

Original Research Paper

The Use of α -MF Signal Peptide Without Spacer for Producing Insulin Aspart Precursor in *Pichia pastoris* KM71

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Abstract: A spacer peptide (Glu-Ala repeats) connects N-terminal of a polypeptide precursor to the signal peptide of α -mating factor (α -MF) from *Saccharomyces cerevisiae*. During the polypeptide precursor secretion, the signal peptide will be first cleaved by signal peptidase in the endoplasmic reticulum, followed by Kex2 endopeptidase and finally the spacer peptide is removed by dipeptidyl aminopeptidase in the Golgi apparatus. In the case of insulin precursor, unfortunately, mixture forms of the insulin precursor containing a spacer peptide and the correct insulin precursor is observed in *Pichia pastoris*. To overcome this problem, a gene encoding insulin Aspart precursor without the spacer peptide has been constructed and its expression has been investigated in this study. The gene of insulin Aspart precursor has been successfully expressed extracellularly in *P. pastoris* KM71 with molecular mass of 6306.8 Da as determined by LC-ESI-MS. The expression level of insulin Aspart precursor is affected by aeration, cell density and methanol as an inducer. In shake flask production, the insulin Aspart precursor was optimally produced by 3% methanol induction every 24 h for two days, at initial cell density (OD₆₀₀) of 60 and aeration (ratio of culture volume to flask volume) of 1:25. Based on Western blot analysis, there is no intracellular insulin Aspart precursor detected. Therefore, the spacer peptide is not essential for insulin Aspart precursor secretion in *P. pastoris* and removal of spacer resulted in insulin Aspart precursor homogeneity.

Keywords: Spacer Peptide, Insulin Aspart Precursor, *Pichia pastoris*, Protein Secretion

Introduction

Insulin is the first approved recombinant protein for therapeutic practice by the United State Food and Drug Administration (USFDA) in 1982 (Leader *et al.*, 2008). Further developments of insulin are the introduction of rapid-acting insulin analogue in 1990 and long-acting basal analogue in early 2000 (Tibaldi, 2014). Insulin Aspart is one of the rapid-acting insulin analogues in which proline (A28) is substituted to aspartic acid. This amino acid substitution increases the repulsive force between chains and inhibits dimerization of insulin, resulting in a more rapid absorption to circulation after subcutaneous injection (Baeshen *et al.*, 2014; Tibaldi, 2014).

Commercial recombinant insulin is mainly produced in *Escherichia coli* and *Saccharomyces cerevisiae*

(Baeshen *et al.*, 2014). Among other alternative yeast expression systems, a methylotrophic yeast *Pichia pastoris* has become a preferable host because of its excellent features (Baeshen *et al.*, 2014). *P. pastoris* has been reported to have an equal capacity for expressing and secreting human insulin precursor compared to *S. cerevisiae* (Kjeldsen *et al.*, 1999). Furthermore, large scale human insulin production in *P. pastoris* has been studied. Human insulin precursor is successfully produced using fed-batch fermentation with protein yield to be 2.26-3.8 g/L (Gurramkonda *et al.*, 2010; Polez *et al.*, 2016; Xie *et al.*, 2008).

The signal peptide of the α -mating factor (α -MF) is mostly used for insulin precursor in *P. pastoris* (Daly and Hearn, 2005; Wang *et al.*, 2001). The α -MF signal peptide is composed of pre-pro-peptide followed by dibasic Kex2 cleavage site (-KR) and a spacer peptide

consisting of two or three dipeptides of Glu-Ala (Kurjan and Herskowitz, 1982). The cleavage of signal peptide occurs in the three consecutive steps, namely the pre-peptide is cleaved by signal peptidase in the endoplasmic reticulum; the pro-peptide is then cut by Kex2 endopeptidase; and the Glu-Ala repeats is finally removed by dipeptidyl aminopeptidase A in the Golgi apparatus (Lin-Cereghino *et al.*, 2013). However, a partial cleavage of Glu-Ala spacer from N-terminal of the insulin precursor could occur in *S. cerevisiae* since a low activity of dipeptidyl aminopeptidase. It is reported that 89% of the secreted insulin precursor is in the form of Glu-Ala-Glu-Ala-insulin precursor, 2% of Glu-Ala-insulin precursor, while only 9% is in the correct form (Thim *et al.*, 1986). The role of a spacer in secretion of insulin precursor is still unclear, whether it helps the Kex2 endoprotease cleavage or secretion directly in *P. pastoris* (Kjeldsen *et al.*, 1999). It is predicted that the Kex2 cleavage is influenced by the amino acids following the -LEKR-, sequence in which a study of the purified Kex2 endoprotease shows a significantly increase activity in the presence of Arg residue preceding the cleavage site (Brenner and Fuller, 1992).

This paper describes the use of α -MF signal peptide without a spacer for producing insulin Aspart precursor in *P. pastoris* KM71. A linker -RWR- is introduced to connect the A and B chain of insulin Aspart precursor. The gene encoding insulin Aspart precursor is codon optimized for *P. pastoris* and expressed under a strong and highly regulated *AOX1* promoter. Our findings indicate that insulin Aspart precursor is successfully expressed extracellularly despite no spacer peptide addition in the α -MF signal peptide.

Materials and Methods

Strain and Materials

Escherichia coli TOP10 F' as the general cloning host and *P. pastoris* KM71 as the expression host were obtained from Thermo Fisher Scientific, USA. A gene encoding insulin Aspart Precursor (ASP) was chemically synthesized and inserted to pPICZ α A by GenScript, USA. Forward primer AOX1 (5'-GACTGGTTCCAATTGACAAGC-3') and reverse primer AOX1 (5'-GCAAATGGCATTCTGACATCC-3') were purchased from Invitrogen (USA).

Restriction enzymes, Spectra LR Protein Marker and Gene Ruler 1 Kb DNA Ladder were purchased from Thermo Fischer Scientific, USA. *Pfu* DNA polymerase was obtained from Agilent, USA. *Taq* DNA polymerase, dNTPs and MgCl₂ were purchased from KAPA, USA. ATP Plasmid DNA Mini Kit (ATPBioTech Inc., Taiwan) was employed for plasmid isolation. GenJET Gel

Extraction Kit (Fermentas Life Sciences, AS) was used for DNA purification from gel agarose. Mouse insulin antibody mAb and Goat anti-mouse IgG antibody (H&L) [HRP] were supplied by GenScript, USA. The other chemicals for culture mediums, reagents and buffers were purchased from Merck (USA), Bio Basics (Canada) and Sigma-Aldrich (USA).

Transformation of *P. pastoris* KM71

P. pastoris KM71 was used to express the synthetic ASP gene encoding insulin Aspart precursor. The recombinant plasmid pPICZ α A-ASP (Fig. 1) was linearized by restriction enzyme *SacI* at 37°C for 4-6 h, then analysed by agarose electrophoresis. The linearized plasmid was then purified from the agarose gel. A volume of 5 μ L containing 400 ng of purified plasmid was used to transform 80 μ L of *P. pastoris* KM71 competent cells. The competent cells were prepared according to Cregg and Russell (1998). The transformation of *P. pastoris* competent cells were carried out by electroporation on condition 1.5 kV, 25 μ F and 200 Ω . A volume of 1 mL YPD (1% yeast extract, 2% peptone and 2% dextrose) was immediately added, and the mixture was then transferred to a sterile microtube and incubated at 30°C for 2-4 h without shaking. An aliquot of 25-100 μ L cells was grown on YPD plates containing 100 μ g/mL zeocin and incubated at 30°C for 2-3 days.

Screening of Transformants

To screen the *P. pastoris* transformants, colonies were grown on YPD plates containing zeocin with a higher concentration (500-2000 μ g/mL). Survived colonies on the highest zeocin concentration were selected and screened for Mut^s or Mut⁺ phenotypes by direct PCR using the AOX1 primers. To select and confirm the ability of *P. pastoris* transformant to express insulin Aspart precursor, colony blot was performed (Annibali *et al.*, 2014). Colonies, grown on YPD plate containing 100 μ g/mL zeocin, were transferred to a nitrocellulose membrane. The membrane with attached colonies was transferred to MMH plate (1.34% yeast nitrogen base, 4 \times 10⁻⁵% biotin, 2% methanol, 2% agar) then incubated at 30°C for 24 h. The membrane was taken out from the plate and washed by TBST (20 mM Tris-Cl; 150 mM NaCl pH 7.6, 0.1% Tween 20) until the colonies were washed out. The membrane was blocked by 5% skimmed milk in TBST for 1 h at room temperature, then washed three times with 10 mL TBST. The membrane was further incubated with Mouse insulin antibody mAb (1:500 in TBST) overnight at 8-10°C, then washed three times with 10 mL TBST. The membrane was incubated again with Goat anti-mouse IgG antibody (H&L) [HRP] (1:1000 in TBST) for 1 hour at room temperature, washed three times with 10 mL TBST, then 5 mL ChromoSensor™ One solution TMB substrate (GenScript, USA) was added, shaken at low speed at room temperature until color formed.

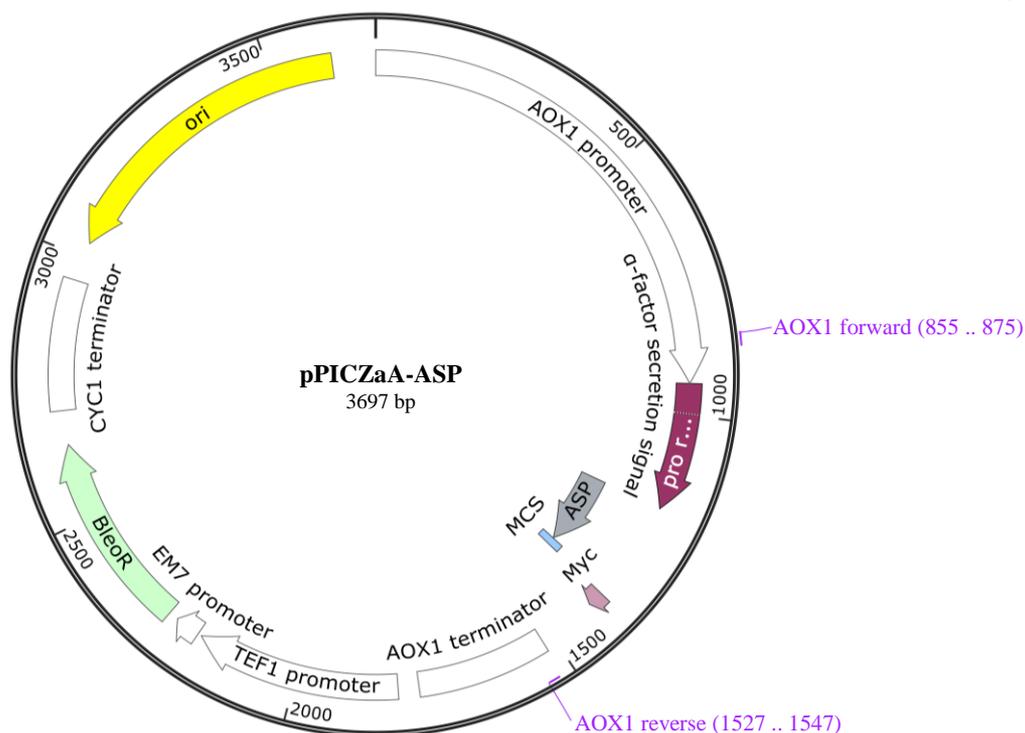


Fig. 1: The vector map of pPICZ α A-ASP

Expression of Insulin Aspart Precursor

P. pastoris harboring insulin Aspart precursor gene was grown in 10 mL YPD containing 100 μ g/mL zeocin with shaking at 250 rpm at 30°C for 18 h. A volume of culture was transferred to 50 mL buffered glycerol-complex (BMYG) medium which contained 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5} % biotin, and 1% glycerol in 500 mL flask to obtain an OD₆₀₀ of 0.2. The culture was further grown for 24 h. The cells were harvested and resuspended in the medium of BMYG (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% yeast nitrogen base, and 4×10^{-5} % biotin). The culture was induced by methanol every 24 h and further grown on shaking incubator at 225 rpm and 30°C. After 48 h of production, the culture was harvested by centrifugation at 3000 \times g for 15 minutes. The cells were stored at -20°C and the supernatant was kept at 4°C for further analysis.

Optimization of methanol concentration to induce the expression of insulin Aspart precursor was carried out using various final methanol concentration (0.5, 1, 2 and 3%). A volume of methanol concentration was fed to 5 mL of BMYG culture in 50 mL flask to obtain each final concentration. The effect of initial cell density was studied by applying different initial OD₆₀₀ of BMYG culture (OD₆₀₀ of 45, 60, 75, 90 and 105). The expression was induced by 3% methanol, at the same

aeration (ratio culture volume to flask volume 1:10). A various ratio volume of BMYG culture to flask volume (10 mL in 250 mL flask, 25 mL in 250 mL flask and 25 in 500 mL flask) was used to investigate the effect of aeration for insulin Aspart precursor expression level. The study was performed at the same initial OD₆₀₀ of 60 with 3% of final concentration of methanol induction.

SDS-PAGE and Western Blot

The cell culture supernatant was precipitated by TCA according to Koontz (2014) prior to SDS-PAGE. Intracellular protein was isolated by TCA lysis as described in Baerends *et al.* (2000). The protein was dissolved in sample buffer and boiled for 10 minutes before loading. All samples were loaded according to OD₆₀₀ of each cell culture. SDS-PAGE was conducted as described by Schagger (2006). The gel was stained by Coomassie blue staining, except the gels for further analysis with Western blot. All SDS-PAGE images were further analyzed by densitometer (TotalLab, England). Protein from the SDS-PAGE gel was transferred to nitrocellulose membrane using the eBlot Protein Transfer System (GenScript, USA). The membrane was blocked, incubated with antibodies and developed by ChromoSensor™ One solution TMB substrate with the same procedure of colony blot (*see Screening of Transformant section*).

LC-ESI-MS Intact Mass Analysis of Insulin Aspart Precursor

The LC-ESI-MS intact mass analysis was undertaken at Australian Proteome Analysis Facility (APAF), Macquarie University, Australia. The Mass spectrometer QExacte Plus (ThermoScientific, USA) equipped with an electron-spray ionizer was used to acquire the ESI-MS data.

One mg of dried sample obtained from TCA precipitation of the cell culture supernatant was reconstituted in 100 μ L 0.1% formic acid. A dilution of 1:50 was prepared in 0.1% formic acid. Sample (10 μ L) was injected onto the analytical column (Waters XBridge BEH C4, 2.1 \times 100 mm, 300 \AA , 3.5 μ m) at 40 $^{\circ}$ C with a flow rate of 200 μ L/min. Protein was eluted from the column using a linear solvent gradient from mobile phase A (0.1% formic acid): mobile phase B (0.1% formic acid and 99.9% acetonitrile) of 85:15 to 55:45, over 10 min. After elution, the column was cleaned with 90% buffer B for 2 min and then equilibrated with 15% buffer B for 2 min before next sample injection.

The reverse phase LC eluent was subjected to positive ion electrospray analysis in Full MS mode. The FT mass range was set 400-2,800 m/z, 3.5 kV spray voltage, sheath gas 25, aux gas 5, in-source CID 30 eV, orifice temperature 275 $^{\circ}$ C, resolution 17,500, AGC target 3 \times 10⁶, S-lens RF level set to 80, summed 10 microscans, maximum ion injection time 150 milliseconds.

The intact mass raw files were processed using Xcalibur. Expected masses were calculated using BioPharma Finder (v3.0). The deconvoluted monoisotopic masses were calculated using the online ESIprot calculator.

Results and Discussion

The Expression Cassette of Insulin Aspart Precursor

The synthetic *ASP* gene encodes a polypeptide consisting of 4 amino acids of Kex2 cleavage site -LEKR- followed by insulin Aspart precursor, comprising of the B chain 1-30 amino acids, linker -RWR- and the A chain 1-21 amino acids (Fig. 2A). The synthetic *ASP* gene was codon optimized for expression in *P. pastoris* resulting in codon adaptation index of 0.857 and GC content of 41.8%. The analysis was carried out using CAIcal server at <http://genomes.urv.es/CAIcal/> (Puigbò *et al.*, 2008) and the codon preference of *P. pastoris* was obtained from <https://www.kazusa.or.jp/codon/>. The gene was fused in-frame with the pre-pro region of α -factor signal peptide and expressed under the regulation of methanol inducible *AOX1* promoter (Fig. 2B).

Colony Screening of P. pastoris Harboring Insulin Aspart Precursor Gene

Screening of *P. pastoris* harbouring *ASP* gene was carried out by colony PCR. It was found that five out of

nine transformants contained *ASP* (Fig. 3), as indicated by the presence of 692 bp band correlated with the size of flanking region of the *AOX1* promoter inserted with the *ASP* gene (Fig. 1). Moreover, *P. pastoris* transformants carrying *ASP* was subjected to colony blot screening against insulin antibody to determine their ability in producing Asp (data not shown). One colony showing high intensity was then selected for further analysis.

Optimization of Insulin Aspart Precursor Production

Insulin Aspart precursor production in shake flask culture was optimized in terms of methanol concentration, cell density and aeration. The SDS-PAGE analysis of the culture supernatant showed the appearance of 6.3 kDa protein band after methanol induction, but no protein band with the same molecular mass was detected on culture supernatant without methanol induction (Fig. 4). Methanol concentration of 0.5, 1, 2 and 3% were used to induce the cultures every 24 hours for 2 days. The expression level of Insulin Aspart precursor increased as the supplied methanol concentration was leveled up, in which 3% methanol resulted in the highest insulin Aspart precursor expression (Fig. 5). Our finding is in agreement with Kupculik and Sevela (2004) which stated that specific product formation rate is strongly dependent on methanol level. The phenotype of colony used for insulin Aspart precursor production is Mut^S which has slow ability to utilize methanol as sole carbon source. Mut^S phenotype enables methanol accumulation for promoter activation in shake flask culture since the methanol is supplied once daily (Gandier and Master, 2018). The provided methanol in the medium will not be rapidly utilized to accelerate its growth but will be used instead for promoter induction.

The effect of cell density (OD₆₀₀) on the expression of insulin Aspart precursor was then investigated. The result showed that the expression of insulin Aspart precursor increased as cell density (OD₆₀₀) of 45 was levelled up to 60 and started to decline afterward (Fig. 6). The concentration of protein product in extracellular medium is proportionally correlated to the cell density (Macauley-Patrick *et al.*, 2005). However, after the cell density reached OD₆₀₀ of 60, the expression level of insulin Aspart precursor decreased as the cell density declined.

In this study, the same aeration and methanol concentration was applied throughout the production. It is possible that the expression of insulin Aspart precursor is low due to the methanol and oxygen shortage. Higher cell concentration requires higher oxygen uptake (Çelik *et al.*, 2009). Moreover, shake flask system has limitation in controlling the methanol uptake by the cells (Macauley-Patrick *et al.*, 2005).

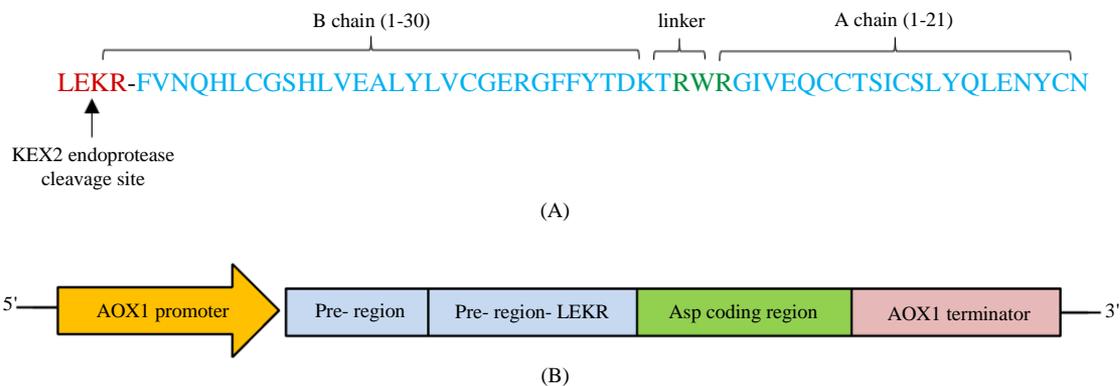


Fig. 2: (A) Amino acid sequences of insulin Aspart precursor; (B) The expression cassette of insulin Aspart precursor

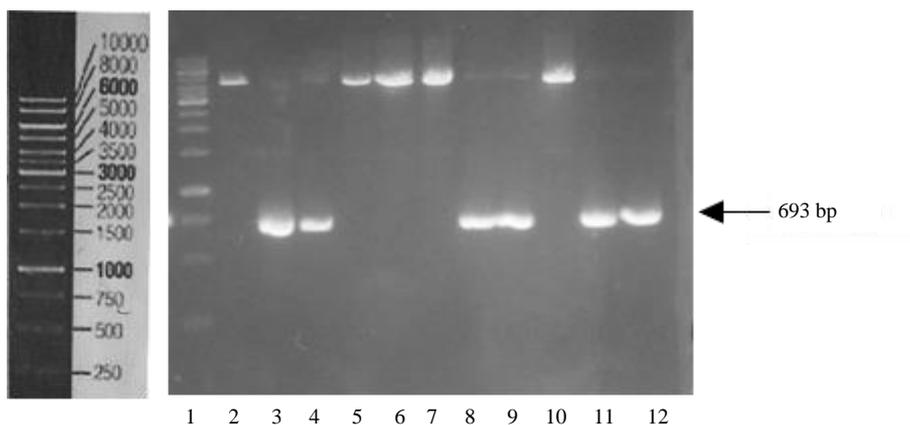


Fig. 3: Colonies of *P. pastoris* harbouring insulin Aspart precursor gene. Lane 1, 1 kb DNA ladder; lane 2, *P. pastoris* KM71; lane 3, positive control using pPICZaA-ASP; lanes 4 to 12, *P. pastoris* KM71 transformants with the colony carrying the *Asp* gene was indicated by ~693 bp of PCR product

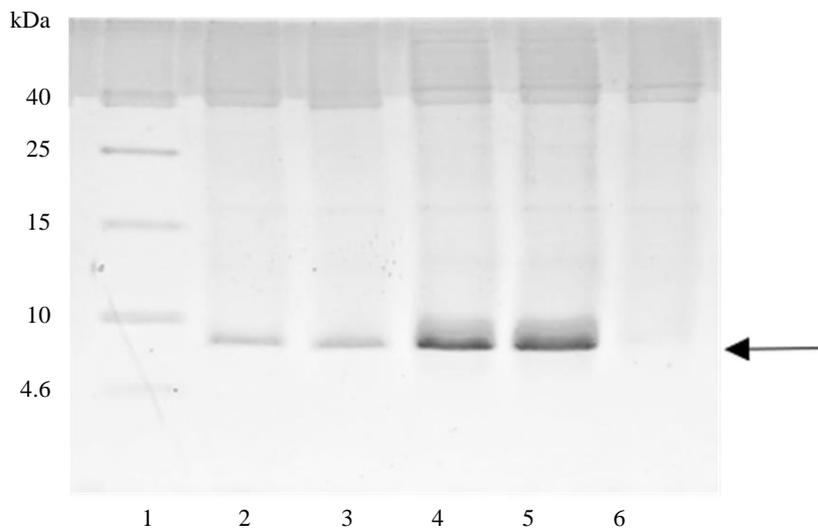


Fig. 4: Profiles of extracellular insulin Aspart precursor expressed at various concentration of methanol. Lane 1, protein marker; lanes 2 to 5, methanol concentration of 0.5, 1, 2 and 3%, respectively; lane 6, without methanol

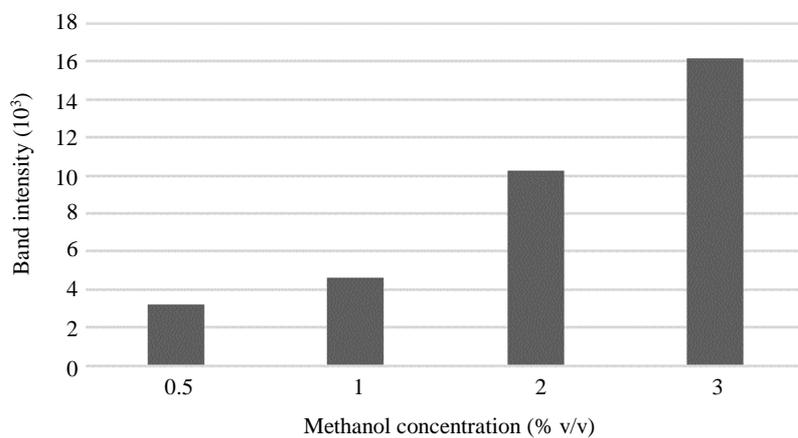


Fig. 5: Band intensity of insulin Aspart precursor expression at various concentrations of methanol

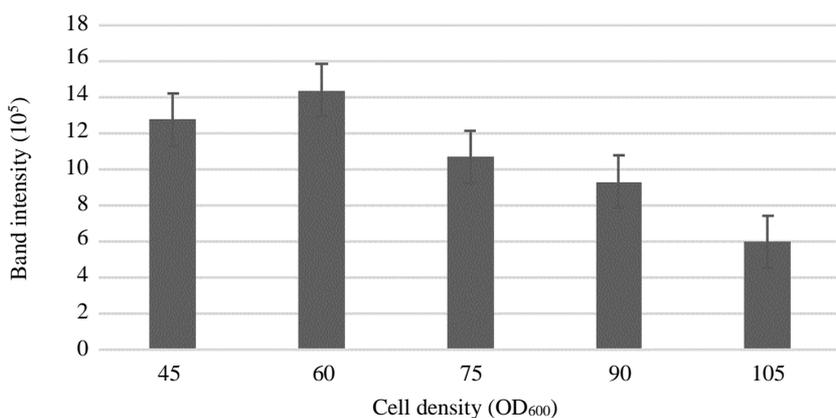


Fig. 6: Band intensity of insulin Aspart precursor expression at various cell density (OD₆₀₀)

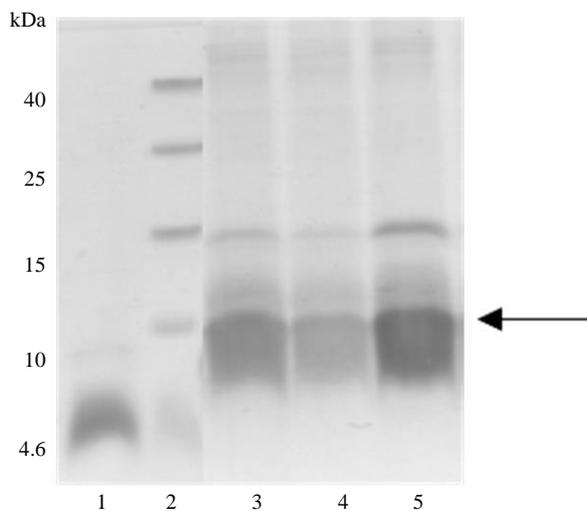


Fig. 7: Profiles of extracellular insulin Aspart precursor produced on various aeration. Lane 1, commercial bovine insulin; lane 2, protein ladder; lanes 3 to 5, ratio of culture volume to flask volume of 1:20, 1:10, 1:25, respectively

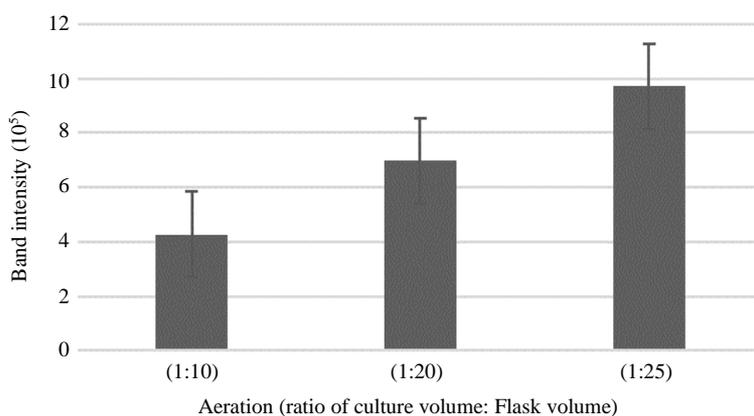


Fig. 8: Band intensity of insulin Aspart precursor expression in various aeration

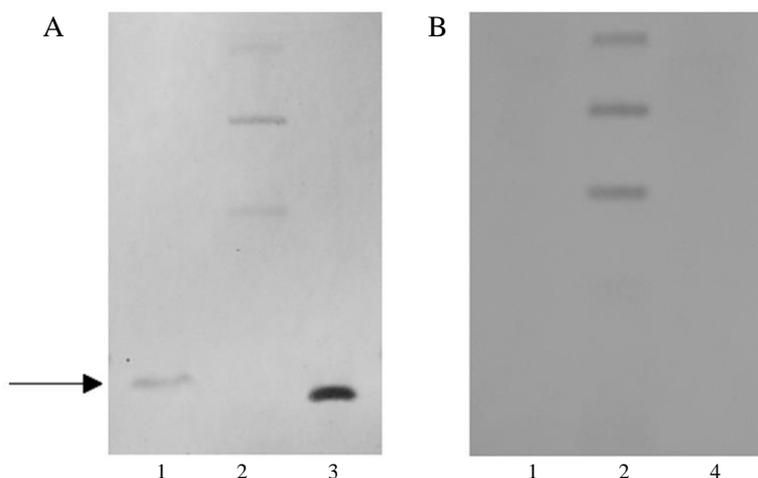


Fig. 9: Western blot of culture supernatant; (A) and intracellular fraction; (B) against insulin antibody. Lane 1, *P. pastoris* KM71/pPICZαA-ASP; lane 2, protein marker with molecular masses of 40, 25, and 15 kDa; lane 3, commercial bovine insulin; lane 4, *P. pastoris* KM71

Furthermore, the effect of aeration to insulin Aspart precursor expression was investigated by applying various ratios of culture volume to flask volume. A gradual increase in insulin Aspart precursor expression was observed as the aeration ratio was risen from 1:10 to 1:25 (Fig. 7 and 8). Aeration is related to oxygen supply which is required for the growth of *P. pastoris* and heterologous protein expression. In shake flask, oxygen supply can be increased by raising the shaking speed or reducing culture volume (Yu *et al.*, 2014).

The Secretion of Insulin Aspart Precursor

The secretion of insulin Aspart precursor was studied using Western blot analysis of extracellular and intracellular protein. According to Western blot analysis, insulin Aspart precursor was found in the culture medium and was not detected in the intracellular fraction (Fig. 9). This result indicates that

the α -MF signal peptide has been removed completely despite no spacer addition.

Further analysis by LC-ESI-MS showed that the molecular mass of insulin Aspart precursor is 6306.8 Da and no other form of extracellular insulin Aspart precursor was detected (Fig. 10). The result suggested that the removal of spacer -EAEA- leads to insulin Aspart precursor secretion in its correct form and has no impact to the integrity of the product.

Kjeldsen *et al.* (1999) reported a different result of human insulin precursor expression in *S. cerevisiae*. The spacer was also removed from the constructed gene and a linker -SDDAK- was used to connect the C-terminal of B chain to the N-terminal of A chain of human insulin lacking its B30 amino acid. The result revealed an incomplete cleavage of the α -MF signal peptide and produced the hyperglycosylated fusion protein of α -MF signal peptide and insulin precursor to the culture medium.

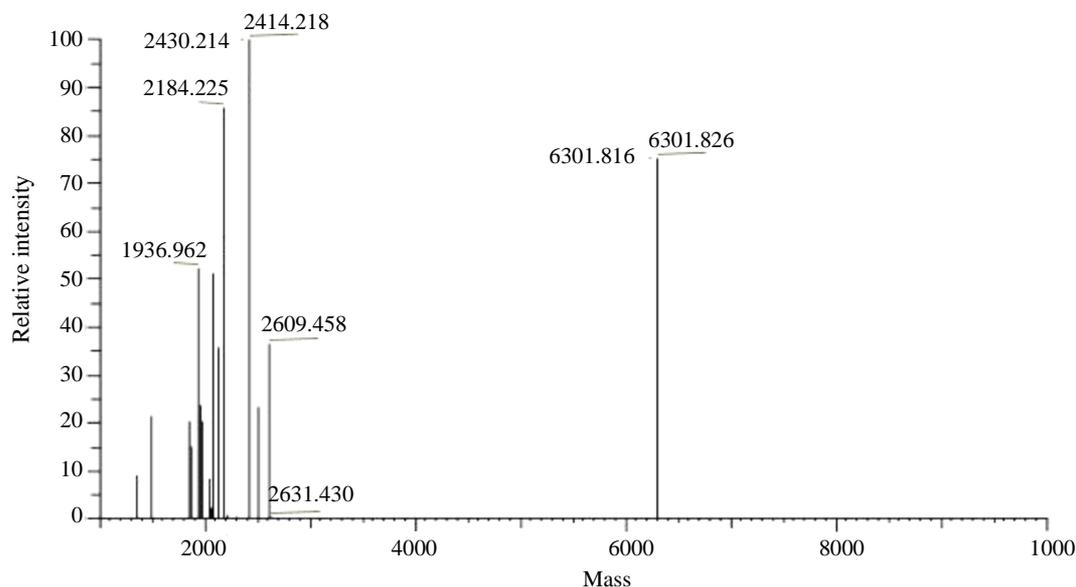


Fig. 10: ESI Mass spectrum of insulin Aspart precursor

It is possible that the use of different host cells leads to different signal peptide processing result. Although the core secretion pathway function in yeast is similar on genomic level (Delic *et al.*, 2013), the morphological differences between *P. pastoris* and *S. cerevisiae* are observed (Papanikou and Glick, 2009). Generally, *P. pastoris* produces heterologous protein more efficiently compared to that of *S. cerevisiae*, due to these morphological differences (Zahrl *et al.*, 2017). In terms of spacer function, our result showed that the presence of spacer -EAEA- is not necessary for insulin Aspart precursor secretion in *P. pastoris*, while the spacer is required in *S. cerevisiae*.

Conclusion

The gene encoding insulin Aspart precursor was successfully expressed extracellularly in *P. pastoris* KM71. The optimal condition for shake flask production of insulin Aspart precursor was achieved at 3% methanol induction every 24 h for two days with initial cell density (OD_{600}) of 60 and aeration (ratio of culture volume to flask volume) of 1:25. Only the correct form of insulin Aspart precursor with molecular mass of 6301.8 Da was observed suggesting that the cleavage of the α -MF signal peptide without -EAEA- spacer is completed and the removal of the spacer resulted in insulin Aspart precursor.

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Authors Contribution

Popi Asri Kurniatin: Performed all experiments, conducted data analysis and prepared the manuscript.

Fifi Fitriyah Masduki: Contributed in Western blot data analysis.

Neni Nurainy: Participated in production optimization and data-analysis.

Ihsanawati: Handled data analysis and prepared the manuscript.

Dessy Natalia: Designed the whole experiments, conducted data analysis and prepared the manuscript.

Ethics

All authors read and approved the final version of this manuscript. There are no ethical issues to declare that could arise after the publication of this manuscript.

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