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On-line methanol sensor system development for recombinant human serum albumin production by *Pichia pastoris*

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An on-line methanol sensor system was developed using a methanol probe, methanol sensor unit and peristaltic pump. The system was commanded using data acquisition (DAQ) and LabVIEW software. Calibration of the methanol sensor system was done in a medium environment with yeast cells during cells adaptation to methanol metabolism after glycerol feeding was stopped. The correlation equations between voltage output signal from the methanol sensor unit and residual methanol in culture broth were created with third order polynomial regression. This developed system was implemented for on-line methanol control in recombinant human serum albumin (rHSA) protein production by *P. pastoris* KM71 at methanol levels of 4 and 10 g/l with controlled fluctuations at 13.0 and 11.3% of oscillation, respectively. The accumulated amounts of recombinant protein from two levels of methanol concentration controls (4 and 10 g/l) were similar but the proteins were produced at a different rate related with methanol concentration in the broth. Therefore, the control at 10 g/l methanol had a higher production rate (0.53 mg-protein/g dry-cell·h) than 4 g/l methanol control (0.38 mg-protein/g dry-cell·h) as it reached the maximum protein concentration in a shorter time, even though its cell yield was less than that of 4 g/l methanol control. At the end of the experiments, the high cell density environment caused both cell and protein reduction by cell autolysis and protease degradation. However, the protein decrease could be prevented by taking protein induction at a low temperature and a pH where protease does not function.

Key words: Methanol monitoring, methanol sensor, on-line methanol, *Pichia pastoris*, recombinant human serum albumin.

INTRODUCTION

Human serum albumin (HSA) produced in the liver is a major protein component of human blood plasma. With a molecular weight of 66.5 kDa, HSA comprises about one-half of the blood serum protein (approximately 40 g/l)

(Huang et al., 2005; Kaoru, 2006; Belew et al., 2008). HSA functions as a carrier protein for steroids, fatty acids and thyroid hormones and plays a role in stabilizing extracellular fluid volume in blood (Huang et al., 2005; Belew et al., 2008). It was used clinically as a therapeutic agent in hypoalbuminemia or traumatic shock (Kobayashi et al., 2000a; Watanabe et al., 2001; Huang et al., 2005; Ohya et al., 2005; Kaoru, 2006) and applied to stabilize blood volume during surgery and during shock or burn cases (Dong et al., 2012). It was also used for the formulation of protein therapeutics, vaccine formulation and manufacturing, coating of medical devices and drug delivery (Zhang et al., 2013). In 2011, the worldwide demand for HSA was estimated to be more than 500 tons/year (He et al., 2011) and the demand has since been increasing. Traditionally, HSA is produced by fractionation from human plasma but this method is limited by human blood supply and has the risk of contamination of blood-derived pathogens (Kobayashi et al., 2000a; Watanabe et al., 2001; Ohya et al., 2005; Kaoru, 2006; Belew et al., 2008). Therefore, recombinant compounds from genetically modified organisms were used to solve these problems (Daly and Hearn, 2005; Kaoru, 2006). Recombinant human serum albumin (rHSA) has been produced in various expression systems, including *Escherichia coli* (Latta et al., 1987), *Saccharomyces cerevisiae* (Sleep et al., 1990), *Bacillus subtilis* (Saunders et al., 1987), *Kluyveromyces lactis* (Saliola et al., 1999), *Pichia pastoris* (Kobayashi et al., 2000a, b; Watanabe et al., 2001; Ohya et al., 2005; Belew et al., 2008; Sohn et al., 2010; Stadlmayr et al., 2010; Dong et al., 2012), transgenic animals (Barash et al., 1993) and transgenic plants (Huang et al., 2005; He et al., 2011; Zhang et al., 2013).

P. pastoris is a methylotrophic yeast which is widely used to produce various recombinant proteins. The popularity of *P. pastoris* as a host for the production of recombinant proteins has drawn attention due to several advantages (Sohn et al., 2010; He et al., 2011; Çelik and Çalik, 2012; Krainer et al., 2012; Potvin et al., 2012; Garcia-Ortega et al., 2013; Fickers, 2014; Byrne, 2015; Çalik et al., 2015). The main reasons are that the genetics of *P. pastoris* can be easily manipulated to express foreign proteins and can perform post-translational modifications presented in higher eukaryotes. Moreover, *P. pastoris* can be grown to very high cell densities (Krainer et al., 2012; Fickers, 2014; Byrne, 2015), in some instances reaching 200 g/l (Heyland et al., 2010). In addition, *P. pastoris* has few natively secreted proteins at relatively low concentrations and can simplify subsequent purifications of the secreted recombinant protein (Potvin et al., 2012). Heterologous protein expression from *P. pastoris* is normally produced by a three-stage fermentation process (Çelik and Çalik, 2012; Potvin et al., 2012; Looser et al., 2015). The first stage is batch fermentation where *P. pastoris* is cultured on glycerol. Fed batch culture, the second stage, starts when glycerol in the initial step is depleted, glycerol is fed into the culture in order to prolong the growth and increase the yeast cells to a higher density. The third stage is the induction stage where protein production is induced by the addition of methanol. Methanol as the inducer of the *AOX1* promoter has been commonly used to express the heterologous protein in the recombinant *P. pastoris*. Not only the inducing chemical, but methanol is also poisonous to the *P. pastoris* 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 (Minning et al., 2001; Hong et al., 2002; Lee et al., 2003b; Potvin et al., 2012). To achieve these constrains, methanol monitoring and control are very important. Gas-liquid chromatography (GC) and high performance liquid chromatography (HPLC) are both expensive and hardly implemented on-line (Guarna et al., 1997; Hong et al., 2002; Potvin et al., 2012; Gonçalves et al., 2013). However, methanol concentration could be alternatively indicated by dissolved oxygen (DO) monitoring. When cells are actively grown with available carbon sources, DO in the broth is used up and the level becomes very low. When the carbon source is depleted, the DO level immediately increases. If the carbon source is provided, the DO again decreases (Lee et al., 2003a). These phenomena are used to control the DO tension range and can be applied to methanol feeding (Lee et al., 2003a; Lee et al., 2003b). If DO tension reaches above a set value, then the methanol is fed into the bioreactor and it is stopped when DO tension falls below a lower set value. The DO value can rise to reach the upper set point again when the methanol is completely depleted, then the feeding cycle resumes. In this way, the concentration of methanol fluctuates; it might be kept under control and sometimes reaches toxic levels (Minning et al., 2001). However, this technique is rather complicated to implement (Irani et al., 2015) and cannot be used in glycerol-methanol mixed feeding (Hong et al., 2002). In addition, it is not appropriate for application if the rate of methanol assimilation is slow which exhibits Mut^S and Mut^- phenotypes because yeast cells might be exposed to non-inducing levels of methanol (Guarna et al., 1997; Hong et al., 2002). In order to solve both insufficient and excessive methanol feeding, an on-line methanol monitoring system was developed by many researchers (Guarna et al., 1997; Katakura et al., 1998; Zhou et al., 2002; Schenk et al., 2007). The system was capable of

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managing methanol feeding and was able to keep residual methanol in broth cultures at a constant level (Guarna et al., 1997; Hellwig et al., 2001; Suwannarat et al., 2013).

In this study, a genetically modified Mut^S *P. pastoris* KM71 strain, which produces and secretes rHSA, was used as a model to study on-line methanol control in heterologous protein expression processes.

MATERIALS AND METHODS

Organism

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

Media

Yeast extract peptone dextrose (YPD) media contained 10 g yeast extract, 20 g peptone and 20 g dextrose per liter of deionized water. Basal salt media (BSM) contained 26.7 ml 85% H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4.7H_2O$, 4.13 g KOH, 50.0 g glycerol and 6.7 ml PTM1 trace salt in deionized water 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 to a total volume of 1 L. The PTM1 trace salt was sterilized by filtration.

Preparation of inoculums

P. pastoris stored at -80°C was used to inoculate a starter culture in YPD media 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 same condition as before until reaching an OD_{600} of 20. The BSM inoculums were then transferred aseptically to 1 L of BSM (working volume) in a 2 L bioreactor (BIOSTAT B, B. Braun Biotech International, Melsungen, Germany). The volume of inoculum used in all the experiments was 10% of the working volume of the bioreactor.

Batch fermentation

The batch fermentations were performed with 1 L BSM in a 2 L bioreactor. The temperature was set to 30°C and pH was maintained at 5 by using 25% NH_4OH and 85% H_3PO_4. Dissolved oxygen was kept above 20% saturation by using cascaded control of agitation to maintain dissolved oxygen at the set value. Aeration was supplied at 2 vvm and pure oxygen was used and mixed with the air if the stirrer could not control this value. Foaming was monitored and controlled by an antifoam sensor which would occasionally add antifoam (Antifoam 204, Sigma, Deisenhofen, Germany) into the culture broth to prevent excessive foaming during fermentation.

Fed batch fermentation

When glycerol in BSM was depleted after the batch fermentation stage, the culture was exponentially fed with glycerol feed medium (50% w/v glycerol with 15 ml/l PTM1) by using predetermined exponential feeding rate calculated by Equation 1 according to d'Anjou and Daugulis (1997) and Jahic et al. (2002) until the cell concentration reached 100 g/l. The yeast cell concentration was calculated with Equation 2 to predetermine the glycerol feeding time.

$$F = \frac{\mu_{set}}{S_0 Y_{X/S}} X_0 V_0 e^{u_{set}(t-t_0)} \quad (1)$$

$$X = \frac{X_0 V_0}{V} e^{u_{set}(t-t_0)} \quad (2)$$

Where F is glycerol feed rate (l/h), X is biomass concentration in dry weight (g/l), X_0 is biomass concentration in dry weight at initial feeding (g/l), V is the medium volume (l), V_0 is the medium volume at initial feeding (l), S_0 is the substrate (glycerol) concentration in inlet feed (g/l), $Y_{X/S}$ is the yield coefficient biomass per substrate, glycerol (g/g), μ_{set} is the specific growth rate set point (h^{-1}), t is the run time (h) and t_0 is the initial feeding time (h). The controlled condition was the same as in batch stage.

Protein induction

After reaching the predetermined cell density (100 g/l), glycerol feeding was stopped and the culture was left for 4 h for the starvation phase. Methanol with 15 ml/l PTM1 was then fed into the bioreactor in order to induce rHSA expression. An initial pulse of methanol was firstly fed into the bioreactor to a level of 10 g/l and left for 4 h. During this time, both methanol concentration and voltage output signal were monitored for methanol probe calibration. After that, the methanol feed was controlled by on-line methanol sensor. The temperature was set to 22°C (Anasontzis and Penã, 2014) and the pH to 6.00 (Kobayashi et al., 2000a) during the induction phase.

Sample analysis

Samples were taken during fermentation and centrifuged at 9000 rpm for 5 min at 4°C to separate yeast cells from fermented broth. Yeast cell concentration was determined by measuring OD_{600} and then converted to dry cell weight by OD-dry cell weight correlation. Glycerol and methanol concentrations in the fermented broth were analyzed by HPLC (Shimadzu Ltd., Tokyo, Japan). The column used for HPLC was an Anemex HPX-87 H Column (Bio Rad) and the temperature was set at 45°C in combination with 0.5 mM sulfuric acid as mobile phase and a flow rate of 0.6 ml/min. Detection was done using a refractive index detector. Total protein concentration in the harvested broth was analyzed by Bradford assay and bovine serum albumin (BSA) was used as standard protein (Suwannarat et al., 2013). Samples from the culture supernatant were analyzed by SDS-PAGE on 12% gels according to standard protocols. The protein in the SDS-PAGE gels was visualized by Coomassie blue staining with Imperial™ Protein Stain (Thermo Fisher Scientific).

On-line methanol sensor development

Methanol detector and sensor units used in this study were developed by Reven Biotech Inc., Canada. The system of these devices was described by Guarna and co-workers (1997). The methanol was controlled using external control mode and

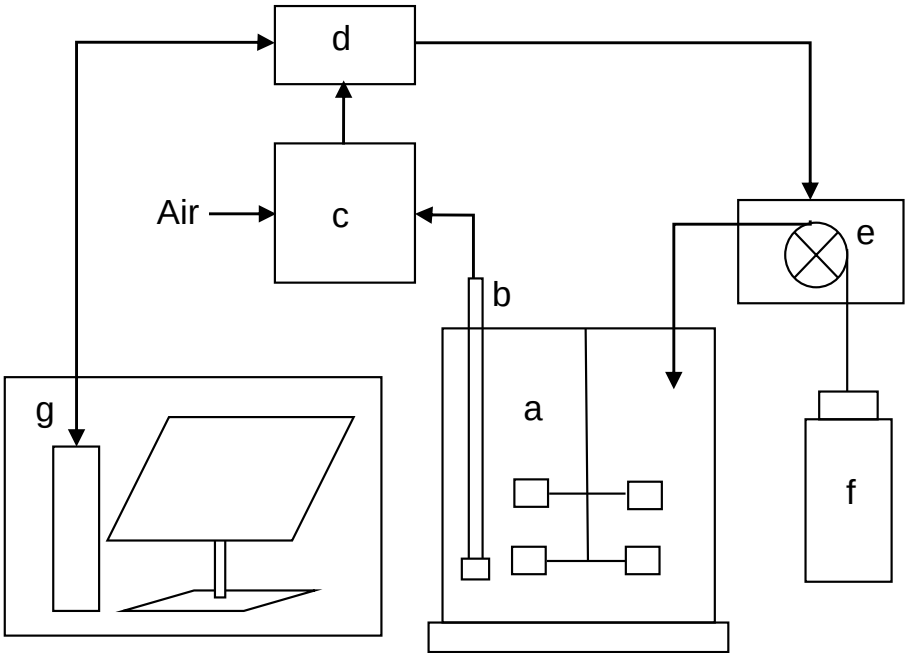

Figure 1. Schematic diagram of the developed methanol controlling system. **(a)** Bioreactor. **(b)** Methanol probe. **(c)** Methanol sensor unit. **(d)** Data acquisition (DAQ) device. **(e)** Peristaltic pump. **(f)** Methanol feed reservoir. **(g)** Computer set. Arrows indicate direction of signal and mass flow.

commanded by data acquisition (DAQ) with LabVIEW software version 7.0 of National Instruments Corporation, USA, providing a control program and user interface. The methanol sensor system was set up as shown in Figure 1. The methanol probe was immersed in the broth and connected with the methanol sensor unit by gas flow connection which supplied dry clean air to flush out volatile methanol gas to the Figaro model TGS 822 SnO_2 organic vapor sensor mounted in a plexiglass housing (in methanol sensor unit) (Guarna et al., 1997). The output from sensor was a voltage signal transmitted via the RS 232 port to the DAQ device controlling the peristaltic pump by on/off control. As shown in Figure 2, the diagram of methanol on-line instruction by the DAQ application with LabVIEW software controlled methanol concentration in the broth; when the measured methanol concentration was lower than a set value, the peristaltic pump for methanol feeding medium was stimulated for a cyclic time (adjusted through user interface at 5 s for this study) and stopped to wait for a new signal or new methanol concentration (if the concentration was still below the set point, the pump was stimulated again). This operation was taken until the methanol concentration reached the set point. The sensor was turned on for 8 h or more before induction phase for stabilizing voltage baseline. Sensor calibration was required by making a correlation between voltage output signal and methanol residue in fermented broth detected with off-line HPLC.

Methanol sensor calibration

Calibration was done during the yeast cells accommodation to methanol metabolism in the induction stage (during 4 h after the initial methanol pulse at 10 g/l) by monitoring the voltage output signal from the methanol sensor device and the residual methanol concentration in culture broth detected by off line HPLC. The correlation of these observed data were set up with the third order polynomial regression (Guarna et al., 1997) using the curve fitting function in Excel program, as shown in Figure 3.

RESULTS AND DISCUSSION

All the experiments were initiated by batch fermentation using 1 L working volume BSM in a 2 L bioreactor; the results are shown in Figure 4. The biomass (dry cell weight) of initial yeast cells increased from 0.5 to 24 g/l within 30 h, while glycerol was assimilated and decreased from 40 g/l until it was depleted. After glycerol had been depleted, the fed-batch stage was started in order to prolong the growth phase of *P. pastoris* and increased yeast cells to a high density. In this stage, yeast cells were fed more glycerol with glycerol feed medium (50% w/v glycerol with 15 ml/l PTM1) into the bioreactor. The feed pattern of glycerol addition depended on exponential growth of cells, therefore, the feeding strategy was done based on Equation 1 (d'Anjou and Daugulis, 1997; Jahic et al., 2002). Glycerol feeding time and cell concentration could be calculated by Equation 2. During the fed-batch stage, the μ_{set} was set at 0.08 h^{-1} to avoid metabolic overflow (Suwannarat et al., 2013; Looser et al., 2015). When the yeast cells reached the predetermined concentration, 100 g/l (at 54^{th} h) in the bioreactor, the glycerol feed was stopped and, thus, the fed-batch stage was finished. After 4 h where the cells used up left over glycerol residual and some metabolites,

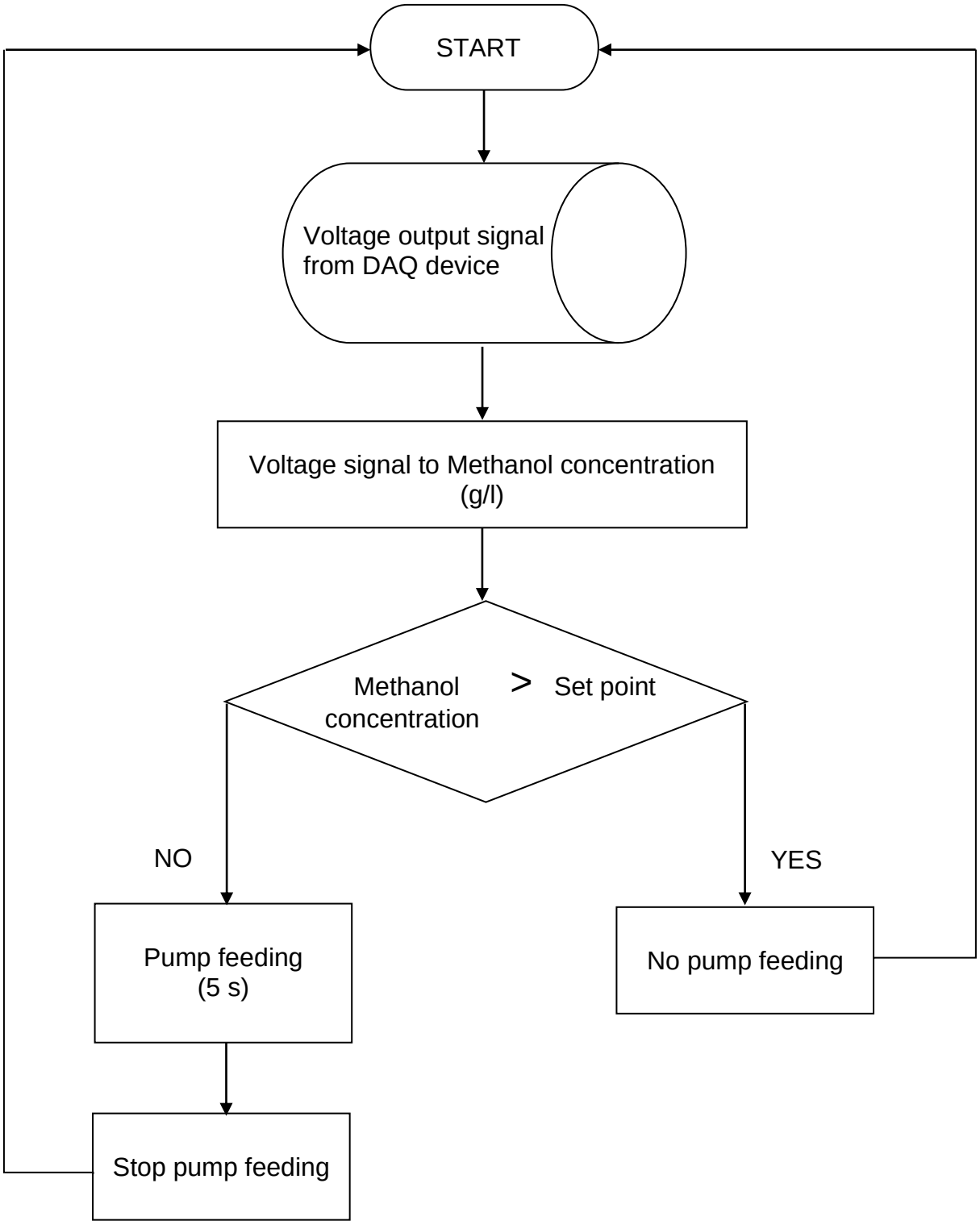

Figure 2. Schematic diagram of on-line methanol control system flow chart.

the induction phase was started at the 58th h by adding methanol to a concentration of about 10 g/l, in order to activate the *AOX1* promoter to express the rHSA protein. The solution was then left for a few hours (4 h) before continuing the experiment. Normally, the initiated methanol concentration in the bioreactor was used at 2-4 g/l (d'Anjou and Daugulis, 2001; Trinh et al., 2003; Potvin et al., 2012), however, 10 g/l methanol was used for this

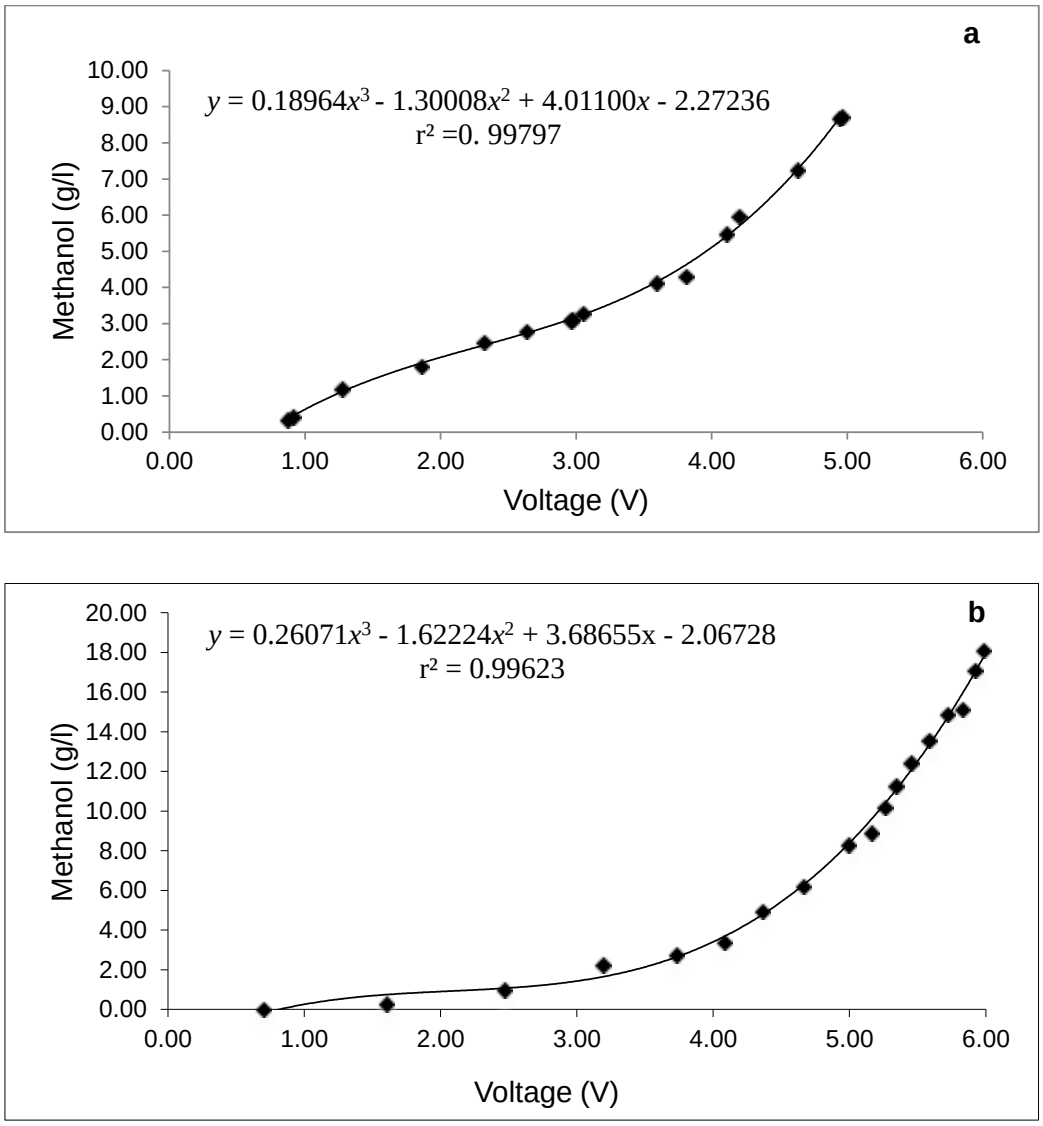

Figure 3. Correlation of voltage signal and methanol residue; *y* is methanol concentration (g/l), *x* is voltage signal (V) and r^2 is correlation coefficient.

study. The higher concentration of methanol used in this study not only activated the *AOX1* promoter and induced rHSA expression, but was also used to calibrate the methanol sensor probe and devices. It had been reported that the culture medium did not influence the calibration where the characteristics of the probe were virtually the same measuring in either methanol-water solution or culture medium with or without cells (Guarna et al., 1997), however, both fermentation medium and biomass affected the calibration curve according to Ramon and co-workers (2004), which corresponded to the preliminary methanol sensor test (data not shown). During the 4 h period, where yeast cells adapted themselves from glycerol to methanol metabolism in substrate change, the calibration was taken by monitoring the voltage output signal from methanol sensor devices and methanol decrease was detected by off-line HPLC. Therefore, the correlation between residual methanol in culture broth (by off-line HPLC) and voltage output signal measured by methanol sensor devices was created. As non-linearity of the sensor response (Guarna et al., 1997), the correlation was set up with the third order polynomial regression obtained by using the curve fitting function in Excel program (as shown in Figure 3). In Experiment 1, the correlation equation was:

$$y = 0.18964x^3 - 1.30008x^2 + 4.01100x - 2.27236 \quad (3)$$

Where, *x* is voltage output signal form methanol sensor

device (volt, V) and *y* is residual methanol concentration in culture broth (g/l). The correlation curve is shown in Figure 3a and the correlation coefficient (r^2) is 0.99797. After calibration, this correlation was used for methanol concentration control in the induction stage of Experiment 1 at set point, 4 g/l. The methanol control system (Figure 1) was commanded by DAQ with LabVIEW program providing the user interface of methanol concentration monitoring and control. The methanol residual in broth was still detected to evaluate the on-line control using off-line HPLC. In 4 g/l methanol induction (Experiment 1), the methanol control was in the range of 4 ± 0.52 g/l which was 13.0% of oscillation (the result was illustrated by ▲ symbol in Figure 4a). The methanol concentration used for protein induction in Experiment 1 was controlled at 4 g/l according to the best result for *P. pastoris* strain KM71 producing human growth hormone (hGH) (Suwannarat et al., 2013). Varying the inducing methanol concentration, the other experiment (Experiment 2) was operated with 10 g/l methanol control, in induction stage as the preliminary study showed that *P. pastoris* strain KM71 could endure methanol concentration more than 10 g/l and the report by Bushell and co-workers (2003) showed that the *AOX1* promoter was fully induced at 10 g/l methanol. However, Kupcsulik and Sevella (2004) investigated a range of methanol concentrations from 0.45-8.85 g/l that did not show characteristics of metabolic inhibition, but out of this range was not studied. Therefore, the methanol control at 10 g/l in the induction stage was chosen for Experiment 2. Likewise in Experiment 1, the methanol sensor probe and devices were also calibrated during the 4 h where cells accommodated to methanol metabolism. The correlation equation and calibration curve shown in Figure 3b with r^2 at 0.99623 for Experiment 2 was:

$$y = 0.26071x^3 - 1.62224x^2 + 3.68655x - 2.06728 \quad (4)$$

Using Equation 4 for Experiment 2, the methanol level in the bioreactor was on-line controlled at 10 g/l; however, off-line HPLC also still analyzed residual methanol to assess the control of methanol sensor devices. The on-line methanol control at 10 g/l methanol induction experiment varied in the range of 10 ± 1.13 g/l which was 11.30% of oscillation (the result was illustrated by ▲ symbol in Figure 4b). The fluctuation of methanol control in this study was not more than 13% which could be acceptable, but could be improved next time by adjusting the methanol feeding pump times (via the user interface) to less than 5 s (used in this study). Comparing Equation 3 and 4, the correlation between residual methanol in culture broth and voltage output signal of these equations were dissimilar in each calibration. Consequently, it was necessary to calibrate the methanol sensor probe and devices before use, especially for the procedure with both culture medium and biomass which corresponds to Ramon et al. (2004). It demonstrated that the on-line methanol sensor system developed in this study could implement methanol level control in high cells density of recombinant protein production. The results of methanol control in Experiment 1 and 2 (Figure 4a and b, respectively) showed that the highest cells and protein concentrations were respectively 162.15 and 5.98 g/l at the 156th h (98th h of induction) in Experiment 1 and 152.13 and 5.94 g/l at the 132nd h (74th h of induction) in Experiment 2. The accumulated protein secreted into culture broth was alike but in different production rate accordingly as shown in Figure 4, where the protein concentration curve in 10 g/l methanol induction (Figure 4b) was more incline than the curve of 4 g/l methanol induction (Figure 4a), therefore, the protein reached its maximum in a shorter time. This showed that the specific productivities of secreted protein (calculated at maximum protein concentration) were 0.38 and 0.53 mg-protein/g dry-cell·h in 4 and 10 g/l methanol controls, respectively. As a consequence, in the non-inhibitory methanol level, recombinant protein production rate was increased relative to the methanol concentration corresponding to Katakura et al. (1998), Khatri and Hoffmann (2006) and Damasceno et al. (2004). The secreted protein with HSA protein of about 80-86% (from previous studies) was analyzed, the rHSA was compared with standard HSA (67 kDa) by SDS-PAGE, as shown in Figure 5, which showed the same molecular size. In this study, high methanol levels did not significantly affect protein production in spite of cell yield which decreased corresponding to Bushell et al. (2003) and Schenk et al. (2007), hence cell yield in higher methanol induction was less than the lower methanol induction, but it reached the maximum cell concentration in a shorter time. After, methanol feeding was stopped because the assimilation was not taken due to the yeast cells entering stationary phase reaching its maximum, therefore the growth began to slow which was caused by a limitation of some other components (d'Anjou and Daugulis, 2000) and some metabolites produced by the cells. In the high cell density culture environment, cell autolysis and proteolytic degradation made the yeast cells and protein to decrease (Cregg et al., 2000), therefore, yeast cells decreased continuously after the maximum growth at the end of the experiment which is shown in Figure 4. However, secreted proteins were not reduced owing to the condition of induction at low temperature (22°C) which could reduce proteolysis (Potvin et al., 2012; Gonçalves et al., 2013; Anasontzis and Penã, 2014). In addition, pH 6 was not appropriate for protease enzyme activity in rHSA production (Kobayashi et al., 2000a) then the proteolytic activity could be minimized by optimizing pH and temperature during cultivation (Curvers et al., 2001).

Conclusion

The on-line methanol sensor system was developed by commanding the methanol detector and sensor unit using

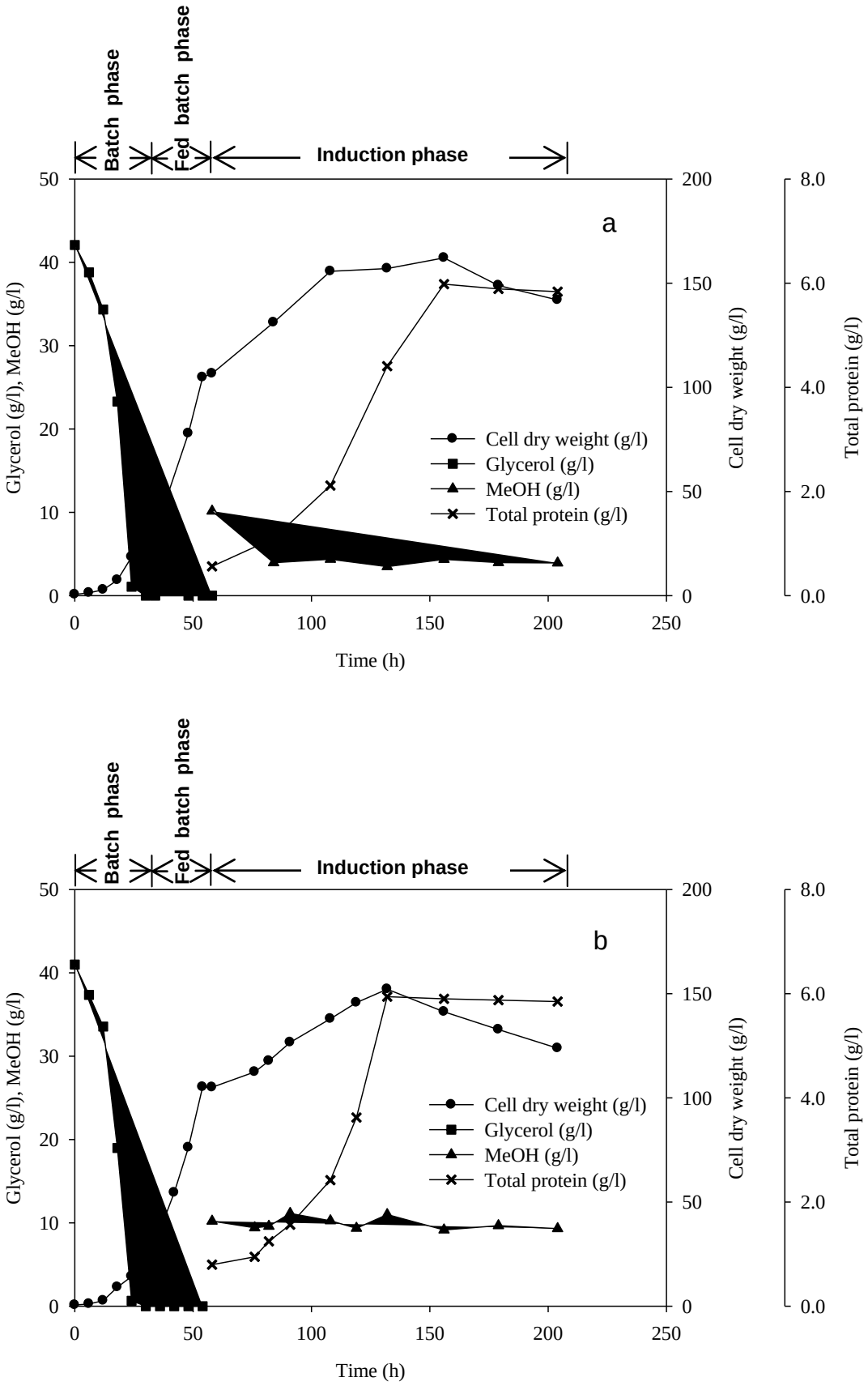

Figure 4. *P. pastoris* growth behavior. a) inducing at 4 g/l methanol; b) inducing at 10 g/l methanol.

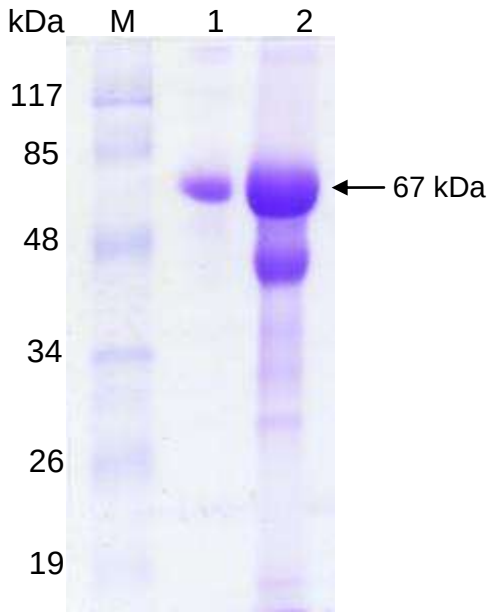

Figure 5. SDS-PAGE analysis of the *P. pastoris* KM71 produced HSA protein. Lane 1, standard HSA (67 kDa); lane 2, supernatant.

data acquisition (DAQ) with LabVIEW software providing a control program and user interface. This developed system was capable of managing the methanol feed and was able to keep residual methanol in broth culture at a constant level. The fluctuation of methanol controls were 13.0 and 11.3% at inducing methanol levels 4 and 10 g/l, respectively. In the non-inhibitory methanol level, the production rate of recombinant protein increased with methanol concentration in the culture broth despite cell yield reduction, therefore the protein reached the maximum in a shorter time. The specific productivities of secreted protein were 0.38 and 0.53 mg-protein/ g dry-cell-h in 4 and 10 g/l methanol controls, respectively. Cell lysis and proteolytic degradation caused a decrease in cells and proteins in high cell density environments, but the protein degradation in this study could be prevented by induction at a low temperature (22°C) and pH 6 which was not appropriate for protease activity.

Conflict of Interests

The authors have not declared any conflict of interests.

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