Stability analysis of a *Pichia pastoris* recombinant clone expressing human insulin precursor

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Abstract. Current recombinant human insulin production utilizes two major expression systems, *Escherichia coli* and *Saccharomyces cerevisiae*-based expression systems. Methylotrophic yeast, *Pichia pastoris*, has appeared as a promising alternate yeast recombinant host for insulin precursor (IP) expression due to its ability to produce high titers, efficient secretion, and growth to very high cell densities. Similar to the *S. cerevisiae* system, *P. pastoris* secreted soluble IP into the culture supernatant. In the previous study, we have established *P. pastoris* recombinant clones harboring synthetic insulin precursor (IP) expression cassette integrated into their genomes through homologous recombination. It is essential to verify that the expression cassettes of the IP encoding gene remain stably integrated with the genome during such prolonged methanol induction. Therefore, we aimed to analyze the stability of one recombinant clone (CL-4) expressing the human insulin precursor by verifying the stable integration of the IP expression cassette into the genome by PCR, and the IP protein expression after prolonged methanol induction over 70 generations. We found that the expression cassette was stably integrated into the genome of the CL-4 recombinant clone and the IP expression was sustained after 72 generations of cultivations in the culture and induction media without antibiotic selection.

1. Introduction

Diabetes is a severe chronic disease characterized either by the inability of the pancreas to produce a sufficient amount of insulin hormone which regulates blood sugar or glucose level, or due to the body ineffectively use the insulin it produces [1]. The global prevalence of diabetes has been increasing over recent decades worldwide. It was estimated in 2015 that 415 million people suffer from diabetes, 5 million deaths attributable to diabetes and the number is predicted to reach 642 million by 2040 [2]. Since insulin and its analogs currently applied for the effective treatments of diabetes there will be increasing demand for insulin in the coming decades.
The recombinant insulin proteins expressed in various organisms including *Escherichia coli*, yeast, and others [3]. However, current human insulin production predominantly employs *E. coli* and yeast-based expression systems (mainly *Saccharomyces cerevisiae*) [4]. In *E. coli* system, the insulin precursor (IP) is overexpressed in the form inclusion bodies which required solubilization and oxidative refolding [5], whereas, in yeast-based expression system, the soluble IP is secreted into the culture supernatant and converted into human insulin through enzymatic reactions [6].

The methylotrophic yeast, *Pichia pastoris*, can be employed as a host for IP expression. Several studies have reported the secretory expression of IP protein in *P. pastoris* [4, 6 - 10] with the highest secreted IP in the fermentor scale reached up to 4.51 g/L [6]. There are various advantages of using *P. pastoris* as a recombinant host such as inexpensive culture-medium, fast-growth, easy purification, regulated under the control of the strong alcohol oxidase promoter, high secretory ability, rarely secreted other protein than the expression product [11] and the fermentation process has been widely studied [6]. The obvious advantage of using *P. pastoris* over *S. cerevisiae* is that the recombinant proteins produced can be efficiently expressed without over-glycosylation [3, 12].

In our previous study, we have established the *P. pastoris* CL-4 recombinant clone expressing IP secreted into the culture supernatant [13]. Therefore in this study, we attempted to assess the CL-4 recombinant clone stability in expressing IP and confirmed its genetic stability after 72 generations of cultivations and methanol inductions.

2. Materials and Methods

2.1. Strain

*P. pastoris* recombinant clone No. 4 used in this study harbor IP cassette was established in our previous study [13] and derived from *P. pastoris* X33 strain (Invitrogen, Carlsbad, CA).

2.2. Growth evaluation of *P. pastoris* recombinant clone

In order to define one generation of CL-4 recombinant clone, we conducted growth evaluation of the CL-4 in three different media used in this study i.e. yeast extract peptone dextrose (YPD) medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% w/v glucose], buffered glycerol-complex (BMGY) medium [2% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate pH 6.0, 1.34% (w/v) YNB, 4 x 10⁻⁵% (w/v) biotin, 1% (v/v) glycerol] and buffered methanol-complex (BMMY) medium [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate pH 6.0, 1.34% (w/v) YNB, 4 x 10⁻⁵% (w/v) biotin, 2% (v/v) methanol]. A single colony of CL-4 was grown in 3 mL YPD medium at 30°C in a a shaking incubator (250 rpm) for 24 h. The cells were harvested by centrifuging at 3,000 × g for 5 min at room temperature. Cell pellet was resuspended and subcultured in 15 mL of YPD, BMGY, and BMMY medium. Culture was placed in a 100 mL flask incubated at 30°C in a shaking incubator (250 rpm). The cell density (OD₆₀₀) was measured from 0 – 6 h incubation.

2.3 Stability analysis of IP expression in *P. pastoris* recombinant clone

The stability analysis of IP expression in *P. pastoris* recombinant clone was conducted without zeocin selection in the culture and induction medium. Two colonies of CL-4 *P. pastoris* recombinant clone from Yeast Extract peptone dextrose (YPD) agar plate [1% (w/v) yeast extract, 2% (w/v) peptone, 2% w/v glucose, 2% (v/v) agar], were inoculated into two tubes of 2 mL YPD medium and shaken at 250 rpm and 30°C for 24 h. Cells were harvested by centrifuging at 1,500-3,000 × g for 5 min at room temperature. The cell pellet was then inoculated into 10 mL BMGY medium in a 100 mL flask and grown at 30°C in a shaking incubator (250 rpm) for 24 h. One-milliliter aliquots were taken at the end of the 24 h culture to inoculate culture medium for the next generation. While the remaining cells were harvested by centrifuging at 3,000 × g for 5 min at room temperature. Cell pellet was resuspended to an OD₆₀₀ of 10 in 10 mL BMMY medium to induce expression. The culture was placed in a 100 mL flask. A 100% methanol was added to a final concentration of 2% methanol every 24 h to maintain
induction. After 3 d of methanol induction, the supernatant was transferred to a separate tube and stored at −20°C until ready to assay. The IP expression cycle was repeated using 1 ml of BMGY culture of earlier batch until it reached the fifth batch where each batch has 12 generations differences.

2.4. Genetic stability analysis of the CL-4 recombinant clones after prolonged methanol induction
Genomic DNAs of the CL-4 recombinant clone were extracted from the cell pellet of 72 h post methanol induction cultures of the batch I - V using Yeastar Genomic DNA Kit (Zymo Research Corp., Irvine, USA) following the manufacturer’s instructions. The integrant, IP cassette constructed in the pD902 vector, in the genome of 72 h post methanol induction cultures of the batch I - V were validated by PCR using an AOX1 specific primer set consisted of AOX1F (5'-GACTGGTTCCAATTGACAAGC-3') and AOX1R (5'-GCAAATGGCATTCTGACATCC-3'). The primers are listed in the EasySelect™ Pichia Expression Kit manual (Invitrogen 2010). PCR amplification was conducted using HotStar Taq Master mix Kit DNA polymerase (Qiagen). PCR amplification with AOX1 primer set was carried out in a 25 μL mixture (total volume) containing ~50 ng of P. pastoris genomic DNA, 12.5 HotStar Taq Master Mix, and 0.2 μM of each primer. We used the following PCR conditions: 95°C for 15 min; then 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min; followed by the last cycle at 72°C for 10 min. The amplicon of the predicted IP cassette was then sequenced (1st Base DNA sequencing service, Malaysia). Clustal multiple sequence alignment was carried out using DnaMan1.

2.5. Protein analysis and quantification
Protein analysis was conducted by SDS-PAGE analysis. The supernatant of culture broth samples was analyzed by denaturing 15% polyacrylamide gel electrophoresis using the Tricine buffer system [14]. Samples (20 μL) were mixed with an equal volume of Tricine sample buffer, mixed and boiled for 15 min. Samples were loaded on to the gel (20 μL per lane) and electrophoresed. The separated polypeptides were stained using Coomassie Brilliant Blue Solution (BioRad) for SDS-PAGE analysis. Each gel was destained in 15 mL destaining solution I [methanol 40%, acetic acid 7%, water 53%] for 30 minutes and in 20 mL destaining solution II [methanol 5%, acetic acid 7%, water 88%] for about 3 hours. Protein concentration in Tricine SDS-PAGE was quantified using ImageJ software by applying lysozyme as standard ranging from 0.03 to 2 mg/mL.

3. Results and Discussion
3.1. Growth evaluation of the CL-4 recombinant clone
Genetic stability refers to the integrity of the production strain during the cultivation process to the limit of the production population doubling level (PDL) [15]. To determine the doubling time of CL-4 recombinant clone, we conducted growth evaluation in YPD, BMGY, and BMMY medium as these media used as culture and induction medium of IP expression. There are three phenotypes of P. pastoris host strains which differ related to their ability to utilize methanol: the wild type or methanol utilization plus phenotype (Mut+), and those resulting from deletions in the AOX1 gene [methanol utilization slow (Mut−)], or both AOX genes [methanol utilization minus (Mut−)]. The wild type strain X33 is one of the most widely used P. pastoris strains besides the GS115 (his4) which both known as Mut+ strains [16]. The recombinant strain used in this study was previously identified as Mut+ phenotype derived from X33 wild type strain [13]. The growth temperature of P. pastoris is 28–30°C for liquid cultures, plates, and slants. Doubling time of log-phase Mut+ or Mut− Pichia in YPD is ~2 hours (Invitrogen, 2010). Since the P. pastoris CL-4 recombinant clone was identified as a Mut+ phenotype with pD902-IP integrated into its genome without AOX1 gene disruption. Here, we confirmed that the CL-4 recombinant clone has a similar doubling time around 2 hours in three different media YPD, BMGY, and BMMY in a shaking incubator of 250 rpm at incubation
temperature 30°C as shown in figure 1. Therefore in this study, one generation was defined as a two-hour incubation of an initial inoculum with particular cell density in a culture medium of YPD or BMGY at 30°C in a shaking incubator of 250 rpm.

**Figure 1.** Growth curve of CL-4 recombinant clone in YPD, BMGY, and BMMY medium. The cell densities were doubled every 2 hours. Squares, the CL-4 growth on YPD medium; Diamonds, the CL-4 growth on BMGY medium; Triangles, the CL-4 growth on BMMY medium.

### 3.2. Stability analysis of the IP expression

In our previous study, an IP expression cassette consisted of truncated α-factor signal peptide-spacer peptide (EEAEAEAPK)-insulin B chain (1-29)-short connecting peptide (DGK)-insulin A chain (1-21) was constructed in a Pichia integrative vector pD902-IP (3921 bp). The pD902-IP vector was integrated into the P. pastoris X33 genome through homologous recombination. One of the recombinant clones (CL-4) was confirmed to secrete IP protein in the culture supernatant, which has a size of ~7 kDa. The predicted molecular weight size of secreted IP is 7053 Da, comprising of 63 amino acids [13]. Since the CL-4 recombinant clone had higher IP expression compared to other clones, in this study we analyzed the IP expression stability of the CL-4 recombinant clone. The IP expression was conducted in five batches representing 24 – 72 generations. In each batch culture, the CL-4 recombinant clone was cultivated in the BMMY induction medium with 100% methanol added to a final concentration of 2% methanol every 24 h to maintain induction. The supernatant was collected from the culture 72 h post-methanol induction. Figure 2A shows the SDS-PAGE of culture supernatant 72 h post methanol induction from the five batches. It shows an IP band of ~7 kDa from 24 to 72 generations culture supernatant. Even though after 24 generations the CL-4 had no or very little amount of secreted IP protein, the IP expression was observed after 36 – 72 generations. This result revealed that the IP expression was sustained after 72 generations of cultivation in BMGY and BMMY medium without antibiotic selection. Based on the ImageJ analysis applying lysozyme as standards showed that the expression level varied among generations. The highest expression level was achieved after 60 generations where the IP productivities were around 0.2 mg/10⁸ cells (figure 2B, table 1)
Figure 2. A) SDS-PAGE of the culture supernatants of CL-4 recombinant clone at 24 – 72 generations in 15% SDS-PAGE gel (M= molecular weight polypeptide standards, lane 1 and 2= 24 generations, lane 3 and 4= 36 generations, lane 4 and 5= 48 generations, lane 6 and 7 = 60 generations, lane 8 and 9 = 72 generations B) Expression levels analyzed by imageJ.
Table 1. The amount of secreted IP (mg/L) in the culture supernatant of CL-4 recombinant clone from 24 – 72 generations

| Number of Generation from initial inoculum | Colony | Starting cell density (OD_{600}) | Final cell density (OD_{600}) | Final cell density (cells/mL x 10^8) | Secreted IP (mg/L) | Yield of IP (mg/10^8 cells) |
|-------------------------------------------|--------|---------------------------------|------------------------------|-------------------------------------|--------------------|-----------------------------|
| 24                                        | 1      | 10.13                           | 15.79                        | 7.9                                 | -584.7             | -                           |
|                                           | 2      | 10.03                           | 18.23                        | 9.1                                 | -345               | -                           |
| 36                                        | 1      | 9.65                            | 20.81                        | 10.4                                | 245.3              | 0.02                        |
|                                           | 2      | 7.75                            | 20.45                        | 10.2                                | 307.3              | 0.03                        |
| 48                                        | 1      | 13.45                           | 21.81                        | 10.9                                | 361.4              | 0.03                        |
|                                           | 2      | 12.02                           | 21.07                        | 10.5                                | 1366.5             | 0.13                        |
| 60                                        | 1      | 9.09                            | 18.49                        | 9.2                                 | 1923.1             | 0.20                        |
|                                           | 2      | 9.00                            | 18.69                        | 9.3                                 | 1926.4             | 0.21                        |
| 72                                        | 1      | 10.11                           | 21.28                        | 10.6                                | 335.6              | 0.03                        |
|                                           | 2      | 9.23                            | 21.28                        | 10.6                                | 305.8              | 0.03                        |

*The final cell density (cells/mL x 10^8) was calculated from the amount of the final cell densities (OD_{600}) where one OD_{600} = ~5 \times 10^7 cells/ml.

3.3. IP cassette stability in the genome of CL-4 recombinant clone after prolonged methanol induction

The colonies of each batch were subjected to genetic validation after the cultivation in the BMMY medium for 3 days. The genetic validation was confirmed by PCR using specific primer pair of the \(AOX1\) gene (AOX1F/AOX1R). The PCR of the 10 colonies assessed for the IP expression stability resulted in two fragments of the \(AOX1\) gene (~2000 bp) and IP cassette (~500 bp) (figure 3A). It confirmed that the CL-4 recombinant clone is a \(Mut^+\) phenotype and the CL-4 recombinant clone maintained the integrated foreign pD902-IP in its genome after 24 – 72 generations of the cultivation in the culture (YPD, BMGY) and induction (BMMY) media without antibiotic selection. The \(Mut^+\) phenotype strain grows normally on methanol since the integrative vector is designed to integrate into its chromosomal \(AOX1\) locus by single crossover to avoid the \(AOX1\) gene disruption [17]. Sequencing analysis was conducted for the confirmation of the 500 bp fragments of 10 colonies from the 24 – 72 generations. The 511 bp PCR fragments of 10 colonies had a 100% identity to the 849-1359 bp position of pD902-PI. Figure 3B shows multiple sequence alignment of the 511 bp of PCR products amplified from PCR confirmation of the 10 colonies against the 511 bp (849-1359 bp) of the pD902-PI sequence. The IP cassette position in the pD902-PI vector is from 944 to 1314 bp. It revealed that the 10 colonies from 24 – 72 generations harbor correct IP cassette in their genomes.
Figure 3. Genetic validation of the CL-4 recombinant clone after prolonged methanol induction. A) PCR confirmation of 24 – 72 generations [M = Marker 1 kb DNA ladder; 24.1 and 24.2 = two colonies of 24 generations; 36.1 and 36.2 = two colonies of 36 generations; 48.1 and 48.2 = two colonies of 48 generations; 60.1 and 60.2 = two colonies of 60 generations; 72.1 and 72.2 = two colonies of 36 generations; G1 = initial inoculum; WT = P. pastoris X33 wild type], B) Multiple sequence alignment of IP-cassette sequences of 10 colonies from 24 – 72 generations compared to the IP sequence of initial inoculum (G1) and the synthetic IP sequence harbored by pD902-IP expression vector (IP-ref). The figure shows ~150 bp representatives of 511 bp aligned sequences which showed 100% identity.
4. Conclusions

The *P. pastoris* CL-4 recombinant clone stably expressed the IP protein and maintained the integrated IP cassette in its genome from 24 – 72 generations of cultivation and methanol induction. The CL-4 recombinant clone can be employed for the IP production in the fermentor scale.

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