COMBINED AGE AND SEGREGATED KINETIC MODEL FOR PENICILLIN FED-BATCH CULTIVATION

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Abstract: In mycelia cultivation, the morphology of filamentous microorganisms changes greatly along with the cultivation time, which causes variations in mass transfer and metabolic behavior of the microorganisms. To give an insight into the morphology-associated time-variant process dynamics in a simple way, a cell age model is proposed for *Penicillium chrysogenum* fed-batch cultivation . With help of this model, the average ages of the segregated cell populations may be estimated. The age model is further combined with a segregated penicillin model found in the literature. The combined model system seems to work well which is shown by the verification results with commercial penicillin fed-batch cultivations.

Keywords: Biotechnology, fermentation, modelling, parameter estimation, penicillin.

1. INTRODUCTION

The complexity of *Penicillium chrysogenum* fed-batch cultivation is closely associated with the morphological changes or the aging process of the microorganism: formation of hyphae and pellets, segmentation of mycelia, formation of vacuoles, and, at the later phase of cultivation, lysis of cells. It is therefore very difficult to build a mathematical model with fixed parameters which can cover all growth phases of the microorganism during a fed-batch cultivation. However, there are some ways to simplify the problem encountered (Nielsen, 1993; Tiller *et al*., 1994). In the work done by Tiller *et al.* (1994), the biomass is morphologically divided into three parts: growing cells, non-growing cells and autolysed cells. Monod-type kinetics are assumed and a "mean culture age", which is approximately proportional to the cultivation time, is introduced to take the influence of the aging process of the biomass into account. This model is verified with laboratory scale experiments, but failed to explain the commercial penicillin cultivations shown in this paper which result in a high product concentration and a long, efficient productive period of up to 280h. In this contribution the model of Tiller et al. (1994) will be modified in several aspects. At first, instead of autolysed biomass, a segregated biomass of metabolically inactive cells is introduced. Second, two more mass balance equations for both (i) the hydrolysis-requiring sugar and (ii) the unusable sugar are introduced to describe the high reducible

sugar concentration in the commercial production medium. Finally, the "mean culture age" is substituted by the average ages of the segregated biomass. For this purpose, a simple cell age model is established. This age model delivers the average age of growing cells, non-growing cells and the intact productive cells - which are

defined as the sum of growing and non-growing cells. The combined cell age- and segregated kinetic model for *P. chrysognum* was tested on one pilot scale fed-batch cultivation and two industrial scale charges.

2. MODIFICATION OF THE SEGREGATED KINETIC MODEL

By denoting k_{12} and k_{vac} as the conversion rate of cells from their growing state to their non-growing state and from the non-growing state to the metabolically inactive state, and by denoting A_1, A_2 and A as the average ages of growing cells, non-growing cells and the intact productive cells, respectively, the original segregated kinetic model of Tiller *et al*. (1994) is modified as follows:

(a) k_{12} is assumed as a bilinear function of A_1 and product concentration P; k_{vac} is assumed to be a linear function of A_2; the specific penicillin production rate decreases as A increases.

(b) Di- and polysaccharides found in industrial medium, forming a fraction of the hydrolysis-requiring R_S, must undergo hydrolysis before being metabolized. A hydrolysis model for this part of sugars is included. In addition, a small fraction of sugars in industrial medium, R_U, is found to be unusable by the microorganisms. An additional balance equation for it is introduced.

Modification (a) is drawn according to natural laws of living beings while modification (b) is mainly industrial cultivation-associated. Usually, R_U and R_S are dependent on the substrate types and the sterilization techniques applied. Even though glucose is used as the main carbon source in penicillin production, R_U and R_S are not so small to be negligible because of the impurities in industrial glucose and addition of other carbon sources. The hydrolysis process can be modeled by a Monod-type kinetic, where the concentration of hydrolases may take the value of the growing cells' concentration. It should be pointed out that modification (b) is only necessary when the residual sugar concentration is measured as the total reducible one.

The modified model equations are given in detail by Eqs. (1)-(20). For the meaning of symbols and their units reference is made to the nomenclature at the end of the paper. The dynamics of growing and non-growing cell concentrations (X_1 and X_2, respectively) in fed-batch cultivation are given by:

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

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

The evolution of the concentrations of directly usable sugar (S_D), unusable sugar (S_U) and hydrolysis-requiring sugar (S_S) are described by:

$$dS_D / dt = (r_{sg} - \mu_S / Y_{XS}) X_1 - (\nu / Y_{PS} + m) X + F_S S_R (1 - R_S - R_U) / V_F - DS_D \quad (3)$$

$$dS_U / dt = F_S S_R R_U / V_F - DS_U \quad (4)$$

$$dS_S / dt = -r_{sg} X_1 + F_S S_R R_S / V_F - DS_S \quad (5)$$

The concentration of pharmamedium (PM), penicillin (P) and metabolically inactive biomass (W) is changing as follows:

$$dPM / dt = -\mu_{PM} X_1 / Y_{XPM} - DPM \quad (6)$$

$$dP / dt = \nu X - k_h P - DP \quad (7)$$

$$dW / dt = k_{vac} X_2 - DW \quad (8)$$

The other balance and kinetics equations are:

$$dV_F / dt = F_S + F_{Pre} + F_{Ammo} - F_o \quad (9)$$

$$X = X_1 + X_2 \quad (10)$$

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

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

$$\mu_S = \mu_{S\max} S_D / (K_S + S_D) \quad (13)$$

$$\mu_{PM} = \mu_{PM\max} PM / (K_{PM} + PM) \quad (14)$$

The hydrolysis rate of di- and polysaccharides is modeled with Monod-type kinetics:

$$r_{sg} = r_{sg\max} S_S / (K_{S_S} + S_S) \quad (15)$$

k_{12} and k_{vac} are modified by following equations:

$$k_{12} = f_{12} A_1 P \quad (16)$$

$$k_{vac} = f_{vac} A_2 \quad (17)$$

The specific penicillin formation rate (ν) is determined by the overall specific growth rate μ, but it is subject to a decrease as the average age of intact productive cells increases:

$$\nu = \nu_{\max} f(\mu) / (1 + K_A A) \quad (18)$$

Where, $\mu=(\mu_S+\mu_{PM})X_1/(X_1+X_2)$; $f(\mu)$ usually equals 1.0 except when $\mu > 2\mu_p$ (see nomenclature), then $f(\mu)=0$. For *OUR* and *CPR* calculations the same expressions as in Tiller *et al*. (1994) are used:

$$OUR = [(\mu_S + \mu_{PM}) X_1 Y_{OX} + (\nu Y_{OP} + m_O) X] / 32 \quad (19)$$

$$CPR = [(\mu_S + \mu_{PM}) X_1 Y_{CX} + (\nu Y_{CP} + m_C) X] / 44 \quad (20)$$

The average ages of the different cell sub-populations will be estimated by the following cell age model.

3. CELL AGE MODEL FOR *P. CHRYSOGENUM*

$(\mu_S+\mu_{PM})X_1V_F\Delta t$

Growing phase

$X_1(1)$ $X_1(2)$ .... $X_1(i)$ .... $X_1(m)$

$k_{12}X_1V_F\Delta t$

Non-growing phase

$X_2(1)$ $X_2(2)$ .... $X_2(j)$ .... $X_2(m)$ .... $X_2(n)$

$k_{vac}X_2V_F\Delta t$

Metabolically inactive phase Waste container

Fig. 1. Schematic description of the cell age model

The discrete cell age model is schematically shown in Fig. 1. The growing cells and non-growing cells are assumed to be distributed in a series of cell age intervals with width of Δt (hours). $X_1(i)$ represents growing cells (weight in grams) in the ith age interval and $X_2(j)$ non-growing cells in the jth age interval. Hence, the growing cells in the ith age interval, for example, have an age of $i\times\Delta t$ hours. $i=1,2, ..., m$ and $j=1,2, ..., n$, where m and n take the maximal value of i and j which satisfies $X_1(i)>0$ and $X_2(j)>0$, respectively. At each simulation step – the step length is also set to Δt – all cells are shifted from one interval to the next according the following mechanism:

$$X_1(i+1)\leftarrow X_1(i) \qquad i=2,3, ..., m \qquad (21)$$

$$X_2(j+1)\leftarrow X_2(j) \qquad j=2,3, ..., n \qquad (22)$$

$$X_1(1)= (\mu_S+\mu_{PM})X_1V_F\Delta t \qquad (23)$$

$$X_2(1)=0 \qquad (24)$$

The new-born cells during Δt are logically assumed to enter the first age interval of the growing state so that Eq. (23) is obtained. In the meantime, the oldest growing cells will fall into 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 the cells in the mth age interval in the growing phase will fall into the same age interval m in the non-growing phase. If $X_1(m)$ is less than $k_{12}X_1V_F\Delta t$, the deviation will be supplemented by $X_1(m-1)$, $X_1(m-2)$, ... , but again, their ages maintain unchanged. The oldest non-growing cells, amounting $k_{vac}X_2V_F\Delta t$, will fall into the metabolically inactive state – waste container. The age distribution of the metabolically inactive cells is not of interest and is therefore not logged in the model.

The changes of the reactor volume caused by the inlet (feeding of substrates, precursors and other nutrient solutions) have obviously neither influence on the cell age distribution nor on the absolute values of $X_1(i)$ and $X_2(j)$. The effluent (intermediate harvesting) results in no changes of the cell age distribution, however, the cell mass will be decreased by a factor of $(1-F_o\Delta t/V_F)$. In reality there will be a dispersion of age distribution over the individual cell age intervals. To take this effect into account, a weighted moving average (Deckwer et al, 1991) is introduced. This results in a smoothed age distribution. Now A_1, A_2 and A may be easily calculated by Eqs. (25)-(27).

$$A_1 = \sum_{i=1}^{m} iX_1(i) \,/\, \sum_{i=1}^{m} X_1(i) \qquad (25)$$

$$A_2 = \sum_{j=1}^{n} jX_2(j) \,/\, \sum_{j=1}^{n} X_2(j) \qquad (26)$$

$$A = \{\sum_{i=1}^{m} iX_1(i) + \sum_{j=1}^{n} jX_2(j)\} \,/\, \{\sum_{i=1}^{m} X_1(i) + \sum_{j=1}^{n} X_2(j)\} \qquad (27)$$

Evidently, the cell age model described above introduces no extra parameters. The only thing which should be predetermined is the initial distribution of growing and non-growing cells. This will be dealt with in the next section.

4. VERIFICATION OF THE COMBINED MODEL SYSTEM

Three fed-batch cultivations with the same commercial strain are used to verify the modified model system. The first one is a pilot scale fed-batch cultivation while the other two are industrial charges. The pilot bioreactor has a volume of 4000 l whereas the production bioreactor is in excess of 100,000 l. The inoculation ratio is ca. 10% for all cultivations. The feeding streams during fed-batch operation included carbon source, phenyl acetic acid and ammonium sulfate solutions. Foam is controlled by adding vegetable oil. As intermediate harvesting, the spent medium is periodically withdrawn after 60h. No significant autolysis of biomass is observed during the scheduled cultivation period. Samples are taken usually every 4 hours for assays of residual sugar and penicillin titres. The dry biomass concentration is measured only occasionally.

For simulations, initial conditions have to be defined. For the three cultivations, the initial X, S and P are known, however, the initial S_S, S_U and PM are not measured. They take the estimated values of 2.0, 1.0 and 2.0 g l^{-1}, respectively. It is very difficult to determine the initial age distributions (i.e. distribution shape and the percentage) of growing and non-growing cells. Fortunately, like in case of baker's yeast (Deckwer *et al.*, 1991; Yuan *et al.*, 1993), simulations revealed that the initial shapes of the distribution (uniform, increasing or decreasing) have only little influences on the aging process during the main cultivation. Therefore, a uniform initial distribution is assumed. The ratio of growing to non-growing cells is found to have an affect on A_1 and A_2 within the first 60 hours of the main cultivation, but not thereafter. Hence, one fourth of the initial biomass is assumed to consist of non-growing

cells and the rest are considered to be growing cells. The width of the cell age interval Δt is set to 0.2h, which proved to be the largest one possible without affecting the simulation accuracy.

Table 1 Cultivation dependent parameters

Parameters	Units	Cultivation No. 1	2	3
R_S	–	0.05	0.055	0.04
Y_{CP}	g g^{-1}	*)	3.39	3.33
Y_{CX}	g g^{-1}	*)	1.0	1.0
Y_{OP}	g g^{-1}	*)	3.57	3.33
Y_{OX}	g g^{-1}	*)	1.0	1.0
ν_{max}	h^{-1}	0.0080	0.0078	0.0079

*) No measurements for gas balance are available

Although in Eq. (1)-(20) more than twenty model parameters are present, the number of cultivation dependent parameters is considerably lower. Table 1 shows the cultivation dependent parameters estimated from process data, while the values of other relatively invariant model parameters are given in the nomenclature. Fig. 2 shows the comparison of the model simulations and the measurements of the state variables

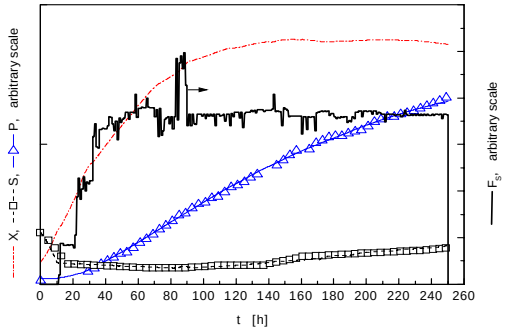
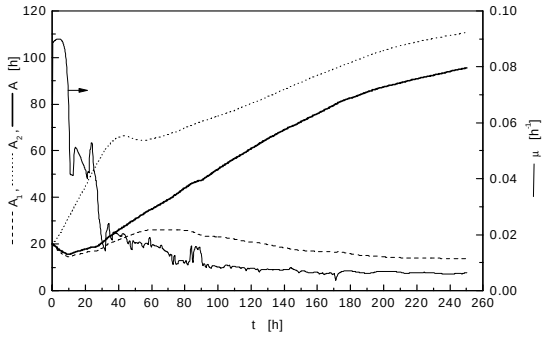


Fig. 2. Substrate feeding rate (thick solid line), simulated state variables (lines) and their measurements (symbols) for cultivation 1.



for cultivation 1, where the thick solid line indicates the substrate feeding rate. For reasons of confidentiality, real ordinate scales have been removed. Clearly, simulations by the combined model system fit the penicillin concentration and residual sugar concentration very well (there are no measurements of biomass for cultivation 1). Fig. 3 illustrates the simulated overall specific growth rate μ and the average ages of different cell sub-populations. The average age of growing cells A_1 reaches its maximum at about 60h and decreases thereafter. The average age of non-growing cells A_2 and of the intact productive cells A increases monotonously but not linearly with cultivation time. After 250h of cultivation, the average age of the intact productive cells is about 95 hours.

Similar satisfactory agreement between simulated state variables X, S and P and their measurements may be found in cultivations 2 and 3, as shown by Figs. 4 and 7. Figs. 5 and 8 illustrate the comparison between simulated and measured *OUR* and *CPR* . Again, a good agreement may be found. This agreement may be a favorable indication of the validity of the modified model structure. Figs. 6 and 9 show the average ages of different cell populations for these two charges. They have similar time courses as those of cultivation 1.

Fig. 3. Simulated average ages and overall specific growth rate for cultivation 1.

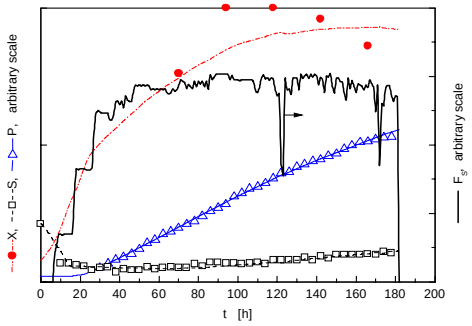


Fig. 4. Substrate feeding rate (thick solid line), simulated state variables (lines) and their measurements (symbols) for cultivation 2.

Fig. 5. Overall specific growth rate, hexose concentration, simulated *OUR* and *CPR* (lines) and their measurements (symbols) for cultivation 2.

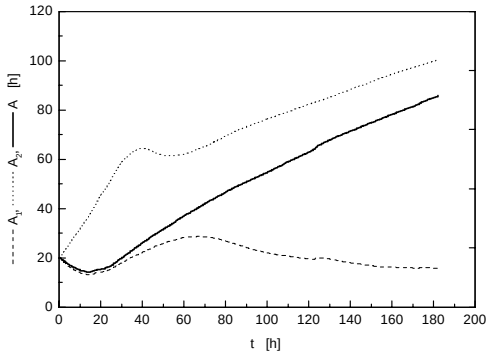


Fig. 6. Simulated average ages and overall specific growth rate for cultivation 2.

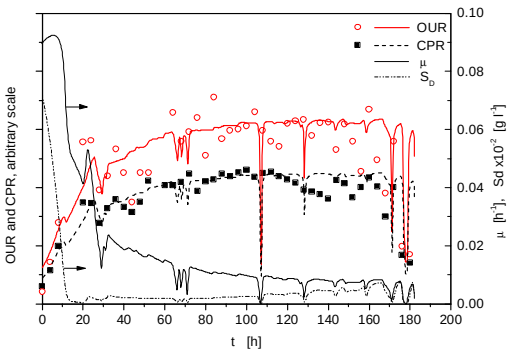


Fig. 8. Overall specific growth rate, hexose concentration, simulated *OUR* and *CPR* (lines) and their measurements (symbols) for cultivation 3.

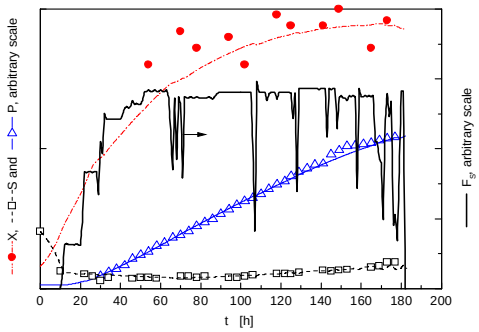


Fig. 7. Substrate feeding rate (thick solid line), simulated state variables (lines) and their measurements (symbols) for cultivation 3.

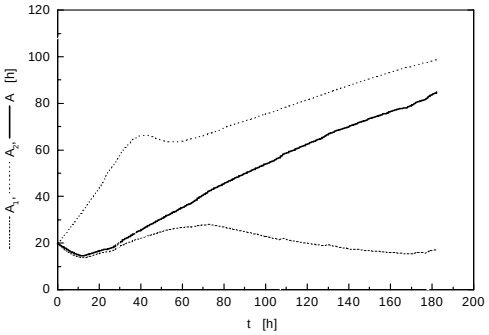


Fig. 9. Simulated average ages and overall specific growth rate for cultivation 3.

5. DISCUSSION AND 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 metabolically most active part of the hyphen, whereas aged, often vacuolized parts are relatively inactive. Therefore 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 done by relating the conversion rate k_{12} and k_{vac} to the average age of related cell sub-populations and by introducing an age associated inhibition term to the specific product formation rate ν.

The initial cell age distribution was proven by simulations (not shown here) to only have a small influence on the cell aging process during the major part of the cultivation. In penicillin production the biomass may grow ca. 8-fold after 60h of cultivation compared with its initial value and ca. 16-fold before termination. During this propagation process, variations of the initial cell age distribution are damped so greatly that they can hardly be recognized in the main production phase. The residual sugar concentration *S* in Figs. 2, 4 and 7 has a clear increasing trend. According to the model simulation, the total reducible sugar in spent medium after 180h of cultivation consists of 10% of directly usable hexose, 60% of hydrolysis-requiring sugar and 30% unusable sugar. Actually, the hydrolysis-requiring sugar at this stage may also be regarded as unusable sugar since it could be hardly hydrolyzed by cells which are becoming less and less active. Besides a small amount of impurity (cellulose for example) in industrial glucose, the unusable sugar may probably arise from the sterilizing process for substrate. The introduction of the hydrolysis reaction, Eq. (15), seems to be necessary because of the wide use of other di- and polysaccharides in the antibiotics industry. The two kinetic parameters in this equation may be varied depending on the type of carbon source and microorganism used. The modified substrate model equations (3)-(5) gave a good description of the initial responses and the accumulating trend of the residual sugar.

The cell age model proposed in this paper is a qualitative one. It is difficult to experimentally verify the aging process of mycelium and its effect on the kinetics. However, the input-output data fitting results reveals that the combined segregated age model is a reasonable approximation of the real process. The cell age model itself provides a possibility to estimate the average ages of different cell populations. Further incorporation of these average ages into the combined model system, see Assumption (a) and Eqs. (16)-(18), follows actually the natural law for most living beings: active and productive during youth and middle age; less

productive because of high age; and the transformation between youth, middle age and old depends, besides nutrient and other environmental factors, mainly on the average age of the population. In other words, it is acceptable that cell age has some effect on the kinetics of the microorganism. The age model proposed here is simple in structure and introduces no extra parameters. The cell age model can be expected to be applied to other kinds of mycelium cultivations because the cell age seems to have a general influence on the metabolic behavior of fungal mycelia.

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7. NOMENCLATURE

A	average age of the intact productive cells, h
A_1	average age of growing cells, h
A_2	average age of non-growing cells, h
CPR	carbon dioxide production rate, mole l^{-1} h^{-1}
D	dilution rate, h^{-1}
f_{12}	constant in k_{12}, 0.000085, h^{-2}
F_{Ammo}	feeding rate of ammonium sulfate, l h^{-1}
F_o	withdrawn rate of spent medium, l h^{-1}
F_{Pre}	feeding rate of precursor solution, l h^{-1}
F_S	feeding rate of carbon source, l h^{-1}
f_{vac}	constant in the expression of k_{vac}, 0.00003, h^{-2}
K_A	constant in the expression of ν, 0.004, h^{-1}
k_h	penicillin decay rate, 0.0015, h^{-2}
k_{12}	conversion rate of cells from growing phase to non-growing phase, h^{-1}
K_{PM}	Monod constant for pharmamedium, 2.0, g l^{-1}
K_S	Monod constant for hexose, 0.4, g l^{-1}
K_{Ss}	Monod constant for hydrolysis-requiring sugar, 0.2, g l^{-1}
k_{vac}	conversion rate of cells from non-growing phase to metabolically inactive phase, h^{-1}
m	maintenance coefficient for hexose, 0.01, h^{-1}
m_C	maintenance coefficient for CO_2, 0.01, h^{-1}
m_O	maintenance coefficient for O_2, 0.01, h^{-1}
OUR	oxygen uptake rate, mole l^{-1} h^{-1}
P	penicillin concentration, g l^{-1}
PM	pharmamedium concentration, g l^{-1}
R_S	fraction of hydrolysis-requiring sugars in feed
r_{sg}	hydrolysis rate of di- and polysaccharides, h^{-1}
r_{sgmax}	maximum hydrolysis rate, 0.0032, h^{-1}
R_U	fraction of unusable sugars in feed, 0.01
S	reducible sugar conc. in medium, g l^{-1}
S_D	hexose or directly usable sugar conc. in medium, g l^{-1}
S_R	reducible sugar concentration in feed, g l^{-1}
S_S	hydrolysis-requiring sugar concentration in medium, g l^{-1}
S_U	unusable sugar concentration in medium, g l^{-1}
t	cultivation time, h
V_F	liquid volume in bioreactor, l
X	biomass concentration, g l^{-1}
X_1	concentration of growing cells, g l^{-1}
$X_1(i)$	growing cells in the *i*th age interval, g
X_2	concentration of non-growing cells, g l^{-1}
$X_2(j)$	non-growing cells in the *j*th age interval, g
Y_{CP}	yield of CO_2 per penicillin produced, g g^{-1}
Y_{CX}	yield of CO_2 per dry biomass produced, g g^{-1}
Y_{OP}	O_2 uptake per penicillin produced, g g^{-1}
Y_{OX}	O_2 uptake per dry biomass produced, g g^{-1}
Y_{PS}	yield of penicillin on sugar, 1.2, g g^{-1}
Y_{XS}	yield of dry biomass on sugar, 0.61, g g^{-1}
Y_{XPM}	yield of biomass on pharmamedium, 0.6, g g^{-1}
Δt	width of cell age intervals and discrete simulation steps, h
μ	overall specific growth rate, h^{-1}
μ_P	max. growth rate at which the maximum product formation may maintained, 0.035, h^{-1}
μ_{PM}	specific growth rate on pharmamedium, h^{-1}
μ_{PMmax}	maximum value of μ_P, 0.05, h^{-1}
μ_S	specific growth rate on sugar, h^{-1}
μ_{Smax}	maximum value of μ_S, 0.06, h^{-1}
ν	specific penicillin formation rate, h^{-1}
ν_{max}	maximum value of ν, h^{-1}

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