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Research Article

Influence of Co-feeding Methanol-sorbitol Ratio on Production of Human Insulin Precursor Expressed by Mut$^+$ *Pichia pastoris*

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ABSTRACT

An increasing number of diabetic patients and the demand for insulin encourage the development of recombinant insulin production on a large scale. Human insulin precursor (HIP) expressed by Mut$^+$ *Pichia pastoris* using methanol as an inducer was developed. However, methanol above 5% (w/v) or 1.56 M is toxic for the host. Sorbitol was introduced as a co-substrate with methanol. To our knowledge, the study of methanol/sorbitol co-feeding on human insulin precursor (HIP) expression by Mut$^+$ *Pichia pastoris* in a bioreactor has yet to be reported. This study aimed to investigate the influence of the methanol-sorbitol co-feeding ratio on the expression of HIP expressed by *P. pastoris* X33 Mut$^+$. The study was conducted by comparing the cultivation of *P. pastoris*/pD902-IP Mut$^+$ in a 10-liter bioreactor under three conditions: feeding 100% methanol, mass ratio of MeOH:sorbitol 12:1 and 3:1. The oxygen consumption of methanol/sorbitol is less than the methanol feeding. The mass ratio of MeOH:sorbitol 12:1 produced the highest HIP titer (1326.5 mg/L), 1.5 times higher than methanol feeding, the lowest specific growth rate, but the highest specific productivity at the induction phase. MeOH:sorbitol mass ratio 3:1 produced the highest dry cell weight (DCW) amount (96 g/L). These results suggested that an appropriate ratio of sorbitol-methanol can be a choice to replace methanol feeding in a Mut$^+$ *P. pastoris*.

1. Introduction

Diabetes mellitus is a metabolic disease that is characterised by increasing blood sugar levels above the normal level because insulin is not produced or insulin receptor is insensitive. Globally, 537 million people were reported as diabetes patients in 2021. This number is predicted to increase up to 782 million in 2045 (International Diabetes Federation 2021). As a response to the trend, the global insulin market in 2023 reached 19.97 billion USD and will increase from 2024 to 2031 (Data Bridge 2024). Production of recombinant insulin by *Pichia pastoris* as a methylotrophic yeast has been widely employed (Annibali *et al.* 2014; Siew and Zhang 2021). The superior features of *Pichia*, such as the ability to grow in a defined medium to achieve high-density culture, having a proper eukaryotic post-translational modification, and producing a high level of heterologous protein, are described in many reviews (Baeshen *et al.* 2014; Byrne 2015; Barone *et al.* 2023). *P. pastoris* has a robust and tightly regulated alcohol oxidase1 (AOX1) promoter that is driven by methanol to induce heterologous protein expression (Özçelik *et al.* 2019). A Mut$^+$ strain of *P. pastoris* has *AOX1* and *AOX2* genes in its genome. This strain is sensitive to the availability of methanol because both *AOX1* and *AOX2* genes are active (Jia *et al.* 2017). This feature makes this strain a choice to host heterologous protein production under methanol regulation (Carly *et al.* 2016).

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Methanol, as a sole carbon source, has some disadvantages, such as consuming a high level of oxygen and producing high heat during cultivation (Wang *et al.* 2019). Methanol is also toxic to the cell when the concentration is above 5% (w/v) or equal to 1.56 M (Karbalaei *et al.* 2020). To overcome those issues, several carbon sources, such as glucose, glycerol, or sorbitol, were used in combination with methanol to reduce the adverse effects of methanol (Azadi *et al.* 2018). Among glucose and glycerol, sorbitol was the potential co-substrate with methanol because it is a non-repressive carbon source for the AOX1 promoter (Azadi *et al.* 2017). The mixed feeding of sorbitol and methanol can increase the expression of a porcine insulin precursor (PIP) in the flask scale (MeOH:sorbitol = 10:1 mass ratio) (Zhu *et al.* 2013). However, 1:1 C-mol MeOH/C-mol sorbitol (equal to MeOH: sorbitol = 1:1 mass ratio) did not increase the expression of host cell protein (Wang *et al.* 2019). To determine the optimal ratio of MeOH:sorbitol for HIP expression, cultivation of HIP host was conducted in two mass ratios, MeOH:sorbitol 12:1 and 3:1. The ratio was selected based on the previous studies to increase HIP expression above the pure methanol feeding.

Insertion of the recombinant plasmid into the genome of *P. pastoris* may affect the physiology of the yeast. In this research, we used an integrative vector pD902-IP carrying recombinant HIP cassette in the construct of 3921 bp plasmid. The theoretical size of the human insulin precursor is 7053 Da (Nurdiani *et al.* 2018). The vector was then inserted into the genome of *P. pastoris* X33. The selected strain *P. pastoris* X33/pD902-IP clone 4 (CL4) was confirmed as Mut^+ phenotype (Nurdiani *et al.* 2020). Cultivation of the strain using continuous methanol feeding in 1 L and 10 L bioreactor reported earlier with the highest HIP titer were 267 mg/L and 928 mg/L, respectively (Putro *et al.* 2022; Puspitasari *et al.* 2023). A preliminary study of co-feeding sorbitol with methanol as a mixed inducer of HIP in flask scale resulted in a comparative expression of HIP with the sole methanol feeding (Putro *et al.* 2023). Furthermore, the effect of sorbitol mixed feeding in the same concentration to various numbers of porcine insulin precursor copy genes resulted in different responses (Chen *et al.* 2017). However, the effect of the sorbitol ratio in mixed feeding on the production of HIP in bioreactors has yet to be studied. Thus, this study aimed to evaluate the influence of the sorbitol-methanol co-feeding ratio on the output of HIP in a fed-batch bioreactor. The growth kinetics of the producer strain were determined to establish process parameters for scaling up cultivation.

2. Materials and Methods

2.1. Strain and Media

A recombinant human insulin precursor gene in an integrative vector, pD902-IP (provided by ATUM), inserted in the genome of *P. pastoris* X33 (Invitrogen) was developed and selected earlier (Nurdiani *et al.* 2018; Nurdiani *et al.* 2020). The strain chosen, *Pichia pastoris* X33 Mut^+ pD902-IP clone 4 (or CL4), was employed in this research. The composition of Yeast Peptone Dextrose (YPD) medium was prepared according to (Kurniatin *et al.*2019), and the composition of media half concentration of Basal Salt Medium (½ BSM) supplemented with trace mineral salts (PTM1) was based on Wu *et al.* (2019).

2.2. Cultivation in Bioreactor

2.2.1. Preculture Preparation

Preculture was prepared from the working cell bank (WCB) of the selected strain. An amount of 500 µL of WCB was inoculated into 40 ml of YPD medium supplemented with 100 µg/ml zeocin (modified from Puspitasari *et al.* 2023). The culture was then incubated at 30°C and 250 rpm for 24 hours. The cells were then inoculated into ½ BSM medium and incubated at the condition above for 48 hours. Four hundred milliliters of inocula of ½ BSM medium was transferred into 3.6 L of ½ BSM media supplemented with PTM1 4.35 ml/L ½ BSM media in a 10 L bioreactor (MDFT-10N, BE Marubishi).

2.2.2. Batch cultivation

In the batch cultivation stage, the temperature was set at 30°C. Aeration and agitation were set at 2 L/min and 300-500 rpm, respectively. Dissolved oxygen (DO) was observed, and the pH culture broth was maintained at pH 5 with 25% (v/v) ammonia (Puspitasari *et al.* 2023). Antifoam 205 (Sigma Aldrich) was added when high foam was formed. Batch cultivation was finished when the DO spike occurred.

2.2.3. Protein Induction

After the DO spike, the inducer was supplied into the culture, and the temperature was lowered to 28°C. Pure methanol (A1) or a combination of mass ratio 12:1 MeOH:sorbitol (A2) and 3:1 MeOH:sorbitol (A3) supplemented with PTM1 12 ml/L was continually fed to a final concentration of 0.6 g/L/h. Samples were collected twice a day. The culture broth was centrifuged at 10,000 rpm and 4°C for 10 minutes; then, the supernatant was stored to be analyzed and purified.

2.3. Analytical Procedures

2.3.1. Cell Density Determination

DCW determination followed the previous work (Puspitasari *et al.* 2022) with a modification at the time of centrifugation. One milliliter of broth was centrifuged at 14,000 g for 10 minutes, and the cell pellet was washed with 1 ml reverse osmosis water before being dried in the oven at 90°C for 2 hours. It was then cooled in a desiccator for 30 minutes before weighing (Puspitasari *et al.* 2023).

2.3.2. Determination of HIP concentration

HIP quantification was done by separating the supernatant from the cell pellet and then filtering with 0.45 µm. Ten microliter of sample was loaded into an analytical RP-HPLC column (Shodex RP18-415, C18, 150 × 4.6 mm, 6 µm). The temperature column was 25°C. Elution was done with mobile phase A (0.1% v/v TFA in water) and mobile phase B (0.1% TFA in acetonitrile) with a gradient of mobile phase B as follows: 15-30% B (0-6 minutes); 30-100% B (6-18 minutes), which was modified from Puspitasari *et al.* (2023) by changing elution time of each gradient with the same total elution time. The flow rate was 1 ml/min. The eluted sample was monitored at 214 nm. HIP concentration in the sample was quantified using a standard curve of human insulin (Sigma).

2.3.3. Determination of Glycerol, Methanol, and Sorbitol Concentration in Broth Culture

Samples were centrifuged at 14,000 rpm, 10 minutes, and 4°C and filtrated with 0.45 µm. Ten microliters of filtrate sample were then loaded into the Aminex HPX-87 H column (Biorad). The mobile phase was H_2SO_4 0.008 N, with a flow rate of 0.7 ml/min (Puspitasari *et al.* 2023). The column temperature was 35°C. A refractive index detector was used to detect the content of glycerol, methanol, and sorbitol in eluted samples. The standard curve of glycerol, methanol, and sorbitol was used to quantify the content of that carbon source in the samples (Puspitasari *et al.* 2022).

2.4. Kinetics Determination

The following equation calculated the specific growth rate (µ):

$$\mu = (\ln(D_t V) - \ln(D_0 V_0)) / (t - t_0) \quad (1)$$

Where D_t is the concentration of biomass at a particular time (g/L) while D_0 is initial biomass concentration (g/L), V is the working volume in a specific time (L), V_0 is the initial working volume (L), t is cultivation time (h), and t_0 is initial time (h) (Looser *et al.* 2015). After the HIP concentration was known, the yield of product per biomass can be determined by plotting the HIP amount in the sample (mg) versus biomass amount (g) at the same time point. The gradient of that curve is the coefficient of yield product per biomass ($Y_{p/x}$).

Specific product formation rate (q_p) can be calculated with the following equation:

$$q_p = \mu Y_{p/x} \quad (2)$$

Where µ is the specific growth rate (h^{-1}), and $Y_{p/x}$ is the yield of product per biomass (mg/g).

3. Results

3.1. Dissolved Oxygen (DO) Profile of CL4

The percentage of DO was observed to evaluate the metabolic rate of the culture and determine the time to add inducer. In all processes (A1 to A3), the DO percentage exhibited a decrease from the initial point, followed by a sudden increase (DO spike) (Figure 1). At this point, the glycerol batch phase was ended, and the protein induction phase was started. After the inducer was added, the percentage of DO for methanol feeding (A1) was mainly under 100%, while for mixed feeding methanol and sorbitol (A2 and A3), the DO percentage was above 100% (Figure 1).

3.2. Cell density Determination

The cultivation process resulted in the biomass of yeast and secreted proteins. Biomass of CL4 was measured as dry cell weight (DCW) to evaluate each cultivation process. At the beginning of the process, the DCW of A1 was the highest among all. However, at the time above 90 hours, the DCW of A2 and A3 were above the DCW of A1 cultivation until the harvesting time (Figure 2A). At the harvesting time, the DCW concentration of A2 and A3 was 1.3 and 1.8 times higher than A1 (69 g/L, 96 g/L, and 52.8 g/L, respectively) (Figure 2B).

3.3. Determination of HIP Concentration

The HIP was expressed after the inducer was added. Based on the gene construct, the HIP was secreted into the media as an extracellular protein. The concentration of HIP was measured to investigate the effect of each inducer on the expression of HIP. The HIP titer profile of A1 and A2 cultivation is similar, except that in the harvesting time of HIP, A1 decreased, so the highest

HIP titer was produced by A2 cultivation (Figure 3A). However, the A3 cultivation with a bigger portion of sorbitol in the feeding mixture produced lower HIP than the pure methanol (A1) (Figure 3A). At the end, HIP concentration of A1, A2 and A3 were 891.1 mg/L, 1326.5 mg/L and 432.4 mg/L, respectively. This means the HIP of A2 was 1.5 times higher than A1, while the HIP of A3 was 0.5 times lower than A1 (Figure 3B).

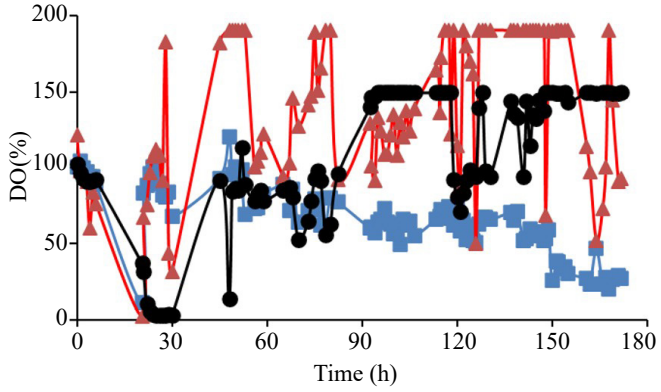

Figure 1. Dissolved oxygen (DO) profile of cultivation CL4 in a bioreactor. (■): A1, feeding methanol 100%; (●): A2 (12:1 MeOH/sorbitol, g/g) and (▲): A3 (3:1 MeOH/sorbitol, g/g)

3.4. Determination of Glycerol, Methanol, and Sorbitol Content in the Culture

In the cultivation, glycerol, methanol, and sorbitol were used as carbon sources to support the growth of the yeast. Methanol and sorbitol were added when the glycerol was exhausted in the media. Glycerol in all strategies (A1, A2, and A3) was totally consumed before methanol (A1) or methanol/sorbitol mixture (A2 and A3) was added to the media (Figure 4A). The methanol in the A1 strategy was entirely consumed, whereas the methanol in the A2 and A3 strategies was detected for some time points and subsequently was depleted (Figure 4B). Sorbitol in A2 was totally consumed, while in A3, it was presented at a one-time point and exhausted for the remaining time (Figure 4C).

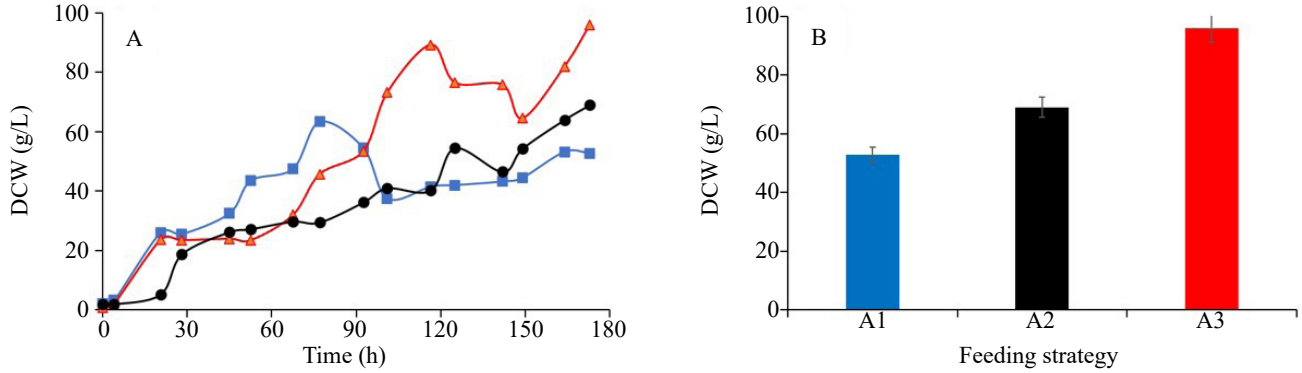

Figure 2. Dry cell weight (DCW) profile of CL4 in bioreactor 10 L. (A) DCW concentration during cultivation process. (B) DCW concentration at harvesting time. (■): A1, feeding methanol 100%; (●): A2 (12:1 MeOH/sorbitol, g/g) and (▲): A3 (3:1 MeOH/sorbitol, g/g)

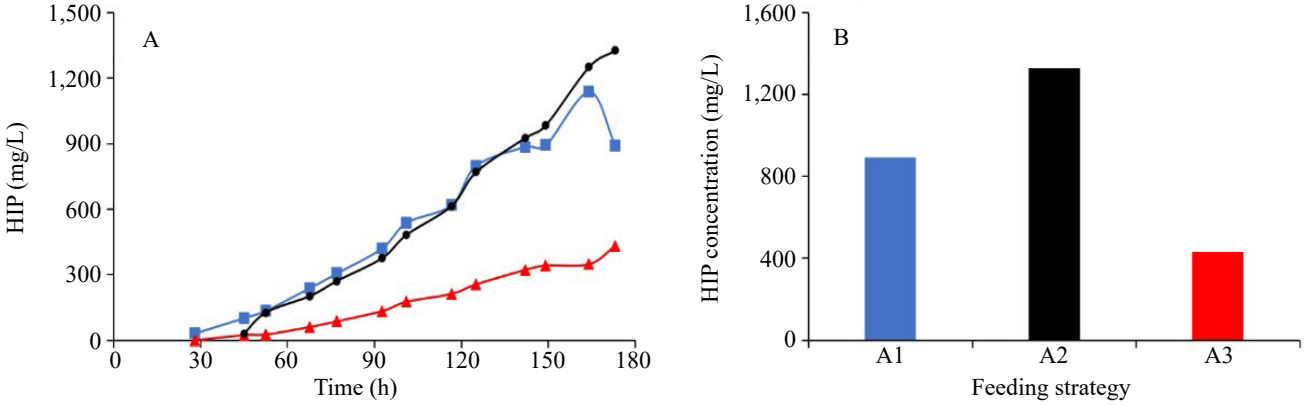

Figure 3. Human insulin precursor (HIP) profile expressed by CL4 in bioreactor 10 L. A: HIP concentration during cultivation. B: HIP concentration at harvesting time. (■): A1, feeding methanol 100%; (●): A2 (12:1 MeOH/sorbitol, g/g) and (▲): A3 (3:1 MeOH/sorbitol, g/g)

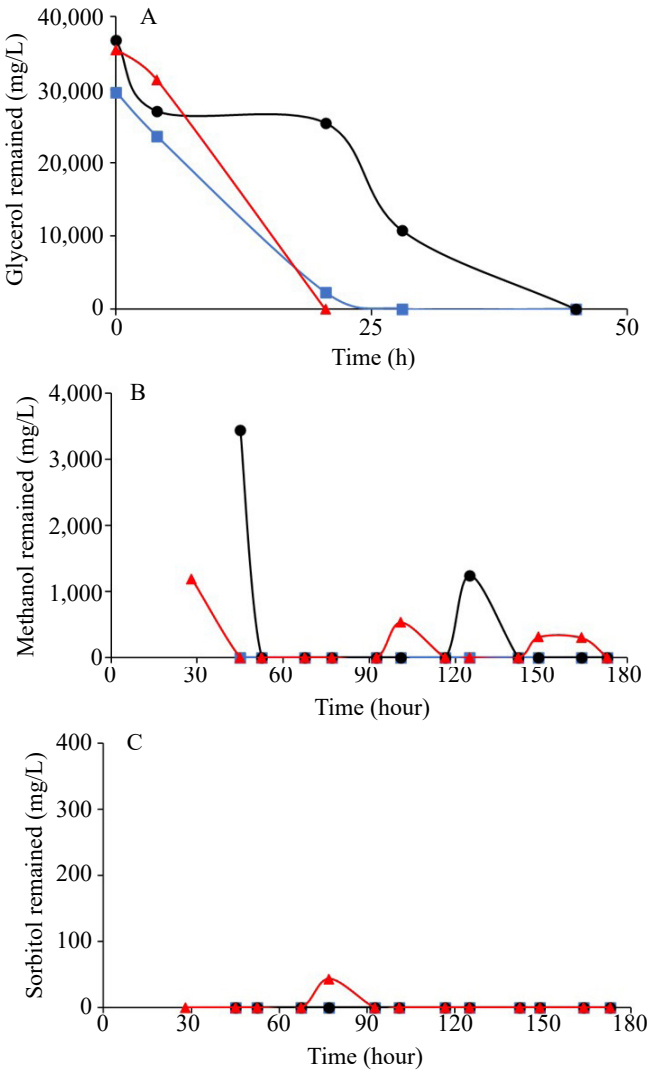

Figure 4. Carbon source content profile in the culture . A: Glycerol. B: Methanol. C: Sorbitol. (■): A1, feeding methanol 100%; (●): A2 (12:1 MeOH/sorbitol, g/g) and (▲): A3 (3:1 MeOH/sorbitol, g/g)

3.5. Determination of Strain-specific Parameter

Recombination of heterologous gene construct may affect the growth performance of host yeast. The optimal scale-up process can be designed by knowing the strain characteristic, such as the maximum specific growth rate (μ_{max}). The μ_{max} on glycerol of CL4 in all cultivation processes was 4.5-8.8 times higher than its μ_{max} on methanol. Gene construct and culture conditions may affect the production kinetics. Product formation kinetics is characterized by the optimal specific growth rate, $\mu(q_{p,\ max})$, specific productivity (q_p), and production kinetics profile. The $\mu(q_{p,\ max})$ of CL4 in the A2 condition was the lowest, while its $q_{p,\ max}$ was the highest compared with the A1 and A3 processes (Table 1).

The production kinetics of A2 are similar to that of A1, which has bell-shaped kinetics (Figure 5). This means the optimal production achieved is not at the maximal growth but at the μ of 0.011 and 0.007 for A1 and A2, respectively (Table 1 and Figure 5). Differently, the production kinetics of A3 exposed a linear relation between qp and its μ. The optimal production was achieved at the maximal μ, which was 0.015 (Table 1 and Figure 5).

4. Discussion

Large-scale production of recombinant insulin is needed to fulfill the increasing demand of the market. High cell density cultivation in the bioreactor is then developed to obtain a high amount of protein target. In the process, the percentage of dissolved oxygen dynamically changed depending on the culture condition. The DO concentration in the cultivation broth is a result of the relationship between oxygen transfer rate (OTR) (from the gas to liquid and the culture into the cells) and oxygen uptake rate (OUR) of the yeast for growth, maintenance, and production (Garcia-Ochoa and Gomez 2009). This means when the DO concentration is high, the OUR is low.

DO profile reflects the metabolism state of the yeast. In methanol metabolism, oxygen is needed to convert

Table 1. Kinetics characteristics of *P. pastoris* Mut$^+$ pD902-IP

	Cultivation condition	μ_{max} glycerol (h^{-1})	Product formation kinetics		
			μ-range (h^{-1})	$\mu(q_{p,\ max})$ (h^{-1})	$q_{p,\ max}$ (mg g^{-1} h^{-1})
A1	MeOH 100%	0.128	0.004-0.021	0.011	0.011
A2	12:1 mass ratio MeOH/ sorbitol	0.090	0.006-0.020	0.007	0.007
A3	3:1 mass ratio MeOH/ sorbitol	0.132	0.002-0.015	0.015	0.015

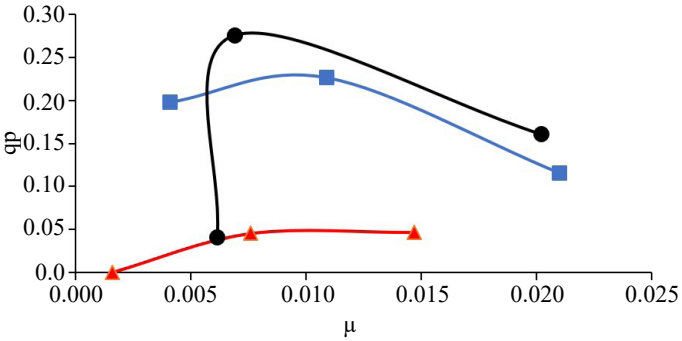

Figure 5. Production kinetics profile of CL4 in bioreactor 10 L. (■): A1, feeding methanol 100%; (●): A2 (12:1 MeOH/ sorbitol, g/g) and (▲): A3 (3:1 MeOH/sorbitol, g/g)

methanol into formaldehyde when methanol induces the expression of *AOX* genes (Jia *et al.* 2017). In the co-feeding strategy, the amount of methanol was reduced, so the consumption of oxygen in the culture was decreased. When the OUR decreases and OTR is constant, the DO value will be high. This result is in accordance with previous work, which reported that OUR of pure methanol feeding was higher than the mixture (Wang *et al.* 2019). The DCW concentration of A2 and A3 is higher than A1 (Figure 2B). This result is similar to previous work reported (Wang *et al.* 2019). The A3 strategy, which has a higher sorbitol concentration, resulted in the highest DCW concentration. However, it did not result in the highest HIP expression because the methanol portion decreased in the mixture. Interestingly, in the A2 strategy, where the sorbitol concentration is lower than A3, the DCW value is under the DCW of A3, but the HIP concentration is higher than A3. The HIP expression was driven by methanol because the HIP genes were located after the pAOX1 promoter (Nurdiani *et al.* 2018). In sole methanol feeding, methanol was used to synthesize both cells and protein. In the co-feeding strategy, sorbitol serves as an energy supplier, so methanol was optimally used for AOX transcription activities (Katla *et al.* 2020). Sorbitol should be in a low concentration and not accumulate in the broth to maintain the methanol consumption rate (Jia *et al.* 2017). In addition, glucose and glycerol were not selected because of their repression activity to AOX1 promoter (Wang *et al.* 2016). Sorbitol as co-substrate with methanol in an appropriate ratio can be a better inducer for heterologous protein expression under pAOX1 regulation in a Mut^+ strain.

Specific growth rate (μ) can indicate the condition of a strain after the insertion of a recombinant gene. The rate will be slower than the wild type if the recombination process impairs the existing genes. *Pichia pastoris* grows faster in glycerol than methanol as the μ_{max} glycerol is higher than μ_{max} methanol (Pentjuss *et al.* 2023). The maximum specific growth rate, μ (q_p, $_{max}$), of the A2 condition was the lowest compared with the A1 and A3 processes (Table 1). This results in accordance with Heyland *et al.* (2011), which states that the best producer strain normally grows slower.

An optimal cultivation process on a large scale can be designed by determining strain characterization, such as its μ_{max} in each carbon source. Biomass production can be controlled by setting the feeding rate to reach a particular μ value under its maximum value (Chongchittapiban *et al.* 2016). An optimal feeding rate can prevent substrate accumulation in the culture and yield a desirable protein concentration. The strain characteristic can be used to design optimal processes in scale-up by applying acceptable process parameter values, such as oxygen transfer rate (OTR) of the large scale in the preliminary experiment (Carly *et al.* 2016). In this work, glycerol was used to produce biomass at the beginning of the cultivation process. A methanol or methanol/sorbitol mixture was added after all glycerol had been depleted in order to prevent repression of glycerol in the AOX1 promoter. In the A2 and A3 processes, methanol was detected for a period (Figure 4B), which may be attributed to the reduction in methanol consumption rate by the yeast due to the availability of sorbitol in the media. However, at the end of the process, all methanol and sorbitol were consumed.

The relationship between qp and μ (production kinetics) reflects the equilibrium between various processes in a cell until the product is secreted (Looser *et al.* 2015). The bell-shaped kinetics of A1 and A2 (Figure 5) are similar to the porcine insulin precursor. This means the optimal production achieved is not at the maximal growth. The more pronounced bell-shaped production kinetic needs more tight control of μ during the cultivation process to avoid a worthy decrease in q_p (Garrigós-Martínez *et al.* 2019). Differently, the production kinetics of A3 (Figure 4) exposed a linear relation between q_p and its μ. This type of kinetics is known as growth coupled, and it is also recorded in the cultivation of Avidin (Looser *et al.* 2015).

In conclusion, the addition of co-substrate sorbitol to methanol fermentation has been demonstrated to affect the growth of biomass, the rate of oxygen consumption, and, as a consequence, the expression of the human insulin precursor. A higher sorbitol ratio in the 3:1 mass ratio MeOH:sorbitol resulted in an increased biomass amount. However, the expression of HIP did not exhibit a linear correlation with biomass growth. Nevertheless, a lower sorbitol ratio in 12:1 mass ratio MeOH:sorbitol resulted in the highest expression of HIP. These findings illustrate that the incorporation of sorbitol as a co-substrate with methanol has the potential to replace a pure methanol feeding strategy, thereby enhancing the production of the human insulin precursor. However, the present study employed only two mass ratios; therefore, it is not possible to conclude that the 12:1 MeOH:sorbitol ratio is the optimal ratio. It is recommended that cultivation be conducted with more than two mass ratios and that the ratio be adjusted to create a smaller gap to determine the optimal ratio. The expression of HIP can be increased by applying the kinetic data to design an optimal feeding rate in the next cultivation.

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