HAYATI
Journal of Biosciences

Vol. 31 No. 2, March 2024 248-255
DOI:10.4308/hjb.31.2.248-255
ISSN: 1978-3019
EISSN: 2086-4094

Cloning and Extracellular Expression of Glargine in *Pichia pastoris*

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ARTICLE INFO

Article history:
Received February 6, 2023
Received in revised form November 1, 2023
Accepted November 8, 2023

KEYWORDS:
Diabetes,
glargine,
human insulin,
insulin analogues,
Pichia pastoris

ABSTRACT

Patients with diabetes mellitus increase significantly every year. The increasing number of people with diabetes mellitus results in increased insulin requirements. There are two types of insulin used for diabetes mellitus treatment: human insulin and insulin analogues. *Escherichia coli*, *Pichia pastoris*, *Saccharomyces cerevisiae*, or *Hansenula polymorpaha* has been used to produce human insulin and insulin analogues. *Pichia pastoris* can produce glargine in large quantities, and the insulin protein produced will be secreted outside the cell to facilitate the purification process. The advantage of glargine has a long working time of up to 24 hours. Hence, glargine is more effective because patients with diabetes receive glargine injections only once daily. The research started with cloning the glargine gene, transforming pPICZαA-G plasmid into *Pichia pastoris*, and testing glargine production. 20 recombinant *Pichia pastoris* colonies were selected and regenerated. Eight recombinant *Pichia pastoris* colonies were tested for glargine production, and six colonies were detected producing glargine by electrophoresis SDS-PAGE gel stained with Coomassie blue. This study aims to produce glargine using *Pichia pastoris* as an expression system capable of producing glargine extracellularly, thus simplifying the purification process.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disease characterized by high glucose levels in the blood and glucose intolerance due to insulin deficiency, insulin resistance, or both (Mane *et al.* 2012; Baynest 2015; Rachdaoui 2020; Wszola *et al.* 2021). Insulin is a hormone produced by pancreatic cells to control blood glucose by regulating the use and storage of glucose (Ahmad 2014; Gupta *et al.* 2015; Rahman *et al.* 2021). The leading cause of insulin deficiency is damage to pancreatic cells, which function to produce insulin (Gururaj Setty *et al.* 2016; Liu *et al.* 2018). In addition, insulin resistance also causes DM. Insulin resistance reduces the ability of insulin to stimulate glucose utilization or decreases the response of target cells, such as muscles, tissues, and the liver, to physiological insulin levels (Basukala *et al.* 2018; Gotham *et al.* 2018; Sorli 2014).

Two types of insulin for treating DM are human insulin and insulin analogues. Insulin analogues are minor modifications of human insulin through genetic engineering to alter the pharmacokinetics, absorption profile, and duration of action of insulin. Insulin analogues consist of fast-acting insulin analogues (aspart, lispro and glulisine) and long-acting insulin analogues (glargine, detemir, and degludec. Glargine, as long-acting insulin analogue has two peptide chains containing 53 amino acids. Chain A consists of 21 amino acids, and chain B consists of 32 amino acids. Glargine modifies human insulin by extending the C terminus of the B chain by adding two arginine residues after the B30 position and

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replacing asparagine with glycine at the A21 position (Sleigh 1998; Tibaldi 2008; Hilgenfeld *et al.* 2014; Gururaj Setty *et al.* 2016; Shen *et al.* 2019; Hirsch *et al.* 2020). Amino acid modification of glargine forms amorphous precipitates in the subcutaneous tissue at neutral pH, inhibiting absorption and prolonging action duration. Glargine has an onset of 90 minutes and a long working time of up to 24 hours. Hence, glargine is more effective because patients with diabetes receive glargine injections only once daily (Sleigh 1998; Tibaldi 2008; Hilgenfeld *et al.* 2014; Gururaj Setty *et al.* 2016; Shen *et al.* 2019; Hirsch *et al.* 2020). In the United States, from 2016 to 2020, the percentage of glargine use consistently accounted for about half (47.1%-53.0%) of total medications. In 2020, glargine accounted for 52.6% of the 4.9 million total (Sarkar *et al.* 2021).

Production of human insulin and insulin analogues can use *Escherichia coli, Saccharomyces cerevisiae, Pichia pastoris*, and *Hansenola polymorpha* as expression systems (Baeshen *et al.* 2014; Sandow *et al.* 2015; Hwang *et al.* 2016; Stubbs *et al.* 2017; Riggs 2021; Sekaringtyas *et al.* 2021). However, *Escherichia coli* is preferred because it produces large amounts of insulin with low production costs (Allen *et al.* 2019). Like *Escherichia coli, Pichia pastoris* grows fast, is easy to handle and manipulate genetically. This yeast produces fully folded recombinant proteins, their glycosylation of recombinant proteins is almost the same as mammalian cells, and they produce active proteins (Gurramkonda *et al.* 2010; Baeshen *et al.* 2014; Baghban *et al.* 2019). Another reason is that *Pichia pastoris* can produce large amounts of recombinant protein and be secreted, thus facilitating purification (Gurramkonda *et al.* 2010; Baeshen *et al.* 2014; Baghban *et al.* 2019). This study aims to produce glargine using *Pichia pastoris* as an expression system capable of producing glargine extracellularly, thus simplifying the purification process.

2. Materials and Methods

2.1. Plasmid and Strain

The glargine gene (U9162FE120-2) codon-optimized for *Pichia pastoris* was synthesized by GenScript and inserted into the pPICZαA plasmid (Figure 1). *Pichia pastoris* was from Invitrogen Co., Ltd.

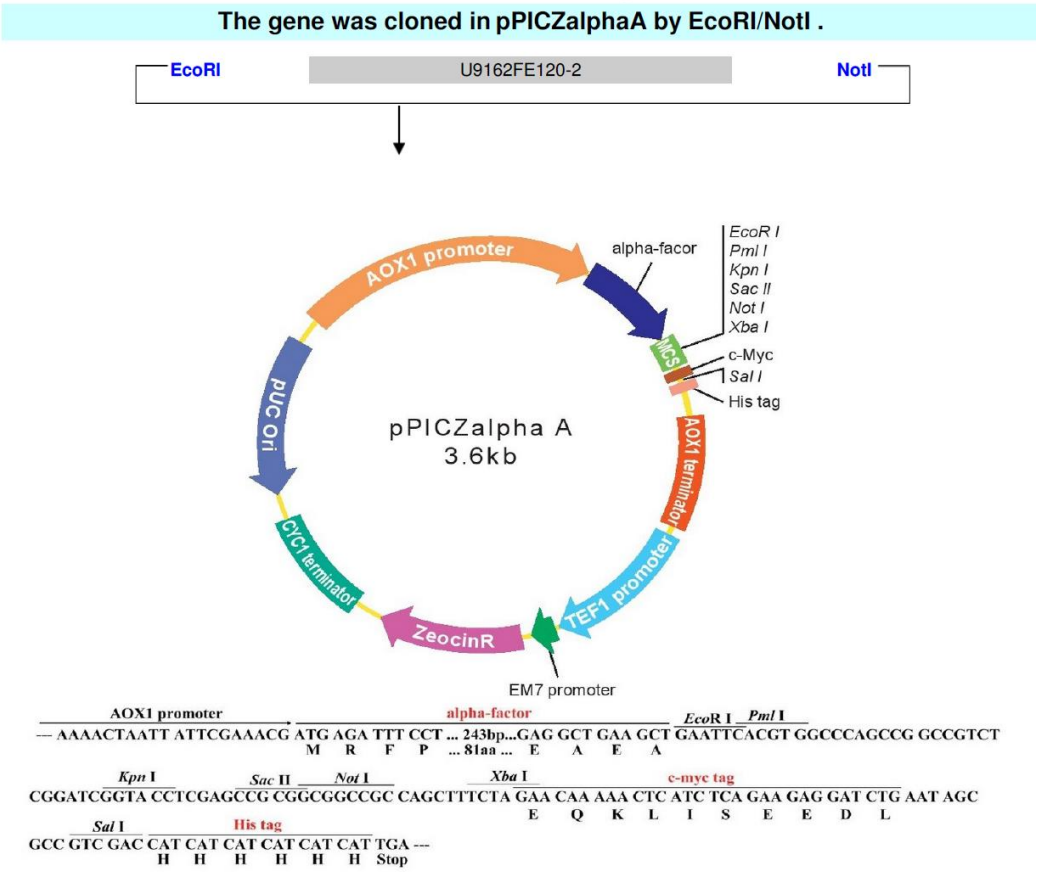


Figure 1. Map of pPICZαA-G plasmid

2.2. Isolation and Characterization of pPICZαA-G plasmid

Escherichia coli culture was grown overnight in Luria Bertani medium. 1.5 ml was taken, put into a 1.5 ml tube, and centrifuged (14,000-16,000 g, 1 minute, room temperature). The pellet was suspended in 200 µL PD1 Buffer, added 200 µL PD2 Buffer, and stirred slowly by inverting the tube ten times. The suspension was incubated (room temperature, 2 minutes), added 300 µL PD3 Buffer, was mixed immediately by inverting the tube ten times, and centrifuged (14,000-16,000 g, 3 minutes, room temperature). The supernatant was put into the PDH column in a 2 ml tube and centrifuged (14,000-16,000 g, 1 minute, room temperature). The supernatant in a 2 ml tube was discarded. The PDH column was put back into a 2 ml tube, 400 µL Buffer W1 was added, and centrifuged (14,000-16,000 g, 1 minute, room temperature). The supernatant in a 2 ml tube was discarded, the PDH column was put into a 2 ml tube, and 600 µL Wash Buffer was added to the PDH Column. The PDH column was centrifuged (14,000-16,000 g, 1 minute, room temperature). The supernatant in a 2 ml tube was discarded, and the PDH column was put back into a 2 ml tube. The PDH column was centrifuged (14,000-16,000 g, 3 minutes, room temperature). PDH column was transferred to a new 1.5 ml tube, added 50 µL Elution Buffer, incubated (2 minutes), and centrifuged (14,000-16,000 g, 2 minutes, room temperature). The isolated pPICZαA-G plasmid was digested by SacI, confirmed by electrophoresis and sequencing. pPICZαA-G plasmids were run on 2% agarose gel with 0.5X TAE buffer stained in 1 µL SYBR Green at 50 V for 50 minutes. The purified pPICZαA-G plasmid was sequenced to confirm the glargine gene in pPICZαA-G plasmid. The PCR product of *Pichia pastrois* genome was examined by DNA sequencing using F AOX1 primer (5´-GACTGGTTCCAATTGACAAGC-3´) to confirm the presence of the synthetic glargine gene in pPICZαA-G plasmid. R AOX1 primer (5´-GCAAATGGCATTCTGACATCC-3´) (Anonimous 2010) was also used to validate the presence of the synthetic glargine gene in pPICZαA-G plasmid. The sequencing reaction contained 8.0. µL reaction mix, 2 µL purified PCR product, 1 µL AOX1 primer, and 9 µL deionized water. The above mixture is loaded into the PCR machine for thermal cycling. The conditions of thermal cycling were 96°C (1 minute), 25 cycles at 96°C (10 seconds), 50°C (5 seconds) and 60°C (4 minutes). The resulting thermal cycling of sample was purified, loaded, sequenced with an Applied Biosystems machine, and analyzed.

2.3. Electroporation of pPICZαA-G plasmid into *Pichia pastoris*

Overnight *Pichia pastoris* 0.5 ml was grown in 100 ml of YPD till OD_{600} = 1.3 – 1.5. The culture was centrifuged (1,500 g, 5 minutes, 4°C). Pellets were resuspended by 500 ml sterile water (4°C) and centrifuged (1,500 g, 5 minutes, 4°C). Pellets were resuspended by 250 mL sterile water (4°C) and centrifuged (1,500 g, 5 minutes, 4°C). Pellets were resuspended by 20 ml 1 M sorbitol (4°C) and centrifuged (1,500 g, 5 minutes, 4°C). Pellets were suspended by 1.5 ml 1 M sorbitol (4°C). 80 µL of the suspension was added to 5 µg of linearized pPICZαA-G plasmid, placed in a sterile cuvette, incubated (94°C, 5 minutes), and pulsed by an electroporator (1,500 V and 5 ms). The suspension in the cuvette was added 1 ml of 1 M sorbitol (4°C). The suspension was transferred by a sterile 15-ml tube and incubated at 30°C without shaking for 1-2 hours. 50 µL of suspension were grown, labeled YPDS medium with 100 µg/ml Zeocin (selected medium), incubated for 3–10 days at 30°C. 10–20 colonies were selected and grown on fresh YPD or YPDS medium with 100 µg/ml Zeocin.

2.4. Characterization of recombinant *Pichia pastoris*

Recombinant *Pichia pastoris* was characterized by PCR and DNA sequencing. PCR amplified the glargine gene in the genome of *Pichia pastoris*. The PCR reaction mixture included the selected genome of recombinant *Pichia pastoris*, 12.5 µL GoTag Green Master Mix, 2 µL forward primer 10 µM, 2 µL reverse primer 10 µM, 1 µL DNA template 50 ng/µL, and 7.5 µL PCR-grade water. The PCR cycling: 2 minutes of denaturation at 95°C, 30 cycles of denaturation at 95°C for 30 seconds, 30 seconds of annealing at 52°C, and 30 seconds of extension at 72°C. A final extension step at 72°C for 5 minutes. PCR products were run on 2% agarose gel with 0.5X TAE buffer stained in 1 µL SYBR Green at 50 V for 50 minutes and sequenced to confirm glargine gene was integrated into the genome of *Pichia pastoris*. The DNA sequence of the synthetic glargine gene from Genscript: TTCGTTAACCAACA-TT TGTGTGGTTCTCATTTGGTTGAAGCATTGTATTTGGTT TGTGGTGAAAGAGGTTTCTTTTATACTCCAAAAACAAG AAGAGGTATCGTTGAACAATGTTGTGTACTTCTTGTTC ATTGTATCAATTAGAAAATTACTGTGGTTAA. The PCR product of *Pichia pastoris* genome was examined by DNA sequencing using primer specific for glargine gene 5'F (5'-ACCAACATTTGTGTGGTTCTCA-3') to confirm the presence of the synthetic glargine gene

in the recombinant *Pichia pastoris* genome. Primer R (5'-TGTTCAACGATACCTCTTCTTGT-3') was also used to validate the presence of the partial synthetic glargine gene in recombinant *Pichia pastoris*. The DNA sequencing procedure to detect the glargine gene in the genome is the same as procedure 2.2.

2.5. Production of Glargine

Eight colonies of recombinant *Pichia pastoris* were inoculated in each Erlenmeyer containing 10 ml BMGY at 30°C in an incubator shaker (250 rpm) until OD_{600} = 2-6. The cultures of recombinant Pichia pastoris were centrifuged (1,500–3,000 g, 5 minutes, room temperature). Pellets (OD_{600} > 1.0) were resuspended in BMMY medium and grew (30°C) in a shaker incubator (250 rpm) overnight. The concentration of 0.5% methanol is maintained by adding methanol every 24 hours. After 72 hours of incubation, the cultures were harvested, centrifuged (1,500–3,000 g, 5 minutes, room temperature) and glargine in the supernatant was characterized by SDS-PAGE gel electrophoresis. The supernatant was analyzed by SDS-PAGE 15% polyacrylamide gel-electrophoresis and tricine buffer system (Haider *et al.* 2012). 20 µL of sample was mixed with 20 µL of sample buffer and heated for 10 minutes. 20 µL of the above mixture was loaded into the gel and electrophoresed. Electrophoresis results were stained with Coomassie blue.

3. Results

Result of pPICZαA-G plasmid isolation was electrophoresed in 1% agarose gel. The size of the pPICZαA-G plasmid was about 3800 bp (Figure 2), confirming the existence of glargine gene in pPICZαA-G plasmid by sequencing. First sequencing used F AOX1 primer (5´-GACTGGTTCCAATTGACAAGC-3´) and second sequencing used R AOX1 primer (5´-GCAAATGGCATTCTGACATCC-3´). The sequencing results with F AOX1 primer and R AOX1 primer were combined and aligned with optimized glargine gene. The DNA sequencing showed that DNA sequence from pPICZαA-G plasmid was 100% similar to the DNA sequence of synthetized glargine gene (Figure 3).

The recombinant *Pichia pastoris* grew on selected medium after 3 days of incubation but *Pichia pastoris* without pPICZαA-G plasmid did not grow on selected medium (Figure 4). 20 colonies of recombinant *Pichia pastoris* were selected and grew on selected medium

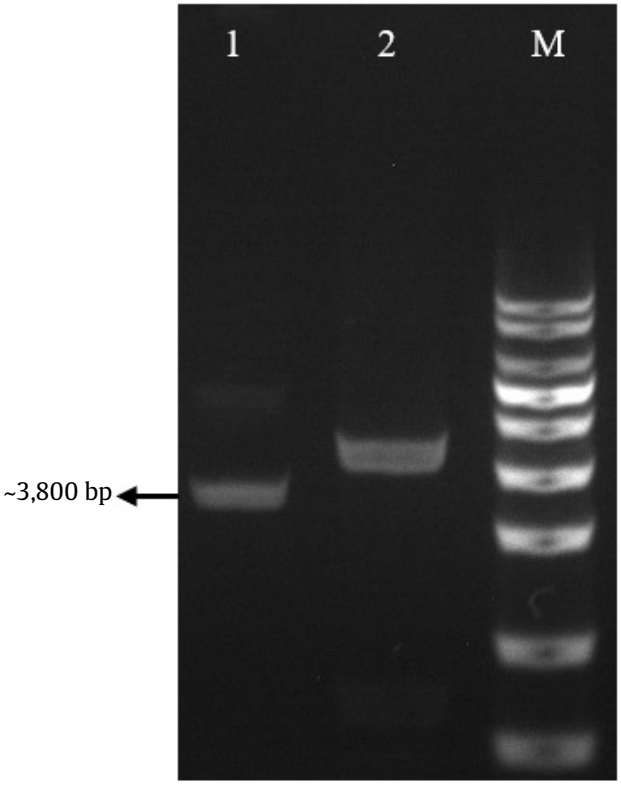


Figure 2. Agarose electrophoresis of pPICZαA-G Plasmid. 1. Linearized pPICZαA-G plasmid; 2. pPICZαA-G plasmid; M. 10 kb DNA Ladder (GenScript M101O, USA)

agar (Figure 5). In this experiment, 20 colonies were randomly selected from tens to hundreds of transformed *Pichia pastoris* colonies in a petri dish for experimental reproducibility and representativeness of the sample for statistical significance.

The PCR examination of the recombinant *Pichia pastoris* using specific primers for the synthetic glargine gene resulted in the amplification of a single band, confirming the successful integration of the pPICZαA-G plasmid containing the synthetic glargine gene into *Pichia pastoris* genome. The PCR validates that the recombinant strain contains the desired gene, with the PCR product containing the desired gene being 102 base pairs long (Figure 6). DNA sequencing results show a complete match (100% sequence identity) between the PCR product and the synthetic glargine gene sequence (Figure 7).

Pichia pastoris produced extracellular glargine in broth of fermentation medium. The size of glargine was about 10 KDa (Figure 8).

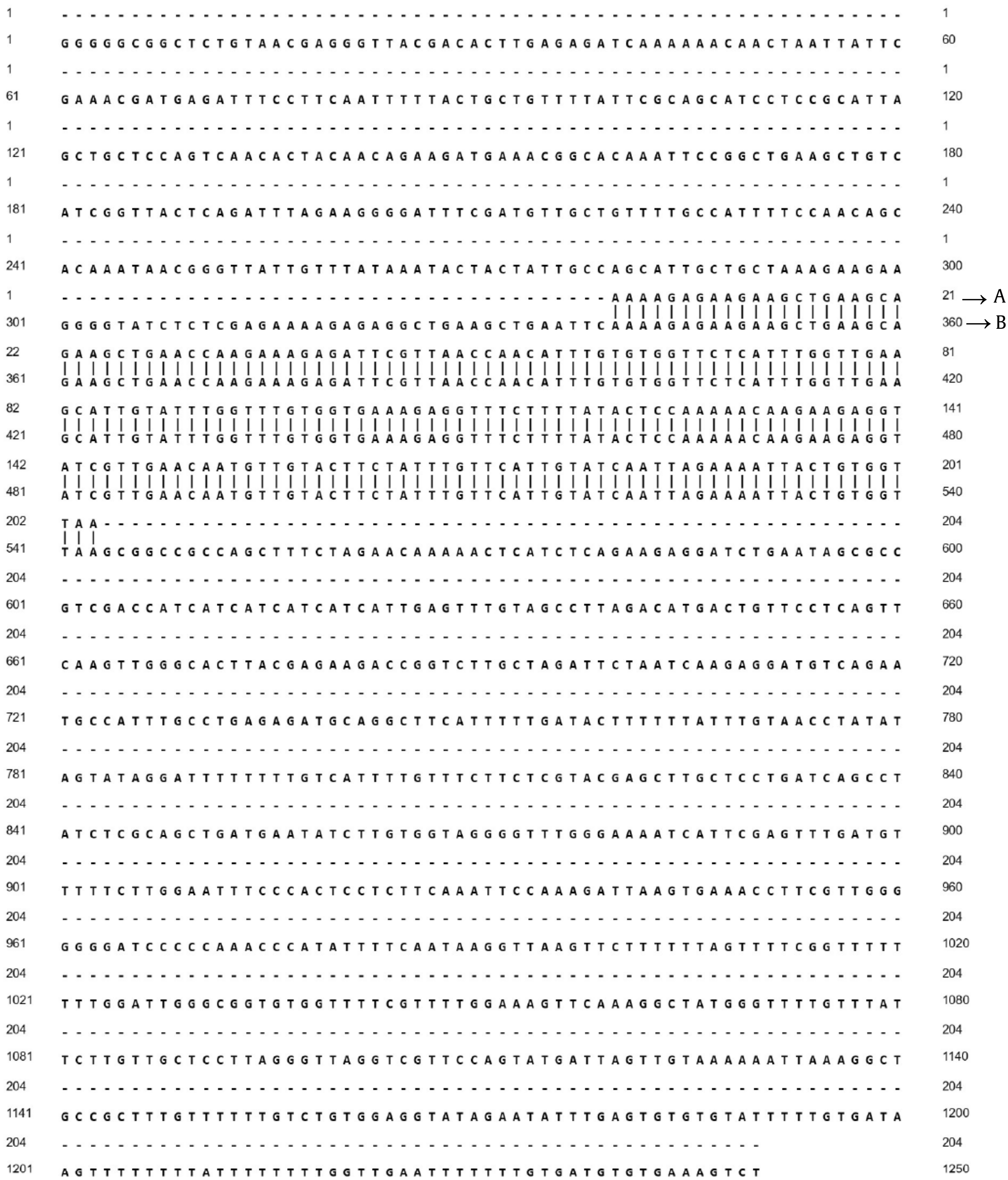
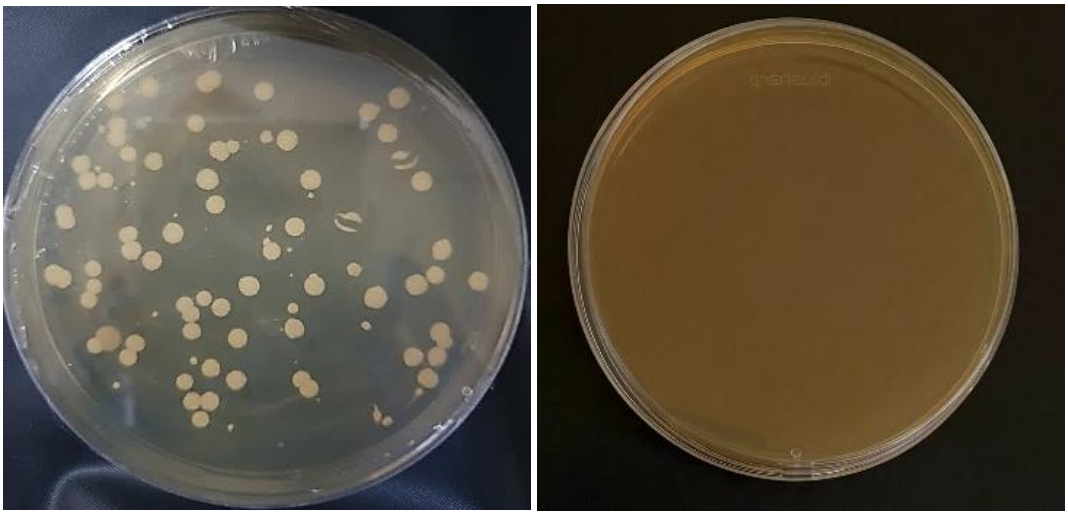


Figure 3. The sequence alignment from synthetized glargine gene (A) and PCR product of pPICZαA-G plasmid (B)



A B

Figure 4. (A) The recombinant *Pichia pastoris*, (B) negative control plate (*Pichia pastoris* without plasmid)

Figure 5. The recombinant *Pichia pastoris* on the selected medium

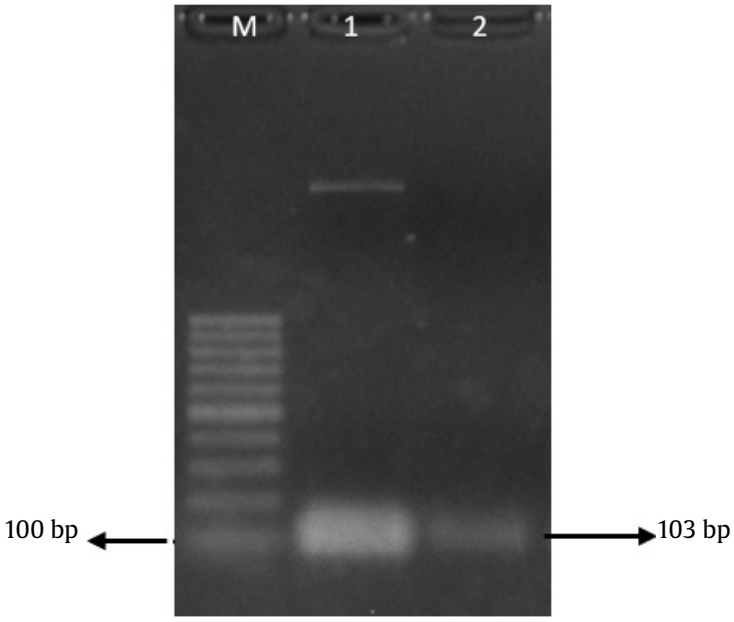


Figure 6. Agarose electrophoresis of PCR product. M. 100 bp Ladder (SMOBIO DM2100, Taiwan); 1. Partial of glargine gene from pPICZαA-G plasmid (Positive Control); 2. Partial of glargine gene from genome of *Pichia pastoris*

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1    ----------------------------------------TTCGTTAACCAACATTTGTGTGGTTCTCA   29 → A
1    AGCTGAAGCAGAAGCTGAACCAAGAAAGAGATTCGTTAACCAACATTTGTGTGGTTCTCA   60 → B
30   TTTGGTTGAAGCATTGTATTTGGTTTGTGGTGAAAGAGGTTTCTTTTATACTCCAAAAAC   89
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
61   TTTGGTTGAAGCATTGTATTTGGTTTGTGGTGAAAGAGGTTTC----------------   103
90   AAGAAGAGGTATCGTTGAACAATGTTGTACTTCTATTTGTTCATTGTATCAATTAGAAAA   149
103  ----------------------------------------------------------   103
150  TTACTGTGGTTAA   162
103  -------------   103
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Figure 7. The sequence alignment from PCR product of genome *P. pastoris* (A) and synthetized glargine gene (B)

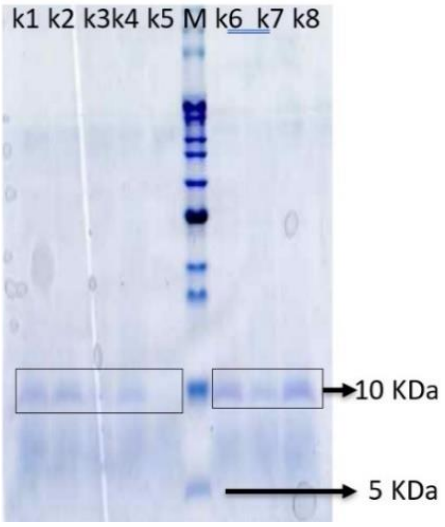

Figure 8. SDS-PAGE of glargine in SDS-PAGE gel. K1-K8 = glargine from supernatant of *Pichia pastoris* culture (colony 1-8) post methanol induction (72 h) M. Polypeptide molecular weight standards (SMOBIO PM2700, Taiwan)

4. Discussion

Digestion with a specific enzyme was used to confirm the inserted gene in a plasmid and determine the plasmid's molecular weight size. pPICZαA-G plasmid can be digested by PstI, ClaI, PmLI, EcoRI, BsmBI, SfiI, Asp718, XhoI, KpnI, SacII, XbaI,NotI, BamHI, BglI, PmeI, BstX, and SacI (Figure 1). In this study, SacI was used to digest pPICZαA-G plasmid. This plasmid has a unique site so that linear pPICZαA-G plasmid integrates into AOX1 locus on *Pichia pastoris* genome. In addition to SacI, PmeI and BstXI digest the pPICZαA-G plasmid to produce a linear pPICZαA-G plasmid with a unique site that can integrate at the AOX1 locus of *Pichia pastoris* genome. The size of linearized pPICZαA-G plasmid from result of isolation was about 3,800 bp (Figure 2). Further confirmation was carried out by DNA sequencing of the glargine gene in pPICZαA-G plasmid using AOX1 primer. Sequencing results showed that the glargine gene was in pPICZαA-G plasmid (Figure 3).

Zeocin is a bleomycin or phleomycin class of antibiotics isolated from *Streptomyces verticillus* (Anonimous 2010). The mechanism of action of zeocin is to bind and cut DNA so that the cell dies. Zeocin is toxic to bacteria, fungi (including yeast), plants and mammalian cells. The pPICZαA-G plasmid has a resistance gene to zeocin (Figure 1). The zeocin resistance gene (sh ble gene) produces a protein that binds to zeocin so that zeocin is inactive (Gatignol *et al.* 1988; Trastoy *et al.* 2005). Recombinant *Pichia pastoris* contains the plasmid pPICZαA-G, so it can grow on YPD selection media containing zeocin (Figures 4 and 5).

The recombinant *Pichia pastoris* were examined for the presence of glargine gene by PCR. The genome of recombinant *Pichia pastoris* has the same glargine gene as the plasmid pPICZαA-G (Figure 6). The DNA sequencing of the PCR product from the recombinant *Pichia pastoris* genome was 100% identical to the synthesized glargine gene (Figure 7).

Recombinant *Pichia pastoris* can produce recombinant proteins intracellularly or extracellularly. In this study, recombinant *Pichia pastoris* has a signal peptide factor derived from *Saccharomyces cerevisiae* (from pPICZαA-G) so that glargine is secreted out of cells of recombinant *Pichia pastoris.* Eight recombinant *Pichia pastoris* were tested for glargine production. Six colonies of recombinant *Pichia pastoris* (k1, k2, k4, k6-k8) produced glargine, which was about 10 KDa (Figure 8). For further confirmation, the glargine needs to be analyzed by Western Blot. Western Blot involves separating the protein, transferring it to a membrane, and detecting glargine as a target protein by a specific antibody. Western blot can provide accurate, specific, and repeatable results (Meftahi *et al.* 2021).

Acknowledgements

We thank the National Research and Innovation Agency (BRIN) and Educational Fund Management Institution (LPDP) for providing financial support. This work was also supported by Padjadjaran University.

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