not have been a limiting factor, as TKN values for cotton stalks in culture media were approximately 30.7 mM nitrogen which exceeds lower nitrogen level limits of 2.4 mM [14]. In contrast, ligninolytic enzyme production may be induced by lignin itself after depleting easily digestible cellulose and hemicellulose fractions. Thus, the composition and the structural rigidity of lignocellulosic biomass may affect ligninase secretion and delignification efficiency. Results also suggest that aeration or oxygen supplementation may greatly influence not only fungal growth but also lignin degradation during fungal pretreatment of lignocellulosic biomass [18].

Furthermore, high selectivity for lignin degradation can be achieved by choosing different fungal species such as *P. ostreatus* and *C. subvermispora* or genetically modifying *P. chrysosporium* to lack the ability to produce cellulase [58–60]. However, based on the observations in this research and previous studies, the energy gain from lignin catabolism alone might be too low to support the normal fungal growth and metabolic activities [52]. In order to provide carbon/energy sources other than cellulose/hemicellulose, supplementation with low grade sugar/starch wastes may be feasible alternatives. Engineered microbial species that use only hemicellulose as carbon/energy source could be another possible solution. However, this approach may reduce the availability of xylose as a fermentable sugar. Further investigations into other mechanisms that control the metabolic activities of delignification will also help to optimize the fungal pretreatment process.

Conclusions

In this study, a set of mathematical equations have been proposed for describing the holocellulose consumption, lignin degradation, cellulase, and ligninolytic enzyme production associated with the growth of *P. chrysosporium* on cotton stalks for 14 days. Extended Monod equations were applied in order to capture the condition where both holocellulose and lignin were used as substrates. Additionally, substrate consumption equations were used to predict the effects of substrate utilization on fungal biomass growth, product synthesis, and maintenance of microbial cells. Luedeking–Piret equations were utilized to describe enzyme productions associated with utilization of substrates. The models were established in three different phases (I: day 0–4, II: day 4–7, III day 7–14) based on assumptions on fungal growth and substrates consumption. The models yielded sufficiently accurate predictions for

fungal growth, holocellulose consumption, lignin degradation, and ligninolytic enzyme production, but fair predictions for cellulase production. This study provides a better understanding of the fungal delignification process and offers insights on improving the efficacy of microbial pretreatment technology.

Acknowledgments The authors would like to thank Dr. Van Den Truong (Dept of Food Science, NCSU), Roger Thompson (USDA-ARS, NCSU) and Rachel S. Huie (Dept of Biological and Agricultural Engineering, NCSU) for generously helping with TKN and O_2/CO_2 analysis.

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