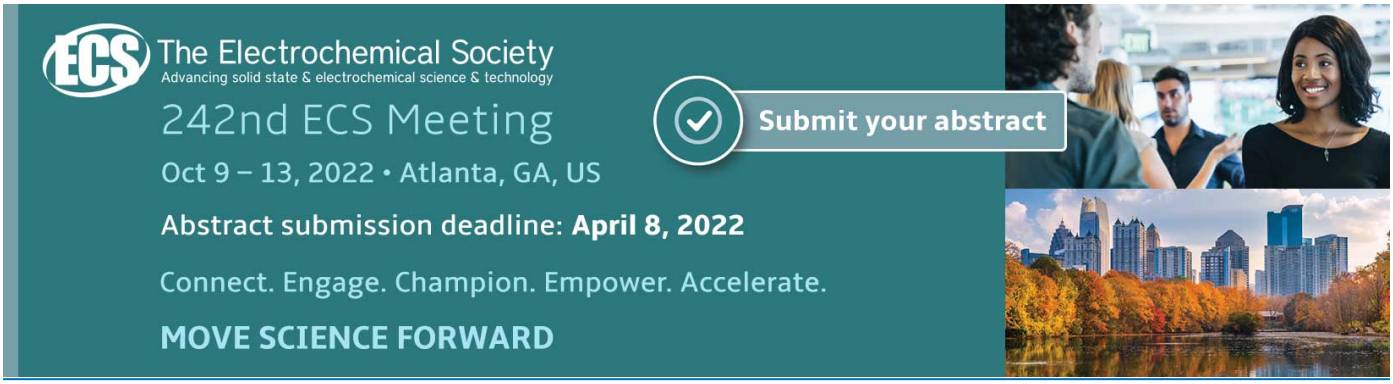
Transformation and characterization of human insulin precursor gene in *Pichiapastoris* X-33



Transformation and characterization of human insulin precursor gene in *Pichia pastoris* X-33

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Abstract. The case of diabetes increases significantly and has been projected to reach 592 million people in 2035. Consequently, the necessity of insulin will rise manifold and an efficient production system for insulin production is required to meet the market demands. The human insulin precursors that enzymatically converted to human insulin can be produced using *Escherichia coli*, *Saccharomyces cerevisiae*, or *Pichia pastoris*. In this study, *Pichia pastoris* is used for production human insulin precursor because the resulting recombinant protein can be folded accordingly and secreted to the external environment of the cell that simplifies the purification process. The study was initiated with the insertion of a synthetic gene of human insulin precursor into the pPICZαA to create recombinant pPICZαA-IP plasmid. The recombinant plasmid was transformed into *Escherichia coli* Top10 which then isolated and digested by the *SacI* enzyme. The linearize pPICZαA-IP plasmid was transfected into *Pichia pastoris* X-33 by electroporator. The result of transformation process, a total of 20 colonies of *P. pastoris* X-33 were selected and inoculated in YPD agar medium containing Zeocin. The two colonies of *P. pastoris* were characterized by PCR and sequencing showed that the recombinant pPICZαA-IP plasmid was successfully integrated into selected colonies of *P. pastoris*.
Keywords: *Escherichia coli*, *Pichia pastoris*, human insulin precursor, pPICZαA-IP plasmid, transformation

1. Introduction

Over the half last century, the insulin demand increases considerably at the same time with the prevalence of diabetic patients every year. According to the WHO, the number of people suffer from diabetes mellitus is predicted to grow to 592 million in 2035 and this upward trend led diabetes to become one of the deadly non-communicable diseases in the future [1]. The International Diabetes Federation (IDF) estimated the annual global health care costs for diabetes at $850 billion in 2017 [2]. Consequently, the requirement of insulin rises significantly, and an efficient expression system is required for the production of insulin to fulfil the market demand.

Currently, the production of recombinant human insulin uses *Escherichia coli*, *Saccharomyces cerevisiae*, and *Pichia pastoris* for therapeutic use in humans. Even though *E. coli* is more widely used to produce recombinant proteins due to the high growth rate, simple to handle, fast-growing and relatively inexpensive. However, *E. coli* has disadvantages such as the formation of inclusion bodies, endotoxin contamination, and poor secretion [3]. On the other hand, the *S. cerevisiae* system currently gives the most

world supply of insulin because the insulin precursor (IP) is directly produced extracellularly in the correct conformation [4]. However, the human insulin produced in *S. cerevisiae* needs the multiple steps, up to fifteen steps of purification process and standard recovery [5].

Pichia pastoris becomes the promising yeast host for recombinant protein expression because of its ability to reach high cell densities by its robust methanol-inducible alcohol oxidase 1 (AOX1) promoter and simple development process, which provide to high quality and a high percentage of recombinant proteins are both intracellular and secretory [3]. Moreover, compared to *S. cerevisiae, P. pastoris* secretes small amounts of the native protein, thereby simplifying the purification process of secreted recombinant proteins and produces a recombinant protein that is not hyperglycosylated [3, 6].

Several studies have used *P. pastoris* for IP production. Nurdiani et al. [6] used pD902 plasmid as a vector to insulin precursor production in *P. pastoris* [6]. pPICZαA plasmid used by Gurramkonda et al. [7] and Baeshen et al. [8] for insulin precursor production in *P. pastoris*. Polez et al. [5] and Abdulkareem [9] used pPIC9k plasmid for inserting insulin precursor gene. The main difference with previous studies was using the synthetic insulin precursor gene resulting from codon optimization for expression in *P. pastoris*, so it is expected to produce the highest possible value expression level of IP.

In this study, we used a synthetic gene of human IP, which was inserted into pPICZαA plasmid. Then the electroporator was used to transform the linearized pPICZαA-IP plasmids into *Pichia pastoris* X-33. The colonies of *P. pastoris* contained pPICZαA-IP plasmids that were characterized by polymerase chain reaction (PCR), gel agarose electrophoresis, and sequencing.

2. Materials and methods

2.1. Codon optimization of IP gene

OptimumGene™ codon optimization analysis was used for codon optimization of IP gene for expression in *Pichia pastoris*. The parameters of optimization are codon usage bias, GC content, CpG dinucleotides content, mRNA secondary structure, cryptic splicing sites, premature plyA sites, internal chi sites and ribosomal binding sites, negative CpG islands, RNA instability motif, repeat sequences, and restriction sites.

2.2. Strains and plasmids

Pichia pastoris yeast strain X-33 and the pPICZαA plasmid were purchased from Invitrogen Co., Ltd. The human insulin precursor gene in pPICZαA plasmid and were synthesized by GenScript. *Escherichia coli* Top10 was used to amplify these plasmids.

2.3. Transformation of pPICZαA-IP plasmid into E. coli Top10

Before subsequent transformation into *Pichia pastoris* X-33, the pPICZαA-IP plasmid was transformed into *E. coli* Top10 by calcium chloride heat shock method [10]. The pPICZαA-IP plasmid was added into the competent cells and incubated in a cold box containing ice for half an hour. *E. coli* Top10 competent and plasmid in the tube were incubated on 42°C for one and a half minutes (heat shock) and incubated in a cold box containing ice for 5 minutes. A pre-warmed LB low salt medium was added into cells and incubated in a shaker incubator at 37°C with a rotation speed of 200 rpm for 60 minutes. Cells of *E. coli* Top10 were centrifuged at 4000 rpm for 5 minutes at room temperature. The cell pellets were resuspended with 200 µL LB low salt and spread in LB low salt plates containing Zeocin™ (100 µg/mL). The plates were covered with aluminium foil and cultured at 37°C for 18 hours. The single colony of transformant was observed and selected for plasmid isolation.

2.4. Isolation of pPICZαA-IP plasmid

Plasmid DNA of pPICZαA-IP was isolated using the Presto™ Mini Plasmid Kit [11]. The single transformant of *E. coli Top10* was grown in 10 mL LB low salt medium containing 100 µg/mL Zeocin and cultured overnight at 37°C with a rotation speed of 200 rpm. The cultured bacterial cells of *E. coli* Top10 were centrifuged at a rotation speed of 14-16,000 × *g* for 60 seconds at room temperature then

discarded the supernatant. The cell pellet was entirely resuspended by adding 200 µL of PD1 Buffer using a vortex. The resuspended sample was mixed gently with 200 µL of PD2 buffer by inverting the sample in the tube ten times for cell lysis then allowed to stand at room temperature for 120 seconds to ensure the lysate is homogeneous. PD3 Buffer (300 µL) for neutralization was added and mixed immediately by inverting the sample in the tube ten times. Cells were centrifuged at 16-20,000 × *g* for 5-8 minutes at room temperature. During the centrifugation, a PDH Column was placed in a 2 mL collection tube. The supernatant was added completely to the PDH Column and centrifuged at 14-16,000 × *g* for 30 seconds at room temperature. Then the flow-through was thrown away, and the PDH column was placed back in the 2 mL collection tube. Wash buffer (600 µL) was added into the PDH Column and centrifuged at 14-16,000 × *g* for half a minute at room temperature. The flow through was thrown away and the PDH Column was placed back in the 2 mL Collection Tube. To dry the matrix column, the collection tube with PDH Column was centrifuged at 14-16,000 × *g* for 3 minutes at room temperature then the dried PDH Column was transferred to a new 1.5 mL microcentrifuge tube. 50 µL of Elution Buffer was added to the column matrix's center and incubated for at least 2 minutes to allow Elution Buffer to be completely absorbed. The tube with column matrix was centrifuged at 14-16,000 × *g* for 120 seconds at room temperature to elute the purified plasmid. The isolated plasmid was digested using *SacI* restriction enzyme followed by the confirmation of the linearized recombinant plasmid using electrophoresis on agarose gel.

2.5. Transformation of pPICZαA-IP into Pichia pastoris

The electroporator was used to transform the linearized pPICZαA-IP plasmids into *Pichia pastoris* X-33 [12]. In order to obtain optimum integration of the recombinant construct into *Pichia* genome, approximately 5–10 µg of pPICZαA-IP plasmid DNA was linearized with *SacI*. The *SacI* is an enzyme to cut one time in the 5´ AOX1 region to linearize either pPICZ or pPICZα. Agarose gel electrophoresis was used to check the linearization of the plasmid by using a small aliquot of the plasmid digestion mix. The transformation process was initiated by preparing the competent cells of *P. pastoris* X-33. *P. pastoris* X-33 strain was cultured into 2.5 mL of YPD medium at 30°C, 250 rpm for overnight. The overnight culture was inoculated into 250 mL of YPD medium in 1000 mL Erlenmeyer and allowed to grow overnight again at 30°C, 250 rpm to reach an OD600 = 1.3 – 1.5. The cells were harvested by centrifugation at 1,500 × *g* for 5 minutes at 4°C and the pellet was resuspended with 250 mL of ice-cold, sterile water. The cells were centrifuged at 1,500 × *g* for 5 minutes at 4°C and the pellet was resuspended with 125 mL of ice-cold, sterile water. The cells were centrifuged again at 1,500 × *g* for 5 minutes at 4°C and the pellet was resuspended with 10 mL of ice-cold 1 M sorbitol. After that, the cells were centrifuged at 1,500 × *g* for 5 minutes at 4°C and the pellet was resuspended in 1 mL of ice-cold sorbitol 1 M for a final volume of approximately 1.5 mL. The competent cells of *P. pastoris* X-33 should be kept on ice and used immediately. The competent cells (80 µL) were mixed with 5 – 10 µg of linearized plasmid DNA (in 5 – 10 µL sterile water) and transferred to an ice-cold 0.2 cm electroporation cuvette as well as incubated on ice for 2 minutes. The cells were pulsed using the manufacturer's instructions for *S. cerevisiae* (Gene Pulser XcellTM Electroporation Systems, Bio-Rad) with a charging voltage of 1500 V and time constant 5 ms. Immediately, 1 mL of 1:1 ice-cold 1 M sorbitol and YPD medium was added to the cuvette. The cuvette contents were transferred to a sterile 50 mL tube and incubated at 30°C, 225 rpm for 90 minutes. The tube was centrifuged at 3,000 × *g* for 5 minutes to pellet the cells. The pellet was resuspended in 200 µL of YPD medium and spread 40 µL each on separate, labelled YPD plates containing 100 µg/mL zeocin. The plates were incubated at 30°C for 3 days until colonies grew as many as 20 single colonies were selected and grown on YPD plates containing 100 µg/mL zeocin.

2.6. Characterization of selected Pichia pastoris colonies

The *Pichia pastoris* transformant colonies were screened on YPD medium containing a gradually rising concentration of zeocin from 100 µg/mL to 2000 µg/mL which aims to identify the multicopy clones. Two colonies of *P. pastoris* were selected and characterized by polymerase chain reaction (PCR) and

sequencing. Confirmation of the integration of linearized vector pPICZαA-IP into *Pichia pastoris* genome was performed by PCR. IP gene was amplified by KOD-Plus- (Toyobo, Japan) with a specific primer for IP amplification. The application of IP gene with a pair of primers specific for IP gene was carried out in a 50 µL reagent mixture (total reaction volume) containing the selected colony of *Pichia pastoris*, 5 µL 10x Buffer for KOD-Plus-, 2 mM dNTPs, 25 mM $MgSO_4$, 0.3 µM of each primer, x µL PCR grade water, and 1.0 U KOD-Plus- under the following PCR cycling conditions: 94°C for 2 min; then 30 cycles at 94°C for 15 sec, 52°C for 30 sec and 68°C for 30 sec; followed by last cycle at 12°C for ∞. The PCR products were electrophoresed on 1.5% agarose gel and sequencing analysis with 5' Pichia primer (5'-gactggttccaattgacaagc-3') to confirm of IP gene into pPICZαA-IP plasmid and F (5'-agctgaagcagaagctgaacca-3') for confirmation partial IP gene into *P. pastoris* recombinant clones.

3. Results

3.1. Codon optimization of IP gene

A wide variety of factors regulate and influence gene expression levels. In this codon optimization, as many of these factors (codon usage bias, GC content, CpG dinucleotides content, mRNA secondary structure, cryptic splicing sites, premature plyA sites, internal chi sites and ribosomal binding sites, negative CpG islands, RNA instability motif, repeat sequences, and restriction sites) are used as possible to produce the highest possible value expression level of IP. The result of codon optimization obtained 45 codons from 66 codons of IP gene (figure 1).

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Optimized 1    AAAAGAGAAGAAGCTGAAGCAGAAGCTGAACCAAAGTTCGTTAACCAACATTTGTGTGGT
Original  1    AAGAGAGAAGAAGCTGAAGCTGAAGCTGAACCAAAGTTTGTGAACCAACACCTGTGCGGC
Optimized 61   TCTCATTTGGTTGAAGCATTGTATTTGGTTTGTGGTGAAAGAGGTTTCTTTTATACTCCA
Original  61   TCACACCTGGTGGAAGCTCTCTACCTAGTGTGCGGGGAACGAGGCTTCTTCTACACACCC
Optimized 121  AAAGCTGCAAAAGGTATCGTTGAACAATGTTGTACATCTATTTGTTCATTGTATCAATTA
Original  121  AAGGCTGCTAAGGGCATTGTGGAACAATGCTGTACCAGCATCTGCTCCCTCTACCAGCTG
Optimized 181  GAAAATTACTGTAATTAA
Original  181  GAGAACTACTGCAACTAA
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Figure 1. Result of codon optimization of IP gene for expression in *Pichia pastoris*.

3.2. Transformation of pPICZαA-IP plasmid into E. coli Top10 and isolation of pPICZαA-IP plasmid

The human IP gene cassette was inserted into pPICZαA plasmid (figure 2). The IP cassette consisted of *S. cerevisiae* truncated α-factor secretory signal located at 5' terminus of the IP expression cassette which contains Kex2 cleavage site (LEKR) at its carboxy-terminal part, followed by a spacer peptide (KREEAEAEAEPK) to increase efficiency Kex2 processing and secretion [13]. The IP encoding gene was constructed in *Pichia* integrative vector pPICZαA-IP with 198 bp. The pPICZαA-IP plasmid was transformed into *E. coli* Top10 and the transformant of *E. coli* harbouring pPICZαA-IP plasmid was digested by *SacI* restriction enzyme which resulted in one band size of ~3800 bp (figure 3).

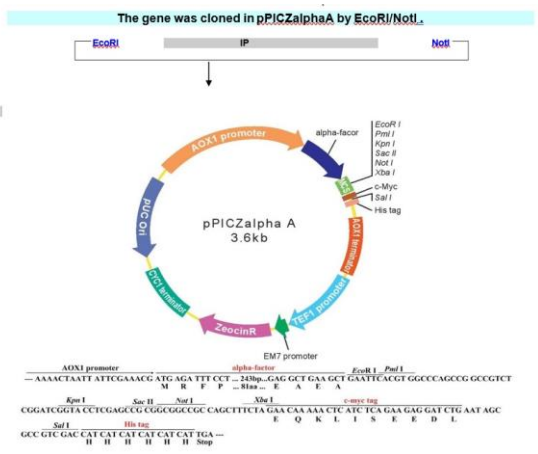

Figure 2. Map of the pPICZαA-IP plasmid. The PI gene was inserted in EcoRI and NotI sites in MCS region of pPICZαA-IP plasmid [14]

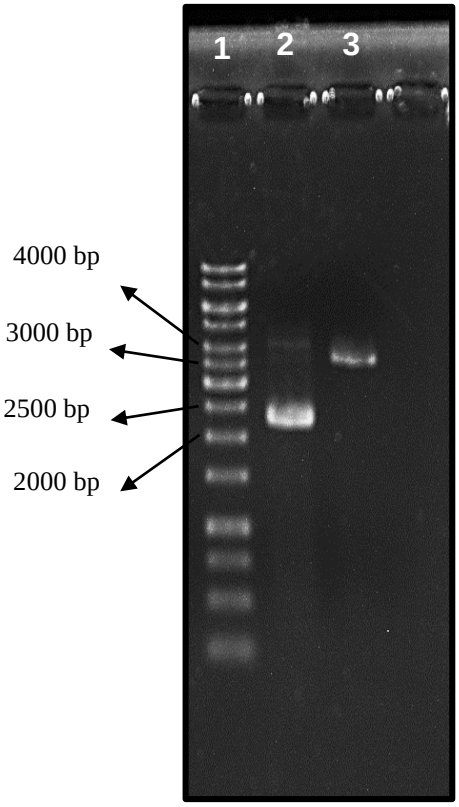

Figure 3. Electophoregram of the pPICZαA-IP digested by *SacI* enzyme: 1) 1kb Ladder; 2) pPICZαA-IP; 3) pPICZαA-IP digested by *SacI*.

The concentration level and purity of DNA pPICZαA-IP plasmid was measured using BioDrop. The plasmid DNA concentration was determined by measuring the absorbance at a wavelength of 260 nm. The measurement at a wavelength of 280 nm is also required to determine the presence of protein contamination, as well as at a wavelength of 320 nm to detect other contaminants. The results of absorbance measurement can be observed at table 1. The alignment process was carried out between the sequences pPICZαA-IP plasmid and the optimized Insulin sequences (figure 4).

Table 1. The results of the absorbance measurement of pPICZαA-IP plasmid using Biodrop

Parameter	Result
A230	0.130 A
A260	0.260 A
A280	0.134 A
A320	0.002 A
A260/A230	2.012
A260/A280	1.951
Concentration of DNA pPICZαA-IP plasmid	258.5 ng/µL
Total number of DNA pPICZαA-IP plasmid	12,925 ng

3.3. Transformation of linearized pPICZαA-IP plasmid into P. pastoris X-33

The *SacI* linearized pPICZαA-IP plasmid was transformed into *P. pastoris* X-33 electrocompetent cells to facilitate single crossover recombination at the *AOX1* locus. Transformants were spread in YPD medium containing zeocin (100 µg/mL). After 3 days of incubation, transformant colonies grew on the sample plate (figure 5). After this step, 20 single transformant colonies on the sample plates were selected and inoculated on YPD plates containing100 µg/mL zeocin (figure 6).

```
                      340       350       360       370       380       390
IP plasmid     AAAAGAGAAGAAGCTGAAGCAGAAGCTGAACCAAAGTTCGTTAACCAACATTTGTGTGGT
               ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
IP gene        AAAAGAGAAGAAGCTGAAGCAGAAGCTGAACCAAAGTTCGTTAACCAACATTTGTGTGGT
                       10        20        30        40        50        60

                      400       410       420       430       440       450
IP plasmid     TCTCATTTGGTTGAAGCATTGTATTTGGTTTGTGGTGAAAGAGGTTTCTTTTATACTCCA
               ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
IP gene        TCTCATTTGGTTGAAGCATTGTATTTGGTTTGTGGTGAAAGAGGTTTCTTTTATACTCCA
                       70        80        90       100       110       120

                      460       470       480       490       500       510
IP plasmid     AAAGCTGCAAAAGGTATCGTTGAACAATGTTGTACATCTATTTGTTCATTGTATCAATTA
               ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
IP gene        AAAGCTGCAAAAGGTATCGTTGAACAATGTTGTACATCTATTTGTTCATTGTATCAATTA
                      130       140       150       160       170       180

                      520
IP plasmid     GAAAATTACTGTAATTAA
               :::::::::::::::::
IP gene        GAAAATTACTGTAATTAA
                      190
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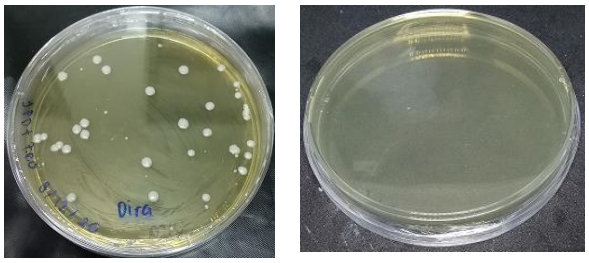
Figure 4. The result of sequence alignment from isolated pPICZαA-IP plasmid and Optimized IP gene in pPICZαA (synthetic gene).



a) b)

Figure 5. The result of the transformation of *P. pastoris* X-33 in YPD plate containing 100 μg/mL zeocin: a) Transformant of *P. pastoris* X-33 colonies on the sample plate; b) Negative control plate (competent cells of *P. pastoris* X-33 without plasmid).

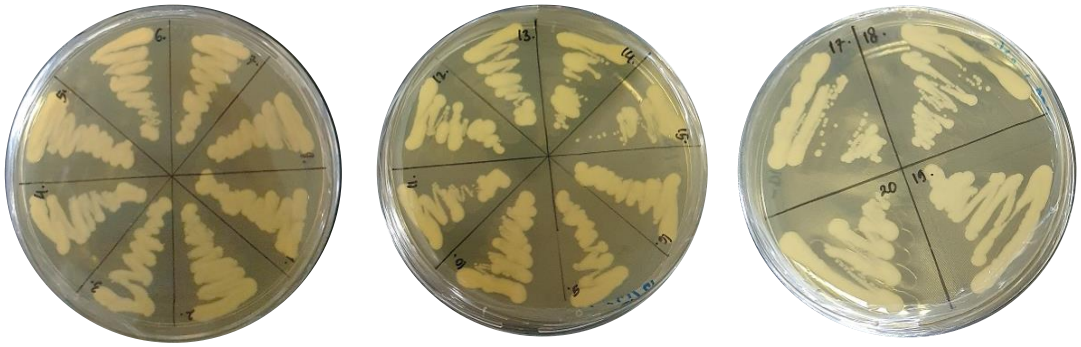


Figure 6. The selected 20 transformants of *P. pastoris* on the YPD plates containing 100 μg/mL zeocin.

3.4. Characterization of selected Pichia pastoris colonies

The transformant colonies were cultured in YPD plates containing the various concentrations of zeocin from 100 μg/mL to 2000 μg/mL to identify accepted multicopy recombinant strains. The result shows

that in figure 7, all of 20 transformant colonies were able to grow on YPD plates containingzeocin up to the highest zeocin concentration (2000 µg/mL).

PCR with specific primer pair for IP gene was used to confirm the integration of the IP gene into *P. pastoris* genome (figure 8). Colony PCR of two *P. pastoris* transformants (no. 1 and 2) resulted in only one band DNA of IP gene (~120 bp).

The sequencing results showed that the PCR product of *P. pastoris* genome (~120 bp) was 100% identical to the sequence of pPICZαA-IP plasmid (figure 9). IP-PCR product didn't show the whole inserted IP gene, and it only showed a part of targeted sequence according to the primer used.

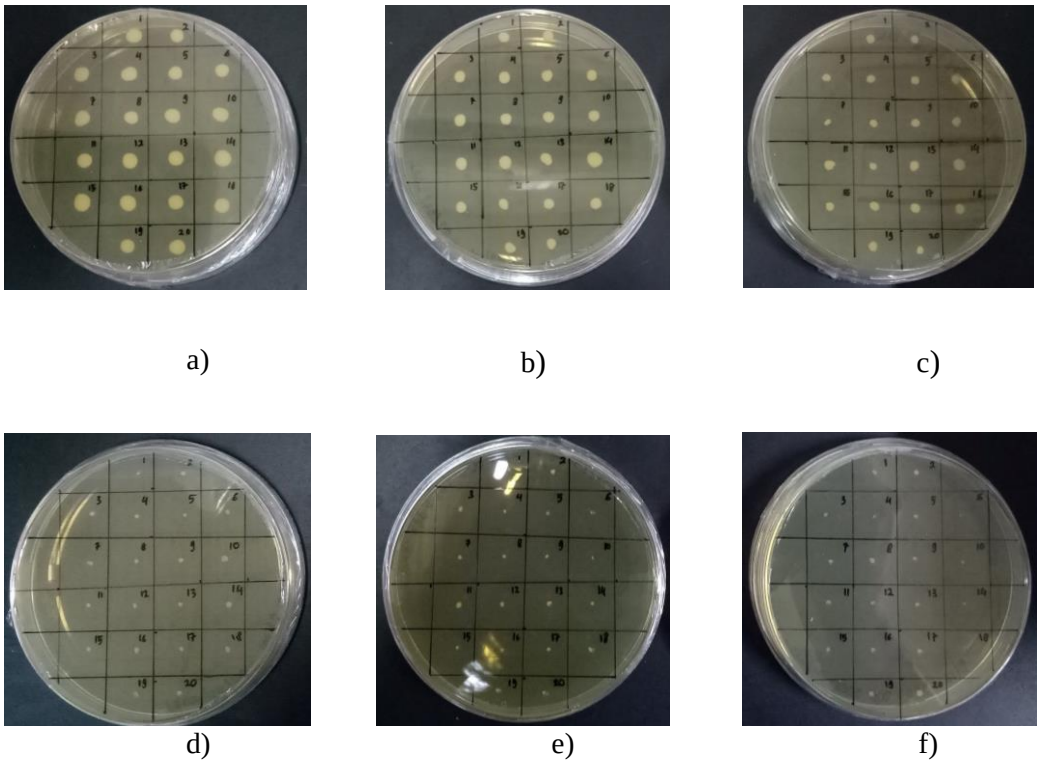


Figure 7. The selected 20 transformants of *P. pastoris* on YPD plates containing variation of zeocin concentration: a) 100; b) 200; c) 500; d) 1000; e) 1500; f) 2000 µg/mL

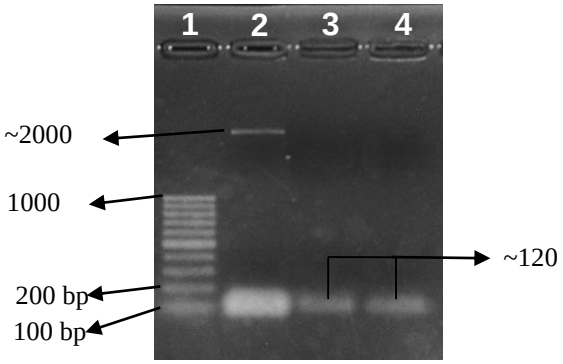


Figure 8. Electropherogram of PCR product from *P. pastoris* recombinant clones: 1) 100 bp Ladder; 2) PCR product from reference pPICZαA-IP plasmid; 3) PCR product from *P. pastoris* no.1; 4) PCR product from *P. pastoris* no.2

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

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

            140
IP gene     GT
            ::
PCR product GT
```

Figure 9. The result of sequence alignment from PCR product and optimized PI gene

4. Discussion

The pPICZαA-IP plasmid was transformed into *E. coli* Top10 by calcium chloride ($CaCl_2$) before subsequent transformation into *Pichia pastoris* X-33. The ice-cold calcium chloride solution stimulates the binding of the plasmid to the cell surface and the plasmid enters the cell after a short heat shock [15]. Thus, the plasmid that has entered the competent cell will increase in number along with the microbial cell division. The *E. coli* Top10 competent cells that have been formed will then be used for the transformation of the pPICZαA-PI plasmid [16].

The pPICZαA-IP plasmid was linearized by the SacI enzyme before being used for the transformation into *Pichia pastoris* to linearize DNA from circular to linear, thus the plasmid DNA could be integrated in *P. pastoris* genome during the transformation process. The result of linearization was confirmed using gel electrophoresis in figure 3. As can be observed, before linearization there was a circular DNA band at 2 – 2.5 kb. After linearization with SacI enzyme, a circular DNA was linearized into a linear DNA with a band at approximately 3 – 4 kb, which complied with the DNA band target at 3.8 kb.

After confirming the isolated pPICZαA-IP plasmid DNA with gel electrophoresis, the concentration level and purity of the plasmid DNA was measured using BioDrop. The absorbance reveals the concentration and purity of the DNA. The purity DNA was measured by comparing the absorbance at wavelengths of 260 and 280 nm. Pure DNA has a value between 1.7 to 2.0. It can be seen that pPICZαA-IP plasmid DNA had an A260/A280 ratio value was 1.951. Thus, the isolated pPICZαA-IP plasmid DNA met the requirements of good purity and quality of DNA, without any RNA or protein contaminants. It means that the plasmid DNA can be used for the next downstream process.

The sequencing result of pPICZαA-IP plasmid using the Sanger Sequencing method obtained the nucleotide sequence of pPICZαA-PI plasmid with a nucleotide length of 1406 bases and it was used to the sequence alignment process with the optimized Insulin sequence. As can be observed in figure 4 that the optimized insulin sequence has been inserted into the pPICZαA-IP plasmid in *E. coli* Top10.

Plasmid or vector transformation is the process by which exogenous DNA is transferred into *Pichia pastoris* X-33 as the host cell. The transformation method of pPICZαA-IP plasmid into *P. pastoris* was electroporation which is a short electrical pulse is used to make the yeast cell temporarily permeable to plasmid [17]. The permeable cell membrane of the yeast was able to take up plasmid, thus the plasmid would increase gradually in line with the cell division. The *SacI* linearized pPICZαA-IP plasmid was transformed into *P. pastoris* X-33 by electroporator, inoculated on YPD plates containing100 μg/mL zeocin, and finally incubated at 30°C for 3 days. It is known that pPICZαA plasmid is a type of resistant plasmid which has a gene confers resistance to zeocin [14]. As can be seen in figure 5 that the colonies of recombinant *P. pastoris* grew on YPD medium + zeocin. It indicates the successful transformation of pPICZαA-IP recombinant plasmid to *P. pastoris* X-33. Moreover, all the selected 20 single transformant colonies also grew on YPD medium + zeocin (figure 6) before further confirmed by PCR and sequencing analysis.

To identify multicopy recombinant strains, the transformants were cultured in YPD medium containing various zeocin concentrations from 100 to 2000 μg/mL. As can be observed in figure 7, all of 20 transformant colonies were able to grow at the highest concentration of zeocin (2000 μg/mL), with the size variation of the colonies that grew at each concentration of zeocin. The higher concentration of zeocin in the YPD plate, the smaller colony size would grow. This result represents that the transformants can be identified as multicopy clones (> 2 copy integrants) because of the clone survival at the zeocin concentration up to 500 μg/mL suggests a copy number of 2 [18]. The multicopy of IP gene into transformant increases expression level of IP [19-21].

Two colonies (the 1st and the 2nd colony) of 20 transformant colonies were analyzed by PCR and sequencing to check for the presence of pPICZαA-IP plasmid in *P. pastoris* genome. The PCR products were electrophoresed on 1.5% agarose gel (figure 8). As can be seen that plasmid pPICZαA containing the insulin precursor gene (~120 bp) had been transfected in *P. pastoris* and ready to be expressed.

The result of sequencing analysis for the confirmation of the 120 bp bands of two selected transformant colonies showed that the sequence of PCR product had 100% identical with the optimized PI gene. Figure 9 reveals that the insulin gene from transformant colony no. 1 could be detected by PCR.

5. Conclusion

PCR and sequencing analysis results showed that pPICZαA-IP plasmid DNA had been integrated successfully into selected colonies of *Pichia pastoris* X-33. The expression, purification, and bioconversion of insulin precursor to insulin will be carried out in the future research.

Acknowledgments

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