International Journal on Advanced Science Engineering Information Technology

Vol.12 (2022) No. 3
ISSN: 2088-5334

Evaluating Pulses and Modified Fed-batch Feeding of Methanol to Increase Expression Level of Human Insulin Precursor in *Pichia Pastoris* High-Density Cultivation

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***Abstract*— Production of sufficient insulin at a more affordable price is necessary. The increase in the number of people living with diabetes puts more burden on healthcare and the economy. *P. pastoris* is a promising host to produce human insulin precursors at a high yield in minimal medium and secretes low levels of endogenous protein impurities. Production of the precursor involves several parameters, including glycerol concentration, culture density, methanol concentration, and medium composition. This study evaluated the effect of those parameters on protein expression in the flask culture. Subsequently, fermentation in the bioreactor was carried on according to the information obtained from flask culture. Methanol feeding to induce protein expression was undertaken by pulses and fed-batch modes. The fed-batch method was modified from a standard technique by incorporating constant flow rates with variable feed concentrations. Cell density was determined based on optical density measurement at 600 nm and dry cell weight. Tricine SDS-PAGE and reversed-phase HPLC conducted protein analysis. The pulse feeding produced higher precursor concentrations at ~445 mg/L than modified fed-batch feeding at ~267 mg/L. However, the modified fed-batch feeding can be an alternative to producing human insulin precursors when a standard fed-batch feeding with variable flow rates and 100% (v/v) methanol feed is difficult to apply.**

***Keywords*— Diabetes; human insulin precursor; *Pichia pastoris*; bioreactor; fed-batch.**

Manuscript received 5 Mar. 2021; revised 10 Jun. 2021; accepted 11 Jul. 2021. Date of publication 30 Jun. 2022.

I. INTRODUCTION

The world diabetes prevalence was projected to increase significantly, from 463 million in 2019 to 700 million in 2045 [1]. It was predicted that from 2015 to 2030, the increase in economic burden caused by diabetes accounted for USD 0.9 trillion [2]. Those facts raise awareness about producing sufficient insulin at a more affordable price. Insulin is a polypeptide hormone-containing 51-amino acid that was formerly isolated from the porcine pancreas. The advance in technology on DNA recombinant has enabled the production of recombinant human insulin [3]. The major viable routes in large-scale production involve bacteria and yeast as microbial hosts to produce insulin precursors [4], [5].

Although *Saccharomyces cerevisiae* is the most preferred yeast-based system, research and development have brought other alternatives recently [6]. One of the comparable alternatives is *P. pastoris* which has some beneficial features. The cell densities can reach up to ~200 g/L of cell dry weight [4] or 500 g/L wet cell weight [7]. Production of a human insulin precursor (HIP) utilizing P. pastoris was reported to achieve concentrations up to 4.51 g/L [4], [7]. Other capabilities of *P. pastoris* which attract industrial interest are having a robust and tightly regulated alcohol oxidase 1 (AOX1) promoter and the capacity to exhibit post-translational modifications, such as glycosylation and disulfide bond formation. The AOX1 promotes the utilization of methanol as a sole carbon source to produce high-level protein expression. Commercial kits for this expression system are available [8]. Moreover, less extensive glycosylation in *P. pastoris* than in S. cerevisiae creates additional benefits in reducing immunogenicity [9].

The HIP production involves several important parameters, including glycerol concentration, initial culture density, methanol concentration, dissolved oxygen, and medium composition. Glycerol affects specific growth rates and subsequently influences protein expression [10], [11]. Culture density at initial methanol induction can contribute to the higher or lower expression of the protein [12], [13]. Methanol and oxygen should be controlled to maintain productivity and avoid severe methanol accumulation. Limited methanol supply may reduce protein expression due to low induction intensity. At the same time, a long-time oxygen limitation could also reduce metabolic activities. On the other hand, the excess of methanol may lead to cell damage and hence lower protein yield [14], [15]. To induce protein expression, methanol can be fed by using the pulse method [7], [16], [17] or fed-batch mode [4], [7], [18]. Challenges in selecting methanol feeding strategies still exist and are limited by methanol feeding process control [19]. Hence evaluation of the feeding strategies is essential.

The minimal media, also called basal media for fermentation using *P. pastoris* are available as standard [7], [20] and modified basal media [4]. However, problems might still occur regarding the unbalance of nutrient composition, salt precipitation, and high osmotic pressure [7], [21]. We previously applied modified basal media in the flask culture, resulting in no or low HIP protein concentration (unpublished data). Therefore, evaluation of an appropriate medium is still important.

We have selected several clones of *P. pastoris* X-33, which confirmed to secrete HIP from the previous study [22]. The expression vectors were developed in our laboratory [23]. Preliminary optimization using the rich medium of BMGY and BMMY was done by involving pH, temperature, and methanol concentration. This study evaluates the HIP production at flask culture and bioreactor by using a basal medium. The concentration of basal medium was varied, and glycerol concentration, starting culture density, and methanol feeding profiles in flask cultivation. The influence of those factors on cell growth and HIP expression was evaluated. According to the information resulting from flask culture, fermentation in the bioreactor was carried on. We employed pulses and fed-batch methanol feeding in the bioreactor. Fed-batch feeding was modified by using constant flow rate - increasing feeding concentration as an alternative to a standard method, constant feeding – increasing flow rate to overcome instrument limitation.

II. Materials and Method

A. Strain and Media

Recombinant *P. pastoris* strain X-33/Mut$^+$, expressing HIP under the control of AOX1 promoter, was used. The *P. pastoris* genome was integrated with recombinant plasmid pD902-IP, which carries the gene encoding for the HIP. Cultivations to produce HIP were conducted in basal media (BSM), which were suitable for *P. pastoris*, namely BSM-1 and BSM-2 [4], [7], respectively, with a little modification. Glycerol concentration was varied from 10 to 90 g/L. Glycerol, basal medium, and trace salts were sterilized by autoclaving. Subsequently, trace salts were filter sterilized. Preculture for cultivation was prepared in YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L dextrose), containing 100 µg/mL zeocin.

B. Expression of the HIP in Flasks Culture

Preculture was prepared by inoculating the glycerol stock of *P. pastoris* into YPD medium. The volume ratio of glycerol stock to YPD culture was 1:20. The culture was grown in a shaking incubator at 30 °C and 250 rpm for 24 h. The cells were harvested by centrifugation at 3000 × g for 5 min at room temperature. The pellets were resuspended in the basal medium containing trace salt solution and glycerol. The concentration of basal medium was varied in the range of 25-100% (v/v). Glycerol concentration was varied from 10 to 90 g/L. Incubation of culture was carried on at 30 °C and 250 rpm. After 24 h, the cells were harvested and centrifuged as above, and the pellets were transferred into a basal medium containing trace salts solution and methanol. The concentration of basal medium was varied in the range of 25-100% (v/v). Methanol (100%) was added at the beginning of induction and every 24 h at a final concentration range of 1-3% (v/v). Samples were collected at least at the start point and 72 h of methanol induction to analyze cell density and protein. The culture broth was centrifuged at 10.000 rpm and 4 °C for 10 min. Subsequently, the supernatant was stored in separate tubes at -20 °C for protein assays.

C. Expression of the HIP in Bioreactor

Preculture was prepared in the flask by inoculating 0.5 mL glycerol stock of *P. pastoris* into 10 mL YPD medium. The culture was grown in a shaking incubator at 30 °C and 250 rpm for 24 h. The cells were harvested by centrifugation at 3000 × g for 5 min at room temperature. The pellets were resuspended in flask containing 100 mL of 50% BSM-2, 0.435% (v/v) trace salts, 8.7×10^{-5}% (w/v) biotin, and 40 g/L glycerol. Incubation was carried out at 30 °C and 250 rpm. After 24 h, the culture was inoculated into a bioreactor (Eppendorf BioFlo-120) containing 50% BSM-2 and 40 g/L glycerol at a total volume of one liter. Trace salts and biotin were added at concentration of 0.435% (v/v) and 8.7×10-5% (w/v) respectively. Antifoam 205 (Sigma Aldrich) was added at 0.01% (v/v). Fermentation was undertaken by controlling temperature, pH, dissolved oxygen (DO), aeration, and agitation. The temperature of the medium is maintained at 28 °C utilizing a heating plate and circulated chilled water around the vessel jacket. The level of pH was monitored and maintained at pH 5 by the addition of liquid ammonia 12.5% (v/v) and H_3PO_4 1 M. Flowrate of air was adjusted at 2 L/min. Dissolved oxygen was set at a minimum of 30% and cascaded with agitation speed at the range of 300-500 rpm. Methanol induction was conducted in pulses and fed-batch mode, starting after 24 h fermentation. Methanol % (v/v) in pulse feeding was added every 24 h, according to Table 1. Feed containing 30% methanol (v/v) was supplied at 0.1 ml/min flow rate during induction in fed-batch feeding. Feeds with 30, 40, and 50% (v/v) methanol concentrations were supplied in another fed-batch mode at the same flow rate. Trace salts and biotin were added to methanol feed at 12 mL of 100% methanol per liter. Samples were collected at the start point and every 24 h of methanol induction. The culture broth was centrifuged at 10.000 rpm and 4 °C for 10 min. Subsequently,

the supernatant was stored in separate tubes at -20 °C for protein assays.

D. Analytical Methods

Cell growth was determined by measuring OD_{600} and dry cell weight (DCW). In the measurement of DCW, the sample (1 mL) was centrifuged at 10.000 rpm for 10 min. The pellet was dried in an oven at 80 °C for 24 h and weighed. The purity of the HIP was determined by using the Tricine SDS-PAGE method described elsewhere [23]. The concentration of the HIP was determined semi-quantitatively by using ImageJ [24]. A series concentration of lysozyme was used to establish a standard curve. The HIP was quantified using reversed-phase HPLC (RP-HPLC), incorporating an Agilent HPLC system equipped with an autosampler and UV-Vis detector. Samples were filtered (0.2 µm) and mixed with an equal volume of mobile phase A [0.02 % (v/v) TFA in MilliQ water], then loaded into Jupiter Phenomenex C4 (300 Å, 250 mm L × 4.6 mm ID, 5 µm). Elution was conducted at 0.8 mL/min flow rate by using mobile phase A and B [0.02 % (v/v) TFA in acetonitrile] with gradient method as follow: 0 - 20%B (0 - 6 min), 20 - 46%B (6 - 32 min). The column effluent was monitored at 214 nm, while the column temperature was maintained at 25 °C. The standard curve for quantification was established by using insulin from the bovine pancreas.

III. Results and Discussion

A. Expression of the HIP in Flask Culture

1) Effect of Glycerol Concentration: The effect of glycerol on cell growth and HIP expression was studied in 10 mL flask cultures using BSM-1 medium. Glycerol concentration of up to 90 g/L was used as a carbon source during cell growth. After 24 h of the growth phase, cells were harvested and transferred into fresh BSM-1 containing 2% (v/v) of methanol. Cultures were grown until 72 h. The image of SDS-PAGE analysis showed that glycerol 10 and 30 g/L resulted in distinct protein bands compared to that of 60 and 90 g/L (Fig 1).

Glycerol concentration of more than 40 g/L could be toxic for the microorganism. However, other studies reported the ability of P. pastoris to grow at a high concentration of glycerol at 95.2 g/L [4]. High glycerol concentration will generally result in higher cell density and protein yield. However, it might also lead to ethanol accumulation as a bioproduct of glycerol utilization. It was predicted that ethanol consumption before methanol utilization could reduce protein secretion [25]. It was also indicated that the efficiency of glycerol utilization might be lower at higher concentrations. The optimum glycerol concentration for cell growth might depend on the target protein, strain, and transcriptional profiles [26], [27].

2) Effect of Basal Medium Concentration: The medium basal concentration effect was studied using BSM-2. Glycerol concentration of 40 g/L was used during the growth phase. After 72 h of methanol induction, the highest increase of cell density was observed at the OD_{600} from the culture with 100% BSM-2 compared to other concentrations (Fig 2). The protein band of the SDS-PAGE gel image of the sample from 100% BSM-2 culture was visible compared to the others.

The BSM-2 in this study is a minimal medium that contains a specific salt formulation [7], [20]. Limitations and excess nutrients could restrict the cell growth of *P. pastoris* [28] or the quality of expressed protein [29]. The higher concentrations of medium components, such as vitamins, histidine, EDTA, and Triton X-100 could positively affect protein yield. However, higher salt concentrations, for instance, KH_2PO_4 and $MgSO_4.7H_2O$ had negative effects [21].

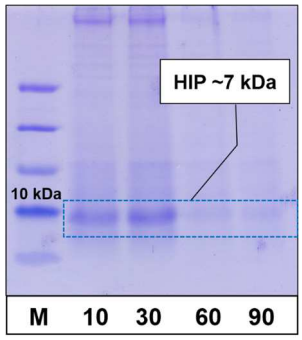
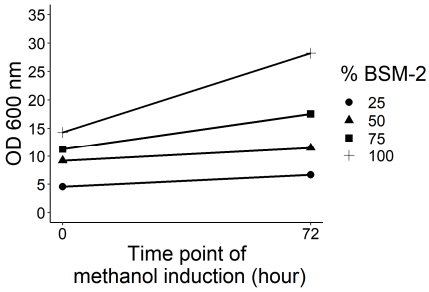


Fig. 1 SDS-PAGE gel image of HIP produced from flask cultures in BSM-1 medium incorporated different glycerol concentrations (10 – 90 g/L) during the cell growth phase. Samples were taken after 72 h of methanol induction.

(a)



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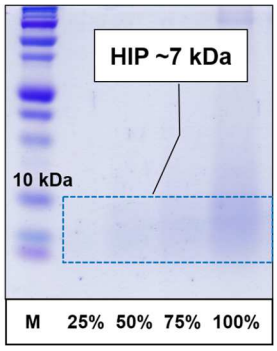

Fig. 2 Optical density and SDS-PAGE image of 10 mL flask culture. (a) OD_{600} (average, n=3) at the beginning and end of methanol induction (72 h). Methanol induction was conducted every 24 h at 2% (v/v). (b) Image of Tricine SDS-PAGE analysis result.

Other studies also revealed that the culture with the lowest salt concentration resulted in the highest growth rate [7], [30]. The high osmotic pressure at higher salt concentrations was predicted to limit cell growth and protein production. Therefore, a reduced concentration of basal medium can be evaluated at a larger scale to increase cost-efficiency.

3) Effect of Starting Culture Density: Starting OD_{600} on HIP expression was examined in BSM-1 and BSM-2 media, involving starting OD_{600} of ~ 15 and ~ 28. A fifty percent concentration of BSM-2 [7] was compared to a 100% concentration of BSM-1 [4]. There were higher increases in

OD_{600} from cultures with lower starting OD_{600}, after 72 h of methanol induction than from higher starting OD_{600} (Fig 3). There was an indication that at a higher cell density, the metabolisms were mainly targeted for protein secretion rather than for biomass generation [12]. In the report, cultures with initial densities below 40 OD_{600} nm U/mL exhibited a higher density after 48 h.

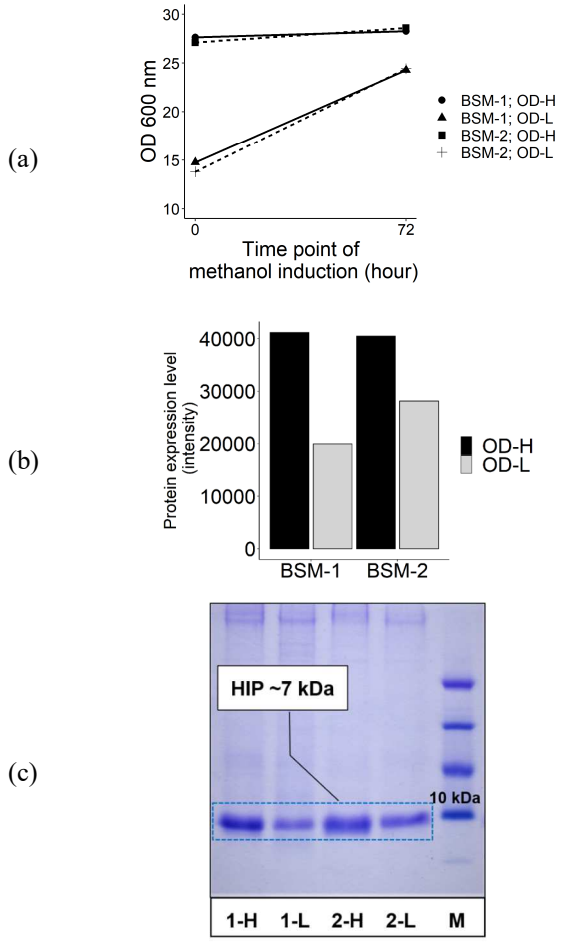

Fig. 3 Effect of starting OD*600* on *P. pastoris* grown in 10 ml of culture utilizing BSM-1 and BSM-2 media. (a) OD_{600} at the point of start and after 72 h of methanol induction. OD-L = lower starting OD_{600} of (~15); OD-H = higher starting OD_{600} of (~28). Solid line: BSM-1; dashed line: BSM-2. (b) The expression level of the HIP. (c) SDS-PAGE gel image of the experiment; 1-H and 1-L = BSM-1 of high and low starting OD_{600} respectively; 2-H and 2-L = BSM-2 of high and low starting OD_{600}, respectively. Samples were taken after 72 h of methanol induction.

In the current study, the accumulated HIP at 72 h resulted from the culture with higher starting density in both basal media was higher than that of the lower starting density culture, as represented by the intensity of the SDS-PAGE protein band (Fig 3b). At high starting density, BSM-1 and BSM-2 produced a comparable protein expression level.

The results indicated that expression of HIP could be increased by leveling up starting OD_{600} of culture to some point or by increasing inoculum volume. However, a study reported that a higher inoculum volume of 5% resulted in lower expression than that of 2% inoculum volume [13].

4) Effect of Methanol Concentration in Batch Induction Mode: Methanol induction was undertaken in the pulse method. In this study, a constant methanol concentration of 2% (v/v) and of gradual increase from 1 to 3% (v/v) were applied in a 125 mL culture containing 50% BSM-2. Pulse induction of methanol was also conducted at concentrations 0.5 - 2% in other studies [7], [16], [17].

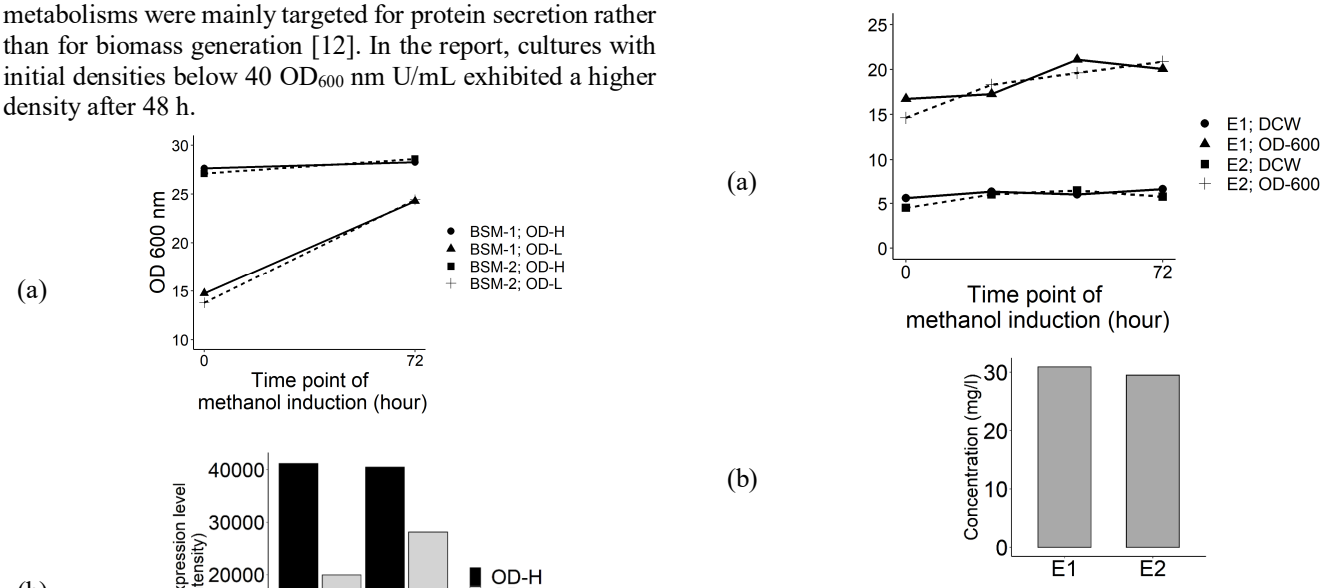

Fig. 4 Effect of methanol concentration on *P. pastoris* grown in 125 mL of culture utilizing BSM-2 medium. (a) OD_{600} and DCW (mg/L). (b) The concentration of HIP determined by RP-HPLC. Methanol concentrations (v/v) at 0, 24, and 48 h were 1, 2, and 3% respectively for E1 and 2% constantly for E2. The experiment was conducted in duplicate.

The profile of OD_{600} and DCW between the two experiments after 72 h methanol induction were quite similar (Fig 4a). Figure 4b showed that HIP concentrations after 72 h methanol induction were relatively low. Compared to bioreactors, lower aeration performance in shaking flasks might limit methanol utilization, resulting in lower cell density and protein expression. In the induction period, methanol has roles as a carbon source for biomass growth as well as an inducer for protein secretion. The metabolism of methanol demands a high amount of oxygen [15]. Despite that HIP concentration only differs slightly between the two experiments, higher HIP from E1 might indicate that a gradual increase of methanol concentration allowed the better adaptation of cells to methanol. Several studies addressed the importance of methanol adaptation by applying a stepwise increase of methanol concentration [4], [7], [18]. The effect of methanol concentration was evaluated further in the bioreactor.

B. Expression of the HIP in Bioreactor

1) Methanol Adaptation in Pulses Feeding Mode: To evaluate the effect of methanol adaptation in a larger scale of batch cultivation, the HIP production was undertaken in a one liter working volume fermentor. Glycerol concentration and methanol feeding profile are described in Table 1. Fermentation F1, F2, and F3 were aimed to study methanol adaptation, while F4 evaluated the effect of glycerol increase on HIP yield. Methanol concentration in fermentation F2 was higher at all induction periods than F1. Methanol concentration in F3 and F4 was the same as F2, but the initial induction in fermentation F3 was carried on at 48 h of fermentation time or delayed for 24 h compared to F1 and F2.

The concentration of HIP resulting from F1 at 48 and 72 h after initial induction was higher than F2 (Table 2). The lower concentration of methanol in F1 might result in better adaptation. Cell density might be insufficient for higher methanol concentration at the initial induction stage. As a result, accumulated methanol could be toxic and limit protein expression [4]. Fermentation F3 produced a higher HIP level at all time points than F1 and F2 (Fig 5). The delay of initial induction in F3 might result in total glycerol consumption. The reduced metabolic activity when glycerol was depleted in F3 was indicated by a sudden increase (spike) of dissolved oxygen (DO) at around 48 h (Fig 6b). The profile of DO in fermentation F1 was different from F2. Initial induction in F1 was carried on when glycerol level was still high. The absence of DO spike projected it at around 24 h (Fig 6a). A high concentration of glycerol at the early step of induction could influence protein expression. Metabolism of glycerol might result in ethanol as a by-product. This might reduce the performance of enzymes involved in methanol utilization, including the AOX1 promoter. Ethanol consumption might hamper protein secretion [25], [31].

Several periods of DO spike at around 100% DO, followed by several hours of high DO plateau profile can be seen in Figure 6b. The plateau could indicate the methanol starvation period. Some studies reported that methanol starvation periods might influence protein production. At certain hours, especially at the initial induction period, it could assist *P. pastoris* adaptation to avoid methanol accumulation. However, a long starvation period might reduce protein yield [10], [32]. DO spike at initial induction and methanol starvation periods in F3 was predicted to contribute to higher HIP production compared to F1 and F2.

The difference of glycerol effect between 40 g/L and 60 g/L was evaluated in fermentation F3 and F4. At the fermentation time of 24 h, when methanol induction started, DCW did not differ significantly (Fig 7). The increase in glycerol concentration did not result in a drastic rise of DCW during 24 h. Glycerol addition of 60 g/L might cause a slower growth of the cells. Another study revealed that involving 60 g/L glycerol resulted in a lower specific growth rate compared to 20 and 40 g/L. However, by adding 60 g/L glycerol, DCW at the point after glycerol was depleted and at maximum DCW were higher [10]. A similar phenomenon occurred in this study, where the DCW of F4 was higher than F3 after 24 h of fermentation (Fig 7). The HIP concentration of F4 surpassed that of F3 after 96 h of induction or at 120 h of fermentation time (Fig 5). At the earlier induction phase of F4, the excess of glycerol might be consumed prior to methanol to increase the biomass and repressed the AOX1 promoter to secrete HIP [33]. The induction level raised after glycerol was entirely consumed.

TABLE I
THE CONCENTRATION OF GLYCEROL AT A GROWTH PHASE AND METHANOL AT PULSE INDUCTION PHASE APPLIED IN ONE-LITER FERMENTATION

Fermentation	Glycerol (g/L)	Methanol (% v/v) added at fermentation time point					
		24 h	48 h	72 h	96 h	120 h	144 h
F1	40	1	2	3	-	-	-
F2	40	2	3	4	-	-	-
F3[(a)]	40	-	2	3	4	4	4
F4	60	2	3	4	4	4	-

[(a)]initial induction was delayed for 24 h.

TABLE II
OD_{600}, DCW, AND HIP CONCENTRATION OF FERMENTATION F1 AND F2 RESULTED FROM THE VARIATION OF METHANOL CONCENTRATIONS

Time after initial induction (h)	OD_{600}		DCW (g/l)		PHI concentration (mg/L)	
	F1	F2	F1	F2	F1	F2
24	53.3	53.7	27.3	25.0	0.3	0.6
48	56.2	51.1	28.5	26.4	110.1	10.2
72	64.2	59.1	31.2	31.2	221.9	66.9

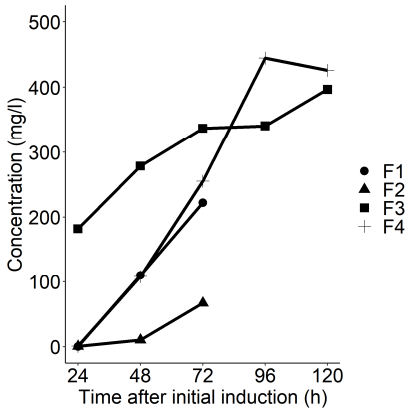

Fig. 5 Concentration of HIP resulted from pulses methanol induction, as determined by reversed-phase HPLC.

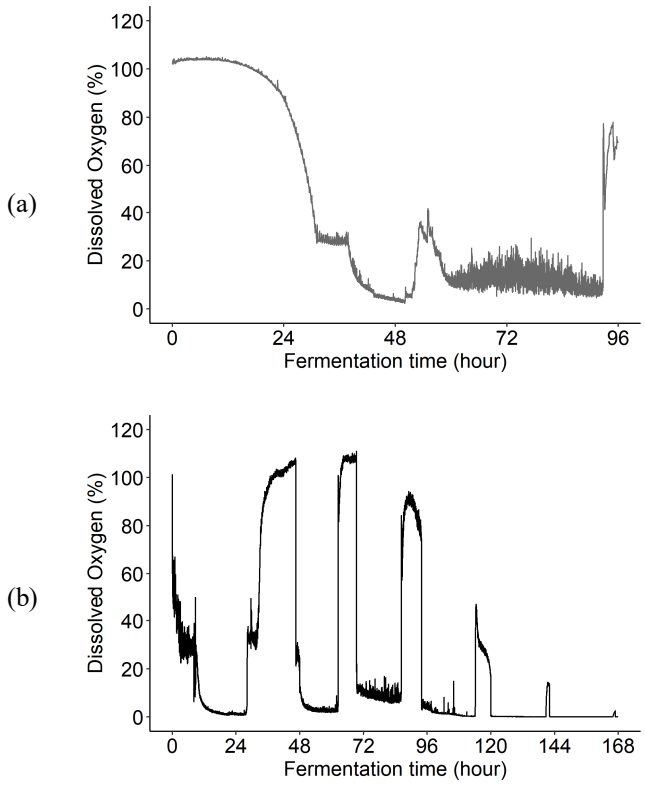

Fig. 6 Dissolved oxygen (DO) profile during fermentation. (a) DO profile of F1. (b) DO profile of F3.

2) Fed-batch Induction of Methanol: Methanol induction in fed-batch mode was purposed to supply methanol continuously while minimizing methanol accumulation. In this study, fed-batch induction employed methanol feed with constant and different concentrations over the induction

period. Methanol feed at 30% (v/v) was supplied at 0.1 mL/min flow rate in fermentation F5. Fermentation F6 involved methanol addition at the same flow rate but at feed concentrations of 30, 40, and 50% at the fermentation time points of 24, 48, and 72 h, respectively. The concentration of HIP resulting from fed-batch feeding (Fig 8) was lower than that from pulse feeding. For instance, the maximum HIP concentration from F4 and F5 were 445 and 267 mg/L, respectively. DCW in F6 decreased from the beginning of induction, although it leveled up at the end of fermentation (Fig 7).

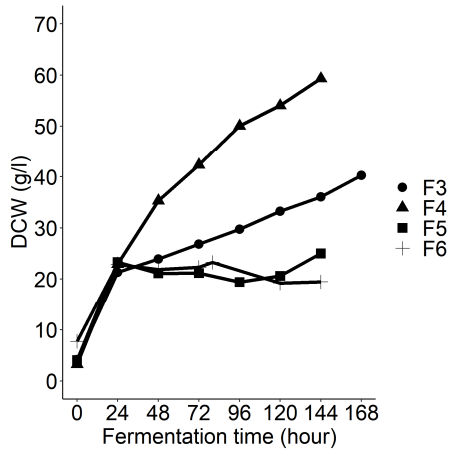

Fig. 7 DCW of *P. pastoris* culture in bioreactor cultivation.

The overall increase of DCW in F5 was significantly lower compared to F3 and F4. One possible cause of lower cell density was the dilution effect of continuous methanol supply. Culture volume at the end of fermentation could reach around 2 liters, compared to pulse feeding which was around 1 liter. Compared to F6, DCW of F5 increased from fermentation time 96 to 144 h, but the opposite profile occurred in F6. It is predicted that the methanol toxicity level in F6 was higher than F5 due to higher feed concentration. This might be detrimental to cells, hence limiting culture density and HIP production (Fig 8) [4], [7].

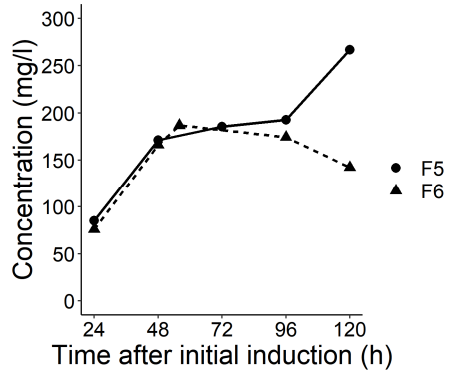

Fig. 8 Concentration of HIP resulted from fed-batch methanol induction, as determined by reversed-phase HPLC.

Methanol induction profile might result in variation of proteolytic degradation. Methanol metabolism demands a high amount of oxygen and could release hydrogen peroxide and formaldehyde as by-products. Those by-products might lead to cell death [34] and subsequently, proteases were released from dead cells. Proteolysis level could be higher when methanol is accumulated at limited oxygen environment. Proteolytic degradation could be limited by applying DO-stat strategy. Methanol is added when DO level increase above the setpoint [35]. The next study must control methanol concentration following the specific growth rate during the induction period.

IV. Conclusion

The higher amount of glycerol and methanol in pulse feeding contributed to the increase of HIP expression. However, allowing culture adaptation to methanol concentration is necessary by controlling methanol concentration during induction time based on culture density. It is predicted that the dilution effect and higher concentration of methanol in the fed-batch feeding method led to lower cell density and HIP expression. The best HIP concentrations in one-liter fermentation were ~267 and ~445 mg/L for feed-batch and pulse feeding. Fed-batch feeding with a gradual increase of methanol feed concentration can be an alternative to producing HIP in a bench-scale bioreactor. These methods can be implemented when fed-batch feeding with increasing flow rates, and 100% methanol feed is difficult to apply. The next study should incorporate other on/off-line measurements for better control of methanol feedings, such as measurement of methanol and carbon dioxide concentration.

Acknowledgment

This work was funded by The Indonesian Institute of Sciences (LIPI), The Ministry of Research and Technology/National Research and Innovation Agency (BRIN), and The Managing Agency of Endowment Fund for Education (LPDP), Republic of Indonesia.

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