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Research Article

Applicability of the constitutive *GAP* promoter for recombinant human insulin hormone production in *Pichia pastoris*

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ABSTRACT

Insulin is a hormone regulates glucose homeostasis in the body. In this study, the precursor of the human insulin hormone was expressed under the constitutive Glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter. The expression vector was transferred into the *P. pastoris* X33 strain by electroporation. After the protein expression study in 5L scale bioreactor, the samples were analysed by SDS-PAGE and ELISA methods. In this study, 11 mg/ L of protein was produced in the bioreactor at the end of production. The MALDI-TOF analysis confirmed that the protein produced was a precursor of the human insulin hormone. At the end of the study, it can be said that a bioprocess has been developed under bioreactor conditions to produce human insulin precursors by using constitutive *GAP* promoter of *P. pastoris*.

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INTRODUCTION

The incidence of diabetes in recent years has increased the need for insulin. Since the early 1920s, diabetes has been treated with insulin derived from the pancreas of porcine and bovine. The average daily dose for a patient treated with insulin is approximately 40 to 60 U, or 1.4 to 2.1 mg. In the industrialised part of the world, 130,000 mega U insulin are used annually to treat diabetic patients, and the demand for insulin is increasing by 3-4% every year [1].

Human insulin is primarily responsible for regulating glucose metabolism and is produced in the β-cells of the pancreatic islets. It is a polypeptide with a molecular weight of 5808 Da, consisting of two chains, one 21 amino acids long (chain A) and the other 21 amino acids long (chain B), linked by two disulphide bridges [2].

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In the past, insulin was obtained from animals, but in the last 20 years it has been altered by biological and chemical reactions and has begun to be obtained semi-synthetically in a form similar to human insulin. However, with advances in molecular biology and biotechnology, biosynthetic human insulins have been started to be produced by recombinant DNA technology using bacteria and yeasts and have been widely used by diabetic patients that match the structure of insulin produced by the human body. Although *Saccharomyces cerevisiae* is still an expression system for recombinant protein production, alternative yeasts such as *Pichia pastoris* [3,4], *Hansenula polymorpha* [5,6], *P. methanolica* [7] have been used and occupy an important place in recombinant heterologous protein production.

P. pastoris is a unicellular, methylotrophic yeast and can multiply rapidly in inexpensive cultures like bacteria. This eukaryotic microorganism can perform post-translational modifications such as glycosylation, polypeptide folding and disulfide bond formation, which are necessary for the recombinant expression of eukaryotic proteins but cannot be performed by bacteria [8]. This makes this microorganism one of the most important microorganisms, especially for the recombinant production of eukaryotic proteins. In the production of heterologous proteins, *P. pastoris* expression systems offer scalable cell densities with easy genetic manipulation. Compared to *S. cerevisiae*, the *P. pastoris* system produces higher amounts of correctly folded and glycosylated recombinant protein as well as better secretion yield [9,10]. In addition, the presence of vectors with promoters such as the highly inducible *AOX1* and structural *GAP* provides great flexibility and convenience in the design of host vectors for recombinant protein production.

P. pastoris can utilise many different carbon sources during its growth, such as ethanol, methanol, glycerol, mannitol, glucose, sorbitol, alanine and trehalose. However, the presence of some carbon sources (such as glycerol, ethanol, acetate and glucose) in the environment suppresses the utilisation of other carbon sources such as methanol. However, in the study conducted by Inan and Meagher (2001), it was observed that trehalose, sorbitol, alanine and mannitol did not have catabolite suppression effects on the *AOX1* promoter, which is frequently used in recombinant protein expression [11]. The opposite is true for the *GAP* promoter. In a study by Baumann et al. [12] it was shown that some ethanol produced during recombinant protein production with the structural promoter *GAP* had no repressive effect on this promoter or even increased the level of protein production with the *GAP* promoter. The *AOX1* promoter has an important advantage and is highly preferred for recombinant protein production due to its high cell densities as a result of its induction with methanol and its high efficiency. However, the strength of induction of the *AOX1* promoter relies entirely on the use of methanol as a carbon source. Methanol is obtained from petrochemical sources that are not suitable for use in certain food products and additives. Methylotrophic yeasts produce hydrogen peroxide (H_2O_2) through methanol metabolism, which can cause proteolytic degradation of recombinant proteins through oxidative stress [13,14]. Methanol, a toxic and flammable chemical, is considered a potential hazard in large-scale fermentations. In addition, the use of glucose by the microorganism to produce this metabolite increases the oxygen demand of the microorganism by a factor of 3 to 4 [15], and the use of so much oxygen during production undesirably increases the temperature of the bioreactor [16], which has a negative effect on the expression of foreign genes [9].The high oxygen demand during production and the reduction of the heat released by the utilisation of this oxygen increase the production price. In addition, if the heat exchange and oxygen transfer capacity are low, it causes difficulties in large-scale production. Therefore, instead of using methanol-dependent promoters such as *AOX1*, another route is taken, namely the use of promoters that do not require methanol for induction. *GAP*, a constitutive promoter that does not require methanol induction, can achieve high expression levels without methanol [17]. There are many studies showing that this promoter is a very strong structural promoter and achieves high expression levels during heterologous protein production. For example, Goodrick et al. [18] produced active recombinant chitinase protein up to 400mg/L in a 1.5 L bioreactor under the *GAP* promoter. In addition, it was shown in shake flask experiments that 38 kDa pro-aqualizine protein can be produced in an amount of 1g/L [19]. Another study reported that the expression of the mammalian membrane transport proteins hPEPT1 and rPEPT2 is 5 -fold higher under the *GAP* promoter than under the *AOX1* promoter [20]. Although the *AOX1* promoter generally reaches high expression levels, these examples have shown that in some cases the use of the *GAP* promoter gives better results than the *AOX1* promoter. In addition, in expression systems using the *GAP* promoter, continuous production of recombinant proteins is ensured without the need to add methanol and this system is known to be very suitable for large-scale fermentation of heterologous proteins.

The main objective of this study was the extracellular production of human insulin using the *P. pastoris* expression system. Therefore, the recombinant insulin protein gene was cloned into the PGAPZαA vector and expression of the recombinant protein was carried out under the constitutive *GAP* promoter, which does not require methanol for induction. To our knowledge, this is the first study producing recombinant human insulin by using constitutive *GAP* promoter.

MATERIALS AND METHODS

Plasmids, Strains and Culture Media

Competent *E. coli* XL1-Blue strain and *P. pastoris* X33 strain were used in cloning and protein expression studies, respectively. pGAPZαA plasmid (Invitrogen, CA, USA) was used for the construction of expression vectors. YPD (1% yeast extract, 2% peptone, 2% glucose) and BYED (3%

yeast extract, 2% glucose, 1.34% yeast nitrogen base without amino acids (YNB), $4x10^{-5}$ g/L biotin, 100 mM potassium phosphate, pH 6) were used as general culture media for *P. pastoris* strains. Depending on the resistance gene on the plasmid used, appropriate antibiotics were added to the liquid and solid media used.

Construction of Expression Vector

The insulin precursor (IP) gene was cloned into the pGAPZαA vector from *EcoR*I and *Xba*I restriction sites to construct pGAPZα-IP expression vector. The *EcoR*I and *Xba*I digested products were ligated using the Rapid DNA Dephos&Ligation kit (Roche) and then transformed into the *E. coli* XL1-Blue strain. The pGAPZα-IP expression vector was linearised using the *Avr*II restriction enzyme and the linear vector was transformed into the electrocompetent cells of *P. pastoris* X33. The transformed cells were selected on YPD agar plates containing 100mg/mL and 500mg/mL zeocin. Gene integration and gene copy number of the transformed cells were determined by real-time PCR analysis.

Real-time PCR Analysis

Primers GAPPromrtF-TAGCAGCCCAGGGATGGA and GAPPromrtR-TCCGCCCGCTATTATTGC, AktinrtF-TATGCCGGTTTCTCCTTACC and AktinrtR-CACGGACGATTTCTCTCTCTCA were used to amplify the *GAP* promoter region and the actin gene, respectively, in the PCR reaction. The reaction mixture was prepared according to the Maxima SYBR Green/ROX qPCR master mix (Thermo Scientific) protocol and the RT-PCR conditions consisted of an initial melting step at 95°C for 10 minutes followed by 40 cycles of melting at 95°C for 15 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. The genomic DNA of the X33 strain was used as calibrator and the actin gene as reference. The gene copy number was calculated using the following formula:

Gene copy number=$2^{-\Delta\Delta Ct}$

ΔΔCt= ΔCt target gene-ΔCt calibrator gene

ΔCt target gene=Ct target gene- Ct reference gene

ΔCt calibrator gene=Ct calibrator gene- Ct reference gene

Protein Expression Studies in Shake Flask

Transformed *P. pastoris* X33 cells were first grown in YPD broth medium overnight in a shaking incubator at 28°C and 200 rpm. The next day, growing cells were transferred to 30 mL of BYED medium at 28°C and 200 rpm. It was developed for protein expression under the control of the *GAP* promoter for 40 hours in a shaking incubator. At the end of the 40th hour, the culture was harvested by centrifugation at 5000 g at 4°C and the supernatant samples were stored at -20°C until used for total protein, SDS-PAGE and ELISA analysis.

Protein Expression Studies in 5 L Bioreactor

A 5 L bioreactor (Sartorius Stedim BIOSTAT B) was used to produce recombinant insulin hormone on a large scale under the control of the constitutive *GAP* promoter. The frozen culture was transferred to 100 mL of BMGY (10 g/L yeast extract, 20 g/L peptone, 13.4 g/L YNB, 4 $4x10^{-5}$ g/L biotin, 10% glycerol, 100 mM potassium phosphate, pH 6) medium and incubated at 28 °C for approximately 20 hours. This culture was used to inoculate 2 L of basic salt medium (26.7 mL/L H_3PO_4 (85%), 0.93g/L $CaSO_4$, 18.2 g/L K_2SO_4, 14.9 g/L $MgSO_4.7H_2O$, 4.13 g KOH, 40 g/L glycerol, 2 mL/L 5% antifoam). 4.35 mL/L of the PTM1 salt was added to the medium.

The fermentation was carried out in two stages; the first stage consists of the batch phase and the second stage consists of the fed-batch phase. In the batch phase, the glycerol in the medium was utilised by the cells. The batch phase was continued for about 20 hours and concluded with the peak of dissolved oxygen (DO). The sudden increase in DO is an indication that glycerol, the carbon source in the medium, is depleted. Following the depletion of glycerol, the fed-batch stage, in which mainly protein expression takes place, was initiated, in which 50% (w/v) glucose solution was fed. The feed rate was gradually increased from 3 mL/L/s to 18 mL/L/s during the first 24 h of fermentation and kept constant at 18 mL/L/s until the end of fermentation. The fermentation temperature was kept constant at 28°C and the pH value at 5. The pH of the fermentation was controlled with 26% (v/v) NH_4OH and the same chemical was used as a nitrogen source. The oxygen content was maintained at 20% saturation by stirring and adding air and pure oxygen (1.5 vvm). pH and oxygen concentration were determined using appropriate probes (Hamilton, Reno, Nevada). The supernatants were collected at specified time intervals during fermentation and analysed to determine protein production.

SDS-PAGE Analysis

SDS-Polyacrylamide Gel Electrophoresis was applied by preparing a gradient gel (3.5% polyacrylamide loading gel and 10% polyacrylamide separating gel, 0.8 mm) in an OWL P8D8 (Thermo Scientific, IL, USA) device. 15 µL of the supernatant of samples, 12 µL of SDS gel loading buffer (125mM Tris-Cl, pH 6.8, 37.5% glycerol, 10% SDS, 0.025% Coomassie blue R-250) and 3 µL of 1M DTT was mixed vortex for 1 minute and then kept at 70°C for 10 minutes. After vortexing samples, 15 µl of each sample were loaded into the SDS gel. Two different buffers were used for electrophoresis, as cathode buffer (0.1M Tris, 0.1M Tricine and 0.1% SDS) and anode buffer (0.2M Tris-Cl, pH 8.9). The electrophoresis process was carried out by filling the cathode buffer between the two gels and the anode buffer in the electrophoresis tank and running it at 150 V for 60 minutes. The gel was then stained with a staining solution (0.1% Coomassie blue R-250, 10% acetic acid, 20% methanol, 70% distilled water) for 1 hour on an orbital shaker. The excess dye was removed with a destaining solution (10% acetic acid, 50% methanol and 40% distilled water) for 1 hour, and then the gel was washed with distilled water

for 5 minutes and imaged with the Odyssey imaging system (Li-Cor, NE; USA).

ELISA Analysis

The human insulin ELISA kit (Sigma-Aldrich) was used to determine the amount of protein produced. First, dilutions of the standard (lyophilised human insulin) were prepared according to the kit's protocol, and the absorbance values obtained from the standards were used to prepare a standard calibration curve to be used in the calculations. The prepared standards and the supernatant samples obtained from the fermentation were added in an amount of 100 µL to the microtiter plate pre-coated with human insulin antibody and incubated for 2.5 hours on an orbital shaker at room temperature. Then 300 µL of the1x washing solution was added to the wells of the microtiter plate and the washing procedure was repeated 4 times. After 100 µL of the detection antibody (biotinylated anti-human insulin antibody) was added to the wells and incubated in a shaker at room temperature for 1 hour. The washing step was repeated at this stage to remove the unbound detection antibody. Next 100 µL of HRP-streptavidin solution was added to the wells to bind to the detection antibody and incubated in the shaker for 45 minutes. After repeating the wash step at this stage, 100 µL of the TMB substrate solution was added to the wells to form a specific signal colour and incubated in the dark in a shaker at room temperature for 30 minutes. After, 50 µL of the stop solution was added to stop the reaction, and the absorbance values were read at 450 nm wavelength in the microplate reader without wasting time and the protein amounts were calculated.

Purification of Human Insulin

Ni^{+2} or Cu^{+2} immobilized metal affinity chromatography (IMAC) is used for the purification of human insulin protein. For this purpose, IMAC purification was performed using a Ni^{+2}-loaded resin. To purify the insulin protein in the supernatant sample, 25 mL equilibration buffer (20 mM sodium phosphate, 300 mM sodium chloride (PBS) 10 mM imidazole, pH7.4) was added to 25 mL supernatant and 500 µL Ni-NTA resin (Thermo Scientific) was added. The proteins were attached to the resin by spinning for 3.5 hours. Then this mixture was loaded onto the column and eluted. The first fraction collected was designated as the flow-through. After this step, they were washed with wash buffers containing 25 mM, 50 mM and 75 mM imidazole, respectively, and the 1st, 2nd and 3rd wash samples were collected. Then 3 different elution were made with elution buffers containing 100 mM, 200 mM and 300 mM imidazole and the 1st, 2nd and 3rd elution samples were collected and analysed in SDS-PAGE.

MALDI-TOF Analysis

MALDI-TOF verification of the produced protein was made with the MALDI TOF-MS/MS (Shimadzu, Manchester, England). After the purified protein samples were separated on the SDS-PAGE gel, the protein band of human insulin precursors was cut from the gel and tryptic peptide digestion was performed in accordance with the Proteoextract all-in one trysin digestion kit (Calbiochem, Germany) protocol. The samples prepared after tryptic peptide digestion were given to the MALDI TOF-MS/MS and analyzed.

RESULTS AND DISCUSSION

Selection of Transformants

P. pastoris X33 transformants containing the pGAPZα-IP vector were selected on YPD agar plates containing zeocin (100mg/mL). Gene integration and copy number of the expression cassette were determined by RT-PCR. Among ten transformants, three clones were identified that contained a single copy of the expression cassette, and transformant #4 was selected for further investigation.

Protein Production of the pGAPZα-IP Clone in the Bioreactor

Protein production was first done in a shake flask experiment with the pGAPZα-IP clone, and then insulin was produced on the scale of the 5L bioreactor. The single copy of the gene-containing clone was used for the expression of the recombinant human insulin. Protein production was carried out under hypoxic conditions, at a constant temperature of 28°C, pH of 5 and ethanol concentration of 0.75% and lasted for a total of 120 hours. After the batch phase, the fed-batch phase was initiated and fed with a 50% (w/v) glucose solution. The feeding rate of the glucose solution was gradually increased from 3 mL/L/s to 18 mL/L/s during the first 24 hours of fermentation and kept constant at 18 mL/L/s until the end of fermentation. Samples obtained during fermentation were analysed by SDS -PAGE. The expected band size of the produced protein is 6.8 kDa. Insulin production was observed between the 70th and 120th hour of fed-batch fermentation (Figure 1).

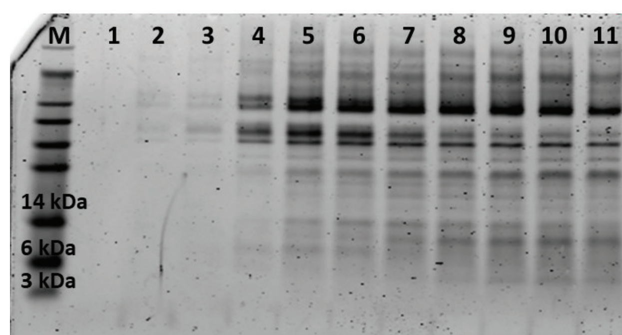

Figure 1. SDS-PAGE analysis of the culture supernatant of *P. pastoris* X33 transformant containing the pGAPZa-IP vector. M: SeeBlue Plus2 prestained protein ladder, 1: Batch 0 time, 2: Batch time end, 3: Fed-batch 0 time, 4: 12 h, 5: 30 h, 6: 40 h, 7: 70h, 8: 85 h, 9: 100 h, 10: 110 h; 11: 120 h.

Protein Purification and Protein Amount Determination

Purification of insulin precursors from the 120-hour supernatant obtained at the end of fermentation was performed by IMAC and the purified samples were analysed by SDS-PAGE (Figure 2). The 120-hour fermentation sample of the pGAPZ-IP clone containing a single copy of the expression cassette was purified and the elution sample obtained from both the fermentation supernatant sample and after purification was analysed by using the ELISA method. It was found that the supernatant sample contained 11 mg/L protein and the elution sample contained 60 mg/L protein after 120 hours.

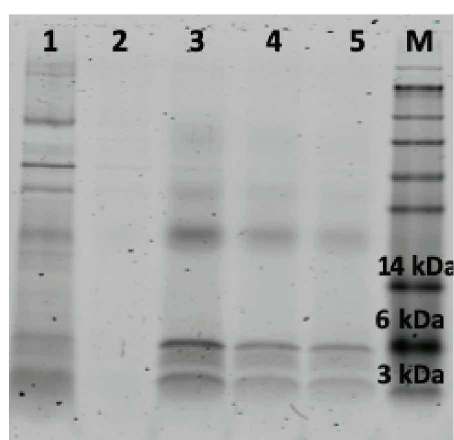

Figure 2. SDS gel showing the purification of the 120-hour supernatant sample. 1. Flow through, 2. Wash, 3. 1st elution, 4. 2nd elution, 5. 3rd elution, M. SeeBlue Plus2 prestained protein ladder.

After the fermentation supernatant was purified, the protein band of human insulin precursors was cut from the SDS-PAGE gel and analysed by MALDI TOF-MS/MS, and it was confirmed that the protein produced was human insulin hormone precursors.

Insulin is a hormone used to treat diabetes. With the increase in the incidence of diabetes in the world, the demand for insulin is also increasing. It is well known that obtaining insulin by conventional methods is both costly and very low in terms of the amount of product obtained. Therefore, in order to meet the increasing demand, scientists have started to produce the insulin hormone recombinantly, using expression systems such as bacteria and yeast, and studies on this topic continue to this day. Recently, the methylotrophic yeast *P. pastoris* has been used quite frequently for recombinant protein production. In this study, using the *P. pastoris* expression system, which has many advantages over bacteria and other yeast expression systems, an attempt was made to produce human insulin hormone under the control of the structural *GAP* promoter.

Within the scope of this study, different clones were created, and expression studies were carried out to produce insulin hormone under the control of the *GAP* promoter. For this purpose, the IP expression cassettes pGAP-HSA-IP-NS and pGAP-HSA-IP were first created and after their transformation into wild X33 strain, protein production was carried out. In the construction of these expression cassettes, HSA was used as a secretion signal sequence to secrete the produced protein from the cell. Recombinant proteins with *P. pastoris* are either produced in the cytosol within the cell or secreted from the cell by secretion signals. *P. pastoris* secretes low levels of endogenous proteins and the culture medium does not contain any additional proteins, as the heterologous protein secreted outside the cell constitutes most of the total protein in the growth medium. Therefore, extracellular secretion serves as the first step of purification in separating foreign proteins from cellular proteins. A signal sequence is needed for targeting the secreted foreign protein to the secretory pathway. The α-factor pre-pro peptide of *S. cerevisiae* has been successfully used as a secretory signal sequence. Alternative signal sequences such as HSA (human serum albumin), PHO (Acid Phosphatase), PIR1 (Protein with Internal Repeats) are also used. When used as the pre-pro HSA signal sequence, the bovine pancreatic trypsin inhibitor was synthesised in *P. pastoris* to yield its native N-terminal sequence [21]. Xiong et al. [22] produced the human lysozyme and showed that HSA is a good signal sequence that promotes heterologous protein secretion in *P. pastoris*. In another study, the long-lived HSA and interferon α-1 fusion protein pre-pro HSA signal sequence was used to produce a protein with very high antiviral activity and secreted in *P. pastoris* [23]. All these studies show that pre-pro HSA can be used as a good signal sequence in *P. pastoris*. In our study, no extracellular protein secretion was observed in the clones (pGAPZ-HSA-IP) produced with the HSA signal sequence, so the proteins produced remained inside the cell. These results therefore indicated that the HSA secretion signal was not suitable for extracellular production of human insulin hormone precursors under the control of the constitutive *P. pastoris GAP* promoter. Therefore, it was decided to replace the HSA secretion signal with the "α mating" secretion signal sequence, and protein production was continued by creating pGAPZα-IP-6XHis clone. Production was carried out under two different bioreactor conditions with the pGAPZα-IP-6XHis clone. In the first bioreactor experiment, the temperature was 28°C, pH 5 and glucose was fed under the condition that DO was kept constant at 30%, while in the second bioreactor experiment, 50% glucose was fed at the temperature 28°C, pH 5 and ethanol 0.75% under hypoxic conditions. However, in the productions carried out with these clones, it was observed that the protein produced remained in the cell due to the addition of His-taq. Then, it was decided to create a pGAPZα-IP clone and isolate the DNA of the transformants formed after the clone was created. The gene copy numbers of the clones were determined by RT-PCR using isolated genomic DNAs. It was found that the resulting clones contained 1 and 2 copies of the expression

cassettes. The clones containing one copy and two copies of the expression cassettes were taken separately for protein production in the bioreactor under different fermentation conditions. First, the clone containing two copies of the expression cassettes was fed with 50% glucose at a specific growth rate of 0.05 and fermentation was carried out at a pH of 3.5, a temperature of 28°C and a dissolved oxygen concentration of 30%, and it was found that the protein produced had a dimer structure (results not shown). As a result of ELISA analysis, it was found that 1.2 mg/L of protein was produced extracellularly. In the other fermentation with the same clone, all conditions were kept constant as in the first fermentation, but the pH of the fermentation medium was adjusted to 5 and production was carried out, but extracellular protein expression could not be detected. Fermentations were carried out with the clone containing a single copy of the expression cassette under the same conditions, but no extracellular expression was observed. Thereupon, another fermentation was performed with the clone containing a single copy of the expression cassette in a hypoxic environment, keeping the temperature constant at 28 °C, pH 5 and ethanol concentration of 0.75%. This time extracellular production was observed in SDS-PAGE analysis and by ELISA analysis. It was found that 11 mg/L protein was produced. Protein purification was performed from the culture supernatant, and it was found that 60 mg/L protein was in the elution sample. Although some studies suggest that high copy number is one of the most important factors that increase the efficiency of protein expression [24,25], there are also studies in the literature that show the opposite [26,27]. Due to the inability to perform post-translational modifications such as folding, disulphide bridge formation and signal processing in the endoplasmic reticulum [10,28], the large amount of protein produced in high copy number clones cannot be secreted and remains in the cell [29]. When the study was initially designed, the aim was to produce human insulin hormone precursors as a fusion protein that, contrary to the literature, does not contain spacer peptides. However, the experiments showed that extracellular secretion of the human insulin precursors is only guaranteed if they contain an spacer peptide such as "Glu Glu Ala Glu Ala Glu Ala Glu Pro Lys (EEAEAEAEPK)". Previously, Kjeldsen et al. [30] used the yeasts *P. pastoris* and *S. cerevisiae* to express insulin and compared these two yeasts. Synthetic prepro-leader sequences were designed for IP secretion in *S. cerevisiae* and these sequences were shown to accelerate IP secretion in *P. pastoris*. Various spacer peptides were used in the fusion protein, and it was shown in this study that the use of these peptides increased the yield of fermentation products of IP in *P. pastoris*. The highest efficiency was obtained in the expression vector formed by the intermediate peptide "Glu Glu Ala Glu Ala Glu Ala Glu Pro Lys (EEAEAEAEPK)" together with the α-factor signal sequence. As a result of this study, a similar result was obtained, and extracellular protein production was observed in the expression vector generated by using the spacer peptide "Glu Glu Ala Glu Ala Glu Ala Glu Pro Lys (EEAEAEAEPK)" together with the α-factor signal sequence.

Gurramkonda et al. [4] reported that, 1.5 g/L pure human insulin hormone was produced under the control of methanol-induced *AOX1* promoter. The another study showed that when producing recombinant human insulin under the control of the *AOX1* promoter, the modulating of the inoculum density and methanol induction increases protein production. According to the results of this study, the concentration of recombinant IP has been determined as 2.267 mg/L when the 10 times higher cell density and 3% methanol induction [31]. The studies about the human insulin production is going on. Constructing different expression vectors affects recombinant protein production. For this reason, scientists construct an expression vector using different secretion signals in *P. pastoris* and investigate its effect on extracellular recombinant protein production. For example, the truncated α-factor secretion signal sequence significantly increased the efficiency of recombinant human insulin hormone under the control of the *AOX1* promoter [32]. Considering the studies in the literature, it is seen that the *AOX1* promoter is frequently used in insulin production studies in *P. pastoris*. Therefore, this study, unlike others, has shown for the first time that the *GAP* promoter can be used in the production of human insulin hormone. Like other studies, it will be possible to produce higher amounts of human insulin protein with the *GAP* promoter by making modifications to both the expression vector and the fermentation conditions.

CONCLUSION

The recombinant human insulin was produced using constitutive *GAP* promoter. pGAPZα-IP expression vector was constructed and transformed into the compotent *P. pastoris* cell. Protein expression studies carried out at the level 5L bioreactor. The results show that:

- *GAP* promoter has the potential to produce human insulin. In research, where an inducible promoter is preferred for the production of recombinant proteins, it can therefore be used as a substitute. The spacer peptide is important for the extracellular secretion of the produced human insulin protein.
- In this study 11 mg/L protein was produced at the bioreactor level.
- In future studies, to increase the amount of protein produced, fermentation conditions need to be optimized. In addition, the necessary analysis must be carried out for the produced protein to be used therapeutically.

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AUTHORSHIP CONTRIBUTIONS

Authors equally contributed to this work.

DATA AVAILABILITY STATEMENT

The authors confirm that the data that supports the findings of this study are available within the article. Raw data that support the finding of this study are available from the corresponding author, upon reasonable request.

CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

There are no ethical issues with the publication of this manuscript.

REFERENCES

[1] Kjeldsen T. Yeast secretory expression of insulin precursors. Appl Microbiol Biotechnol 2000;54:277–286. [CrossRef]

[2] Ahmad B. Pharmacology of insulin. Br J Diabetes Vasc Dis 2004;4:10–14. [CrossRef]

[3] Kjeldsen T, Frost-Petterson A, Hach M. Secretory expression and characterization of insulin in Pichia pastoris. Biotechnol Appl Biochem 1999;29:79–86. [CrossRef]

[4] Gurramkonda C, Polez S, Skoko N, Adnan A, Gabel T, Chugh D, et al. Application of simple fed-batch technique to high-level secretory production of insulin precursor using Pichia pastoris with subsequent purification and conversion to human insulin. Microb Cell Fact 2010;9:1–11. [CrossRef]

[5] Weydemann U, Keup P, Piontek M, Strasser AWM, Schweden J, Gellissen G, et al. High-level secretion hirudin by Hansenula polymorpha-authentic processing of three different preprohirudins. Appl Microbiol Biotechnol 1995;44:377–385. [CrossRef]

[6] Gellissen G, Melber K. Methylotrophic yeast Hansenula polymorpha as production organism for recombinant pharmaceuticals. Arzneimittelforschung 1996;46:943–948.

[7] Raymond CK, Bukowski T, Holderman SD, Ching AFT, Vanaja E, Stamm MR. Development of the Methylotrophic Yeast Pichia methanolica for the Expression of the 65 Kilodalton Isoform of Human Glutamate Decarboxylase. Yeast 1998;14:11–23. [CrossRef]

[8] Hagenson MJ. Production of recombinant proteins in the methylotrophic yeast Pichia pastoris. Bioprocess Technol 1991;12:193–212.

[9] Cereghino JL, Cregg JM. Heterologous protein expression in the methylotrophic yeast Pichia pastoris. FEMS Microbiol Rev 2000;24:45–66. [CrossRef]

[10] Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM. Heterologous protein production using the Pichia pastoris expression system Yeast 2005;22:249–270. [CrossRef]

[11] İnan M, Meagher MM. Non-Repressing Carbon Sources for Alcohol Oxidase (*AOX1*) Promoter of Pichia pastoris. J Biosci Bioeng 2001;92:585–589. [CrossRef]

[12] Baumann K, Maurer M, Dragosits M, Cos O, Ferrer P, Mattanovich D. Hypoxic fed-batch cultivation of pichia pastoris ıncreases specific and volumetric productivity of recombinant proteins. Biotechnol Bioeng 2008;100:177–183. [CrossRef]

[13] Hilt W, Wolf DH. Stress-induced proteolysis in yeast. Mol Microbiol 1992;6:2437–2442. [CrossRef]

[14] Xiao AF, Zhou XS, Zhou L, Zhang YX. Improvement of cell viability and hirudin production by ascorbic acid in Pichia pastoris fermentation. Appl Microbiol Biotechnol 2006;72:837–844. [CrossRef]

[15] Mateles RI. Calculation of oxygen required for cell production. Biotechnol Bioeng 1971;13:315–328. [CrossRef]

[16] Lin J, Panigraphy D, Trinh LB, Folkman J, Shiloach J. Production process for recombinant human angiostatin in Pichia pastoris. J Ind Microbiol Biotechnol 2000;24:31–35. [CrossRef]

[17] Waterham HR, Digan ME, Koutz PJ, Lair SV, Cregg JM. Isolation of the Pichia pastoris glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. Gene 1997;186:37–44. [CrossRef]

[18] Goodrick JC, Xu M, Finnegan R, Schilling BM, Schiavi S, Hoppe H, Wan NC. High-level expression and stabilization of recombinant human chitinase produced in a continuous constitutive Pichia pastoris espression system. Biotechnol Bioeng 2001;74:492–497. [CrossRef]

[19] Olezdzka G, Dazbrowski S, Kur J. High levels expression, secretion and purification of the thermostable aqualysin I from Thermus aquaticus YT-1 in Pichia pastoris. Protein Expr Purif 2003;29:223–229. [CrossRef]

[20] Döring F, Theis S, Daniel H. Use of the glyceraldehydes-3-phosphate dehydrogenase promoter for production of functional mammalian membrane transport proteins in the yeast Pichia pastoris. Biochem Biophys Res Commun 1998;250:531–535. [CrossRef]

[21] Yang L, Dong W, He J, Ren X, Yan W. Expression and purification of natural N-terminal recombinant bovine pancreatic trypsin inhibitor from Pichia pastoris. Biol Pharm Bull 2008;31:1680–1685. [CrossRef]

[22] Xiong R, Chen J, Chen J. Secreted expression of human lysozyme in the yeast Pichia pastoris under the direction of the signal peptide from human serum albümin. Biotechnol Appl Biochem 2008;5:129–134. [CrossRef]

[23] Tian S, Li Q, Yao W, Xu C. Construction and characterization of a potent, long-lasting recombinant human serum albumin-interferon a1 fusion protein expressed in Pichia pastoris. Protein Exp Purif 2013;90:124–128. [CrossRef]

[24] Vassileva A, Chugh DA, Swaminathan S, Khanna N. Effect of copy number on the expression levels of hepatitis B surface antigen in the methylotrophic yeast Pichia pastoris. Protein Expres Purif 2001;21;71–80. [CrossRef]

[25] Norden K, Agemark M, Danielson JA, Alexandersson E, Kjellbom P, Johanson U. Increasing gene dosage greatly enhances recombinant expression of aqua porins in Pichia pastoris. BMC Biotechnol 2011;11:1–12. [CrossRef]

[26] Hohenblum H, Gasser B, Maurer M, Borth N, Mattanovich D. Effects of gene dosage, promoters,and substrates on unfolded protein stress of recombinant Pichia pastoris. Biotechnol Bioeng 2004;85:367–375. [CrossRef]

[27] Türkanoğlu Özçelik A. Comparison of multicopy pro-microbialtransglutaminase encoded gene expression in Pichia pastoris. Acta Alimentaria 2019;48:384–392. [CrossRef]

[28] Shen Q, Wu M, Wang HB, Naranmandura H, Chen SQ. The effect of gene copy number and coexpression of chaperone on production of albumin fusion proteins in Pichia pastoris. Appl Microbiol Biot 2012;96:763–772. [CrossRef]

[29] Hohenblum H, Borth N, Mattanovich D. Assessing viability and cell-associated product of recombinant protein producing Pichia pastoris with flow cytometry. J Biotechnol 2003;102:281–290. [CrossRef]

[30] Kjeldsen T, Frost-Petterson A, Hach M. Secretory expression and characterization of insulin in Pichia pastoris. Biotechnol Appl Biochem 1999;29:79–86. [CrossRef]

[31] Nurdiani D, Nuruliawaty Utami H, Putro EW, Kusharyoto W. Enhancement in human insulin precursor secretion by Pichia pastoris through modification of expression conditions. Hayati J Biosci 2022;29:22–30. [CrossRef]

[32] Utami N, Nurdiani D, Hariyatun H, Putro EW, Patria FP, Kusharyoto W. Full-length versus truncated α-factor secretory signal sequences for expression of recombinant human insulin precursor in yeast Pichia pastoris: a comparison. J Genetic Eng Biotechnol 2023;21:3–15. [CrossRef]