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# THE DEVELOPMENT OF HUMAN INSULIN PRECURSOR EXPRESSED FROM *P.PASTORIS*-X33 IN A 10L-FERMENTER

**Dian Japany Puspitasari[1a)], Hendi Saleh Permana[2b)], Rudiyono[3c)], Anis Herliyati Mahsunah[4d)]**
[1]Departemen Biologi-Institut Pertanian Bogor, Jalan Agatis Kampus IPB Dramaga, Bogor, +62-251-8622833
[2]Direktorat Pengelolaan Laboratorium, Fasilitas Riset dan Kawasan Sains dan Teknologi-BRIN
[3]Pusat Riset Bahan Baku Obat dan Obat Tradisional-BRIN
[4]Pusat Riset Mikrobiologi Terapan-BRIN, Jl. Raya Jakarta-Bogor Km. 46, Cibinong, Bogor, Jawa Barat 16911
e-mail: [a)]japanydian@apps.ipb.ac.id, [b)]hend022@brin.go.id, [c)] rudi012@brin.go.id, [d)]anis003@brin.go.id

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## ABSTRACT

Human insulin is a hormone that regulate blood glucose in human. Due to the vital role of insulin and the trend of diabetic case globally, insulin demand has been rise in the world. Indonesia as a country that had diabetic patient more than 10 million people in 2017 is still imported raw material of human insulin. The development of production process of human insulin is needed in order to support government objective to be independence in medicine aspect. Human insulin precursor (HIP) expressed from a *Pichia pastoris* X33/pD902-IP had been developed and optimised in a small scale. However, the scaling up in fermenter 10L has not been studied. Using a 10L-fermenter the fermentation system of *P.pastoris* X33/pD902-IP was developed. Fermentation 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 1-3% for 48 hours. The dry cell weight (DCW) and HIP titer were 72 g/L and 192 mg/L, respectively. This development is the first fermentation process of HIP in fermenter 10L in Indonesia.

**Keywords**: insulin, precursor, fermenter, development

## INTRODUCTION

Insulin is a regulatory hormone that assists cells to take glucose into the cell. Type 1 diabetic and late phase type 2 diabetic patients needs insulin to manage their blood glucose level. Due to the vital role of insulin and the rising trend of diabetic patient globally, insulin demand has been raised. It is predicted in 2027, insulin global market will be up to 55, 066 million USD (Data Bridge, 2021).

Insulin is naturally produced by pancreas, however, in diabetic case, insulin cannot be produced or cannot work properly in its receptor, or both (Ahmad, 2014). Nowadays, insulin can be produced recombinantly using several hosts, namely *E.coli*, *S.cereviceae*, mammalian cells and *P.pastoris*. *P.pastoris* has been a popular choice to be a host for several recombinant protein (M. N. Baeshen et al., 2016; Jiao et al., 2018; Tripathi et al., 2015). Due to its features such as able to secrete non native protein, able to do post translational modification and produse less immunogenic protein (Baeshen et al., 2014; Karbalaei et al., 2020; Vogl et al., 2018).

In the previous study, human insulin precursor (HIP) has been generated in *P.pastoris* X33/pD902-IP (Nurdiani et al., 2018). The expression of this protein had been confirmed in shake flask culture and 2L-fermenter. However, the scale up of HIP has not been studied in a 10L-fermenter. This paper will discuss development of HIP production process in a 10L-fermenter.

## RESEARCH METHOD

*P.pastoris* X-33/pD902-IP was obtained from research group of Dini Nurdiani (Nurdiani et al., 2018).

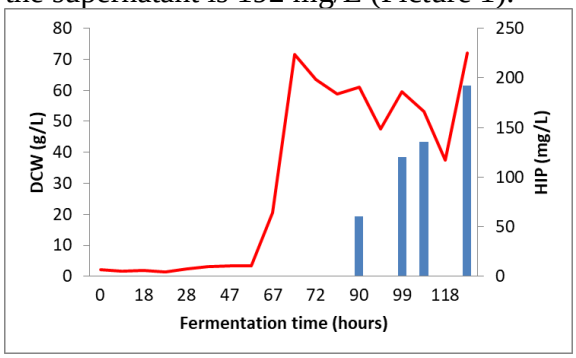
Cultivation of *P.pastoris* X-33/pD902-IP clon 4 (CL4) was conducted as follow: a single colony of CL4 from a YPD agar contain zeocin 100 µg/mL (Nurdiani et al., 2022) was inoculated into 40 mL YPD liquid contain zeocin 100 µg/mL. The culture was grown in a shaking incubation at 250 rpm and 30°C for 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 media (Wu et al., 2019) supplemented with 4,35 mL PTM1 trace salts/L (Invitrogen Corporation, 2002) of ½ BSM, 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 media in a 10 L Fermenter. Agitation was started at 150 rpm for 18 h and then raised gradually into the speed of 500 rpm. Aeration started at 2 L/h and then maintained at 3 L/h. Dissolved oxygen (DO) was maintained above 20%, and the temperature was set at 28°C (fermentation time 0 - 43 h; 66-120 hours) and 30°C (fermentation time at 44-65 hours). 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 1-3% (v/v) after the gliserol 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 10000 rpm and 4°C for 10 minutes to be stored at -80°C for further analysis (Wu et al., 2019).

Samples that collected were then analysed to determine the amount of biomass, dry cell weight (DCW) and the titer of HIP produced. The measurement of DCW followed Kocaefe-Özsen et al., (2022) with some modification: samples were centrifuged 14000 rpm for 5 minutes. The pellets were dried in an oven at 90°C for 2 hours and keep in dessicator for 30 minutes before weighted. The method to quantify HIP was done using a reversed-phase HPLC (RP HPLC) as in Putro et al., (2022).

## RESULT AND DISCUSSION

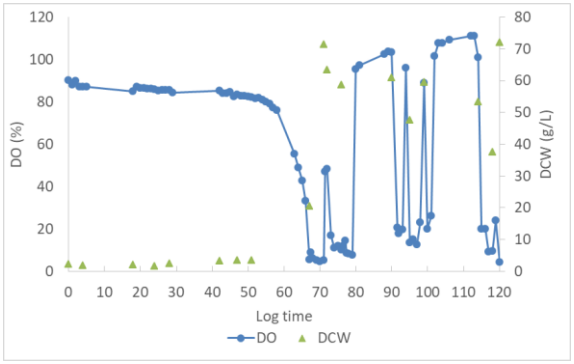
Cultivation in fermenter 10L stopped at 120 hours which consist of 72 hours glycerol batch phase and 48 hours methanol induction phase. The amount of biomass produced was 72 g/L and the HIP titer of the supernatant is 192 mg/L (Picture 1).




Picture 1. Biomass and HIP production of P.pastoris X33/pD902-IP in fermenter 10L. Red lines indicate DCW and blue bars indicate HIP titer.

At 48 hours of induction, DCW of this work is higher than the previous work (Putro et al., 2022), which are 72 and 28,5 g/L, respectively. Titer of HIP in this work at 48 hours induction was also higher than the previous work, 192 mg/L and 110.1 mg/L, respectively. Those results confirmed that the scaling up of this protein achieved the purpose of scale up production which is to demonstrate fermentation process in larger scale to has the same productivity and quality as in the small scale (Yang, 2014).

Interestingly, we observed a plateau phase in DO record graph several hours after methanol pulse feeding was stopped (Picture 2). This might because methanol as the carbon source was exhausted from the media, so the microbes stopped their metabolism and the oxygen in the media was not consumed (Invitrogen Corporation, 2002).



Picture 2. Dissolved oxygen profile during process

Microbes was starving, thus the biomass of the cell in several hours after that had decreased. Interestingly, if the methanol feeding is continued, the number of biomass increased again (picture 2). Thus, we speculate if the feeding strategy set as continuos feeding, 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 500 mg, while the pulse feeding-methanol strategy resulted in 445 mg (Putro et al., 2022).

Since insulin is vital for diabetic patient and Indonesia still imported the raw material (Permen 2017), the development of production system for insulin is a need. The strain used in this work is developed in country (Nurdiani et al., 2018) as well as the fermentation system in the small scale (Nurdiani et al., 2022; Putro et al., 2022). However, the scaling up of this strain has not been studied. After establishing a developed fermentation system at industrial scale, the production of insulin in Indonesia can be done.

## SUMMARY

The development of human insulin precursor fermentation system in a 10L-fermenter had been done. This is a novel development of human insulin precursor in Indonesia at fermenter scale. The process resulted in biomass amount and HIP titer, 72 g/L and 192 mg/L, respectively. These results are higher than the previous research. However, those results are not the optimal results. The fermentation process can be optimised in the next work to achieve higher results and optimal process condition for HIP production.

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