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DOI
10.1016/j.cesx.2020.100085

Publication date
2020

Document Version
Final published version

Published in
Chemical Engineering Science: X

Chemical Engineering Science: X 8 (2020) 100085

Contents lists available at ScienceDirect

Chemical Engineering Science: X

journal homepage: www.elsevier.com/locate/cesx

Mathematical modelling for the optimization of cellulase production using glycerol for cell growth and cellulose as the inducer substrate

Lucas Gelain [a,b,*], Luuk van der Wielen [a,c], Walter M. van Gulik [a], José Geraldo da Cruz Pradella [d], Aline Carvalho da Costa [b]

[a] Delft University of Technology, Department of Biotechnology, Van der Maasweg 9, 2629HZ Delft, the Netherlands
[b] University of Campinas, School of Chemical Engineering, Av. Albert Einstein, 500, Campinas, Brazil
[c] University of Limerick, Bernal Institute, V94 T9PX Limerick, Ireland
[d] Federal University of São Paulo, Institute of Science and Technology, Av. Cesare Mansueto Giulio Lattes, 1201 S. J. Campos, Brazil

ARTICLE INFO

Article history:

Keywords:
Mathematical modelling
Trichoderma harzianum
Cellulose
Glycerol
Bioprocess
Optimization of cellulase production

ABSTRACT

Cellulase production can be divided into two steps: growth stage; followed by an induction stage. To develop a mathematical model for the optimization of this strategy, two sets of experiments were performed in batch mode for parameter estimation. One set of experiments was performed to evaluate the influence of glycerol regarding cell growth (initial concentrations of 5, 10, 15 and 20 g/L). The other set of experiments considered the induction stage using cellulose as the substrate (initial concentrations of 5, 10, 20, 30 and 40 g/L). Two feeding strategies were simulated to maximize cellulase production using glycerol to maintain a high cell concentration. The first simulation used a discrete feed and the second used a continuous feed of cellulose. The mathematical model proposed allows maintaining a high cell concentration and the addition of optimal small amounts of the inducer substrate to prevent inhibition of enzyme production.

1. Introduction

In the biotechnological production of biofuels, such as ethanol and other chemicals using lignocellulosic materials, hydrolysis is one of the most important steps of the process. The enzymes used in the hydrolysis step (cellulase) can be produced by filamentous fungi of the genus *Trichoderma*, which is well adapted for bioprocesses (Strakowska et al., 2014).

The importance of cellulase production goes beyond its use in lignocellulose hydrolysis. Recent market reports show that cellulase has increasingly been used in many industrial applications, such as coffee processing, winemaking, fruit juice production, paper and pulping as well as laundry detergents and the production of cleaning and washing agents (Jayasekara and Ratnayake, 2019). These authors also cite applications in agriculture and medical area.

The production of cellulase has been determined as non-growth associated (Gelain et al., 2015). Thus, separation of the process into two-stage, allowing the optimization of the growth stage and the production stage separately may result in increased process performance. Delabona et al. (2016) showed that a two-stage process consisting of growth of *T. harzianum* on glycerol, followed by induction with sugarcane bagasse pretreated led to an important increase in productivity and cellulase activity. The authors suggested that the increase in production was due to a greater number of active tips of mycelia as well as long hyphae, which increased protein secretion capacity. Additionally, glycerol is reported being a "neutral" carbon source (Ilmén et al., 1997), thus could prevent catabolite repression to occur.

Studies on decreasing cellulase production costs are important since productivity and enzymatic activity in the growth cultures are in general low. To achieve this goal, mathematical modelling becomes an important tool as mathematical models can be used to develop optimal strategies. Therefore, the development of mathematical models for the two-stage process described in Delabona et al. (2016) could be an interesting approach aiming at the maximization of the cellulase productivity in fed-batch mode since the model can be used to suggest optimal feeding strategies.

In this work, a mathematical model for cellulase production using glycerol for cell growth and cellulose as the inducer substrate is proposed and used to simulate optimal feeding policies in a fed-batch cellulase production process. The experiments were performed in batch mode using different initial concentrations of

* Corresponding author.
E-mail address: lgelain@ucs.br (L. Gelain).

https://doi.org/10.1016/j.cesx.2020.100085
2590-1400/

the substrates and then the data were used to estimate the parameters for the mathematical model. After that, the mathematical model was adapted for fed-batch mode and used to simulate optimal feeding policies for the production of cellulase. The simulations consider the consumption of glycerol for cell growth and the consumption of cellulose for cellulase production. The strategies proposed allow maintaining a high cell concentration using a "neutral" substrate and the addition of optimal small amounts of the inducer substrate to prevent inhibition of enzyme production.

2. Materials and methods

2.1. Microorganism

The wild strain *Trichoderma harzianum* P49P11 was used in this study. The strain was isolated from the Amazon forest (Delabona et al., 2012). It was grown on potato dextrose agar at 29 °C for 5 days and then used for inoculum preparation.

2.2. Culture conditions

The culture conditions were prepared according to Gelain et al. (2015). The spore suspension of *T. harzianum* was transferred to a 2 L shake flask containing per litre: glucose, 10 g; peptone, 1 g; Tween 80, 1 mL; saline solution, 50 mL. After 60 h of cultivation at 29 °C and 200 rpm in a shaker (New Brunswick Scientific innova44), 10% (v/v) was transferred to a 3 L bioreactor (New Brunswick Scientific BioFlo 115) containing per litre: glycerol, 5, 10, 15, or 20 g; or cellulose (Celufloc 200^{TM}, Celuflok Ind. Com., Brazil), 5, 10, 20, 30 or 40 g; peptone, 1 g; Tween 80, 1 mL; saline solution, 50 mL. The solution of Mandels was used (Mandels and Reese, 1957), in g/L: KH_2PO_4, 20; $(NH_4)_2SO_4$, 14; urea, 3; $MgSO_4{\cdot}7H_2O$, 3; $CaCl_2$, 3; $FeSO_4{\cdot}7H_2O$, 0.05; $ZnSO_4{\cdot}7H_2O$, 0.014; $MnSO_4{\cdot}H_2O$, 0.016; $CoCl_2$, 0.02. Batch mode experiments were performed in duplicate with a working volume of 1.9 L. The inocula used for the experiments using glycerol were prepared separately from the inocula used for cellulose conditions. Additionally, the duplicates were carried out by using different inocula. For parameter estimation, an average of the cell concentration from the inocula was used that resulted in an initial cell concentration of 0.4 ± 0.02 g/L for the experiments using cellulose, and 0.5 ± 0.03 g/L for the experiments using glycerol. The temperature was controlled at 29 °C and the pH was controlled at 5.0 ± 0.5 by the addition of an aqueous solution of NH_4OH (1:3) and 0.4 M H_2SO_4. The stirring speed was kept between 200 and 300 rpm, and the airflow between 0.48 and 0.7 vvm to prevent dissolved oxygen to drop below 30%. The value of 30% of dissolved oxygen was used as a standard value for all experiments to guarantee an excess of dissolved oxygen present in the growth medium. Furthermore, 1 mL/L of polypropylene glycol antifoaming agent (P2000, Dow Chemical, Brazil) was added. One experiment was performed starting in batch mode with 15 g/L of glycerol, and after 24 h, a mass of cellulose was added resulting in 20 g/L inside the bioreactor. This experiment was named repeated batch. The media were sterilized at 121 °C for 30 min.

2.3. Analytical procedures

The samples were taken at 8, 12, 24, 32, 48, 72 and 96 h for the experiments in batch mode for the visualization of the profiles of cell, substrate and enzymes. Cellulase activity was determined using the filter paper activity assay (Ghose, 1987). Reducing sugars were measured by the DNS method (Miller, 1959). The method for the estimation of beta-glucosidase activity was adapted from Zhang et al. (2009). The cellulose and mycelium concentrations for the experiments using cellulose were determined according to Ahamed and Vermette (2009). The enzymes are present in the supernatant of the samples and can be separated from the cell and residual concentration of cellulose by centrifugation. More details of these analytical procedures are described in Gelain et al. (2015).

For glycerol assays, 10 mL of culture broth was withdrawn and centrifuged (3000x g for 20 min). The supernatant was used to measure glycerol concentration and the pellet was dried (70 °C) until constant weight for the determination of cell concentration. Glycerol was measured using the column Aminex HPX-87H 300x7.8 mm (BIO-RAD), a flow rate of 0.6 mL/min, isocratic conditions, and H_2SO_4 as the eluent for 30 min. The equipment was the Agilent 1260 Infinity with an infrared detector.

2.4. Mathematical methods

Parameter estimation and simulations were performed using Matlab R2013b. The differential equations were solved by the *ode8* function, the objective function was minimized by the *fmincon* function using the interior-point algorithm, and the *interp1* was used for interpolation. The simulations of the equations were performed using Simulink (Matlab). The optimization of feeding strategies was performed according to Becerra (2004). The experiments with initial glycerol concentrations of 5, 10 and 20 g/L and initial cellulose concentrations of 10, 20 and 30 g/L were employed for parameter estimation. The experiment with 15 g/L of glycerol was used for validation of the mathematical model using glycerol as the substrate and the experiments with 5 and 40 g/L of cellulose were used for extrapolation analysis of the mathematical model using cellulose as the substrate. The repeated batch was used to test the prediction capacity of the model using cellulose as the substrate. The objective function described in Andrade et al. (2013) was used for parameter estimation (Eq. (1)). The mathematical model and simulation platforms used in this project are available in Gelain (2020).

$$f = \sum_{n=1}^{np} \left[\frac{(X_n - X_{en})^2}{{X_{em}}^2} + \frac{(S_n - S_{en})^2}{{S_{em}}^2} + \frac{(P_n - P_{en})^2}{{P_{em}}^2} \right] \quad (1)$$

where, X_{en}, S_{en} and P_{en} are the experimental data, X_n, S_n and P_n are the concentrations provided by the model, X_{em}, S_{em} and P_{em} are the maximum measured concentrations of cell, substrate and product, respectively, and *np* is the number of samples.

3. Results and discussion

3.1. Mathematical modelling

The substrate consumption rate was considered dependent on cell growth rate and proportional to mycelium concentration (C_X) (Eqs. (2) and (3)). Eq. (2) describes the consumption of glycerol (C_G) for cell growth and Eq. (3) describes the consumption of cellulose (C_C) for cell growth and has also a second term that describes the consumption of cellulose by active cells (C_{Xact}). Active cells are an attempt at cell segregation, wherein this case, they are considered as being the part of cells responsible for synthesizing enzymes. This term considers that part of the cells is being activated due to the presence of an inducer (cellulose). These equations consider a dilution rate (D), which represents the feeding of glycerol. Eq. (3) has also a term corresponding to continuous feeding of cellulose through an inflow rate, u (g/h). It was considered that there was no dilution effect inside the bioreactor when cellulose (solid material) was added. For the batch condition, the

dilution rate (D) is equal to zero as well as the inflow rate of cellulose (u). Parameters are displayed in Tables 1 and 2.

$$\frac{dC_G}{dt} = -\alpha'\left(\frac{dC_X}{dt}\right)_g C_X + D(C_{G,f} - C_G) \tag{2}$$

$$\frac{dC_C}{dt} = -\alpha\left(\frac{dC_X}{dt}\right)_g C_X - \beta\left(\frac{dC_{Xact}}{dt}\right)_g C_{Xact} + \frac{u}{V} - DC_C \tag{3}$$

where, α' is the constant of glycerol consumption, α is the constant of cellulose consumption for cells, β is the constant of cellulose consumption for active cells, $C_{G,f}$ is the glycerol concentration from the feed, and V is the volume.

Eq. (4) describes the cell growth rate depending on the substrate (C_S = cellulose (C_C) or glycerol (C_G)) according to the *Monod* equation. The cell growth rate has an inhibition term dependent on cell concentration according to the logistic equation for population growth (Fujikawa et al., 2004). It was assumed that there was a control of cell growth based on cell concentration where C_{XmS} is considered the maximum cell concentration allowed by the environment. Eq. (5) represents the cell growth for the conditions using glycerol and Eq. (6) describes the cell growth for the conditions using cellulose as the substrate. Eq. (6) does not consider the dilution rate because it was only used to describe cell growth in batch conditions.

$$\left(\frac{dC_X}{dt}\right)_g = \mu_{XmS}\left(\frac{C_S}{C_S + k_S}\right)\left(1 - \frac{C_X}{C_{XmS}}\right)C_X - DC_X \tag{4}$$

$$\left.\frac{dC_X}{dt}\right|_G = \mu_{XmG}\left(\frac{C_G}{C_G + k_G}\right)\left(1 - \frac{C_X}{C_{XmG}}\right)C_X - C_X(\mu_{XmdG} + D) \tag{5}$$

$$\left.\frac{dC_X}{dt}\right|_C = \mu_{XmC}\left(\frac{C_C}{C_C + k_C}\right)\left(1 - \frac{C_X}{C_{XmC}}\right)C_X - \mu_{XmdC}\left(\frac{C_C}{C_C + k_{Cd}}\right)C_X \tag{6}$$

where, μ_{XmS}, μ_{XmG}, μ_{XmC} are the maximum specific cell growth rates, k_S, k_G, k_C are the *Monod* constants for cell growth, C_{XmS}, C_{XmG}, C_{XmC} are the maximum cell concentrations, μ_{XmdG}, μ_{XmdC} are the maximum specific rates for cell death and k_{Cd} is the *Monod* constant for cell death for cellulose conditions. The *Monod* constant for cell death for glycerol conditions was considered zero by the parameter estimation. Subscripts *S*, *C* and *G* correspond to substrate, cellulose and glycerol, respectively.

Velkovska et al. (1997) proposed that the cellulase production by *Trichoderma reesei* Rut C30 using cellulose was not associated with cell growth. They developed a mathematical model with cell segregation where first the formation of a primary mycelium responsible for a high substrate consumption rate occurs, followed by the formation of a secondary mycelium. This secondary mycelium was considered responsible for cellulase synthesis. This segregation was also considered in this project and the cells responsible for enzyme production were named active cells. Eq. (7) describes the active cell growth rate and Eq. (8) includes the deactivation rate (k_{da}).

Table 1
Parameters for the mathematical model using glycerol as the substrate.

μ_{XmG}	Maximum specific cell growth rate (h^{-1})	0.23
k_G	Monod constant for cell growth (g/L)	2
C_{XmG}	Maximum cell concentration (g/L)	25.8
μ_{XmdG}	Maximum specific rate for cell death (h^{-1})	0.04
α'	Constant of glycerol consumption (g (of C_G) L/ g^2 (of C_X))	0.38
D	Dilution rate (h^{-1})	0.0006
$C_{G,f}$	Glycerol concentration in the feed (g/L)	1 200
C_X	Cell concentration (g/L)	
C_G	Glycerol concentration (g/L)	

Table 2
Parameters for the mathematical model using cellulose as the substrate.

μ_{XmC}	Maximum specific cell growth rate (h^{-1})	0.48
k_C	Monod constant for cell growth (g/L)	6
C_{XmC}	Maximum cell concentration (g/L)	12
μ_{XmdC}	Maximum specific rate for cell death (h^{-1})	0.095
k_{Cd}	Monod constant for cell death (g/L)	0.44
q_{Fm}	Maximum specific rate for cellulase production (FPU/g (of C_{Xact}) h)	23.5
C_{Fm}	Maximum cellulase activity (FPU/L)	2 513
k_{iF}	Inhibition constant for cellulase production $(g/L)^2$	1.91
k_{dF}	Deactivation constant for cellulase (h^{-1})	0.002
q_{Bm}	Maximum specific rate for beta-glucosidase production (U/g (of C_{Xact}) h)	63.42
C_{Bm}	Maximum beta-glucosidase activity (U/L)	5 013
k_{iB}	Inhibition constant for beta-glucosidase production $(g/L)^2$	3.97
k_{dB}	Deactivation constant for beta-glucosidase (h^{-1})	0.0011
μ_{em}	Maximum specific growth rate for active cells (g (of C_{Xact}) / g h)	0.25
k_{Ce}	Monod constant for active cell growth (g/L)	2.84
$C_{Xact\,m}$	Maximum active cell concentration (g/L)	5.76
S_{iF}	Concentration of cellulose that inhibits cellulase production (g/L)	10
S_{iB}	Concentration of cellulose that inhibits beta-glucosidase production (g/L)	8
k_{da}	Deactivation constant for active cells (h^{-1})	0.38
a	Inhibition control for cellulase	0/0.15
b	Inhibition control for beta-glucosidase	0/0.15
α	Constant of cellulose consumption for cells (g (of C_C) L/ g^2 (of C_X))	0.069
β	Constant of cellulose consumption for active cells (g (of C_C) L/ g^2 (of C_{Xact}))	0.21
u	Inflow rate of cellulose (g/h)	
V	Volume (L)	
C_X	Cell concentration (g/L)	
C_{Xact}	Active cell concentration (g/L)	
C_C	Cellulose concentration (g/L)	
C_F	Cellulase activity (FPU/L)	
C_B	Beta-glucosidase activity (U/L)	

$$\left(\frac{dC_{Xact}}{dt}\right)_g = \mu_{em}\left(\frac{C_C}{C_C + k_{Ce}}\right)\left(1 - \frac{C_{Xact}}{C_{Xact\,m}}\right)C_X - DC_{Xact} \tag{7}$$

$$\frac{dC_{Xact}}{dt} = \mu_{em}\left(\frac{C_C}{C_C + k_{Ce}}\right)\left(1 - \frac{C_{Xact}}{C_{Xact\,m}}\right)C_X - C_{Xact}(k_{da} + D) \tag{8}$$

where, μ_{em} is the maximum specific growth rate for active cells, k_{Ce} is the *Monod* constant for active cell growth, $C_{Xact\,m}$ is the maximum active cell concentration and k_{da} is the deactivation constant for active cells.

Cellulase (C_F) and beta-glucosidase (C_B) production rates have an inhibition term dependent on enzymatic activity, and a second, dependent on cellulose concentration (C_C) (Equations (9) and (10), respectively). The enzyme production rates are proportional to active cell concentration. Parameters a and b represent logical controls regarding the inhibition influence by cellulose according to another parameter, S_{iF} and S_{iB}, respectively, and when cellulose concentration is above these values (S_{iF} and S_{iB}), a and b are equal to 0.15, adding an inhibition effect on the enzyme production rate. Otherwise, a and b are equal to zero. S_{iF} and S_{iB} are cellulose concentrations that inhibit the production of cellulase and beta-glucosidase, respectively. The values of a and b were manually adjusted according to the residual value of the objective function (Eq. (1)). They were the only parameters kept constant during parameter estimation. Values between 1 and 0 were tested.

$$\frac{dC_F}{dt} = q_{Fm}\left(1 - \frac{C_F}{C_{Fm}}\right)\left(\frac{1}{aC_C{}^2/k_{iF} + 1}\right)C_{Xact} - C_F(k_{dF} + D) \tag{9}$$

$$\frac{dC_B}{dt} = q_{Bm}\left(1 - \frac{C_B}{C_{Bm}}\right)\left(\frac{1}{bC_C{}^2/k_{iB} + 1}\right)C_{Xact} - C_B(k_{dB} + D) \quad (10)$$

where, q_{Fm} is the maximum specific rate for cellulase production, C_{Fm} is the maximum cellulase activity, k_{iF} is the inhibition constant for cellulase production, k_{dF} is the deactivation constant for cellulase, q_{Bm} is the maximum specific rate for beta-glucosidase production, C_{Bm} is the maximum beta-glucosidase activity, k_{iB} is the inhibition constant for beta-glucosidase production and k_{dB} is the deactivation constant for beta-glucosidase.

Eq. (11) describes the variation in the volume (V).

$$\frac{dV}{dt} = DV \quad (11)$$

3.2. Results for mathematical modelling using glycerol in batch mode

The experiments varying the initial concentrations of glycerol (5, 10 and 20 g/L) in the batch mode were employed for parameter estimation for the growth stage. Eqs. (2) and (5) were used. The initial conditions for parameter estimation were 0.5 g/L of cell and 5, 10 and 20 g/L of glycerol. The experiment using 15 g/L of glycerol was used to validate the model. The results of the fit (continuous lines) and the experimental data are shown in Fig. 1. Error bars correspond to the sample standard deviation.

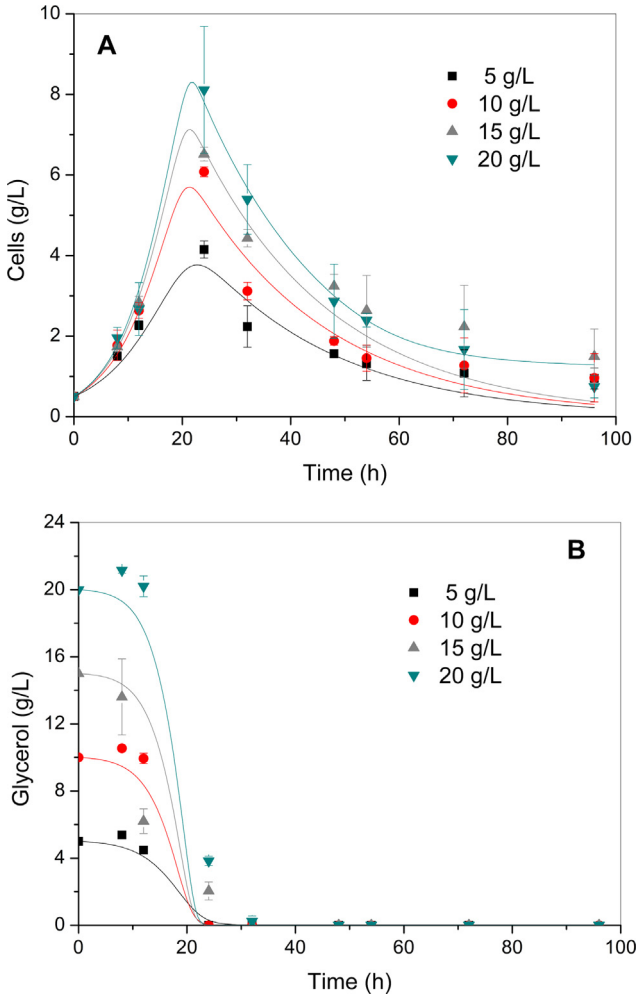

Fig. 1. Fit of the model (continuous lines) for cell concentration (A) and glycerol concentration (B).

The fit of the model proposed for cell growth followed the profiles of the experimental data. Glycerol consumption was fast for all the conditions and the model described the profiles based on the experimental data available. Nevertheless, more samples during the variation period in glycerol concentration would have contributed to improving the fit and the visualization of the profiles of glycerol consumption. The data from 15 g/L condition was not used for parameter estimation and the simulation of this condition predicted the profiles of cell and glycerol concentrations. The parameter estimation considered the average values from the duplicates of the experimental data and the model does not include the influence of the experimental errors. Parameters are shown in Table 1.

3.3. Results for mathematical modelling using cellulose in batch mode

Assays with initial concentrations of cellulose of 10, 20 and 30 g/L were used for parameter estimation. Eq. (3) was used for cellulose consumption, Eq. (6) for cell growth, Eq. (8) for active cell concentration and Equations (9) and (10) for enzyme production. The initial conditions for parameter estimation were 0.4 g/L of cell, 10, 20 and 30 g/L of cellulose and the enzymatic activities of cellulase and beta-glucosidase were considered zero. Fig. 2A, 2B and 2C show the fit of the model for cell growth, substrate consumption and cellulase production using 10, 20 and 30 g/L of cellulose, respectively, and Fig. 2D shows the fit of beta-glucosidase activity for those three conditions. The fit of the model proposed for cell growth, cellulose consumption and enzyme production followed the profiles of the experimental data. Parameters are shown in Table 2.

According to parameter estimation using cellulose, the maximum concentration of cells and active cells allowed in the bioreactor are 12 g/L and 5.76 g/L, respectively. The maximum cellulase and beta-glucosidase activities are 2 513 FPU/L and 5 013 U/L, respectively. According to parameter estimation using glycerol (Table 1), the maximum concentration of cells allowed in the bioreactor is 25.8 g/L. The use of glycerol with cellulose could allow the increase in the cell concentration compared to experiments only using cellulose, creating the possibility of also increasing the concentration of active cells, which could provide an increase in enzyme synthesis.

3.4. Prediction capacity of the mathematical model

For extrapolation of the mathematical model using cellulose as the substrate, the 5 and 40 g/L conditions were used. These experiments were not included in parameter estimation. The simulations are shown in Fig. 3A and 3B for cell growth, cellulose consumption, and cellulase production. Fig. 3C shows the beta-glucosidase simulation. Simulation for the 40 g/L condition indicates a good fit for cell concentration, substrate consumption and beta-glucosidase production. For the 5 g/L condition, the model could only predict well the substrate consumption and beta-glucosidase activity. Cellulase production was overestimated, but follows the same profile of the experimental data.

A simulation was also performed to predict the production of enzymes in the repeated batch, which started with glycerol for the batch phase (15 g/L), followed by one feeding of cellulose after 24 h. This experiment was not included in parameter estimation. The cellulose feeding resulted in a concentration of 20 g/L inside the bioreactor. Only cellulose consumption was considered for the simulation. Assuming that the cellulose consumed was mainly for cellulase synthesis, Eq. (3) was used to represent the substrate consumption rate considering the cellulose consumption for cell growth ($\alpha(dC_X/dt)_g C_X$) and u equal to zero. The results of the simulation are presented in Fig. 3D only after the feeding of cellulose.

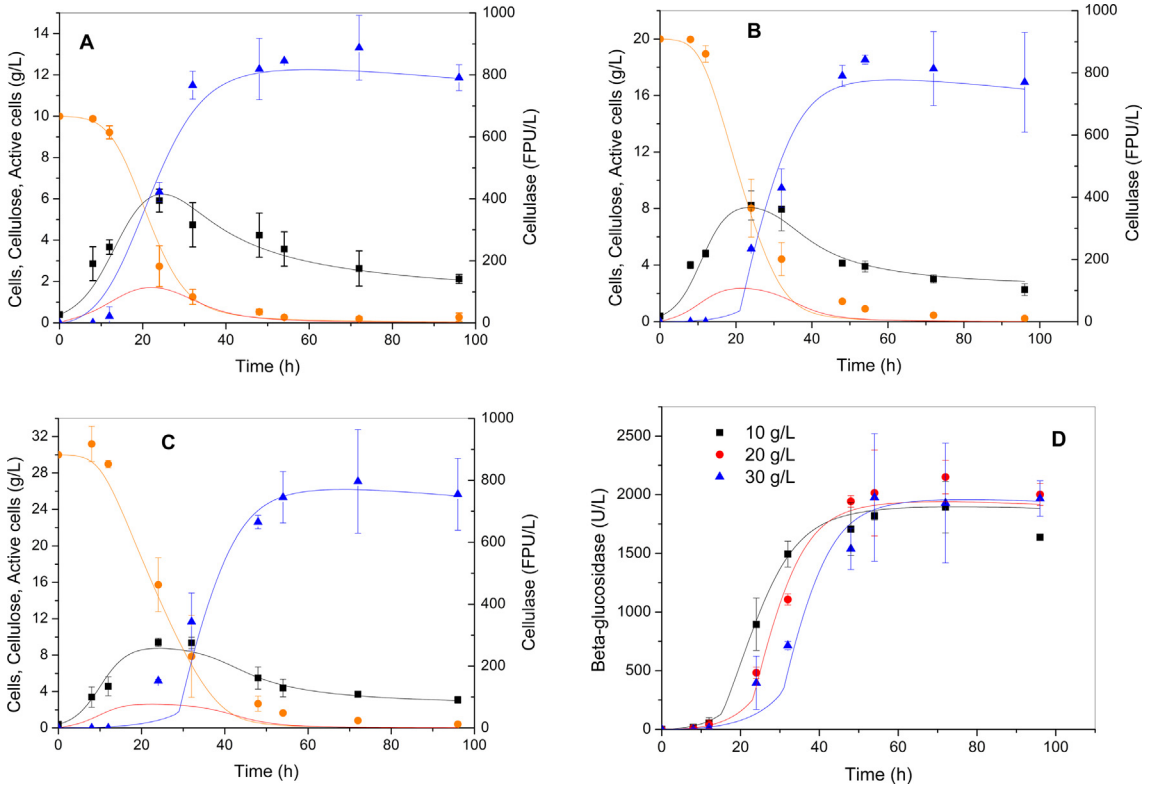

Fig. 2. Fit of the model for the assays using 10 (A), 20 (B) and 30 g/L of cellulose (C), (●) cellulose, (■) cells, (▲) cellulase activity, (—) active cell simulation. Fit of the model for beta-glucosidase activity (D).

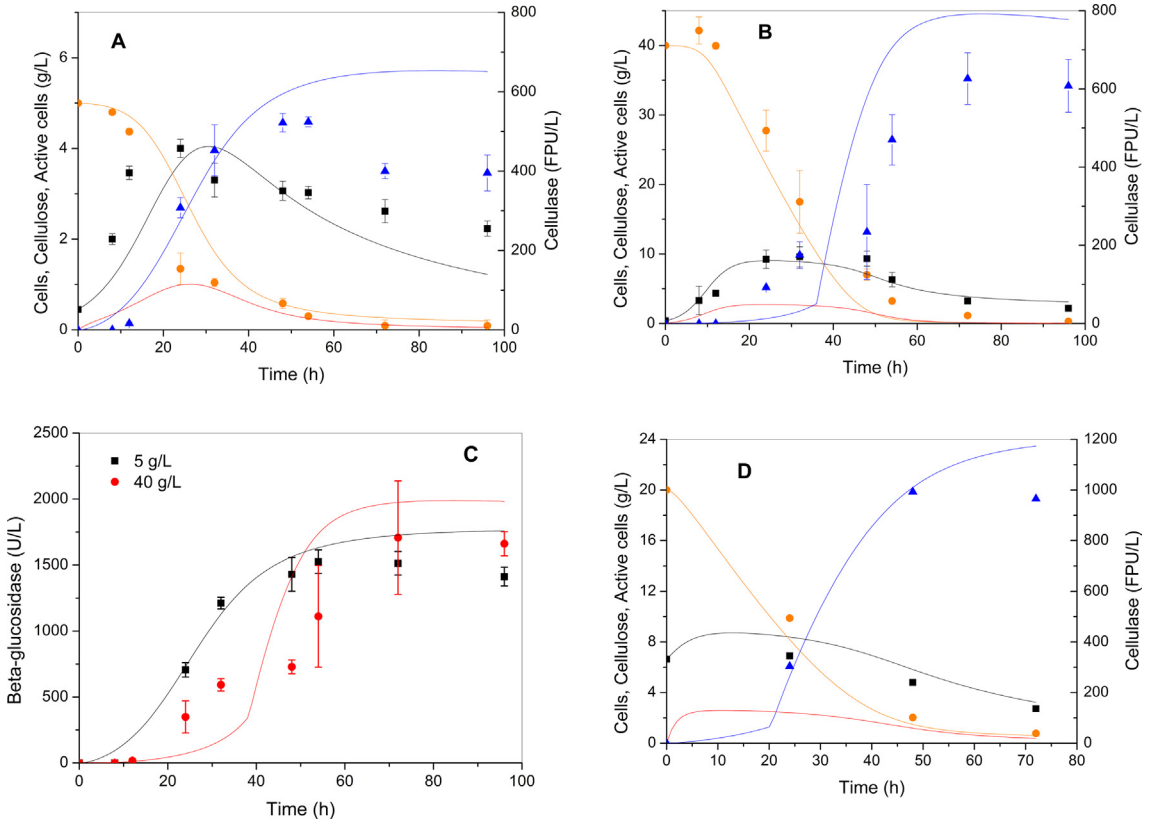

Fig. 3. Extrapolation of the mathematical model for the assays using 5 (A) and 40 g/L of cellulose (B) and simulation of beta-glucosidase activity (C). Fit of the model for the repeated batch (D) starting with glycerol (15 g/L, data not presented) for cell growth and then cellulose for cellulase induction (20 g/L), (●) cellulose, (■) cells, (▲) cellulase activity, (—) active cell simulation.

The batch phase using glycerol is not presented. It can be seen that the model could represent well the production of cellulase, cell growth and cellulose consumption.

Fig. 3D, shows the induction of cellulase production after an initial concentration of glycerol. The induction stage started with 20 g/L of cellulose and 6.6 g/L of cells. It can be observed that the cell concentration did not increase due to the presence of cellulose. Therefore, cellulose presence only influenced the production of cellulase, and this influence was represented in the model by active cells.

3.5. Simulation of strategies for cellulase maximization

The mathematical model was developed to simulate optimal feeding concentrations of the inducer substrate (cellulose) to provide the highest cellulase activity. The model was used to simulate one condition where there is a continuous feeding of glycerol and a discrete feeding of cellulose and one condition where there is a continuous addition of cellulose and glycerol from the feed. The glycerol feeding is to keep the cell at the desired concentration, and cellulose consumption is only for enzyme production. In these simulations, cellulose consumption does not influence cell growth rate because glycerol was considered as the carbon source more easily available for cell growth. Cellulose was only considered to be used to produce active cells.

According to Ilmén et al. (1997), glycerol is considered a "neutral" substrate, meaning that it might not repress or induce cellulase synthesis. Thus, it was considered that the presence of glycerol as an initial substrate concentration or as a continuous inflow rate, would not interfere with the cellulase production rate and the parameters estimated in the batch will still be suitable for fed-batch mode using cellulose as the inducer.

For the optimization of cellulose feeding, first, it was considered a discretization of the feed. For this purpose, in Simulink (Matlab), the integrator block had the "External reset: rising" option active, which enables the reset of the integrator when the feed increases due to an addition of a mass of cellulose. In every feed, the integrator block considers the cellulose inside the bioreactor plus the mass of cellulose added and uses this value as the initial substrate concentration to initiate the integration again. Eq. (3) was used to optimize the cellulose feeding using the discretization approach considering the cellulose consumption for cell growth ($\alpha(dC_X/dt)_g C_X$) and u equal to zero.

Another possibility of optimizing cellulose feeding is considering an inflow rate of cellulose. For this purpose, Eq. (3) was used considering the cellulose consumption for cell growth equal to zero. This equation allows the generation of an optimal feeding profile considering u (g/h) as the manipulated variable.

An algorithm described in Becerra (2004) was used for the optimization of the continuous feed of cellulose. The method considers the manipulated variable as parameters and performs parameter estimation using the Simulink (Matlab). The manipulated variable is represented by a matrix containing two columns. The first column is the time changing according to a fixed integration step and the second corresponds to parameters (manipulated variable) that change according to an objective function.

The discrete feeding was optimized following an adapted algorithm from Becerra (2004), where is this case, the optimization occurs at one value of the manipulated variable at a time, keeping the remaining values static in the matrix. Once this first value of the manipulated variable is optimized based on the objective function, the algorithm moves to the next one, and after building up an optimal profile, the algorithm repeats the single optimization at a time until satisfying a condition. This alteration was an attempt to optimize a range of values of the manipulated variable instead of optimizing all of them for every integration step. For example, optimization of the manipulated variable every 8 h, not every integration step (integration step = 1 h).

The first simulation (Fig. 4B, 4C and 4E) starts with 10 g/L of cellulose, 1 g/L of glycerol and 0.4 g/L of cells. It considers a constant dilution rate of 0.0006 h^{-1} from a glycerol solution of 1 200 g/L to keep the cell concentration close to 7 g/L. A discrete feed of cellulose was optimized to provide the maximum cellulase activity at 96 h. The cellulase activity at 96 h was the objective function to be maximized. Eq. (2) was used for glycerol consumption, Eq. (3) for cellulose consumption ($\alpha(dC_X/dt)_g C_X$ and $u = 0$), Eq. (5) for cell growth, Eq. (8) for active cell growth and Eqs. (9) and (10) for enzyme production. Glycerol consumption was considered for the cell growth rate, and cellulose consumption was only considered for enzyme production. The maximum concentration of cellulose allowed inside the bioreactor was 10 g/L. The volume increased from 0.9 to 0.95 L.

The second simulation (Fig. 4B, 4D and 4F) starts with 1 g/L of cellulose and follows the same considerations described earlier, but in this case, it considers a continuous feed of cellulose (u) described by Eq. (3) ($\alpha(dC_X/dt)_g C_X = 0$). The cellulase activity at 47.5 h was the objective function to be maximized. The simulations using the cellulase activity at 47.5 and 96 h as the objective functions provide similar maximum cellulase activities, however, at 47.5 h, the simulation provides higher activity for beta-glucosidase.

Fig. 4A summarizes the possible interactions that the model provides to simulate strategies using glycerol for cell growth and cellulose for active cell growth. The active cells are responsible for producing enzymes. Cellulose concentration influences the production of enzymes according to the inhibition parameters. The simulations of enzyme production are described in Fig. 4B. According to the simulation for the optimization of cellulase production, the discrete and continuous feeding generated the same enzymatic activity. Therefore, if a continuous feeding device is not available, discrete feeding could be an alternative to provide similar production of the target enzymes.

Fig. 4C and 4D present the cell, active cell and glycerol concentrations for the discrete and continuous feeding strategies, respectively. Cell growth provides the same profile since the cell concentration was considered dependent on the glycerol concentration for both strategies with the same dilution rate. The slight differences regarding the active cell concentration have resulted from the cellulose feeding. Fig. 4E and 4F show cellulose concentrations and feeding concentrations. For the discrete feeding strategy (Fig. 4E), the bars represent the concentrations of cellulose inside the bioreactor considering the current volume. For the continuous feeding strategy (Fig. 4F), the optimization algorithm provided an optimal profile of cellulose feeding (g/h).

The strategies proposed here are examples to demonstrate some possibilities that can be exploited in future works. If the experiments show that a continuous addition of glycerol prevents the consumption of cellulose and consequently the induction of enzymes, then glycerol can only be used until the achievement of the desired cell concentration. For this purpose, the dilution rate can be calculated to provide the desired cell concentration at steady-state using Eqs.s (2) and (5). First, the dilution rate is isolated from Eq. (2) (Eq. (12)) and also from Eq. (5) (Eq. (13)). The desired cell concentration is assigned in both equations and a concentration of glycerol should be found that satisfies the equality between D_1 and D_2.

$$D_1 = \frac{\alpha' \mu_{XmG}}{\left[(C_{G,f} - C_G) + \alpha' {C_X}^2\right]} \left(\frac{C_G}{C_G + k_G}\right)\left(1 - \frac{C_X}{C_{XmG}}\right) {C_X}^2 \tag{12}$$

$$D_2 = \mu_{XmG}\left(\frac{C_G}{C_G + k_G}\right)\left(1 - \frac{C_X}{C_{XmG}}\right) - \mu_{XmdG} \tag{13}$$

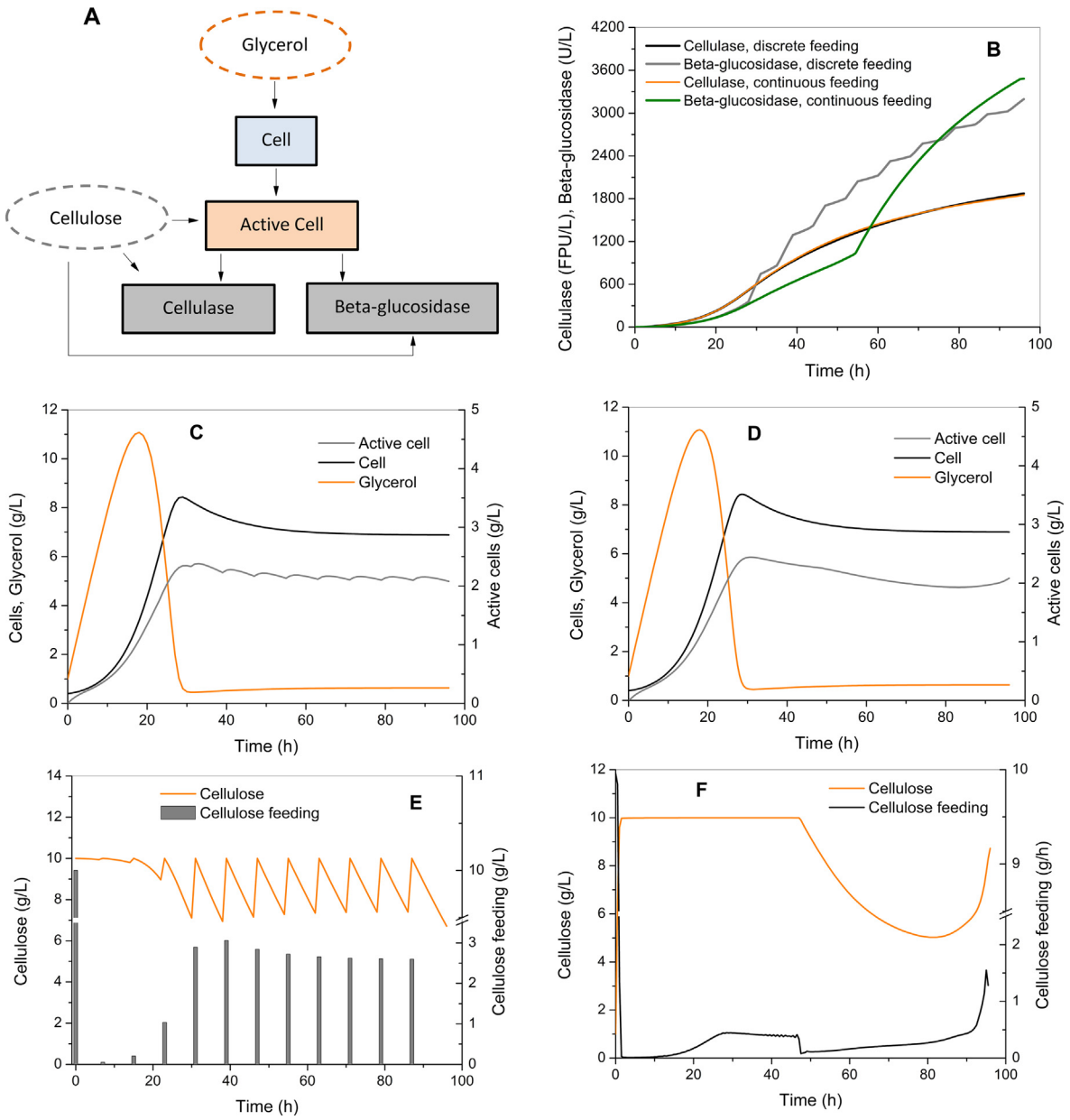

Fig. 4. Diagram of the simulation strategy (A), cell growth depends on glycerol concentration, active cell growth depends on cell and cellulose concentrations, cellulase and beta-glucosidase production depend on the active cell and cellulose concentrations. Simulated strategies using a continuous feed of glycerol with a discrete or continuous feed of cellulose, cellulase and beta-glucosidase activities (B), cell, active cell and glycerol concentrations using a discrete feed of cellulose (C) and a continuous feed of cellulose (D), cellulose concentration and a discrete feed of cellulose (concentration of the feed inside the bioreactor) (E) and a continuous feed of cellulose (F).

The advantages of the model designed in this project include the possibility of optimizing the feed of the inducer substrate using a discrete or continuous approach. To date, it was not found in the literature works proposing mathematical models for the optimization of cellulase production using a discrete feeding. The inhibition parameters of the enzyme production equations can easily be changed according to new experiments to describe strains less repressed. The feed of glycerol can be a constant value or follow a specific inflow rate dependent on other components such as cell concentration and time.

3.6. Analysis of enzyme production

Table 3 shows a comparison of productivities between the batch conditions using 10 and 20 g/L of cellulose, the repeated batch and the results of the simulations described earlier. The repeated batch results indicated similar productivity than the batch experiments using 10 and 20 g/L of cellulose. Perhaps, the repeated batch strategy provoked inhibition of enzyme production. In the repeated batch, there was only one feed corresponding to 20 g/L of cellulose inside the bioreactor, but a better strategy could be a small continuous or periodic addition of the inducer substrate to prevent inhibition of enzyme production. This strategy was represented by the simulations using a continuous and discrete feed of cellulose, which indicates the obtaining of higher productivity of cellulase (FPU/L h) than the batch and repeated batch analysed in this project.

The cellulase production rate of 75 FPU/L h is considered a desired productivity that can be used in industrial processes (Himmel et al., 1999). Thus, the mathematical model developed in this work can be adapted for other microorganisms and inducer substrates (such as sugarcane bagasse) aiming at the development of new strategies to keep increasing the productivity of cellulase until the achievement of the desired value.

Table 3
Cellulase production rate ($\Delta C_F/\Delta t$) and beta-glucosidase production rate ($\Delta C_B/\Delta t$) from 0 to 72 h. Specific cellulase production rate ($\Delta C_F/\Delta t C_{X_m}$) and specific beta-glucosidase production rate ($\Delta C_B/\Delta t C_{X_m}$) from 0 to 72 h considering the maximum cell concentration (C_{X_m}).

	Batch		R. batch[a]	Batch (model)		Simulation		
	10 g/L	20 g/L		10 g/L	20 g/L	Repeated batch	Continuous feeding	Discrete feeding
$\Delta C_F/\Delta t$(FPU/L h)	12.3	11.3	13.4	11.3	10.7	16.3	22.5	22.5
$\Delta C_B/\Delta t$(U/L h)	26.3	29.9	26.3	26.3	27.1	29.7	34.1	35.9
$\Delta C_F/\Delta t C_{X_m}$(FPU/g h)	2.1	1.4	1.9	1.8	1.3	1.9	2.7	2.7
$\Delta C_B/\Delta t C_{X_m}$(U/g h)	4.4	3.6	3.8	4.2	3.4	3.5	4	4.3

[a] Repeated batch, starting with 15 g/L of glycerol and fed once with cellulose at 24 h (20 g/L).

4. Conclusions

A mathematical model for glycerol and cellulose conditions was developed and presented a good fit for the majority of the experimental data. Although extrapolation analysis indicated limitations, the model can still predict the profiles of the experimental data using cellulose for conditions out of the range of concentrations used for parameter estimation. Analysis of the repeated batch has indicated that the mathematical model can predict the profiles of experiments at first using glycerol for cell growth then cellulose for enzyme production. Simulations of strategies were presented as possibilities that can be exploited in future works. The model and strategies were developed as tools to be used for cellulase maximization and to be adapted for less repressed strains and other substrates.

CRediT authorship contribution statement

Lucas Gelain: Software, Investigation, Writing - original draft, Writing - review & editing. **Luuk Wielen:** Visualization, Conceptualization. **Walter M. Gulik:** Validation, Conceptualization. **José Geraldo Cruz Pradella:** Methodology, Resources, Conceptualization. **Aline Carvalho Costa:** Supervision, Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This project was supported by the São Paulo Research Foundation (FAPESP), process number 2014/22537-9, the Brazilian National Council for Scientific and Technological Development (CNPq), process number 142478/2014-8, Brazilian Biorenewables National Laboratory (LNBR), the University of Campinas (UNICAMP) and Delft University of Technology (TU Delft). The authors would like to thank Deise Juliana da Silva Lima for assistance in the preparation of the experiments.

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