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Optimization of Synthetic Glucose Oxidase Gene Using A Recombinant Combination Strategy in Pichia Pastoris GS115

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Abstract

Glucose oxidase (e.C1.1.3.4) is a flavoenzyme that catalyzes the oxidation of β-D-glucose to D-gluconolactone and hydrogen peroxide. The D-gluconolactone hydrolyzed spontaneously produces gluconic acid. Glucose oxidase has been widely used in chemical, food industries, and medicine. In this study, the glucose oxidase gene was identified in the chromosomal DNA of Aspergillus niger and isolated by PCR techniques. The glucose oxidase gene was then synthetically designed, optimized, cloned, and transferred into suitable hosts. The constructed clones were selected to modify the best production and combined promoter systems to produce glucose oxidase enzymes. In this design, the PCR product fragment was cloned into pPIC9 plasmids and transferred into Pichia pastoris GS115 by electroporation. The constructed strain was named pp.pPIC9/GOX. This strain was selected as a host for the other synthetic construct called pGAPZ/GOX. The strain pp.pPIC9/GOX was transformed as the host for the pGAPZ/GOX construct containing the glucose oxidase gene, which is controlled by the pGAPZa promoter with a self-inducing promoter and the newly constructed strain named pp.pPGAPZ/PIC9/GOX. The expression and comparison of the glucose oxidase activity were investigated by combining methanol and self-induction promoter in both pp.pPIC9/GOX and pp.pPGAPZ/PIC9/GOX strains simultaneously, which led to higher glucose oxidase gene activity in the new strain. Using the methanol induction promoter with the simultaneous expression of self-induction promoter in separate constructs and same strains increased the glucose oxidase gene activity.

Keywords*:* Glucose Oxidase; Pichia Pastoris GS115; Recombinant Combination Strategy.

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Introduction

Glucose oxidase (beta-di-glucose: oxygen 1-oxide and reductase 4, 3, 1, and 1 EC) catalyzes the oxidation of β-di-glucose along with molecular oxygen. This reaction consists of two separate steps: 1) Substrate oxidation and enzyme reduction in the reduced phase and 2) Enzyme re-oxidation in the oxidation phase [1]. Muler (1928), for the first time, reported the activity of this enzyme in an extract of Aspergillus niger culture, and after that, this enzyme was purified directly from Aspergillus strains [2]. However, commercial glucose Oxidase enzymes are derived from fungal sources, such as Aspergillus and Klebsiella strains [3, 4].

Aspergillus niger glucose oxidase is an enzyme that consists of two subunits with a molecular weight of 150 kDa, including FAD molecules attached directly to the enzyme in full configuration [5, 3]. Whittingtonin (1990) produced a high level of a recombinant enzyme by simulating the enzyme with expression in Saccharomyces cerevisiae strains [6, 7]. In another study, Kim et al. purified this enzyme from Aspergillus niger ACMO4 [8].

The application of this enzyme is used in the chemical industry to produce organic acids, including gluconic acid applicable in health and beauty industries, such as toothpaste, mouthwash, liquid soap, face cream, and protective lotion for skin and hair [9, 5]. This Acid is obtained from different sources, such as bacteria or fungi, and can be intracellular, extracellular, or attached to cell membranes [10, 11].

The Pichia pastoris expression system is beneficial for producing complex proteins that require post-translational modifications (such as disulfide bonds) [12]. Alcohol oxidase 1 (AOX1) gene, promoter of AOX1, is known as one of the most potential promoters and stimuli of eukaryotic transcription in Pichia Pastoris system [13]. It was developed successfully as a drug inhibitor of Calicrin in 2009 [14]. A high cell density in the culture medium was achieved (200 g / l dry weight)[15]. However, Saccharomyces cerevisiae proteins remain predominantly in plasma with the ability to make post-translational changes, such as polypeptide structure, disulfide bond formation, and glycosylation [16].

The content of intracellular proteins in the extracellular environment is relatively low; however, the purification of secreted proteins is relatively easy compared to the purification of cellular proteins [17, 18]. One of the advantages of this system is the expression of recombinant protein in the absence of mannose transferase, which is highly allergenic and is present in the yeast Saccharomyces cerevisiae [19], and no endotoxin and phage contamination was found in this system [20].

In the Pichia Pastoris system, the genome can accept a foreign gene and produce recombinant proteins [21] with the expression system available as a kit. [12]. However, Pichia pastoris is not an ideal host in all respects, and there have been cases of low production performance or inability to express such genes [22]. Pichia pastoris system cannot be modified after human gene translation [16] as, in some cases, added sugars to human proteins that are not naturally found in these proteins [23].

Another common problem with dysfunctional secretion in complex secreted proteins is methanol as the flammable elements in media [24], and storing the large amounts required for these processes is undesirable. Moreover, methanol is mainly obtained from petrochemical sources that cannot be used to produce food and additives [25].

The other promoter (GAP promoter) is powerful and durable, a cost-effective method for the large-scale production of recombinant proteins [26]. The amount of recombinant protein produced using the GAP promoter has been shown to be equal to or greater than that produced by the AOX promoter [27]. The glyceraldehyde 3-phosphate dehydrogenase gene is conserved in many organisms, including Pichia pastoris. The GAP gene encodes the known protein glyceraldehyde 3-phosphate dehydrogenase, which is used to express high levels of recombinant proteins in Pichia pastoris [28]. This promoter is designed to express high levels of the gene in Pichia pastoris yeast. Simulated genes are regulated by the pGAPZαA expression transporter promoter, which can produce more products than the resulting expression vector. However, the function of any foreign protein expressed in Pichia yeast also depends on the toxicity degree of the recombinant protein produced for the host [29].

Recent studies demonstrated that recombinant protein technology combines AOX1 with GAP promoters [30]. A statistically significant difference in cell growth intensity and host protein secretion was almost twice more than the GAP promoter [31]. One of the key criteria for producing foreign proteins is to use an appropriate promoter to increase the transcription efficiency in gene expression [32]. In Pichia pastoris, promoters AOX1 and AOX2 were identified as the original natural promoters. Subsequently, in Pichia pastoris, three phenotypes appeared. The success of the Lactobacillus Pieris expression system is mainly due to the presence of the AOX promoter system [33].

The AOX promoter system is strongly stimulated by methanol and blocked by other carbon sources, such as

glycerol and glucose [21, 29]. In a methanol-containing medium as a carbon source, AOX mRNA contains more than five percent RNA storage [21]. In other words, when grown as a carbon source in a methanol-containing medium, the abundance of the enzyme alcohol oxidase-1 can reach 30% of the cell protein concentration [21, 32]. This shows the excellent potential of the alcohol oxidase 1 promoter, which makes up 85% of the alcohol oxidase activity [34-37].

In contrast, the enzyme alcohol oxidase 2 is controlled by a weaker promoter, called the alcohol oxidase 2 promoter, which accounts for 15% of the alcohol oxidase activity in the cell [35, 38]. In addition, new promoters have been isolated for the expression of foreign genes and used economically, including glyceraldehyde phosphate dehydrogenase [22, 29, 33], Formaldehyde hydrogenase [22, 29], and Dihydroxyacetone synthetase (DHAS) [39]. These promoters are present in the yeast Pichia pastoris [28].

Biochemical studies have shown that the use of methanol requires specific metabolic pathways with several different enzymes [40]. In this pathway, the formaldehyde dehydrogenase converts formaldehyde to formic acid and carbon dioxide [41, 42]. The presence of methanol, formaldehyde, and formic acid together with glucose and ethanol stimulate the synthesis of methanol metabolizing enzymes [40, 43, 44]. The main enzymes in this reaction are formaldehyde dehydrogenase, format dehydrogenase, and dihydroxyacetone synthase [45].

In this study, two combined promoter systems are used for the high production of glucose oxidase enzyme. The glucose oxidase gene, which contains the natural sequence secretion signal, was cloned into the pPIC9 plasmids and transferred by electroporation into Pichia pastoris genome to prepare pp.pPIC9/GO host. Then, another construct with the pGAPZa promoter, which was the self-induced promoter, was transformed into the pp.pPIC9/GO host to make the final strain named pp.pGAPZ/pPIC9/GO. The expression of glucose oxidase genes was compared in both pp.pPIC9/GO and pp.pGAPZ/pPIC9/GO strains.

Materials and Methods

Chemical solutions

Chemical solutions in this study include 4-amino antipyrine, sodium dodecyl sulfate, phenol, glycerol, methanol, ethanol, bermophenol blue, hydrochloric acid, glacial acetic acid, agar, ammonium sulfate (APS), sodium chloride (Nacl), Glycine, Kumasi Blue, Trisbazi, Acrylamide, Glucose, H2O2, TCA, (SDS), beta mercaptoethanol, sodium hydroxide (NaoH), and phenylmethyl sulfonyl fluoride (PMSF). We purchased the solustions from Sigma.

Antibiotics

Ampicillin and zeosin antibiotic (Invitrogen) at a 100 mg / ml concentration were used. To prepare a concentration of 100 mg / ml, we dissolved 100 mg of antibiotic powder in 1 ml of sterile distilled water and stored it in a freezer)

Antibodies

Rabbit serum immunized with pure recombinant glucose oxidase produced in E. coli was used as the primary antibody and HRP (Proxidase Anti Rabit) as the secondary antibody in this study. Cyto Matin Gene Company provided the antibodies.

Strains

Yeast pichia pastoris GS115, provided by the National Institute of Genetic Engineering and Biotechnology, and was used in this study.

Plasmids

Two types of plasmids, Ppic9 and PpGAPZa, were obtained from the National Institute of Genetic Engineering and Biotechnology and used in this work.

Enzymes

Peroxidase and glucose oxidase enzymes purchased from Sigma were used in this study.

Markers

The protein molecular weight marker was purchased from the National Institute of Genetic Engineering and Biotechnology (NIGEB) protein purification group.

Culture medium

YPD liquid culture medium: Ypd liquid culture medium contains 1 g Yeast Extract, 2g Pepton, and 2ml Glucose20%, and the desired medium reaches 100 ml volume (filter the glucose solution separately and autoclave the rest of the culture medium at 121 ° C for 15 minutes). After reaching 50 ° C, it was added to the antibiotic ampicillin. All materials were provided by Sigma Company.

YPD solid culture medium: YPD solid culture medium contains 1 g yeast Extract, 2 g pepton, 2 ml Glucose 20%, and Agar 1.5 g. Dilute the medium to 100 ml (filter the glucose solution separately and autoclave the rest of the culture medium at 121 ° C for 15 minutes). After reaching 50 ° C, it is added to the antibiotic ampicillin. Then, we pour about 20 to 25 ml per plate, and after hardening, the medium is stored at 4

° C. All materials were provided by Sigma Company.

BMGY culture medium: BMGY is prepared using the following combination: yeast Extract 1%, Pepton 2%, YNB 1.34%, Potassium Phosphate buffer M 0.1 PH: 6, Biotin 5-10. 4% and 1% Glycerol. After bringing the pH to 6, it is autoclaved at 121 ° C for 15 minutes (YNB solution must be filtered).

BMMY culture medium: BMMY culture medium includes yeast Extract 1%, pepton 2%, potassium phosphate buffer 0.1M (PH: 6, biotin 5-10. 4%, YNB 1.34%, and methanol 1%. All materials are provided by Sigma Company.

Solutions required to determine enzyme activity

Solutions required to determine the enzyme's activity include 8gr NaCL phosphate buffer, 0.2g kcl, NA2HPO4 1.44gr, DW 800cc PH: 7.4, KH2po4 0.24g, phenol 6%, peroxidase enzyme, and 4-aminopyrine.

Preparation of Pichia Pastoris susceptible cells for electroporation

The following method was adhered to base on the protocol recommended by Invitrogen. Pichia pastoris colonies were cultured in 5 ml YPD medium in Falcon at 30 °C overnight. Then, we added 2 µl of overnight culture to 500 ml of fresh medium and incubated overnight in Erlenmeyer to increase the OD of the cells to 1.5-3.1. The cells were then centrifuged at 1500 ° C for 5 minutes at a temperature of 4 °C, and the supernatant was discarded. The collected precipitate was again mixed with 500 ml of sterile cold water. We then centrifuged the cells, discarded the supernatant, mixed the precipitate in 20 ml of cold sorbitol, and placed the cells on ice.

Electroporation

To integrate the plasmid containing the recombinant protein gene in the genome Pichia pastoris, the Invitrogen method was used. We mixed 80 µl of the susceptible cell of Pichia pastoris with 5-10 µg of linearized DNA in 5-10 µl of sterile water to 0.2 and transferred it to pre-ice cold electroporation (0° C). Next, we incubated the cell contents on ice for 5 minutes and applied the electroporation according to the parameters of Saccharomyces cerevisiae yeast, which is a recommended electroporation device (220Ω, 25F25, V1500). Immediately, we added 1 ml of ice-cold sorbitol to the cuvette and mixed the resulting solution. The cuvette contents were transferred to a sterile 15 ml falcon and incubated at 30 ° C without shaking for 1-2 hours. Then, we poured 25, 50, 100, and 200 microliters of Falcon contents on YPD plates containing 100 / g / ml of Zeosin antibiotic and spread them on the plate with a pasteurizer pipette. The plates were incubated for 2–4 days at 30 ° C to form colonies. We then selected 10-20 colonies and transferred them to fresh YPD-Agar plates containing 100 / g / ml zeocin antibiotic. Finally, the transformed cells were purified.

Preparation of SDS-PAGE gel electrophoresis

SDS-PAGE (SDS poly Acrylamide Gel Electrophoresis) was used to separate and determine the molecular weight of proteins. To prepare this gel, 30% acrylamide + 0.8% bisacryl amide, 1.5 M tris base solution = 0.4 + (pH% SDS (buffer X 4 lower gel), electrode buffer or tank buffer (1000 ml), sample buffer (2X), and 10% ammonium persulfate were used.

Enzyme assay method

The enzyme assay was performed using the Omodeo method. In the presence of glucose oxidase, glucose was converted to Glucono Delta Lactone, and H2O2 was produced. Due to the reaction of phenol and enzyme peroxidase and the bonding between phenol and 4 amino antipyrines, a red substance called quinoaimine was produced. The absorption was calculated at 500 nm.

Results

Construction of pp.pPIC9 / GO strain

In our previous study, pPIC9 / GO construction was developed by the research team to express the glucose oxidase gene in Pichia pastoris. After confirmation, this construct was transferred into the P. pastoris GS115 cells, and the clone was named pp.pPIC9 / GO. This clone was used as the host for the next construction.

Designing and Optimization of the glucose oxidase and pGAPZ promoter sequences synthetically in pGAPZaA vector to make a novel pGAPZαA / GO construct

The following sequences were designed, optimized, and synthesized for the glucose oxidase gene and cloned from the Xho1 and Not1 sites in the pGAPZαA vector. The construct was named pGAPZαA / GO. Figure 1 shows the complete sequences of pGAPZαA / GO construction. This construct was transferred into the pp.pPIC9 / GO Steain.

The synthetic gene in pGAPZaA: Confirmation and location

The location (A) and the frame confirmation (B) of the glucose oxidase gene in the pGAPZαA vector are shown in Figure 2.

pGAPZαA- XhoI – GO—NOT1 – XbaI:

AGATCTTTTTTGTAGAAATGTCTTGGTGTCCTCGTCCAATCAGGTAGCCATCTCTGAAATATCTGGCTCCGTTGCAACTCCGAACG
ACCTGCTGGCAACGTAAAATTCTCCGGGGTAAAACTTAAATGTGGAGTAATGGAACCAGAAACGTCTCTTCCCTTCTCTCTCCTTC
CACCGCCCGTTACCGTCCCTAGGAAATTTTACTCTGCTGGAGAGCTTCTTCTACGGCCCCCTTGCAGCAATGCTCTTCCCAGCATTA
CGTTGCGGGTAAAACGGAGGTCGTGTACCCGACCTAGCAGCCCAGGGATGGAAAAGTCCCGGCCGTCGCTGGCAATAATAGCGG
GCGGACGCATGTCATGAGATTATTGGAAACCACCAGAATCGAATATAAAAGGCGAACACCTTTCCCAATTTTGGTTTCTCCTGACC
CAAAGACTTTAAATTTAATTTATTTGTCCCTATTTCAATCAATTGAACAACTATTTCGAAACGATGAGATTTCCTTCAATTTTTACT
GCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAG
CTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTA
TAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCT**CTCGAG**AAGAGACAGACCTTGTTGGTTTCCTCCTTGGT
TGTTTCTTTGGCTGCTGCTTTGCCACACTACATCAGATCCAACGGTATTGAGGCTTCCTTGCTGACTGATCCAAAGGACGTTTCCGG
TAGAACCGTCGACTACATTATTGCTGGTGGTGGTTTGACCGGTTTGACTACTGCTGCTAGATTGACTGAGAACCCCAACATCTCCG
TTTTGGTTATTGAGTCCGGTTCCTACGAATCCGACAGAGGTCCAATTATCGAGGACTTGAACGCTTACGGTGACATCTTCGGTTCT
TCCGTTGATCACGCTTACGAGACTGTTGAGTTGGCTACCAACAACCAGACTGCCTTGATTAGATCCGGTAACGGTCTTGGTGGTTC
CACTTTGGTTAACGGTGGTACTTGGACTAGACCACACAAGGCTCAAGTTGACTCTTGGGAGACTGTTTTCGGTAACGAAGGTTGG
AACTGGGACAACGTTGCTGCTTACTCCTTGCAAGCTGAAAGAGCTAGAGCACCAAACGCTAAGCAAATTGCTGCTGGTCACTACT
TCAACGCCTCTTGTCATGGTGTTAACGGTACTGTTCATGCCGGTCCAAGAGATACTGGTGATGACTACTCTCCAATCGTCAAGGCT
TTGATGTCTGCCGTTGAGGATAGAGGTGTCCCTACCAAGAAAGACTTCGGTTGTGGTGATCCACACGGTGTCTCTATGTTTCCAAA
CACCTTGCACGAGGACCAGGTTAGATCAGACGCTGCTAGAGAATGGTTGCTGCCAAACTACCAAAGACCAAACTTGCAGGTCCTG
ACTGGTCAGTACGTTGGTAAGGTTTTGCTGTCCCAGAACGGTACTACTCCAAGAGCTGTTGGTGTTGAGTTCGGTACTCACAAGGG
TAACACCCACAACGTTTACGCCAAGCACGAAGTTTTGTTGGCTGCAGGTTCTGCTGTTTCCCCAACCATTTTGGAATACTCCGGTA
TCGGTATGAAGTCCATCTTGGAGCCATTGGGTATCGACACCGTTGTTGATTTGCCAGTCGGTTTGAACTTGCAAGACCAGACTACT
GCCACCGTCAGATCCAGAATTACTTCTGCTGGTGCTGGTCAAGGTCAGGCTGCTTGGTTTGCTACTTTCAACGAAACCTTCGGTGA
CTACTCCGAAAAGGCTCACGAGTTGTTGAACACCAAGTTGGAGCAATGGGCTGAAGAGGCTGTTGCTAGAGGTGGTTTTCACAAC
ACTACCGCCTTGTTGATCCAGTACGAGAACTACAGAGACTGGATCGTCAACCACAACGTCGCTTACTCTGAGTTGTTCTTGGATAC
TGCTGGTGTTGCCTCTTTCGACGTTTGGGATTTGTTGCCATTCACCAGAGGTTACGTCCACATCTTGGATAAGGACCCATACTTGCA
CCACTTCGCTTACGACCCTCAGTACTTCTTGAACGAGTTGGACTTGTTGGGTCAAGCTGCTGCTACTCAGTTGGCTAGAAACATTT
CTAACTCCGGTGCCATGCAGACTTACTTCGCCGGTGAAACTATTCCAGGTGACAACTTGGCTTACGATGCTGACTTGTCTGCTTGG
ACTGAGTACATCCCATACCACTTCAGACCTAACTACCACGGTGTTGGTACTTGCTCCATGATGCCAAAAGAAATGGGTGGTGTTGT
TGACAACGCCGCCAGAGTTTACGGTGTTCAAGGTTTGAGAGTCATCGACGGATCCATTCCACCAACTCAAATGTCCTCTCACGTCA
TGACCGTTTTCTACGCTATGGCCTTGAAGATCTCCGACGCTATTTTGGAGGACTACGCCTCCATGCAGTAA**GCGGCCGC**T**CTAGA**A
CAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATTGAGTTTTAGCCTTAGACATGACTGT
TCCTCAGTTCAAGTTGGGCACTTACGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCATTTGCCTGAGA
GATGCAGGCTTCATTTTTGATACTTTTTTATTTGTAACCTATATAGTATAGGATTTTTTTTGTCATTTTGTTTCTTCTCGTACGAGCTT
GCTCCTGATCAGCCTATCTCGCAGCTGATGAATATCTTGTGGTAGGGGTTTGGGAAAATCATTCGAGTTTGAGTTTTTCTTGGTATT
TCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAGACCTTCGTTTGTGCGGATCCCCCACACACCATAGCTTCAAAATGTTTCTA
CTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTTCAAAACACCCAAGCACAGCATACTAAATTTTCC
CTCTTTCTTCCTCTAGGGTGTCGTTAATTACCCGTACTAAAGGTTTGGAAAAGAAAAAAGAGACCGCCTCGTTTCTTTTTCTTCGTC
GAAAAAGGCAATAAAAATTTTTATCACGTTTCTTTTTCTTGAAATTTTTTTTTTTAGTTTTTTTCTCTTTCAGTGACCTCCATTGATA
TTTAAGTTAATAAACGGTCTTCAATTTCTCAAGTTTCAGTTTCATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGTTCATTAGA
AAGAAAGCATAGCAATCTAATCTAAGGGCGGTGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACAAGGT
GAGGAACTAAACCATGGCCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCCGGAGCGGTCGAGTTCTGGACC
GACCGGCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACGACGTGACCCTGTTCATCAGCGCGG
TCCAGGACCAGGTGGTGCCGGACAACACCCTGGCCTGGGTGTGGGTGCGCGGCCTGGACGAGCTGTACGCCGAGTGGTCGGAGG
TCGTGTCCACGAACTTCCGGGACGCCTCCGGGCCGGCCATGACCGAGATCGGCGAGCAGCCGTGGGGGCGGGAGTTCGCCCTGCG
CGACCCGGCCGGCAACTGCGTGCACTTCGTGGCCGAGGAGCAGGACTGACACGTCCGACGGCGGCCCACGGGTCCCAGGCCTCG
GAGATCCGTCCCCCTTTTCCTTTGTCGATATCATGTAATTAGTTATGTCACGCTTACATTCACGCCCTCCCCCCACATCCGCTCTAA
CCGAAAAGGAAGGAGTTAGACAACCTGAAGTCTAGGTCCCTATTTATTTTTTTATAGTTATGTTAGTATTAAGAACGTTATTTATA
TTTCAAATTTTTCTTTTTTTTCTGTACAGACGCGTGTACGCATGTAACATTATACTGAAAACCTTGCTTGAGAAGGTTTTGGGACGC
TCGAAGGCTTTAATTTGCAAGCTGGAGACCAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTG
CTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGAC
TATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCT
TTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCT
GTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTA
TCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTA
ACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTG
ATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAA
GATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGCATGAGATC

Figure 1. The sequence of the Synthetic glucose oxidase gene and pGAPZαA vector. The Glucose oxidase gene cloning sites of Xho1 and Not1 are marked in red.

Confirmation of the constructed plasmids in cloning vector by gel electrophoresis

After verifying the sequencing frames in both constructs for the glucose oxidase gene under the control of AOX and PGAPZ promoters, we transferred the constructs into relative hosts through electroporation, and the screening pPGAPZαA / GO colonies were isolated due to zeocin-resistant colonies.

A

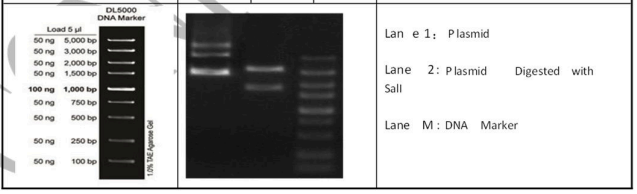
Certificate of Analysis

Gene Name	XhoI-Gene-XbaI	Order No.	704030-9
Lot No.	V12080	Cloning Vector	pGAPZ αA
Cloning Sites	XhoI(first)--XbaI	Insert Size	1841 bp
C ompetence	TOP10	Vector Resistance	Zeocin

QC Results

Test Items	Specifications	Results
Insert Sequence	Insert sequence results consistent with target	Pass
Vector Sequence	Flanking sequence consistent with expected	N/A
ORF Across Junction	Correct and consistent with target	N/A
Restriction Digest	Expected fragment sizes observed	N/A
PCR Amplification	Correct without non - specific bands	Pass
DNA Quantity/Quality	Actual (by A 260)	5μg
	Concentration (n/a if lyophilized)	N/A
	Purity (A 260 /A 280 = 1.8 - 2.0)	Pass
	# of Tubes	1
	Matrix	TE (lyophilized)
Endotoxin Test	Verified, <0.1 EU/ μg (Endo-Free Preps Only)	N/A
Appearance	Clear, no visible particles	Pass
Label	Correct and white	Pass
Comments	-	-

Restriction Digestion Map



Certified by: M

B

Figure 2. The location and confirmation of the cloned gene in the pGAPZαA / GO construct: (A) circular map of pGAPZαA / GO , (B) the confirmation certificate of the correct cloning of the synthetic gene in pGAPZαA / GO by the manufacturer company

Five Zeusen-resistant colonies were isolated for later studies. The extraction of PIC9 / GO and PGAPZαA / GO constructs is confirmed by the electrophoresis gel in Figure 3.

The pGAPZαA/Go construct was transformed using the electroporation method into the pp.pPIC9 / GO

strain. In this new strain, both AOX1 and GAP promoters were combined for recombinant proteins separately in the same host for the simultaneous expression system. However, many genes were used as markers in piciha pastoris. Some markers are related to the histidine biosynthesis pathway. However, it was not applicable in this work as the pPIC9 construct previously was cloned in the host, and the histidine selection mutant was impossible. Therefore, another selective marker, zeocin, was used to screen transformed cells to ensure plasmid survival through subsequent generations. The transformed colonies were isolated based on ziocin marker in screening and after repeated passages in plates containing plates ziocin antibiotic marker. The recombinant strain named pp.pGAPZ/pPIC9/GO.

SDS Page and DOT BLOT of strains pp.pPIC9 / GO and pp.pGAPZ / pPIC9 / GO

Analyzing SDS-PAGE protein profiles and enzymatic activity in yeast culture medium containing recombinant construct showed that the cloned gene was translated into protein and included a band of 65.9 kDa. This is similar to the glucose oxidase standard protein size in both clones of pp.pPIC9 / GO and pp.pGAPZ / pPIC9 / GO (Figure 4 A). The strains containing the recombinant plasmids ppPIC9 / GO and pp.pGAPZ / pPIC9 / GO secreted the enzyme glucose oxidase into the culture medium, which is a more accurate method to detect the enzyme in both strains. For this purpose, a dot blot was used to identify specific proteins through monoclonal or polyclonal antibodies (Figure 4 B).

Dot blot of glucose oxidase was used to identify GO enzyme in both strains containing recombinant plasmid pPIC9 / GO and pGAPZ / pPIC9 / GO. Line 1- Dot blot glucose oxidase related to the pp.pPIC9 / GO strain, Line 2,4 – Pichia Pastoris GS115 cells lacking glucose oxidase gene as negative the control. Line 3- Dot blot of Glucose oxidase enzyme as a positive control, Line 5 - Pichia Pastoris GS115 cells without glucose oxidase gene strain as the negative control (B).

Comparison and evaluation of the enzymatic activity of pp.pPIC9 / GO and pp.pGAPZ / pPIC9 / GO strains

The enzymatic activity of pp.pPIC9 / GO and pp.pGAPZ / pPIC9 / GO strains was compared based on culture duration using buffer phosphate at 82 ° C and 225 rpm. Enzyme production by the ppic9-GOX strain is shown in Figure 5 A. The standard glucose oxidase curve and the amount of enzyme production are depicted. The linear slope equation obtained from the standard diagram is shown in Figure 5 B: Y = 0591/1 X +0073/0.

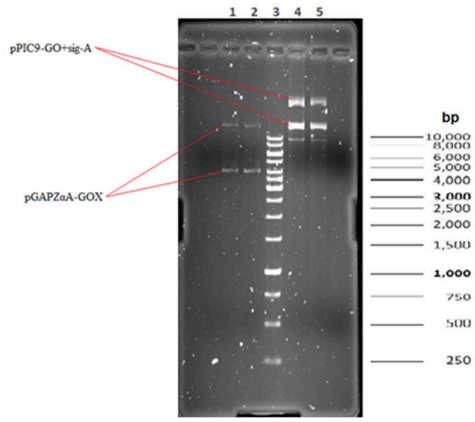
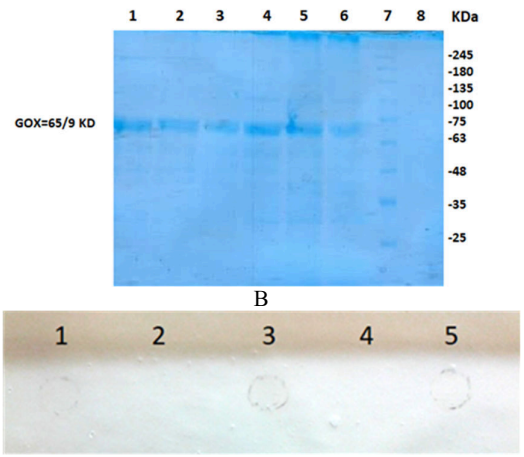


Figure 3. Extraction of pPIC9 / GO and pGAPZαA / GO plasmid structures, Lines 1 and 2 of the pGAPZαA / GO plasmid construct, Line 3- Molecular weight marker 1 Kb, Lines 4 and 5 of the pPIC9 / GO plasmid construct, pGAPZαA / GO structure transfer to host pp.pPIC9 / GO

A



B

Figure 4. Protein profiles in Polyacrylamide gel electrophoresis of glucose oxidase gene expression from pp.pPIC9 / GO and pp.pGAPZ / pPIC9 / GO strains .Lines 1, 2, and 3 belong to pp.pGAPZ / pPIC9 / GO strain, lines 4, 5 and 6 belong to pp.pPIC9 / GO strain, line 7 corresponds to protein molecular marker, and line 8 belongs to p.Pasrtoris GS115 strain (A).

The highest level of glucose oxhdase activity was obtained in pp.pGAPZ / pPIC9 / GO strain, 0.1 and pp.pPIC9 / GO strain, 0.06, based on optical density according to following linear equation:

Y = 0591/1 × (0.1) +0073/0 = 0.11321

Y = 0591/1 × (0.06) +0073/0 = 0.07084

Glucose oxidase production was estimated in

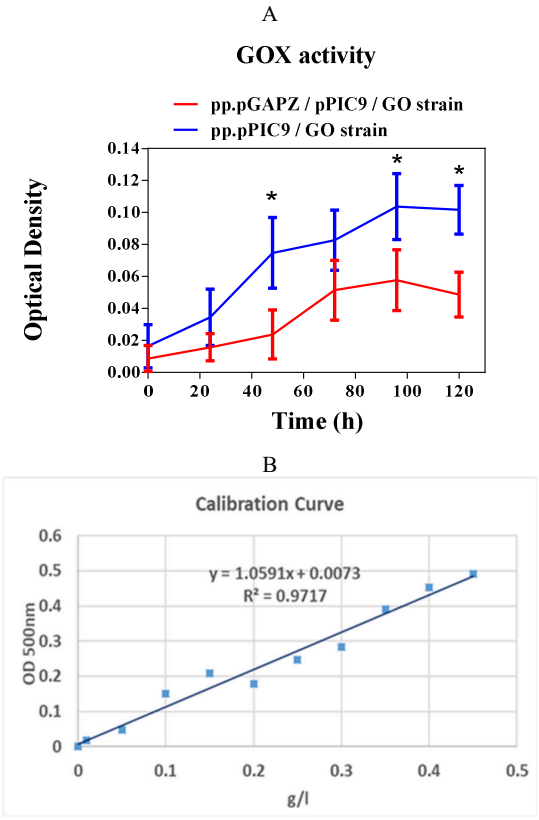


Figure 5. Comparison of glucose oxidase activity (A): Top legend, the activity of glucose oxidase in pp.pGAPZ / pPIC9 / GO strain, and down legend, the activity of glucose oxidase in pp.pPIC9 / GO strain based on culture durations. (B) Standard curve for the GOX activity. * indicate significant differences between the activity of glucose oxidase in pp.pGAPZ / pPIC9 / GO strain with that of in pp.pPIC9 / GO strain. (* P value ≤ 0.05).

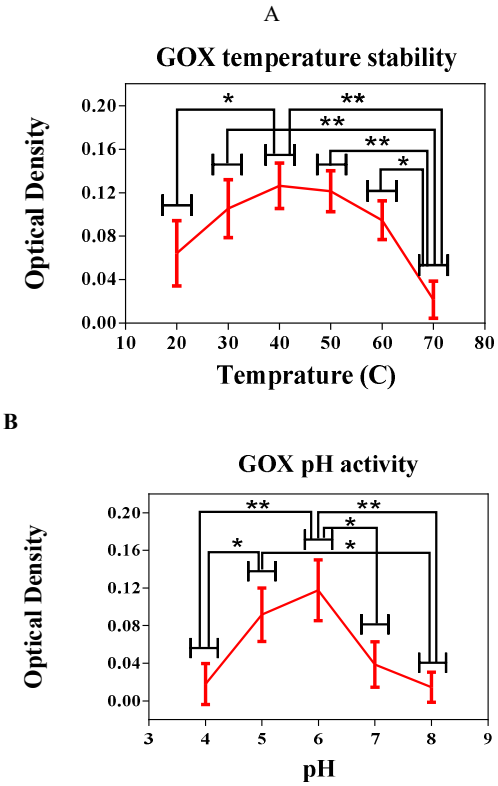


Figure 6. The glucose oxidase enzyme activity of the recombinant pp.pGAPZ / pPIC9 / GO strain at different temperatures (A), and the glucose oxidase enzyme activity of the recombinant pp.pGAPZ / pPIC9 / GO strain at different pH (B). * indicate significant differences between different temperature and dissimilar pH (* P value ≤ 0.05, ** P value ≤ 0.01, *** P value ≤ 0.001).

pp.pGAPZ / pPIC9 / GO strain, 0.11321 g / l, and pp.pPIC9 / Go strain, 0.07084 g / l based on the linear equation.

The effect of pH and temperature on novel recombinant pp.pGAPZ / pPIC9 / GO strain

The enzyme solution of glucose oxidase was exposed for 2 hours at different temperatures, and pHs and enzyme activity were investigated. To examine the temperature effects, we exposed the Glucose oxidase solution to 20 ° C to 70 ° C temperatures for 95 minutes. The enzyme activity was then measured. The relative activity of the enzyme was drawn in Excel software (Figure 6 A). The results showed that the enzyme had the highest activity at 40 ° C to 50 ° C temperatures. For the PH effects, the enzyme activity was measured in different PH solutions (4 to 8) for 95 minutes. The relative activity of the enzyme was drawn in Excel software (Figure 6 B). The result showed that the enzyme had the highest activity at pH 6.

Discussion

Glucose oxidase is an enzyme with many applications in natural and healthy food industries and non-toxic preservatives (to remove glucose and oxygen from food and non-alcoholic beverages). In addition, glucose oxidase is one of the essential enzymes in clinical diagnosis and biosensor technology.

In recent decades, glucose oxidase has been produced primarily by cellular enzymes in the wild-type Aspergillus niger, which requires high oxygen and glucose concentrations from a carbon source. Immediately after enzyme production, glucose is

converted to gluconic acid, a crucial substrate for cell growth and glucose oxidase production. This flavoprotein reduces molecular oxygen to peroxidase and oxidizes beta glucose to glucono delta lactone. The reduced form of glucose oxidase is re-oxidized by molecular oxygen. Expanded hydrogen peroxide is decomposed into water and oxygen by catalase as a result of the reaction. In fact, glucose oxidase (beta-glucose: oxygen - oxygen oxidoreductase EC 1.1.3.4) is a FAD-dependent glycoprotein that catalyzes the oxidation of beta-glucose to diglucone 1 and 5 lactones, which remove hydrogen from glucose.

Many researchers have studied the enzymatic conversion of glucose to gluconic acid by glucose oxidase, the mechanism of action, properties, and molecular structure of GOx. As we know today, this enzyme is actually a homodimer with a molecular weight of 150-180 kDa. This molecule is composed of two strong bands of FAD molecules. Decomposition of two subunits is performed only in denaturation conditions and is accompanied by the absence of the FAD factor. The gene of glucose oxidase enzyme contains 1818 bp nucleic acid in Aspergillus niger, which lacks introns and has post-translational changes, such as glycosylation.

This enzyme catalyzes glucose to glucono delta lactone in the presence of oxygen. Glucose oxidase and catalase are involved in this reaction system. Glucose oxidase was first purified from the extract of Aspergillus niger, but later, several microorganisms were selected for industrial production of this enzyme on a pilot scale [6, 7, 8, 46].

This enzyme is used as a biological sensor for glucose, a preservative in the food industry, and organic acids production in the baking, washing, medical, and cosmetic industries [3]. Recent research shows that transferring glucose oxidase gene to plants can lead to plant resistance to pests [47]. Thus, enzyme production belongs to a large company, and its use is not allowed in other countries or companies due to a patent.

Different research groups have performed isolation, cloning, and expression of this gene. A cDNA library was prepared, and the enzyme gene was isolated from Aspergillus niger NRRL-3 [46]. Using 800 bp cDNA, we screened 12,000 recombinant phages, and the coding region of the isolated is 1815 bp, encoding 605 amino acids, of which 21 terminal amino acids are related to secretory signals and 584 to enzymes. They did not report any areas as introns. They also showed a pyrimidine-rich region before starting the 12-bp codon and suggested that this region replace the TATA and the CAAT box.

In another study, this enzyme was isolated from Aspergillus niger ATCC 9029 by a genomic library and cDNA, and the gene was cloned into two plasmids. It was demonstrated that the gene contained 1815 nucleotides encoding 605 amino acids, which make up the 21 primary amino acids of the secretory signal. They also did not report introns, but the lateral region of this strain was quite different from that of Aspergillus niger NRRL-3 in the previous study [6].

In other research work, the gene of this enzyme was obtained by producing a genomic library from the wild strain Aspergillus niger ACMO4 in Korea. The isolated gene with 16 different nucleotides and 10% specific activity was more similar to the glucose oxidase 9029 genes [8]. This gene was isolated by amplifying through PCR optimization on genomic DNA extracted from Aspergillus niger ATCC 9029 as a model. Although culturing and extracting genomic DNA from filamentous fungi is difficult, this study showed that ultrasound and hydrolysis in liquid nitrogen could provide sufficient genomic DNA for PCR. However, in 2002, a simple and effective method was reported by Cassago et al. to isolate DNA from filamentous fungi [48]. The primers were based on gene sequences reported [6, 46].

In general, yeast secretes a small amount of natural protein in culture medium compared to filamentous fungi. This feature makes the yeast an attractive host for protein production because, in the end, products that facilitate easy refining and protein breakdown are reduced. Thus, the recombinant protein forms a large part of the total protein in the expression medium. However, the cytoplasmic expression usually leads to high expression levels due to the lack of potential restriction on the secretory pathway. This type of expression also has negative aspects. For example, in many cases, the structure and process of proteins, such as the formation of disulfide bonds, have severe limitations in cytoplasmic expression. Likewise, the breakdown of cells to extract proteins requires other steps in the product processing because the yeast cell walls are very strong. Through this process, the materials produced by cell destruction must be purified, and the final product is only part of the cell components as only one percent of the proteins inside the cell are target proteins.

In this study, the enzyme was produced under the GAP promoter, which is permanently induced by the GAPDH gene and the effect of short sequences of aromatic amino acids on bed-to-bed binding. Previously, in a similar study, when an enzyme was produced under the PIC9 promoter, a set of engineering hosts was used to produce recombinant proteins that did not contain proteases and protein-degrading enzymes.

The production has reached the best scale. In this study, enzyme production from GS115 strain Pichia pastoris was used, and the focus in Pichia pastoris GS115 strain was on the presence of vacuolar protease as a major problem. Vacular protease is an important factor for protein breakdown and instability of recombinant protein produced in culture medium.

Production of recombinant glucose oxidase was estimated in pPIC9 / GO constructs and pGAPZαA/GO constructs. The reason for the decrease in pPIC9/GO strain is related to the type of recombinant promoter, given that the main focus of the project was to investigate the performance of two combined forms of the promoter, methanol-induced and self-induced promoter [49]. For this purpose, two constructs for expressing the glucose oxidase gene in Pichia pastoris were investigated. These constructs are designed to express the gene in Pichia pastoris with a natural genetic sequence signal (the source of this gene was Aspergillus niger) and transferred into the genus Pichia pastoris GS115. Then, the recombinant production of glucose oxidase and its expression were investigated, and the results of SDS-page and dot blot showed that the construct with the leader sequences is more suitable to produce glucose oxidase. This construct was selected to purify after optimizing the recombinant enzyme conditions and then studied for biochemical properties. The type of enzyme was similar to glucose oxidase isolated from other fungal sources in terms of physical and biochemical properties.

Protein proteolysis during inadequate secretion of complex extracellular proteins is one of the limiting factors of enzyme production. An important feature in most Pichia pastoris of the GS115 strain is the presence of vacuole proteases. This was one of the problems of this project. Vacuole proteases are crucial factors in protein breakdown. For example, some proteins are produced unstable in the culture medium of pichia pastoris because they are sensitive to proteases and are rapidly degraded by them. Primarily, in large-scale culture conditions, these enzymes are found in large quantities in the culture medium due to high cell density [33].

The following methods are recommended to protect recombinant proteins and can partially alleviate proteases effects.

1- Adding a complementary amino acid or peptide to the growth medium.

2- Using a buffer with pH:3 in which the amount of protein degradation is reduced.

3. The use of host strains that produce little protease.

4- Murusugi et al. reported that applying skim milk powder to induce culture medium protected the recombinant protein against protease enzymes [12].

5- Using ammonium ions or L-arginine-hydrochloride (ammonium phosphate).

6- Replacing the culture medium with a fresh culture medium at the production phase to prevent proteases accumulation (of course, when the vector is pGAPZα, it uses only the YPD culture medium [21].

7- Reducing induction time and low temperature of 23 degrees Celsius (low temperature stabilizes the cell membrane and reduces the amount of protease release from the cell [21].

Kex2p is one of the most common proteases produced by the yeast Pichia Pasturis, a membrane protein located at the end of the Golgi apparatus. Due to the pathway of protein secretion in this system and cleavage at the GLU-ALa site and s the glucose oxidase gene sequence, this protease may be produced in the strains used in this study [21].

The secretion could be under the influence of some factors, such as:

1- Gene (when the number of copies of this number exceeds a certain limit, the secretory pathway is blocked).

2- Strain phenotype

3- Secretory signal

4- Sequence (expression with AOX2 promoter has a wide efficiency in the secretion of recombinant proteins)

5- Processing

6-Proteolysis, fermentation, and glycosylation. Also, the amount of secretion depends on the nature and structure of the protein.

Unfortunately, there is no way to predict the effectiveness of this signal in Pichia pastoris. Experiments usually require trial and error to find the best secretion signal for a particular protein [12]. The potent and inducible AOX1 promoter is often used to express recombinant proteins in the yeast Pichia pastoris. One of the problems of this promoter is continuously adding methanol as an inducer in the culture medium of expression and increasing the risk of combustion and production costs [3].

The GAP promoter has a continuous system with higher efficiency in less time than a semi-continuous system [12]. This promoter is expressed with sugar and does not require methanol or a change of culture medium [33]. It is expected that with the help of an efficient secretory signal and the removal of proteases from the culture medium by changing the host strain, the relevant strain will be suitable for industrial uses.

Using the pGAPZαA expression vector would reduce oxygen consumption, increase the cell's response

to oxidative stress, and improve cell viability. In addition, the structural promoter is continuously active and replicating and expressing the gene, and the glucose oxidase gene is sequentially amplified with increasing the construct function.

The glyceraldehyde 3-phosphate dehydrogenase (pGAP) promoter is a permanent promoter and does not require methanol induction, and the recombinant protein produced at the pGAPZαA expression vector will be equal to or greater than the amount of recombinant protein produced by pAOX. Due to the low level of intracellular protein secreted by the yeast, we designed the alpha secretory signal (α-MF) to produce the recombinant protein and its appropriate stability and flexibility. In addition, a histidine biosynthesis marker was added to the vector to ensure continued growth in future generations and a zeocin antibiotic resistance marker to ensure plasmid survival.

In another process, AOX1 and GAP promoters were used in combination to produce recombinant proteins. The PIC9 expression vector had an AOX1 promoter, and the combination of the two vectors increased the performance by about one and a half times. The optimal conditions for enzyme production were pH 6 and temperature 30 ° C. However, previous studies estimated that the production of recombinant glucose oxidase in pGAPZαA / GO and pGAPZαA / GO with heptapeptide was 0.234 g / l 0.135 g / l, respectively. The use of heptapeptide reduces enzyme production due to the structure's function related to the favorable conditions for the stability of the recombinant enzyme. Nevertheless, in the combined strain of this study, it seems to be even less than pp.pGAPZαA / GO.

The integration of the pGAPZαA / GO construct from gene entry in the GAP promoter region results from a crossover causes one or more copies of the gene upstream or downstream of the site in the PGAP region pGAPZ or pGAPZα promoters. Enter GAP. The PGAP promoter and target genes cause resistance to zeosin and antibiotic markers in the host. The same is true for nonlinear plasmids, albeit at a lower frequency [35]. Therefore, due to the strain's resistance to 100 to 200 mg of the antibiotic zeocin, it can be expected that at least one copy of the gene has entered the pGAPZα induction promoter. On the other hand, due to gene insertion into the AOX1 (GS115) or aox1: ARG4 (KM71) site, the crossover may occur in three regions (AOX1). It happens that this action probably causes the presence of one or more gene copies in the construct [35]. With this explanation, it is difficult to posit that one or more copies of the glucose oxidase gene entered the host in the pp.pPIC9 / GO strain at AOX1 (GS115) or aox1: ARG4 (KM71). However, this strain is not necessarily self-induced.

The results showed that during the use of methanol, with the simultaneous expression of self-induction, the activity of the alcohol oxidase promoter decreases, which can overcome oxidative stress. Furthermore, cell survival during the culture process is improved. By combining metabolic engineering and antioxidant performance factors, glucose oxidase production in Pichia pastoris may increase [50].

The main problem with using glucose oxidase isolated from a native source is that the microorganism producing the enzyme may have contaminants not suitable for specific uses of the desired protein. Because glucose oxidase is used to preserve food and its main production source, Aspergillus niger is highly allergenic and not approved for use in the food industry. In addition, precise purification methods of this enzyme are very expensive. Glucose oxidase is also an intracellular enzyme that makes it difficult to extract and purify this enzyme. These problems can be solved by producing this enzyme in recombinant systems [3].

In 2019, Rezaeipour investigated the enzymatic activity and protein expression of recombinant enzymes produced in two recombinant constructs pGAPZαA / GOX and pGAPZαA / GOX together with heptapeptidra. The production of recombinant glucosidase enzyme was reported in native pGAPZαA / GOX and pGAPZαA / GOX constructs with heptapeptide of 0.234 g / l and 0.135 g / l, respectively. The decrease in producing the structure of heptapeptidra is related to the optimized conditions for the production of recombinant enzymes in the pGAPZαA / GOX structure [4]. In the present study, the production of glucosidase enzyme in pp.pGAPZ / pPIC9 / GO structure was estimated at 0.11321 g / l, and in pp.pPIC9 / Go structure was estimated at 0.07084 g / l.

Although the production of recombinant enzyme is less than that of the two pGAPZαA / GOX constructs in both constructs of the present study, the researchers expected that at least in the simultaneous use of two pIC and PGAPZ promoters, the enzyme activity would be higher than one-promoter condition. In comparison with Rezaipour's study (2019), the activity of enzymes is due to different study conditions, including culture medium and environmental factors. In Rezaeipour's study, the use of YNB and YPD media made a significant difference in the production or activity of glucose oxidase [4].

On the other hand, recombinant protein in the host (Picaya pastoris) can be expressed intracellularly or extracellularly. Because this yeast secretes low intracellular proteins, the recombinant protein is often the majority of the proteins produced in the

environment. The recombinant protein delivery to the culture medium is an important step in purification. In Rezaeipour's study (2019), one or more stages of purification were used, but in the present study, purification was not used. The supernatant of the culture medium was used directly for enzymatic activity [4].

Bliad (2018) examined two constructs of Ppic9 / Gox + sig and Ppic9 / Gox-sig (with and without guide sequence). Under the control of pIC9 promoter for the expression of recombinant glucose oxidase gene in Pichia pastoris, the glucose oxidase enzyme was reconstructed and examined. It has also performed partial purification of the enzyme as in the case of Rezaeipour (2019). The results of SDS page and immunoblott showed that the structure with the guide sequence was more suitable in terms of glucose oxidase production. In the study of Bliad (2018), although several stages of purification were used, and only enzymatic activity was measured by a calorimetric method, there was no significant difference in comparison with Rezaeipour calorimetric activities and enzymatic activity curves. In addition, these two studies used different inducers in terms of induction. We should note that the samples of both studies have passed several purification stages, and then the enzymatic activity was measured.

The present study aimed to investigate the production and activity of glucose oxidase enzyme simultaneously under the control of two different promoters in terms of induction and to evaluate the enzyme activity quantitatively without using purification steps. We also directly study the production and activity of enzymes from the supernatant.

In 2014, Lee and colleagues recommended a hybrid strategy to improve production. The group, which pursued commercial production goals, used the pPIC9k promoter to express glucose oxidase while cloning more copies of glucose oxidase to increase the production scale. However, their main problem with the high production was the high-density fermentation of oxygen, which they sought to reduce oxygen consumption. The team used a hybrid strategy involving simultaneous feeding of mannitol and two-step methanol induction and simultaneous expression of a general transcription activator. They increased production by 58.1 times in a three-liter fermentation compared to controls. They used the GCN4 gene to increase protein levels by up to 2.8-fold compared to controls. Although the scale of the present work was at a research level, the results of Lee et al. (2010) might be a good model to enhance production efficiency when considered together with our findings [50].

Considering that at least one copy of the glucose oxidase gene has entered the pp.pPIC9 / GO strain, we think that the amount of glucose oxidase production or activity in this strain is similar to our findings.

Nevertheless, in the case of the pp.pGAPZ / pPIC9 / GO strain, due to the arrival of a copy or possibly other versions of the glucose oxidase gene with an entirely different promoter, which is self-induced and without the need for extracellular induction, its integration is different from the locus. Besides, more production was expected than the amount produced in this strain. However, due to the current production of this strain and its resistance to the antibiotic Ziosin, at least one copy of this gene could be expressed in this strain, but that this strain has another copy of the glucose oxidase gene under control and alkali. It is methanol, and we expected the activity to be at least doubled, but it could not be achieved for several reasons. First, the interference of the activities of two promoters due to different repressors, activators, substrates. And different environmental and intracellular optimizing factors could decrease protein production and enzyme activity.

Conclusion

An important feature of Pichia pastoris of the GS115 strain is the presence of protease vacuoles, which was one of the problems of this project. Vacuole proteases are crucial factors in protein breakdown. For example, some proteins are unstable in the Pichia pastoris medium because they are sensitive to proteases and are rapidly degraded. Especially in large-scale culture conditions, these enzymes are found in abundance in the culture medium due to high cell density. Several cases have been reported as successful strains in reducing the breakdown of some foreign proteins. Strains with this property can grow in high cell concentrations with minor cell destruction, but their growth rate is slow. These strains have poor survival and are difficult to deform [12].

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