Expression Process Study for Human Proinsulin Production Using Novel Recombinant Pichia pastoris (Postprint)

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Abstract

Abstract: The use of Pichia pastoris as a heterologous expression host for synthesizing human insulin precursor has been widely applied in laboratory research and industrial production. Current studies primarily employ the natural methanol-inducible AOX1 promoter, using methanol as the sole carbon source for induced fermentation production of insulin precursor. However, during high-density fermentation of Pichia pastoris, methanol metabolism is characterized by high oxygen consumption and heat generation, coupled with complex feeding control strategies, which limits the scale-up of fermentation. Based on our previous work on the transcriptional regulation design of the AOX1 promoter, this study proposes to drive insulin precursor gene expression using an artificially designed high-efficiency constitutive transcriptional regulation device CSAD_5, and develops a fermentation process using glucose as the carbon source to resolve the issues of heat generation, oxygen consumption, and process control inherent in methanol-based systems. Furthermore, by enhancing selection pressure to increase heterologous gene copy number, a recombinant Pichia pastoris strain with high insulin precursor expression was obtained. Through optimized culture conditions at the 5 L bioreactor scale, the insulin precursor titer reached 1.85 g/L at 108 h, representing the highest reported level for human insulin precursor production using glucose as the carbon source. This study provides novel insights and approaches for the industrial production of insulin precursor and the application of Pichia pastoris.

Full Text

Preamble

Heterologous Expression of Human Insulin Precursor in a Newly Engineered *Pichia pastoris*

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Abstract

Pichia pastoris is one of the most widely used heterologous expression hosts for synthesizing human insulin precursors in both laboratory research and industrial production. Currently, induction fermentation primarily relies on the naturally methanol-inducible AOX1 promoter, utilizing methanol as the sole carbon source. However, during high-cell-density fermentation of *P. pastoris*, methanol metabolism is associated with high oxygen consumption, substantial heat generation, and complex feeding control strategies, which limit process scale-up. Based on our previous work on transcriptional regulation design of the AOX1 promoter, this study employed a synthetic, efficient constitutive transcriptional signal amplification device, CSAD_5, to drive insulin precursor gene expression and developed a glucose-based fermentation process to address the challenges of heat generation, oxygen consumption, and process control inherent in methanol systems. By enhancing selection pressure to increase heterologous gene copy number, we obtained a high-producing recombinant *P. pastoris* strain. Using an optimized cultivation process, insulin precursor titer reached 1.85 g/L at 108 h in a 5-L bioreactor, representing the highest reported level for human insulin precursor production using glucose as carbon source. This research provides novel insights and methodologies for industrial insulin precursor production and broader applications of *P. pastoris*.

Keywords: *Pichia pastoris*; insulin precursor; fermentation process; high-producing strain screening

Introduction

Insulin is a small protein hormone secreted by pancreatic -cells that regulates blood glucose homeostasis [1] and serves as an essential therapeutic for insulin-dependent diabetes mellitus [2], commanding enormous market demand. Current production methods typically employ genetic engineering approaches using *Escherichia coli* or yeast expression systems to produce human insulin precursor, which is subsequently processed into active insulin [3].

The *Pichia pastoris* expression system is among the most widely utilized platforms for heterologous protein production [4], offering facile genetic manipulation, stable heterologous gene inheritance [5], established methods for multi-copy

integration screening [6,7], mature post-translational modifications compared to prokaryotic systems [8], and simple medium composition for high-density fermentation that facilitates product purification [9]. In 2006, *P. pastoris* was designated as Generally Recognized As Safe (GRAS) by the U.S. FDA [10], with over 5,000 heterologous proteins successfully expressed to date [5].

P. pastoris has been extensively investigated for insulin precursor production [11,12]. Current research predominantly employs methanol as the sole carbon source and inducer, leveraging the efficient AOX1 promoter (PAOX1) to drive insulin precursor expression [13]. PAOX1 strictly depends on methanol induction with a single regulation mode and is strongly repressed by various common carbon sources [14,15], restricting available carbon sources for industrial production. Methanol-based large-scale production suffers from high oxygen demand, excessive heat generation, and complex feeding control strategies [8,16,17], while metabolic byproducts such as H2O2 can cause target protein degradation [18]. To mitigate methanol dependence for high-level heterologous protein expression, researchers have identified methanol-independent promoters including PGAP, PICL1, and PPHO89, though their expression capacity remains weaker than PAOX1 and cannot sustain high-efficiency production [19,20]. Previous reports demonstrated PGAP-based insulin precursor expression under non-methanol conditions, but titers were low, reaching only 0.3 g/L at the 30-L scale—merely 30% of PAOX1 levels [21]. Other studies have engineered PAOX1 to enable insulin precursor expression using conventional carbon sources like glucose, yet low expression levels and complex processes remain unresolved [22]. Therefore, the key challenge lies in maintaining high-efficiency heterologous protein expression while eliminating methanol dependence.

Our laboratory previously designed and constructed a transcriptional signal amplification device, CSAD, that enables high-level target gene expression using various carbon sources including glucose, glycerol, and methanol. This design employed the LacI/lacO interaction system from the *E. coli* lactose operon, fusing the PAOX1 transcriptional activator Mit1 activation domain with LacI and replacing the native PAOX1 upstream regulatory sequence with lacO sequences [23]. Building upon this foundation, we further optimized the design to obtain an enhanced device, CSAD_5. This study utilized CSAD_5 to drive insulin precursor expression, screened a single-copy expression strain, developed a glucose-based fermentation process for high-level insulin precursor production at the bioreactor scale, and further explored the industrial potential of *P. pastoris* for insulin precursor production by enhancing antibiotic selection pressure.

Materials and Methods

1.1.1 Strains

Pichia pastoris GS115 was purchased from Invitrogen. The single-copy strain WT-IP, expressing insulin precursor under PAOX1 control, and the single-copy strain CSAD_5-IP, expressing insulin precursor under CSAD_5 control, were

obtained by linearizing the pPIC9K-IP and pCSAD_5-IP plasmids with SalI, respectively, followed by electroporation into GS115 competent cells and screening. The CSAD_5-IP1 through CSAD_5-IP10 series of strains were obtained using the method described in Section 1.2.4.

1.1.2 Culture Media

The composition and preparation of YPD, YND, YNM, MGY, BSM basal medium, PTM1 trace elements, G418 antibiotic, and biotin followed the *Pichia pastoris* instruction manual (www.thermofisher.com).

1.1.3 Equipment

The 5-L bioreactor used in this study was an FGM-5L (Type III) bioreactor from Shanghai Guoqiang Bioengineering Equipment Co., Ltd. The HPLC system was an Agilent 1260 with Agilent ChemStation analysis software.

1.2.1 HPLC Detection of Insulin Precursor

Insulin precursor was quantified by high-performance liquid chromatography (HPLC) [22]. The system consisted of an Agilent 1260 chromatograph with a C18 column (KromasilTM, Sweden, 250 mm × 4.6 mm × 5 m, 100 Å spherical silica). Mobile phase A was 0.1% trifluoroacetic acid (TFA) and mobile phase B was 100% acetonitrile. The column temperature was maintained at 26°C with an injection volume of 20 L and flow rate of 1 mL/min. Detection was performed at 280 nm. The column was equilibrated with 15% B, and the elution program was: 0–20 min, 15–60% B; 20–25 min, 60–100% B.

1.2.2 Shake-Flask Fermentation of Insulin Precursor Strains

Strains from glycerol stocks were inoculated into serum bottles containing YPD medium and cultivated to logarithmic phase. Cells were harvested by centrifugation at 12,000 g for 2 min, washed twice with sterile water, and inoculated into 500-mL baffled flasks containing 50 mL of YNM or YND medium at a final OD600 of 1. Carbon sources were supplemented every 24 h to their initial concentrations, and 1-mL samples were collected at intervals. Samples were centrifuged at 12,000 g for 5 min, and the supernatant was analyzed for insulin precursor titer. All *P. pastoris* strains were cultivated at 30°C with shaking at 200 rpm.

1.2.3 5-L Bioreactor Fermentation of Insulin Precursor Strains

Seed Culture: For inoculum preparation, strains were cultivated in shake flasks. MGY medium was used for seed culture during glycerol batch fermentation, while YND medium was used for glucose batch and fed-batch cultures. Glycerol stock cultures were first inoculated into serum bottles containing YPD medium and grown to logarithmic phase as primary seed. Primary seed was

then transferred at a 1:40 ratio into 300 mL of MGY or YND medium and cultivated to logarithmic phase as secondary seed. The secondary seed was transferred entirely into a 5-L bioreactor containing 3 L of basal salt medium BSM (supplemented with PTM1 at 4.5 mL/L).

Methanol Fermentation Process: The initial fermentation phase commenced with 40 g/L glycerol in the medium. Upon glycerol depletion, indicated by a sharp increase in dissolved oxygen (DO), glycerol feeding was initiated until wet cell weight (WCW) reached approximately 250 g/L, at which point glycerol feeding ceased. After a 0.5-h starvation period, methanol feeding was initiated. Anhydrous methanol was manually added (4 mL) and feeding paused; after DO dropped and recovered, the process was repeated after 0.5 h before switching to automatic methanol feeding with gradual rate increases to the desired level. Dissolved oxygen was maintained at 30–50% by controlling agitation (200–1000 rpm) and aeration (2–6 L/min). Pure oxygen was supplied when both agitation and aeration reached maximum capacity. pH was controlled using an automated peristaltic pump connected to the bioreactor, with ammonium hydroxide addition. The pH was maintained at 5.0 during batch phase and 3.5 during induction phase. Temperature was controlled at 30°C throughout fermentation.

Glucose Fermentation Process: The initial fermentation phase began with 40 g/L glucose. After glucose depletion and completion of the batch phase, 50% (w/v) glucose solution was fed at a constant limiting rate until fermentation completion. Dissolved oxygen, pH, and temperature control were identical to the methanol fermentation process.

1.2.4 Screening of High-Yield Strains

Following the method described in the Multi-Copy *Pichia* Expression Kit (Invitrogen), the pCSAD_5-IP plasmid was linearized with SalI and electroporated into GS115 competent cells. Recovery cultures (50, 100, 150, 200, and 250 L) were plated on YPD agar containing 0.5, 1, 1.5, 2, and 3 mg/mL G418, respectively, and incubated at 30°C. Transformants were cultivated to logarithmic phase, diluted to OD600 = 0.1, and 4- L aliquots were spotted on YPD plates with varying G418 concentrations. The maximum tolerable concentration was defined as the highest G418 concentration at which growth was observed, yielding a series of strains with different G418 tolerance levels.

1.2.5 Data Analysis

All bar graphs were generated using GraphPad Prism 6.0 (GraphPad Software), and all line graphs were created using Origin 8.0 (OriginLab). All wet cell weight and insulin precursor titer data represent the mean of three independent experiments. Statistical significance was determined using Student' s t-test.

Results

2.1 Shake-Flask Expression of Single-Copy Insulin Precursor Strains

To evaluate the insulin precursor expression capability of the single-copy strain CSAD_5-IP, shake-flask fermentation was performed alongside the WT-IP strain using the method described in Section 1.2.2. The WT-IP strain was induced with 0.5% (v/v) methanol, while CSAD_5-IP was cultivated under 0.5% and 1% (v/v) methanol conditions, as well as 0.5%, 1%, and 2% (w/v) glucose conditions. As shown in [Figure 1: see original paper], CSAD_5-IP produced 4.9 mg/L insulin precursor at 0.5% (v/v) methanol, which was higher than the 1% (v/v) methanol condition and comparable to WT-IP performance under methanol induction. The highest titer of 6.2 mg/L was achieved with CSAD_5-IP at 1% (w/v) glucose, representing an approximate increase over WT-IP under methanol conditions. These results demonstrate that CSAD_5-IP successfully expresses insulin precursor using both methanol and glucose carbon sources, with glucose-based production exceeding that of the wild-type strain under methanol induction. To further enhance target protein titers and evaluate industrial potential, both methanol- and glucose-based fermentation processes were investigated at the 5-L bioreactor scale.

2.2.1 Methanol Fermentation Process Analysis for Single-Copy Strain in 5-L Bioreactor

Low-flow methanol feeding strategies have been shown to reduce oxygen consumption and heat generation during fermentation [24]. To investigate the effects of lower methanol feeding rates on CSAD_5-IP growth and product formation, three different methanol feeding rates of 3.33, 6.67, and 12.0 mL/(h · L broth) were evaluated using the fermentation protocol described in Section 1.2.3. The WT-IP strain was maintained at the optimal methanol feeding rate of 12.0 mL/(h · L broth).

As illustrated in [Figure 2: see original paper], increasing methanol feeding rates correlated with improved wet cell weight and insulin precursor production in CSAD_5-IP. At 12.0 mL/(h · L broth), CSAD_5-IP exhibited the fastest growth rate, comparable to the wild-type strain, achieving a maximum insulin precursor titer of 0.32 g/L at 100 h. However, this represented only 50% of the WT-IP maximum titer (0.64 g/L). These results indicate that under 5-L bioreactor methanol-based conditions, CSAD_5-IP yields were relatively low, necessitating exploration of alternative processes.

2.2.2 Exploration of Glucose Fermentation Process for Single-Copy Strain in 5-L Bioreactor

To examine the effects of different glucose feeding rates on CSAD_5-IP growth and product formation, 50% (w/v) glucose solution was fed at rates of 11.5, 15.5, and 21.5 mL/(h · L broth) following the batch phase using the protocol described in Section 1.2.3.

As depicted in [Figure 3: see original paper], a glucose feeding rate of 21.5 mL/(h·L broth) resulted in maximum insulin precursor titer of 0.55 g/L at 92 h, after which productivity declined. This rate significantly accelerated cell growth, with wet cell weight exceeding 600 g/L by 76 h. However, growth subsequently slowed, reaching 645 g/L at 100 h, accompanied by substantial increases in broth viscosity and severe mass transfer limitations that prevented dissolved oxygen maintenance above 30%, forcing premature fermentation termination. These findings indicate that high glucose feeding rates are detrimental to both product formation and process control. The lowest wet cell weight and insulin precursor titer were observed at 11.5 mL/(h · L broth), yielding 0.42 g/L at 108 h. An intermediate feeding rate of 15.5 mL/(h · L broth) produced the optimal result, achieving 0.65 g/L at 100 h, establishing this as the preferred glucose feeding rate.

2.2.3 Comparison of Glucose and Methanol Fermentation Processes

As shown in [Figure 4: see original paper], at the 5-L scale, CSAD_5-IP growth under glucose feeding at 15.5 mL/(h·L broth) was comparable to WT-IP growth under methanol conditions. The CSAD_5-IP strain achieved maximum insulin precursor titer of 0.65 g/L at 100 h, equivalent to the WT-IP maximum under methanol induction.

Regarding process control, methanol toxicity necessitates strict control of methanol feeding during the carbon source transition (glycerol to methanol) for WT-IP, a complex and time-consuming process (~8 h) requiring substantial expertise. In contrast, CSAD_5-IP fermentation eliminates carbon source switching, utilizing glucose as the sole carbon source throughout with easily controlled feeding rates and simplified operation. For oxygen demand, as shown in [Figure 5: see original paper], WT-IP required pure oxygen (99.999% purity) supplementation beginning at 48 h when wet cell weight reached 268 g/L at 44 h and aeration/agitation limits were reached. Pure oxygen flow gradually increased to two-thirds of total gas flow. Conversely, CSAD_5-IP required no pure oxygen throughout fermentation, with aeration never reaching maximum capacity. Previous reports indicate that glucose-based *P. pastoris* high-density fermentation generates substantially less heat than methanol-based processes (ΔHS, methanol = -22.70 kJ g ^{1}; ΔHS, glycerol = -17.98 kJ g ^{1}; ΔHS, glucose = -15.58 kJ g ^{1}) [25,26]. In summary, the CSAD_5-IP glucose-based process achieves insulin precursor titers comparable to the WT-IP methanol system while offering significant advantages in process control, oxygen consumption, and heat generation. Given that heterologous genes in CSAD_5-IP are single-copy, increasing target gene dosage based on this glucose fermentation process could further enhance insulin precursor titers.

2.3.1 Shake-Flask Expression of High-Yield Insulin Precursor Strains

Previous studies have demonstrated that increasing antibiotic selection concentration is an effective method for obtaining high-copy transformants [27]. Using

the approach described in Section 1.2.4 and the Multi-Copy *Pichia* Expression Kit (Invitrogen), ten recombinant *P. pastoris* strains were screened and designated CSAD_5-IP1 through CSAD_5-IP10.

These strains were fermented in shake flasks using 1% (w/v) glucose as carbon source. As shown in [Figure 6: see original paper], insulin precursor expression increased progressively with higher antibiotic selection concentrations. The CSAD_5-IP10 strain, selected on 3.0 mg/mL G418, achieved the highest titer of 15.6 mg/L, representing a 1.5-fold improvement over the single-copy CASD_5-IP strain.

2.3.2 5-L Bioreactor Expression of High-Yield Insulin Precursor Strain

The CSAD_5-IP10 strain was selected for insulin precursor production in a 5-L bioreactor using the optimized glucose fermentation process. As depicted in [Figure 7: see original paper], CSAD_5-IP10 achieved maximum insulin precursor titer of 1.85 g/L at 108 h. This represents the highest reported level for human insulin precursor production using glucose as carbon source, surpassing the previous record of 1.28 g/L [20]. Regarding growth, this strain exhibited similar growth kinetics to the wild-type strain during the first 80 h, maintaining wet cell weight around 550 g/L from 80 h to process completion while insulin precursor titers continued to increase. Throughout the entire fermentation, neither aeration nor agitation reached maximum capacity, and no pure oxygen supplementation was required, demonstrating that this high-yield strain not only significantly improves productivity but also simplifies process control, making it more suitable for industrial application.

Discussion

China has the world' s largest diabetic population, with a continuously rising trend [28]. As an essential therapeutic for insulin-dependent diabetes, insulin commands substantial market demand. Therefore, increasing insulin production, optimizing manufacturing processes, and reducing production costs hold significant economic and scientific value. The *P. pastoris* expression system, as a major platform for heterologous protein production, has been applied to human insulin precursor manufacturing due to its inherent advantages [12]. Previous research has focused primarily on methanol-inducible PAOX1 promoter-based production [13].

In PAOX1-regulated fermentation processes, glycerol is typically used as the initial carbon source to build biomass before switching to methanol for protein induction upon glycerol depletion [29]. Methanol toxicity necessitates strict feeding control, resulting in a complex and time-consuming process [8]. Methanol-based fermentation generates high heat and oxygen demand [17], and methanol' s volatile, hazardous nature poses risks during handling and storage, imposing numerous limitations on industrial applications. Celik et al. [30] employed

methanol/sorbitol co-feeding during the induction phase to reduce methanol usage, but productivity was inferior to methanol-only induction. Wang et al. [24] developed a low-flow methanol feeding process based on PAOX1 transcription factor engineering, alleviating heat and oxygen issues while maintaining high-level expression, but the methanol-based process paradigm remained unchanged. Wang et al. [31] also developed a glucose/glycerol alternating feed strategy to avoid methanol, but could not achieve glucose as the sole carbon source. This study utilized the efficient constitutive amplification device CSAD_5 to drive insulin precursor expression, establishing a glucose-based fermentation process that uses glucose as the sole carbon source throughout with easily controlled feeding rates, simplified operation, low oxygen demand, and reduced heat generation, offering clear industrial advantages. Li et al. [21] employed the native constitutive promoter PGAP for non-methanol-based human insulin precursor expression, but achieved low titers with a maximum of only 0.3 g/L at the 30-L scale. In contrast, by increasing selection pressure, this study obtained a high-yield strain that produced 1.85 g/L human insulin precursor in a 5-L bioreactor using the developed process, representing the highest reported level for glucose-based production. Overall, this study maintains high-level human insulin precursor expression while eliminating methanol dependence, providing new perspectives for industrial production and *P. pastoris* applications.

This study generated a series of high-yield strains through increased selection pressure. As shown in [Figure 6: see original paper], insulin precursor expression increased with antibiotic tolerance concentration, suggesting that further antibiotic concentration escalation could potentially yield even higher-producing strains. Sawanan et al. [32] demonstrated that secreted insulin precursor titers in *P. pastoris* are influenced not only by transcription levels but also by protein secretion efficiency. Liang et al. [33] improved insulin precursor production by co-expressing SNARE components that facilitate Golgi-to-plasma-membrane trafficking, enhancing heterologous protein secretion. Additionally, protein folding and degradation significantly impact heterologous protein yields. Gustavo et al. [34] identified the endoplasmic reticulum chaperone Kar2/Bip as crucial for proper heterologous protein folding in *P. pastoris*. Sreenivas et al. [35] reduced insulin glargine degradation by disrupting the KEX1 gene. Therefore, future work building upon this study could further increase human insulin precursor production through strategies including: additional high-copy-number strain screening, overexpression of SNARE components to enhance secretion efficiency, overexpression of Kar2/Bip to reduce misfolding, and KEX1 deletion to minimize product degradation.

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Note: Figure translations are in progress. See original paper for figures.