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SHORT COMMUNICATION

A simple unstructured model-based control for efficient expression of recombinant porcine insulin precursor by *Pichia pastoris*

Hai-feng Hang*, Wen Chen*, Mei-jin Guo*,,†, Ju Chu*, Ying-ping Zhuang*, and Siliang Zhang*,†**

*State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, P.R. China
**Department of Biotechnology, Jiangxi Agricultural University, 10 Yingshang Road, Nanchang 330045, P.R. China
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Abstract−Based on the fact that *Pichia* cell growth follows a Monod equation under the condition of methanol concentration limitation, a kinetics model of recombinant methylotrophic yeast *Pichia pastoris* expressing porcine insulin precursor (PIP) was developed in the quasi-steady state in the induction phase. The model revealed that the relationship between specific growth rate (μ) and substrate methanol concentration was in accord with the Monod equation. The fermentation kinetic parameters maximum specific growth rate (μ_{max}), saturation constant (K_s) and maintenance coefficient (M) were estimated to be 0.101 h^{-1}, 0.252 g l^{-1}, and 0.011 g MeOH g^{-1} DCW h^{-1}, respectively. The unstructured model was validated in methanol induction phase with different initial cell densities. Results showed that the maximum specific protein production rate ($q_{p.max}$) of 0.098 mg g^{-1} DCW h^{-1} was achieved when μ was kept at 0.016 h^{-1}, and the maximum yield of PIP reached 0.97 g l^{-1}, which was 1.5-fold as that of the control. Therefore, the simple Monod model proposed has proven to be a robust control system for recombinant porcine insulin precursor production by *P. pastoris* on pilot scale, which would be further applied on production scale.

Key words: *Pichia pastoris*, Porcine Insulin Precursor, Monod Modeling, Methanol Feeding Strategy

INTRODUCTION

As a diabetes drug, especially for the treatment of insulin-dependent diabetes mellitus (IDDM), insulin is still indispensable even though some less effective drugs are available. The recombinant human insulin has been produced at large-scale using *Escherichia coli* [1,2] and *Saccharomyces cerevisiae* [3]. Economically, an alternative way to produce recombinant human insulin is to express the porcine insulin precursor first, and then allow the *in vitro* processing with trypsin alone to obtain the mature insulin molecule in two steps*:* removal of Ala-Ala-Lys spacer and addition of threonine in B30 via a transpeptidation reaction [3-5].

Although human insulin is successfully expressed in *E. coli*, it always forms inclusion bodies that should be dealt with by denaturing and refolding after fermentation, resulting in very low recovery. Regarding conventional yeast *S. cerevisiae*, its expression level is also limited because of its own limitations including difficulty in high-density growth, no powerful and regulated promoters for expression, etc [6]. However, the methylotrophic yeast *Pichia pastoris* has been widely reported as a suitable expression system for therapeutic protein production [7,8]. Heterologous protein expression under the control of P_{AOX1} allows for the design of precisely controlled high-cell density cultivation strategies for heterologous protein production, and is structured in two main phases [9]: The first phase is to obtain a high-substrate biomass yield and the second phase is to induce foreign protein expression in the presence of methanol. The methanol concentration in the medium affects both the level of foreign protein production and the growth of *P. pastoris* cells probably due to the intracellular accumulation of formaldehyde and H_2O_2 through methanol metabolism pathway [10]. Besides, owing to the very complex non-linear dynamics of *P. pastoris* system, the optimal control parameters during the bioprocess may vary significantly with the operation conditions (e.g., temperature, pH, and culture medium), phenotype, strain physiology [11], and specific characteristics of the heterologous protein of interest, such as cell toxicity, stability and protease sensitivity [12]. Thus, different methanol feeding strategies in the protein induction phase have been applied to optimize the protein production, e.g., exponential feeding strategy [13], closed-loop DO (Dissolved Oxygen)-stat [14], constant specific growth rate control [15]. Meanwhile, several mathematical modeling methodologies have been also developed: the method of dynamic programming with optimal specific growth rate [16], minimal-variance-controller and a semi-continuous Kalman-filter [17], a stoichiometric mass balance-based macrokinetic model [18] and the online methanol consumption rate-based methanol feeding model [9]. However, some of the methanol feed approaches described in previous studies cannot be universally applied to any foreign protein expression by *Pichia* system. Therefore, in this work an unstructured Monod model was developed to control methanol feed rate for high PIP yield based on the fact that *Pichia* cell growth follows a Monod equation under methanol concentration limitation [11,19,20]. It was proved that a simple Monod model was useful for PIP production in fed-batch culture. The highest PIP yield level achieved was 1.5 times as high as that of the control.

MATERIALS AND METHODS

1. Microorganism

P. pastoris GS115/PIP strain was constructed as follows. A por-

†To whom correspondence should be addressed.
E-mail: guo_mj@ecust.edu.cn, siliangz@ecust.edu.cn
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cine insulin precursor (PIP) gene *pip* was artificially synthesized based on PIP nucleotide sequence. Plasmid was obtained after inserting a *pip* gene into a modified vector pPIC9K (GenBank No. Z46233) between the *Xho*I and *Not*I sites between the AOX1 promoter and the signal mating factor-α. After *Sal*I digestion, the linearized plasmid *pPIC9k/PIP* was integrated into the *His4* locus of *P. pastoris* chromosome according to *Pichia* Expression Kit, Invitrogen, CA, USA), then GS115/PIP strain with a Mut^+ phenotype was selected to perform the following fermentation experiments.

2. Inoculum Preparation

Preculture in shake-flask was performed as follows. A well-isolated single colony of *P. pastoris* GS115/PIP grown on a yeast peptone dextrose (YPD) plate(1% yeast extract, 2% polypeptone, 2% dextrose, and 2%g agar) was used to inoculate a 500-ml shake flask containing 50 ml BMGY (buffered minimal glycerol-complex medium) composed of 1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 0.004% (w/v) biotin, 1% (w/v) glycerol, then incubated at 250 rpm, 30 ºC. After 24 h of incubation, 10% (V/V) of the preculture was inoculated into a 5 *l* fermenter (FMS-5, NCBio, Shanghai, P.R.China) with 2.5 *l* YPD medium. It was run at 30 ºC, pH 5.0 (controlled with 28% ammonium hydroxide), 1 VVM air-flow rate, and dissolved oxygen tension (DOT) above 25% air saturation with stirring speed regulation. The culture was grown overnight at 30 ºC, stirring rate 500 rpm, and a constant air flow rate of 1 VVM. After carbon substrate depletion, the culture was used as the second seed culture.

3. Fed-batch Fermentation in 50-L Fermenter

The second seed culture was used to inoculate (10%, v/v) a 50-*l* bioreactor (Model FUS-50L(A), NCBio, P.R.China) containing 25 *l* basal salts fermentation medium (g l^{-1}) ($CaSO_4$, 0.93; K_2SO_4, 18.2; $MgSO_4 \cdot 7H_2O$, 14.9; KOH, 4.13; glycerol, 40, and 85% w/v H_3PO_4, 26.7 ml l^{-1}) with 4.0 ml l^{-1} PTM1 trace salts (6 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3 g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20 g $ZnCl_2$, 65 g $FeSO_4 \cdot 7H_2O$, 0.2 g biotin and 5 ml H_2SO_4 per liter). DOT and pH were monitored by a DO probe and a pH electrode (Mettler Toledo), respectively. It was run at 30 ºC, pH 5.0 (controlled with 28% ammonium hydroxide), 1 VVM air-flow rate, and DOT above 25% saturation with stirring speed regulation.

A three-phase fermentation protocol (glycerol batch, glycerol fed-batch, methanol fed-batch) was used. During glycerol batch phase, operation parameters were controlled at pH 5, 30 ºC, and DO maintained at 25%. After a sharp DO increase, the glycerol fed-batch phase was initiated at a constant flow rate of 15 ml l^{-1} h^{-1} with 50% (v/v) glycerol until the dry cell weight (DCW) reached about 50.6-64.4 g l^{-1}. In methanol fed-batch phase, 100% methanol with 4.0 ml PTM1 per liter was fed into the fermenter with varying feed rate according to the estimated specific growth rate.

4. Cell Optical Density (OD_{600}) Determination

OD_{600} was determined spectrophotometrically at 600 nm after appropriate dilution of a sample with deionized water. Meanwhile, the deionized water was used as control. One unit of OD_{600} is equivalent to 0.23 g of dry cell weight (DCW) per liter.

5. Porcine Insulin Precursor Concentration Assay

Porcine insulin precursor (PIP) concentration was analyzed by HPLC (HP1100), employing a .4.6×250 mm MacroSep C8 column (ES, West Berlin, USA). A gradient of solution A from 100% to 45% with solution B increasing from 0 to 55% (v/v) was injected into the column at a flow of 1.0 ml min^{-1} in 50 min. Solution A was composed of 10% (v/v) acetonitrile, 0.1% (v/v) trichloroacetic acid (TCA). Solution B was composed of 80% (v/v) acetonitrile and 0.1% (v/v) TCA.

6. Methanol Concentration Assay

Methanol concentration was determined by gas chromatography (GC920, Shanghai, China). The chromatography column (2 m in length and 2 mm at diameter) was packed with chromosorb 101 (Dikma, Lampoc, CA, USA). Flow rates of the carrier gas (Nitrogen, N_2), fuel gas (Hydrogen, H_2) were controlled at 15 ml min^{-1} and 10 ml min^{-1}, respectively. The flame ionisation detector, injector and the column oven were maintained at 135 ºC , 170 ºC and 135 ºC, respectively.

7. Determination of O_2 and CO_2 in Exhaust Gas

Oxygen and carbon dioxide concentration in the exhaust gas outlet were determined on-line by Magnos 4G and Uras 3G (H&B, Germany), respectively. Oxygen uptake rate and carbon dioxide evolution rate were calculated on-line.

8. Calculation of Specific Rates

Specific growth-rate (μ, h^{-1}), specific substrate consumption rate (q_s, $g_{methanol}\ g_{biomass}^{-1}\ h^{-1}$), specific PIP production rate (q_p, $g_{PIP}\ g_{biomass}^{-1}\ h^{-1}$) and methanol feeding rate (R_{MeOH}, ml h^{-1}) were calculated as described by Cos [21] using suitable smoothing routines (Matlab 6.1 Curvefit Toolbox, The Mathworks Inc., Natick, USA) and mass balances applying complete data sets with coincident off-line biomass (OD_{600}), substrate, and PIP concentration data. Briefly, biomass, substrate, and PIP concentration data were smoothed first. After that, the first derivatives of the smoothed curves were obtained. Feed-flow rate was time-averaged between consecutive off-line biomass samples. Total volume was estimated taking into account feed-flow rate including carbon sources (methanol), base solutions, as well as volume samples. Finally, the mass balances of the bioprocess using fed-batch operation were set up, in which several reaction rates were obtained as follows:

$$\mu = \frac{d(XV)}{(XV)dt} = \frac{\Delta(XV)}{X_0 V_0 \Delta t} \tag{1}$$

$$Q_s = q_s \times \int_0^t (XV)dt \tag{2}$$

$$q_p = \frac{\Delta(PV)}{X_0 V_0 \Delta t} \tag{3}$$

$$R_{MeOH} = \frac{\mu XV}{\rho q_s} \tag{4}$$

where X_0 is the initial biomass concentration in methanol feed phase (g l^{-1}), S is the substrate concentration of the broth (g l^{-1}), S_0 is the inlet substrate concentration (g l^{-}), P is the PIP concentration (g l^{-}), t is the time (h), ρ is the methanol density (g l^{-1}) and V is the total volume of the culture (*l*).

RESULTS AND DISCUSSION

1. Typical Time Course of Cell Growth and PIP Production in Fed-batch Culture

As shown in Fig. 1, there were three stages throughout recombinant *Pichia* fed-batch culture. Firstly, after nearly 1 h lag stage, ex-

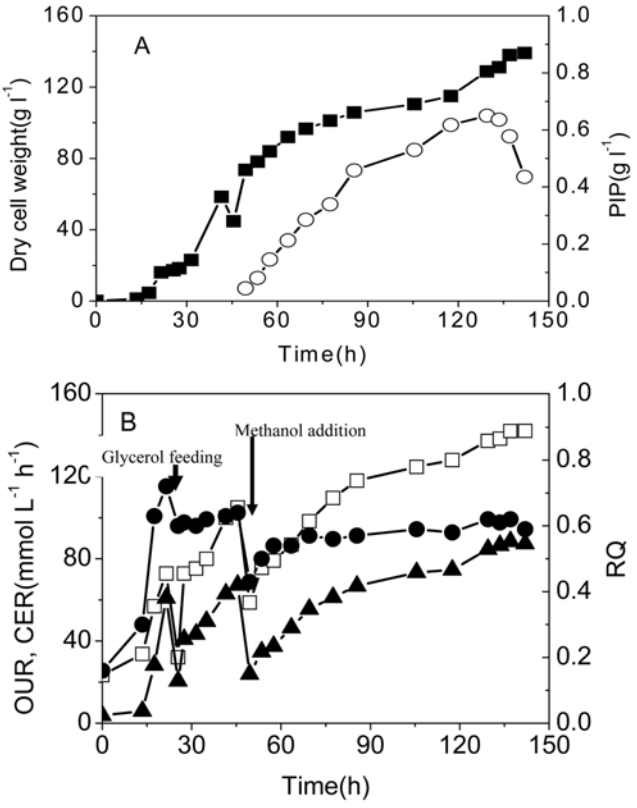

Fig. 1. Typical time course of fed-batch culture of *P. pastoris*. A three-phase fermentation protocol (glycerol batch, glycerol fed-batch, methanol fed-batch) was used. It was run at following conditions: pH and temperature were controlled at 5.0 and 30 °C, respectively, and DO was maintained at 25% with agitation speed and airflow rate (1-2 VVM).
A: Time course of dry cell weight (closed square) and PIP yield (open circle); B: Bioprocess profile of physiological parameters oxygen uptake rate (OUR, open square), carbon dioxide evolution rate (CER, closed triangle) and respiratory quotient (RQ, closed circle).

ponential growth occurred from 1 h to 22 h of cultivation, was observed from off-line parameter DCW (dry cell weight) and on-line parameters OUR (oxygen uptake rate) and CER (carbon dioxide evolution rate). Meanwhile, respiratory quotient (RQ) displayed an approximately linear trend, and its peak value was around 0.72. Then, glycerol fed-batch was initiated by glycerol addition until DCW reached around 80 g l^{-1}. During this stage DCW, OUR and CER increased rapidly; however, RQ maintained constant at 0.62±0.05 due to the adoption of limiting glycerol feeding strategy. Cell biomass yield ($Y_{x/s}$) on glycerol at this stage was 0.59 g g^{-1}, which was higher than that of 0.45 g g^{-1} at glycerol batch stage. At the third stage with methanol addition, cell optical density continuously increased accompanying the rise of OUR and CER. The highest OUR and CER values of 142.08 mmol l^{-1} h^{-1} and 88.47 mmol l^{-1} h^{-1} were achieved at 135 h of culture (Fig. 1B), respectively, which were 35% and 32% higher than those at the glycerol feed stage. This phenomenon has not been reported before, to our knowledge; the possible explanation is that it was caused by high foreign gene dosage (6-8 copies PIP). RQ was found to keep around 0.55±0.05, and PIP yield was approximately coincident with high cell density, increased almost linearly to 0.6 g l^{-1} at 135 h, followed by rapid decline, which was likely due to cell lysis and protease degradation [22].

2. Cell Growth Modeling in PIP Induction Phase

Owing to methanol limitation and constant RQ in PIP production phase (Fig. 1B), the initial phase of induction was assumed to belong to a pseudo-stationary phase. As described in literature published earlier [13,19], *Pichia* cell growth followed a Monod equation under methanol concentration limitation. Therefore, the Monod equation can be expressed as follows (Eq. (5)).

$$\frac{1}{\mu} = \frac{K_s}{\mu_{max}} \times \frac{1}{S} + \frac{1}{\mu_{max}} \tag{5}$$

Based on typical time course of fermentation at different methanol feed rates, specific growth rate (μ) was calculated to be 0.154 h^{-1} and the average residual methanol concentration (S) throughout the fermentation was 3.05 g l^{-1}. The parameters μ_{max} and K_s were estimated to be 0.101 h^{-1} and 0.252 g l^{-1}, respectively, by using linear regression. A linear regression coefficient (R^2) of 0.96 indicated that the kinetics of cell growth fitted Monod model well.

3. Calculation of Kinetics Parameters in PIP Production Phase

According to substrate mass balance, specific rates were expressed as follows (Eq. (6)):

$$q_s = \frac{\mu}{Y_{X/S}} + m + q_P \tag{6}$$

q_p can be negligible due to very low percentage of methanol substrate being used to synthesize PIP, so Eq. (6) was written:

$$q_s = \frac{\mu}{Y_{X/S}} + m_P \tag{7}$$

q_s obtained at different μ was plotted in Fig. 2. The biomass yield on methanol ($Y_{X/S}$) of 0.386 g g^{-1} and maintenance coefficient (m) of 0.011 g g^{-1} h^{-1} were calculated, respectively, which were higher than 0.306 g g^{-1} and 0.0226 g g^{-1} h^{-1} reported by País [23]. However, the relationship between specific PIP production rate (q_p) and specific growth rate (μ), which was our greatest concern in developing an optimal feeding strategy, is depicted in Fig. 3. The highest $q_{p.max}$ with 0.098 mg g^{-1} h^{-1} was achieved at specific growth rate of

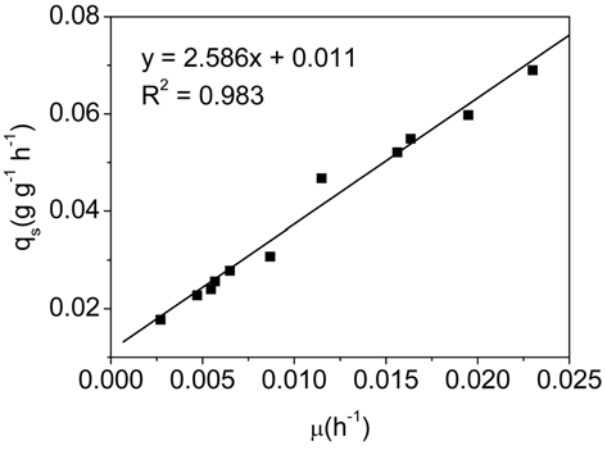

Fig. 2. Linear relationship between specific substrate consumption rate (q_s) and specific growth rate (μ).

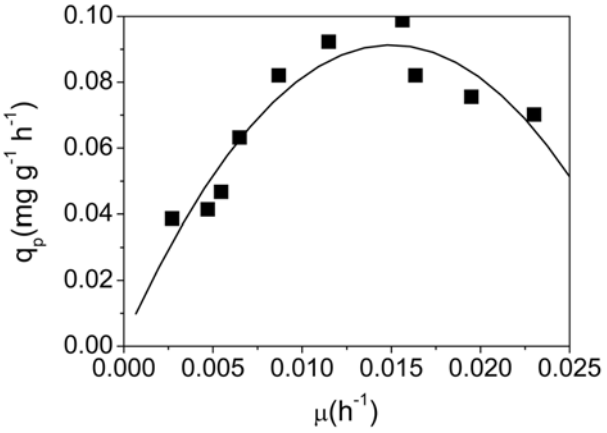

Fig. 3. Relationship between specific PIP formation rate (q_p) (closed square) and specific growth rate (μ) of *P. pastoris* in the methanol induction phase. The trend of specific PIP formation rate was simulated at different specific growth rate (μ), which was displayed as solid line.

$0.016\ h^{-1}$, which was in accordance with that reported earlier [23], while less than optimal μ of $0.0267\ h^{-1}$ [13]. As a result, the optimal μ could vary with different *Pichia* expression systems, which would be related to heterologous protein property, host phenotype and gene dosage, etc. [24]. To attain the highest PIP production level, theoretically, μ should be controlled at $0.016\ h^{-1}$ in methanol induction phase.

4. Validation of the Model

Two experiments with different initial cell densities (58.8 g l^{-1} and 68.6 g l^{-1}) were performed to validate the model in methanol induction phase. μ was approximately maintained at $0.016\ h^{-1}$ by using the methanol exponential feeding strategy. In theory, cell density and PIP concentration were predicted by the integration of Eqs. (1) and (3), respectively, and then compared to the experimental results. As shown in Fig. 4, the actual biomass values and PIP yield were in agreement with their corresponding calculated values with exponential methanol feed mode. The peak PIP concentration, 0.97 g l^{-1} and 0.85 g l^{-1} was achieved with different initial cell densities of 58.8 g l^{-1} and 68.6 g l^{-1} (Fig. 4A), respectively, which were approximately 1.5 times as high as that of the control. In fact, PIP yield, 5.7 mg g^{-1} obtained with initial biomass of 68.6 g l^{-1} was slightly lower than that, 6.6 mg g^{-1} with 58.8 g l^{-1} initial biomass. The fact that cell growth rate at late PIP production stage (50-60 h of methanol induction) was lower than the estimated values (Fig. 4B) was due to the deteriorated culture environment such as protease degradation, sensing quorum, and accumulation of toxic by-products like ethanol [25]. Since all unfavorable conditions mentioned were not considered in the present unstructured model, higher PIP yield could be attained if the proposed model was modified further. Although there were a few drawbacks of the Monod model developed, the PIP yield achieved and PIP yield per gram biomass were approximately 2.5 and 6 folds as compared to the reported results [23]. Therefore, the simple unstructured Monod model was proved to be a robust control system for recombinant porcine insulin precursor (PIP) production by *Pichia*.

Interestingly, it was found that PIP production was 13.4% higher

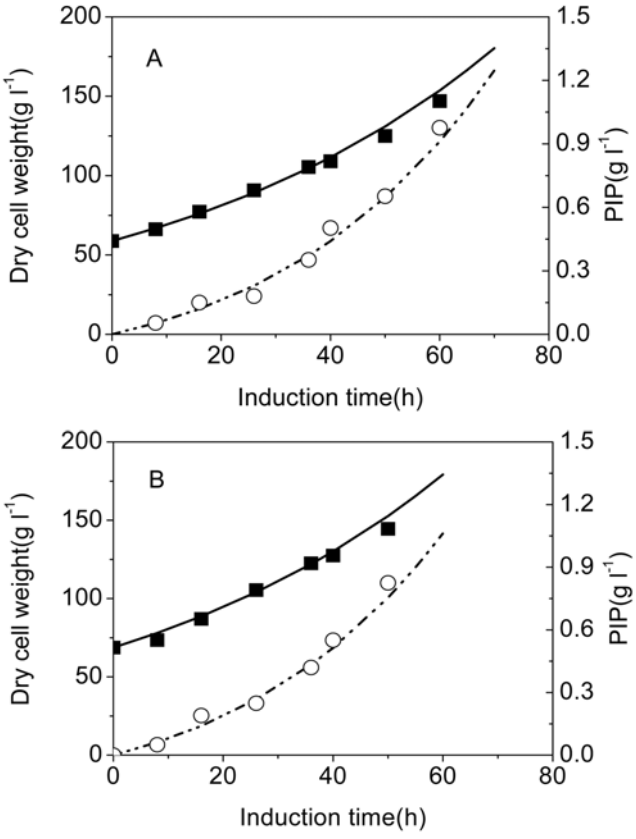

Fig. 4. Comparison of actual values of biomass and PIP yield and their corresponding predicted values (predicted dry cell weight, solid line; predicted PIP yield, dash dot dot line), which were estimated by the cell growth kinetic model with different initiation cell densities, respectively.
A: Profile of biomass (measured dry cell weight, closed square) and PIP yield (measured concentration, open circle) at induction-phase with initiation cell mass of 58.8 g l^{-1}; B: Profile of biomass (the measured dry cell weight, closed square) and PIP yield (the measured concentration, open circle) at induction-phase with initiation cell mass of 68.6 g l^{-1}.

and final cell density was 14.7% lower with lower initial cell density than that with higher initial cell density, which indicated that the control of specific growth rate was an effective way for foreign protein yield enhancement [15] and high cell density strategy was not always effective for the improvement of target protein production.

CONCLUSION

An unstructured Monod model was developed for recombinant porcine insulin precursor production by *P. pastoris* GS115/PIP based on the fact that *Pichia* cell growth follows a Monod equation under methanol concentration limitation. The parameters μ_{max} and K_s were estimated to be $0.101\ h^{-1}$ and 0.252 g l^{-1}, respectively, with a linear regression coefficient (R^2) of 0.96 with the linear regression method. To attain the highest PIP level, the optimal specific growth rate of $0.016\ h^{-1}$ for the methanol fed-batch phase was estimated according to the relationship between specific production rate (q_p) and specific growth rate (μ). It was then validated in methanol induction phase with different initial cell densities, and the peak PIP yield achieved

was 0.97 g l^{-1} with initial cell density of 58.8 g l^{-1} at methanol fed-batch phase, which was 1.5 and 2.5 times higher than those unoptimized and the earlier reported result, respectively.

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NOMENCLATURE

DCW: dry cell weight [g l^{-1}]
K_s : saturation constant [g l^{-1}]
m : maintenance coefficient [g MeOH g^{-1} DCW h^{-1}]
MeOH : methanol
P : PIP concentration [g l^{-1}]
q_s : specific methanol consumption rate [g g^{-1} h^{-1}]
q_p : specific PIP production rate [g g^{-1} h^{-1}]
R_{MeOH} : methanol feed rate [ml h^{-1}]
t : induction time [h]
V : broth volume [l]
$Y_{X/S}$: biomass yield on methanol [g DCW g^{-1} MeOH]
$Y_{P/X}$: PIP yield on biomass [mg protein g^{-1}]
μ : specific growth rate [h^{-1}]
μ_{max} : maximum specific growth rate [h^{-1}]
ρ : methanol density [g l^{-1}]

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