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Optimization of PCR Conditions for Adding *Xho*I Restriction Sites to the Glucose Oxidase Gene of *Aspergillus niger* IPBCC 08.610

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ABSTRACT

Glucose oxidase (GOX) is naturally produced by fungi Aspergillus niger. The GOX enzyme catalyzes the oxidation reaction of β-D-glucose to produce δ-gluconolactone and hydrogen peroxide a (H_2O_2). Hydrolysis of δ-gluconolactone will produce gluconic acid that together its derivatives have benefits in the pharmaceutical field as a drug for mineral deficiencies. A. niger IPBCC 08.610 is a local isolate that produce intracellular GOX with higher yield than extracellularly. GOX can be expressed extracellularly by cloning into the expression vector pPICZαB which has the signal peptide α-mating factor (α-MF). GOX gene construction needs to be done by adding some features such as XhoI restriction sites at the 5′ and 3′ ends, XbaI restriction site at 3' side, and spacer peptide glu-ala-glu-ala at 5' side. This research aims to optimize Polymerase Chain Reaction (PCR) conditions in two stages of amplification, stage I to copy the GOX gene and stage II to add those features so it is hoped that recombinant GOX can increase gluconic acid production. The results of primer concentration optimization showed that primers with a concentration of 10 µM produced clearer PCR amplicons than those with a concentration of 20 µM. The optimal temperature for amplification stage I is 58°C. The amplification stage II annealing temperature was modified with the first ten cycles based on the lowest Tm of primer value, 52°C, and the subsequent 25 cycles based on the highest Tm of primer value, 61°C.

Keywords: *Amplification, annealing, GOX, primer concentration*

1. INTRODUCTION

The pharmaceutical industry put an interest in gluconic acid and its derivatives during the past fifty years. Present estimates place gluconic acid output at 100,000 tons annually, with biotechnological processes accounting for nearly all of it (Climent *et al.* 2011). Gluconic acid derivate, the metal-gluconate compounds has been discovered to treat some metal-deficiency such as hypocalcaemia, hypozincemia, and anemia (Jimenez *et al.* 2015; Yu *et al.* 2017; Trailokya *et al.* 2018). The popular biotechnological process to produce gluconic acid is fermentation glucose using fungi such as *Aspergillus niger* and *Penicillium* sp. *A. niger* is a well-known fungi that produce glucose oxidase, which can lead to the production of gluconic acid and its derivates (Cañete-Rodríguez *et al.* 2016; Dubey *et al.* 2017).

Glucose oxidase (GOX; EC 1.1.3.4) is an oxidoreductase enzyme that can accelerate oxidation and reduction reactions. The GOX enzyme catalyzes the oxidation reaction of β-D-

glucose in the presence of oxygen as an electron acceptor to produce δ-gluconolactone and hydrogen peroxide (H_2O_2) (Ozyilmaz 2019; Khatami *et al.* 2021). The δ-gluconolactone is then hydrolyzed to gluconic acid, also the H_2O_2, with the presence of peroxidase, will decompose into hydrogen and oxygen (Ramachandran *et al.* 2006). The hydrolyzed gluconic acid, with the addition of a base, results in carboxylate salt, namely gluconate. Divalent metals such as Ca^{2+}, Zn^{2+}, Fe^{2+} can be easily bound by this salt to create extremely stable chemical complexes (Cañete-Rodríguez *et al.* 2016).

The extensive use of the GOX enzyme is undoubtedly in line with the consumption of GOX. The local isolate *Aspergillus niger* IPBCC 08.610, which was successfully isolated from Dryobalanops hedgerow litter in the Tarakan area, North Kalimantan, is a potential source for GOX enzyme production in Indonesia. Mutant GOX IPBCC 08.610 gene has been studied to decreasing Km value that has the potential to strengthen the ligand-receptor interaction and boost gluconic acid synthesis (Alimah *et al.* 2022). *A. niger* IPBCC 08.610 produces intracellular GOX enzyme with higher yield than the extracellular GOX enzyme (Putri 2012; Rohmayanti *et al.* 2017; Indriani *et al.* 2018). This is a disadvantage of the GOX enzyme from *A. niger* IPBCC 08.610 because it requires a long process to isolate the intracellular GOX enzyme (Indriani *et al.* 2018). Therefore, genetic engineering methods are needed to help the production of recombinant GOX extracellularly, with an expression system in eukaryotes such as *Pichia pastoris* (Gomes *et al.* 2018).

The GOX-encoding gene was cloned into an expression vector pPICZα (A, B, or C) with the host *P. pastoris.* Vector pPICZα has secretion signal α-mating factor (α-MF) with functions to direct the expression of extracellular secretions (Niu *et al.* 2015). The expression system in *P. pastoris* has been proven to increase the extracellular production of recombinant GOX with higher activity than its native (Kovačević *et al.* 2014; Qiu *et al.* 2016).

The GOX-encoding gene has been cloned into pGEM®T-Easy (Kurniatin *et al.* 2020) and used as a template for the construction of the GOX IPBCC 08.610 gene with two restriction sites, *Xho*I and *Xba*I (Putri 2020). In previous research, digesting and ligating the GOX IPBCC 08.610 gene using *Xho*I and *Xba*I was not successful. This is because the restriction site *Xba*I is affected by Dam methylation in the GATC site. If there are methyl groups in *Xba*I recognition sites, it will block the restriction enzyme to cleave the DNA (Medina *et al.* 2022). Another strategy that can be used is to cleave GOX IPBCC 08.610 gene with the same restriction sites, namely *Xho*I (GOX-Xho). The *Xho*I restriction site on the pPICZα vector allows genes to ligate to the *Xho*I restriction site and in frame with the α-mating factor (α-MF) signal peptide.

The addition of some features in GOX IPBCC 08.610 gene such as *Xho*I restriction sites at the 5' and 3' ends, *Xba*I restriction site at 3’ side, and spacer peptide glu-ala-glu-ala at 5’ side was done using PCR method. This research aims to optimize the PCR conditions, including primer concentration and primer annealing temperature. The constructed GOX-Xho gene is expected to be ligated into the pPICZαB expression vector. The recombinant GOX IPBCC 08.610 is expected to increase the production of gluconic acid. It is hoped that information related to optimizing PCR conditions can increase insight regarding strategies that can be used to improve the quality of amplification using PCR.

2. METHODOLOGY

Optimization of PCR Stage I

The GOX gene used in this research is a gene fragment isolated from previous research (Putri, 2020). Amplification stage I was used to copy the Open Reading Frame (ORF) of the GOX IPBCC 08.610 gene using the primer pair FPI-GOX and RPI-GOX with the sequences described in Table 1. The PCR reagent mixture consisted of 23 µL nuclease-free water (NFW), 25 µL 2X MyTaq HS Red Mix, 0.5 µL primer FPI-GOX (20 and 10 µM), 0.5 µL RPI-GOX (20 and 10 µM), and 1 µL GOX gene as a DNA template. Amplification was carried out for 35 cycles, including initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at a temperature range of 58-62°C for 30 seconds, the extension stage was carried out at 72°C for 90 seconds and final extension at 72°C for 5 minutes. After the PCR cycle, 10 µL of PCR product was analyze using 1% agarose gel electrophoresis, running at 100 Volt for 30 minutes, then visualized using GelDoc (UVITECH). The PCR I product is used as template DNA for amplification stage II.

Agarose 1% Gel Extraction of GOX Gene

The PCR product of amplification stage I contained non-target bands. Therefore, the gene target was purified using the QIAquick® Gel Extraction Kit. The GOX gene amplicon with a size between 2000 – 1600 bp was excised from the gel, then put into a 1.5 mL microtube and then weighed (100 mg gel equal to 100 µL). QG buffer was added three times of the gel volume. The mixture was incubated for 20 minutes on a heat block at 58°C and mixed every 2 minutes using vortex. One volume of isopropanol was added and then mixed by vortex. The QIAquick spin column was placed in a 2 mL tube, and then the mixture was put into the QIAquick spin column and centrifuged for 1 minute at 13,000 rpm for the DNA binding stage.

The DNA binding stage was carried out twice. The supernatant in the 2 mL tube was discarded, then the washing stage continued by adding 750 µL of PE buffer to the QIAquick spin column and allowed the buffer to absorb for 3 minutes. Next, the column was centrifuged for 1 minute at 13000 rpm. The supernatant was discarded, and the empty column was centrifuged again for 3 minutes at 13,000 rpm so that the column was completely dry from the remaining buffer. The column is aired to dry. The column was moved to a 1.5 mL microtube, added with 25 µL of pre-warmed NFW right in the middle of the column and allowed to soak for 5 minutes, followed by centrifuged for 1 minute at 13,000 rpm. The supernatant that passes through the column is Elution 1. Elution 2 is carried out similarly with the addition of 13 µL NFW.

Table 1. Sequences and Tm of Primers Used

Primer	Sequences	Melting temperature (Tm)	Theoretical Tm of overlap sequences (Thermo Fisher Tm calculator)
FPI-GOX	5′AGCAATGGCATTGAAGCCAGCCT-3′	62.2ºC	-
RPI-GOX	5′CTGCATGGAAGCATAATCCGCCAAGA-3′	61.3ºC	-
FPII-GOX-Xho	5′TAACTCGAGAAAAGAGAAGCTGA**AGCTAGCAATGGCATTGAAGCC**-3′	66.4ºC	55.1ºC
RPII-GOX-Xho	5′AAACTCGAGTCATTCTAGAGA**CTGCATGGAAGCATAATCCGCCAA**-3′	66.7ºC	63.9ºC

Note: sequences that are in bold are sequences that complement with the GOX gene.

Table 2. The PCR program of amplification stage II before and after modification

Before modification	Temperature (°C)	Time (min)	Number of cycles
Initial denaturation	95	5	1
Denaturation	95	0.5	
Annealing	62	0.5	35
Extention	72	1.5	
Final extention	72	5	1
After modification	**Temperature (°C)**	**Time (min)**	**Number of cycles**
Initial denaturation	95	5	1
Denaturation	95	0.5	
Annealing	52	0.5	10
Extention	72	1.5	
Denaturation	95	0.5	
Annealing	61	0.5	25
Extention	72	1.5	
Final extention	72	5	1

Optimization of PCR Stage II

Amplification stage II was carried out to add *Xho*I restriction sites at the 5' and 3' ends, an *Xba*I restriction site and stop codon at the 3' end, and a glu-ala-glu-ala spacer at the 5' end. The template DNA was the PCR I product. The primer pairs were FPII-GOX-Xho and RPII-GOX-Xho with the sequences in Table 1. The PCR reagent mixture consisted of 23 µL NFW, 25 µL 2X MyTaq HS Red Mix, 0.5 µL FPII-GOX-Xho primer (10 µM), 0.5 µL RPII-GOX-Xho (10 µM), and 1 µL PCR I product. The PCR settings of amplification stage II before and after modification is shown in Table 2. After the PCR cycle, 10 µL of PCR product was analyze using 1% agarose gel electrophoresis.

3. RESULTS

Contruction of GOX-Xho Gene

Amplification stage I was carried out to copy the Open Reading Frame (ORF) of the GOX IPBCC 08.610 gene using the primer pair FPI-GOX and RPI-GOX with the sequence in Table 1 resulting a size of 1749 bp (Putri, 2020). The annealing of the primer pair FPI-GOX and RPI-GOX was illustrated in Figure 1. Amplification stage II was carried out to add *Xho*I restriction sites at the 5' and 3' ends. The *Xho*I 5’ restriction site followed by oligonucleotide encoded glu-ala-glu-ala spacer was introduced to the GOX gene by primer FPII-GOX-Xho. The *Xho*I 3’ restriction site with an additional *Xba*I restriction site and stop codon was also introduced to the GOX gene by primer RPII-GOX-Xho. The annealing of primer pair FPII-GOX-Xho and RPII-GOX-Xho was illustrated in Figure 1.

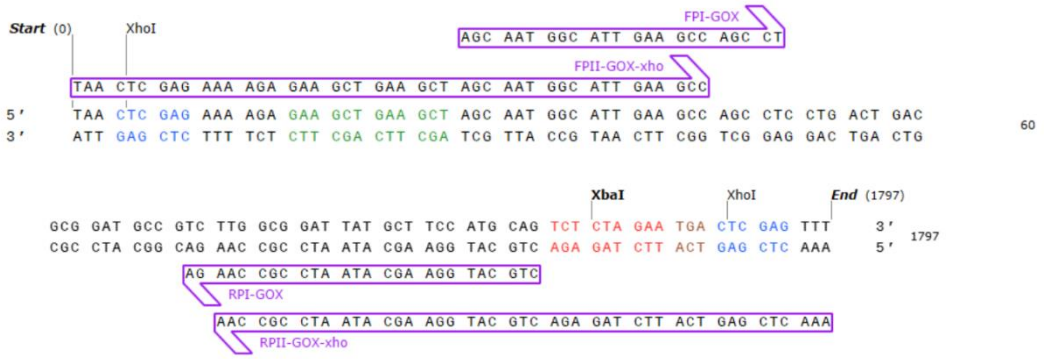


Figure 1. Annealing position of primer pair FPI-GOX and RPI-GOX, FPII-GOX-Xho and RPII-GOX-Xho on GOX gene. Note on highlighted codon, blue: XhoI; green: peptide spacer glu-ala-glu-ala; red: XbaI; and brown: stop codon.

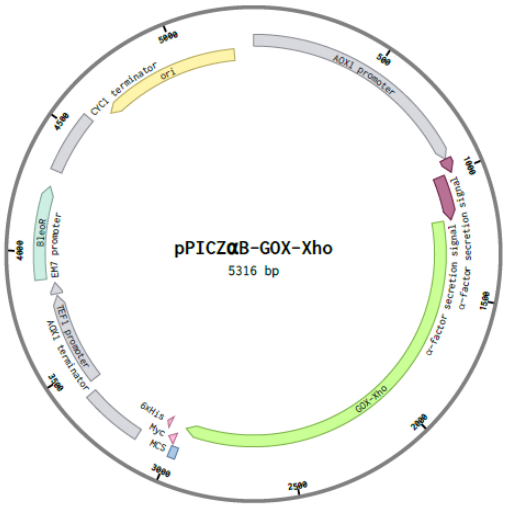


Figure 2. pPICZαB-GOX-Xho map (Invitrogen 2010)

The GOX gene with an additional *Xho*I restriction site will be ligated to pPICZαB as illustrated in Figure 2. The digestion of pPICZαB with *Xho*I removes the Kex2 endoprotease cleaving site and the EAEA spacer in the 5' region. Therefore, the missing features were added to the GOX-Xho gene with the primer FPII-GOX-Xho. It is also necessary to add an *Xba*I restriction site before the *Xho*I 3' end of the GOX gene so that the GOX gene and pPICZαB can be digested with the *Xba*I restriction enzyme, and the GOX gene will be fused to the 6×His-tag as illustrated in Figure 3.

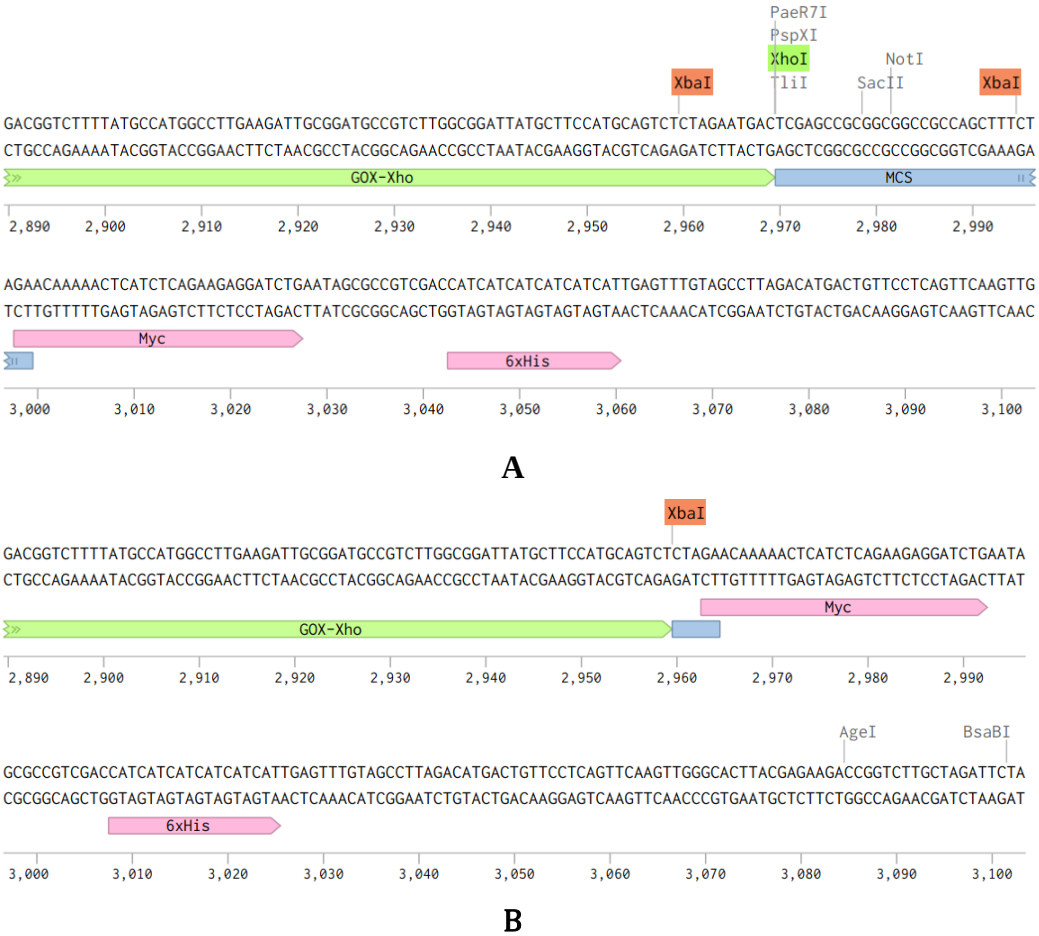


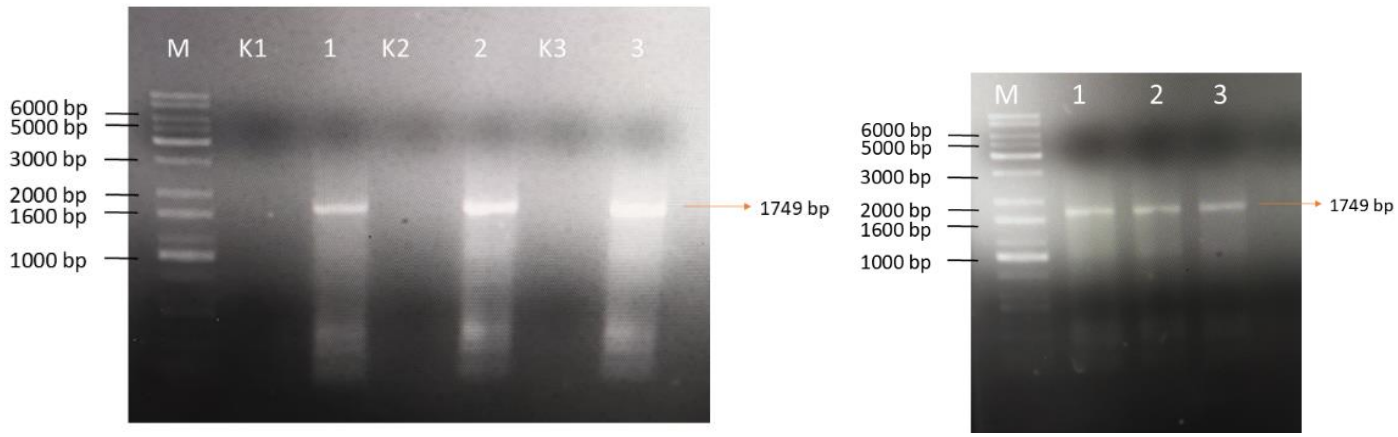
Figure 3. pPICZαB-GOX-Xho (A) before digestion with XbaI (5316 bp) and (B) after digestion with XbaI (5281 bp)

Optimization of PCR Primer Concentration and Temperature of Annealing Stage I

Amplification stage I with primer concentration of 10 µM produced more distinct band compared to it of 20 µM as shown in Figure 4A and 4B. No primer dimers were formed from both variations in primer concentration based on the negative control. The amplification stage I annealing temperature was optimized using gradient PCR with temperature variations of 58°C, 60.28°C, and 62°C. The result reveals that the non-target band was still observed. However, a clearer band was observed at a temperature of 58°C. The non-target product of amplification can be caused by the impurities of the GOX gene template. The size of target amplicons is 1749 bp.

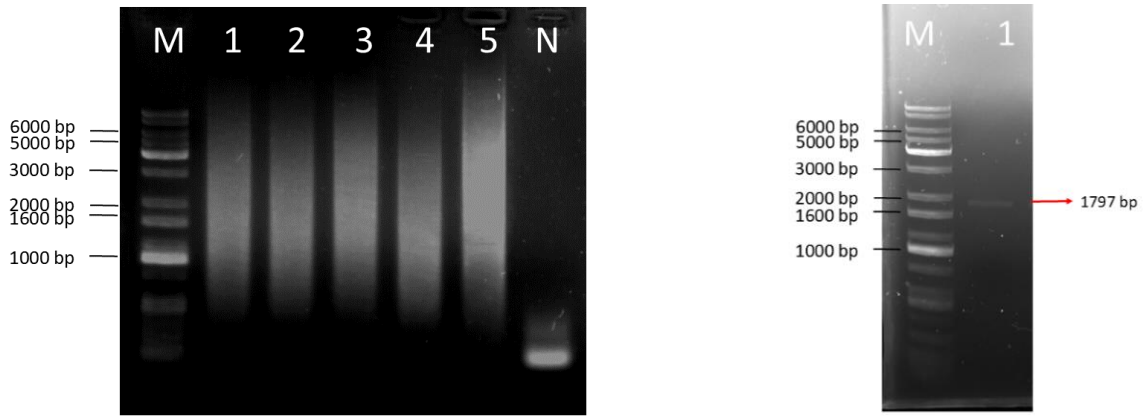
Optimization of Amplification Stage II

Amplification stage II with the annealing temperature of 62°C was unsuccessful to produce the target gene as shown in Figure 5A. The annealing temperature was 62°C based on the Tm value of the primer pair FPII-GOX-Xho and RPII-GOX-Xho. The FPII-GOX-Xho and RPII-GOX-Xho primer sequences that complement with GOX gene shown in Table 1, have different Tm values of 55.1°C and 63.9°C respectively. Therefore, the amplification stage II strategy was modified into the first ten cycles with an annealing temperature of 52°C then the subsequent 25 cycles with an annealing temperature of 61°C. This condition is carried out so that the sequence of primer FPII-GOX-Xho that complement to GOX gene can produce amplicons with the addition of *Xho*I restriction site at the 5' end. The amplicon becomes a template for the RPII-GOX-Xho primer to anneal for the subsequent 25 cycles. Figures 5B are electropherogram of the results of amplification stage II with modification of the annealing temperature, producing an amplicon measuring 1797 bp with a band between 2000 bp and 1600 bp.



A **B**

Figure 4. Electropherograms amplification stage I of GOX gene by gradient PCR with temperature range (S1) 58°C, (S2) 60.28°C, (S3) 62°C, and (K1-K3) negative control using primer concentration (A) 20 µM and (B)10 µM



A **B**

Figure 5. Electropherograms amplification stage II product of GOX-Xho gene on gel agarose 1% with annealing temperature (A) 62°C and (B) modified temperature

4. DISCUSSION

The GOX gene with an additional *Xho*I restriction site will be ligated to pPICZαB so the GOX gene will be fused to signal peptide α-mating factor (α-MF) as illustrated in Figure 2. The signal peptide α-MF consists of a pre-pro-peptide followed by a Kex2 cleavage site and a spacer peptide glu-ala-glu-ala (EAEA). In protein secretion, the peptide signal will be cleaved by the peptidase in the endoplasmic reticulum (ER). The Kex2 endoprotease cleaves the lys-arg in the Golgi apparatus, and the EAEA spacer is cleaved by dipeptidyl aminopeptidase (Ste13), resulting in the mature protein secreted extracellularly (Gaffar 2010).

The addition of an *Xho*I restriction site at the 3' end lead the GOX gene not fusion with a c-myc epitope and polyhistidine tag (6×His-tag). The c-myc epitope permits detection of fusion protein and 6×His-tag facilitates the purification of recombinant proteins (Zhao *et al.* 2015). Fusion with a 6×His-tag can increase the specific activity of the recombinant protein (Adivitiya *et al.* 2016).

A high primer concentration will increase the number of amplicons, both target gene amplicons and non-target bands (Farrar dan Wittwer 2015; Jansson dan Hedman 2019). Substantial primer concentration can disrupt the PCR cycle so that the primer will not anneal to the target area, and non-target bands or primer dimers are formed (Park *et al.* 2020). Non-target products become amplification inhibitors leading to the formation of smaller non-target products than the target gene. In addition, the presence of non-target products causes the polymerization activity of the target gene to decrease due to the reduced amount of polymerase available (Jansson & Hedman, 2019). Inappropriate annealing temperatures can cause the formation of smears and non-target bands (Park *et al.* 2020). The annealing temperature is usually about 5°C below the primary Tm value (Obradovic *et al.* 2013; Silalahi *et al.* 2021). This PCR modification is similar to nested PCR, in which the PCR amplicons from the first cycle will be a template for the subsequent PCR cycles. Still, the difference is that nested PCR uses two sets of primers, but this research only uses one set of primers. The nested PCR was designed to improve the sensitivity and specificity of amplicons (Nazir *et al.* 2019).

In conclusion, *Xho*I restriction sites at 5' and 3' ends was successfully introduced to the GOX IPBCC 08.610 gene by two stage of PCR amplification, as well as *Xba*I restriction site and stop codon at the 3' side, glu-ala-glu-ala spacer at the 5' side resulting gene target of 1797 bp. The optimal condition for

amplification stage I was primer concentration of 10 µM and annealing temperature at 58°C. The optimal annealing temperature of amplification stage II was 52°C for initial ten cycles and the next 25 cycles was 61°C. With this research, recombinant GOX IPBCC 08.610 is expected to produce extracellularly using expression vector pPICZαB using *P. pastoris* as a host, so the production of gluconic acid can be increased.

5. ACKNOWLEDGMENT

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