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PRODUCTION OF HUMAN INSULIN PRECURSOR IN A 10 LITER BIOREACTOR USING A METHANOL PULSE FEEDING

Produksi Prekursor Insulin Manusia pada Bioreaktor 10 Liter Menggunakan Metanol yang Diberikan Secara Berkala

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ABSTRACT
Indonesia as a country that had 19.5 million diabetic patients in 2021 is still importing raw materials of human insulin. The development of human insulin production process is needed to support the government's objective to be independent in medicine aspect. Human insulin precursor (HIP) expressed from Pichia pastoris X33/pD902-IP had been developed and optimized in small-scale cultivation. However, the scaling up in bioreactor 10 L has not been studied. Using a 10 L-bioreactor the fermentation of P. pastoris X33/pD902-IP was developed. Fermentation was done in 120 hours using a basal salt medium (half concentration) for the vegetative and induction media. To induce HIP expression, methanol is fed by pulse strategy with a gradient concentration of 1-3% (v/v) for 48 hours. The dry cell weight (DCW) and HIP titers were 72 g/L and 286 mg/L, respectively. This development is the first fermentation of HIP expressed by P. pastoris X33/pD902-IP in a 10 L-bioreactor.

Keywords: *bioreactor, fermentation, insulin precursor, methanol, Pichia pastoris*

ABSTRAK
Indonesia sebagai negara dengan penderita diabetes 19,5 juta orang pada tahun 2021 masih mengimpor bahan baku insulin manusia. Pengembangan proses produksi insulin manusia diperlukan dalam rangka mendukung tujuan pemerintah untuk mandiri di bidang produksi bahan baku obat. Ekspresi prekursor insulin manusia oleh *Pichia pastoris* X33/pD902-IP telah dikembangkan dan dioptimalkan dalam skala kultur kocok. Akan tetapi, produksinya pada bioreaktor 10 L belum dilakukan. Untuk itu, fermentasi *P. pastoris* X33/pD902-IP dipelajari pada bioreaktor 10 L. Fermentasi dilakukan selama 120 jam dengan menggunakan media garam basal (setengah konsentrasi). Untuk menginduksi ekspresi HIP, metanol diberikan dengan gradien konsentrasi 1-3% (v/v) secara berkala selama 48 jam. Berat sel kering dan titer HIP masing-masing adalah 72 g/L dan 286 mg/L. Proses ini merupakan fermentasi pertama HIP yang dihasilkan *P. pastoris* X33/pD902-IP pada skala bioreaktor 10 L.

Kata Kunci: bioreaktor, fermentasi, metanol, *Pichia pastoris*, prekursor insulin

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INTRODUCTION

Insulin is a regulatory hormone that assists cells in taking glucose into the cell. Type 1 diabetic and late-phase type 2 diabetic patients need insulin to manage their blood glucose level. The number of diabetic people globally was 537 million in 2021 and it is predicted to be 783 million in 2045. In addition, Indonesia was ranked as the fifth country that had 19.5 million diabetic people in 2021 (IDF 2021). Due to the vital role of insulin and the rising trend of diabetic patients globally, insulin demand has been raised. It is predicted in 2027, global insulin market will be up to 55.066 million USD (Data Bridge 2021).

Human insulin is a polypeptide that is formed through several steps after translation. At the first stage, this protein is secreted as preproinsulin, which consist of signal peptide, B chain, C chain and A chain (Vasiljević et al. 2020). In the next step, the signal peptide and C chain are deleted, and the B chain and A chain are connected with disulfide bridges (Bataille 2007). The human insulin precursor (HIP) in this work is a recombinant form that mimics human preproinsulin. It consists of a truncated-α-factor and spacer to replace signal peptide, A chain, B chain and short linker between A and B chain to replace C-peptide. Plasmid pD902-IP carrying HIP cassette was integrated into *Pichia pastoris* X-33 Mut^+ (Nurdiani et al. 2018). Clone 4 of the recombinant yeast (CL4) was selected to be employed in this research (Nurdiani et al. 2020).

P. pastoris has been a popular choice to be a host for several recombinant protein (Tripathi et al. 2015, Baeshen et al. 2016, Jiao et al. 2018). *P. pastoris* has four chromosomes with a genome size of 9,4 Mbp (Bernauer et al. 2021). At the fourth chromosome, promotor *AOX1* (Alcohol Oxidase 1) is located (De Schutter et al. 2009). This promotor regulates protein expression under the control of methanol. It is a strong inducible promotor; thus, many recombinant proteins employ this promotor system to express the desired protein (Özçelik et al. 2019). Due to its features such as able to secrete nonnative protein, able to do post-translational modification and produce less immunogenic protein (Baeshen et al. 2014, Karbalaei et al. 2020, Vogl et al. 2018), high cell density fermentation (HCDF) of *P. pastoris* is an attractive strategy in order to obtain high yield of biomass and protein target (Liu et al. 2016). However, scaling up from a bench scale or smaller volume into higher volume fermenter faces some challenges.

Tank configuration such as the geometry of the tank is one factor that should be considered (Junker 2004). Other parameters such as temperature, pH, airflow rate as linear parameters and agitation as non-linear parameters should be identified in order to achieve the main goal of scale up production (Yang 2014). The successful scale up process results in a consistent large-scale fermentation that meets the expected requirement (Crater and Lievense 2018).

The expression of HIP had been confirmed in 2 L-fermenter (Putro et al. 2021, Putro et al. 2022). However, the scale up of HIP has not been studied in a 10 L-fermenter. In addition, the growth curve of CL4 in fermenter media in shake flask has not been studied. This study consists of cultivation, establishing the growth curve and confirming the expression of HIP from CL4 in the media that will be used in a bioreactor. After confirmation, the strain will be trained in 10 L fermenter to express HIP. The aim of this research was to scale up the production of HIP from shake flask to 10 L-fermenter. Thus, the fermentation method of CL4 in 10 L-fermenter was developed after studied the growth profile of CL4 and confirmed the expression of HIP in the flask scale.

MATERIALS AND METHODS

Location and time

This research was conducted in August-November 2022 at the Laboratory of Biotechnology, Deputy for Research and Innovation Infrastructure-National Research and Innovation Agency (BRIN)

Materials

Recombinant *P. pastoris* X33 integrating human insulin precursor gene into its genome (*P. pastoris* X-33/pD902-IP clone 4, CL4) were employed (Nurdiani et al. 2018). This strain was obtained from Biological Product Development Research Group-BRIN. Cultivation was done in YPD media according to Kurniatin et al. (2019),

the half concentration of basal medium with 4% (w/v) glycerol (½ BSM(G)) according to Zhu et al. (2021) and half concentration of basal medium without glycerol to be added with methanol (1/2 BSM(M)) supplemented with trace mineral, PTM1 according to Invitrogen Corporation (2002)

Cultivation in shake flask

A single colony of CL4 was inoculated into 36 mL YPD medium in flask 250 mL. Culture was incubated for 72 hours. At 25 hr, 20 mL of culture was centrifuged and the cells were transferred into 30 mL ½ BSM(G) in 250 mL flask. Incubation was carried for 72 hours, 30°C and 250 rpm. At 24 hours, 25 mL of culture was harvested and the cells were transferred into 30 mL ½ BSM(M) in flask 250 mL. Methanol was fed at 1%, 2% and 3% at 0, 24 and 48 hours after transferred into ½ BSM(M), respectively (Nurdiani et al. 2022, Putro et al. 2022). Incubation was carried for 72 hours, 30°C and 250 rpm. Samples were taken at start point and every 24 hours at the induction phases. Samples were analyzed for dry cell weight (DCW) and HIP titer determination. Samples were accomplished in quadruplicate.

Cultivation in 10 liter fermenter

Cultivation of CL4 was conducted as follows: a single colony of CL4 from a YPD agar containing zeocin 100 µg/mL (Nurdiani et al. 2022) was inoculated into 40 mL YPD liquid containing zeocin 100 µg/mL. The culture was grown in a shaking incubation at 250 rpm and 30ºC for 24 hours. The cells were harvested by centrifugation 3000 g for 5 min at room temperature. The pellets was resuspended with 400 mL of ½ BSM(G) media (Wu et al. 2019) supplemented with 4.35 mL PTM1 trace salts/L (Invitrogen Corporation 2002) of ½ BSM(G), and incubation was carried at 250 rpm and 30°C. After 24 hours, the cultured was then transferred into a 3.6 L of ½ BSM(G) media in a 10 L fermenter. Agitation was set at the range of 150-500 rpm. Aeration was maintained at the range of 2-4 L/min. Dissolved oxygen (DO) was observed over the time, and the temperature was set at the range of 28-30ºC. The level of pH was monitored and maintained at pH 5 by addition of 12.5% ammonia and 1M of H_3PO_4.

Methanol induction was conducted by adding 100% methanol to a final concentration of 1-3% (v/v) after the glycerol batch phase was finished. Trace salts PTM1 were added in methanol feed at 12 mL/L methanol 100%. Samples were collected at several points. The supernatant was separated from the broth by centrifugation at 10,000 rpm and 4°C for 10 minutes to be stored at -80°C for further analysis (Wu et al. 2019). Samples were accomplished in quadruplicate.

Determination of cell concentration

Dry cell weight was measured by weighting pellet cell after centrifuged 14000 rpm, 5 minutes and 4°C as in (Bredell et al. 2018). The pellet was then dried in oven 90°C for 2 hours then cooled at desiccator for 30 minutes prior to weight (modified from Kocaefe-Özsen et al. 2022).

Protein Analysis

HIP quantification from supernatant culture was done by RP-HPLC modified from Wu et al. (2019) and Putro et al. (2022). Supernatant was separated from culture by centrifugation at 14000 rpm for 5 minutes in 4ºC. Analytical RP-HPLC column Shodex, RP18-415 (5µm C18, 250 x 4.6 mm) was employed to analyse HIP from the supernatant. Mobile phase A was 0.1% (v/v) TFA in water and elution was done with a gradient of mobile phase B (0.1% TFA in acetonitrile) as follow: 15-30%B (0-12 min), 30-100% B (12-18 min). The flow rate was 1 mL/min and the elute fraction was monitored at 214 nm. A standard curve of human insulin (Sigma) was used to quantify HIP concentration in the samples.

Glycerol and methanol determination

Samples were centrifuged 14000 rpm, 5 min, 4ºC. The supernatant was loaded into Aminex HPX-87 H column. The temperature column was set at 35ºC and the mobile phase used was 0.008 N H_2SO_4 with a flow rate of 0.7 mL/min. Detection is carried out using a refractive index detector. Sample chromatograms were compared with standard glycerol and methanol chromatograms to calculate their concentrations (Chongchittapiban et al. 2016).

RESULTS AND DISCUSSION

Cultivation in shake flask

Expression of HIP in shake flask needs to be confirmed before proceeding to the fermenter scale. In addition, the growth curve of CL4 in ½ BSM media was not known previously. Thus, the dry biomass of CL4 from samples in each media was plotted against time to capture the growth curve. Figure 1 shows CL4 growth curve in YPD, ½ BSM(G), and ½ BSM(M). A significant escalation of biomass was observed in ½ BSM(G) rather than in other media.

The expression of HIP was confirmed at flask scale after 72 hours induction with methanol. The HIP titer was 95 mg/L analysed by RP-HPLC. This result is higher than previous study (30 mg/L) in Putro et al. (2022). In addition, DCW of this work (13 g/L) is also higher than the previous result (5 g/L) (Putro et al. 2022). This might because not all the cells in this work were transferred into the new media. This approach resulted in lower size of inoculum. Based on Putro et al. (2022), lower size inoculum resulted in higher improvement in yeast growth compared with the higher size.

Cultivation in 10 liter fermenter

Cultivation was done in a 10 L glass jar fermenter (MDFT-10N, BE Marubishi) supplemented with a compressor to supply air and a chiller to manage the temperature to achieve the desired temperature. This fermenter is a desktop fermenter equipped with Rushton turbine impeller that is suitable for yeast fermentation and cooling coins to reduce the heat produced from the fermentation system (Jossen et al. 2016). The general structure of bioreactor depicts in Figure 2.

When scaling up the process, it is important to consider the geometry similarity of the vessel. The geometry of vessel is represented as ratio of H/D (Figure 2). The geometry of the previous scale (2 L vessel, Eppendorf BioFlo-120) is not similar with the current vessel (10 L vessel, BE Marubishi MDFT-N-10L). Thus, an adjustment in the process needs to be done.

In the beginning, agitation (orange line, Figure 3) was set at 150 rpm to lower the shear stress of CL4. After 5 hours, the agitation was increased gradually to 400 rpm. When the foam formed intensely, the agitation was decreased and then increased gradually into 500 rpm. The aeration (green line, Figure 3) was tuned at 2 L/min at the beginning and when the DO was below 20%, the aeration was increased gradually to 4 L/min before finally set at 3 L/min. However, in carbon limiting condition, when methanol was fed, the DO level (blue line, Figure 3) decreased although the agitation was increased. Thus, in the next hours, the

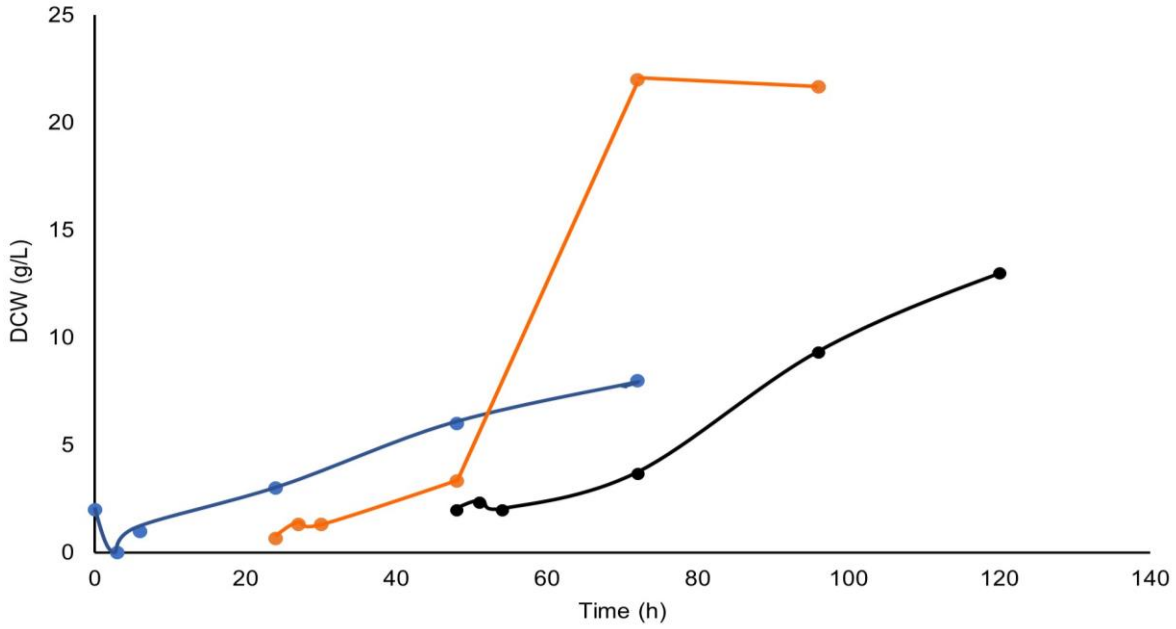

Figure 1. The growth curve of CL4 in 3 different media. Blue line: YPD media. Orange line: ½ BSM(G) media and black line: ½ BSM(M) media

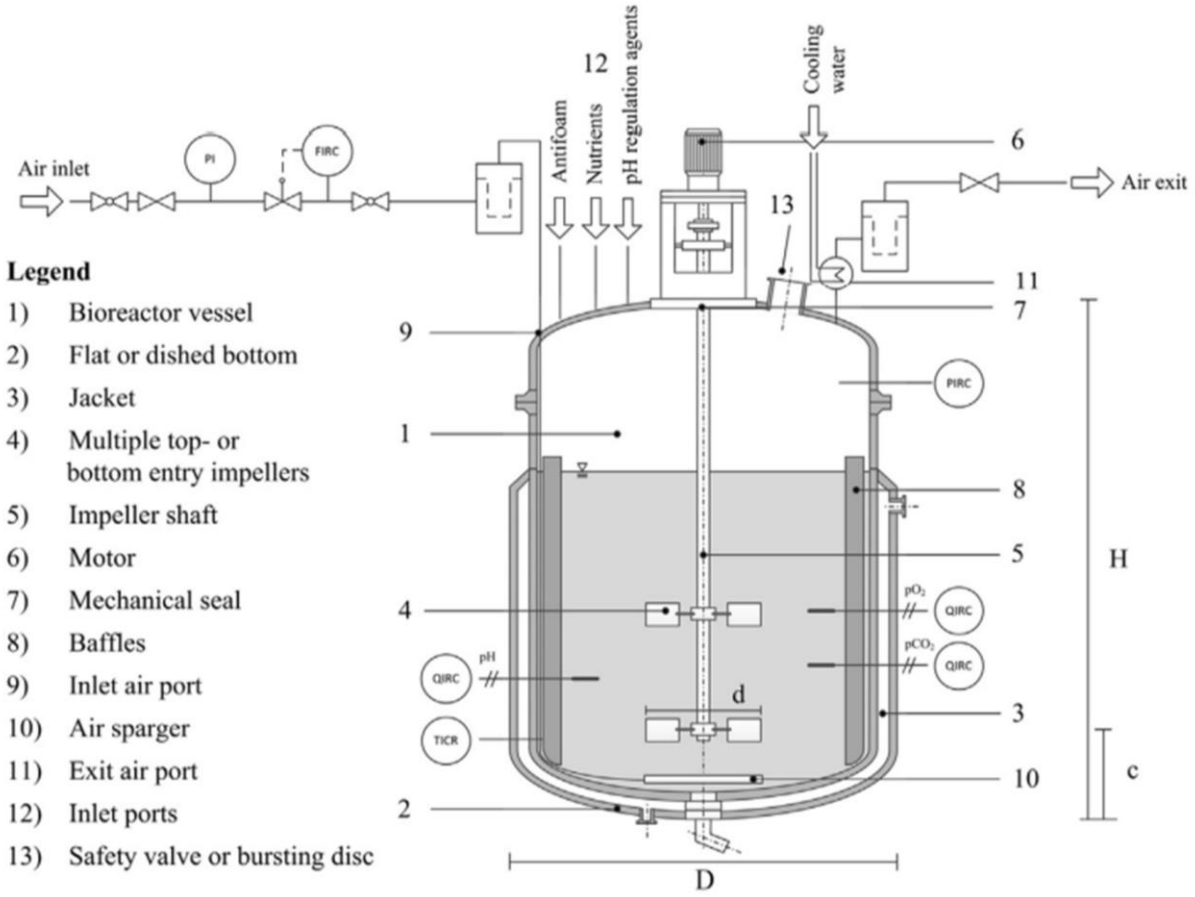

Figure 2. General structure of bioreactor (Jossen et al. 2016)

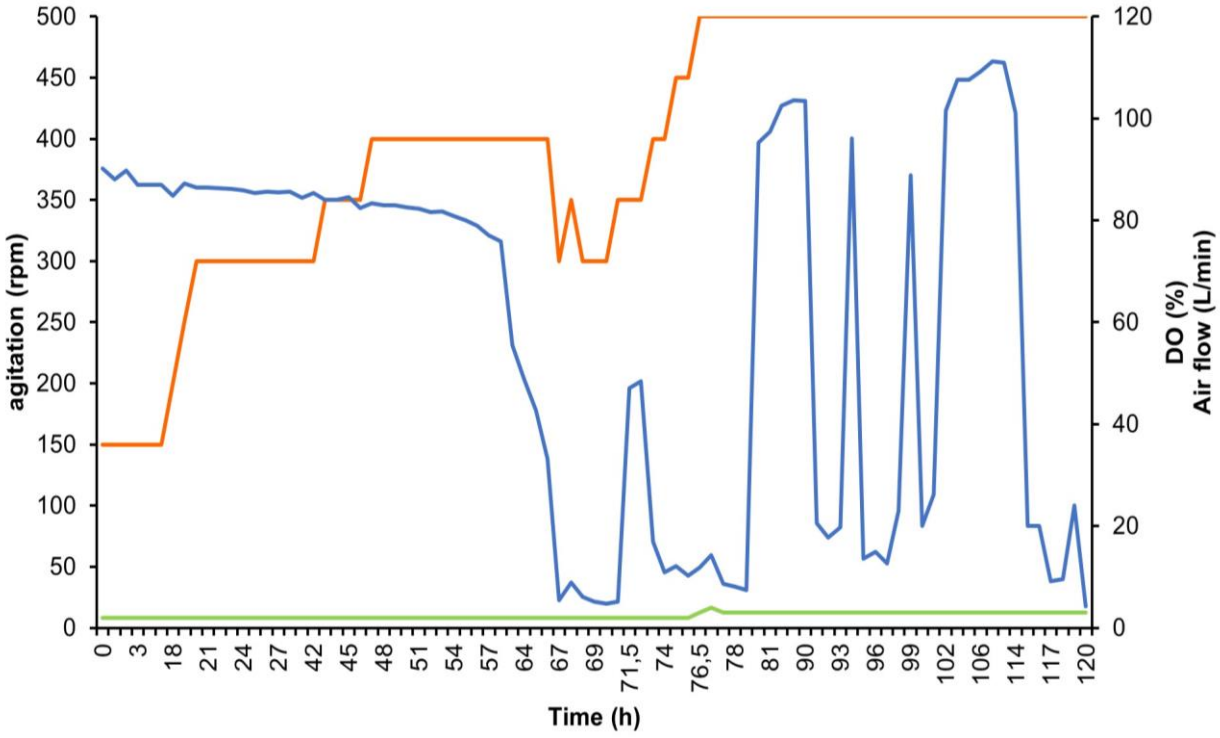

Figure 3. Process parameter profile in fermenter 10 L. Orange line: agitation, blue line: DO, green line: aeration

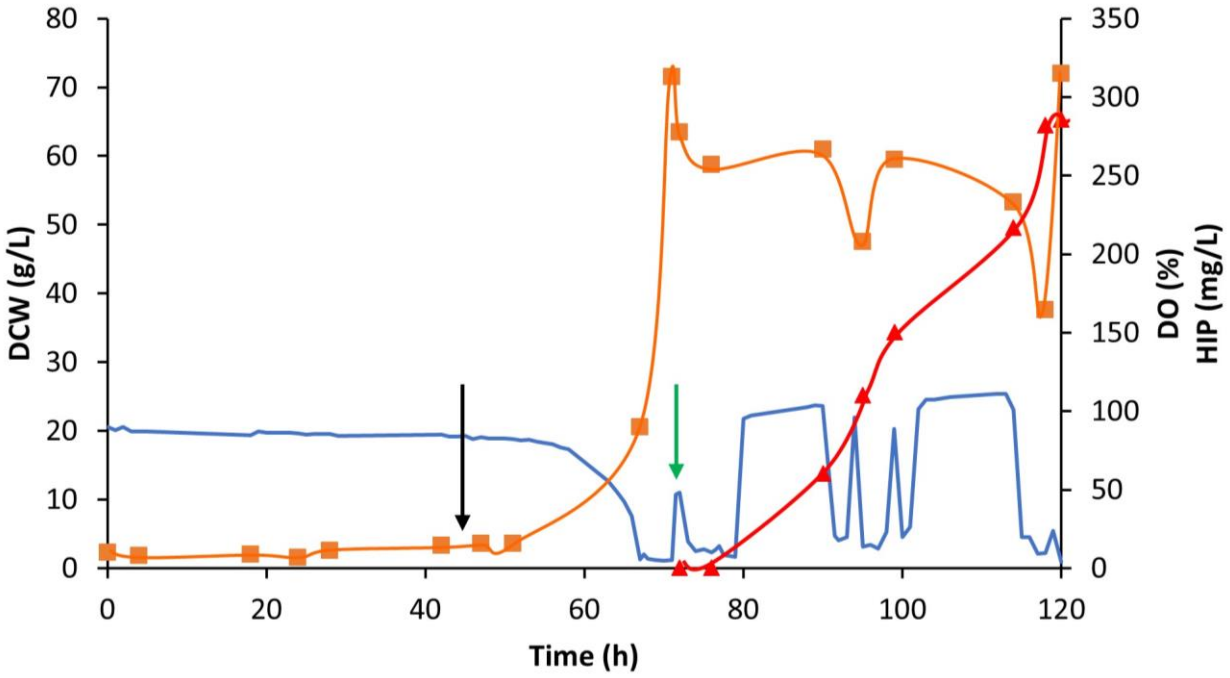

Figure 4. Biomass, DO and HIP production of *P. pastoris* X33/pD902-IP in fermenter 10 L. The plot of DCW (orange line) represents the growth curve of CL4 in bioreactor. Black arrow: 1.7% (v/v) inoculum was added. The HIP expression (red line) started after 76 hours fermentation. DO (blue line) spike indicated by green arrow

agitation remained at maximum speed (500 rpm) and the dynamic change of DO value was observed to feed methanol when the DO above 20%.

Low agitation such as 100 rpm resulted in low shear stress of yeast, while when the agitation speed increases, the shear rate will increase. Shear rate correspondences with cell viability (Vlaev et al. 2016). *P. pastoris* as an obligate aerobe organism (Randone 2014) needs oxygen to metabolize glycerol and methanol (Liu et al. 2016). A sufficient level of oxygen such as above 20% was needed to assure the growth of the yeast in methanol-fed media (Invitrogen Corporation 2002).

The size of inoculum is one factor that affects fermentation performance (Ginovart et al. 2011). Small size of inoculum will prolong the lag time of fermentation. The lag phase of CL4 in the fermenter was more than 24 hours. Thus, at 46 hours fermentation time, a 1.7% (v/v) CL4 culture in ½ BSM(G) media was added to the fermenter (Figure 4, black arrows). This approach resulted in an accelerating log phase that started at 51 hours and finished at 71 hours fermentation. At the end of log phase, DO spike was occurred (green arrow, Figure 4). This indicated that glycerol had finished in the media. Cultivation was then continued with 48 hours methanol induction phase. The final amount of biomass produced was 72 g/L and the HIP titer of the supernatant was 286 mg/L (Figure 4). The trend of HIP production (red line, Figure 4) increases over time while the trend of biomass production (orange line, Figure 4) tends to decrease. This may because the yeast had aged (Ginovart et al. 2011) as a result of the long lag phase in the beginning of the process.

After the end of process, DCW and titer of HIP in this work was 130.8% and 28.9% higher than the previous work (31.2 g/L and 221.9 mg/L), respectively. Those results confirmed that the scale up of this protein achieved the purpose of scale up production which is to demonstrate the fermentation process in larger scale has the same productivity and quality as in the small scale (Yang 2014). However, in another work that occupied *P. pastoris* Mut^+ expressing HIP in ½ BSM media, the titer of HIP was ~2.5 g/L at the similar fermentation time with this work (Wu et al. 2019). They applied glycerol fed batch after glycerol batch phase and continuous methanol feeding in the induction phase. This approach can be applied in the next research to increase the HIP titer of CL4.

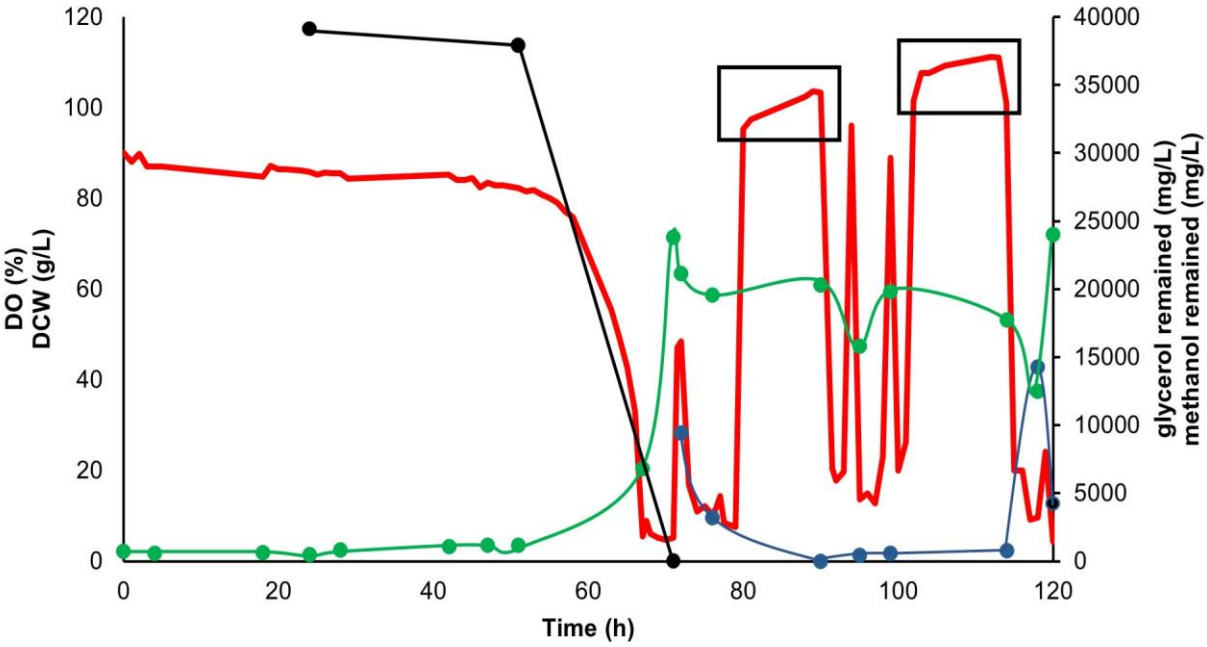

Figure 5. Carbon sources, DO and dry biomass profile during process. Red line: DO. Blue line: methanol, Green line: DCW. Black line: glycerol. Black boxes: plateau phase

In the glycerol batch phase, the glycerol content in the media (black line, Figure 5) decreased sharply when the log phase (green line, Figure 5) happened. At the DO spike, there was no glycerol remained. At this point, methanol feeding was started. In the beginning, culture was adapted to the shift of carbon source from glycerol to methanol, thus methanol was mainly remained in the media (blue line, figure 5). Methanol was fed when the DO level above 20%. However, there were several times points that methanol was not fed. The objective of this procedure was to observe the behaviour of the yeast when methanol fed was stopped for several hours. As results, we observed plateau phases in DO record graph when applied that procedure (Figure 5, black boxes). This was because methanol was exhausted from the media, so the microbes stopped their metabolism and the oxygen in the media was not consumed (Invitrogen Corporation 2002). Microbes was starving, thus the biomass of the cell in several hours after that had decreased. Thus, methanol content remained in media was increased. Interestingly, when methanol was fed again, DO decreased sharply and in the next hours DO increased significantly. This indicated that the culture was in carbon limiting condition, so when methanol was fed, they directly consumed it by taking oxygen in media. As a result of methanol fed after the plateau phase, the number of biomass increased again (Figure 5, green line). It is predicted if the feeding strategy set as continuous feeding, the plateau phase will be eliminated, the biomass amount and titer of HIP can achieve higher result than this current work. In addition, in the previous work, the continuous strategy resulted HIP amount of 534 mg, while the pulse feeding-methanol strategy resulted in 221.9 mg (Putro et al. 2022).

Since insulin is vital for diabetic patient and Indonesia still imported the raw material (Permen Kesehatan 2017), the development of production system for insulin is a necessity. The strain used in this work is developed domestically (Nurdiani et al. 2018) as well as the fermentation system in the small scale (Nurdiani et al. 2022, Putro et al. 2022). The results of this work demonstrate that the expression of HIP from CL4 (clone 4 of *P. pastoris* X-33/pD902-IP) is potential to be optimised in 10 L fermenter. Scale up then can be continued to pilot scale, moreover into industrial scale to produce human insulin in Indonesia.

CONCLUSION

The development of human insulin precursor fermentation in a 10 L-fermenter has been done. This is the first development of the fermentation method for *P. pastoris* X-33/pD902-IP clone 4 at 10 L -fermenter scale.

The process resulted in biomass amount and HIP titer of 72 g/L and 286 mg/L, respectively. These results are higher than the previous research. However, those results are not optimal results. The fermentation process can be optimized in the next work to achieve higher results and optimal process conditions for HIP production.

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REFERENCES

Baeshen MN, Bouback TAF, Alzubaidi MA, Bora RS, Alotaibi MAT, Alabbas OTO, Alshahrani SM, Aljohani AAM, Munshi RAA, Al-Hejin A, Ahmed MMM, Redwan EM, Ramadan HAI, Saini KS, Baeshen NA (2016) Expression and purification of c-peptide containing insulin using *Pichia pastoris* expression system. Biomed Res Int 2016:3423685. doi: 10.1155/2016/3423685

Baeshen NA, Baeshen MN, Sheikh A, Bora RS, Ahmed MMM, Ramadan HAI, Saini KS, Redwan EM (2014) Cell factories for insulin production. Microb Cell Fact 13:141. doi: 10.1186/s12934-014-0141-0

Bataille D (2007) Pro-protein convertases in intermediary metabolism: Islet hormones, brain/gut hormones and integrated physiology. J Mol Med 85:673–684. doi: 10.1007/s00109-007-0167-4

Bernauer L, Radkohl A, Lehmayer LGK, Emmerstorfer-Augustin A (2021) *Komagataella phaffii* as emerging model organism in fundamental research. Front Microbiol 11:607028. doi: 10.3389/fmicb.2020.607028

Bredell H, Smith JJ, Görgens JF, van Zyl WH (2018) Expression of unique chimeric human papilloma virus type 16 (HPV-16) L1-L2 proteins in *Pichia pastoris* and *Hansenula polymorpha*. Yeast 35:519–529. doi: 10.1002/yea.3318

Chongchittapiban P, Borg J, Waiprib Y, Pimsamam J, Tongta A (2016) On-line methanol sensor system development for recombinant human serum albumin production by *Pichia pastoris*. Afr J Biotechnol 15:2374–2383. doi: 10.5897/ajb2015.15122

Crater JS, Lievense JC (2018) Scale-up of industrial microbial processes. FEMS Microbiol Lett 365:fny138. doi: 10.1093/femsle/fny138

Data Bridge (2021) Insulin Market – Global Industry Trends and Forecast to 2027 | Data Bridge Market Research. In: Data Bridge Mark Res Website. https://www.databridgemarketresearch.com/reports/global-insulin-market#. Accessed 14 Oct 2021

De Schutter K, Lin YC, Tiels P, Van Hecke A, Glinka S, Weber-Lehmann J, Rouzé P, Van De Peer Y, Callewaert N (2009) Genome sequence of the recombinant protein production host *Pichia pastoris*. Nat Biotechnol 27:561–566. doi: 10.1038/nbt.1544

Ginovart M, Prats C, Portell X, Silbert M (2011) Exploring the lag phase and growth initiation of a yeast culture by means of an individual-based model. Food Microbiol 28:810–817. doi: 10.1016/j.fm.2010.05.004

IDF (2021) IDF Diabetes Atlas 10th edition. International Diabetes Foundation

Invitrogen Corporation (2002) Pichia Fermentation Process Guidelines. Prog Bot 67:1–11

Jiao L, Zhou Q, Su Z, Xu L, Yan Y (2018) High-level extracellular production of *Rhizopus oryzae* lipase in *Pichia pastoris* via a strategy combining optimization of gene-copy number with co-expression of ERAD-related proteins. Protein Expr Purif 147:1–12. doi: 10.1016/j.pep.2018.02.005

Jossen V, Eibl-Schindler R, Pörtner R, Kraume M, Eibl D (2016) Stirred bioreactors: Current state and developments, with special emphasis on biopharmaceutical production processes. In: Current Developments in Biotechnology and Bioengineering: Bioprocesses, Bioreactors and Controls. Pp 179–215. Elsevier, Amsterdam. doi:

10.1016/B978-0-444-63663-8.00007-0
Junker BH (2004) Scale-up methodologies for *Escherichia coli* and yeast fermentation processes. J Biosci Bioeng 97:347–364. doi: 10.1016/s1389-1723(04)70218-2
Karbalaei M, Rezaee SA, Farsiani H (2020) *Pichia pastoris*: A highly successful expression system for optimal synthesis of heterologous proteins. J Cell Physiol 235:5867–5881. doi: 10.1002/JCP.29583
Kocaefe-Özsen N, Yilmaz B, Alkım C, Arslan M, Topaloglu A, Kısakesen HLB, Gülsev E, Çakar ZP (2022) Physiological and molecular characterization of an oxidative stress-resistant *Saccharomyces cerevisiae* strain obtained by evolutionary engineering. Front Microbiol 13:822864. doi: 10.3389/fmicb.2022.822864
Kurniatin PA, Masduki FF, Nurainy N, Ihsanawati, Natalia D (2019) The use of α-MF signal peptide without spacer for producing insulin aspart precursor in *Pichia pastoris* KM71. Am J Biochem Biotechnol 15:198–207. doi: 10.3844/ajbbsp.2019.198.207
Liu WC, Gong T, Wang QH, Liang X, Chen JJ, Zhu P (2016) Scaling-up fermentation of *Pichia pastoris* to demonstration-scale using new methanol-feeding strategy and increased air pressure instead of pure oxygen supplement. Sci Rep 6:18439. doi: 10.1038/srep18439
Nurdiani D, Hariyatun, Kusharyoto W (2018) Secretory expression of human insulin precursor in *Pichia pastoris* employing truncated α-factor leader sequence and a short C-peptide. Indones J Biotechnol 23:102–108. doi: 10.22146/ijbiotech.38958
Nurdiani D, Hariyatun, Utami N, Putro EW, Kusharyoto W (2020) Selecting *Pichia pastoris* recombinant clones for higher secretion of human insulin precursor into the culture supernatant. IOP Conf Ser: Earth Environ Sci 439:012017. doi: 10.1088/1755-1315/439/1/012017
Nurdiani D, Hariyatun, Utami N, Putro EW, Kusharyoto W (2022) Enhancement in human insulin precursor secretion by *Pichia pastoris* through modification of expression conditions. HAYATI J Biosci 29:22–30. doi: 10.4308/hjb.29.1.22-30
Özçelik AT, Yılmaz S, Inan M (2019) *Pichia pastoris* promoters. Methods Mol Biol 1923:97–112. doi: 10.1007/978-1-4939-9024-5_3
Permen Kesehatan (2017) Peraturan Menteri Kesehatan No. 17 Tahun 2017 tentang Rencana Aksi Pengembangan Industri Farmasi dan Alat Kesehatan
Putro EW, Nurdiani D, Hariyatun, Utami N, Kusharyoto W (2021) Capture and intermediate purification of human insulin precursor from *Pichia pastoris* culture using cation exchange chromatography. IOP Conf Ser: Earth Environ Sci 762:012028. doi: 10.1088/1755-1315/762/1/012028
Putro EW, Nurdiani D, Hariyatun, Utami N, Kusharyoto W, Juanssilfero AB, Silvia RA (2022) Evaluating pulses and modified fed-batch feeding of methanol to increase expression level of human insulin precursor in *Pichia pastoris* high-density cultivation. Int J Adv Sci Eng Inf Technol 12:1001–1007. doi: 10.18517/ijaseit.12.3.15109
Randone BP (2014) Bioprocess Studies of Biomass and Recombinant Protein Production by the Methylotrophic Yeast *Pichia pastoris*. Thesis, University College of London
Tripathi L, Mani S, Raut R, Poddar A, Tyagi P, Arora U, de Silva A, Swaminathan S, Khanna N (2015) *Pichia pastoris*-expressed dengue 3 envelope-based virus-like particles elicit predominantly domain III-focused high titer neutralizing antibodies. Front Microbiol 6:1005. doi: 10.3389/fmicb.2015.01005
Vasiljević J, Torkko JM, Knoch K-P, Solimena M (2020) The making of insulin in health and disease. Diabetologia 63:1981–1989. doi: 10.1007/S00125-020-05192-7
Vlaev SD, Pavlova K, Rusinova-Videva S, Georgieva K, Georgiev D (2016) Agitation effects and kinetic constants of exoglucomannan production by antarctic yeast strain in a stirred tank bioreactor. Chem Biochem Eng Q 30:393-400. doi: 10.15255/CABEQ.2015.2276
Vogl T, Gebbie L, Palfreyman RW, Speight R (2018) Effect of plasmid design and type of integration event on recombinant protein expression in *Pichia pastoris*. Appl Environ Microbiol 84:e02712-17.

doi: 10.1128/AEM.02712-17

Wu J, Gong G, Han S, Zhang W, Hu Y, Xie L (2019) Expression, purification, and characterization of the Degludec precursor DesB30. Protein Expr Purif 161:28–39. doi: 10.1016/j.pep.2019.04.010

Yang X (2014) Scale-up of microbial fermentation process. Pp. 669–675. Bull AT, Junker B, Katz L, Lynd LR, Masurekar P, Reeves CD, Zhao H (Eds). Manual of Industrial Microbiology and Biotechnology, Third Edition. doi: 10.1128/9781555816827.ch47

Zhu W, Xu R, Gong G, Xu L, Hu Y, Xie L (2021) Medium optimization for high yield production of human serum albumin in *Pichia pastoris* and its efficient purification. Protein Expr Purif 181:105831. doi: 10.1016/j.pep.2021.105831