Heterologous Expression of Recombinant Human Insulin Glargine (hIG) in Methylotrophic Yeast *Pichia pastoris*

Neng Herawati*, Yana Rubiyana, Desriani Desriani, and Adi Santoso

Research Center for Genetic Engineering, National Research and Innovation Agency (BRIN) Kawasan Sains dan Teknologi Soekarno, Jl. Raya Jakarta Bogor Km. 46 Cibinong, West Java, Indonesia

*Corresponding author: neng.herawati@brin.go.id

Abstract

World health organization (WHO) announced that diabetic patients increased significantly yearly worldwide. Consequently, the need for insulin becomes very large. *Pichia pastoris*, defined as methylotrophic yeast and a well-known expression and protein production host, is widely used for biopharmaceutical-based drug production. This research aims to synthesize human insulin glargine (hIG) protein in yeast *P. pastoris*. Human insulin glargine is a group of long-acting analogue insulin.We used a synthetic hIG-encoding gene constructed in frame with the truncated α-factor secretion signal in a pD902 expression vector. Recombinant plasmid pD902-hIG was linearized using *Sac*1 enzyme and transformed into *P. pastoris* genome. Multicopy clones were selected on YPDS plates containing Zeocin™ with concentrations of 100; 500; 1,000; and 2,000 µg//mL. Analyses using SDS-PAGE, slot blot, and Western blot showed that recombinant hIG protein had been obtained with a molecular weight of approximately 6,000 Daltons.

Keywords: insulin, glargine, diabetes mellitus, *Pichia pastoris*

Introduction

Diabetes Mellitus (DM) is a well-known disease commonly indicated by high-level sugar in the bloodstream. The rise of blood sugar usually happens when the body is unable to release insulin or consume an adequate amount of insulin and leave the excess in the blood. The number of people with diabetes has risen due to population growth, aging, urbanization,growing overweight prevalence rate, and worsen by minimum physical activities. DM incidence has reached epidemic worldwide, with almost 174 million people living with diabetes. Moreover, it is predicted that the number becomes double by 2030 (Wild *et al*., 2004). Insulin is the main factor for DM type 1 and type 2 treatments. Uncontrolled diabetes triggers coronary heart disease, kidney failure, blindness, amputations, and risk of death. Thus, sufficient insulin supply is crucial to prevent those adverse clinical effects. Insulin utilization for DM treatment has been started since the first time insulin was found by Banting and Best in 1922 (Kaur & Badyal, 2008). It was purified from cows or pigs (Baeshen *et al*., 2014). In 1980, with the aid of DNA recombinant technology, human insulin was firstly developed, replacing animal insulin for human DM therapy.

Insulin glargine is an analog, long-acting insulin that consists of 52 amino acids. It first launched in Germany in 2000, followed in England and USA. It is used for diabetes type 1 and 2 treatments (Joseph & Donner, 2015; Barnet, 2006). Two modifications were made to human insulin for approaching insulin analog development. The first modification was done by adding 2 arginine molecules at the C terminal of the B-chain. The second modification is the replacement of glycine molecule with asparagine residue, at position 21 of the A-chain. Pharmacokinetic and pharmacodynamic studies confirmed that a single injection of insulin glargine leads to 24-hours action without any undesirable peaks (Barnet, 2006; Chakkarwar & Manjrekar, 2005). Insulin glargine also known to reduce hypoglycemia. Insulin glargine has a molecular weight of 6,063 Daltons (Chatterjee *et al*., 2006). Chemical structure examination of insulin found that A-chain and B-chain of insulin glargine are connected by disulfide bonds bridging chain A7 with B7, as well as A20 and B19. The third disulfide bond located in A-chain connecting A6 and A11 (Gualandi-

Signorini & Giorgi, 2001; Derewenda *et al*., 1989; Joshi *et al*., 2007). The total of amino acids residues of A-chain is 21, while B-chain is calculated to have 30 residues.

Pichia pastoris protein expression system has several advantages for recombinant protein production, such as major secretion protein of interest because *P. pastoris* produce a minimal amount of its endogenous proteins. Undoubtedly, it provides ease during protein harvest. Other advantages are straightforward transformation and easy selection methods comparable to bacterial expression system; there are no endotoxin and bacteriophage contamination (Li *et al*., 2007; Rabert *et al*., 2013; Cereghino & Cregg, 2000; Herawati *et al*., 2018); notably, secretion of target protein. The presence of AOX1 (alcohol oxidase 1) promotor controls protein recombinant expression by induction mechanism (Skoko *et al*., 2003; Cereghino & Cregg, 2000); manage to grow in high cell densities (> 100 g/L dry cells mass) in the fermentor and simple medium culture (Brierley, 1998; Li *et al*., 2007); it provides post-translational modifications, such as protein folding, glycosylation, acylation, proteolytic regulation, and targeting to subcellular compartments (Jahic *et al*., 2002; Ahmad *et al*., 2014; Schwarzhans *et al*., 2016).

In *P. pastoris* expression system, insulin glargine is expressed as a single-chain precursor consisting of an N-terminal B-chain followed by an A-chain. Glargine precursor was digested with trypsin to separate A- and B-chains, which were then converted into 2 chains (Hazra *et al*., 2009). Trypsin specifically cut lysin and arginine residues of the C terminal (Olsen *et al*., 2004). Because the B-chain sequence of insulin glargine has lysin and arginine residues, the number of contaminant fragments was eliminated by trypsin. Glargine has unique amino acid sequences whereas on its B-chain settled two arginine amino acids (RR), which can be treated with kex2p and change single chain precursor into 2 chains (Sreenivas *et al*., 2015). This research aims to synthesize extracellular expression of human insulin glargine (hIG) using methylotrophic yeast to produce biologically active hIG protein for further utilization in DM treatments in Indonesia.

Materials and Methods

Gene Construction. Genetic code of hIG DNA sequence and signal sequences of Mat-α secretion factors from yeast *Saccharomyces cerevisiae* was synthesized by ATUM (USA) gene synthesis service provider in pD902 (pD902: 2922912) expression vector. The gene construct of hIG/pD902 (Figure 1) was verified using gene sequencing analysis.

Plasmid Cloning Vector. The pD902-hIG plasmid was transformed into *E. coli* DH5α competent cells using the heat shock method. Recombinant *E. coli* cells were grown on solid Luria Broth (LB) medium that already contain Zeocin™ at a concentration of 25 µg/mL, incubated at 37ºC for 18-24 hours. The selected single colonies was regenerated in liquid LB medium with 25 µg/mL of Zeocin™, optimum temperature was set at 37ºC and was incubated for 18-24 hours at 150 rpm. Plasmid isolation was carried out with a HiSpeed plasmid Midi Kit from Qiagen. The DNA concentration was measured using a Nanodrop™ spectrophotometer prior to the linearization process using *Sac*1 (Thermo scientific) restriction enzyme.

***P. pastoris* Transformation and Confirmation.** The GS115 yeast strain (Invitrogen, CA, USA) was used for hIG expression. Competent cells preparation followed modified method of Cereghino *et al.* (2005). Linearized of pD902-hIG plasmids were transformed into *P. pastoris* competent cells by electroporation. Using 0.2 cm cuvette (Biorad), recombinant cells were recovered for 1 hour in 1 M sorbitol at 30ºC, then spread on YPDS medium (1 % yeast extract, 2 % peptone, 2 % dextrose, 1 M sorbitol, and 2 % agar) with 100 µg/mL of Zeocin, incubated at 30 ºC for 2 days. After 2 days of incubation, single colonies that grew on YPDS medium was then purified by re-growing in the same medium and same condition (YPDS agar + 100 µg/mL Zeocin™ for 2 days).

Selected colonies were grown on liquid YPD medium contain 100 µg/ml Zeocin™ and incubated at 30ºC for 24 hours. A part of the cultures were then preserved into glycerol stock, while the rest was isolated the genomic DNA using Yea Star Genomic DNA Kit (Zymo Research). The obtained genomic DNAs were further confirmed by PCR using a pair of 5'aox and 3'aox primers (Nurdiani *et al*., 2018).

Restriction analysis of genomic DNA was also carried out using *Eco*R1, *Not*1, and *Xba*1 restriction enzymes. DNA bands of PCR product and restriction enzymes analysis were visualized on agarose gel electrophoresis using a UV trans-illuminator. Analysis of DNA Sequence was also performed to confirm the results of integration.

Transformants Selection. The transformants selection process was carried out by growing single colonies on solid YPDS medium containing 4 variations of Zeocin™ concentrations (100; 500; 1,000; and 2,000 µg/mL) at 30ºC for 2-10 days.

Protein Expression. Protein expression was conducted by growing selected transformant colonies into buffered complex medium containing glycerol (BMGY contain 1 % yeast extract, 2 % peptone, 100 mM potassium phosphate pH 6, 1.34 % YNB, 4×10^5 % biotin, and 1 % glycerol) at the temperature of 30 ºC and shaking at 200 rpm until the culture reaches OD_{600}= 2-6 (±16-18 hours). Cells were harvested by centrifugation at 12,000 rpm for 5 minutes. The supernatant was discarded, and obtained pellet was grown (OD_{600}=1) in a buffered methanol–complex medium (BMMY) (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate pH 6, 1.34 % YNB, 4×10^5 % biotin). Furthermore, the cultures were grown at 30 ºC, 200 rpm for 24 hours. Supernatants containing desired protein, were collected and stored for protein expression analysis.

Protein Analysis. Protein analysis was done using the SDS-PAGE method following published article by Schägger (2006). The concentration of separating gel to separate proteins was 16 %, Commassie Brilliant Blue (CBB) R-250 staining (Biorad) was used for protein visualization on acrylamide gel after electrophoresis. Slot Blot analysis was performed on nitrocellulose membrane using monoclonal antibody anti-insulin (mouse). The visualization of protein target on the nitrocellulose membrane was carried out using NBT-BCIP staining substrate. Western blots were performed using a monoclonal anti-insulin antibody (Sigma Aldrich) as the primary antibody and an anti mouse IgG alkaline phosphatase-linked whole antibody (Promega, Madison, WI, USA) as the secondary antibody. The bands were detected by BCIP/NBT Kit color development substrate (life technologies, USA).

Phenotype Assay. Phenotype assay was conducted by growing recombinant *P. pastoris* on a solid medium with minimal medium containing methanol and/or histidine (MM (H)) (1.34 % YNB, 4×10^{-5} % biotin, 0.5 % methanol, 0.0004 % histidine, 2 % agar) and a minimal medium containing glycerol and/or histidine (MD (H)) (1.34 % YNB, 4×10^{-5} % biotin, 1 % glycerol, 0.0004 % histidine, 2 % agar). Based on the speed of methanol use, the phenotype of *P. pastoris* was divided into Mut+ and Muts. Observation of sample growth rate was done every 24 hours.

Results

Gene Construction.

The construction of the hIG gene expression cassette in the pD902 vector is shown in Figure 1, and the vector map after expression cassette was fused in pD902 (ATUM) is shown in Figure 2.

Plasmid Cloning.

Plasmid cloning process pD902-hig into *E. coli* is successful because recombinant *E. coli* can be grown in media with Zeocin™ supplementation. The growing colonies are regenerated in liquid LB media containing Zeocin for plasmid isolation purposes. Successful plasmid isolation is indicated by the presence of a band on the gel electrophoresis. The results of plasmid cloning are shown in Figure 3. The isolated plasmid was then linearized. The results of the plasmid linearization are shown in Figure 4.

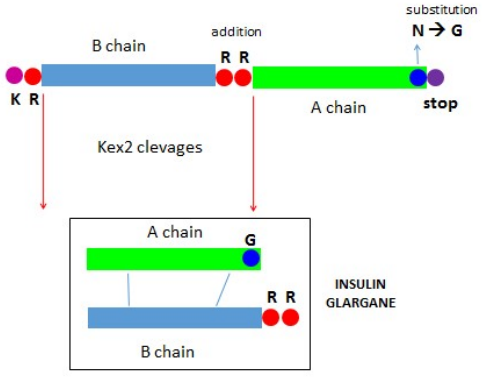

Figure 1. Gene construction for direct secretion of two insulin glargine chains in *P. pastoris.*

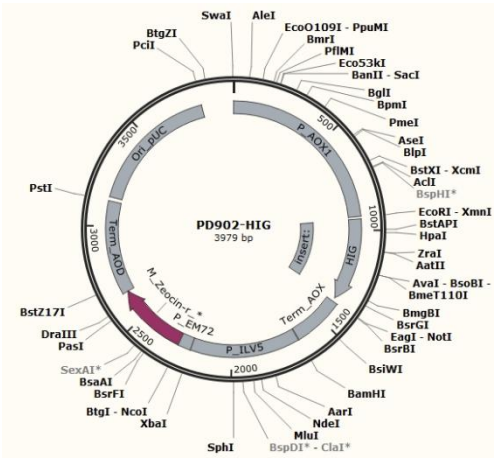

Figure 2.Vector map of pD902-hIG.

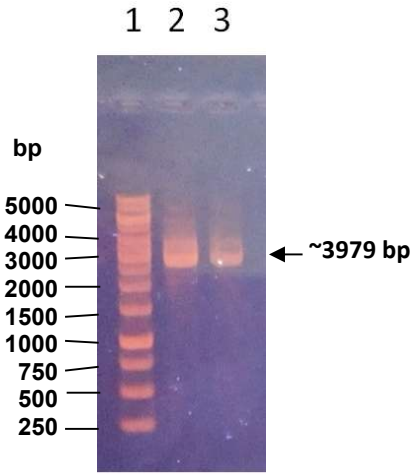

Figure 3. The pD902-hIg plasmid DNA bands. Lane 1:1 kb DNA Ladder, 2-3: plasmid DNA.

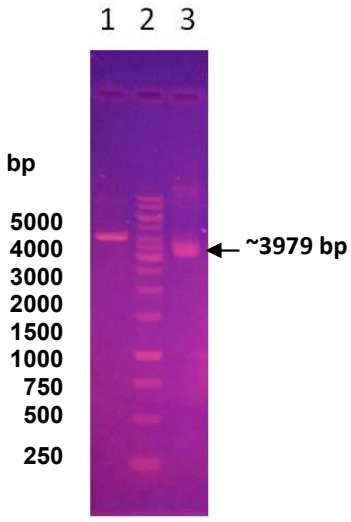

Figure 4. Linearized plasmid DNA bands of pD902-hIG. Lane 1: linear plasmid, 2: 1 kb DNA Ladder, 3: circular plasmid.

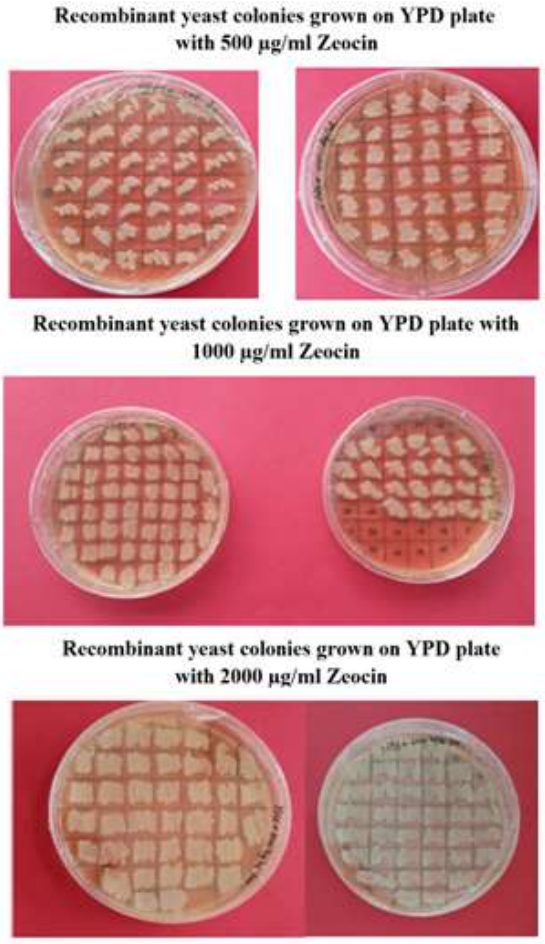

Figure 5. Transformants selection with Zeocin™.

P. pastoris Transformation, Selection, and Confirmation.

The transformation was carried out using *P. pastoris* GS115 strain. The selected clones grew in YPDS plate containing 100 ug/mL Zeocin™ was replated in YPDS plates containing 500; 1,000; and 2,000 ug/mL Zeocin™ gradually (Figure 5). The colonies from 2,000 ug/mL Zeocin™ plate were cultured, followed by genomic DNA extraction from the cell pellet to confirm the positive transformant. The DNA was then digested using various restriction enzymes, as shown in Figure 6. The conformation of positive transformant was also performed by PCR analysis using AOX primers. The amplified DNA at 568 bp indicated that the insulin glargine gene has been successfully integrated into *P. pastoris* genome (Figure 7).

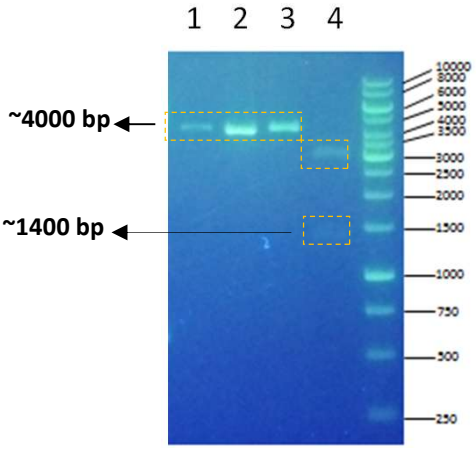

Figure 6. Restriction enzyme analysis of pD902-hIG. Lane 1-4: plasmid digestion using *Eco*R1; *Not*1; *Xba*1 and *Eco*R1; and *Xba*1, respectively. 5: 1 kb DNA Ladder.

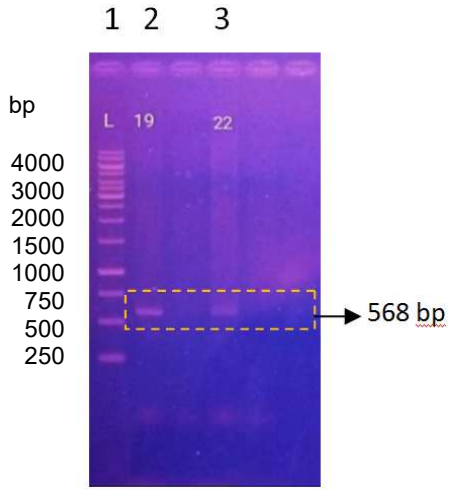

Figure 7. Amplicon gene insertion from recombinant *P. pastoris* genome. Lane 1: 1 kb DNA Ladder, 2: recombinant *P. pastoris* clone 19, 3: recombinant *P. pastoris* clone 22.

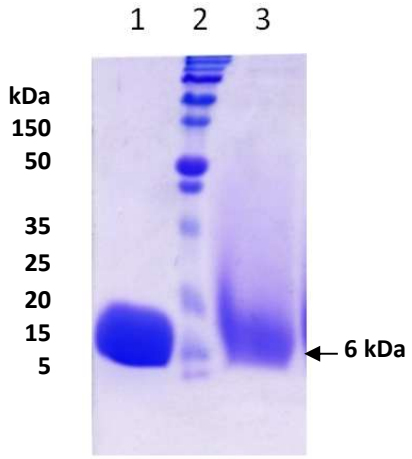

Figure 8. Expression analysis by SDS-PAGE. Lane 1: insulin glargine standard (Lantus), 2: protein marker, 3: sample hIG protein from *P. pastoris.*

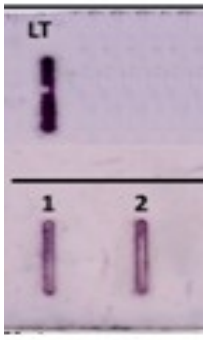

Figure 9. Slot blot analysis of hIG protein. LT: insulin glargine (Lantus) as a positive control, 1-2: hIG protein from *P. pastoris*.

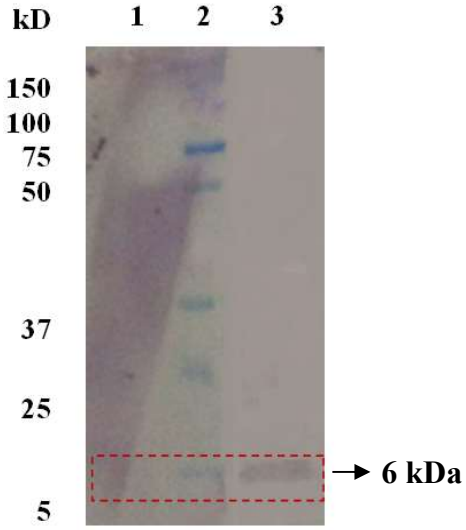

Figure 10. Western blot analysis of hIG protein. Monoclonal antibody specific for insulin was used as the primary antibody. Lane 1: insulin glargine standard (Lantus), 2: protein marker (Bio Rad), 3: sample of hIG protein from *P. pastoris*.

Protein analysis.

Supernatant cultures secreted by *P. pastoris* cells were characterized by their molecular weight and protein identity through SDS-PAGE, Slot blot, and Western blot methods, respectively. The result of hIG protein analysis are shown in Figures 8, 9, and 10, respectively.

Discussion

The construction of the hIG gene expression cassette in pD902 vector is shown in Figure 1. The DNA sequence of Mat-α *S. cereviseae* is integrated into the vector whichispositionedbefore the B-chain of the glargine gene.In this construct, between B-chain and A-chain is not connected by C-chain. This strategy refers to a publication conducted by

Sreenivas *et al*. (2015). The expectation is that insulin glargine will be directly expressed by *P. pastoris* cells in the form of two chains.

The B-chain and A-chain are combined to form one open reading frame (ORF) in constructing the gene that expresses insulin. At position 5'of the B-chain, there are lysine (K) and arginine ® amino acids. Meanwhile, at position 3' of the B-chain, two arginine (RR) amino acid are inserted. The function of RR here has 2 objectives, firstly, RR is part of the B-chain and secondly, RR along with KR is a motif that is the substrate of the Kex2 protein produced in the host cell we use (*P. pastoris*). The ORF ends with a stop codon which is located at position 3'in A-chain. It is known that *P. pastoris* expresses Kex2 protein, which has KR or RR as the substrate. The specification of this substrate makes ORF expressed by *P. pastoris* to be cut by Kex2, which is produced in *P. pastoris* to form B-chain and A-chain. The expression cassette for this research is pD902 (ATUM) (Figure 2). Propagation process of pD902-hIG plasmids into *E. coli* cells was successful, as demonstrated by the growth of colonies on LB solid medium containing 25 µg/mL Zeocin™. This evidence shows that recombinant *E. coli* already contains pD902-hIG plasmids Zeocin™ resistant marker; thus, *E. coli* can grow on these selection medium.

Some colonies were then regenerated in a liquid LB medium containing Zeocin™ for plasmid isolation. Successful plasmid isolation was demonstrated by the presence of DNA bands on agarose gel electrophoresis (Figure 3). Obtained DNA concentration after being measured by Nanodrop™ spectrophotometer was 1,913 ng/µL. Subsequently, plasmids were linearized using *Sac*1 restriction enzyme (Figure 4) before being transformed into *P. pastoris* genome. Linearization results (Figure 4) confirms that pD902-hIG plasmid was successfully linearized with the length of ±3,979 bp as expected.

The successful transformation was confirmed by digestion of pD902-hIG using *Sac*1, *Not*1, *Xba*1, and *Eco*R1 restriction enzymes. The digestion reaction was arranged with single and double (a pair) of restriction enzymes. The results of restriction process are shown in Figure 5.

The methylotrophic yeast *P. pastoris* is a host for the production of recombinant proteins (Cereghino & Cregg, 2000; Cregg *et al.*, 2000). Linearized recombinant plasmids that have been integrated into theGS115 genome of *P. pastoris* cells can grow on YPDS medium containing 100 µg/mL Zeocin™, which means plasmid was successfully integrated into the yeast genome; thus, cells can produce Zeocin resistance protein. The morphology of *P. pastoris* colonies was seen in around shape with a creamy white color (picture is not displayed). Based on the growth ability of recombinant *P. pastoris* in a YPD medium containing Zeocin, it can be concluded that transformation process was successful.

Transformation of the GS115 strain with a linear recombinant plasmid construct is a homologous recombination at the AOX1 locus. Most transformants can be classified as Mut+. However, the presence of AOX1 sequences on plasmids allows recombination to occur in the 3'AOX 1 region, disrupts the wild-type of AOX1 gene and produces Mut^S transformers (Cregg *et al*., 2000). MDH and MMH solid medium tests were carried out to determine which transformants were Mut+ (data was not shown).

Transformation of recombinant *P. pastoris* with the insertion gene produces several single colonies. Recombinant multicopy selection of colonies was conducted to select the highest hIG protein-producing clone. The selection was carried out on a solid YPDS medium containing Zeocin at concentrations of 100, 500; 1,000; and 2,000 µg/mL (Easy select™ *P. pastoris* Expression Kit). The selection results are shown in Figure 6. Survived clones grown on medium with the highest Zeocin concentrations (2,000 µg/mL) are considered to have exceptional protein expression.

Confirmation of gene insertion, which was integrated into *P. pastoris* genome was carried out in two stages; isolation of the recombinant *P. pastoris* genome and amplification of inserted genes by PCR using 5'aox and 3'aox primer pair. Recombinant *P. pastoris* that carry inserted gene will produce an extend of 568 bp amplicon size (325 bp AOX1 gene and 243 bp insertion gene size) (Figure 7).

Supernatant cultures secreted by *P. pastoris* cells were characterized by their molecular weight and protein identity through SDS-PAGE, Slot blot, and Western blot methods, respectively. The pD902 expression vector used in this study has an AOX1 promoter whose regulated by the methanol as an inducer. The GS115 strain used is classified as Mut+ (methanol utilization positive) phenotype, which is very responsive to methanol as a carbon

source (Cereghino & Cregg, 2000). Molecular weight analysis results with CBB staining confirms ± 6 kDa band size. This size is comparable with molecular weight of Lantus (commercial insulin glargine) as a protein control (Figure 8). The preliminary test of protein identity using monoclonal anti-insulin antibodies also shows the presence of protein dots on the nitrocellulose membrane (Figure 9). Protein identity was verified by Western-Blot with a specific anti-insulin monoclonal antibody (Figure 10).

Conclusion

This study has successfully carried out recombinant expression of hIG protein using GS115 strain (Mut^+) *P. pastoris*. Molecular weight analysis with SDS-PAGE shows that glargine protein has been secreted by *P. pastoris* yeast cells as host. However, currently we are still looking for optimum conditions to perform analysis on protein identity using Western blotting method, and proteins characterization using the LC-MS.

Acknowledgements

We thank Lailatul Qodria for her assistance in protein analysis.

References

Ahmad, M., Hirz, M., Pichler, H., & Schwab, H. (2014). Protein expression in Pichia pastoris: recent achievements and perspectives for heterologous protein production. *Applied Microbiology and Biotechnology, 98*(12), 5301-5317.

Baeshen, N. A., Baeshen, M. N., Sheikh, A., Bora, R. S., Ahmed, M. M. M., Ramadan, H. A., ... & Redwan, E. M. (2014). Cell factories for insulin production. *Microbial cell factories*, *13*(1), 1-9.

Barnett, A. H. (2006). Insulin glargine in the treatment of type 1 and type 2 diabetes. *Vascular Health and Risk Management, 2*(1), 59.

Brierley, R. A. (1998). Secretion of recombinant human insulin-like growth factor I (IGF-I). In Pichia protocols (pp. 149-177). Humana Press.

Cereghino, J. L., & Cregg, J. M. (2000). Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS microbiology reviews, 24*(1), 45-66.

Cereghino, J. L., Wong, W. W., Xiong, S., Giang, W., Luong, L. T., Vu, J., ... & Lin-Cereghino, G. P. (2005). Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast *Pichia pastoris*. *Biotechniques, 38*(1), 44-48.

Chakkarwar, P. N., & Manjrekar, N. A. (2005). Insulin glargine: A long acting insulin analog. *Journal of Postgraduate Medicine, 51*(1), 68.

Chatterjee, S., Tringham, J. R., & Davies, M. J. (2006). Insulin glargine and its place in the treatment of Types 1 and 2 diabetes mellitus. *Expert Opinion on Pharmacotherapy, 7*(10), 1357-1371.

Derewenda, U., Derewenda, Z., Dodson, G. G., Hubbard, R. E., & Korber, F. (1989). Molecular structure of insulin: the insulin monomer and its assembly. *British Medical Bulletin, 45*(1), 4-18.

Gualandi-Signorini, A. M., & Giorgi, G. (2001). Insulin formulations-a review. *European Review for Medical and Pharmacological Sciences, 5*, 73-84.

Hazra, P., Dave, N., Kannan, V., Tiwari, S., Goel, A., Iyer, H., ... & Patale, M. B. (2009). A method of obtaining purified heterologous insulins expressed in yeast. Paten, WO/2009/104199 A1.

Herawati, N., Kusumawati, A., & Santoso, A. (2018). Pichia pastoris: Sel Ragi Untuk Produksi Protein Rekombinan. *Berita Biologi, 17*(2), 91-102.

Invitrogen, 2010. EasySelect™ Pichia Expression Kit. A manual of methods for expression of Recombinant roteins using pPICZ and pPICZα in *Pichia pastoris*

Jahic, M., Rotticci-Mulder, J. C., Martinelle, M., Hult, K., & Enfors, S. O. (2002). Modeling of growth and energy metabolism of Pichia pastoris producing a fusion protein. *Bioprocess and Biosystems Engineering, 24*(6), 385-393.

Joseph, J. J., & Donner, T. W. (2015). Long-term insulin glargine therapy in type 2 diabetes mellitus: a focus on cardiovascular outcomes. *Vascular Health and Risk Management, 11*, 107.

Joshi, S. R., Parikh, R. M., & Das, A. K. (2007). Insulin-history, biochemistry, physiology and pharmacology. *Journal-Association of Physicians of India, 55*(L), 19.

Kaur, J., & Badyal, D. K. (2008). Newer insulins. *JK Science Journal of Medical Education & Research*, *10*(3), 107-111.

Li, P., Anumanthan, A., Gao, X. G., Ilangovan, K., Suzara, V. V., Düzgüneş, N., & Renugopalakrishnan, V. (2007). Expression of recombinant proteins in *Pichia pastoris*. *Applied Biochemistry and Biotechnology, 142*(2), 105-124.

Nurdiani, D., Hariyatun, H., & Kusharyoto, W. (2018). Secretory expression of human insulin precursor in *Pichia pastoris* employing truncated α-factor leader sequence and a short C-peptide. *Indonesian Journal of Biotechnology, 23*(2), 102-108.

Olsen, J. V., Ong, S. E., & Mann, M. (2004). Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Molecular &Cellular Proteomics, 3*(6), 608-614.

Rabert, C., Weinacker, D., Pessoa Jr, A., & Farías, J. G. (2013). Recombinants proteins for industrial uses: utilization of Pichia pastoris expression system. *Brazilian Journal of Microbiology, 44*, 351-356.

Schägger, H. (2006). Tricine–SDS-PAGE. *Nature Protocols, 1*(1), 16-22.

Schwarzhans, J. P., Wibberg, D., Winkler, A., Luttermann, T., Kalinowski, J., & Friehs, K. (2016). Integration event induced changes in recombinant protein productivity in *Pichia pastoris* discovered by whole genome sequencing and derived vector optimization. *Microbial Cell Factories, 15*(1), 1-15.

Skoko, N., Argamante, B., Grujičić, N. K., Tisminetzky, S. G., Glišin, V., & Ljubijankić, G. (2003). Expression and characterization of human interferon-β1 in the methylotrophic yeast *Pichia pastoris*. *Biotechnology and Applied Biochemistry, 38*(3), 257-265.

Sreenivas, S., Krishnaiah, S. M., Govindappa, N., Basavaraju, Y., Kanojia, K., Mallikarjun, N., ... & Sastry, K. N. (2015). Enhancement in production of recombinant two-chain Insulin Glargine by over-expression of Kex2 protease in *Pichia pastoris*. *Applied Microbiology and Biotechnology, 99*(1), 327-336.

Wild, S., Roglic, G., Green, A., Sicree, R., & King, H. (2004). Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes care, 27*(5), 1047-1053.