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Combined Age and Segregated Kinetic Model for Industrial-scale Penicillin Fed-batch Cultivation

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Abstract This paper proposes a cell age model for *Penicillium chrysogenum* fed-batch cultivation to supply a qualitative insight into morphology-associated dynamics. The average ages of the segregated cell populations, such as growing cells, non-growing cells and intact productive cells, were estimated by this model. A combined model was obtained by incorporating the average ages of the cell sub-populations into a known but modified segregated kinetic model from literature. For simulations, no additional effort was needed for parameter identification since the cell age model has no internal parameters. Validation of the combined model was performed by 20 charges of industrial-scale penicillin cultivation. Meanwhile, only two charge-dependent parameters were required in the combined model among approximately 20 parameters in total. The model is thus easily transformed into an adaptive model for a further application in on-line state variables prediction and optimal scheduling.

Keywords: cell age, segregated kinetic model, model validation, state variables prediction, penicillin cultivation

INTRODUCTION

In penicillin fermentation, the morphological changes or aging process of the microorganism are commonly observed. Such morphological changes include the formation of hyphae and pellets, segmentation of mycelia, formation of vacuoles, and, at the later phase of cultivation, lysis of cells. These changes cause variations in the mass transfer and metabolic behavior of the microorganisms. Many morphology related studies have been reported [1-3]. In the model of Tiller *et al.* [2], biomass is morphologically divided into three parts: growing cells, non-growing cells and autolyzed cells. In this research, the average ages of growing cells, non-growing cells and the total intact productive cells (defined as the sum of growing and non-growing cells) were estimated by a simple cell age model. These estimated ages were further used to modify the transition rate of cells from one category to another as well as the specific product formation rate calculated through Tiller's model. The combined cell age and segregated kinetic model for *P. chrysogenum* was then validated with the data of industrial penicillin fed-batch fermentation.

Besides cell mass, ages of segregated cell populations play an important role because it may impact both cell growth and product formation. However, cell ages are extremely difficult to model so that in the literature, when such variables are concerned, they often presented as the function of the cultivation time [2]. While in the age model presented in this article, cells in a growing phase and a non-growing phase carry their natural age. The new born cells carry a natural age of 0 h and fall into the age interval of $[0, \Delta t]$ of the growing phase. The oldest growing cells fall into the non-growing phase but without changing their age. Similarly, only the oldest non-growing cells may fall into the metabolically inactive phase. The amount of new born cells is calculated by the segregated kinetic model, while the amount of phase shifting cells is assumed to be dependent on the average age of related cell sub-populations. Moreover, the specific penicillin formation rate is assumed to decrease as the average age of intact productive cells increases. The combined cell age and segregated kinetic model fits the industrial penicillin fermentation well and its adaptive form will be of great practical utility for on-line state variables prediction and optimal scheduling.

MATERIALS AND METHODS

Data (20 batches) of industrial scale fed-batch cultivations were obtained from a Chinese pharmaceutical company and applied to verify the combined model system. The production bioreactor had a volume of 50,000 L and the inoculation ratio was approximately 10%. Typically, a cultivation started from a batch phase. 10 h later, carbon

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source (mainly glucose) and phenyl acetic acid solutions were fed into the bioreactor. After another 30 h, ammonia and ammonium sulfate solutions were added. Vegetable oil was used to control foam. For intermediate harvesting, the spent medium was periodically withdrawn every 16 h during the second half of the cultivation period.

Samples were taken every 4 h for the assays of penicillin, residual sugar and phenyl acetic acid concentration. The measurement error is estimated by 2~3%. The biomass concentration (dry weight) was only measured occasionally and the measurement error was very high (7~8%). The penicillin concentration was determined by HPLC. For the assay of the reducible sugar concentration, samples was hydrolyzed by HCl first, then determined by titration. Biomass concentration is determined by vacuumed drying at 80°C and -750 mmHg for 24 h.

RESULTS AND DISCUSSION

Segregated Kinetic Model

Based on Tiller's segregated kinetic model [2], the adapted model contains the following modifications:

(a) both cell growth and product formation are assumed to be cell age dependent and
(b) mass balance models for hydrolysis-required sugars (disaccharides and polysaccharides), unusable sugars as well as the hydrolysis kinetic model are introduced, since the carbon source used in industry may contain these sugars.

Modification (a) is based on the principle that cells are active and productive during youth and middle age, while less productive in old age. Moreover, the transformation from a growing to a non-growing phase, as well as from a non-growing to a metabolically inactive phase, depends not only on nutrient and other environmental factors, but also mainly on the average ages of the corresponding cell populations. Modification (b) mainly results from industrial cultivation where the percentages of hydrolysis-required sugars and unusable sugars are usually dependent on the substrate types and the sterilization techniques applied. It should be pointed out that modification (b) is only necessary when the residual sugar concentration is measured as the total reducible sugar.

Eqs. (1)~(18) present the whole segregated kinetic model, of which Eqs. (1)~(3) are from the original model of Tiller, while others are modified to different extent. The meanings of the symbols and their units are referred to in the Nomenclature section of the paper. The growing and non-growing cells with concentrations X_1 and X_2, respectively, as well as the concentration of intact productive cells ($X = X_1 + X_2$):

$$\frac{dX_1}{dt} = (\mu_S + \mu_{PM})X_1 - (D + k_{12})X_1 \quad (1)$$

$$\frac{dX_2}{dt} = k_{12}X_1 - (D + k_{vac})X_2 \quad (2)$$

$$X = X_1 + X_2 \quad (3)$$

The concentrations of directly usable sugar (S_D), unusable sugar (S_U) and hydrolysis-requiring sugar (S_S) are described by Eqs. (4)~(6).

$$\frac{dS_D}{dt} = (r_{sg} - \frac{\mu_S}{Y_{XS}})X_1 - (\frac{\nu}{Y_{PS}} + m)X + F_S S_R (1 - R_S - R_U)/V_F - DS_D \quad (4)$$

$$\frac{dS_U}{dt} = F_S S_R R_U / V_F - DS_U \quad (5)$$

$$\frac{dS_S}{dt} = -r_{sg}X_1 + F_S S_R R_S / V_F - DS_S \quad (6)$$

The concentrations of pharma-medium (PM) and penicillin (P), the metabolically inactive biomass (W) as well as the medium volume (V_F) in the bioreactor are described as follows:

$$\frac{dPM}{dt} = -\frac{\mu_{PM}}{Y_{XPM}}X_1 - DPM \quad (7)$$

$$\frac{dP}{dt} = \nu X - k_h P - DP \quad (8)$$

$$\frac{dW}{dt} = k_{vac}X_2 - k_{PM}W - DW \quad (9)$$

$$\frac{dV_F}{dt} = F_S + F_{Pre} + F_{Ammo} + F_{AS} - F_o - \alpha V_F \quad (10)$$

In Eq. (10), F_S, F_{Pre}, F_{Ammo}, and F_{AS} stand for the feeding rate of the carbon source, precursor, ammonia and ammonium sulfate, respectively. F_o represents the withdrawal rate due to intermediate harvesting. α stands for the evaporation coefficient caused by aeration. The total reducible sugar concentration (S), the dilution rate (D), the specific growth rate based on glucose (μ_S) as well as the specific growth rate based on pharma-medium (μ_{PM}) are described in the following equations:

$$S = S_D + S_S + S_U \quad (11)$$

$$D = (F_S + F_{Pre} + F_{Ammo} - \alpha V_F)/V_F \quad (12)$$

$$\mu_S = \mu_{Smax}\frac{S_D}{K_S + S_D} \quad (13)$$

$$\mu_{PM} = \mu_{PMmax}\frac{PM}{K_{PM} + PM} \quad (14)$$

The hydrolysis rate of di- and polysaccharides is mod-

eled with Monod kinetics [4] in Eq. (15). The hydrolase concentration is assumed to be proportional to the growing cell concentration, see Eq. (6).

$$r_{sg} = \frac{r_{sg\,max} S_S}{K_{S_s} + S_S} \tag{15}$$

The dependence of k_{12} and k_{vac} on cell age is described by Eqs. (16) and (17).

$$k_{12} = f_{12} A_1 \tag{16}$$

$$k_{vac} = f_{vac} A_2 \tag{17}$$

The specific penicillin formation rate, ν, is determined by the overall specific growth rate μ, but it may decrease as the average age of intact productive cells increases. Therefore, it is modeled as:

$$\nu = \nu_{max} \frac{f(\mu)}{1 + K_A A^2} \tag{18}$$

where $f(\mu)$ is the same as in Tiller's model [2]: $f(\mu) = 1$ except when μ exceeds a critical value of $2\mu_P$. The overall specific growth rate, μ, is defined as $\mu = (\mu_S + \mu_{PM})X_1/(X_1 + X_2 + W)$. The average ages of the different cell sub-populations, A_1, A_2 and A, are estimated by the cell age model described in the next section.

Cell Age Model for *Penicillium chrysogenum*

Fig. 1 schematically shows the discrete cell age model, in which the growing cells and non-growing cells are assumed to be distributed in a series of cell age intervals with a width of Δt (hours). $x_1(i)$ represents the growing cells (weight in grams) in the i^{th} age interval, and $x_2(j)$ symbolizes the non-growing cells in the j^{th} age interval. $i = 1,2, \ldots\ldots, m$ and $j = 1,2, \ldots\ldots, n$, where m and n take the largest values of i and j, which satisfy $x_1(i)>0$ and $x_2(j)>0$, respectively. The growing cells in the i^{th} age interval have an age of $i \times \Delta t$ hours. The simulation step length was set as Δt, and then at each simulation step all cells are shifted from one interval to the next according to the following mechanics:

$$x_1(i+1) \leftarrow x_1(i), \qquad i=2,3, \ldots, m \tag{19}$$

$$x_2(j+1) \leftarrow x_2(j), \qquad j=2,3, \ldots, n \tag{20}$$

$$x_1(1) = (\mu_S + \mu_{PM}) X_1 V_F \Delta t \tag{21}$$

$$x_2(1) = 0 \tag{22}$$

The newborn cells during Δt are logically assumed to enter the first age interval of the growing phase, which results in Eq. (21). Meanwhile, the oldest growing cells enter the non-growing phase in such a way that their total amount equals $k_{12}X_1V_F\Delta t$, but their ages remain unchanged. This means that cells leaving the m^{th} age interval in the growing phase will fall into the m^{th} age interval in the non-growing phase. If $x_1(m)$ is less than $k_{12}X_1V_F\Delta t$, the difference will be supplemented by $x_1(m-1)$, $x_1(m-2)$, ... , but their ages remain also unchanged. The oldest non-growing cells amounting to $k_{vac}X_2V_F\Delta t$ fall into the metabolically inactive phase or the waste container.

The changes of the reactor volume caused by the inlet (feeding of substrates, precursors and other nutrient solutions) have neither an obvious influence on the cell age distribution nor on the absolute values of $x_1(i)$ and $x_2(j)$. The withdrawal *via* intermediate harvesting does not change the cell age distribution, but the cell mass is decreased by a factor $(1-F_o\Delta t/V_F)$. In this case, $x_1(i)$ and $x_2(j)$ should be corrected with Eqs. (23) and (24).

$$x_1(i) \leftarrow (1-F_o\Delta t/V_F)x_1(i), \qquad i=1,2, \ldots, m \tag{23}$$

$$x_2(j) \leftarrow (1-F_o\Delta t/V_F)x_2(j), \qquad j=1,2, \ldots, n \tag{24}$$

In reality, cell cycle progression can be a random process [5]. To take this into account, a five-point weighted moving average is introduced to smooth the cell distribution. The weighting factors are 0.05, 0.2, 0.5, 0.2, and 0.05. Details on moving average can be found in [6]. Now A_1, A_2 and A can be easily calculated by Eqs. (25)~(27). Evidently, no extra parameters are introduced in the cell age model Eqs. (21)~(27).

$$A_1 = \sum_{i=1}^{m} i\Delta t x_1(i) \Big/ \sum_{i=1}^{m} x_1(i) \tag{25}$$

$$A_2 = \sum_{j=1}^{n} j\Delta t x_2(j) \Big/ \sum_{j=1}^{n} x_2(j) \tag{26}$$

$$A = \{\sum_{i=1}^{m} i\Delta t x_1(i) + \sum_{j=1}^{n} j\Delta t x_2(j)\} / \{\sum_{i=1}^{m} x_1(i) + \sum_{j=1}^{n} x_2(j)\} \tag{27}$$

Model Validation

Data (20 batches) from industrial cultivations are used for model validation. For lack of measurements, initial PM is assigned an empirically estimated value of 20 g/L and the initial ratio $S_D : S_S : S_U$ is estimated as 4 : 2 : 1. These are verified to be a suitable approximation by the good fitting results obtained during the first 10 h for S, see Figs. 2 and 3. The initial cell age distributions, including the distribution shape and the percentages of growing and non-growing cells, are determined by try and error. Similar to the case of baker's yeast [6,7], the initial distribution is set to uniform. On the other hand, one fourth of the initial biomass is assumed as non-growing cells and the rest as growing cells. The width of the cell age interval, Δt, is set to 0.1 h, which has been proved to be the largest time that did not affect simulation accuracy.

Although Eqs. (1)~(18) contain approximately 20 model

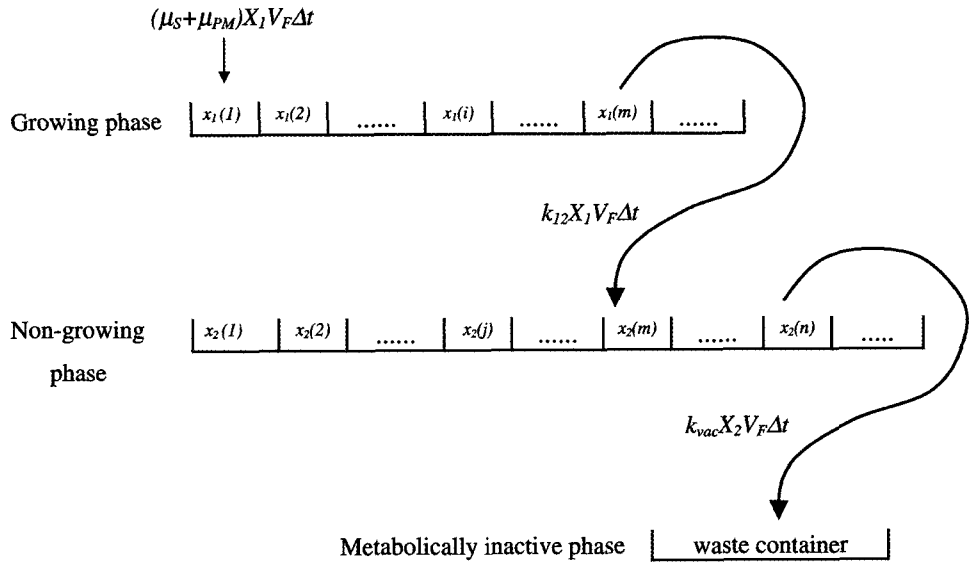

Fig. 1. Schematic description of the cell age model.

parameters, the number of strongly charge-dependent parameters is found to be limited to only two: the maximal specific growth rate on sugar (μ_{Smax}) and the maximum specific penicillin formation rate (ν_{max}). Table 1 lists the identification results of some constant model parameters. The rest may be found in the Nomenclature section of the paper. The Simplex method [8] is used for identification of the two charge-dependent parameters. The objective function for lumped parameter identification is given in Eq. (28), where *NP* and *NS* are the number of sampling points of a charge for the assay of penicillin and substrate, respectively. An equal weight is attached to the two terms *P* and *S* since the measurement error for these two variables is similar. Table 2 lists the identified charge-dependent parameters for each of the 20 charges.

$$sse = \sum_{i=1}^{NP}\left(P_{ms}(i) - P_m(i)\right)^2 + \sum_{j=1}^{NS}\left(S_{ms}(j) - S_m(j)\right)^2 \tag{28}$$

To quantify a model discrepancy, a relative fitting error for the penicillin concentration at the i^{th} sampling point, $e_{fit}(i)$, is calculated by Eq. (29).

$$e_{fit}(i) = \frac{P_{ms}(i) - P_m(i)}{P_m(i)} \tag{29}$$

Subsequently, a quality-index for model validation is defined as the averaged relative fitting errors, $\bar{e}_{fit}$ see Eq. (30), where q is the number of measurement points contained in the timeperiod of interest.

$$\bar{e}_{fit} = \sqrt{\frac{\sum_{i=1}^{q} e_{fit}(i)^2}{q}} \tag{30}$$

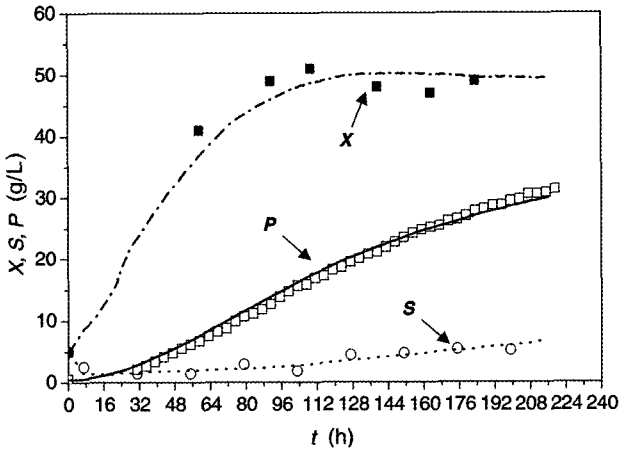

Fig. 2. Simulated state variables (lines) and their measurements (symbols) for charge 4, a batch with the average fitting error of 3.7% during the second half of cultivation.

For a more accurate analysis, the industrial-scale penicillin cultivation is divided into two phases: (1) the earlier phase of cultivation corresponding to $t \leq 96$ h and (2) the later phase of cultivation corresponding to $t > 96$ h. The averaged relative fitting errors in these two phases are denoted by $\bar{e}_{fit,1}$ and $\bar{e}_{fit,2}$, respectively. Table 2 shows the results.

From Table 2 it is found that $\bar{e}_{fit,1}$ is as high as 12.1%. This should be mainly attributed to the strong quality fluctuations of the preculture, which is normally uncontrollable. However, the second half of cultivation is described very well by the model with a mean $\bar{e}_{fit,2}$ of only 2.0%. This is highly emphasized because great attention should be paid to the second half of cultivation to ensure that optimal scheduling is carried out during this period.

Figs. 2 and 3 show comparisons of the model simula-

Table 1. Relatively constant model parameters

Para.	Unit	Value	Para	Unit	Value
f_{12}	h^{-2}	8.5×10^{-5}	Y_{CP}	g/g	3.36
f_{vac}	h^{-2}	7.5×10^{-5}	Y_{CX}	g/g	1.0
K_A	h^{-2}	8.0×10^{-5}	Y_{OP}	g/g	3.45
K_{PM}	g/L	2.0	Y_{OX}	g/g	1.0
K_S	g/L	0.4	Y_{PS}	g/g	1.2
K_{Ss}	g/L	0.2	Y_{XS}	g/g	0.61
k_h	h^{-2}	0.0015	Y_{XPM}	g/g	0.60
r_{sgmax}	h^{-1}	0.0032	μ_{PMmax}	h^{-1}	0.05

Table 2. μ_{Smax} and ν_{max} obtained by lumped identification and the resulting fitting errors

Charge No.	μ_{Smax} (h^{-1})	ν_{max} (h^{-1})	$\bar{e}_{fit,1}$ (%)	$\bar{e}_{fit,2}$ (%)
1	0.061	0.0075	14.2	1.4
2	0.069	0.0065	16.6	2.0
3	0.061	0.0074	12.3	3.6
4	0.060	0.0073	16.6	3.7
5	0.069	0.0067	8.9	1.9
6	0.061	0.0079	10.2	1.2
7	0.060	0.0079	17.0	2.3
8	0.061	0.0076	14.2	1.5
9	0.061	0.0076	18.7	1.8
10	0.061	0.0078	12.4	1.5
11	0.069	0.0064	7.3	1.9
12	0.065	0.0064	6.9	2.0
13	0.068	0.0069	14.0	2.0
14	0.068	0.0069	12.0	1.5
15	0.068	0.0065	3.8	1.9
16	0.069	0.0068	15.2	3.2
17	0.075	0.0062	12.0	1.5
18	0.069	0.0066	11.0	2.1
19	0.069	0.0066	8.6	1.0
20	0.069	0.0066	10.5	2.1
Mean	0.066	0.0070	12.1	2.0

tions of the state variables X, S and P and their measurements for two selected charges: one with $\bar{e}_{fit,2}$ of 3.7% (charge 4), and the other with an average $\bar{e}_{fit,2}$ of 1.9% (charge 5). These figures show that the combined model fits the penicillin concentration and residual sugar concentration very well, even for the worst case (Fig. 2). With respect to the biomass, model validation is difficult with a lack of sufficient data.

As an industrial application-oriented model, two important factors can not be under estimated. The first factor is evaporation and medium carryover by aeration.

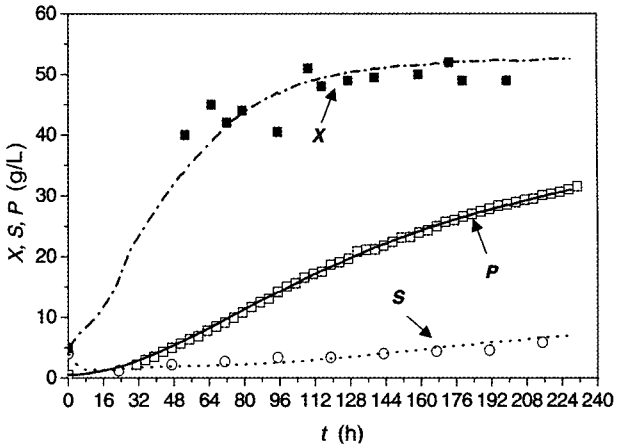

Fig. 3. Simulated state variables (lines) and their measurements (symbols) for charge 5, a batch with the average fitting error of 1.9% during the second half of cultivation.

When using a bioreactor with a volume of 50,000 L and an aeration rate of 1.5 vvm, vapor loss can be as high as 1,000 L a day. This was accounted for by the term αV_F in Eq. (9). The evaporation coefficient α is estimated to be 0.0008~0.0012 $LL^{-1}h^{-1}$. The second factor is the impurity of industrial glucose and/or hydrolysis required sugar. Clearly, the modified carbon source balance model, Eqs. (3)~(5), explains the initial responses and accumulating trends of the residual sugar very well, see Figs. 2 and 3.

To evaluate the model's accuracy more elaborately, it is found that for charge 5 (Fig. 3) the fitting results are good throughout the cultivation. However, for charge 4 (Fig. 2) the modeled product concentration is too high to compare the measurements before 144 h, and afterwards they become too low. Such discrepancies are referred to as a systematic error of the model. In the case of on-line uses, a rolling identification of the sensitive and time-variant model parameters μ_{Smax} and ν_{max} will be carried out, so that the systematic error will be reduced. This leads to the adaptive form of the model, which will be applicable for model-based feeding control and optimal scheduling of the process.

CONCLUSION

For the filamentous biomass in penicillin cultivations, a distinction can be made between young and aged fractions of mycelium mass. The growing tip is the most metabolically active part of the hyphen, whereas aged, often vacuolyzed parts are relatively inactive. It is reasonable to assume that the age of the segregated biomass will have an influence on cell growth and product formation. In the combined age and segregated kinetic model, this is interpreted by correlating the conversion rates k_{12} and k_{vac} with the average age of related cell sub-populations and by introducing an age associated depression term to the specific product formation rate.

In penicillin production, the biomass may multiply to

circa 8-fold of its initial value after 60 h of cultivation and circa 10-fold before termination. During this propagation process, possible influences of the initial cell age distribution are erased dramatically and hardly recognized in the main production phase. The cell age model presented here is a qualitative model. The aging process of mycelium and its effect on kinetics is difficult to verify experimentally. In this study, validation of the model is carried out with the most common method of input-output verification using only the measurable data. Taking the typically sizeable fluctuations of the final penicillin concentrations of the 20 testing charges (*ca.* 15%) into account, the qualitative and quantitative data fitting results shown in Table 2 and Figs. 2 and 3 reveal that the combined age and segregated kinetic model is a reasonable approximation of the real process.

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NOMENCLATURE

A, A_1, A_2	average age of total productive cells (sum of growing and non-growing cells), growing cells as well as non-growing cells (h)
CPR	carbon dioxide production rate (mole $m^{-3}h^{-1}$)
D	dilution rate (h^{-1})
$e_{fit}(i)$	relative fitting error for i^{th} penicillin measurement point (%)
$\bar{e}_{fit,1}$	average of relative fitting errors during the earlier phase of cultivation ($t \leq 96$h), (%)
$\bar{e}_{fit,l}$	average of relative fitting errors during the later phase of cultivation ($t > 96$h), (%)
$F_{Ammo}, F_{AS}, F_{Pre}, F_S, F_o$	feeding rate of ammonia solution, ammonium sulfate solution, precursor, carbon source as well as withdrawal rate (h^{-1})
f_{12}, f_{vac}, K_A	model parameters (h^{-2})
K_{PM}, K_S, K_{Ss}	Monod constant for pharma-medium, hexose and hydrolysis-requiring sugar (g/L)
k_{12}	conversion rate of cells from the growing phase to the non-growing phase (1/h)
k_h	penicillin decay rate (h^{-2})
k_{vac}	conversion rate of cells from the non-growing phase to the metabolically inactive phase (h^{-1})
m	maintenance coefficient for hexose, 0.01 (h^{-1})
OUR	oxygen uptake rate (mole $m^{-3}h^{-1}$)
P	penicillin concentration (g/L)
PM	pharma-medium concentration (g/L)
R_S	fraction of hydrolysis-requiring sugars in total reducible sugar in feed, 0.05 (–)
R_U	fraction of unusable sugars in total reducible sugar in feed, 0.01 (–)
r_{sg}	hydrolysis rate of di- and polysaccharides (h^{-1})
r_{sgmax}	maximum hydrolysis rate (h^{-1})
S, S_D	total reducible as well as directly usable sugar concentration in spent medium (g/L)
S_R	total reducible sugar concentration in feed (g/L)
S_S, S_U	hydrolysis-requiring sugar as well as unusable sugar concentration in spent medium (g/L)
t	current cultivation time (h)
V_F	liquid volume in bioreactor (L)
X_1, X_2, X	growing, non-growing as well as total intact cell (biomass) concentration (g/L)
$x_1(i)$	mass of growing cells in the i^{th} age interval (g)
$x_2(j)$	mass of non-growing cells in the j^{th} age interval (g)
Y_{CP}, Y_{CX}	yield of CO_2 per penicillin as well as per dry biomass produced (g/g)
Y_{OP}, Y_{OX}	O_2 uptake per penicillin as well as per dry biomass produced (g/g)
Y_{PS}, Y_{XS}	yield of penicillin as well as biomass on sugar (g/g)
Y_{XPM}	yield of dry biomass on pharma-medium (g/g)
Δt	width of cell age intervals and discrete simulation steps (h)
α	evaporation coefficient, 0.0008~0.0012 ($LL^{-1}h^{-1}$)
μ	overall specific growth rate (h^{-1})
μ_P	maximum overall specific growth rate at which the maximum product formation may be maintained, 0.035 (h^{-1})
μ_{PM}, μ_S	specific growth rate on pharma-medium as well as on sugar (h^{-1})
μ_S	specific growth rate on sugar (h^{-1})
ν	specific penicillin formation rate (h^{-1})

Suffixes

ms	model simulated value
m	measurements
max	maximum value of the related variable

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