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Full Length Research Paper

Comparison between constant methanol feed and on-line monitoring feed control for recombinant human growth hormone production by *Pichia pastoris* KM71

Yardrung Suwannarat[1], Sornpichai Saeseaw[2], Niwat Chanasutthiprapa[3] and Anan Tongta[1]*

[1]Division of Biotechnology, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, 49 Soi Tientalay 25, Bangkhuntien-Chaitalay Road, Takham, Bangkhuntien, Bangkok 10150 Thailand.
[2]Department of Chemical Engineering, Faculty of Engineering, King Mongkut's University of Technology Thonburi, 126 Pracha-utid Road, Bangmod, Toongkru, Bangkok 10140 Thailand.
[3]Industrial Park Center, King Mongkut's University of Technology Thonburi, 49 Soi Tientalay 25, Bangkhuntien-Chaitalay Road, Takham, Bangkhuntien, Bangkok 10150 Thailand.

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Two methanol feeding methods, namely constant methanol feed and on-line monitoring feed control by methanol sensor were investigated to improve the production of recombinant human growth hormone (rhGH) in high cell density cultivation of *Pichia pastoris* KM71 in 2 L bioreactor. The yeast utilized glycerol as a carbon source for cell growth and yeast cells were accumulated to high cell density. Cell dry weight concentration around 140 to 150 g/l was obtained before entering the methanol induction period. Methanol was applied to express rhGH after high cell accumulation. The constant methanol feed rate at 0.009 l/h was applied to the cultivation to compare with the on-line monitoring feed control of methanol concentration. The highest amount of rhGH around 501 mg/l was obtained by using on-line monitoring feed control of methanol at the set point of methanol concentration at 4.0 g/l. After achieving the best result from the on-line monitoring feed control method, the experiment was further carried out to investigate the optimal methanol concentration. On-line controlling of methanol concentrations at 2.0, 4.0 and 8.0 g/l were investigated. The result shows that high amount of rhGH was achieved by controlling the methanol level at the set point of 4.0 g/l.

Key words: Recombinant human growth hormone, *Pichia pastoris*, fed-batch cultivation, methanol feeding control.

INTRODUCTION

Recombinant human growth hormone (rhGH) is a commercial pharmaceutical product that has been utilized to treat children with growth hormone deficiency, children with chronic renal failure, girls with Turner's syndrome and adults with growth hormone deficiency. Numbers of researches were reported about the production of rhGH from different microorganisms such as *Escherichia coli* (Shin et al., 1998; Zhang et al., 1998; Castan et al., 2002), *Bacillus subtilis* (Tuncer et al., 2009), *Saccharomycess cerevesiae* (Teug et al., 1991), *Pichia pastoris* (Ecamilla-Trevino et al., 2000; Eurwilaichitr et al., 2002; Orman et al., 2008; Orman et al., 2009) and Chinese hamster ovary (CHO) (Catzel et al., 2003). *E. coli* cannot produce post translational modifications such as glycosylation and disulfide bond formation because *E. coli* is a prokaryotic cell. Therefore, eukaryotic cell such as *S. cerevisiae* and *P. pastoris* have been proposed to produce rhGH. *P. pastoris* is a good candidate for recombinant protein expression because

*Corresponding author. E-mail: anan.ton@kmutt.ac.th or yardrung@yahoo.com. Tel: 02-470-7564.

of its remarkable advantages such as the ability to grow to high cell density in a defined medium, ability to perform many post translational modifications and ability to produce high level of desired protein (Creg et al., 2000; Cereghino and Cregg, 2000; Cereghino et al., 2002). Normally, *P. pastoris* utilized glycerol as a carbon source for cell growth and yeast cells were accumulated to high cell density. Methanol was applied to express protein after high cell accumulation. Regulation and induction of recombinant protein by methanol has been shown to be simple, easy to scale-up and cost effective for industrial fermentations (Cregg et al., 1993). However, high concentrations of methanol are toxic to *P. pastoris* because of the accumulation of formaldehyde and hydrogen peroxide inside the cells (Murray et al., 1989; Zhang et al., 2000). An accurate regulation of the methanol concentration in high cell cultivation of *P. pastoris* is necessary to prevent the accumulation of methanol to levels that are toxic to the cells. Some authors showed that methanol concentration between 2 g/l and 3.5 g/l are optimal for protein production (Cunha et al., 2004; Schenk et al., 2007) and some authors presented that methanol concentrations above 3.6 g/l inhibit the yeast growth and lead to cell death (Jahic et al., 2006). Therefore, the methanol concentration should be considered and maintained constant during the induction phase in order to obtain the high production of desired protein (Chiruvolu et al., 1997). Methanol monitoring methods including off-line and on-line measurements have been developed to be used in *P. pastoris* cultivation. Chromatographic methods such as gas chromatography (GC), high performance liquid chromatography (HPLC) that used in a batch or pre-programmed methanol addition strategies are common off-line methanol monitoring methods. Due to these off-line methods being time-consuming, the on-line monitoring method has been proposed. Several on-line monitoring methods have been proposed to determine methanol concentration. Guarna and co-workers developed an on-line methanol sensor to monitor methanol concentration in shake flask cultures of *P. pastoris* to produce a recombinant mouse endostation (Guarna et al., 1997). Surribas and coworkers proposed an automated sequential injection analysis (SIA) method to monitor the methanol concentration for the production of heterologous lipase (Surribas et al., 2003). Flame ionization detectors (FIDs) and fourier transform mid-infrared spectroscopy (FTIR) have also been used for the on-line monitoring of methanol in *P. pastoris* cultivation (Gurramkonda et al., 2009; Crowley et al., 2005). On-line probes for the monitoring of methanol concentration in *P. pastoris* are commercially available. However, the reading signal from the devices may be affected by other volatile compounds such as ethanol and ammonium (Ramon et al., 2004). In this study, comparison between the methanol feeding methods was investigated to obtain high production of rhGH by high cell density cultivation of *P. pastoris* KM71 which is Mut^S phenotype. The productions of rhGH by varying the methanol concentration by on-line control method were also investigated.

MATERIALS AND METHODS

Organism, culture medium and substrate

P. pastoris strain KM71 was obtained from the Thailand National Center for Genetic Engineering and Biotechnology (BIOTEC). YEPD medium: 5 g yeast extract, 10 g peptone and 20 g dextrose; basal salts medium (BSM): 0.9 g $CaSO_4$, 18 g K_2SO_4, 14 g $MgSO_4$, 4 g KOH, 26 ml H_3PO_4 (85% stock), 40 g glycerol; glycerol solution; 50% glycerol (w/w) with 10 ml PTM1/l glycerol and methanol solution:100% methanol with 10 ml PTM1/l methanol were used

Methanol control equipments

Methanol sensor (probe and detector) was purchased from Raven Biotech Inc., Canada. Methanol control was performed using LabVIEW, National Instruments Corporation, USA.

Inoculum preparation

1 ml of *P. pastoris* stock culture was refreshed and grown overnight in 100 ml YPED. 2 ml of *P. pastoris* from YPED was further grown in 100 BSM until OD_{600} reached 2 to 6. Both of YEPD and BSM culture were incubated at 30°C with 200 rpm shaking rate. The BSM culture was transferred into 2 L bioreactor (BIOSTAT B, B. Braun Biotech International, Melsungen, Germany) containing 1 L of BSM.

Exponential feeding in glycerol fed-batch cultivation

Cultivation was carried out at 30°C. Aeration was set at a value between 2 to 4 vvm. Agitation was set between 400 and 1200 rpm to maintain dissolved oxygen (DO) levels at above 20%. The headspace pressure was maintained at ambient. The pH was adjusted to 4.5 by addition of 25% NH_4OH solution or 85% H_3PO_4 solution. Antifoam 204 (Sigma, Deisenhofen, Germany) was added when required to prevent excessive foaming. The glycerol batch phase was continued until the glycerol was exhausted, which was indicated by a sharp rise in DO level. Then, the glycerol fed-batch phase was started using an exponential feed rate as shown in Equation 1.

$$F = \frac{\mu_{set}}{S_0 Y_{X/S}} X_{10} V_0 e^{\mu_{set}(t-t_0)} \quad (1)$$

Where, S_0 is glycerol concentration in the feed reservoir (g/l), t is time of the exponential feed, (h), t_0 is starting time of the exponential feed (h), V_0 is liquid volume at the starting time of the exponential feed (l), X_{10} is the cell concentration at the starting time of the exponential feed (g/l), $Y_{X/S}$ is yield coefficient cell mass per substrate (g/g) and μ_{set} is controlled specific growth rate (1/h).

Methanol addition methods

Constant feed

Small amount of methanol was fed to the culture to make a final concentration of 4.0 g/l. After methanol was allowed to be consumed by cells for 4 h, methanol was constantly feed to the culture.

On-line feed control by methanol sensor

Methanol sensor (probe and detector) was calibrated at the time of induction by manually adding known amount of methanol to the culture with a peristaltic pump and determining the voltage signal from a detector. A calibration was verified by withdrawing samples and measuring the methanol concentration off-line by HPLC (Shimadzu Ltd., Tokyo, Japan). After calibration, methanol concentrations were controlled at set point of 2.0, 4.0 and 8.0 g/l based on on-line measured methanol concentrations by voltage signal of the methanol sensor. In the induction period of both methods, pH and temperature were adjusted to be 4.0 and 22°C, respectively.

Off-line methanol and glycerol analysis

Methanol and glycerol concentrations were analyzed using high-performance liquid chromatography (HPLC; Shimadzu Ltd., Tokyo, Japan) on an Aminex®HPX-87H Ion Exclusion Column (300 nm × 7.8 mm, Bio-Rad Laboratories, USA). Three millimolar of sulfuric acid at a flow rate of 0.6 ml/min was used as the mobile phase. Methanol and glycerol concentrations were detected by measurement of the refractive index detector. Concentrations of methanol and glycerol in each sample were compared to their standard curves.

Protein analysis

Samples of the fermentation culture were taken periodically to determine cell density and protein production. The protein patterns were analyzed by electrophoresis on 15% SDS-PAGE under denaturing conditions according to standard protocols (Laemmli, 1970). The SDS-PAGE gels were visualized by coomassie blue stain. The rhGH concentration was determined by using the Image J program.

RESULTS AND DISCUSSION

Effect of methanol control strategy

Two methanol feeding control methods, namely constant feed (Experiment 1) and on-line monitoring feed control by methanol sensor (Experiment 2) were investigated in order to determine the optimal method to produce high level concentration of rhGH in 2 L bioreactor. The glycerol batch phase was first utilized for cell growth and cell accumulation. After glycerol was depleted which was indicated by the DO spike, cell dry weight concentration at 23.9 g/l (grams of dry cell weight per liter) and 20.8 g/l were obtained at 36 h of cultivation in Experiments 1 and 2, respectively. The DO spike indicated that the initial glycerol no longer exists in the culture which was confirmed by HPLC. The specific biomass product ($Y_{X/S}$) was around 0.6 (g biomass/g glycerol) in both experiments. To obtain high cell density, glycerol was added by using the exponential feed. After DO spike was indicated, the culture was left without glycerol addition for 4 h before the exponential feed to allow the culture to use up all the remaining glycerol and other by-products. This feeding strategy had an advantage for maximum accumulation of cells by controlling the cell specific growth rate at 0.09 h^{-1} which was lower than the maximum specific growth rate (0.19 h^{-1}) to prevent the accumulation of glycerol during the fed-batch phase. Oxygen was supplemented into bioreactor and mixed with air at the ratio of 1:1 to prevent the accumulation of glycerol and maintain the DO level at approximately 20%. Glycerol feed was stopped and the residual glycerol was allowed to be consumed by cells for 2 h before entering the induction period. The initial cell dry weight concentrations at 135.7 and 138.7 g/l were obtained in Experiments 1 and 2 at the end of the glycerol fed-batch phase.

To prepare cells for the methanol induction period in Experiment 1, small amount of methanol was added to the culture until the final concentration reached approximately 4.0 g/l. The exposure of cells to methanol led to slightly decline of cell concentrations. Cell growth resumed after a short period of adaptation around 3 to 4 h. The successful adaptation to methanol was also determined by the resumption of ammonia uptake. The initial methanol no longer existed in culture after 4 h that was indicated by the DO spike. Methanol was added again to the culture by the constant feeding rate at the flow rate of 0.009 l/h (3.47 g/h). A cultivation profile of Experiment 1 is presented in Figure 1A. The amount of rhGH was detected after 17 h of induction time and increased linearly until 26 h of induction. However, the gradual decrease of rhGH concentration was observed after 26 h of induction time. The highest amount of rhGH concentration was 300 mg/l at 26 h of induction and it decreased to be 283 mg/l at the end of the process (57 h of induction time). The reduction of rhGH concentration could have occurred from the accumulation of methanol and most of the methanol may be used for cell growth instead of inducing protein production (Katakura et al., 1998; Min et al., 2010; Wu et al., 2011). As showed in this study, cells growth was observed after 26 h of induction time which indicate that cells prefer to utilize methanol for cell growth rather than induced protein.

The higher production of rhGH concentration was achieved by applying on-line methanol monitoring to control methanol concentration in Experiment 2. The cultivation in the glycerol batch and glycerol fed-batch periods were performed as described in Experiment 1 to obtain high cell density up to 138.7 g/l. Small amount of

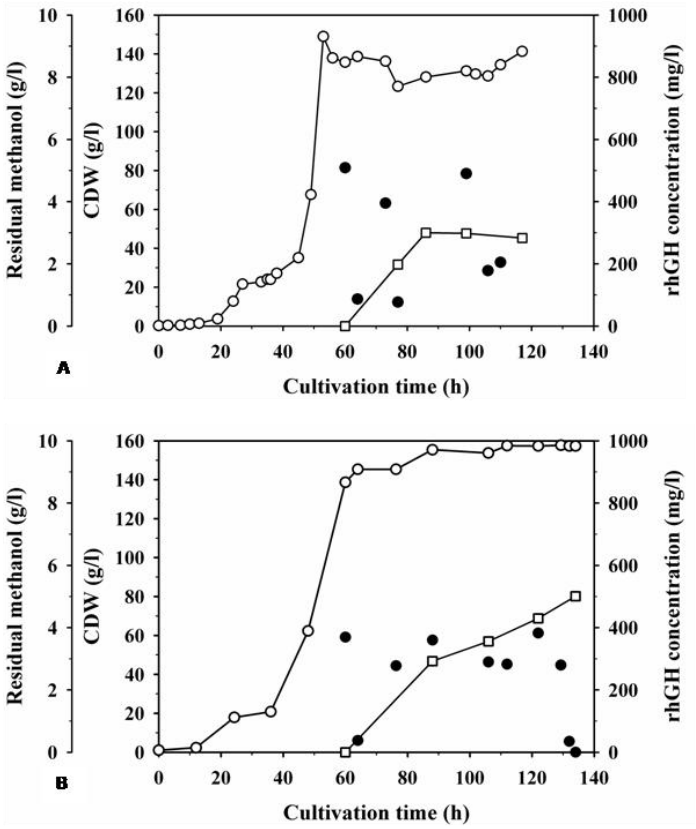

Figure 1. Time course of cell dry weight concentration (—○—), rhGH concentration (—□—), and residual methanol (●) in the high cell cultivation of *Pichia pastoris* KM71 of experiment 1 (A), experiment 2 (B), and SDS-PAGE analysis of culture broth at different induction time (C). Lane 1, marker; lane 2 to 4, standard at different concentration; lane 5 to10, supernatant at 74, 62, 46, 28, 16 and 0 h of induction time, respectively.

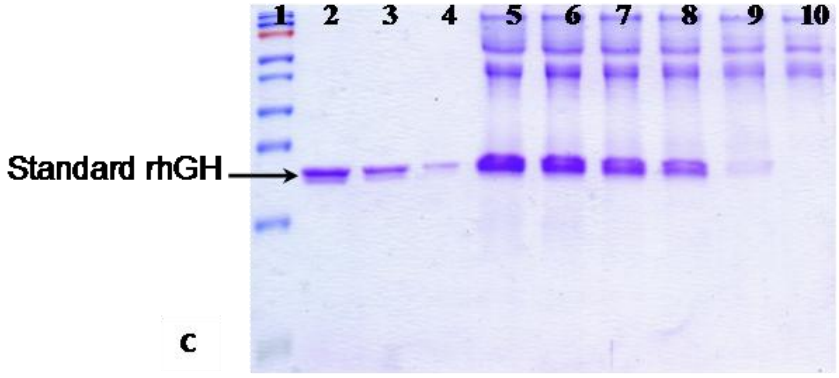

Figure 1. Continued.

methanol was added to the culture till the final concentration reached approximately 4.0 g/l to initiate cells and sensor calibration. Samples were taken at time interval to determine concentrations of methanol by HPLC. After 4 h, methanol was added to fermentation culture by on-line controlling at the set point of 4.0 g/l using methanol sensor and the LabView program. Time course of cultivation profile and SDS-PAGE analysis of

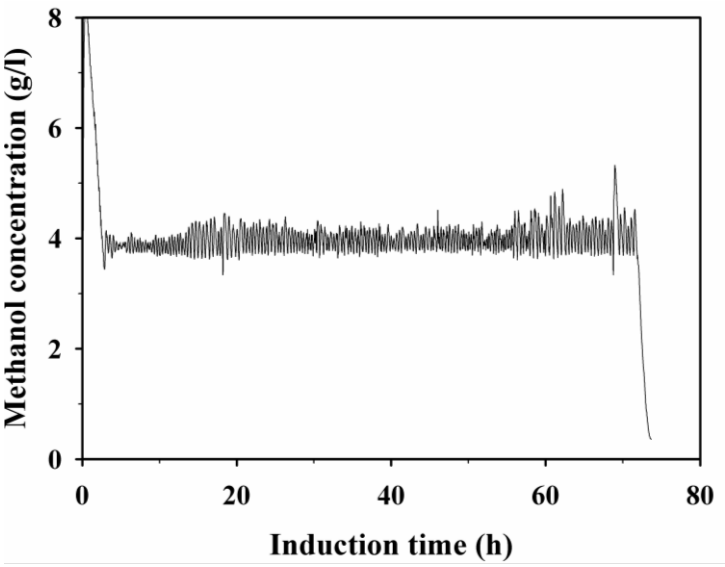

Figure 2. Methanol concentration at a set point of 4 g/l.

Table 1. Comparison of parameters for rhGH production by two different methanol feeding methods.

Parameter	Experiment	
	1	2
Initial cell concentration (g/l)	135.77	138.70
Average methanol concentration (g/l)	3.47	4.00
Ratio of methanol to initial cell concentration (g/g)	0.026	0.029
Final cell concentration (g/l)	141.32	158.27
Maximum rhGH concentration (mg/l)	300	501
Total consumed methanol (g/l)	187.91	232.5
Yield of DCW on methanol (g/g)	0.029	0.084
Yield of rhGH on methanol (mg/g)	1.60	2.15
rhGH productivity (mg/l·h)	5.26	6.77
Average specific rhGH production rate (mg/g) cell·h)	0.039	0.049
Induction period (h)	57	74

supernatant samples taken from Experiment 2 are presented in Figure 1B and C, respectively. The results show that the concentration of rhGH increased gradually over 74 h of the induction time. Off-line HPLC analysis showed that methanol concentrations ranged between 3 to 4 g/l which was a good agreement with on-line methanol measurements. Figure 2 shows the methanol concentration at the set point of 4.0 g/l. Table 1 shows the comparison of some parameters between two methanol feeding methods for rhGH production. The results show that yield of rhGH production on methanol, rhGH productivity and maximum rhGH concentration of Experiment 2 were higher than that of Experiment 1. The maximum rhGH concentration obtained from Experiment 2 was higher than that of Experiment 1 around 40%.

Effect of methanol concentration

To compare the recombinant protein production, high cell density cultivations of *P. pastoris* KM71 to express rhGH by using different methanol concentrations were investigated. During the induction period, methanol was fed to the fermentation culture and kept constant at set points of 2.0, 4.0 and 8.0 g/l, respectively, by continuous feeding based on the signal from the methanol sensor. A LabView program was used to control the methanol concentration. Time courses of cell dry weight concentration and rhGH production from the different methanol concentrations are presented in Figure 3. The results show that rhGH concentration increased with time by controlling methanol at the set point of 4.0 g/l. The

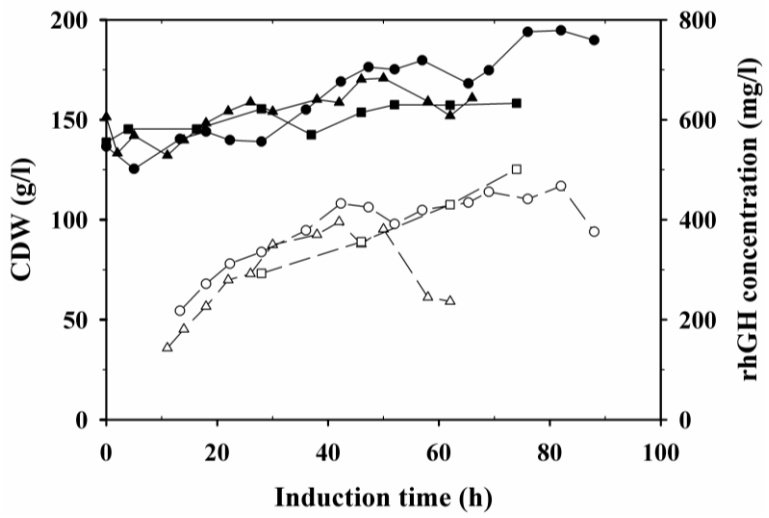

Figure 3. Time course of cell dry weight (CDW) concentration at set points of 2 g/l (—●—), 4 g/l (—■—), 8 g/l (—●—) and rhGH concentration at set points of 2 g/l (—●—), 4 g/l (– □ –), and 8 g/l (– △ –).

Table 2. Comparison of parameters for rhGH production at different methanol concentrations.

Parameter	Methanol concentration (g/l)		
	2	4	8
Initial cell concentration (g/l)	136.61	138.70	151.43
Average methanol concentration (g/l)	2.0	4.0	8.0
Ratio of methanol to initial cell (g/g) (concentration (g/g)	0.015	0.029	0.052
Final cell concentration (g/l)	189.81	158.27	160.93
Maximum rhGH concentration (mg/l)	467	501	396
Total consumed methanol (g/l)	264.5	232.5	220
Yield of cell dry weight on methanol (g/g)	0.201	0.084	0.043
Yield of rhGH on methanol (mg/g)	1.77	2.15	1.80
rhGH productivity (mg/l·h)	5.30	6.77	6.00
Average specific rhGH production rate (mg/g) cell·h) cell·h)	0.039	0.049	0.040
Induction time (h)	88	74	66

maximum rhGH concentration around 501 mg/l was obtained and it was higher than that of set points 2 and 8 g/l. At the set point of 2.0 g/l, rhGH concentration increased at the beginning of the induction period but decreased gradually after 42 h of induction time. When methanol concentration was controlled at the set point of 8.0 g/l, rhGH production was inhibited and the yield decreased clearly after 50 h of induction time. This could be due to the metabolic stress when the cells encountered the high methanol concentration (Wang et al., 2009; Gurramkonda et al., 2009; Wu et al., 2011). This incident may bring about physiological changes that negatively affected process performance (Surribas et al., 2007).

Table 2 presents the comparison of fermentation parameters at different methanol concentrations. High yield of cell dry weight on methanol around 0.201 g/g was observed at the methanol set point of 2.0 g/l which indicate that cells preferred to use methanol for growth rather than protein production. Although the initial cell concentration at the set points of 2.0 and 4.0 g/l were nearly the same, cell density could grow up to 189.8 g/l at the set point of 2.0 g/l while only 158.2 g/l was observed at the set point of 4.0 g/l. The high yield of rhGH on methanol and the rhGH productivity were achieved at the set point of 4.0 g/l. This high yield would suggest that rhGH production by *P. pastoris* KM71 was both economical and feasible. At the set point of 8.0 g/l, the initial cell density was higher than that of the set points 2.0 and 4.0 g/l but the maximum rhGH concentration was

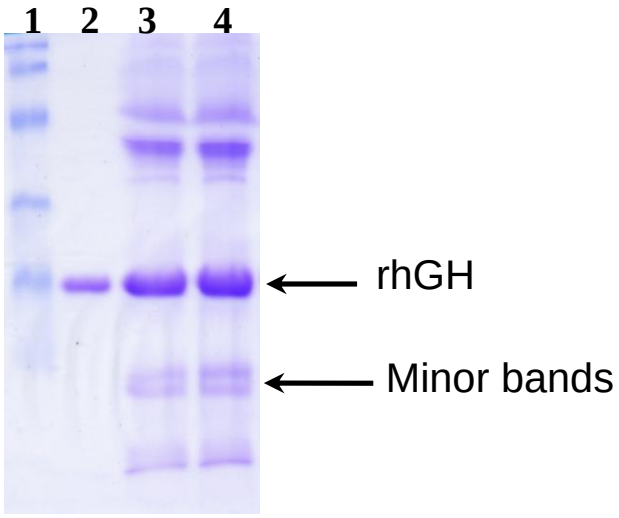

Figure 4. SDS-PAGE analysis of culture broth at different induction time (8% methanol concentration). Lane 1, marker; lane 2, standard; lane 3 to 4, supernatant at 62 and 66 h of induction time.

lower. It was possible that high concentration of methanol could affect the production of the recombinant protein. Some minor bands did appear for rhGH subsequent to the longer induction time as showed in Figure 4, suggesting that some proteolytic degradation may be produced by *P. pastoris* during the induction period (Jahic et al., 2006; Surribas et al., 2007). Moreover, the results show the effect of the ratio of methanol to initial cell concentration to rhGH production. The highest production of rhGH was achieved with the ratio of methanol to initial cell at 0.029 g/g or controlled methanol concentration at 4.0 g/l. The ratio of methanol to the initial cell concentration could be used to optimize the protein production process (Wang et al., 2009). Compared with the previous report (Ecamilla-Trevino et al., 2000), the cultivation of *P. pastoris* KM71 using high cell density fed-batch cultivation in 2 L bioreactor and on-line monitoring of methanol concentration generated a significantly higher level of rhGH concentration.

Conclusion

In conclusion, two methanol feeding strategies and the appropriate methanol concentration during the production of rhGH from *P. pastoris* KM71 in 2 L bioreactor were investigated. The results show that high rhGH production was achieved with the methanol feeding strategy by on-line monitoring and controlling of the methanol concentration at 4.0 g/l in the high cell density cultivation. This result is consistent with the suggestion to maintain methanol concentration in Mut^S strain cultivation at 4.0 g/l or below (Rosenfeld, 1999; Cos et al., 2006). The knowledge obtained from a small scale could be used for the production of rhGH in a commercial scale production.

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