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Research Article

Cloning of the *GOX-Xho* Gene IPBCC 08.610 into Plasmid pTA2 and Its Characterization

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Abstract

Glucose oxidase (GOX) from *Aspergillus niger* catalyzes the oxidation of β-D-glucose to δ-gluconolactone and hydrogen peroxide, making it valuable for industrial applications. Intracellular GOX exhibits higher activity than its extracellular counterpart. This study focuses on enhancing the extracellular production of GOX through recombinant DNA technology. This study aimed to reconstruct the GOX gene by adding *Xho*I sites at both ends, inserting a glu-ala-glu-ala spacer at the 5' end, and introducing an *Xba*I site at the 3' end. These modifications facilitate the cloning of the GOX-Xho gene into the pTA2 vector and its subsequent ligation into the pPICZαB expression vector, allowing for extracellular production of GOX through fusion with the α-mating factor (α-MF) signal peptide. The GOX-Xho gene was successfully amplified, cloned, and characterized. The pTA2-GOX-Xho recombinant plasmid was verified through sequencing and restriction analysis, confirming the present and correct orientation of the 1797 bp GOX-Xho gene. However, sequencing revealed several point mutations, necessitating further computational analysis to predict their impact on the enzyme's structure and function before recombinant protein expression.

Keywords: *Aspergillus niger*, gene cloning, glucose oxidase, pTA2 vector, sequencing

1. INTRODUCTION

Glucose oxidase (GOX; EC 1.1.3.4) is an oxidoreductase enzyme that catalyzes the oxidation reaction from β-D-glucose in producing δ-gluconolactone and hydrogen peroxide (H_2O_2). Although animals and plants can produce GOX enzymes, microorganisms are preferred sources due to their higher yield and cost-effectiveness[1]. GOX enzymes can be produced by several species of fungi, including *Penicillium*, *Talaromyces*, and *Aspergillus*. Among these, *Aspergillus niger* is predominantly utilized in industrial applications[2]. A promising isolate for GOX production is *A. niger* IPBCC 08.610, sourced from the Dryobalanops plants in Tarakan, South Kalimantan.

This locale isolates *A. niger* IPBCC 08.610 and demonstrates significant potential for large-scale GOX production. Nonetheless, the specific activity of the GOX enzymes produced intracellularly was higher than that of the enzyme produced extracellularly. The specific activity of GOX produced intracellularly by this isolate exceeds 1000 U/mg, while extracellular production yields less than 50 U/mg[3]. Despite the preference for extracellular enzyme production in industrial settings due to reduced purification steps and lower production costs[4], the high intracellular-specific activity necessitates alternative production methods. Recombinant enzyme production, involving gene cloning and expression in a suitable vector, is a viable approach to meet this need. Before proceeding, it is necessary to clone the gene into the cloning vector to ensure the incorporation of additional features, such as the restriction site.

The GOX IPBCC 08.610 gene was cloned into the pGEM®T-Easy vector[5], incorporating two restriction sites, XhoI and *Xba*I[6]. Previous attempts to digest and ligate the GOX gene using XhoI and XbaI were unsuccessful due to Dam methylation

interference at the XbaI recognition site[7]. An alternative strategy involves cleaving the GOX gene. It used XhoI sites, facilitating alignment with the α-mating factor (α-MF) signal peptide for extracellular production. Following the construction of the GOX-Xho gene, it was ligated into the pPICZαB expression vector. Additional features were introduced into the GOX IPBCC 08.610 gene using PCR, including XhoI sites at both the 5' and 3' ends, an XbaI site at the 3' side, and a Kex2 cleavage site with a spacer peptide (glu-ala-glu-ala) at the 5' side.

The gene was then characterized before ligation into the expression vector. The pTA2 cloning vector, featuring a single thymine (T) overhang, was selected for its efficiency in rapid and straightforward cloning processes[8]. The pTA2 vector, measuring 2981 bp[8], was used to clone the GOX gene, which has an Open Reading Frame (ORF) of 1749 bp[6]. It is hypothesized that the pTA2-GOX-Xho recombinant plasmid will have a length of about 4700 bp. This research aims to clone the GOX-Xho gene into the pTA2 plasmid, characterize the cloning results, and transform the recombinant plasmid into *Escherichia coli* DH5α cells. The transformation process will be validated using a white-blue selection method. The study involves the construction of the GOX-Xho gene, sequencing, and restriction patterns analysis of the pTA2-GOX-Xho recombinant plasmid to confirm the plasmid's size and integrity.

2. RESEARCH METHODS

Materials

The research utilized *E. coli* DH5α cells. The vector employed was Target Clone/pTA2 (TOYOBO). The primer pair used was FPII-GOX-Xho and RPII-GOX-Xho, with sequences provided in Table 1. The enzymes included the restriction enzyme XbaI kit (Thermo Scientific), RNAse A (Thermo Scientific), 2X MyTaq™ HS Red Mix (Bioline), and the T4 DNA Ligase kit (Vivantis). The medium used was Luria Bertani (LB: 1% tryptone, 1% NaCl, 0.5% yeast extract: for agar plate, it was added 1.5% bacteriological agar), SOC Outgrowth medium (NEB: 2% vegetable peptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). Other reagents used were ampicillin, IPTG (Isopropyl β-D-1-thiogalactopyranoside), X-gal (5-bromo-4-kloro-3-indolyl-β- d -galactopyranoside), solution I (1 M glucose, 1 M Tris-Cl, 0.5 M EDTA, and distilled water), solution II (0.2 N NaOH, 1% SDS, and distilled water), solution III (5 M potassium acetate, glacial acetic acid, and distilled water), chloroform, isoamyl alcohol, EtOH, TE buffer pH 8.0, isopranol, QIAquick PCR Purification kit, *nuclease-free water* (NFW), agarose gel (TOPVISION), and TAE 50× buffer (Thermo Scientific).

Table 1. Sequences of primer pair FPII-GOX-Xho dan RPII-GOX-Xho designed using https://www.ncbi.nlm.nih.gov/tools/primer-blast/

Primer	Sequences
FPII-*GOX-Xho*	5'-TAA**CTCGAG***AAAAGAGAAGCTGAAGCT*AGCAATGGCATTGAAGCC-3'
RPII-*GOX-Xho*	5'-AAA**CTCGAG**TCATTCTAGAGACTGCATGGAAGCATAATCCGCCAA-3'

Notes: bold font: *Xho*I restriction site, italic font: Kex2 cleavage site and spacer peptide EAEA, underline font: *Xba*I restriction site.

Addition of restriction sites into *GOX-Xho* gene using PCR

PCR amplified the GOX gene fragment[6] was amplified by PCR. The PCR reagent mixture consisted of 23 µL NFW, 25 µL 2X MyTaq HS Red Mix, 0.5 µL primer FPII-GOX-Xho (10 µM), 0.5 µL primer RPII-GOX-Xho (10 µM), and 1 µL GOX gene fragment. PCR conditions are shown in Table 2[9]. PCR products were visualized using 1% agarose gel electrophoresis and purified using the QIAquick PCR Purification Kit.

Ligation of the GOX-Xho gene into the Target Clone plasmid (pTA2)

The GOX-Xho gene fragment was quantified using Nanodrop Spectrophotometer to determine DNA purity based on the $A_{260/280}$ and $A_{260/230}$ ratio values. Ligation is carried out with a ratio of DNA insert:vector, namely 1:1. The ligation reagent mixture consisted of 1 µL pTA2 (50 ng/µL), 4 µL GOX-Xho gene (6.3 ng/µL), 2 µL 10X Buffer Ligase, 2 µL T4 DNA ligase (100 U), and NFW to a final volume of 20 µL. The mixture was incubated at 16 ˚C overnight and then inactivated at 65 ˚C for 15 minutes[10].

Transformation of pTA2-GOX-Xho into *E. coli* DH5α

A 10 µL ligation mixture was added to 50 µL of *E. coli* DH5α competent cells in a 1.5 mL microtube. The transformation is carried out using the heat shock method. Cells in 1.5 mL microtubes were incubated on ice for 30 minutes, subjected to heat shock treatment at 42 ˚C for 90 seconds, and incubated again on ice for 2 minutes. A total of 150 µL of SOC

Medium was added to the culture, which was then incubated for 3 hours at 37 °C. Subsequently, 100 µL of the culture was spread on LB agar plates containing 100 µg/mL ampicillin, IPTG, and X-Gal. The plates were incubated overnight at 37 °C[10].

Table 2. PCR conditions of GOX-Xho gene amplification[9]

Stage	Temperature (°C)	Time (minutes)	Number of cycles
Initial denaturation	95	5	1
Denaturation	95	0.5	
Annealing	52	0.5	10
Extension	72	1.5	
Denaturation	95	0.5	
Annealing	61	0.5	25
Extension	72	1.5	
Final extension	72	5	1

Isolation of Plasmid pTA2-GOX-Xho

White colonies that grew on LB agar containing ampicillin, IPTG, and X-gal were cultured in 3 mL of LB broth with ampicillin and incubated overnight at 37 °C. One milliliter of this culture was transferred to a 1.5 mL microtube and centrifuged at 13,000 rpm for 2 minutes, after that, the supernatant was discarded. The pellet was resuspended in 150 µL of alkaline lysis solution I and homogenized by vortexing. Next, 200 µL of alkaline lysis solution II was added, and the tube was inverted 4-6 times. It was followed by adding of 300 µL of alkaline lysis solution III, inverting the tube 4-6 times, and centrifuging at 13,000 rpm for 10 minutes. Subsequently, 300 µL of the supernatant was transferred to a new 1.5 mL microtube. An equal volume (300 µL) of cold isopropanol was added, the tube was inverted 4-6 times, incubated at -80 °C for 15 minutes, and centrifuged at 13,000 rpm for 10 minutes. The supernatant was discarded, and 600 µL of 70% ethanol was added, followed by another centrifugation at 13,000 rpm for 10 minutes. After discarding the supernatant, the tube was air-dried. Finally, 25 µL of TE-RNase buffer was added to the microtube. The plasmids were then visualized on a 1% agarose gel[10].

Purification of the pTA2-GOX-Xho Plasmid

A total of 180 µL of 10 mM Tris-Cl buffer pH 8 was added to the microtube containing pTA2-GOX-Xho, then 200 µL of chloroform: isoamyl alcohol (24:1) was added. The mixture was homogenized by vortexing for 1 minute and then centrifuged at 12,000 rpm for 10 minutes. 180 µL of liquid phase was transferred to a new 1.5 mL microtube. 5 M potassium acetate was added until the final concentration of the mixture was 0.75 M, and then the mixture was homogenized. A 2.5 volume of 100% EtOH was added to the mixture and then centrifuged at a speed of 12,000 rpm for 20 minutes at a temperature of 4 ˚C. The supernatant was discarded, and then 300 µL of 80% EtOH was added and homogenized by vortexing. The mixture was centrifuged at 12,000 rpm for 20 minutes at 4 ˚C. The supernatant was discarded, and the pellet was air-dried. A total of 25 µL of 10 mM Tris-Cl buffer pH 8 was added to the microtube[10].

Confirmation of the GOX-Xho gene using PCR

PCR confirmed the presence of the GOX-Xho gene in the pTA2-GOX-Xho plasmid. The PCR reagent mixture consisted of 23 µL NFW, 25 µL 2X MyTaq HS Red Mix, 0.5 µL FPII-GOX-Xho (10 µM), 0.5 µL RPII-GOX-Xho (10 µM), and 1 µL plasmid pTA2-GOX-Xho. PCR conditions are shown in Table 2. The PCR products, a visual representation of our findings, were carefully visualized using 1% agarose gel electrophoresis.

Analysis of Restriction Patterns

The digestion reagent mixture with *Xba*I consisted of 5 µL (1 µg) pTA2-GOX-Xho, 2 µL10X Tango Buffer, two µL *Xba*I (10 U/µL), and NFW to a volume of 20 µL. The mixture was incubated at 37 ˚C for 7 hours and continued overnight at room temperature[11]. Restriction results were visualized using 1% agarose gel electrophoresis. The purified pTA2-GOX-Xho plasmid was quantified using a NanoDrop Spectrophotometer.

Determination of the Nucleotide Sequence of Recombinant Plasmid

Nitrogen base sequencing was carried out using the T7 Promoter forward primer and the T3 binding site reverse primers. The isolated plasmids were sent to First Base Company in Malaysia to determine the nucleotide sequence.

3. RESULTS AND DISCUSSION

Addition of restriction sites into *GOX-Xho* gene using PCR

The GOX gene consists of 1818 bp, which includes 66 bp signal peptide, 1749 bp open reading frame (ORF), and three bp stop codons. PCR amplification of the GOX gene using primers FPII-GOX-Xho and RPII-GOX-Xho resulted in a 1797 bp amplicon. It includes the 1749 bp ORF and 48 bp of additional sequences, which encompass *Xho*I restriction sites, a Kex2 cleavage site and glu-ala-glu-ala (EAEA) peptide spacer at the 5' side, and an *Xba*I restriction site and stop codon at the 3' side. The electropherogram of GOX-Xho gene amplification is shown in Figure 1.

The additional *Xho*I sites introduced by PCR ensure that the GOX gene can be ligated into the

pPICZαB expression plasmid in frame with the α-mating factor (α-MF) signal peptide, enabling extracellular secretion of the GOX enzyme. Digestion of pPICZαB with *Xho*I removes the Kex2 cleavage site and the EAEA spacer peptide, which contributes to the precise cleavage of pre-pro-proteins and maintains the proper arrangement and folding of the protein of interest. It ensures efficient processing and secretion within the *Pichia* expression system[12]. Therefore, the endoprotease Kex2 cleavage site and spacer peptide EAEA were added to the GOX gene. The addition of an *Xho*I restriction site at the 3' end of the GOX prevents fusion with the c-myc epitope and a polyhistidine tag (6×His-tag) on pPICZaB which facilitates the purification of the recombinant protein[13]. An *Xba*I restriction site was added before the *Xho*I restriction site at the 3' end to enable gene fusion with the c-myc epitope and 6×His-tag.

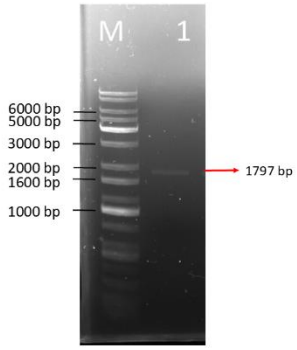

Figure 1. Electropherogram of GOX-Xho gene. DNA Ladder (M), GOX-Xho gene amplicon (1).

Ligation of the GOX-Xho gene into the Target Clone plasmid (pTA2b)

The purified GOX-Xho gene was then quantified using a NanoDrop Spectrophotometer to determine the gene concentration and DNA purity based on the absorbance ratio of 260 nm to 280 nm ($A_{260/280}$) and the absorbance ratio of 260 nm to 230 nm ($A_{260/230}$). Table 3 shows the GOX-Xho gene quantification data. DNA purity is determined based on the $A_{260/280}$ ratio with a range of 1.8-2.0 and the $A_{260/230}$ ratio of 2.0-2.2[14]. The GOX-Xho gene has an $A_{260/280}$ ratio value of 2.05, which indicates that the GOX-Xho gene is pure from contaminants that have absorbance at a wavelength of 280 nm, such as phenol and protein[10]. However, the $A_{260/230}$ ratio was low at 0.06, likely due to contaminants such as EDTA and Guanidine HCl from the purification process that absorbs a wavelength of 230 nm[10].

The purified GOX-Xho gene was ligated into the pTA2 plasmid using a 1:1 ratio of plasmid to insert. Plasmid pTA2 has a size of 3 kb, while the GOX-Xho gene is 1.8 kb. pTA2 is a linear cloning plasmid with a hanging thymine (T) base, compatible with PCR products generated by Taq DNA Polymerase, which adds an adenine (A) overhang at the 3' end[15,16]. The ligation was facilitated by T4 DNA ligase, forming phosphodiester bonds between the DNA ends[17].

Table 3. Quantification of GOX-Xho and pTA2-GOX-Xho genes using NanoDrop Spectrophotometer

DNA	Concentration (ng/µL)	$A_{260/280}$	$A_{260/230}$
GOX-Xho	6.3	2.05	0.06
pTA2-*GOX-Xho*	220.7	1.75	1.12

Transformation of pTA2-GOX-Xho into *E. coli* DH5α

Blue and white selection is carried out to select for the presence of recombinant plasmids in bacterial cells. The successful transformation resulted in white colonies due to the disruption of the lacZ gene, preventing β-galactosidase activity and thus the hydrolysis of X-gal substrate (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) into blue compound 5,5'-dibromo-4,4'-dichloro-indigo[18].

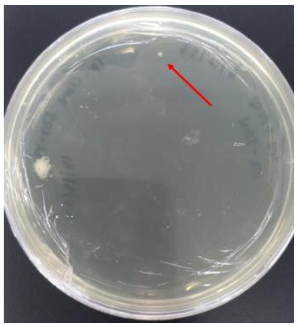

Figure 2. The transformation results of pTA2-GOX-Xho *on E. coli* DH5α

Isolation and Purification of Plasmid pTA2-GOX-Xho

Isolation of pTA2-GOX-Xho from *E. coli* DH5α was performed using the alkali lysis method and purification by extraction method with organic solvents, namely chloroform and isoamyl alcohol. The purified plasmid pTA2-GOX-Xho shown in Table 3 has a concentration of 220.7 ng/µL and an $A_{260/280}$ ratio value of 1.75, which indicates that the plasmid is pure from contaminants such as phenol and protein[10]. However, the $A_{260/230}$ ratio was low at 1.12, likely due to contaminants such as EDTA and Guanidine HCl[10].

The Alkali lysis method yielded higher quantities of plasmid DNA than other methods, such as boiling lysis and miniprep with kits. Unfortunately, it has the highest RNA contamination[19] as shown in Figure 3A. RNA contamination can be digested using RNAse and then purified by liquid-liquid extraction with organic solvents, namely chloroform and isoamyl alcohol. Contaminants other than DNA, such as

proteins, lipids, and cell debris, will be in the organic phase, while DNA will be in the water phase[20]. Plasmid DNA that has been purified will be free from RNA and other contaminants, as shown in Figure 3B.

The linear pTA2 plasmid is 2981 pb in size, and while the results of plasmid isolation show that *E. coli* DH5α cells transformed pTA2-GOX-Xho produce circular plasmids as seen from the electropherogram of plasmid isolation results formed two bands. The two bands are the conversion of circular plasmids in relaxation conditions into supercoiled plasmids so that migration on agarose gels produces more than one band[21]. The presence of these two bands indicates that gene ligation with plasmids successfully produces circular DNA.

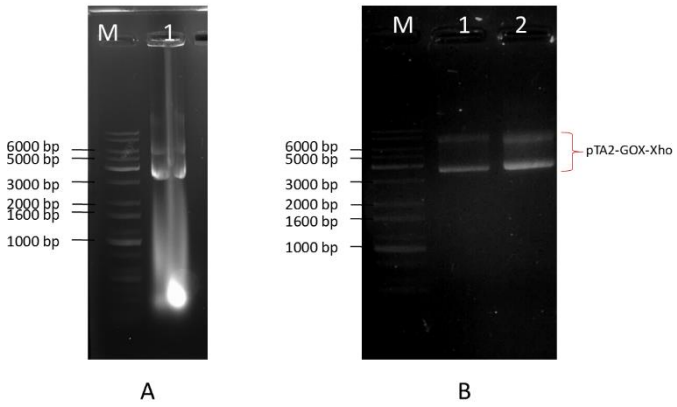

Figure 3. Electropherograms of pTA2-GOX-Xho isolated from *E. coli* DH5α DNA Marker (M), before adding RNAse (A) and after purification (B).

Confirmation of the GOX-Xho gene using PCR

PCR amplification confirmed the presence of the GOX-Xho gene in the pTA-GOX-Xho template, yielding a specific 1797 bp amplicon consistent with the positive control (Figure 4). Non-target amplicons were likely due to suboptimal annealing temperatures[22,23].

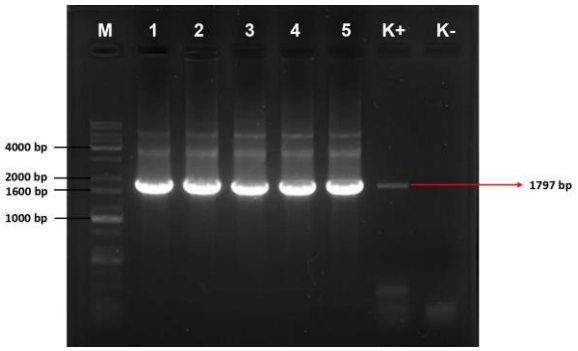

Figure 4. Electropherogram of GOX-Xho gene amplification. DNA Marker (M), pTA-GOX-Xho as a template (1-5), positive control GOX gene fragment (K^+), and negative control (K^-).

Analysis of Restriction Patterns and Nucleotide Sequence of Recombinant Plasmid

The restriction pattern of pTA2-GOX-Xho was analyzed using *Xba*I. An *Xba*I restriction site is in the pTA2 multi-cloning site, located several bases downstream of the cloning insert region. *Xba*I digestion can be used to determine the orientation of the GOX-Xho gene cloned into pTA2 because the GOX-Xho gene has an *Xba*I restriction site on its 3' end. If the GOX-Xho gene clone is positively oriented (5' to 3'), digestion with *Xba*I will produce a single band measuring 4.7 kb consisting of 2.9 kb of pTA2 and 1.8 kb of the GOX-Xho gene. Conversely, if the GOX-Xho gene clone is negatively oriented (3' to 5'), digestion with *Xba*I will produce two bands, each measuring 2.9 kb of pTA2 and 1.8 kb of the GOX-Xho gene. A single band of 4.7 kb indicated the positive orientation of the GOX-Xho gene within the plasmid, as shown in Figure 5.

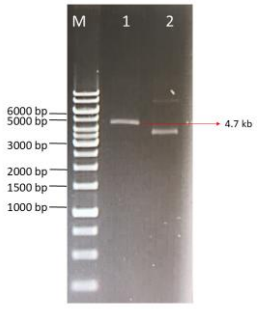

Figure 5. Electrophoregram restriction with *Xba*I DNA Marker (M), aftrer restriction (1) and before restriction (2).

The orientation of the GOX-Xho gene was confirmed by sequencing. The sequencing of pTA2-GOX-Xho was carried out using the universal T7 Promoter forward primer and T3 Binding Site reverse primer. These primers were used to verify the presence and orientation of the

GOX-Xho gene. The sequencing results indicated that the gene was inserted in a positive orientation. Sequencing with the T7 Promoter forward primer revealed the presence of *Hind*II, *Eco*RV, and *Eco*RI restriction sites within the multiple cloning site area, as well as the GOX-Xho gene, which included an additional *Xho*I (C^TCGAG) restriction site at the 5' end, Kex2 cleavage site (AAAAGA) and a spacer peptide EAEA (GAAGCTGAAGCT), as shown in Figure 6A. Sequencing with the T3 Primer Binding Site reverse primer identified *Xba*I, *Spe*I, *Bam*HI, *Sma*I, *Pst*I, and *Eco*RI restriction sites within the multiple cloning site, along with the GOX-Xho gene featuring the addition of *Xho*I and *Xba*I (T^CTAGA) restriction sites at the 3' end as depicted in Figure 6B. The sequencing results confirmed that the GOX-Xho gene is 1797 bp, comprising 1749 bp of ORF and 48 bp of additional features.

BLAST (Basic Local Alignment Search Tool) analysis is used to identify nucleotide sequence similarities based on local alignment between the query sequence and the target sequence found in the database[24]. The analysis revealed that the GOX-Xho sequence in pTA2-GOX-Xho shares similarities with the GOX gene of *A. niger* IPBCC 08.610 (99.66%), *A. niger* Z-25 (98.40%), and *A. costaricaensis* CBS115574 (98.34%). Figure 7 illustrates the alignment of GOX-Xho amino acids with those of *A. niger* IPBCC 08.610 (AYH52696.1).

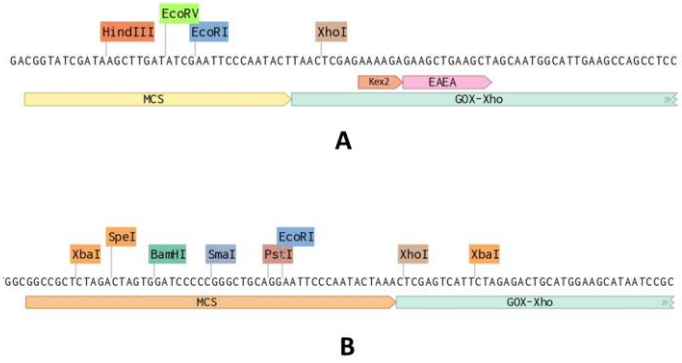

Figure 6. pTA2-GOX-Xho sequence
Forward primer T7 Promoter (A) and reverse primer T3 Primer Binding Site (B)

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Page 1 GOX-Xho Amino Acid, AYH52696.1 glucose oxidase [Aspergillus niger]

                1                                                                                                   112
GOX-Xho A... MQTLLVSSLVVSLAAALPHYIRSNGIEASLLTDPKDVTGRTVDYIIAGGGLTGLTTAARLTENPNITVLVIESGSYESDRGPIIEDLNAYGDIFGSSVDHAYETVELATNNQ
GOX-Xho A... ----------------------SNGIEASLLTDPKDVTGRTVDYVIAGGGLTGLTTAARLTENPNITVLVIESGSYESDRGPIIEDLNAYGDIFGSSVDHAYETVELATNNQ
AYH52696.... MQTLLVSSLVVSLAAALPHYIRSNGIEASLLTDPKDVTGRTVDYIIAGGGLTGLTTAARLTENPNITVLVIESGSYESDRGPIIEDLNAYGDIFGSSVDHAYETVELATNNQ
.....................................................................................................................

                113                                                                                                 224
GOX-Xho A... TALIRSGNGLGGSTLVNGGTWTRPHKAQVDSWETVFGNEGWNWDSVAAYSLQAERARAPNAKQIAAGHYFNASCHGLNGTVHAGPRDTGDDYSPIVKALMSVVEDRGVPTKK
GOX-Xho A... TALIRSGNGLGGSTLVNGGTWTRPHKAQVDSWETVFGNEGWNWDSVAAYSLQAERARAPNAKQIAAGHYFNASCHGLNGTVHAGPRDTGDDYSPIVKALMSVVEDRGVPTKK
AYH52696.... TALIRSGNGLGGSTLVNGGTWTRPHKAQVDSWETVFGNEGWNWDSVAAYSLQAERARAPNAKQIAAGHYFNASCHGLNGTVHAGPRDTGDDYSPIVKALMSVVEDRGVPTKK
.....................................................................................................................

                225                                                                                                 336
GOX-Xho A... DLGCGDPHGVSMFPNTLHEDQVRSDAAREWLLPNYQRPNLQVLTGQYVGKVLLSQNATTPRAVGVEFGTHKGNTHNVYAKHEVLLAAGSAVSPTILEYSGIGMKSILEPLGI
GOX-Xho A... DLGCGDPHGVSMFPNTLHEDQVRSDAAREWLLPNYQRPNLQVLTGQYVGKVLLSQNATTPRAVGVEFGTHKGNTHNVYAKHEVLLAAGSAVSPTILEYSGIGMKSILEPLGI
AYH52696.... DLGCGDPHGVSMFPNTLHEDQVRSDAAREWLLPNYQRPNLQVLTGQYVGKVLLSQNATTPRAVGVEFGTHKGNTHNVYAKHEVLLAAGSAVSPTILEYSGIGMKSILEPLGI
.....................................................................................................................

                337                                                                                                 448
GOX-Xho A... DTVVDLPVGLNLQDQTTSTVRSRITSAGAGQGQAAWFATFNETFGDYTEKAHELLNTKLEQWAEEAVARGGFHNTTALLIQYENYRDWIVKDNVAYSELFLDTAGVASFDVW
GOX-Xho A... DTVVDLPVGLNLQDQTTSTVRSRITSAGAGQGQAAWFATFNETFGDYTEKAHELLNTKLEQWAEEAVARGGFHNTTALLIQYENYRDWIVKDNVAYSELFLDTAGVASFDVW
AYH52696.... DTVVDLPVGLNLQDQTTSTVRSRITSAGAGQGQAAWFATFNETFGDYTEKAHELLNTKLEQWAEEAVARGGFHNTTALLIQYENYRDWIVKDNVAYSELFLDTAGVASFDVW
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                449                                                                                                 560
GOX-Xho A... DLLPFTRGYVHILDKDPYLRHFAYDPQYFLNELDLLGQAAATQLARNISNSGALQTYFAGETIPGDNLAYDADLSAWVEYIPYNFRPNYHGVGTCSMMPKEMGGVVDNAARV
GOX-Xho A... DLLPFTRGYVHILDKDPYLRHFAYDPQYFLNELDLLGQAAATQLARNISNSGALQTYFAGETIPGDNLAYDADLSAWVEYIPYNFRPNYQGVGTCSVMPKEMGGVVDNAARV
AYH52696.... DLLPFTRGYVHILDKDPYLRHFAYDPQYFLNELDLLGQAAATQLARNISNSGAMQTYFAGETIPGDNLAYDADLSAWVEYIPYNFRPNYHGVGTCSMMPKEMGGVVDNAARV
.....................................................................................................................

                561                                 605
GOX-Xho A... YGVQGLRVIDGSIPPTQMSSHVMTVFYAMALKIADAVLADYASMQ
GOX-Xho A... YGVQGLRVIDGSIPPTQMSSHVMTVFYAMALKIADAVLADYASMQ
AYH52696.... YGVQGLRVIDGSIPPTQMSSHVMTVFYAMALKIADAVLADYASMQ
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Figure 7. Alignment of GOX-Xho amino acids with GOX amino acids of *A. niger* IPBCC 08.160 (AYH52696.1). Red mark indicates the position of the mutation on GOX-Xho gene.

The BLAST analysis results indicate that the similarity percentage between GOX-Xho and the GOX gene of *A. niger* IPBCC 08.610 did not reach 100%. This difference can be attributed to several factors:66 bp in the signal peptide and three bp in stop codons from the GOX gene, an addition of 48 bp of features in the GOX-Xho gene, and the presence of several mutations in the GOX-Xho gene. Mutations are alterations in the sequence of nucleotide bases in a gene, manifesting as point mutations—changing one nucleotide base to another—or as insertions and deletions. It causes shifts in the nucleotide sequence DNA polymerase in synthesizing and replicating polynucleotides, cause nucleotide sequencemismatches[25].

The GOX-Xho gene exhibited several point mutations, leading to changes in amino acids, notably at positions I23V, M480L, H516Q, and M523V, as shown in Table 4. The active site of GOX is located at residues glutamic acid (E)412, H516, and H559. Notably, H516 is crucial for facilitating proton transfer from glucose. Mutations can alter enzyme properties, affecting both structure and function, potentially enhancing flexibility and catalytic activity[26].

Computational methods such as molecular dynamics suggest that changing the amino acid H559 to aspartic acid (D) could theoretically reduce the Km value, which is beneficial for gluconic acid production[27]. Similarly, modifying the amino acids H516 to arginine (R) and aspartic acid (D) may increase the Km value, potentially enhancing GOX activity in enzymatic fuel cells[28]. For instance, a mutation in the active site of GOX from *Penicillium amagasakiense*, changing R516 to Q516, resulted in a 120-fold increase in the apparent Km value[29]. Computational modeling is essential for predicting how mutations affect the structure and activity of GOX.

Table 4. Amino acid changes of the GOX-Xho gene before and after mutation

Mutant	Amino acid		Codon position *ORF	Codon	
	Before mutation	After mutation		Before mutation	After mutation
I23V	I23	V23	65-69	ATC	GTC
M480L	M480	L480	1436-1439	ATG	TTG
H516Q	H516	Q516	1571-1574	CAT	CAA
M523V	M523	V523	1592-1593	ATG	GTG

Notes: I = isoleucine, V = valine, M = methionine, L = leucine, H = histidine, Q = glutamine

4. CONCLUSIONS

The GOX-Xho gene was successfully modified using the PCR method, incorporating additional features. This includes the insertion of the *Xho*I restriction sites at both 5' and 3' ends, a Kex2 cleavage site, an EAEA peptide spacer at the 5' end, an *Xba*I restriction site, and a stop codon at the 3' end, resulting in a sequence of 1797 bp comprising an ORF of 1749 bp and 48 bp of additional features. Restriction analysis of pTA2-GOX-Xho with *Xba*I indicated that the GOX-Xho gene was inserted in a positive orientation. Sequencing results identified mutations in the GOX-Xho gene, highlighting the importance of further investigation into their effects on the enzyme's structure and function before proceeding with recombinant protein expression.

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