Simulation of Diaxuc Production of Cephalosporin C by *Cephalosporium acremonium*: Lag Model for Fed-Batch Fermentation

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We extend a previously reported model (Chu, W. B.; Constantinides, A. Biotechnol. Bioeng. 1988, 32, 277–288) for the batch fermentation of cephalosporin C under the diauxic growth of *Cephalosporium acremonium* on glucose and sucrose to a fed-batch system. For this purpose, a novel lag model is proposed for diauxie, which has two functional forms, each embodying the dependence of lag on total cell mass and secondary substrate concentration. This lag model is applicable for batch simulations for arbitrary initial glucose and sucrose concentrations. We used the previously reported batch data to perform locally optimized fed-batch simulations. When applied to fed-batch fermentations, multiple lag times were accounted for. These studies showed that fed-batch fermentations (under the restriction that cell mass concentration did not exceed 25 g/L) could be more productive than simple batch runs. A representative result for a glucose-pulse fed-batch run at optimal cephalosporin production is a productivity of 4.22 mg of cephalosporin C/L-h and a production yield of 9.25 mg of cephalosporin C/g of total sugar used.

Introduction

Cephalosporin C is a naturally produced β-lactam antibiotic (4, 21) which acts against both gram-positive and gram-negative bacteria. It is a starting point in the manufacture of more potent cephalosporins for parenteral use in treating human infections caused by penicillinase-producing bacteria (23). Annual sales of all types of cephalosporins accounted for about 20% of the $16 billion of antibiotics sold in the U.S. in 1987 (29). Cephalosporins are produced by *C. acremonium* and *Streptomyces clavuligerus*. The biosynthesis of cephalosporin C has been well-characterized (1, 9, 10, 19, 21, 26, 27, 28, 29).

Cephalosporin C fermentation by *C. acremonium* is characterized by morphological differentiation and repression by glucose. In submerged culture, *C. acremonium* shows various morphological cell types in fermentation: long slender smooth hyphae; moderately swollen hyphal segments; highly swollen hyphal fragments; unicellular arthrospores, either large spheres or small ellipsoids; and smaller, elongated conidia (16, 18, 20). As described above, glucose represses the enzyme responsible for the production of cephalosporin C. However, it is a rapidly metabolized carbon source and allows rapid cell growth. On the other hand, sugars such as sucrose and lactose are less rapidly metabolized than glucose but do not appreciably repress the formation of cephalosporin C. Thus a combination of glucose with either sucrose or lactose is a useful feedstock in these fermentations.

At the beginning of the fermentation, when glucose is being used by the cells, the majority of morphological forms are long, slender hyphae. The amount of cephalosporin C produced prior to the complete depletion of glucose is small (15). Immediately after the depletion of glucose, production of cephalosporin C starts, accompanied by extensive fragmentation of long swollen hyphal segments into short swollen forms that ultimately become differentiated into arthrospores and conidia (16, 18). Since formation of cephalosporin C ceases with the disappearance of long highly swollen hyphal fragments (16), these morphological forms are thought to be primarily responsible for cephalosporin C production.

Kinetic models for describing the production of secondary metabolites can be based on the age distribution of the microorganisms (6, 22) or on the overall biosynthetic mechanism (e.g., as shown for cephalosporin B by Matsumura et al. (18), Chu and Constantinides (3), and Mainberg and Hu (12)). Matsumura et al. (17) developed a kinetic model to describe the production of cephalosporin C by *C. acremonium* considering morphological differentiation, induction of cephalosporin C production by methionine, and repression by glucose. Mainberg and Hu (12) described cephalosporin biosynthesis in *S. clavuligerus* and provided a rationale for modifying the biosynthetic machinery of cephalosporin through gene cloning. Recombinant DNA technology for strain improvement and knowledge of regulatory control of biosynthetic operons have been reported to be useful to improve production of cephalosporin C by *C. acremonium* (5, 8, 23, 24). The determination of rate-limiting enzymes facilitates these genetic manipulations (13, 14).

Chu and Constantinides (9) developed a mathematic model for batch fermentations using protocryptic *C. acremonium* strain CW-19 (ATCC 36228) grown in a complex medium containing 25 g/L glucose and 30 g/L sucrose. A 2-L glass fermentor was used which was provided with aeration, agitation, and temperature and pH controllers. Their model was used to determine optimal pH and temperature profiles from batch fermentation data.

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Table 1. Estimated Values of Parameters at pH 6.3 and 32 °C Obtained by Chu and Constantinides (3) from Experimental Batch Fermentation Data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>( m_{\text{bi}} )</td>
<td>0.02267</td>
<td>(h (^{-1}))</td>
</tr>
<tr>
<td>( m_{\text{bi}} )</td>
<td>0.01100</td>
<td>(h (^{-1}))</td>
</tr>
<tr>
<td>( m_{\text{bi}} )</td>
<td>0.06256</td>
<td>(h (^{-1}))</td>
</tr>
<tr>
<td>( \sigma_{\text{p}} )</td>
<td>0.00411</td>
<td>(h (^{-1}))</td>
</tr>
<tr>
<td>( \delta_{\text{p}} )</td>
<td>0.00066</td>
<td>(h (^{-1}))</td>
</tr>
<tr>
<td>( \delta_{\text{a}} )</td>
<td>24.7724</td>
<td>(h (^{-1}))</td>
</tr>
<tr>
<td>( \beta )</td>
<td>2.15757</td>
<td>(h (^{-1}))</td>
</tr>
<tr>
<td>( \gamma )</td>
<td>0.01076</td>
<td>(h (^{-1}))</td>
</tr>
<tr>
<td>( K_{S} )</td>
<td>10.1990</td>
<td>(g/L)</td>
</tr>
<tr>
<td>( Y_{H} )</td>
<td>0.46188</td>
<td></td>
</tr>
<tr>
<td>( m_{\text{bi}m} )</td>
<td>0.04269</td>
<td>(h (^{-1}))</td>
</tr>
<tr>
<td>( K_{S} )</td>
<td>0.1</td>
<td>(g/L)</td>
</tr>
<tr>
<td>( I_{1} )</td>
<td>20</td>
<td>(g/L)</td>
</tr>
<tr>
<td>( I_{2} )</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>( a )</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>( \alpha )</td>
<td>0.04</td>
<td>(g/L) (^{-1}) h (^{-1})</td>
</tr>
</tbody>
</table>

This paper extends the doxic batch model, developed by Chu and Constantinides (3), to fed-batch culture and provides a mechanistic framework for describing the relative contributions of the two sugars to cephalosporin C production. For this purpose, a generalized lag model is proposed.

Mathematical Model

We first summarize the model of Chu and Constantinides (3). Biomass accumulation of thin hyphae (\( X_{H} \)) and thick walled cells (\( X_{T} \)) is given by

\[
X_{H} + X_{T} = X_{T} \tag{1}
\]

\[
\frac{dX_{H}}{dt} = \mu_{H}X - \mu_{H}X_{H} - \delta_{H}X_{H} \tag{2}
\]

\[
\frac{dX_{T}}{dt} = \mu_{T}X_{H} - \delta_{T}X_{T} \tag{3}
\]

All symbols are defined in the Notation. The values of the various constants obtained by Chu and Constantinides are given in Table 1. Enzyme accumulation is given by

\[
\frac{dE}{dt} = \alpha_{\text{E}}X_{H} - \beta E \tag{4}
\]

and glucose (\( S_{1} \)) and sucrose (\( S_{2} \)) uptake is expressed as

\[
\frac{dS_{1}}{dt} = -\mu_{S}X_{H} \tag{5}
\]

\[
\frac{dS_{2}}{dt} = -\mu_{S}X_{H} - \frac{m_{S}S_{2}X_{H}}{Y_{S_{2}} + S_{2} + I_{S_{1}}} \tag{6}
\]

The specific growth rate for thick-walled cells is

\[
\mu_{T} = \frac{\mu_{S}m_{S}S_{1}}{1 + I_{S_{1}}} \tag{7}
\]

and that for thin-walled cells is

\[
\mu_{H} = \begin{cases} 
\mu_{S}, & t \leq t_{1} \\
\mu_{S}, & t > t_{1} 
\end{cases} \tag{8}
\]

The specific growth rates corresponding to glucose and fructose are

\[
\mu_{S} = \frac{m_{S}S_{1}}{R_{S_{1}} + S_{1}} \tag{9}
\]

and

\[
\mu_{S} = \phi_{\text{lag}} \left[ \frac{S_{1}}{R_{S_{1}} + S_{1}} \right] \tag{10}
\]

where the lag function is given by

\[
\phi_{\text{lag}} = \left( \tan^{-1} \left( \frac{t_{1} - t_{1}}{t_{1} - t_{1}} \right) \right)^{1/2} + \frac{1}{2} \tag{11}
\]

The specific decay rate for thick hyphae is

\[
\delta_{H} = 0.00441 \tag{12}
\]

and the specific decay rate of thin hyphae is

\[
\delta_{T} = \delta_{H} = \frac{\mu_{S}}{t_{1} + I_{S_{1}}} \tag{13}
\]

The product (cephalosporin C) formation is given by

\[
\frac{dP}{dt} = EX_{T} - \gamma P \tag{14}
\]

The \( \phi_{\text{lag}} \) function described in eq 11 has been constructed so that the thin hyphae grow exclusively on sucrose until it has been completely consumed; only then is sucrose used. The fitting parameter \( K_{S} = 318.2 \) was chosen by Chu and Constantinides for a given right value for lag time (34.77 min) for an initial medium consisting of 25 g/L glucose and 30 g/L sucrose. The form chosen for \( \phi_{\text{lag}} \) is basically that of a smoothed step function, going from 0 at \( t_{1} \) (the time at which glucose is fully consumed) to 1 at \( t_{1} + t_{1} \). The fermentation was stopped after 168 h.

The present work is aimed at fed-batch production of cephalosporin C. The theoretical analysis of fed-batch cultures for a limiting substrate has been discussed by Lim et al. (11). However, a more general model for lag is required in order to describe the additional lag caused by the further addition of one or both sugars. In addition, we wish to examine the effect on overall product formation of different initial concentrations of both glucose and sucrose, which also requires a more flexible lag model. For example, when eq 11 is applied to a batch system with initial sucrose concentration of 30 g/L and initial glucose concentration higher than 45 g/L, sucrose is consumed before glucose is fully depleted. This indicates that the lag described by eq 11 is unsuited to varying initial sugar concentrations.

We propose the following simple lag model. Let the concentrations of cells and sucrose at the point of complete glucose depletion be \( C_{0}^{s} \) and \( C_{0}^{a} \). The latter will just be the initial sucrose concentration, since we retain the assumption of Chu and Constantinides that the sucrose is not used at all until the glucose is fully consumed. The lag time, \( t_{lag} \), can be expected to be inversely related to \( C_{0}^{s} \) on the basis of an induction effect: sucrose promotes the formation of those enzymes required to process it (e.g., invertase), and the higher the sucrose concentration (up to a point), the faster the enzyme formation and the shorter the lag time. However, a saturation effect must also be incorporated: beyond a certain limiting sucrose level, the induction period will not become shorter. The specific, rather than the total, sugar concentration is used to give

\[
t_{lag} = t_{max} + A \exp \left( -\frac{C_{lag}^{s}}{C_{lag}^{s}} \right) \tag{15}
\]

Thus, as the specific concentration, \( C_{lag}^{s} \), increases, the lag time decreases and approaches its minimum value of \( t_{âˆš} \) which can be viewed as the minimum time
needed by the cell to generate the additional enzymes needed to process the sucrose. The parameters $K$, $A$, and $\tau_{max}$ can be calculated by fitting eq (15) to fermentation data. However, in the case of cephalosporin C production, only one data point is available from Chu and Constantinides, i.e., the batch data at initial concentrations of 25 g/L glucose and 30 g/L sucrose. We therefore use the simplified model

$$\tau_{max} = \frac{K}{(C_0^s/C_m^s)}$$  \hspace{1cm} (16)

where one parameter, $K$, is set at 0.68 h from Chu and Constantinides' data ($\tau_{max} = 34.8$ h, $C_0^s = 30$ g/L, $C_m^s = 15.6$ g/L).

**Result and Discussion**

The software package SIMBAS (2) is used on a 30386-based PC to simulate the batch model of Chu and Constantinides as well as the fed-batch simulations based on the lag model proposed in this work. This BASIC program uses a fourth-order Runge-Kutta method to solve ODE systems (Appendixes 1 and 2 found in the supporting information). The batch simulation was also run on a Sun 4/480 computer using the stiff ODE solver DVPD in the IMSL package (7). The results from these two computations agreed to three significant places. The initial values of four state variables, total cell, glucose, sucrose, and cephalosporin C concentrations (Table 1), were taken from Chu and Constantinides (3), and their simulation was repeated. Complete agreement was found, and our result is shown in Figure 1 for reference with the fed-batch runs shown later. It can be seen that cell mass is maximized as the glucose is depleted (Figure 1). The cephalosporin C concentration then reaches a peak as the sucrose is depleted, and formation of thick hyphae occurs together with the appearance of expulsion, a rate-limiting, ring expansion enzyme responsible for cephalosporin C formation. The concentration of thick hyphae increases steadily beyond the point of glucose consumption. The total cell mass decreases during the lag period, which reflects the loss of thin hyphae alone. This is because repression of thick hyphae by glucose is no longer present in this period. It was observed that the maximum cell concentration is achieved before the maximum product concentration is reached.

From Figure 1, the local optimum productivity of cephalosporin C was found to be 3.23 mg of cephalosporin C/L-h) and the local maximum production yield was 6.16 mg of cephalosporin C/g of total sugar consumed.

Fed-batch simulations were performed using the same initial conditions as for the batch run. Figures 2 and 3 show fed-batch cultures predicted by the kinetic model. The simulations suggest that feeding sucrose or glucose alone at a suitable rate will raise the production over that of the batch culture. It is assumed that growth medium is present in sufficient quantities to prevent nitrogen or sulfur limitations and that mixing and aeration are not limiting factors. In fed-batch runs, the thick hyphae concentration increases steadily with time. The maximum cell concentration was achieved before the maximum cephalosporin production, as in the batch run.

**Figure 2** shows the simulation results of glucose fed-batch system in a cephalosporin fermentation with an initial feed rate of glucose at 0.27 g/(L-h) (see Appendix 3, supporting information). This corresponds to a fed-batch glucose pulse of 10 g/L from 104 to 109 h. This resulted in optimal production of cephalosporin C (600 mg/L). Our model takes into account the second lag period that occurs due to the complete consumption of the glucose pulse and the consequent return to sucrose consumption. The small hump (at $t = 105$ h) in the cephalosporin concentration during the fed-batch glucose pulse is due to repression by glucose. However, due to extensive development in current cephalosporin strains, glucose repression of production has been eliminated.

**Figure 3** shows the simulation results of sucrose fed-batch system in cephalosporin fermentation with an initial feed rate of sucrose at 0.06 g/(L-h) (see Appendix 3, supporting information). After the introduction of the sucrose pulse, the cell mass continues to grow since the enzyme is not inhibited as in Figure 2. The duration of the pulse, keeping the pulse concentration constant, was chosen so as to maximize local cephalosporin C production (660 mg/L). Since the cells were already using sucrose as a substrate before the fed-batch sucrose pulse, there is no second lag phase. The present definition of $\tau_{max}$ as a function of the concentrations of the cell mass and sucrose makes the model viable for all values of concentrations of sucrose and glucose for both batch and fed-batch systems.
Figure 2. Time course of fed-batch culture in cephalexin C fermentation. The feed rate of glucose was $F = 0.27 \times 0.0442t$; a 6-h feed pulse of 10 g/L began at 105 h.

Figure 3. Time course of fed-batch culture in cephalexin C fermentation. The feed rate of sucrose was $F = 0.06 \times 0.0317t$; a 10-h feed pulse of 20 g/L began at 104 h.

Dissolved oxygen concentration has been shown to be an important parameter in cephalexin C production by C. acroemium (29). The present model (eqs 15 and 16) was based on the experimental data of Chu and Constantinides, who used a 2-L glass fermentor. We restricted the total cell mass concentration to be below 25 g/L in order to avoid incomplete aeration, an assumption consistent with experimental data (17). However, if this restriction could be lifted in practice by improving oxygen transfer rates, much higher production of cephalexin C would become possible. It is important to note that as such a fermentation is scaled-up, the agitation required to maintain oxygen levels could give rise to cell shearing, which might lead to a more stringent upper bound on cell mass for practical fermentation at industrial scale.

Vick et al. (25) reported 1200 mg/L of cephalexin C production for a batch system (with methionine) and 2400 mg/L for a fed sucrose batch system (with methionine) using C. acroemium. Matsunura et al. (15) showed cephalexin C production for a batch system to be 310 mg/L (without methionine) and 400 mg/L (with methionine) using C. acroemium. Chu and Constantinides (3) reported cephalexin C production of 350 mg/L in a batch fermentation in the absence of methionine.

In the present work, the local optimum productivity of cephalexin C for the glucose fed-batch system was determined as 4.22 mg of cephalexin C/(L·h) and the maximum production yield was found to be 9.25 g cephalexin C/g of total sugar consumed. The local optimum productivity of cephalexin C for the sucrose fed-batch system was determined as 4.15 mg of cephalexin C/(L·h), and the maximum production yield was found to be 7.78 g of cephalexin C/g of total sugar consumed. These parameters were found to be comparable with those obtained by Matsunura et al. for a fed-batch system (15).

Figure 4 plots results of simulation calculations carried out at various times between 33 and 125 h. A maximum in cephalexin C concentration occurs when a sucrose pulse is introduced at approximately 104 h. This is because sucrose is introduced later, and it is used up very quickly and few cells are left to make cephalexin. Thus, there is inhibition of cephalexin production.
Figure 4. Simulated effect of initiation time of sucrose and glucose pulses on the maximum cephalosporin C concentration in a fed-batch culture. The cephalosporin concentration was made dimensionless by dividing it by maximum production in batch fermentation. Each point represents a single simulation. Sucrose pulses are depicted by C; the sucrose concentration in pulse was 30 g/L for a duration of 10 h. Glucose pulses are depicted by G; the glucose concentration in pulse was 10 g/L for a duration of 5 h.

Also, since fermentation is stopped at 168 h (for comparison with Chu and Constantinides’ results), introduction of a pulse after 104 h does not allow for the maximum cephalosporin concentration before 168 h.

Figure 4 shows a minimum followed by a maximum in the effect of the initiation time of the glucose pulse to maximum cephalosporin concentration. Thus, if glucose is introduced at about 67.65 h, the time at which lag ends in a batch fermentation, enzyme activity decreases because the growth of thick-walled cells (see eqs 4 and 7) is suppressed. This is energetically wasteful and leads to a minimum in the cephalosporin concentration at 67.7 h. Introduction of the pulse between the time when cell mass is maximum and cephalosporin concentration is maximum (in the batch fermentation) produces the optimal cephalosporin concentration.

Further studies on cephalosporin C production where methionine was the sulfur source were carried out by Matsumura et al. (17). Their model expressed the specific enzyme concentration (mg of enzyme/g of cell) as

\[ \frac{dP}{dt} = \mu P \exp (\chi_4 - \gamma P) \]  

(17)

The enzyme mass balance is then

\[ \frac{dE^*}{dt} = \alpha^* \mu P \chi_{41} - \beta^* E^* \]  

(18)

where \( \alpha^* \) and \( \beta^* \) are first-order rate constants representing enzyme formation and decomposition, rather than enzyme formation rates and decomposition rates as described by \( \alpha \) and \( \beta \) in the original mass balance, eq 4. Matsumura et al. (17) also linked enzyme formation to the concentration of thick, rather than thin, hyphae; since cephalosporin is produced in the thick hyphae. This would lead to replacing \( \chi_{41} \) by \( \chi_7 \) in eq 17 above.

Current cephalosporin production strains have undergone extensive strain development, and therefore, glucose repression of cephalosporin C production has likely been minimized in at least some industrial fermentations. In this paper, we have presented a case study applicable when the production strain and key operating parameters such as oxygen transfer rate governed by the productivity requirements have been selected. We hope that the case study presented in this paper will be useful to process scientists and engineers who are examining application of models for simulation of other types of fed-batch fermentations. In the case of cephalosporin C, this model attempts to illustrate how a proper understanding of the metabolic requirements of \( C. aceromum \) coupled with a favorable operating environment for the bioreaction can improve cephalosporin productivity.

**Conclusions**

A general lag model was incorporated to a previously proposed kinetic model (3). Its parameters, derived from kinetic data on batch cultures, were then applied to a fed-batch culture. The lag time due to transition from rapidly metabolized glucose to less rapidly metabolized sucrose can be calculated for different values of glucose and sucrose concentrations. The resulting simulations of fed-batch culture are useful in specifying optimal substrate feeding conditions with respect to antibiotic production and illustrate how production of the antibiotic could be enhanced by simultaneously fulfilling three conditions: (1) successive formation of swollen hyphal fragments, (2) minimization of catabolite repression by glucose and (3) maintenance of high feed concentrations. The conditions can be satisfied by feeding glucose and sucrose at a suitable rate, which seems rational from the biological point of view. This strategy has been successfully used by Matsumura et al. (17). The simplified model incorporating lag time effects presented here helps to explain the impact of feeding strategy on cephalosporin C productivity.

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**Supporting Information Available**: Appendices 1–3 discussed in the text (5 pages). Ordering information is given on any current masthead page.

**Notation**

- **A**, \( A_1, A_2 \) constants in the proposed lag time expression
- **B**, \( B_1, B_2 \) constants in the exponential fed-batch glucose feed rate expression
- **C**, \( C_5^* \) total cell mass concentration at time at the point of glucose depletion
- **E**, \( E^* \) specific concentration of the rate-limiting enzyme inside the thick wall cells (relative unit, mg/g/h)
- \( E^* \) specific concentration of the rate-limiting enzyme inside the thick wall cells (mg of enzyme/g of cell)
- \( F(l) \) feed rate of substrate in exponential fed-batch culture (g/L/L)
- \( I_1 \) constant of repression of morphological differentiation by glucose
- \( I_2 \) inhibition constant of sucrose use by glucose
- **K** constant in proposed lag time expression
$K_s$ saturation constant related to glucose
$K_{ri}$ saturation constant related to sucrose
$K_a$ constant related to the steepness of the arctangent curve rising from zero
$m_s$ maintenance constant
$P$ concentration of cephalosporin C (mg/L)
$S_1$ glucose concentration (g/L)
$S_2$ sucrose concentration (g/L)
$t$ time (h)
$t_i$ intermediate time when the glucose initially present in the fermenter is fully consumed (h)
$L_{lag}$ lag period
$L_{lag}$ constant in proposed lag expression
$X_{c}$ concentration of total cell mass (g/L)
$X_{c}$ concentration of thin cell mass
$Y_{X}$ cell mass yield factor from glucose
$Y_{X}$ cell mass yield factor from sucrose
Greek Symbols
$\alpha$ growth-link enzyme formation rate
$\beta$ decomposition rate of the growth-link enzyme
$\alpha^*, \beta^*$ first-order rate constants
$\delta_1$ specific decay rate of thin hyphae
$\delta_2$ maximum specific decay rate of thin hyphae
$\delta_3$ specific decay rate of thick wall cells
$\epsilon_1, \epsilon_2$ constants in exponential fed-batch glucose feed rate expression
$\epsilon_3, \epsilon_4$ constants in exponential fed-batch sucrose feed rate expression
$\theta_{int}$ intermittent lag function
$\gamma$ cephalosporin C decomposition rate constant
$\mu_{max}$ specific growth rate of thin hyphae ($X_c$)
$\mu_{max}$ specific growth rate of thin hyphae on glucose
$\mu_{max}$ maximum specific growth rate of thin hyphae on glucose
$\mu_{max}$ maximum specific growth rate of thin hyphae on sucrose
$\mu_{max}$ specific growth rate of thick wall cells ($X_t$)
$\mu_{max}$ maximum specific formation rate of thick wall cells

Literature Cited


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