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Expression of *Pichia pastoris*-Derived Secreted Insulin Precursor

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----------ABSTRACT----------

Insulin has been used as major treatment for patients with diabetes type 1. An increasing demand for insulin each year becomes a challenge to develop an alternative expression system for insulin precursor production. Pichia pastoris as methylotropic yeast is one of yeast expression systems that has been widely used and proven to successfully express a variety of heterologous proteins in high numbers. This research was carried out to express insulin precursor using α-factor signal peptide of S. cerevisiae for insulin precursor secretion process toward the extracellular space in Pichia pastoris. Concentration of methanol induction and expression media were optimized for efficient process of these proteins in Pichia pastoris. Strain KM71H was used as insulin precursor expression host and pPICZα-A as the expression vector. The result of this study shows that recombinant strain of P. pastoris which is KM71Hα-A/PI could express and secrete insulin precursor, indicated by protein band with a molecular mass of 7 kDa in SDS-PAGE. In this experiment, the most optimum condition for secretory expression of insulin precursor was 1% methanol induction in BMM expression medium. SDS-PAGE analysis showed that IP secretion work better and more efficient in BMM medium if compared to BMMY medium.

KEYWORDS:*Insulin Precursor, Pichia pastoris, Methanol Induction, Expression Media, α-factor Signal, Secretory Expression.*

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I. INTRODUCTION

Insulin has been discovered since 80 years ago and still continues to be studied until now in biochemistry and medicine**[1].** Insulin is still the main treatment for patients with type 1 diabetes and the demandcontinues to increase each year together with an increasing number of patients with diabetes[2]. Insulin is a peptide hormone secreted by β-cells from Langerhans islets of Pancreas. Insulin plays a significant role for maintaining glucose homeostasis in human body [3].Firstly, insulin is produced as preproinsulin with 108 amino acids that contain a signal sequence. Mature insulin consists of two polypeptide chains (Chain A and B) with 51 amino acids long. Chain A contains 21 amino acids and chain B contains 30 amino acids with three disulfide bonds [4].

First insulin that used for diabetes treatment was extracted from the pancreas of bovine and porcine. However, conventional insulin has high levels of contamination from the pancreas hormone so it requires a long purification process [5]. Around 1976, there was a scare that demand for insulin will increase dramatically and in 1992, acute deficiency of insulin worldwide occurred, despite the exclusive pig was raised solely to meet the insulin needs of the world[5]. Therefore, a new technology for human insulin production began to emerge by recombinant DNA which involvesmicroorganismas an alternative to conventional insulin.

Human insulin is one of the first therapeutic proteins expressed in microorganisms [6]. The early-generation recombinant insulin from *E. coli* (Humulin-US / Humuline-EU) succeeded in becoming the first recombinant therapeutic protein approved by the Food and Drug Administration (FDA) and commercially available[6-8]. Since then, recombinant insulin has been used as a substitute for animal insulin in the clinic [9].There are two major pathways that can be used, which is intracellular using *E. coli* expression system and extracellular using yeast expression system. Both of them are affordable in term of cost [1]. Insulin precursor that expressed in *E. coli* has a major problem in the formation of inclusion bodies, requiring further processes such as solubilization and oxidative refoldingafter fermentation [2]. The second generation of human insulin

recombinant was using yeast-based expression system for large-scale production, where the insulin precursor (IP) will be immediately secreted into the supernatant culture as soluble proteinand correctly folded[10]. *Saccharomyces cerevisiae* was the most commonly used as conventional yeast for insulin recombinant expression[11]. In 1999, half of the world's insulin needs came from recombinant insulin produced by S. cerevisiae[12]. This system, however, has a long downstream processing, about fifteen steps, and this system is limited in its expression due to lack of powerful and regulated promoters [1].

In recent years, several alternative yeasts have emerged. The methylotropic yeast especially Pichia pastoris has become popular since it has successfully expressed many heterologous proteins[13].P pastoris is a single cell eukaryotic and capable performing plentiful post-translational modification like those performed by high eukaryotic cell (i.e glycosylation, formation of disulfide bond, and processing proteolytic)[13].*P. pastoris* is well known to have many advantage characteristics, including the powerful and tightly regulated promoter, alcohol oxidase 1 (*AOX1*) which is induced by methanol as well as carbon source, easy to handle and manipulate genetically, simple media and cultivation procedure, easy to reach high cell densities and scaling up, growth media cheaper than mammalian tissue culture or baculovirus-based expression system. *P. pastoris* also has expression levels 10 to 100 times higher than other expression systems [14-16].Other advantage characteristics are high expression level, stable protein expression, high level of protein secretion, and no hyperglicosylation[1, 11].This research was carried out to express insulin precursor using α-factor signal peptide of *S. cerevisiae* for insulin precursor secretion process toward the extracellular space in Pichia pastorisstrain KM71H.

II. MATERIALS AND METHODS

Strain and construction of expression vectors

E. coli TOP10F' is the strain that was used in this study as the host of vector cloning. Pichia pastoris KM71H and expression plasmid pPICZα-A were obtained from Invitrogen. Insulin precursor gene was previously inserted into pPICZα-A via the restriction sites XhoIand NotIin-frame with α-factor secretion signal sequence to create recombinant expression-vector of pPICZα-A/PI; this was orderedthroughGeneScript company. Later, pPICZα-A/PI was integrated into *P. pastoris* KM71H's genome and then will be expressed extracellularly throughout this research.

Transformation of *P. pastoris* for secretory expression of insulin precursor

Plasmid pPICZα-A/PI and pPICZα-A were transformed into *E. coli* TOP10F' for cloning purposes with heat-shock method. After that, pPICZα-A/PI and pPICZα-A were isolated using *High-Speed Plasmid Mini Kit Geneaid.* The isolated plasmid then linearized using restriction enzyme *SacI* and transformed into *P. pastoris* KM71H using *Pichia EasyComp™ Kit.* The transformed vector pPICZα-A was used as the expression control in this study. Yeast Peptone Dextrose (YPD) agar medium (1% yeast extract, 2% peptone, 2% glucose, 1.5% agar) containing Zeocin was used to grow transformant cells to select and identify the clone of *E. coli* that had been inserted by the insulin precursor gene. Flask with 500 ml medium were inoculated with 0.5 ml *P. pastoris* culture and grown at 30℃ under 200 rpm shaking for up to 4 h. The culture was then diluted up to 10-50X to obtain optical density of OD_{600} = 0,6 – 1. After dilution, 0,5 ml of culture was sampled and centrifuged at 500 × g for 5 min at room temperature. The pellet then resuspended with 10 ml of solution 1 and further centrifuged at 500 × g for 5 min at room temperature followed by resuspension again with solution 1. The competent cells were storage at -20℃.

For transformation, 50 µl of competent cells were used and added with 5 µl restriction plasmid and 1 ml solution II. The homogenized mixture of cells and solutions was incubated for 1 h at 30℃ and homogenized in every 15 min. After that, the cells were given heat-shock at 42℃ for 10 min. Cells were separated into 2 tubes, each filled with ±525 µl culture with the addition of 1 ml YPD broth. Cells then incubated at 30℃ for 1 h and further centrifuged at 3000 × g for 5 min at room temperature. The pellet cell was resuspended with 0.5 ml solution III. Cells were combined into 1 tube and centrifuged at 3000 × g for 5 min at room temperature. Next, the pellet was resuspended with 100 µl solution III. The transformant cells were spread on the YPD agar medium containing Zeocin 100 µg/ml and incubated for 3-10 days until colonies formed.

The growth condition of culture and production of insulin precursor

A single colony from YPD selective agar medium containing Zeocin was inoculated into 50 ml BMGY in 250 ml flask. The colony in the flask was grown inincubator-shaker at 250 rpm until the culture reach the optical density of OD600 = 2-6 (± 18-20 h). Around this time, the cells would be on the log phase. Cells were harvested using centrifugation at 1500-3000 × g for 5 min at room temperature. Supernatant was removed but the cells pellet was resuspended in 20 ml BMM/BMMY medium to induce expression. Culture was incubated again in incubator-shaker at 250 rpm for 144 h (6 days). Every 24 h, the samples were induced with the various methanol until the final concentration reach 0.8%; 1% and 2%. Before induction, 1 ml of samples were analyzed

using SDS-PAGE to see the expression level of protein. From here, the most optimum induction time to express insulin expression could be decided. The harvested culture was further centrifuged at 13.000 × g for 3 min at room temperature and the supernatant was separated into different tube. Supernatant or crude extract of the expression result was stored at -80℃ until it was ready to be analyzed.

Precipitation using Trichloroacetic Acid (TCA)

Precipitation using TCA were prepared as described previously **[17].** Each 1 ml of protein sample was added with 100 µl of TCA solution (50 g TCA, 30 ml deionized water) and then homogenized. Protein samples then incubated at 4℃ for 30 min for precipitation process. Next, samples were centrifuged at 13.000 × g for 15 min at 4℃, supernatant was removed carefully and fast. Pellet would be seen on the base of the tube in a small amount. Samples were washed two times with 200 µl cold acetone to remove the remnants of TCA. Samples further centrifuged at 13.000 × g for 5 min at 4℃ and supernatant was removed. Protein pellet could be used directly to be analyzed with SDS-PAGE but firstly had to be resuspended in 30 µl of 2X sample buffer.

SDS-PAGE Analysis of Insulin Precursor

The supernatant of protein samples was analyzed using 10% polyacrylamide gels with tricine buffer system in a mixture of 49.5% T and 3% C [18]. The TCA precipitated samples were suspended in 30 µl 2X sample buffer (0.1 M Tris-HCl pH 6.8; 24% glycerol; 8% SDS; 0.2 M DTT; 0.02% Coomassie blue G-250) and added with 1 µl β-mercaptoethanol. Theelectrophoresis was started with the starting voltage of 30 Volt for 40 min or until the samples came out from the stacking gel.

Then, the voltage could be increased gradually but kept maintained so it would not higher than 80 mA. Overall, the time needed for electrophoresis was around 2.5 h. The protein bands were visualized using Coomassie blue R-250 and silverstainning.

III. RESULTS

Construction of recombinant plasmid (pPICZα-A/PI) for insulin precursor expression

Nucleotide sequence, encoding IP gene, was codon-optimized for IP secretory expression in *P. pastoris* and chemically synthesized as described before [12]. At the N-terminus of this sequence, there were four amino acids (LEKR) corresponding to the C-terminus of *S. cerevisiae* α-factor secretory signal sequence, which contains the Kex2 cleavage site. It is also given additional spacer peptide (EEAEAEAEPK) to make the processing and secretion of Kex2 endoprotease more efficient, followed by the insulin B chain (29 amino acids), a short linker (AAK), and the insulin A chain (21 amino acids). This IP gene was inserted to vector plasmid pPICZα-A, in frame with *S. cerevisiae* α-factor secretory signal sequence to generate recombinant plasmid of pPICZα-A/IP. This PI gene brings its own stop codon so the c-myc and His_6 tag will not be expressed. The IP expressed by this plasmid vector are estimated to be~7 kDa with 63 amino acids. The figure 1 showed that the target genes were inserted to pPICZα-A via the restriction sites XhoIand NotI in frame with α-mating factor.

Transformation of *P. pastoris* KM71H

Expression plasmid pPICZα-A/PI was transformed into *E. coli* TOP10F' for cloning and plasmid propagation. As a control, plasmid without pPICZα-A-insert was also transformed. The transformants then were selected in LB-low salt agar medium containing Zeocin (25 µg/ml). The recombinant plasmid pPICZα-A/PI from *E. coli* was isolated and restricted using SacI. The plasmid pPICZα-A which had been inserted with insulin precursor gene has the size of 3.7 kbp (Figure 1). The result from electrophoresis showed that the restricted pPICZα-A/PI would migrate in a slower pace compared to the circular pPICZα-A/PI. They both were supposed to have the same size, but the circular plasmid would be smaller than 3.7 kbp in size [19] (Figure 3A). LinearizedpPICZα-A/PI and pPICZα-A plasmids further transformed into *P. pastoris* KM71H using Pichia EasyComp™ kit and the transformants were selected using YPD agar medium containing Zeocin (100 µg/ml). After 3-10 days, the successfully transformed clones grew (Figure 2).

Some of clones that strove on Zeocin-containing medium were used for small scale fermentation with methanol induction.

PCR evaluation of insulin precursor from *P. pastoris* KM71H transformant

The *P. pastoris* transformants that strove on Zeocin-containing medium were reconfirmed with PCR. The DNA of *P. pastoris* KM71H transformants were firstly isolated using the method from Harju (2004) [20]. The DNA genome was successfully isolated, and the concentration reached 2500.44 ng/ml with the DNA purity (260/280) value of 2.05. The DNA needed to be further analyzed using PCR was only 100-200 ng/ml. The DNA amplification was done with two *AOX1* primers; forward 5'- GACTGGTTCCAATTGACAAGC-3' and reverse 5'- GCAAATGGCATTCTGACATCC -3'. These two primers were used to amplify the *AOX1* region of *P. pastoris* KM71H-αA/PI. The DNA fragment sized ~720 bp was obtained from the amplification of *AOX1* region

of *P. pastoris* KM71HαA/PI and ~589 bp-sized DNA fragment was obtained from the amplification of *AOX1* region of *P. pastoris* KM71H-αA. The obtained DNA fragments were in accordance in size with the pPICZα-A/PI that was used as the positive control (Figure 4).

Expression of Insulin Precursor

Following selection on zeocin medium, recombinant *P. pastoris* KM71HαA/PI which positively containing insulin precursor gene, used for fermentation on 250 ml Erlenmeyer with incubator-shaker. This method using two-step fermentation to express insulin precursor. First step is cell growth phase, which biomass accumulated on BMGY or BMMY medium until cell density reach $OD\lambda_{600}$ = 4-8. This step prevents cells to get poisoning by methanol which is used as expression inducer. In this step, glycerol's function as carbon source. Second step is induction phase for expression where expression of insulin precursor will be inducted by methanol [21]**.** Even though methanol act as inducer of expression insulin precursor, it also acts as carbon source for further biomass production. In expression phase, cell quantity will be still increase but not significant. Fermentation be held on 168 hours with the first 24 hours as cell growth phase and the next 144 hours as induction phase for the expression. At cell growth phase, KM71H-αA/PI which have phenotype Mut^s (methanol utilization slow), will be growth slowly than other strain with phenotype Mut^+ (methanol utilization plus) such as X33 and GS115 strain. In this research, variation of methanol induction (0,8%; 1%; and 2%) was performed using two different expressionmedium (BMMY and BMM). Extracellular and intracellular expression from insulin precursor analyzed by SDS-PAGE.

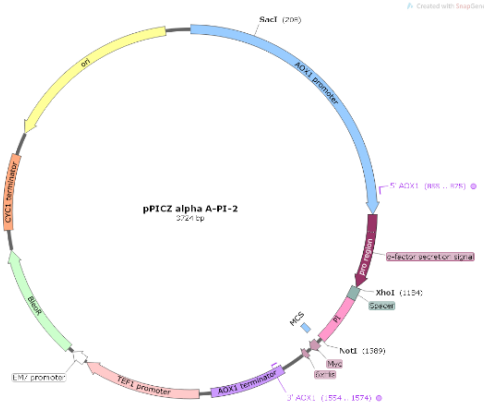

Figure 1. Schematic map of recombinant plasmid pPICZα-A/IP for IP expression. Constructed with SnapGene

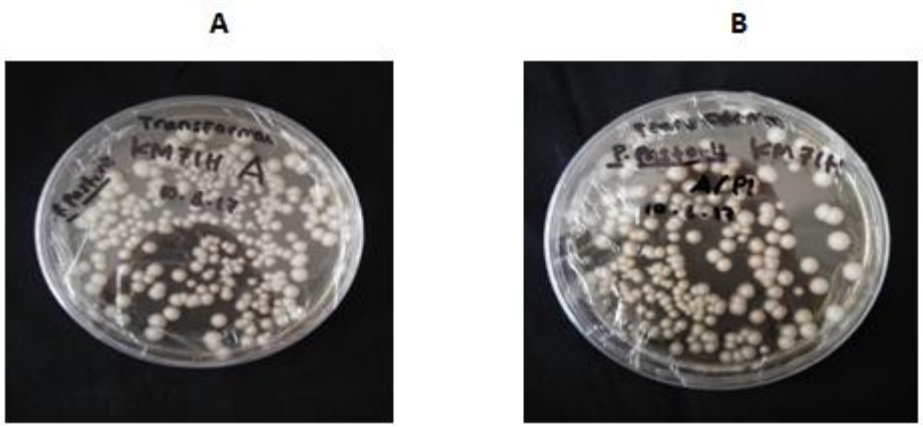

Figure 2. The colonies of recombinant *P. pastoris* that grew on Zeocin-containing medium after 3 days incubation at 28℃. (A) Recombinant *P. pastoris* KM71Hα-A (without the insertion of insulin precursor gene; as a negative control); (B) Recombinant *P. pastoris* KM71Hα-A/PI (with insertion of insulin precursor gene)

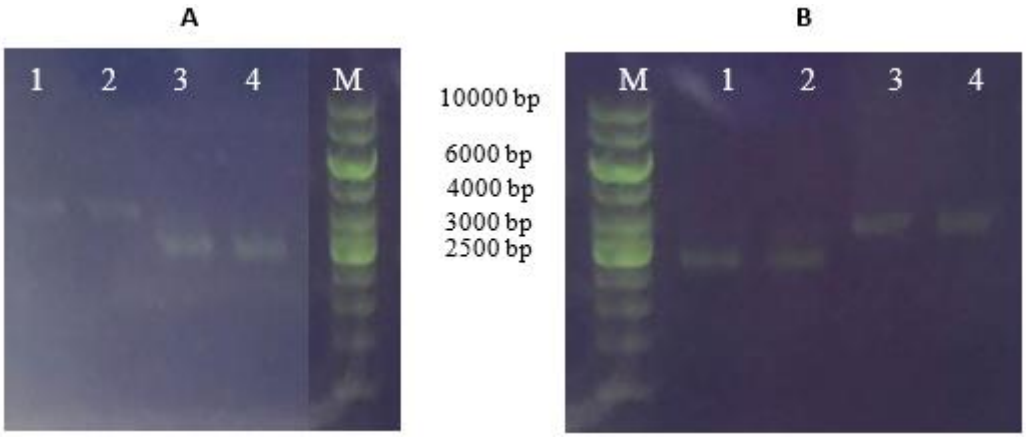

Figure 3. The electrophoresis result of pPICZα-A and expression plasmid pPICZα-A/PI that were isolated from *E. coli* TOP10F' along with linear plasmid that was restricted with SacI. **(A)**Lane 1-2: the restricted linearized-plasmid of pPICZα-A/PI that had the size of ~3.7 kbp. Lane 3-4: circular plasmid of pPICZα-A/PI with the size of ~2.8 kbpisolated from *E. coli* TOP10F'. **(B)** Lane 1-2: the restricted linearized-plasmid of pPICZα-A with the size of ~3.6 kbp. Lane 3-4: pPICZα-A circular plasmid with the size of ~2.7 kbp isolated from *E. coli* TOP10F'.

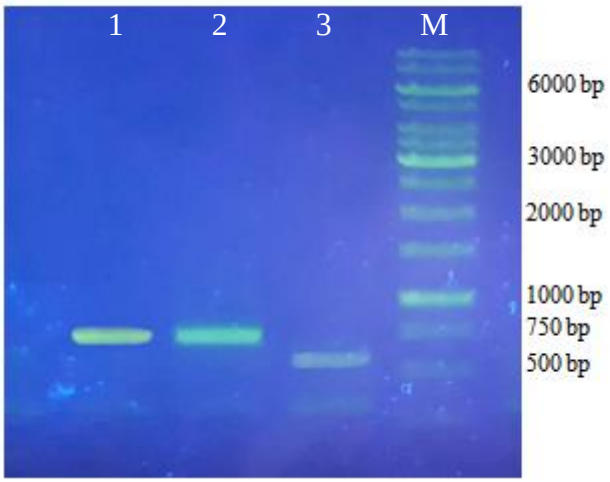

Figure 4. The electrophoresis result of amplified *AOX1* region. Lane 1: pPICZα-A/PI (positive control); Lane 2: the DNA genome of KM71Hα-A/PI with the size of ~720 bp; Lane 3: the DNA genome of KM71Hα-A with the size of ~589 bp.

SDS-PAGE Analysis

Shake-flask expression of IP was performed and after 144 h of induction time with methanol in expression medium (BMM and BMMY), the culture supernatant was analyzed using SDS-PAGE with human insulin as a control (~5,8 kDa). The result showed that recombinant strain KM71Hα-A/PI can expressed IP in both medium with three different concentrations of methanol induction (0.8%; 1%; 2%). But IP that expressed in 0.8% methanol induction has lower level expression compared with the other concentration (data not shown).Culture supernatant from 1% methanol induction in BMM medium was analyzed by SDS-PAGE and displayed a prominent band of ~7 kDa after 72h induction time and the bands were significantly thicken after precipitated with TCA (Figure5A). Result of ImageJ analysis showed that after 72h methanol induction, IP expression was increased by 78.94% and the maximum expression level was attained at 120h (Figure5B**)**.The result of SDS-PAGE analysis for 1% methanol induction in BMMY medium showed PI band of ~7 kDa after 72h with expression level of 70.14% and increased gradually until 120h induction time (Figure 6). In this combination (1%, BMMY), there was no significant difference before and after TCA precipitation. Result of IP expression with 1% methanol induction have the same profile, either in BMM or BMMY, where maximum expression level was achievedafter 120h induction time. For IP expression with 2% methanol induction in BMM media, SDS-PAGE gel showed obvious protein bands of approximately ~7 kDa, with the same molecular size as theoretically predicted. Besides, there was a significant difference after precipitated with TCA (Figure7A). After 72h induction time, expression level of IP reached 78.07%with maximum expression level was attained at 144h

(Figure7B). IP expression was also detected in BMMY medium with 2% methanol induction. SDS-PAGE gel showed there was a clearly IP bands of ~7 kDa at 72 h (Figure8A) and continously increased with the passage of time. Result of ImageJ analysis demonstrated that expression level of IP reached 61.51% at 120 h induction time and obtained maximum expression level at 144 h (Figure8B**)**. Based on SDS-PAGE result, it can be seen that the addition of 2% methanol in both medium expression (BMM and BMMY) generated the same trend, where the maximum expression were obtained at 144 h. Meanwhile, the intracellular IP expression were also analyzed by SDS-PAGE and showed IP bands of ~7 kDa (data not shown). Intracellular expression was higher in BMMY medium than BMM medium. Apparently, some of PI expressed by KM71Hα-A/PI in BMMY medium was not secreted and the PI expression in BMM medium had better secretion process.

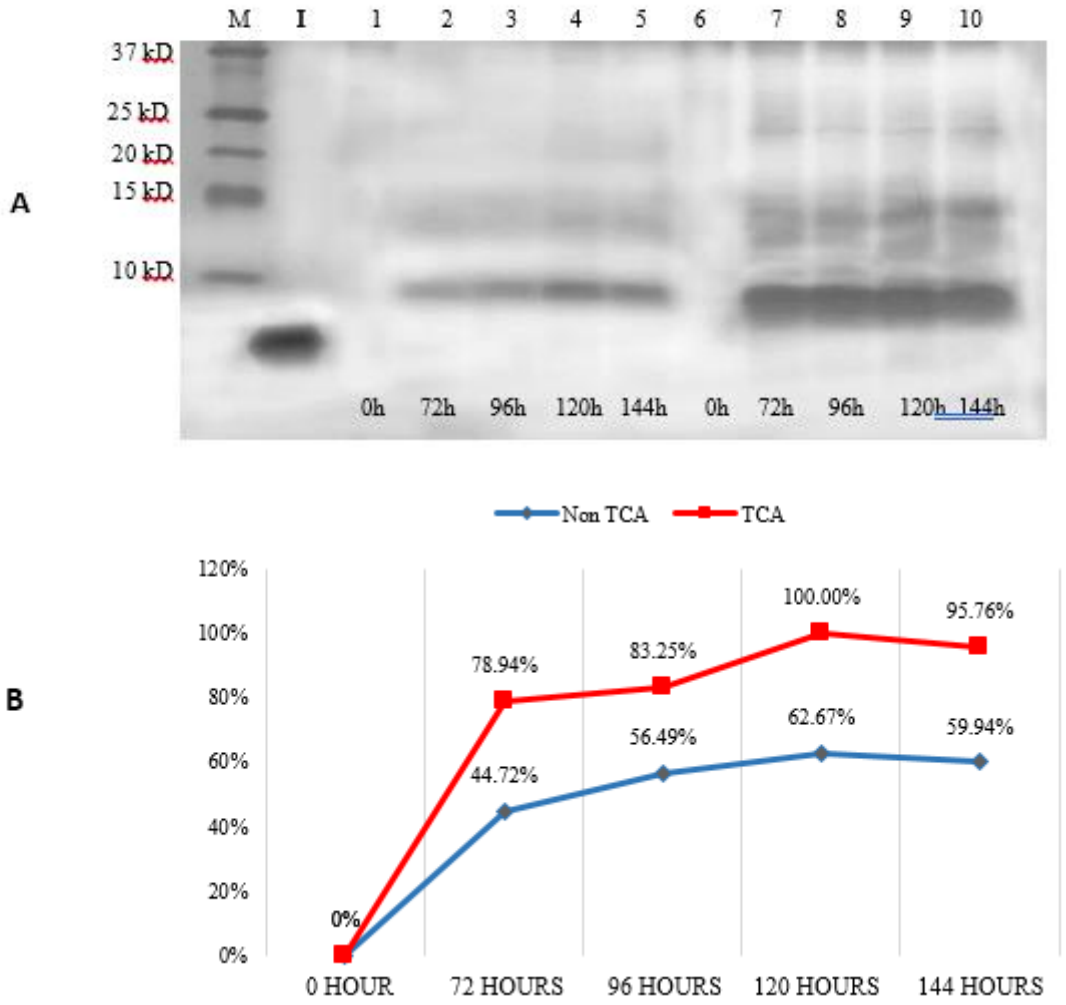

Figure 5 Analysis of culture supernatant with 1% methanol induction in BMM medium. (A) SDS-PAGE gel was visualized by silver staining. M = protein marker; I = insulin control (~5 kDa); Lane 1-5 = culture-supernatant proteins without TCA precipitation that showed PI bands of ~7 kDa; Lane 6-10 = culture-supernatant proteins after TCA protein that showed PI bands of ~7 kDa. (B) ImageJ analysis that displayed an increasing of IP expression level until 120 hours.

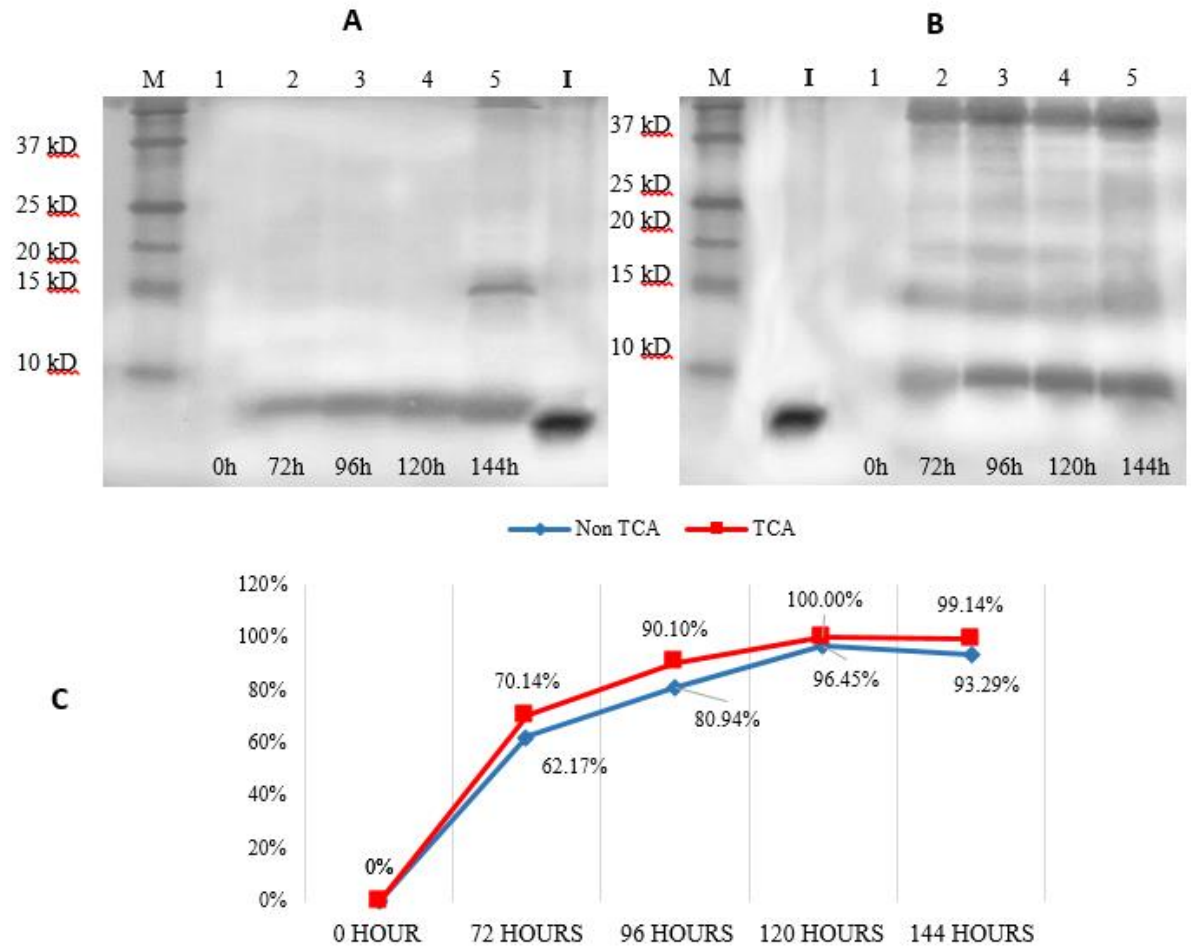

Figure. 6 Analysis of culture supernatant with 1% methanol induction in BMMY medium. SDS-PAGE gels were visualized by silver staining. M = protein marker; I = insulin control (~5 kDa); (A) culture supernatant proteins without TCA precipitation that showed PI bands of ~7 kDa; (B) culture supernatant proteins after TCA precipitation that showed PI bands of ~7 kDa. (C) ImageJ analysis that showed an increasing of IP expression level unt 120 hours.

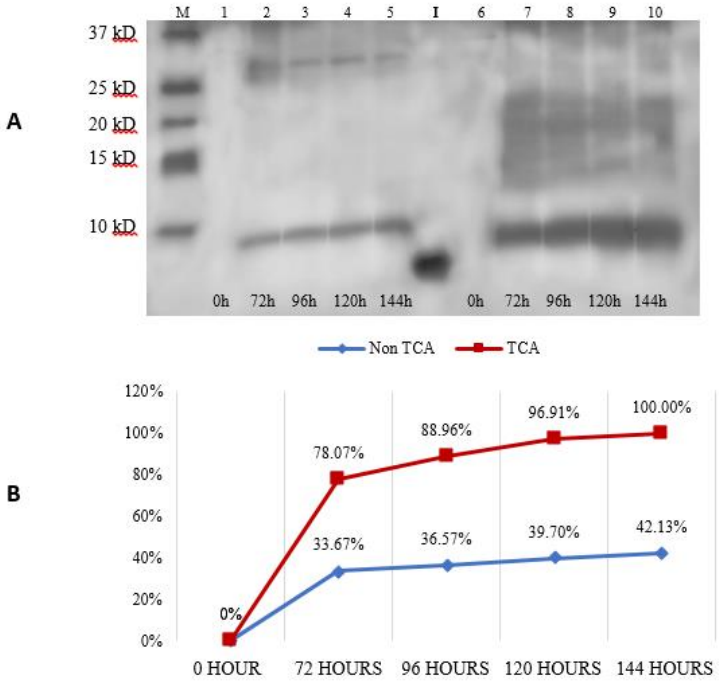

Figure7. Analysis of culture supernatant with 2% methanol induction in BMM medium. (A) SDS-PAGE gel was visualized by silver staining. M = protein marker; I = insulin control (~5 kDa); Lane 1-5 = culture-supernatant proteins without TCA precipitation; Lane 6-10 = cultur- supernatant proteins after TCA precipitation. (B) ImageJ analysis that displayed an increasing of IP expression level during 144 hours.

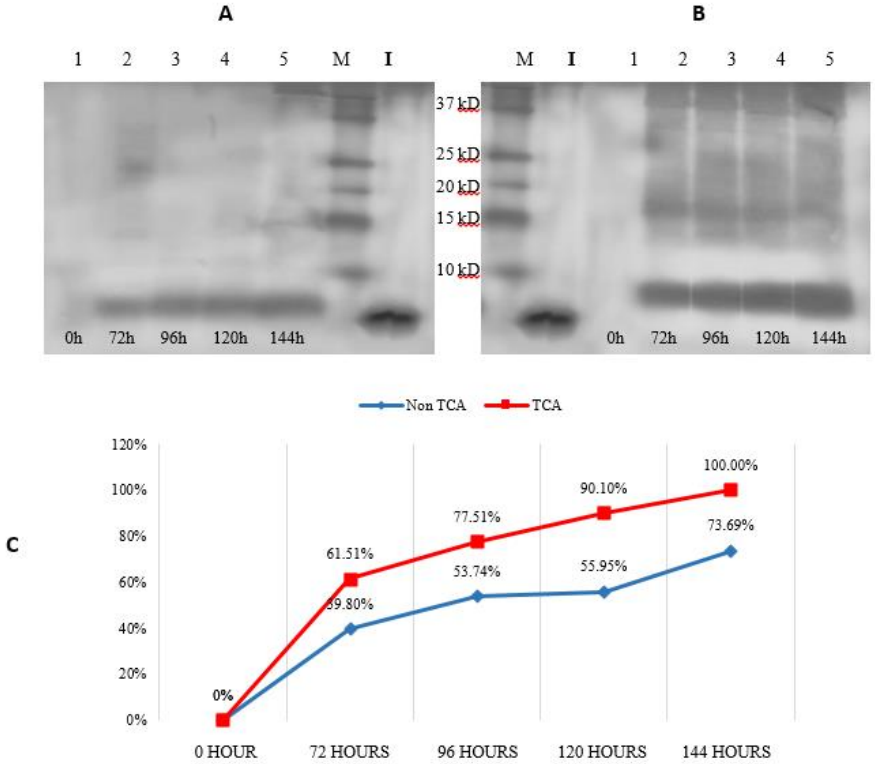

Figure 8 Analysis of culture supernatant with 2% methanol induction in BMMY medium. SDS-PAGE gels were visualized by silver staining. M = protein marker; I = insulin control (~5 kDa); (A) culture-supernatant proteins without TCA precipitation that showed PI bands of ~7 kDa; (B) culture supernatant proteins after TCA precipitation that showed PI bands of ~7 kDa. (C) ImageJ analysis that displayed an increasing of IP expression level until 120 hours.

IV. DISCUSSION

In this research, IP-encoding gene was codon-optimized which aims to increased the amount of functional recombinant protein obtained. Many studies have been conducted to quarry the role of codon bias and it has been proved that significant differences between a gene in native and other expression hosts would cause a decreased in protein expression[22]. The codon optimization of the genes can be performed by replacing the less-used codons with the frequently used codons[23]. Jie et al. (2012) reported that xilanase gene from Thermatoga maritimewas optimized by replacing TCG (Ser), CTC (Leu), AGC (Ser), and GCG (Ala) and the expression of xylanase was increased to 2.8 fold [24].

In this work, IP was expressed in *P. pastoris* KM71H (Mut^s) using α-mating factor from *S. cerevisiae* as secretion signal, a Mut^s strain has severaladvantages compared to Mut^+ strain because it only has one alcohol oxidase encoding gene, which is AOX2 unlike the Mut^+ strain that has two alcohol oxidase (*AOX1* and AOX2) where AOX2 is weaker alcohol oxidase. Because of that KM71H strain doesnot require high concentration of methanol for large-scale compared to Mut^+ strain and grow slower than X33 or GS115 strain (Mut^+). The use of expression medium has effect in secretion process of IP. SDS-PAGE analysis showed that IP secretion worked better and more efficient in BMM medium if compared to BMMY medium. The present of yeast extract and peptone in BMMY medium (classified as rich medium) made protein production faster than in BMM medium, too fast to be efficiently secreted by the machinery of *P. pastoris* KM71H. As the consequence, some IP that were produced by *P. pastoris* still captived in the cells[21]. The present of other proteins was also lower in BMM medium than BMMY medium, indicating that *P. pastoris* secreted a very low endogenous proteins when using BMM medium, it has advantages in the purification process, faster and easier.

Methanol induction also has an important role in IP expression process. Excessive concentrations of methanol can be toxic to cells and too low methanol concentrations may not be sufficient to initiate transcription and induce cell starvation[15]. Based on SDS-PAGE analysis, there was no significant differencebetween two induction concentrations (1% and 2%) but higher methanol concentration could lead to methanol accumulation. However, this accumulation could have negative effects for cell growth and caused cells dead[25].Dead lysed cells were major source of proteolytic activity in culture supernatant which may be able to degrade protein target [26]. Lower methanol induction (1%) in BMM medium obtained optimum IP production at 72 h with expression level of 78.94% and maximum production at 120 h,while 2% methanol induction needed 144 h to reach the peak production. Longer fermentation process mayresult in more proteases released into culture supernatant. Proteolytic activity was found to increase over time in line with the decreased of viable cells[27]. Therefore, the faster and lower induction concentration may be more effective and efficient for secretory expression of IP.

V. CONCLUSION

Secretory expression of IP with α-factor mating signal sequence in *P. pastoris* KM71Hα-A/PI produced more effective and efficient in BMM medium with 1% methanol induction. This combination of media and methanol concentration reached optimum expression at 78.94% after 72 hours induction time throughout this research study.

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