Production and Characterization of Human Insulin Precursor in *Pichia Pastoris* X-33

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Abstract. The prevalence of Insulin Dependent Diabetes Mellitus (IDDM) has increased significantly in the last decades, resulting in an increased demand for insulin. In addition, new routes of oral and inhaled insulin require higher doses thus increasing insulin requirements. Insulin is the primary drug in patients with type 1 diabetes mellitus (T1DM). In some cases, type 2 diabetes mellitus (T2DM) requires insulin for treatment. The researchers have widely developed another expression system to meet the ever-increasing demand for insulin with higher production capacities. Human insulin precursor (HIP) production can use several microorganisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Pichia pastoris*. In this study, *P. pastoris* X-33 was used to produce human insulin precursor. *Pichia pastoris* becomes the promising yeast host for recombinant protein expression because of its ability to reach high cell densities by its robust methanol-inducible alcohol oxidase 1 (AOX1) promoter and simple development process, which provide to high quality and a high percentage of recombinant proteins, both intracellular and secretory. In this study, several zeocin-resistant clones were characterized by PCR and sequencing using a specific human insulin precursor gene to detect plasmid integration into the *P. pastoris* genome. In addition, a test of the effect of zeocin concentration on the growth of the transformation was carried out. The expression of HIP protein in *P. pastoris* X-33 was characterized by SDS-PAGE and Elisa. The result of PCR and sequencing showed that the HIP gene was successfully integrated into selected colonies of *P. pastoris* X-33. All of 20 transformant colonies were able to grow at 100 to 2000 μg per mL and selected colonies of *P. pastoris* X-33 can produce HIP protein.

Keywords: Human precursor insulin · diabetes mellitus · *Pichia pastoris* · expression

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1 Introduction

Diabetes mellitus (DM) is a metabolic disorder disease characterized by hyperglycemia due to insulin deficiency, insulin resistance, or both [1–3]. In 2019, people with DM reached 463 million in the world and are predicted will increase to 700 million by 2045. The total global health cost for DM reaches US$ 760 billion [4, 5]. The prevalence of DM and the life expectancy of DM will continue to increase the need for insulin [6, 7].

The cause of DM is insulin deficiency due to damage to pancreatic cells, which are cells that produce insulin. This condition is known as type1 DM (T1DM). In addition, DM can also be caused by insulin resistance, known as type 2 DM (T2DM). Insulin resistance is a decrease in the ability of insulin to stimulate the use of glucose or a decrease in the response of target cells, such as muscles, tissues, and liver to physiological insulin levels. Insulin has been used for the treatment of people with T1DM since 1922 [8–10]. Insulin is given to T2DM with fasting glucose level $>$ 250 mg/dL, history of pancreatectomy, experiencing T2DM for 10 years, patients with chronic hepatitis, pulmonary tuberculosis, cancer, and reducing the side effects of oral medicines [1, 11, 12].

Insulin is a hormone to convert glucose into energy and maintain or control blood glucose levels [13, 14]. Human insulin is synthesized by pancreatic cells in form of 3 peptides, namely peptides B, C, and A (insulin precursor). Insulin precursor is converted to insulin by enzymatic to remove peptide C. Human insulin is a non-glycosylated dipeptide consisting of peptide B containing 30 amino acids and peptide A containing 21 amino acids, linked by sulfide bonds, and having a molecular weight of 5,808 Da [2, 14–18].

Genetic engineering makes it possible to produce human insulin in microbial cells or other cell systems. In 1978, this technique was developed and used to produce insulin using *Escherichia coli* by Genentech and produced on an industrial scale by Eli Lilly in 1982 [16, 19, 20]. Novo Nordisk produced human insulin using *Saccharomyces cerevisiae* in 1991. In 1995, Sanofi developed a human insulin using *E. coli* and marketed it in 1997 [20, 21]. Today human insulin can be produced by *E. coli. S. cerevisiae*, and *Pichia pastoris.* In this research, *Pichia pastoris* X-33 was used to producing human insulin precursor.

2 Materials and Methods

2.1 Strains and Plasmids

Pichia pastoris X-33 and the pPICZαA plasmid were from Invitrogen Co., Ltd. The human insulin precursor gene was optimization codon for *Pichia pastoris* and synthesized by GenScript. pPICZαA-HIP plasmid contains the human insulin precursor gene (Fig. 1).

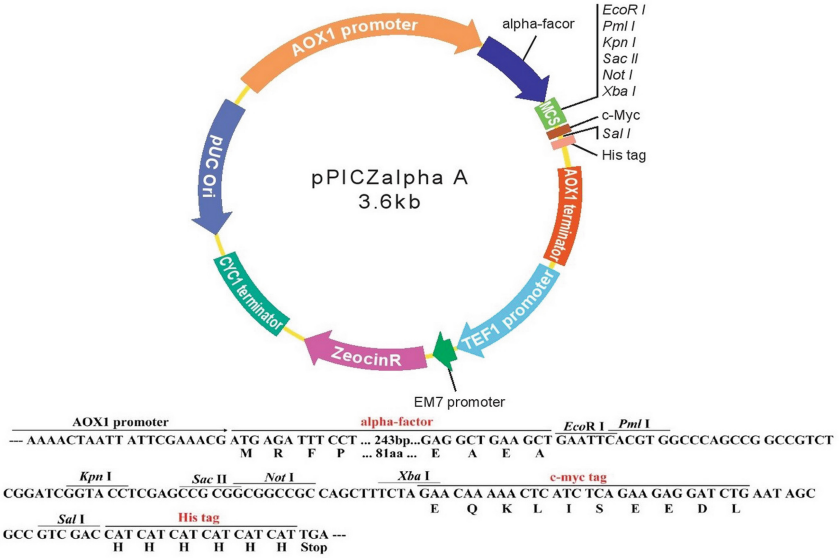

Fig. 1. Map of integrative vector pPICαA-HIP encoding human insulin precursor

2.2 Characterization of Selected Pichia Pastoris Colonies

Transformant of *Pichia pastoris* transformant were grown on YPD medium containing zeocin from 100 μg/mL to 2000 μg/mL for identification of the multicopy clones. Two transformant of *P. pastoris* were tested by polymerase chain reaction (PCR) and sequencing. KOD-Plus kits from Toyobo were used to amplification of HIP gene in *Pichia pastoris* genome. The cycling conditions of PCR was 94 °C for 2 min; then 30 cycles at 94 °C for 15 s, 52 °C for 30 s and 68 °C for 30 s; followed by last cycle at 12 °C for ∞. The PCR products were characterized by electrophoresis on 1.5% agarose gel and to confirm of the integration of pPICZαA-HIP plasmid into *Pichia pastoris* genome.

2.3 Production of Insulin Precursor

A single colony was inoculated in 10 ml of BMGY in a 100 ml baffled flask, grown at 28–30 °C in a shaking incubator (250 rpm) until culture reached an OD600 = 2–6 (approximately 16–18 h). The cells were harvested by centrifuging at 1,500–3,000 × g for 5 min at room temperature. Supernatant was decanted and cells pellets (OD600 of 1.0) was resuspended cell in BMMY medium to induce expression. Culture was placed in a 250 mL baffled flask. The flask was covered by 2 layers of sterile gauze and grown at 28–30 °C in a shaking incubator (250 rpm) for overnight. Methanol was added to a final concentration of 0.5% methanol every 24 h to maintain induction. After 72 h the cultures were harvested, centrifuged at 1500 – 3000 × g for 5 min at room temperature, and human insulin precursor in the supernatant was test by SDS-PAGE gel.

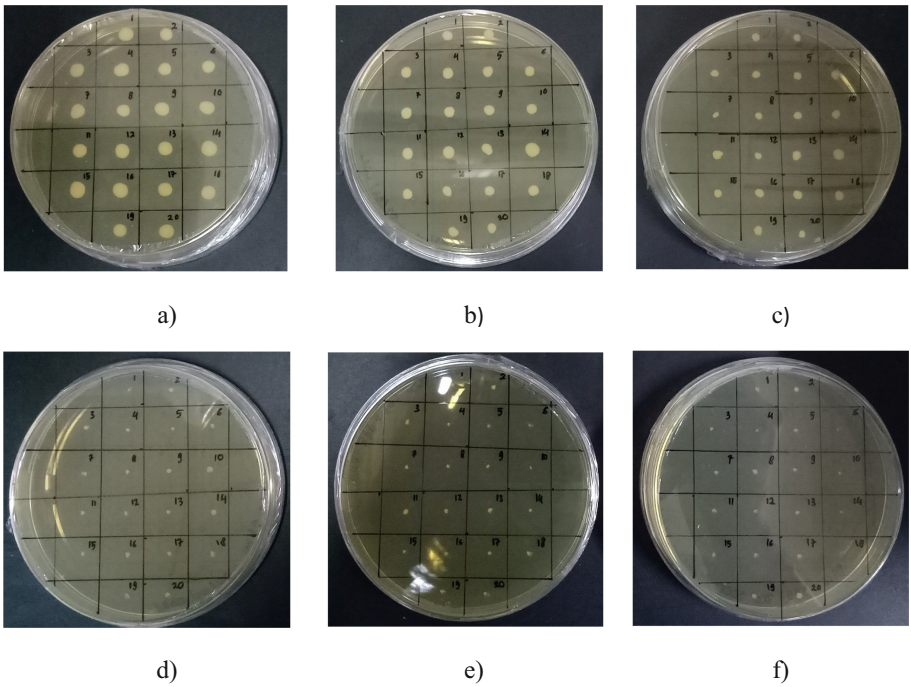

Fig. 2. The selected 20 transformants of *P. pastoris* on YPD plates containing variation of zeocin concentration: a) 100; b) 200; c) 500; d) 1000; e) 1500; f) 2000 μg/mL

3 Results

The use of zeocin markers has the advantage of directly selecting transformants that are hyper resistant to zeocin [22]. The transformant of *Pichia pastoris* were grown in YPD plates containing zeocin 100 - 2000 μg/mL to identify multicopy HIP gene in transformant strains. The copies number the gene is associated with increased production of human insulin precursor and the ability of grown in zeocin as antibiotic selectable marker. *Pichia pastoris* with more copies number of the gene have more integrative vector pPICαA-HIP will increase the production of insulin precursor in and grew in high zeocin concentration because of more zeocin resistance gene [23]. The result shows that all of 20 transformant colonies were able to grow on YPD plates containing all of zeocin concentration (100–2000 μg/mL) (Fig. 2).

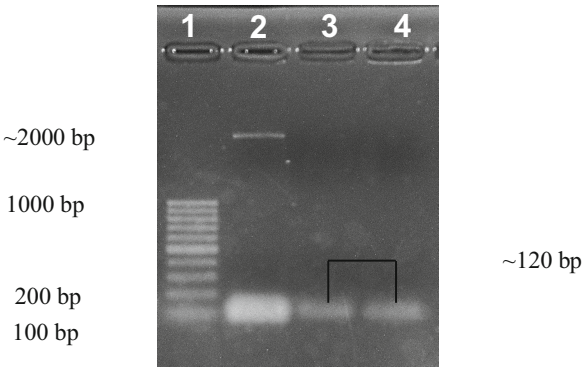

Fig. 3. Electropherogram of PCR product from transformant of *P. pastoris*: 1) 100 bp Ladder; 2) PCR product from reference pPICZαA-HIP plasmid; 3) PCR product from transformant of *P. pastoris* no.1; 4) PCR product from transformant of *P. pastoris* no.2

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                    20        30        40        50        60        70
IP gene      GCAGAAGCTGAACCAAAGTTCGTTAACCAACATTTGTGTGGTTCTCATTTGGTTGAAGCA
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
PCR product  GCAGAAGCTGAACCAAAGTTCGTTAACCAACATTTGTGTGGTTCTCATTTGGTTGAAGCA
                    10        20        30        40        50        60

             80        90       100       110       120       130
IP gene      TTGTATTTGGTTTGTGGTGAAAGAGGTTTCTTTTATACTCCAAAAGCTGCAAAAGGTATC
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
PCR product  TTGTATTTGGTTTGTGGTGAAAGAGGTTTCTTTTATACTCCAAAAGCTGCAAAAGGTATC
                    70        80        90       100       110       120

             140
IP gene      GT
             ::
PCR product  GT
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Fig. 4. The result of sequence alignment from PCR product from transformant of *P. pastoris* and optimized HIP gene from Genscript

Amplification of HIP gene was used to confirm this gene in *P. pastoris* genome. Two transformants of *P. pastoris* (no. 1 and 2) contained HIP gene (~120 bp) and as positive control used pPICZαA-HIP plasmid (Fig. 3).

The sequencing results of PCR product from transformant of *P. pastoris* was 100% identical to the HIP gene sequence (Fig. 4).

The supernatant from the transformant of *P. pastoris* culture contained HIP protein with a size above 7 kDa (24) (Fig. 5).

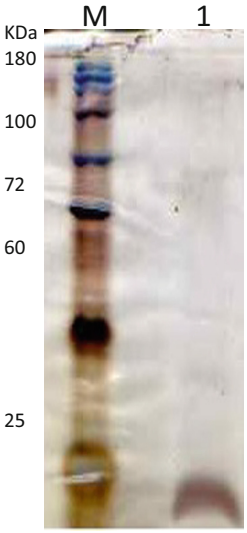

Fig. 5. Human insulin precursor stained with silver nitrate in SDS-PAGE gel. M = Polypeptide molecular weight standards. Lane 1 = culture supernatant of *P. pastoris* recombinant post methanol induction (72 h)

4 Discussion

Vector pPICαA-HIP contains 5'*AOX1* promoter, α-factor secretion signal, multiple cloning site (MCS), *c-myc* epitope, C-terminal polyhistidine tag, *AOX1* transcription (TT) region, *TEF1* promoter, *EM7* promoter, Zeocin resistance gene (*Sh ble*), *CYC1* transcription termination region, pUC origin, and Human insulin precursor gene (HIP gene. Zeocin hyper resistance clones have multicopy of the zeocin resistance gene, which is associated with multicopy of the pPICαA-HIP so the Zeocin hyper resistance clones could grow in high concentrations of zeocin. The growth ability of *P. pastoris* transformants in zeocin concentrations up to 500 g/mL can be used for detection of HIP gene copies in *P. pastoris* transformants. Multicopy clones (>2 copies) were able to grow at zeocin concentrations up to 500 g/mL [25]. In this study, all P. *pastoris* transformants were able to grow on YPD media containing zeocin concentrations up to 2000 g/mL. This shows that all *P. pastoris* transformants contain more than 2 gene copies. The effect of HIP gene copy on *P. pastoris* transformants is directly proportional to the level of HIP expression [23, 26, 27]. Wang et al. reported *P. pastoris* with 6–8 copies HIP gene integrated into the chromosome produced 1.5 g/L IP protein [24]. HIP protein expression increased 13 folds from 19 to 250 mg/L in *P. pastoris* with 11 copies HIP gene [23].

Two colonies of 20 transformant were analyzed by PCR and DNA sequencing to detect the IP gene in the *P. pastoris* genome. The PCR product was electrophoresed on 1.5% agarose gel and showed a band measuring 120 bp equal to the positive control pPICZαA-HIP plasmid (Fig. 3). This indicates that the HIP gene has been successfully integrated into the *P. pastoris* genome. For further confirmation, DNA sequencing was performed. The result of DNA sequencing showed that the DNA sequences of PCR

product were 100% identical to the PI gene. Figure 4, This strengthens the evidence that the HIP gene has been successfully integrated into the *P. pastoris* genome.

The secreted HIP protein is about 7 KDa. The supernatant fermented for 72 h was analyzed by SDS-PAGE to detect HIP protein. The secreted HIP protein in the supernatant fermented can be seen in Fig. 5. For further confirmation, it is necessary to perform Western Blot and assay by HPLC or Elisa.

5 Conclusion

The selected colonies of *P. pastoris* X-33 can produce HIP protein and the production of HIP protein needs to be increased by optimizing the fermentation process.

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