Modeling and Model Based Feeding Control for *Pichia pastoris* fed-batch cultivation

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Abstract: A structured kinetic model, which takes account of major metabolic pathways of glycerol and methanol in *Pichia pastoris*, is presented. Based on the combined kinetic and bioreactor model, feeding profiles of methanol are determined with the aim of maintaining constant specific growth rate during production stage. Compared with the decreasing type of specific growth rate resulted from constant feeding profile in the standard protocol, the constant specific growth rate is believed to be advantageous for improving the productivity. Experimental results indicate that simulations of biomass and protein concentration agree well with the measured data, and the specific growth rates were successfully controlled at various set points.

Keywords: *Pichia pastoris*, Fed-batch cultivation, Modeling, Feeding profile, Model based control

1. Introduction

The specific growth rate of microorganisms has been found to have prominent influence on the productivity in bioprocesses (Jimenez, *et al*., 1997; Chung, 1999). d'Anjou, *et al*. (1997) developed a mass balance and Monod type kinetic model for *P. pastoris* expressing sea raven anti-freeze protein (SR-AFP). Although the measurements agreed with model simulations only qualitatively, the growth associated product formation was revealed. Based on a mass balance model, Kobayashi, *et al*. (2000) obtained the optimal specific growth rate for *P. pastoris* expressing recombinant human serum albumin (rHSA) by dynamic programming method. In the work of Jahic, *et al*. (2002), a kinetic model for *P. pastoris* expressing a fusion protein was proposed to describe cell growth and oxygen consumption. They found that the productivity could be increased by increasing the specific growth rate. In this paper, a structured model for *Pichia pastoris* expressing rHSA is constructed based on the analysis of metabolic pathways of glycerol and methanol. With this model, methanol feeding strategy during production stage is investigated. The aim is to control the specific growth rate at desired set points.

2. Process Description

Recombinant human serum albumin is expressed by *P. pastoris* GS115. The inoculum was grown for 12 to 24 hours until OD_{600} reached 2 to 6. 5-10% inoculum was used for inoculation. Cultivations were carried out in a 30L bioreactor (B.Braun, Germany) with a working volume of 20L at 30℃. pH was maintained at 6.5 by adding 25% ammonia solution, and DO at 30% by adjusting agitation. The solutions of glycerol and methanol were fed with calibrated peristaltic pumps (Watson 101, England). The medium composition was the same as Boze, *et al*. (2001) used.

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The cultivation included a glycerol phase and a methanol phase. The glycerol phase was divided into a batch stage and a fed-batch stage. Cultivation began with the batch stage. Upon the depletion of the glycerol in the batch medium, fed-batch stage commenced by adding glycerol solution at predetermined feeding rates. The glycerol fed-batch culture lasted 16-20 h in order for obtaining high cell density. The methanol phase was subdivided into a 10 h induction stage and a production stage. In the induction stage, methanol was fed with a low initial value in order for the cells to adapt the shifting of carbon source. The majority of rHSA was yielded in the production stage. To determine biomass and protein concentration, samples were collected at intervals of 2 h in the glycerol phase and 4 h in the methanol phase, respectively. Biomass concentration in wet weight was routinely measured. Methanol concentration was measured by HPLC, and the concentration of rHSA was measured with 2D-electrophoresis.

3. Modeling and Validation

3.1 Metabolic flux in the glycerol phase

In the glycerol phase, the main metabolic pathways include phosphorylation, glycolysis, TCA cycle and respiratory chain. The balance equations describing fluxes of metabolites, ATP and NADH in these pathways are presented in Eqs. (1); (2); (4) and (5); (7), respectively (Gancedo, *et al*, 1968; Nevoigt and Stahl, 1997). The formation of the byproduct ethanol (Sonnleitner and Kaeppeli, 1986; Ratledge and Kristiansen, 1987) was neglected in this model for simplification. Eqs. (3) and (6) present the main flux of biomass formation. The assumption was made that ATP was consumed mostly for cell growth and maintenance, as described in Eq. (8). The meaning of the symbols appearing in these equations is explained in the nomenclature.

$$S_{Gly} \xrightarrow{q_{Gly}} S_{GP} \tag{1}$$

$$S_{GP} \xrightarrow{r_S} Pyruvate + ATP + 2NADH \tag{2}$$

$$S_{GP} \xrightarrow{\mu K_{B1}} Cell\ material \tag{3}$$

$$Pyruvate \xrightarrow{r_{Ac}} Acetyl\text{-}CoA + NADH + CO_2 \tag{4}$$

$$Acetyl\text{-}CoA \xrightarrow{r_{TCA}} 2CO_2 + ATP + 4NADH \tag{5}$$

$$Acetyl\text{-}CoA \xrightarrow{\mu K_{B2}} Cell\ material \tag{6}$$

$$1/2O_2 + NADH + P/O\,ADP \xrightarrow{r_{NAD}} P/O\,ATP \tag{7}$$

$$r_{ATP} = \frac{\mu}{Y_{ATP}} + m_{ATP} \tag{8}$$

3.2 Metabolic flux in the methanol phase

In the methanol phase, methanol is first oxidized to formaldehyde (Gellissen, 2000), as described in Eq. (9). The assumption was made that the majority of formaldehyde was condensed with xylulose 5-monophosphate to form glyceraldehydes 3-phosphate (GAP) in an assimilatory pathway, as presented in Eq. (10), where three molecules of formaldehyde are consumed to produce one net molecule of GAP (Gellissen, 2000; Lueers, *et al*., 1998; Cereghino and Cregg, 2000). The rest part is oxidized to formate, and further dissimilated to CO_2 with the generation of reducing power NADH. This is described with Eq. (11). The ratio of formaldehyde catalyzed between dissimilation and assimilation, denoted by φ, is being under investigation. Here, φ was set to 0.25. For simplification, biomass formation was mathematically assumed to be resulted from formaldehyde, see Eq. (12), although it is from GAP metabolically (Gellissen, 2000; Lueers, *et al*., 1998). The metabolism from GAP to pyruvate is presented in in Eq. (13).

$$S_{MeOH} \xrightarrow{q_{MeOH}} S_{For} \tag{9}$$

$$S_{For} \xrightarrow{r_S} \frac{1}{3} GAP - ATP \tag{10}$$

$$S_{For} \xrightarrow{\varphi r_S} CO_2 + 2NADH \tag{11}$$

$$S_{For} \xrightarrow{\frac{1}{3}\mu K_{B1}} Cell\ material \tag{12}$$

$$S_{GAP} \xrightarrow{r_{GAP}} pyruvate + 2ATP + NADH \tag{13}$$

The formation of byproducts during the methanol phase was also neglected, since the specific growth rate was controlled relatively low. Therefore, the metabolic pathways after pyruvate and the respiratory chain were assumed to be the same as those in glycerol phase. It should be pointed out that some model parameters, such as K_{B1}, K_{B2} *etc.*, may take dissimilar values depending on different phases.

3.3 Modeling equations

Based on above statement, the structured model for glycerol phase is presented in Eq. (14), which describes the balances of the carbon source, NADH, ATP and pyruvate. These balance relationships are obtained from Eqs. (1)~(3); (2), (4)~(7); (2), (5)~(8); (2), (4), respectively.

$$\begin{bmatrix} 1 & K_{B1} & 0 & 0 \\ 2 & -4K_{B2} & 5 & -2 \\ 1 & -K_{B2} - \dfrac{1}{Y_{ATP}} & 1 & 2P/O \\ 1 & 0 & -1 & 0 \end{bmatrix} \begin{bmatrix} r_S \\ \mu \\ r_{Ac} \\ q_{O2} \end{bmatrix} = \begin{bmatrix} q_{Gly} \\ 0 \\ m_{ATP} \\ 0 \end{bmatrix} \tag{14}$$

The specific glycerol uptake rate q_{Gly} is described with Monod kinetics, see Eq. (15)

$$q_{S,M} = \frac{q_{Glymax} S_{Gly}}{K_{Gly} + S_{Gly}} \tag{15}$$

However, as a common observation in bioprocesses, it was found that the actual glycerol uptake rate was

much lower than $q_{S,M}$ in the early batch stage. Actually, it is known that Monod kinetics covers only the rapid metabolic regulation, but the pathways for gluconeogenesis are subject to long-term regulation by enzyme induction and repression during batch stage (Bellgardt, 1983). An extended first order closed-loop regulator is introduced to describe the lag phase, which was proposed and well validated by Bellgardt, *et al.* (1986). The regulator model is described with Eq. (16). The actual specific glycerol uptake rate q_{Gly} is obtained according to Eq. (17)

$$\frac{dq_{\lim}}{dt} = k_1(q_{Gly} + q_{\lim 0}) + (-k_2 - \mu)q_{\lim} \quad (16)$$

$$q_{Gly} = \begin{cases} q_{S,M} & if\ q_{S,M} < q_{\lim} \\ q_{\lim} & if\ q_{S,M} \geq q_{\lim} \end{cases} \quad (17)$$

In the methanol phase, the balance equations for carbon source, NADH, ATP and pyruvate are obtained from Eqs. (9)~(12); (4)~(7), (11), (13); (5)~(8), (10); (4), (10), (13), respectively, see Eq. (18).

$$\begin{bmatrix} 1+\varphi & \frac{1}{3}K_{B1} & 0 & 0 \\ \frac{1}{3}+2\varphi & -4K_{B2} & 5 & -2 \\ -\frac{1}{3} & -K_{B2}-\frac{1}{Y_{ATP}} & 1 & 2P/O \\ \frac{1}{3} & 0 & -1 & 0 \end{bmatrix} \begin{bmatrix} r_S \\ \mu \\ r_{Ac} \\ q_{O2} \end{bmatrix} = \begin{bmatrix} q_{MeOH} \\ 0 \\ m_{ATP} \\ 0 \end{bmatrix} \quad (18)$$

The specific protein production rate ρ was assumed to follow the model Eq. (19).

$$\rho = a\mu + b \quad (19)$$

3.4 Bioreactor model

The bioreactor model is established based on mass balance. It includes four balance equations for medium volume, biomass, substrate and product concentrations, see Eqs. (20)~(23), where the coefficient of evaporation α was estimated by 0.0006 l (l h)$^{-1}$ based on the mass balance data of the given equipment.

$$\frac{dV_F}{dt} = F_S + F_{NH3} - F_O - \alpha V_F \quad (20)$$

$$\frac{dX}{dt} = \mu X - \frac{F_S + F_{NH3}}{V_F} X + \alpha X \quad (21)$$

$$\frac{dS}{dt} = \frac{F_S}{V_F} S_R - q_S M_S X - \frac{F_S + F_{NH3}}{V_F} S + \alpha S \quad (22)$$

$$\frac{dP}{dt} = \rho X - \frac{F_S + F_{NH3}}{V_F} P + \alpha P \quad (23)$$

The coupling of the structured kinetic model and the bioreactor model is shown in Fig. 1.

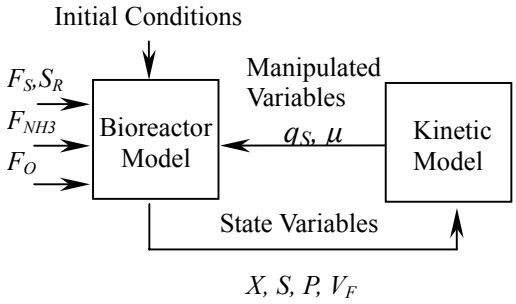

Fig.1 Combined metabolic-bioreactor model

3.5 Validation of the model

Several experiments were carried out to validate the model, and two of them were shown in Fig. 2. It was found that both cell growth and protein production are well described by the model. For confidential reasons, the scale has been removed in this figure as well as in other figures.

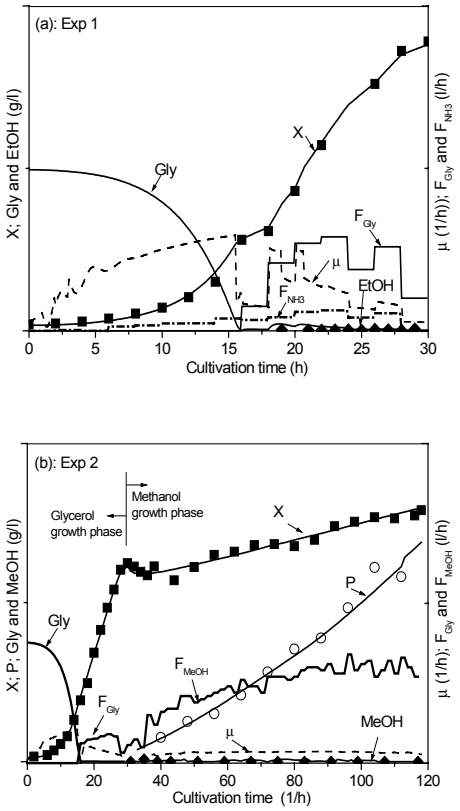

Fig. 2 Comparison of model simulation with measurements. Lines: model simulation; symbols: measurements.

There are seventeen model parameters in Eqs. (14)~(16), (18) and (19). Three of them took fixed values as those for baker's yeast as listed in Table. 1 (Yuan and Bellgardt, 1994). The rest were identified by the Simplex method (Nelder and Mead, 1965), see Tables 2 and 3.

Table 1 Parameters taking fixed values as those for baker's yeast

Para.	Y_{ATP}	P/O	q_{lim0}
Unit	g mol^{-1}	mol mol^{-1}	mol $(gh)^{-1}$
Value	10.5	1.5	0.0006

Table 2 Parameters identified for glycerol growth phase

Para.	q_{Glymax}	K_{Gly}	m_{ATP}	K_{B1}	K_{B2}	k_1	k_2
Unit	mol $(gh)^{-1}$	g l^{-1}	mol $(gh)^{-1}$	mol g^{-1}	mol g^{-1}	h^{-1}	h^{-1}
Exp.1	0.0057	0.05	0.001	0.001	0.014	0.6	0.3
Exp.2	0.0057	0.04	0.001	0.001	0.013	0.5	0.3

Table 3 Parameters identified for methanol growth phase

Para.	$q_{MeOHmax}$	K_{MeOH}	m_{ATP}	K_{B1}	K_{B2}	a	b
Unit	mol $(g)^{-1}$	g l^{-1}	mol $(gh)^{-1}$	mol g^{-1}	mol g^{-1}	-	h^{-1}
Exp.2	0.001	0.16	0.0001	0.015	0.013	0.04	0.0001

4. Model Based Feeding Control

In the literature, exponential type feeding strategy has been proven to be beneficial for improving the recombinant proteins productivity of *Escherichia coli* system (Paalme, *et al*., 1990; Yee and Blanch, 1992). According to the standard protocol, the methanol feeding rate is constant. Such feeding strategy results in a decreasing specific growth rate. This may be one of the reasons for the low productivity of rHSA found in our study (data not shown). Therefore, exponential type methanol feeding profiles are designed with the support of the model. The goal is to maintain the specific growth rate at preset values during the production stage. The sum of squared errors of the specific growth rate between model simulation and the preset value during the whole production stage was chosen as the objective function. First, the increasing type of feeding profile is used during induction stage (30⩽t⩽40h), see Eq. (24). The slope of ω_1 was optimized by Golden Section Search to make the specific growth rate as close as possible to the preset value at 40 h. During production stage (t>40h), the feeding profile shown in Eq. (25) is used. Obviously, the constant ω_2 can be calculated as ($10\omega_1$+12). The parameter ω_3 was then estimated with the same method. Two additional experiments were carried out to validate the control strategy. The results of the control experiments are illustrated in Fig. 3.

$$F(t) = \omega_1 (t - 30) + 12 \qquad 30 \leqslant t \leqslant 40h \qquad (24)$$

$$F(t) = \omega_2 \exp(\omega_3 (t - 40)) \qquad t > 40h \qquad (25)$$

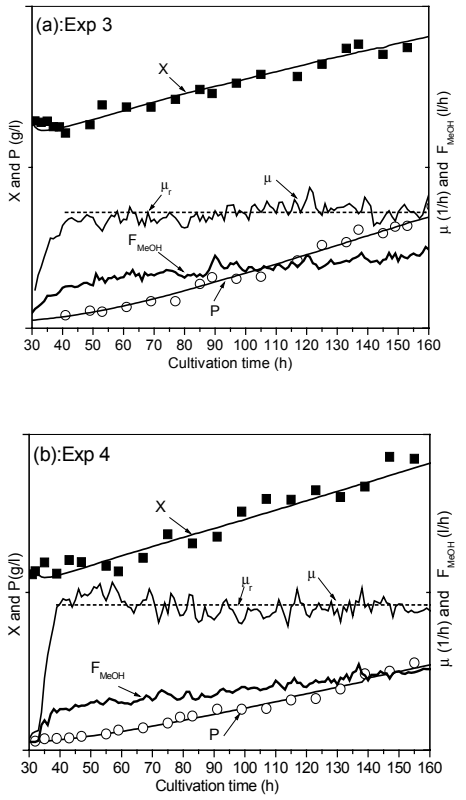

Fig.3 Experiments to test the model based feeding profile. Lines: model simulation; symbols: measurements.

5. Discussion and Conclusion

In this paper, a structured model for *Pichia pastoris* was constructed and validated. Based on the model, the specific growth rate was successfully controlled at predetermined constant levels in the methanol phase. The metabolic model is established based on the simplified flux analysis. It is more complicated in comparison with the mass balance model found in the literature (Kobayashi, *et al*., 2000). However, it enables further investigations on metabolic fluxes. Moreover, this structured model may be applied in those situations, where the set point control of residual methanol concentration is required. It was also found that most model parameters have relative constant values for different experiments, which implies that the model is robust to some extent.

For the methanol phase, Veenhuis, *et al*. (1983) pointed out that the dissimilation of formaldehyde generates the primary part of energy source NADH. That means, the flux of dissimilation plays a significant role in the metabolic network. On the other hand, according to Sibirny *et al*. (1990), the most energy for methanol growth comes from the assimilatory pathway, and the main function of

dissimilation of formaldehyde is to protect the cell from the toxic effect of the accumulated formaldehyde. In this paper, higher flux via assimilatory pathways (corresponding to lower φ) was adopted.

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7. Nomenclature

EtOH	ethanol residual concentration g l^{-1}
F_S	substrate feeding rate l h^{-1}
F_{Gly}	glycerol feeding rate l h^{-1}
F_{MeOH}	methanol feeding rate l h^{-1}
F_{NH3}	ammonia solution feeding rate l h^{-1}
F_O	withdrawal rate cause by sampling l h^{-1}
Gly	glycerol concentration in the medium g l^{-1}
m_{ATP}	maintenance coefficient for ATP mol (g $h)^{-1}$
MeOH	methanol concentration in the medium g l^{-1}
M_S	molecular weight of substrates
P	heterologous protein concentration g l^{-1}
P/O	effectiveness coefficient of oxidative phosphorylation
q_{Gly}	actual specific uptake rate of glycerol mol (g $h)^{-1}$
q_{lim}	specific uptake rate of glycerol obtained from regulator model mol(g $h)^{-1}$
q_{lim0}	initial value of specific uptake rate of glycerol mol(g $h)^{-1}$
$q_{S,M}$	specific uptake rate of glycerol obtained from Monod model mol(g $h)^{-1}$
q_{MeOH}	specific methanol uptake rate mol(g $h)^{-1}$
q_{O2}	specific oxygen uptake rate mol (g $h)^{-1}$
q_S	specific substrate uptake rate mol (g $h)^{-1}$
r_{Ac}	specific acetyle-CoA production rate mol (g $h)^{-1}$
r_{ATP}	specific ATP uptake rate mol(g $h)^{-1}$
r_{GAP}	specific glyceraldehydes-3-phosphate uptake rate mol(g $h)^{-1}$
r_{NAD}	specific NADH uptake rate in respiratory chain mol(g $h)^{-1}$
r_S	specific rate of glycolysis mol(g $h)^{-1}$
r_{TCA}	specific acetyle-CoA uptake rate mol(g $h)^{-1}$
S	substrate concentration in the medium g l^{-1}
S_{MeOH}	extracellular methanol concentration g l^{-1}
S_R	substrate concentration in the feed g l^{-1}
S_{Gly}	extracellular glycerol concentration g l^{-1}
S_{GP}	intracellular glycerol 3-phosphate concentration g l^{-1}
S_{For}	intracellular formaldehyde concentration g l^{-1}
V_F	volume of broth l
X	biomass concentration g l^{-1}
Y_{ATP}	yield coefficient of ATP g mol^{-1}
α	coefficient of evaporation l (l $h)^{-1}$
μ	specific growth rate h^{-1}
μ_r	preset value of the specific growth rate h^{-1}
ρ	specific product formation rate h^{-1}
φ	ratio of formaldehyde consumed between dissimilatory and assimilatory pathways
Suffix max	maximum values of the corresponding parameters or variables
K_{B1}, K_{B2}, K_{Gly}, K_{MeOH}, *a, b,* k_1, k_2	model parameters

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