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Enhancement in Human Insulin Precursor Secretion by *Pichia pastoris* through Modification of Expression Conditions

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ABSTRACT

***Pichia pastoris* is an alternative yeast expression system to produce heterologous proteins. It has excellent characteristics for an industrial cell factory, such as its ability to reach high cell densities, high secretory capacity, and a low level of native proteins. In our previous study, we introduced a synthetic insulin precursor (IP)-encoding gene constructed in a pD902 expression vector into *P. pastoris*. However, the *P. pastoris* recombinant strains expressed a little amount of IP protein. Here, we modified the expression conditions, including inoculum density, methanol concentration, methanol induction time, pH, and temperature, to obtain a higher amount of secreted IP than our previous result. Protein analysis for studying the five parameters was conducted by SDS-PAGE, and the protein amount was estimated by ImageJ applying lysozyme as standard. We successfully enhanced the IP expression by modifying expression conditions. The highest increased of up to 100 folds was achieved when methanol concentration for induction was arranged at 3% (v/v), and the initial cell density for methanol induction was set at an optical density at 600 nm (OD_{600}) of approximately 10 compared to the standard procedure, where the expression was set at 0.5% (v/v) methanol induction and initial cell density at OD_{600} = 1.**

1. Introduction

Human insulin (HI) is a polypeptide hormone consisting of 51 amino acids (aa) residues, which plays an essential role in the regulation of blood glucose levels. Therefore, it is extensively used for the treatment of diabetic patients (Gurramkonda *et al.* 2010). Polypeptide precursor of HI, namely preproinsulin, comprised of 108 aa containing a signal sequence at its N-terminus. The preproinsulin is subsequently cleaved by specific endopeptidase to form proinsulin. Proinsulin is then converted into mature insulin by cleaving 34 aa of C-peptide. The mature insulin has a molecular weight of 5807 Da and contains two polypeptide chains, A- and B-chains, each comprised of 21 and 30 aa, respectively. Two disulfide bonds interlink the A- and B-chains, and the A-chain contains an intra-disulfide bond (Fu *et al.* 2013; Baeshen NA *et al.* 2014). The present recombinant production of HI majorly employed *E. coli* and yeast systems (mainly *Saccharomyces cerevisiae*) (Meehl and Stadheim 2014). *Pichia pastoris* has appeared as an alternate yeast host for HI recombinant production (Kjeldsen *et al.* 1999; Wang *et al.* 2001; Xie *et al.* 2008; Gurramkonda *et al.* 2010; Polez *et al.* 2016; Baeshen *et al.* 2016; Wu *et al.* 2019).

The methylotrophic yeast *P. pastoris* is a high performing expression system for recombinant protein secretion into the culture medium (Cereghino *et al.* 2002). It can produce more than 10 g/l of protein in the culture supernatant (Werten *et al.* 1999). In the recent decade, *P. pastoris* becomes preferable than *S. cerevisiae* for recombinant protein expression system due to its ability to produce high titers by avoiding sugar fermentation to eliminate the toxic fermentative product ethanol. Therefore, it can reach a very high cell density of up to 130 g/l dry cell weight (DCW) similar to the *S. cerevisiae*, which can reach up to 140 g/l (Aw 2013). Furthermore, as a methylotrophic yeast, *P. pastoris* utilizes methanol as a sole carbon source controlled by a strong *AOX1* promoter, showing a 1,000-fold induction upon methanol addition (Cereghino *et al.* 2002).

Methanol plays as an inducer for heterologous protein expression and the carbon source for cell growth with high oxygen requirements (Cereghino and Cregg 2000). Standard heterologous protein

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expression in *P. pastoris* comprised of two stages, which substantially control between the two substrates, glycerol and methanol feeding, during the fermentation period at a preserved temperature of 30°C. The glycerol carbon source is switched to methanol when the cell growth achieved a particular concentration level, and methanol concentration is sustained at a sufficient level to express the heterologous proteins. The standard procedure of heterologous protein production usually associates with several problems, including lower fermentation productivity caused by the extended cell adaptation period after changing the induction setting, the sustainability of foreign protein accumulation at a more extended induction period, and comparably low foreign protein activity. Therefore the optimization of the induction stage process is more challenging than the growth stage (Jin *et al.* 2010).

In our previous study, we have established *P. pastoris* recombinant clone harboring synthetic human insulin precursor encoding gene. However, the IP expression applying standard procedure resulted in a little amount of IP protein yielding ~30 mg/l protein (Nurdiani *et al.* 2018). Here in this study, we aimed to optimize the induction process by modulating five operation variables, i.e. inoculum density, methanol concentration, time point methanol induction, induction pH, and induction temperature to enhance IP protein expression in flask scale.

2. Materials and Methods

2.1. Strains

P. pastoris recombinant strain (CL-4), used in this study, expresses human insulin precursor. It was established in our previous study (Nurdiani *et al.* 2018), derived from *P. pastoris* X33 strain (Invitrogen, Carlsbad, CA).

2.2. Precultures

A single colony of *P. pastoris* recombinant strain from yeast extract peptone dextrose (YPD) agar plate [1% (w/v) yeast extract, 2% (w/v) peptone, 2% w/v glucose, and 2% (v/v) agar] supplemented with Zeocin 100 µg/ml, was inoculated into 10 ml BMGY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mm potassium phosphate pH 6.0, 1.34% (w/v) YNB, 4×10^{-5}% (w/v) biotin, and 1% (v/v) glycerol] in a 100 ml flask and grown at 30°C in a shaking incubator (250 rpm) for approximately 16-18 h. The cells were harvested by centrifuging at 1,500-3,000 × g for 5 min at room temperature. The cell pellet was used for the expression with starting cell density at OD_{600} ≈ 1 in the induction medium.

On the other hand, for all experiments applying inoculum density at OD_{600} ≈ 10 in the induction medium, 2 ml (YPD) medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% w/v glucose] supplemented with Zeocin 100 µg/ml was inoculated with a single colony of *P. pastoris* from a YPD + Zeocin (100 µg/ml) agar plate and shaken at 250-300 rpm and 30°C for 48 h. Cells were harvested by centrifuging at 1,500-3,000 × g for 5 min at room temperature and the cell pellet was transferred into 10 ml BMGY in 100 ml flask and shaken at 300 rpm and 30°C for 24 h.

2.3. Optimization of IP Expression Conditions

We studied the effect of five parameters during methanol induction, i.e., inoculum densities, methanol concentration, time point production, temperature, and pH to enhance the IP expression. Each parameter was assessed individually using a one-factor-at time approach.

The effect of inoculum densities was investigated by applying OD_{600} ≈ 1 and 10 in 10 ml BMMY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mm potassium phosphate pH 6.0, 1.34% (w/v) YNB, 4×10^{-5}% (w/v) biotin, 0.5% (v/v) methanol] in 100 ml flasks. A 100% methanol was added to a final concentration of 0.5% methanol every 24 h to maintain induction. After 3 d methanol induction, the supernatant was harvested by centrifuging at maximum speed for 3 min at room temperature. The supernatant was then transferred to a separate tube and stored at -20°C until ready to assay.

The effect of methanol concentration on the induction phase was investigated by setting the methanol final concentration at 0, 0.5, 1, 2, 3, 4, and 5% (v/v) in 10 ml BMMY medium in 100 ml flasks. The methanol was added at an interval of 24 hours to maintain induction. After 3 days of methanol induction, the supernatant was harvested by centrifugation at maximum speed for 3 min at room temperature. The supernatant was then transferred to a separate tube and stored at -20°C until ready to assay.

For the determination of the time point of IP production, the cell pellets from BMGY culture were resuspended in 25 ml BMMY medium with methanol induction was set in a final concentration of 2% (v/v). In both the BMGY and BMMY medium, the culture was placed in a 250 ml flask. A 100% methanol was added to a final concentration of 2% methanol every 24 h to maintain induction. For the analysis of the expression levels and to determine the optimal time post-induction to harvest, a 1 ml of the expression culture was sampled to a 1.5 ml microcentrifuge tube at an interval of 24 hours until 192 hours after methanol induction.

The supernatant was harvested by centrifugation at maximum speed in a tabletop microcentrifuge for 3 min at room temperature. The supernatant was then displaced into a new 1.5 ml microcentrifuge tube and stored at -20°C until ready to assay.

The effect of induction pH was investigated by setting the 10 ml BMMY medium pH at 4, 5, 5.5, 6, 6.5, 7, and 7.5 in each 100 ml flask. A 100% methanol was added to a final concentration of 2% methanol every 24 h to maintain induction. After 3 d methanol induction, the supernatant was harvested by centrifugation at maximum speed for 3 min at room temperature. The supernatant was then transferred to a separate tube and stored at -20°C until ready to assay.

The effect of induction temperature was investigated by setting the following temperature of 30, 28, and 27°C in each BMMY flask. A 100% methanol was added to a final concentration of 2% methanol every 24 h to maintain induction. After 3 d methanol induction, the supernatant was harvested by centrifugation at maximum speed for 3 min at room temperature. The supernatant was then transferred to a separate tube and stored at -20°C until ready to assay.

2.4. Protein Analysis and Quantification

Protein analysis was conducted by SDS-PAGE and Western blot analysis. The supernatant of culture broth samples was analyzed by denaturing 15% polyacrylamide gel-electrophoresis using a Tricine buffer system (Haider *et al.* 2012). The samples (20 µl) were mixed with an equal volume of Tricine sample buffer, mixed and boiled for 15 min. Samples were loaded on to the gel (20 µl per lane) and electrophoresed, and the separated polypeptides were visualized using Coomassie Brilliant Blue Solution (BioRad) for SDS-PAGE analysis. In Western blot, the gels after electrophoresis were blotted onto nitrocellulose membrane using a wet-type blotting system (Bio-Rad). The transfer buffer consisted of 25 mm Tris-HCl at pH 8.3, 192 mm glycine, 0.05% SDS, and 20% methanol. The blotted membrane was briefly washed in TTBS [TBS (50 mm Tris-HCl at pH 7.4, 150 mm NaCl) containing 0.1% Tween 20] and followed by the blocking step where the membrane was soaked with blocking buffer (1% skim milk and 0.1% BSA in TTBS) for 1 h at room temperature. The membrane was then immersed in the solution of the primary antibody Anti-Insulin/Proinsulin Monoclonal Antibody (ThermoFisher Scientific), shaken for 1 hour at room temperature, followed by incubation at 4°C overnight. After three washing steps with TTBS, the membrane was incubated with the appropriate secondary antibody, anti-mouse IgG (H+L) AP conjugate (Promega), for 1 h at room temperature. The membrane was then washed 3 times with TTBS, air-dried and incubated with NBT-BCIP for 5-10 min in the darkroom.

Protein concentration in tricine SDS-PAGE was quantified using ImageJ software by applying lysozyme as standard ranging from 0.03–4.0 mg/ml.

3. Results

3.1. Effect of Inoculum Densities During Methanol Induction

In our previous study, we obtained Mut+ *P. pastoris* recombinant clone harboring IP encoding gene. We confirmed the IP protein expression in one of *P. pastoris* recombinant clones, CL-4. However, it resulted in a little amount of secreted IP protein, which requires 10 times concentrated culture supernatant for visualization in the SDS-PAGE gel electrophoresis. The standard procedure of *Pichia* expression in a shake flask, according to the manufacturer's instruction, suggests that for Mut+ strain, a starting $OD_{600} \approx 1$ in BMMY medium could use, however, higher cell densities can be applied to increase protein expression. Figure 1a shows the SDS-PAGE electrophoresis results, which revealed IP bands above 6.5 kDa. The imageJ analysis of those IP bands indicated that the IP band of $OD_{600} \approx 10$ has a higher intensity than the IP band of $OD_{600} \approx 1$ (Figure 1b). Subsequent ImageJ analysis of the secreted IP by using lysozyme as standard estimated that the induction started at the $OD_{600} \approx 10$ resulted in 12 folds higher secreted IP than $OD_{600} \approx 1$ (Table 1).

3.2. Effect of Methanol Concentration to the IP Expression

To investigate the effect of methanol concentration on the IP expression. We added 100% methanol for induction to several final concentrations in the BMMY medium ranging from 0.5 to 5.0% (v/v). The other expression condition was set as follows: inoculum density was set at $OD_{600} \approx 10$, induction time was 72 h, induction pH was 6.0, and induction temperature was 30°C. Figure 2a shows the IP bands (above 6.5 kDa) detected in SDS-PAGE analysis of the culture supernatant induced by methanol levels of 0.5 to 5.0%. The secretion of the IP protein was also confirmed by Western blot analysis, which exhibited a single IP band of ~7 kDa (Figure 2b). The expression level gradually increased corresponding to the increase of methanol added up to 3% and gradually decreased by the addition of methanol up to 5% (Figure 2c). Besides inducing IP protein expression, methanol addition in the second phase of cultivation in BMMY medium enhanced cell biomass. As shown in Table 2, the final cell densities of the cultures of the *P. pastoris* recombinant strain added with methanol were higher than the final cell density of the culture without methanol addition (0%).

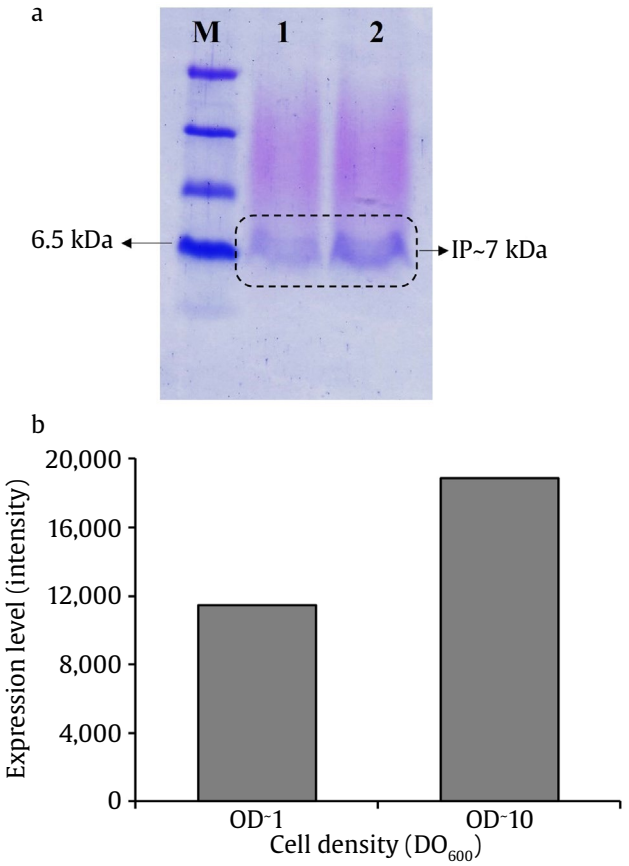

Figure 1. Protein analysis of *P. pastoris* recombinant clone at two different starting OD in BMMY medium. (a) SDS-PAGE of recombinant human insulin precursor in 15% SDS-PAGE gel. M = Polypeptide SDS-PAGE molecular weight standards; lane 1 = expressed IP from *P. pastoris* recombinant strain (CL-4) 72 h post-methanol induction with starting $OD_{600} \approx 1$; lane 2 = expressed IP from *P. pastoris* recombinant strain (CL-4) 72 h post-methanol induction with starting $OD_{600} \approx 10$. (b) The expression level analyzed by using ImageJ

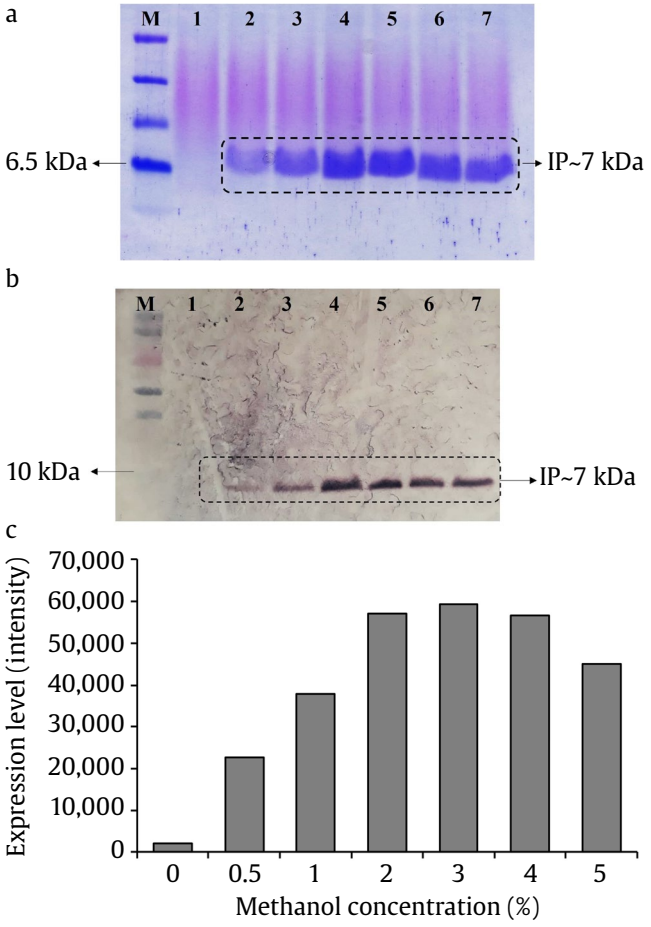

Figure 2. Effect of methanol induction. (a) SDS-PAGE of recombinant human insulin precursor in 15% SDS-PAGE gel. M = Polypeptide SDS-PAGE molecular weight standards; lane 1–7= expressed IP from *P. pastoris* recombinant clone (CL-4) with methanol 100% added for induction to a final concentration of 0, 0.5, 1, 2, 3, 4, and 5% (v/v), respectively. (b) Western blot analysis of expressed IP from *P. pastoris* recombinant clone (CL-4) for methanol induction analysis. M = Prestained precision plus proteinTM dual colour standards; lane 1–7 = expressed IP from *P. pastoris* recombinant clone (CL-4) with methanol 100% added for induction to a final concentration of 0, 0.5, 1, 2, 3, 4, and 5% (v/v), respectively. (c) Expression level analyzed by using ImageJ

Table 1. Effect of starting cell densities to the amount of secreted IP (mg/l) in the culture supernatant

Starting cell densities (OD_{600})	Secreted IP (mg/l)
1.768	23.97
10.57	292.40

3.3. Determination of Time Point Post-methanol Induction to Harvest IP Protein

To determine the time point post-methanol induction to harvest IP protein in shake flask, we observed cell density and IP secretion at several time points from 0 to 192 h post-methanol induction. The expression conditions were set as follows: cell density was started at OD_{600} = 11.82, methanol induction was set at final concentration 2% (v/v), induction pH was 6.0, and induction temperature was 30°C. Figure 3a shows secreted IP in the culture supernatant from 0–192 h post-methanol induction. The IP bands of the culture supernatant of 24-48 h post-methanol induction indicate a molecular weight size above 6.5 kDa. Figure 3b shows that the IP expression gradually increased corresponding to the increase of cell densities up to 72 h post-induction. After 72 h post-induction, the cell was grown slower followed by the decreased secreted IP (Table 3).

Table 2. Effect of methanol concentration to the amount of secreted IP (mg/l) in the culture supernatant

Methanol concentration (%)	Starting cell density (OD_{600})	Final cell density (OD_{600})	Final cell density (cells/ml × 10^8)[a]	Secreted IP (mg/l)	Yield of IP (mg/10^8 cells)
0	10.57	13.49	6.8	-602	-
0.5	10.57	14.38	7.2	422	0.06
1	10.57	15.49	7.8	1189	0.15
2	10.57	15.91	8.0	2147	0.27
3	10.57	15.44	7.7	2267	0.29
4	10.57	14.59	7.3	2128	0.29
5	10.57	14.98	7.5	1550	0.21

[a]The final cell density (cells/ml × 10^8) was calculated from the amount of the final cell densities (OD_{600}) where one OD_{600} = ~5 × 10^7 cells/ml

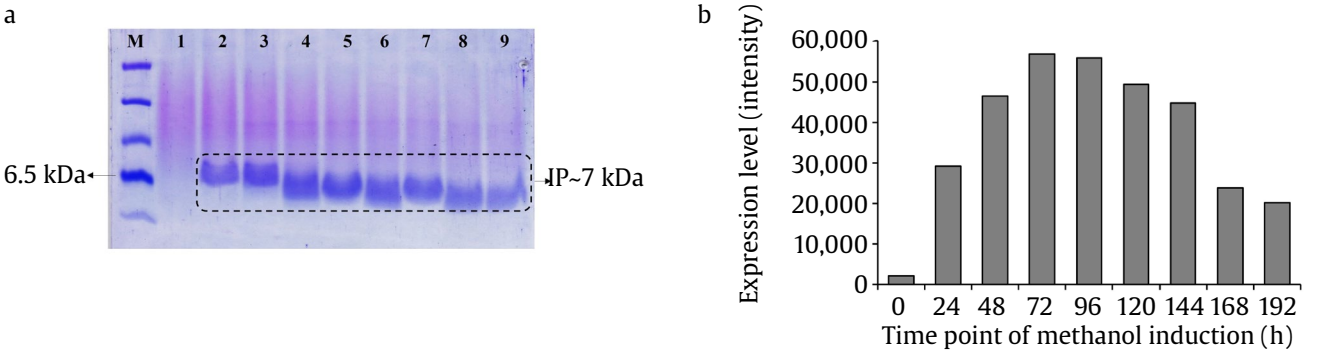

Figure 3. Timepoint of IP production. (a) SDS-PAGE of recombinant human insulin precursor in 15% SDS-PAGE gel. M = Polypeptide SDS-PAGE molecular weight standards; lane 1–9 = secreted IP in the culture supernatant of *P. pastoris* recombinant clone (CL-4) at 0, 24, 48, 72, 96, 120, 144, 168, and 192 h post-methanol induction, respectively. (b) The expression level analyzed by using ImageJ

Table 3. The amount of secreted IP (mg/l) in the culture supernatant at each time point methanol induction

Time point methanol induction (h)	Cell density (OD_{600})	Cell density (cell/ml × 10^8)[a]	Secreted IP (mg/l)	Yield of IP (mg/10^8 cells)
0	11.82	5.91	-601	-
24	15.44	7.72	756	0.09
48	19.54	9.77	1617	0.17
72	19.89	9.95	2135	0.21
96	19.88	9.94	2083	0.21
120	19.97	9.99	1756	0.18
144	20.10	10.05	1532	0.15
168	20.13	10.07	486	0.05
192	21.35	10.68	305	0.03

[a]The final cell density (cells/ml × 10^8) was calculated from the amount of the final cell densities (OD_{600}) where one OD_{600} = ~5 × 10^7 cells/ml

3.4. Effect of Induction pH on IP Production

Here, we studied the effect of pH on the IP expression in *P. pastoris* recombinant strain with induction pH ranging from 4.0 to 7.5. The other expression condition was set as follows: inoculum density was set at OD_{600} = 15.21, induction time was 72 h, and induction temperature was 30°C. Figure 4a depicts that IP bands in SDS-PAGE electrophoresis were detected above 6.5 kDa in all pH range tested. The expression level of IP at pH 4.0-5.5 and 6.5-7.5 have slightly higher intensities than IP at pH 6 (Figure 4b). The estimated concentration of secreted IP at pH 6 was 715 g/l which is the lowest concentration compared to the secreted IPs at pH 4.0-5.5 and 6.5-7.5 which ranging from 817-1,029 g/l (Table 4).

3.5. Effect of Induction Temperature on IP Production

To study the effect of induction temperatures, we conducted IP expression in BMMY induction medium at 27, 28, and 30°C while other expression conditions were set as follows: cell density started

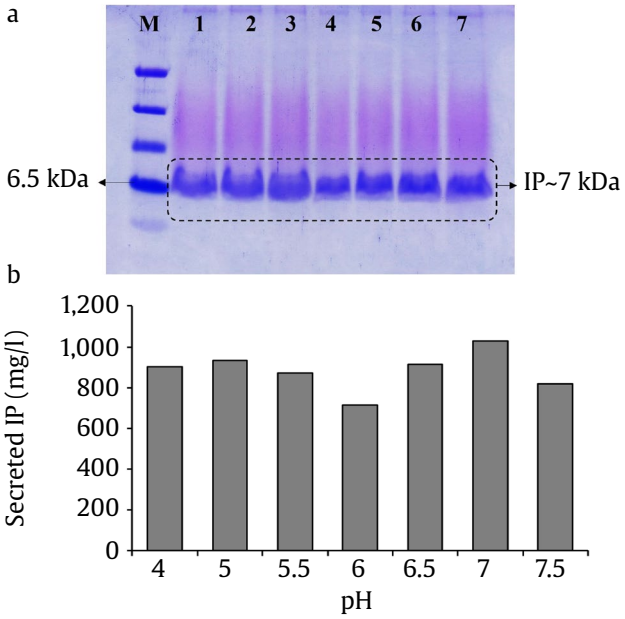

Figure 4. Effect of induction pH on IP production. (a) SDS-PAGE of recombinant human insulin precursor in 15% SDS-PAGE gel. M = Polypeptide SDS-PAGE molecular weight standards; lane 1–7 = expressed IP in the culture supernatant of *P. pastoris* recombinant clone (CL-4) at the pH of 4, 5, 5.5, 6, 6.5, 7, and 7.5, respectively. (b) The expression level analyzed by using ImageJ

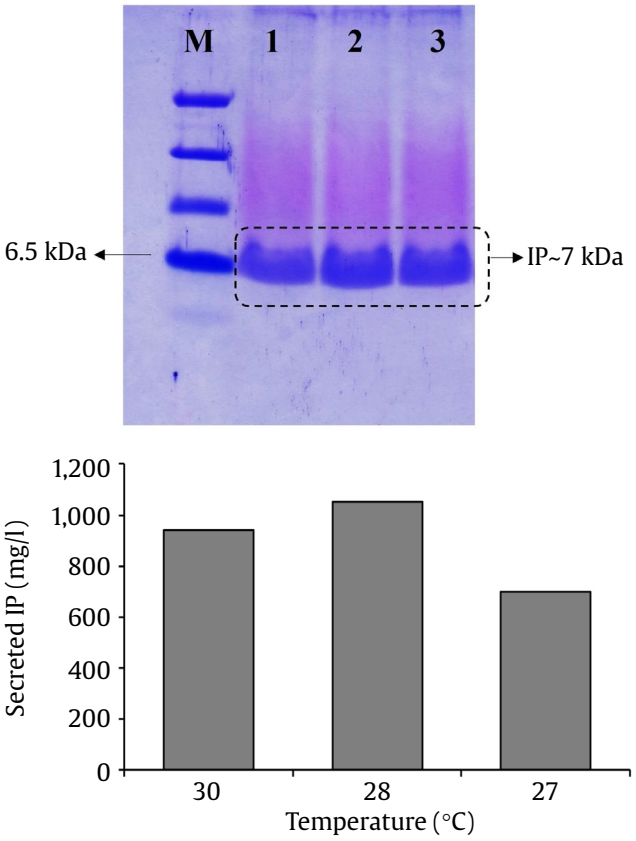

Figure 5. Effect of induction temperature. (a) SDS-PAGE of recombinant human insulin precursor in 15% SDS-PAGE gel. M = Polypeptide SDS-PAGE molecular weight standards; lane 1–3 = expressed IP in the culture supernatant of *P. pastoris* recombinant clone (CL-4) at the temperature of 30, 28, and 27°C, respectively. (b) The expression level analyzed by using ImageJ

at OD_{600} = 14.65, pH 6 and 2% methanol induction. The SDS-PAGE electrophoresis confirmed that the IP bands were above 6.5 kDa in all culture supernatants of three induction temperatures (Figure 5a). The highest IP expression level was observed at 28°C induction temperature with an estimated concentration of IP protein of 1,054 mg/l (Figure 5b, Table 5).

4. Discussion

Commonly, heterologous protein production is conducted in a very high cell density since the protein production practically equivalent to the cell density (Shi *et al.* 2003; Gurramkonda *et al.* 2010; Wu *et al.* 2019). High-cell density cultivation of yeast recombinants for the heterologous protein expression under the control of the *AOX1* promoter (*PAOX1*) consists of two main phases. In the first phase, the yeast usually utilizes glycerol, a non-inducible and repressive substrate, to obtain high biomass yield, and in the second phase, methanol is used as both inducer for foreign protein expression and carbon source (Katakura *et al.* 1998; Trinh *et al.* 2003; Cos *et al.* 2005). Aw (2013) applied $OD_{600} \approx 10$ to start methanol induction for small scale heterologous expression of human serum albumin (HSA) in *P. pastoris*. In this study, we confirmed that higher cell density increased secreted IP. We compared two inoculum densities, $OD_{600} \approx 1$ and $OD_{600} \approx 10$, while other parameters were set as follows: methanol induction was set at 0.5% (v/v), induction time was 72 h, induction pH was 6.0, and induction temperature 30°C. Similarly, Shi *et al.* (2003) investigated the effect of inoculum densities at OD_{600} from 10 to 100 in the ScFv protein expression in a baffled flask. Their study revealed that at higher cell densities, more resources were directed to protein secretion rather than to cell biomass production.

Alcohol oxidase (AOX) enzymes, the first enzymes involved in methanol utilization (Mut) pathway in *P. pastoris*, are encoded by *AOX1* and *AOX2* genes (Juturu and Wu 2018). The *AOX1* gene expression is regulated by *AOX1* strong promoter; hence, it employs to drive the heterologous expression of

Table 4. Effect of induction pH to the amount of secreted IP (mg/l) in the culture supernatant

Induction pH	Starting cell density (OD_{600})	Final cell density (OD_{600})	Final cell density (cells/ml × 10^8)[a]	Secreted IP (mg/l)	Yield of IP (mg/10^8 cells)
4	15.21	19.45	9.73	903	0.09
5	15.21	19.69	9.85	935	0.09
5.5	15.21	19.13	9.57	872	0.09
6	15.21	20.27	10.14	715	0.07
6.5	15.21	20.45	10.23	916	0.09
7	15.21	20.75	10.38	1029	0.10
7.5	15.21	20.69	10.35	817	0.08

[a]The final cell density (cells/ml × 10^8) was calculated from the amount of the final cell densities (OD_{600}) where one OD_{600} = ~5 × 10^7 cells/ml

Table 5. Effect of induction temperature to the amount of secreted IP (mg/l) in the culture supernatant

Induction temperature (°C)	Starting cell density (OD_{600})	Final cell density (OD_{600})	Final cell density (cells/ml × 10^8)[a]	Secreted IP (mg/l)	Yield of IP (mg/10^8 cells)
27	14.65	19.84	9.92	941	0.10
28	14.65	19.95	9.98	1054	0.11
30	14.65	20.51	10.26	700	0.07

[a]The final cell density (cells/ml × 10^8) was calculated from the amount of the final cell densities (OD_{600}) where one OD_{600} = ~5 × 10^7 cells/ml

recombinant proteins. The gene of foreign protein is constructed between the *AOX1* promoter and the *AOX1* terminator in a shuttle vector, which is expected to express the recombinant protein in an equivalent amount (Cereghino and Cregg 2000). There are three phenotypes of *P. pastoris* recombinant strains that can be obtained through homologous recombination of linearized vector harbored targeted foreign protein. First, the Mut+ variants grow fast on methanol, where the two methanol oxidase genes *AOX1* and *AOX2* remain intact. Second, the Muts variants grow slowly on methanol because the *AOX1* gene replaced by the foreign protein and only *AOX2* responsible for methanol utilization, and third, the Mut- variants are unable to grow in the presence of methanol. After all, both *AOX1* and *AOX2* genes are disrupted (Higgins and Cregg 1998). The methanol concentration in the medium influences cell growth and the level of foreign protein production in both Mut+ and Muts phenotypes (Katakura *et al.* 1998). In this study, we observed the enhancement of IP expression corresponded to the addition of methanol concentration (0-5%) in the induction phase. We also found that although the highest secreted IP of 2,267 mg/l was achieved at 3% methanol induction, the highest IP productivity was observed in both methanol concentration of 3% and 4% (0.29 mg/10^8 cells).

Since methanol is used as both carbon source and inducer (Cereghino and Cregg 2000), the cell density was higher with the increase of methanol concentration and gradually decreased at methanol concentration above 2%. Interestingly, 1% and 3% methanol induction had similar cell biomass amount as revealed by the OD_{600}, which were ~15.4. However, 1% methanol induction exhibited a half amount of secreted IP than 3% methanol induction. This may be since, at higher methanol concentration, the cell growth is inhibited and the energy for cell growth can be diverted for heterologous protein production, increasing productivity (Katakura *et al.* 1998). The expression of IP was still observed at 5% methanol induction, but the cell density and the secreted IP decreased to 14.98 and ~1,500 mg/l (Table 2). Katakura *et al.* (1998) reported that significant inhibition of cell growth was observed at methanol concentration of 31 g/l, which mainly due to the toxic effect of methanol on DNA replication and membrane synthesis, but the metabolism and protein biosynthesis were not significantly inhibited. Since 5% (v/v) methanol is equal to 39.5 g/l, it exceeds the toxic level of methanol concentration, which can influence the cell density and heterologous protein expression. Methanol concentration had a significant effect on the IP protein expression. Increasing methanol concentration to 3% resulted in 5 folds higher secreted IP protein compared to the 0.5% methanol concentration (Table 2) and nearly 100 folds higher than the previous expression condition at OD_{600} ≈ 1 and 0.5% methanol concentration.

One of *P. pastoris* distinguish characteristics is the easiness in scaling up the protein expression from shake flask cultures to fermenter cultures, although some proteins tend to have lower expression levels

in the fermenter culture than shake flask (Cereghino and Cregg 2000). For the secreted protein production in bioreactors, cell growth is a notably essential factor, as the concentration of the protein product in the medium is roughly equivalent to the concentration of the cells in the fermentation culture (Cereghino and Cregg 2000). However, contrary to the cell densities, after 72 h methanol induction, the secreted IP expression gradually decreased. This pattern may reveal that the lag and exponential phase of the IP expression occurred until 72 h post-methanol induction. Above 72 h, the IP expression was going through the stationary and decline phase. A shake flask culture is a closed-type culture where there is no substrate addition and has some other limitations such as constant volume, oxygen transfer, and inability to control these factors efficiently (Macauley-Patrick *et al.* 2005). The highest cell density observed at 192 h post-methanol induction (OD_{600} = 21.35); however, at this time point, the secreted IP was lower compared to other time points (24-168 h). The highest secreted IP of 2,135 mg/l was achieved at 72 h post-methanol induction with OD_{600} = 19.89. The highest yield of IP (0.21 mg/10^8 cells) was observed at 72 h and 96 h post-methanol induction (Table 3). Thus, the best time point to harvest IP protein for the batch culture of *P. pastoris* recombinant strain (CL-4) in the shake flask was at 72 h post-methanol induction or can be prolonged to 96 h post-methanol induction.

The pH is one of the controlled parameters besides aeration and carbon source feed rate, which affects protein productivity and activity in fermentation (Macauley-Patrick *et al.* 2005). *P. pastoris* can grow in a wide pH range of 3 to 7, with a minimum effect on the growth rate. However, pH exhibited a significant effect on the secreted recombinant proteins due to protease activity in the fermentation broth (Zhong *et al.* 2004). This study results confirmed the ability of *P. pastoris* to grow in a wide pH range. The highest cell biomass (OD_{600} = 20.75), secreted IP (1,029 mg/l), and IP yield productivity (0.10 mg/10^8 cells) was observed at pH 7 (Table 4). Shi *et al.* (2003) reported that during the fermentation of ScFv in BMMY medium, the lowest levels of protease activity were detected at pH 3, 5, 6, and 8. The enhancement of the secreted IP at pH 7 was 1.4 folds higher than at pH 6.

Temperature is one of the critical operational parameters for improving heterologous protein expression in *P. pastoris* (Wang Yun *et al.* 2009). Previous studies suggested that reducing the temperature below 30°C exhibited a positive effect on heterologous protein production such as relieving cell skeleton lysis and protease secretion (Anasontzis *et al.* 2014; Jin *et al.* 2010; Wang Yun *et al.* 2009). In this study, we observed that lowering induction temperature to 28°C increased 1.5 folds higher secreted IP than at 30°C.

In conclusion, a two-phase cultivation process of *P. pastoris*, which consists of glycerol- and methanol-phase, was optimized to enhance IP protein secretion. Five parameters of operation variables in the methanol induction phase were investigated including inoculum density, methanol concentration, time point methanol induction, pH, and temperature in shake flask culture. This study confirmed the influence of the five investigated parameters on the enhancement of IP protein secretion. A 100 times higher secretion of IP compared to the standard procedure was achieved by modulating inoculum density from OD_{600} ≈ 1 to 10 and methanol induction from 0.5 to 3.0% (v/v). The highest IP concentration attained was ~2,267 mg/l.

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