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Development of simple kinetic models and parameter estimation for simulation of recombinant human serum albumin production by *Pichia pastoris*

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In order to describe and predict the growth and expression of recombinant proteins by using a genetically modified *Pichia pastoris*, we developed a number of unstructured models based on growth kinetic equation, fed-batch mass balance and the assumptions of constant cell and protein yields. The growth of *P. pastoris* on both glycerol and methanol could be represented by Monod kinetic equation. A simple simulation methodology and developed models were shown to satisfactorily describe both growth and production of recombinant human serum albumin (rHSA) using a genetically modified *P. pastoris* Mut^S strain. The obtained parameters from curve fitting were reasonable and could be acceptable. Moreover, the same parameter sets obtained by the experiments indicated the rigidity and consistency of the developed models and fermentation approach of this study. With correlation coefficients (r^2) exceeding 0.99, the models were able to simulate and predict the cell growth behavior and recombinant protein production by *P. pastoris* without requiring complex models.

Key words: Exponential feed, growth modeling, Monod kinetic equation, *Pichia pastoris*, recombinant human serum albumin.

INTRODUCTION

Pichia pastoris is a methylotrophic yeast which has been successfully used for the heterologous expression of a great number of recombinant proteins. The popularity of *P. pastoris* as a host for the production of recombinant

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proteins is due to its several inherent advantages as described in many reviews (Çelik and Çalik, 2012; Gao and Shi, 2013; Gonçalves et al., 2013; Vogl et al., 2013; Fickers, 2014; Byrne, 2015; Çalik et al., 2015; Looser et al., 2015). By combination of powerful genetic techniques, the ability of protein expression and recombinant protein purification at a comparatively low cost, therefore *P. pastoris* is made as a promising system for recombinant protein production. In recombinant protein production, increasing expression and productivity are desired. Therefore, a number of strategies have been employed to increase expression of the recombinant proteins in question, for example, optimizing the fermentation medium (Jungo et al., 2007b; Batista et al., 2013), improving the fermentation method (Bushell et al., 2003; Ohya et al., 2005), different feeding strategies (Sinha et al., 2003; Hu et al., 2007), mixed-substrate feeding (d'Anjou and Daugulis, 2000; Jungo et al., 2007a; Arnau et al., 2011; Zalai et al., 2012) and different oxygen supplementation strategies (Charoenrat et al., 2005; Zhang et al., 2005; Charoenrat et al., 2006). Modeling is another approach without more empirical experiments enhancing and optimizing the production of recombinant proteins. This approach can be successfully applied to describe growth behavior and is used to enhance the productivity of recombinant proteins from both *Escherichia coli* (Baheri et al., 1997) and *Saccharomyces cerevisiae* (Hardjito et al., 1992; Patkar et al., 1993). The modeling is also used for developing an improved fermentation protocol for recombinant *P. pastoris* systems (d'Anjou and Daugulis, 1997). Many model approaches are proposed such as model-based feeding strategy based on mass balance (d'Anjou and Daugulis, 1997; d'Anjou and Daugulis, 2001), macrokinetic modeling based on stoichiometric balance (Ren et al., 2003), model-based specific growth rate (Ren and Yuan, 2005), mix-feed modeling based on growth kinetic (Zhang et al., 2003), growth and protein production modeling based on metabolic flux and metabolic model (Jahic et al., 2002; Sohn et al., 2010; Nocon et al., 2014), growth model optimization using response surface methodology (RSM) (Zhang et al., 2004; Yu et al., 2014) and artificial neural networks (ANNs), fuzzy rule-based systems, or a combination of both (Jenzsch et al., 2006). On these studies, the majorities are complex and require a complicated technical knowledge. However, simple simulation of recombinant microorganisms can be taken by kinetic modeling with the aid of computer software (Vinayagam et al., 2015).

The production of recombinant proteins is normally performed in three-stage fermentation: Batch, fed-batch and induction stage (Çelik and Çalik, 2012; Potvin et al., 2012; Looser et al., 2015). The first stage is batch fermentation where *P. pastoris* is cultured with glycerol. After the initial glycerol is depleted, the glycerol is added to the culture in order to reach high cell density in the second stage. The third stage is the induction stage where expression of the recombinant proteins is induced by methanol.

In this study, the unstructured models based on growth kinetic equation, fed-batch mass balance and constancy of cell and protein yields were developed and constructed following the substrates, glycerol and methanol. The growth model on glycerol is mostly published while the growth model on methanol is rarely due to cell growth of Mut^S strain on this medium, which is very low so is neglected in the model (d'Anjou and Daugulis, 1997). Moreover, in mixed-substrate feeding, the growth on methanol is unnecessary to differentiate from growth on glycerol (d'Anjou and Daugulis, 2001). Therefore, this study proposed the model of growth not only on glycerol but also on methanol. Furthermore, a simple simulation methodology to investigate the behavior of growth and protein production of recombinant microorganisms was also introduced. A Mut^S *P. pastoris* KM71 strain genetically modified to produce and secrete human serum albumin (HSA), a major protein component of human blood plasma, was used as a model for mathematical model development. These models and parameters obtained by simulation methodology could be used as a tool to inspect the recombinant *P. pastoris* fermentation.

MATERIALS AND METHODS

Microorganism

Genetically modified *P. pastoris* KM71 capable of expressing and secreting HSA was used in all experiments. The *P. pastoris* clone created by inserting the coding DNA sequence for mature full length HSA into the expression vector pPICZαA and then integrating into the genome of *P. pastoris* was provided by Dr. Witoon Tirasophon, Mahidol University, Thailand.

Medium

Yeast extract peptone dextrose (YPD) medium contained 10 g yeast extract, 20 g peptone and 20 g dextrose per liter of deionized water. Basal salt medium (BSM) contained 26.7 ml 85% H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4.7H_20$, 4.13 g KOH, 50.0 g glycerol and 6.7 ml PTM1 trace salt in deionized water made up to a total volume of 1 L. The PTM1 trace salt contained 0.5 g $CoCl_2.6H_2O$, 20.0 g $ZnCl_2$, 65 g $FeSO_4.7H_20$, 6.0 g $CuSO_4.5H_2O$, 3.0 g $MnSO_4.H_2O$, 0.1 g KI, 0.2 g $Na_2MoO_4.2H_2O$, 0.02 g H_3BO_3, 5.0 ml H_2SO_4 and 0.2 g biotin in deionized water made up to a total volume of 1 L. The PTM1 trace salt was sterilized by filtration.

Inoculum preparation

P. pastoris stored at -80°C was used to inoculate a starter culture in YPD medium which was subsequently incubated at 30°C and 250 rpm. The starter culture was then used to inoculate 100 ml BSM which was continuously incubated at the aforementioned condition until reaching an OD_{600} of 20 (total OD_{600}). The BSM inocula were then transferred aseptically to 7 L of BSM (working volume) in a 15 L bioreactor (BIOSTAT C, B. Braun Biotech International, Melsungen, Germany). The volume of inocula used in all

experiments was 10% of the working volume of the bioreactor.

Batch fermentation

During the batch fermentation stage, the temperature was set at 30°C and pH maintained at 5.00 by the addition of 25% NH_4OH and 85% H_3PO_4. Dissolved oxygen (DO) was kept above 20% saturation by using cascaded control of agitation. Aeration was supplied at 2 vvm and the air was supplemented and mixed with pure oxygen if the stirrer could not maintain the oxygen levels at the set value. Foaming was monitored by an antifoam probe and antifoam (Antifoam 204, Sigma, Deisenhofen, Germany) was added to the culture in order to prevent excessive foaming during fermentation.

Fed-batch fermentation

After depletion of initial glycerol in the BSM, the glycerol feed medium (50% w/v glycerol in water with 15 ml/l PTM1) was fed according to a predetermined exponential feeding rate described by d'Anjou and Daugulis (1997) and Jahic et al. (2002) until the cell concentration reached 100 g-DCW/l. The conditions were set to the same values as those during the batch fermentation stage.

Protein induction

After reaching the predetermined cell density (100 g-DCW/l), glycerol feed was discontinued and the culture was left for a 4-hour starvation period. Methanol with the addition of 15 ml/l PTM1 was then fed into the bioreactor in order to induce rHSA expression. The initial pulse of methanol was first fed into the bioreactor to a concentration of 4 g-methanol/l (Trinh et al., 2003; Looser et al., 2015) and left for 4 h before the continuous feeding strategies commenced. The temperature was set to 22°C (Jahic et al., 2003; Wu et al., 2012; Gao and Shi, 2013; Gonçalves et al., 2013; Anasontzis and Penã, 2014; Yu et al., 2014) and pH 6.00 (Kobayashi et al., 2000) during the induction phase.

Analysis methods

Samples were taken 5 ml at 6-hourly intervals during both the batch and fed-batch phases and every 24 h during the induction phase. The samples were centrifuged at 9000 rpm (RCF = 9055∗g), 4°C for 5 min and the supernatant was collected for further analysis. Cell concentrations were determined by measuring OD_{600} and then converted to dry cell weight by a correlation of $0.323 \times OD_{600}$ (r^2 = 0.998). Glycerol and methanol concentrations in the medium were analyzed by HPLC (Shimadzu Ltd., Tokyo, Japan) using an Aminex HPX-87H Ion Exclusion Column (Bio Rad) with 0.5 mM sulfuric acid as mobile phase and a flow rate of 0.6 ml/min at 45°C. A refractive index detector was utilized for detection. Total protein concentration in the medium was analyzed by Bradford assay (Suwannarat et al., 2013). The amount of rHSA protein was calculated from the band density, which obtained from SDS-PAGE analysis using 12% gels (according to standard protocols) stained with Imperial™ Protein Stain (Thermo Fisher Scientific), and compared to standard HSA of known concentration using Gene Tools program version 3.06.02. Western blot analysis was performed in order to verify the expression of rHSA protein and the identity of the rHSA bands.

Model development

The models describing the fermentation process were constructed by mass balance on biomass, substrate concentration, recombinant protein production and system volume. The *P. pastoris* KM71 strain used in this study was designated Mut^S which indicated the ability to grow on methanol as well as glycerol. Methanol could be also utilized as an inducer for the expression of recombinant proteins by the *AOX1* promoter (Trinh et al., 2003). The mass balance equations used in this study were described as follows:

$$\frac{dX}{dt} = \mu X \quad (1)$$

$$\frac{dS}{dt} = \frac{F_S}{V} S_0 - \frac{\mu}{Y_{X/S}} X \quad (2)$$

$$\frac{dM}{dt} = \frac{F_M}{V} M_0 - \frac{\mu_M}{Y_{X/M}} X \quad (3)$$

$$\frac{dP}{dt} = \mu_M Y_{P/X} X \quad (4)$$

Where X is biomass concentration in dry weight (g-DCW/l), V is the medium volume (l), F_S is glycerol feed rate (l/h), F_M is methanol feed rate (l/h), S is the substrate (glycerol) concentration (g-glycerol/l), S_0 is the substrate (glycerol) concentration in inlet feed (g-glycerol/l), M is the methanol concentration (g-methanol/l), M_0 is the methanol concentration in inlet feed (g-methanol/l), P is the concentration of recombinant protein secreted into the medium (g-protein/l), $Y_{X/S}$ is the yield coefficient biomass per substrate, glycerol (g-DCW/g-glycerol), $Y_{X/M}$ is the yield coefficient biomass per substrate, methanol (g-DCW/g-methanol), $Y_{P/X}$ is the yield coefficient protein per biomass (g-protein/g-DCW), μ is the specific growth rate on glycerol (h^{-1}), μ_M is the specific growth rate on methanol (h^{-1}) and t is the run time (h).

Monod kinetic equation is widely used for describing the growth of microorganisms and was therefore used to describe the specific growth rate of *P. pastoris* on glycerol (μ) in this study as shown by Equation 5. Methanol was the only energy and carbon source during the induction stage, consequently the specific growth rate on methanol (μ_M) based on the Monod equation could be described as shown in Equation 6 (Curvers et al., 2001):

$$\mu = \frac{\mu_{\max} S}{S + K_S} \quad (5)$$

$$\mu_M = \frac{\mu_{M\max} M}{M + K_M} \quad (6)$$

Where K_S is the Monod saturation constant on glycerol (g-glycerol/l), K_M is the Monod saturation constant on methanol (g-methanol/l), $\mu_{\max}$ is the maximum specific growth rate on glycerol (h^{-1}) and $\mu_{M\max}$ is the maximum specific growth rate on methanol (h^{-1}).

In order to prolong cell growth and attain higher cell density during the fed-batch stage, glycerol was fed exponentially according to Equation 7 to achieve exponential cell growth as

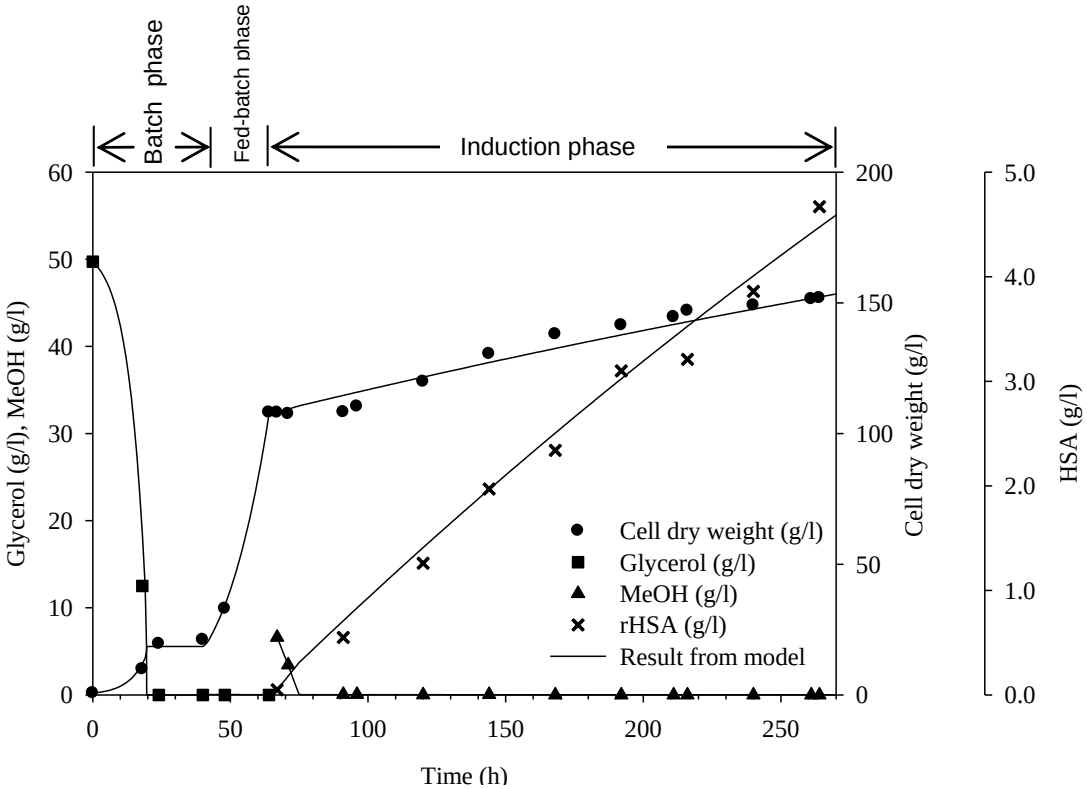

Figure 1. The growth behavior of Mut^S *P. pastoris* KM71 when producing rHSA in RUN 1 experiment. Symbols represent measured data and lines represent simulation. ■ , Glycerol; ● , yeast biomass; ▲, methanol; ×, rHSA

described by d'Anjou and Daugulis (1997) and Jahic, et al. (2002). Equation 8 was used to calculate the yeast cell concentration during the feed batch fermentation and the time required to reach the predetermined final cell concentration during the fed batch fermentation.

$$F_S = \frac{\mu_{set}}{S_0 Y_{X/S}} X_0 V_0 e^{\mu_{set}(t-t_0)} \quad (7)$$

$$X = \frac{X_0 V_0}{V} e^{\mu_{set}(t-t_0)} \quad (8)$$

Where X_0 is biomass concentration in dry weight when the exponential feeding is initiated (g-DCW/l), V_0 is the medium volume when the exponential feeding is initiated (l), μ_{set} is the specific growth rate set point (h^{-1}) and t_0 is the time when the exponential feeding is initiated (h).

A continuous feed pattern was chosen for the methanol feed during the induction stage. The methanol feed rate was calculated based on biomass and the methanol consumption rate according to Equation 9.

$$F_M = \frac{q_M X V}{M_0} \quad (9)$$

Where q_M is specific methanol consumption rate (g-methanol/g-DCW·h).

To solve these differential equations when growing on glycerol and methanol, cell yield, protein yield and the specific methanol consumption rate were assumed to be constant. The models represented as these equations were coded and computed using instructions in Berkeley Madonna program version 9.0.118.

RESULTS AND DISCUSSION

The experiments were performed in a 15 L bioreactor and were initiated as batch fermentation with a working volume of 7 L. In Run 1 experiment, as shown in Figure 1, the yeast biomass increased from 0.67 g-DCW/l at the time of inoculation to 21.04 g-DCW/l at the end of the batch fermentation. During this time period, glycerol was metabolized and was completely consumed within 40 h, decreasing from 49.72 g-glycerol/l. After the initial glycerol in the BSM had been depleted during the batch fermentation stage (at the 40th hour), the subsequent fed-batch stage started by feeding additional glycerol into the bioreactor, thereby prolonging the growth phase of *P. pastoris* and increasing cell density. In order to achieve an exponential growth rate, the feed pattern for the addition of glycerol was calculated using Equation 7. The

Table 1. The predetermined parameters used in simulation of recombinant Mut^S *P. pastoris* KM71 fermentation.

Parameter	Run 1	Run 2
Cell concentration at starting batch culture (g-DCW/l)	0.67	0.59
Glycerol concentration at starting batch culture (g-glycerol/l)	49.72	56.47
Medium volume at initial feeding, V_0 (l)	7.0	7.0
Glycerol concentration in inlet feed, S_0 (g-glycerol/l)	501.67	505.08
Methanol concentration in inlet feed, M_0 (g-methanol/l)	778.15	777.90
Initial feeding time, t_0 (h)	40	40
Specific growth rate set point, μ_{set} (h^{-1})	0.08	0.08
Cell concentration at initial feeding, X_0 (g-DCW/l)	21.04	20.64

parameters used in Equation 7 were obtained from an experiment as shown in Table 1. The yield coefficient on glycerol ($Y_{X/S}$) in Equation 7 was obtained from the change of glycerol and cell concentration over time in batch stage by estimation with the curve fitting function in Berkeley Madonna program version 9.0.118. The $Y_{X/S}$ of *P. pastoris* used in this study was 0.36 g-DCW/g-glycerol. During the exponential feed in fed-batch stage, the μ_{set} (Equation 7) was set at 0.08 h^{-1} (Jenzsch et al., 2006; Suwannarat et al., 2013) to ensure that metabolic overflow would be avoided (Looser et al., 2015). The duration of the exponential feed and the time needed to achieve a certain cell concentration could be calculated using Equation 8, the composed term in Equation 7 representing cell concentration. The cell density targeted in the fed-batch stage was set at 100 g-DCW/l, which achieved in 24 h. In order to activate the *AOX1* promoter and induce expression of rHSA, the induction phase was initiated by the addition of methanol at 4 g-methanol/l (Trinh et al., 2003). An initial pulse of methanol was first given to acclimatize the cells to metabolize methanol. Not only the inducing chemical, methanol is also poisonous to the *P. pastoris* cells because of the accumulation of formaldehyde and hydrogen peroxide, the products of methanol metabolism, inside the cells if it exists at a high concentration (Khatri and Hoffmann, 2006). However, a low methanol concentration is inadequate for protein expression (Gonçalves et al., 2013). Thus, the optimum amount of methanol should be regulated strictly (Potvin et al., 2012). For model development in this study, the continuous methanol feed based on the specific substrate uptake rate was selected (Dietzsch et al., 2011a). Four hours after the initial pulse feed, the continuous methanol feed was initiated and methanol was added to the culture at a rate equal to the specific methanol consumption rate (q_M) of the *P. pastoris* strain used in this study. The q_M had previously been determined by monitoring both methanol and cell concentrations over time in fermentations with constant methanol feed. The data obtained in those experiments gave a q_M of 0.026 g-methanol/g-DCW·h by calculation based on fed-batch mass balance. This value was similar to the study by Dietzsch et al. (2011a, b) in *P. pastoris* Mut^S KM71H strain. The medium volume was drained daily to maintain a constant at 7 L in order to avoid exceeding the capacity of the bioreactor. After the initial methanol pulse, the methanol concentration decreased during the induction phase until the residual methanol was undetectable by HPLC even though methanol was continuously added into the bioreactor according to the calculated feed rate. This was due to limitations of the pump belonging to the bioreactor and in order to avoid accumulation of excess methanol in the bioreactor, the setting had to be slightly lower than the calculation. As shown in Figure 1, the amount of rHSA (× symbol) increased during the induction phase with this methanol feed strategy. Methanol was not present while the methanol feed was operating which was an indication that yeast cells consumed methanol residuals at the feed rate added in (constant q_M, 0.026 g-methanol/g-DCW·h).

In the simulation of *P. pastoris* KM71, Equation 1 to 4 describing the yeast behavior in growth, substrate utilization and recombinant protein production were derived by fed-batch mass balance. The growth kinetic when growing on glycerol (μ) was explained by Monod kinetic equation, shown in Equation 5. In the fed-batch stage, the exponential glycerol feed pattern could be calculated by Equation 7, the cell density and length of the fed-batch stage could be calculated by Equation 8, as described previously. For the induction stage, methanol was the only energy source added into the bioreactor for *P. pastoris*, therefore, the growth of yeast also depended on methanol only. As with the growth on glycerol, Monod kinetic equation was also used for the growth on methanol due to its simplicity and the fact that it did not require complicated parameters. Hence, the kinetic growth on methanol (μ_M) was introduced by Equation 6. During the induction period, the methanol was continuously fed after the initial methanol pulse at 4 g-methanol/l for 4 h. The methanol feed (F_M) presented by Equation 9 was continuously added with constant q_M as described previously. The models represented as these

Table 2. The obtained parameters from simulation of recombinant Mut^S *P. pastoris* KM71 fermentation.

Parameter	Values
Maximum specific growth rate on glycerol, μ_{max} (h^{-1})	0.16
Maximum specific growth rate on methanol, $\mu_{M\,max}$ (h^{-1})	0.003
Yield coefficient biomass per substrate (glycerol), $Y_{X/S}$ (g-DCW/g-glycerol)	0.36
Yield coefficient biomass per substrate (methanol), $Y_{X/M}$ (g-DCW/g-methanol)	0.09
Yield coefficient protein per biomass, $Y_{P/X}$ (g-protein/g-DCW)	0.10
Monod saturation constant on glycerol, K_S (g-glycerol/l)	0.04
Monod saturation constant on methanol K_M (g-methanol/l)	0.01

equations were coded and computed using instructions in Berkeley Madonna program version 9.0.118. The parameters used in simulation are shown in Tables 1 and 2. The parameters in Table 1 were obtained from experimental measurements. As for Table 2, the parameters were obtained using the curve fitter feature in Berkeley Madonna program. The simulation and fermentation results of RUN 1 experiment are shown in Figure 1 where the symbols represent data from the experiment and the lines are derived from the simulation results. The results showed accordance of developed models and that the experiment fit very well. In the induction stage, when methanol was added, it seemed that the yeast had a lag growth due to the effect of diauxic growth where the yeast cell accommodated to methanol. This effect was ignored by the models because of the simplicity of the objective in model development; however, the r^2 between the models and the experimental results exceeded 0.99 in all data sets. In the RUN 1 experiment (Figure 1) the highest concentration of secreted rHSA present in the medium was 4.67 g/l, which occurred after 264 h (197 h of induction) with a simultaneous cell concentration of 151.81 g-DCW/l. The secreted rHSA produced by *P. pastoris* was analyzed by SDS-PAGE and showed the same molecular size as standard HSA (67 kDa), as shown in Figure 2a. By comparing band densities, the rHSA quantity was analyzed with known concentration of standard HSA using Gene Tools program version 3.06.02. Furthermore, the rHSA was also verified with the Western blot analysis and it showed specific binding with antibody as same as standard HSA (Figure 2b).

After 264 h (197 h of induction), the cells entered a stationary phase (data not shown), most likely due to the exhaustion of some essential medium components (d'Anjou and Daugulis, 2000) and/or the accumulation of some metabolites in the medium. The observed decrease in cell growth lowered methanol metabolism, which consequently resulted in the accumulation of excess methanol. It was likely, therefore, that methanol concentration in the bioreactor increased while, simultaneously, the production of rHSA decreased. The experiment was continuously operated until the cell concentration started to decrease. A potential explanation for the decline in cell concentration at these extended durations could be a slower growth rate and methanol consumption combined with a maintained methanol feed resulting in a simultaneous decrease of both cells and protein concentration due to dilution.

When the parameters obtained from this study were compared with the parameters previously reported by other researchers then a number of observations could be made. In d'Anjou and Daugulis (1997, 2000, 2001) studies, they calculated μ_{max} and $\mu_{M\,max}$ for a Mut^S GS115 strain of *P. pastoris* producing and secreting raven anti-freeze protein (SR-AFP) between 0.25 and 0.27 h^{-1} and 0.01 and 0.04 h^{-1}, respectively, where both exceed the values obtained in our experiments (0.16 and 0.003 h^{-1}, respectively). In the review by Looser et al. (2015), μ_{max} of *P. pastoris* GS115 strain were normally in range of 0.20 to 0.29 h^{-1} in all recombinant protein expressions but μ_{max} of *P. pastoris* KM71 strain was not reported. However, μ_{max} of *P. pastoris* Mut$^+$ strain expressing the heavy-chain fragment C of botulinum neurotoxin serotype C (BoNT/C(Hc)) intracellularly as determined by Zhang et al. (2000, 2003) was 0.177 h^{-1}. In addition, μ_{max} determined by Hang et al. (2009) in *P. pastoris* GS115-YY113 with phenotype of Mut^S strain expressing phytase and Cos et al. (2005) in *P. pastoris* X-33 both Mut$^+$ and Mut^S strains expressing lipase were 0.18 h^{-1} which corresponded more closely with the value reported here. For this parameter, the μ_{max} could be compared across the methanol utilization phenotype strains of *P. pastoris* (Looser et al., 2015) because this parameter did not depend on methanol utilization phenotype strains, it commonly presented in any *P. pastoris* strains grew on glycerol. In the review by Looser et al. (2015), $\mu_{M\,max}$ of *P. pastoris* in Mut^S strains in any protein expressions collected were in the range of 0.011 to 0.03 h^{-1} which were similar to d'Anjou and Daugulis (1997, 2000, 2001) studies, however, these values were obtained at 30°C induction temperature which is higher

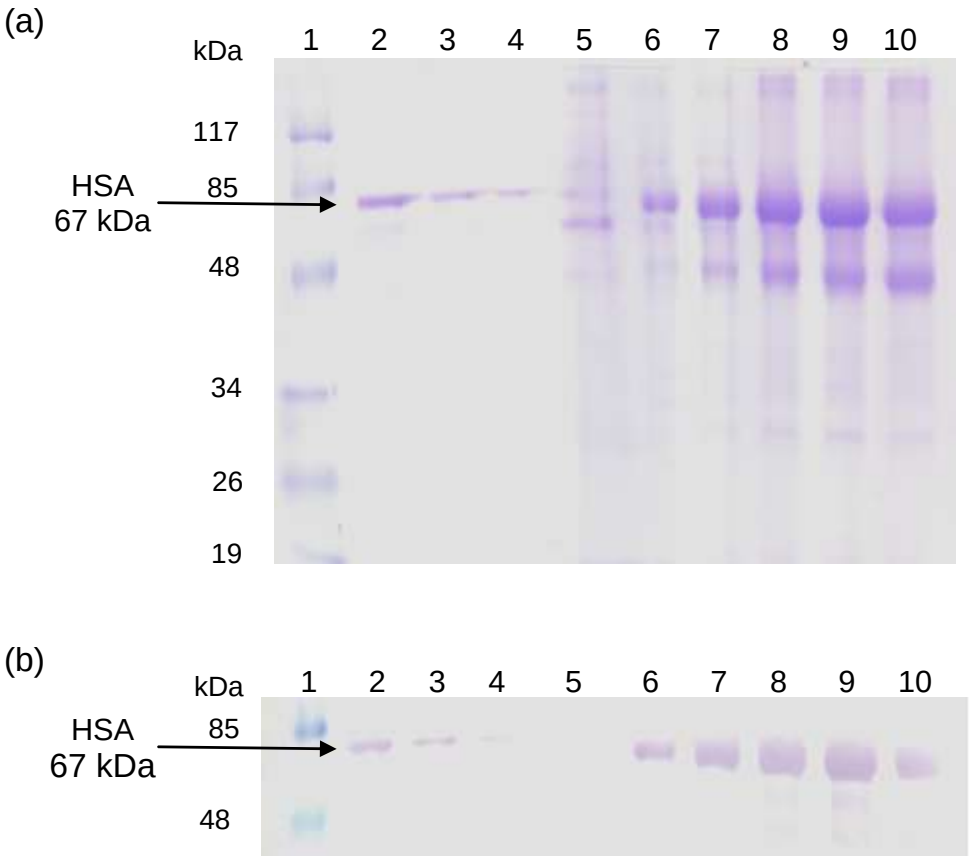

Figure 2. The analysis of rHSA produced by Mut^S *P. pastoris* KM71. (a) The SDS-PAGE analysis at different time of induction. Lane 1, molecular marker; Lane 2, standard HSA (67 kDa) at 0.0504 g/l; Lane 3, standard HSA at 0.0252 g/l; Lane 4, standard HSA at 0.0126 g/l; Lane 5, 0th h; Lane 6, 50th h; Lane 7, 122nd h; Lane 8, 194th h; Lane 9, 271st h; Lane 10, 311st h. (b) The Western blot analysis of SDS-PAGE.

than that of this study (22°C). Therefore, this parameter ($\mu_{M\max}$) could be expected to be larger than that of this study (Jahic et al., 2003). In Zhang et al. (2003) study, $\mu_{\max}$ (0.177 h^{-1}) was similar to this study; they also measured $\mu_{M\max}$ in Mut^S strain of *P. pastoris* GS115 expressing BoNT/C(Hc) at 0.008 h^{-1} which was also closer to the results here (0.003 h^{-1}) than other researchers, but the difference was still obvious. An explanation for the discrepancy could be that the induction stage in this study was performed at 22°C which is lower than the 30°C used in their publication and consequently causes slower growth rates (Curvers et al., 2001; Jahic et al., 2003) corresponding to Arrhenius plot of the growth rate (Jahic et al., 2003). Moreover, the difference in $\mu_{M\max}$ might be due to the use of different strains and the expression of different recombinant proteins. In addition, the cell yield on glycerol ($Y_{X/S}$) and methanol ($Y_{X/M}$) of *P. pastoris* Mut^S strain as described by d'Anjou and Daugulis (1997, 2000, 2001) were between 0.40 and 0.45 g-DCW/g-glycerol and 0.61 and 1.73 g-DCW/g-methanol, respectively. In the review by Looser et al. (2015), $Y_{X/S}$ and $Y_{X/M}$ of *P. pastoris* Mut^S strain were 0.5 to 0.619 g-DCW/g-glycerol and 0.3 g-DCW/g-methanol, respectively. The $Y_{X/S}$, obtained in the current study (0.36 g-DCW/g-glycerol) was fairly close to d'Anjou and Daugulis (1997, 2000, 2001) but $Y_{X/M}$ (0.09 g-DCW/g-methanol) was lower than theirs. It was not strange that the $\mu_{M\max}$ and $Y_{X/M}$ obtained (0.003 and 0.09 g-DCW/g-methanol, respectively) were low as well, because the recombinant protein induction was performed at a lower temperature. Monod saturation constant on glycerol (K_S) calculated in this study was 0.04 g-glycerol/l which differed from the obtained value (0.005 g-glycerol/l) by d'Anjou and Daugulis (1997, 2000) Finally, Monod saturation constant obtained on methanol (K_M) was 0.01 g-methanol/l. Unfortunately, K_M for a Mut^S *P. pastoris* strain was rarely reported in the publications due to overlooking in the slow growth rate on methanol of the strain (d'Anjou and Daugulis, 1997; d'Anjou and Daugulis, 2001). The published K_M parameter was

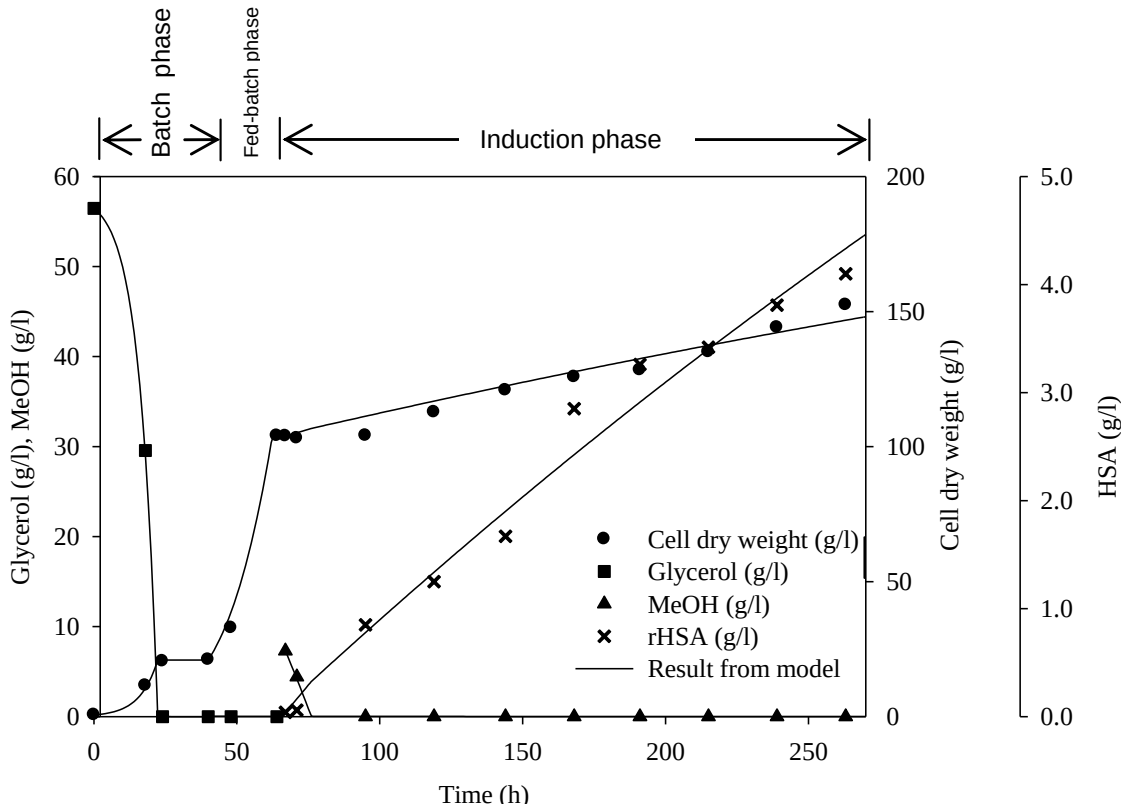

Figure 3. The growth behavior of Mut^S *P. pastoris* KM71 when producing rHSA in RUN 2 experiment. Symbols represent measured data and lines represent simulation. ■ , Glycerol; ● , yeast biomass; ▲, methanol; X, rHSA

reported for a Mut$^+$ strain and was equal to 0.22 g-methanol/l (Curvers et al., 2001).

The Run 2 experiment was operated the same as the Run 1 experiment and the results were similar. As shown in Figure 3, the biomass in batch fermentation increased from 0.59 to 21.04 g-DCW/l within 40 h with glycerol simultaneously decreasing from 56.47 g-glycerol/l until completely consumed. After the batch stage, the fed-batch of exponential glycerol feed started at 40th hour following Equation 7 with μ_{set} at 0.08 h^{-1} for 24 h (calculated by Equation 8) in order to prolong the yeast growth and achieve 100 g-DCW/l high cell density. Henceforth, the rHSA expression was induced by methanol. Four hours after the initial methanol pulse at 4 g-methanol/l, the methanol was continually added following Equation 9 with constant q_M (0.026 g-methanol/g-DCW·h). The methanol residue in the culture broth remained only at the beginning of the feed and then decreased over time until it could not be detected by HPLC; even the methanol was still fed. At the end of the experiment, the concentration of secreted rHSA (× symbol) in the medium at 263 h (196 h of induction) was 4.10 g-methanol/l with a cell concentration of 152.46 g-DCW/l. Furthermore, the results from RUN 2 experiment were used to validate the developed models and parameter set. The consequence from curve fitting showed that the obtained parameters from Run 2 experiment were same as Run 1 (Table 2). By using the parameters in Tables 1 and 2, the Run 2 experiment was simulated according to the proposed models and the results were represented by the lines in Figure 3. As shown in Figure 3, the r^2 in all data sets exceeded 0.99. The simulation results showed that the developed models and the rHSA fermentation approach for this study were rigid and consistent.

Conclusion

In this study, simple models were developed based on growth kinetic equations, fed-batch mass balance and the assumptions of constant cell and protein yields. Monod kinetic equation was used to describe both growths on glycerol and methanol. The developed models fit very well with the experiments with r^2 values exceeding 0.99 in all data sets. The obtained parameters could be reasonably acceptable. Moreover, the models and parameters

were rigid and consistent and could describe and predict cell growth, substrate (glycerol and methanol) utilization and recombinant protein production by *P. pastoris* KM71. Furthermore, the demonstrated simulation methodology in this study could also be used as a tool to study heterologous protein production by recombinant microorganisms where fermentation could be simulated using simple equations and simple methods without the requirement of complex models.

Conflict of Interests

The authors have not declared any conflict of interests.

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