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ORIGINAL ARTICLE

Simultaneous GCN4 co-expression and promoter optimization enhance glucose oxidase production in *Pichia pastoris*

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ABSTRACT

Background and Objectives: This study aimed to enhance glucose oxidase (GOX) production in *Pichia pastoris* GS115 using a novel dual-promoter system, combining the constitutive *glyceraldehyde-3-phosphate dehydrogenase* promoter (p*GAP*) in pGAPZαA with the methanol-inducible *Alcohol oxidase 1* promoter (p*AOX1*) in PIC9.
Materials and Methods: The *GOX* gene from *Aspergillus niger* (ATCC 9029) and a transcription factor, *general control nonderepressible 4* (*GCN4*) gene from *P. pastoris* were co-expressed to mitigate oxidative stress, thereby improving cell viability and enzyme yield. The recombinant construct pGAPZαA-*GOX-GCN4* was transformed into *P. pastoris* GS115 and *P. pastoris* GS115-PIC9 via electroporation. Expression conditions under various temperatures and pH treatments were optimized. We examined glucose oxidase expression by inducing methanol at concentrations of 100% and 5% in BMMY (Buffered Methanol-complex-medium). The highest enzyme levels were observed at pH 6.0, 34°C, and 5% methanol induction. Enzyme validation was performed using SDS and Western blotting.
Results: Co-expression of *GCN4* significantly enhanced GOX production, achieving 16.65 µg/mL (333 U/mL) in *P. pastoris* GS115-PIC9-pGAPZαA-*GOX-GCN4*(2), a 377.4-fold increase over the control, and 11.03 µg/mL (220.6 U/mL) in *P. pastoris* GS115-PIC9-pGAPZαA-*GOX-GCN4*(3), a 249.65-fold increase.
Conclusion: The results demonstrate that *GCN4*'s stress mitigation amplifies the synergy between constitutive and inducible promoters. The dual-promoter strategy offers a robust platform for recombinant protein production.

Keywords: *Pichia pastoris*; Glucose oxidase; *GAP* promoter; *AOX1* promoter; *GCN4*; Dual-promoter expression; Recombinant protein; Methanol induction; Oxidative stress

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INTRODUCTION

Glucose oxidase (GOX, EC 1.1.3.4), a dimeric glycoprotein enzyme, catalyzes the oxidation of β-D-glucose to gluconic acid and hydrogen peroxide, playing a critical role in various industrial and medical applications (1-3). In medical diagnostics, GOX is integral to amperometric glucose biosensors, enabling precise monitoring of blood glucose levels in diabetic patients, with applications in implantable devices for continuous glucose monitoring (1). In the food industry, GOX enhances shelf life by removing residual glucose and oxygen, preventing spoilage and oxidative degradation in products like beverages and baked goods (2, 3). Additionally, GOX is employed in pharmaceutical and biotechnological processes, such as the production of gluconic acid for drug formulations, and in environmental applications, where it aids in degrading endocrine-disrupting compounds, thus mitigating their ecological impact (4, 5). The enzyme is predominantly produced by filamentous fungi, notably *Aspergillus niger* and *Penicillium* species. However, wild-type fungal production is hampered by intracellular enzyme localization, susceptibility to contamination, and complex purification processes, necessitating recombinant expression systems to meet commercial demands.

Recombinant protein production has been explored in diverse hosts, including bacteria (*Escherichia coli*), yeasts (*Saccharomyces cerevisiae*, *Pichia pastoris*), molds, mammalian cells (CHO cells), plant systems, and insect cells. Bacterial systems offer rapid growth and ease of genetic manipulation, but often fail to perform complex eukaryotic post-translational modifications, such as glycosylation, leading to misfolded or inactive proteins. Mammalian cells excel in producing properly folded glycoproteins, but are costly and difficult to scale (6). Plant and insect systems, while promising for certain applications, face challenges in expression consistency and regulatory approval. Yeasts combine the advantages of prokaryotic simplicity with eukaryotic modification capabilities, making them ideal for recombinant protein production (6). *S. cerevisiae*, the first yeast used for this purpose, suffers from plasmid instability, low protein yields, hyper-N-linked glycosylation, and limited scalability, which restricts its use for commercial protein production (7). These limitations have driven the adoption of alternative yeast hosts like *P. pastoris*, which offers superior performance for industrial applications.

Pichia pastoris has emerged as a leading platform for recombinant protein production due to its robust genetic stability, high expression levels, and ability to perform complex post-translational modifications (8-11). Unlike *S. cerevisiae*, *P. pastoris* grows aerobically, avoiding fermentation byproducts such as ethanol and acetic acid, which can inhibit cell growth (12). It achieves high cell densities (up to 200 g/L dry weight) in cost-effective media, facilitating large-scale production (13). *P. pastoris* integrates foreign genes into its genome, ensuring stable expression without plasmid loss, and utilizes inexpensive carbon sources like methanol to induce the *Alcohol oxidase 1* promoter (p*AOX1*) (14-17). The absence of allergenic mannosyl transferase activity in *P. pastoris* results in less immunogenic glycoproteins compared to *S. cerevisiae*, with recombinant protein yields often one to two times higher (12). Furthermore, *P. pastoris* performs eukaryotic modifications, including proper protein folding, disulfide bond formation, glycosylation, and signal sequence processing, ensuring functional recombinant proteins. These features make *P. pastoris* a standard tool for producing proteins for both research and commercial purposes (8-11).

The choice of promoter is a critical determinant of recombinant protein yield in *P. pastoris* (18). The *AOX1* promoter, tightly regulated and inducible by methanol, drives high-level expression in *P. pastoris* GS115 (Mut^+ phenotype) (19). In the presence of methanol, *Alcohol oxidase* in peroxisomes oxidizes methanol to formaldehyde, producing hydrogen peroxide as a byproduct (20). This reaction, while being effective for p*AOX* induction, generates oxidative stress, which can impair cell viability and limit protein production, particularly at high methanol concentrations (1, 21-23). The *glyceraldehyde-3-phosphate dehydrogenase* promoter (p*GAP*), a constitutive promoter, offers an alternative by driving strong expression on glucose or glycerol without methanol induction (16, 24-27). The p*GAP*, derived from a housekeeping gene, supports continuous protein synthesis and often yields recombinant protein levels equal to or greater than those achieved with p*AOX1*, making it advantageous for industrial-scale production (17, 28). Unlike p*AOX1*, p*GAP* does not rely on methanol, reducing oxidative stress and simplifying fermentation processes (29).

Designing an optimal recombinant protein production system in *P. pastoris* requires careful consideration of multiple factors: selecting a host capable of proper folding and post-translational modifications,

choosing an appropriate expression vector with a suitable promoter, optimizing codon usage for efficient translation, incorporating signal sequences (e.g., α-mating factor) for extracellular secretion, and fine-tuning fermentation conditions such as carbon/nitrogen sources, temperature, pH, and aeration (29). This study leverages a dual-promoter strategy, combining the constitutive p*GAP* and inducible p*AOX1* promoters, to maximize the expression of GOX from *A. niger* ATCC 9029 in *P. pastoris* GS115. By integrating the *General Control Non-Derepressible 4* (*GCN4*) gene to enhance cell viability under oxidative stress, this approach aims to overcome the limitations of methanol-induced stress and achieve high GOX yields for industrial and research applications (30).

MATERIALS AND METHODS

Strains, plasmids, and reagents. *Pichia pastoris* GS115 (Mut^+) and the pGAPZαA plasmid were obtained from Invitrogen (Carlsbad, CA, USA). The *P. pastoris*-PIC9 was sourced from the National Institute of Genetic Engineering and Biotechnology (NIGEB, Tehran, Iran). *Escherichia coli* DH5α was used for plasmid propagation. Taq PCR Master Mix was purchased from Ampliqon (Odense, Denmark), and *Pfu DNA Polymerase* from Bioneer (Daejeon, South Korea). Restriction enzymes (*NotI*, *HindIII*, *XbaI*, *XhoI*, and *BinI*), *T4 DNA ligase*, Rapid DNA Ligation Kit, and Agarose Gel DNA Extraction Kit were purchased from Roche (Basel, Switzerland). β-Mercaptoethanol, bisacrylamide, Sodium Dodecyl Sulfate (SDS), TriChloroacetic Acid (TCA), 4-Aminoantipyrine, Coomassie Blue, Bromophenol Blue, Ammonium Persulfate, Peroxidase, and Glucose Oxidase were obtained from Merck (Darmstadt, Germany). Potassium Phosphate Buffer, Sodium Citrate Buffer, and Acrylamide were sourced from Sigma-Aldrich (St. Louis, MO, USA). SYBR Green qPCR Master Mix was purchased from Bio-Rad (Hercules, CA, USA), and the PM1500 ExcelBand All Blue Regular Range Protein Marker from SMOBIO (Hsinchu, Taiwan). Primers for *GOX* and *GCN4* were synthesized by Copenhagen (Tehran, Iran), and the Molecular Biology Kit was obtained from Bio Basic Inc. (Markham, Canada).

Construction of recombinant expression vector. The *GOX* gene from *Aspergillus niger* ATCC 9029 was amplified by PCR and cloned into the *XhoI* and *NotI* sites of pGAPZαA, yielding pGAPZαA-*GOX*. The *GCN4* gene was amplified from *P. pastoris* GS115 genomic DNA using primers designed for an 811 bp fragment and cloned into pGAPZαA-*GOX* at the *NotI* and *HindIII* sites, producing pGAPZαA-*GOX-GCN4*. The alpha-mating factor (α-MF) signal sequence from *Saccharomyces cerevisiae* was included in pGAPZαA for extracellular secretion. Plasmids were propagated in *E. coli* DH5α using LB medium with 50 µg/mL zeocin for selection. The recombinant plasmid was linearized with *BinI* to facilitate genomic integration.

Transformation and expression in *P. pastoris*. The linearized pGAPZαA-*GOX-GCN4* was transformed into *P. pastoris* GS115 and *P. pastoris* GS115-pPIC9 by electroporation (1.5 kV, 25 µF, 200 Ω) using a Bio-Rad Gene Pulser. Transformants were selected on YPD medium (1% yeast extract, 2% peptone, 2% dextrose) containing 100 µg/mL zeocin. Positive colonies were confirmed by PCR using *GOX* and *GCN4*-specific primers. Recombinant strains were cultured in BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34% yeast nitrogen base, 0.00004% biotin, 0.5-2% methanol 5%) for expression studies.

Optimization of expression conditions. Enzyme expression was optimized by culturing recombinant strains in BMMY medium under varying conditions: temperatures (25°C-37°C), pH (5.5-6.0), and methanol concentrations (0.5-3% for 100% methanol; 10% for %5 methanol). Cultures were maintained at 225 rpm in flasks filled to 20% capacity to ensure adequate aeration. Methanol was replenished daily to retain induction. Enzyme activity was measured using a glucose oxidase assay with potassium phosphate buffer, and protein concentration was determined by the Bradford method. Expression was assessed in culture supernatant to confirm extracellular secretion, with no activity detected in cell pellets.

RESULTS

Vector construction and cloning. The *GOX* gene from *Aspergillus niger* ATCC 9029 (1880 bp) was amplified by PCR and cloned into the *XhoI* and *NotI* sites of the pGAPZαA vector (3000 bp), yielding

pGAPZαA-*GOX* (4800 bp). The *GCN4* gene (811 bp) was amplified from *Pichia pastoris* GS115 genomic DNA and cloned into pGAPZαA-*GOX* at the *NotI* and *HindIII* sites, producing pGAPZαA-*GOX-GCN4* (6000 bp). Cloning was confirmed by double digestion with *NotI* and *HindIII*, followed by agarose gel electrophoresis (Figs. 1A and B). The *GCN4* (811 bp) and *GOX* (1880 bp) bands are shown (Fig. 1A), while the sizes of pGAPZαA-*GOX* (4800 bp) and pGAPZαA-*GOX-GCN4* (6000 bp), were confirmed after digestion (Fig. 1B).

Transformation and colony screening. The pGAPZαA-*GOX-GCN4* plasmid was linearized with *BinI* and transformed into *P. pastoris* GS115 and *P. pastoris* GS115-PIC9 by electroporation (1.5 kV, 25 µF, 200 Ω). Transformants were selected on YPD medium containing 100 µg/mL zeocin. Ten to twenty colonies per strain were screened by PCR using *GOX* and *GCN4*-specific primers, confirming successful integration. Three colonies of *P. pastoris* GS115-PIC9-pGAPZαA-*GOX-GCN4* exhibiting high enzyme activity in BMMY medium were selected for further analyses.

Expression optimization. Enzyme expression was optimized by culturing recombinant strains in BMMY medium under varying conditions: temperatures (25°C-37°C), pH (5.5-6.0), and aeration (20% flask volume, 225 rpm). Optimal GOX expression was observed at 34°C and pH 6.0 (Figs. 2A-C). The expression of *P. pastoris* GS115-PIC9-pGAPZαA-*GOX-GCN4* (colony 2) at pH 5.5 and 34°C is presented in (Fig. 2A). The effect of temperature (25°C-37°C) on expression at pH 6.0 is shown in Fig. 2B. Furthermore, Fig. 2C illustrates the expression levels of three colonies at pH 6.0 and 34°C, with colony 2 exhibiting the highest optical density (OD) at 500 nm.

Methanol induction effects. Methanol induction was evaluated at two concentrations: 100% methanol with a daily induction level of 0.5-3%, and 5% methanol with a daily induction level of 10% in BMMY medium. The highest GOX expression was obtained under the 5% methanol condition with 10% daily induction (Fig. 2C). Under these conditions, the strain *P. pastoris* GS115-PIC9-pGAPZαA-*GOX-GCN4* (colony 2) produced 16.65 µg/mL (333 U/mL) after 96 hours, corresponding to a 377.4-fold increase relative to the control (Table 1).

Under the 100% methanol condition, daily induction at 2% yielded the highest expression level at 120 hours (Figs. 3A and B). These figures illustrate the expression profiles across different induction levels with 100% methanol at 96 and 120 hours.

The superior performance of the 5% methanol condition compared with 100% methanol is primarily attributed to reduced methanol toxicity, which better preserves cellular viability and metabolic efficiency.

Protein analysis. SDS-PAGE and Western blotting techniques were employed to validate GOX expression. SDS-PAGE revealed a dominant band at 68-85 kDa in the culture supernatant, consistent with the glycosylated GOX protein (65 kDa in *A. niger*) (Figs. 4A and B). The protein profile of *P. pastoris*

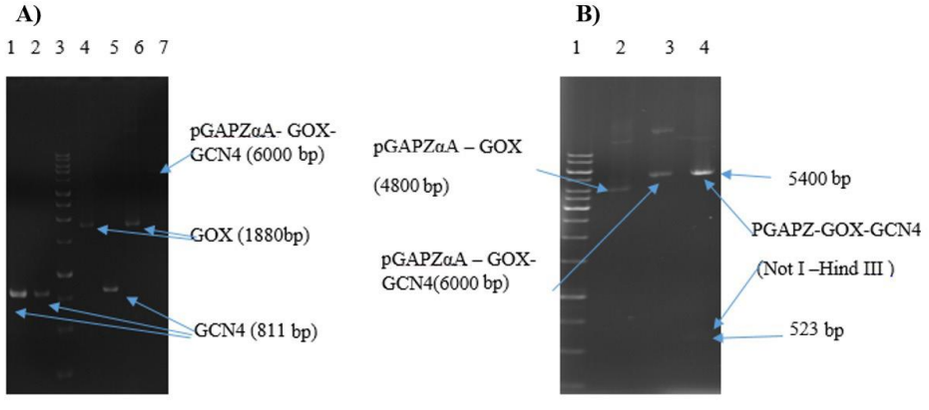

Fig. 1. Cloning confirmation of recombinant vectors by agarose gel electrophoresis. (A) PCR amplification products showing the *GCN4* gene (811 bp) and the *GOX* gene (1880 bp) from *Aspergillus niger* ATCC 9029. (B) Double digestion (*NotI*/*HindIII*) of the pGAPZαA-*GOX-GCN4* vector, yielding 5400 bp and 523 bp fragments, along with the expected bands for pGAPZαA-*GOX* (4800 bp) and pGAPZαA-*GOX-GCN4* (6000 bp).

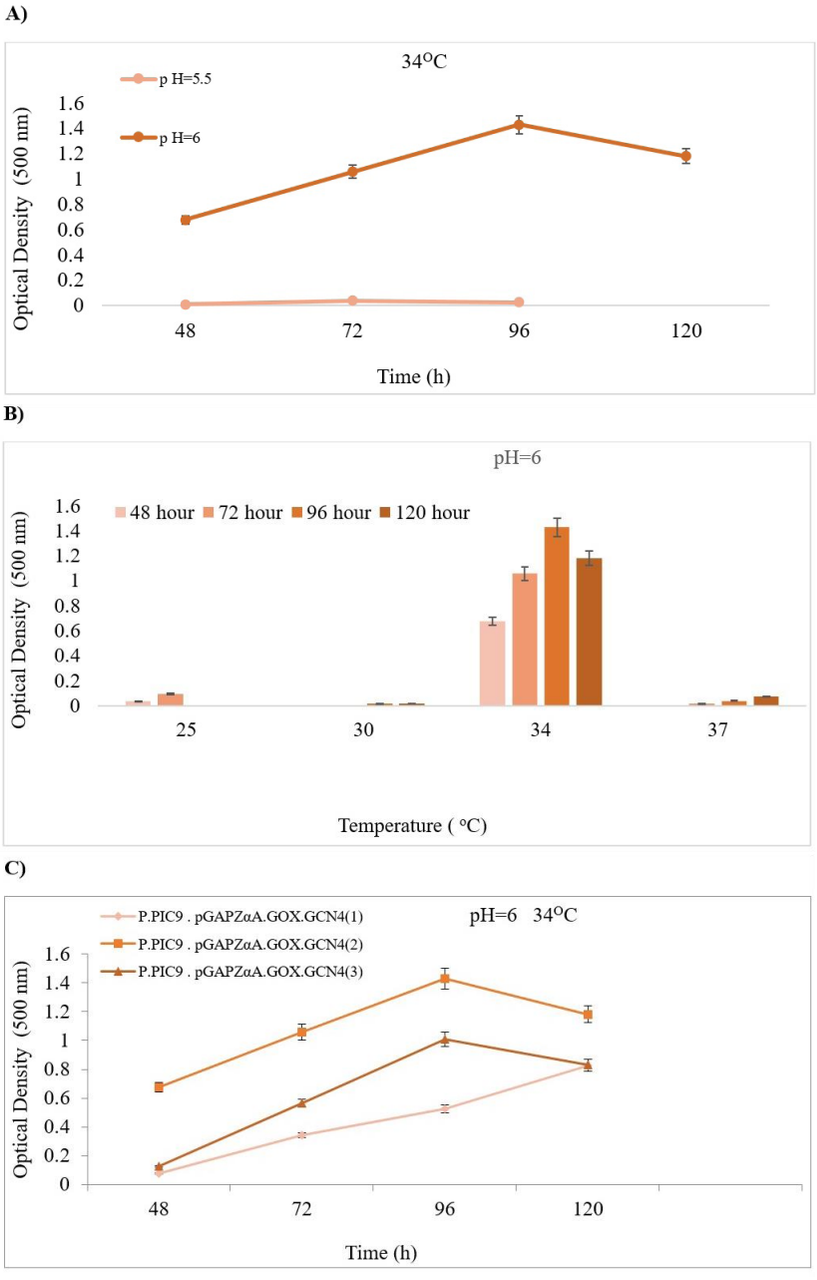

Fig. 2. Optimization of GOX expression in *Pichia pastoris* GS115-PIC9-pGAPZαA-*GOX-GCN4*. (A) Expression in *Pichia pastoris* -PIC9- pGAPZαA -*GOX-GCN4*(2) at pH 5.5 - 6.0 and 34°C, measured by optical density (OD) at 500 nm. (B) Expression at pH 6.0 across temperatures (25°C-37°C) in *Pichia pastoris* -PIC9- pGAPZαA -*GOX-GCN4*(2). (C) Expression at pH 6.0 and 34°C for colonies 1–3, with colony 2 showing the highest OD.

Table 1. Quantitative analysis of GOX activity and fold increase over control in *Pichia pastoris* GS115-PIC9-pGAPZαA-*GOX-GCN4* (1, 2 , 3) , reporting enzyme activity (U/mL), concentration (µg/mL) .

120 hours	96 hours	Sample
23.6	104.45	*P. pastoris* -PIC9- pGAPZαA -*GOX-GCN4* (1)
37.14	377.4	*P. pastoris* -PIC9- pGAPZαA -*GOX-GCN4* (2)
23.71	249.65	*P. pastoris* -PIC9- pGAPZαA -*GOX-GCN4* (3)

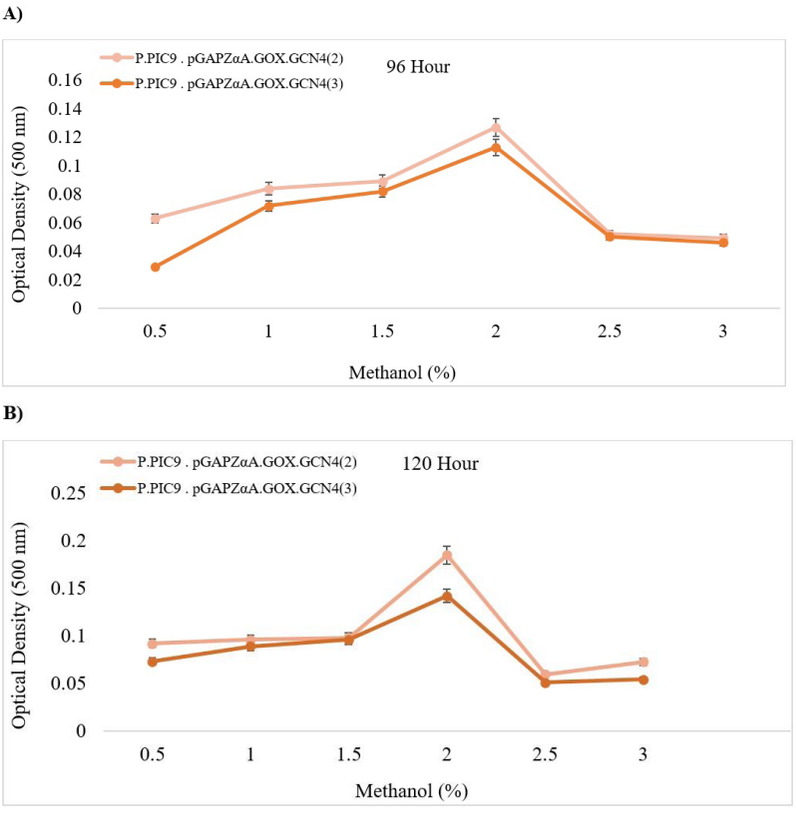

Fig. 3. Effects of methanol induction on GOX expression in *Pichia pastoris* GS115-PIC9-pGAPZαA-*GOX-GCN4* (colony 2).
(A) Expression at 96 hours across methanol concentrations (0.5-3% for 100% methanol).
(B) Expression at 120 hour across methanol concentrations (0.5-3% for 100% methanol).

GS115-PIC9-pGAPZαA-*GOX-GCN4* (colonies 1-3) is shown in Fig. 4A, with no bands observed in host strains lacking GOX. Comparison of strains confirms consistent expression (Fig. 4B). Western blotting using anti-GOX antibodies detected positive bands at similar molecular weights, with expression increasing from 2 to 48 hours of induction (Figs. 5A and B). No GOX activity was detected in cell pellets, confirming the extracellular secretion.

DISCUSSION

Challenges in glucose oxidase production. Glucose oxidase (GOX) is a widely utilized industrial enzyme; however, its production in *Aspergillus niger* remains suboptimal due to intracellular localization, which necessitates extensive extraction and purification procedures that compromise yield and increase processing costs (31). Wild-type strains are additionally vulnerable to microbial contamination, posing significant risks to product integrity, particularly for medical-grade applications. Although *A. niger* generally exhibits higher native GOX productivity than other fungal hosts, inherent scalability constraints limit its industrial applicability. To overcome these limitations, this study employed *Pichia pastoris* GS115 to heterologously express GOX from *A. niger* ATCC 9029, leveraging its efficient secretory pathway to simplify downstream processing and enhance production efficiency (31).

***Pichia pastoris* as a robust expression platform.** *P. pastoris* is globally recognized as an efficient recombinant protein production host due to its ability to reach very high cell densities in cost-effective media and to execute essential eukaryotic post-translational modifications such as N-linked glycosylation and disulfide bond formation (8, 31-33). *Pichia pastoris* offers enhanced genomic stability compared to *Saccharomyces cerevisiae* due to chromosomal integration of heterologous genes. Furthermore, the reduced immunogenicity of glycoproteins produced by *P. pastoris* improves its utility in biomedical enzyme

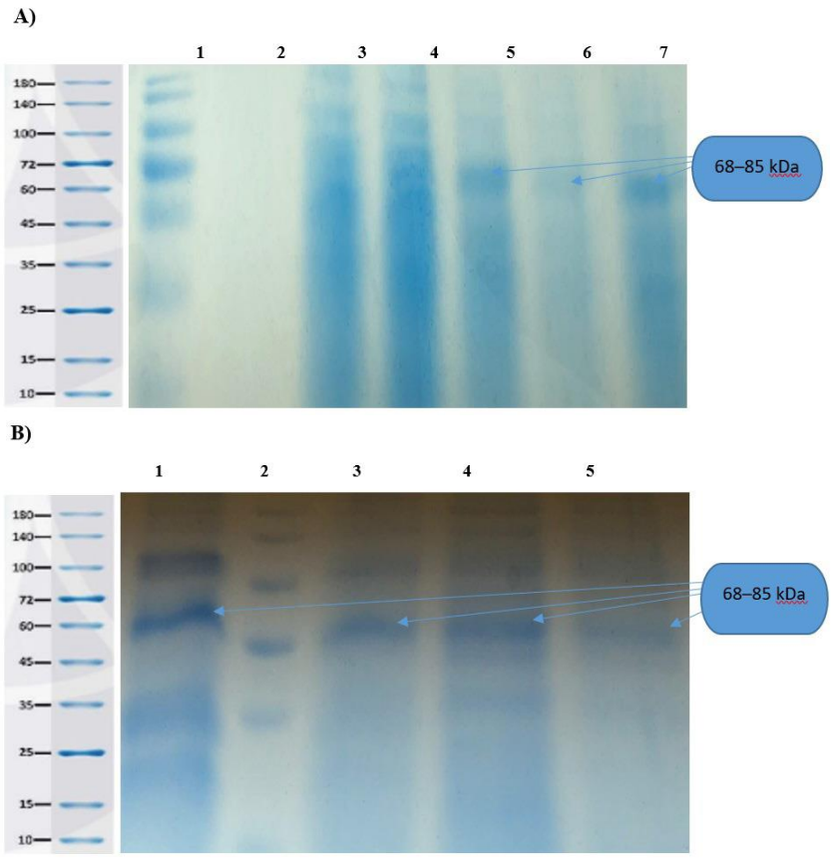

Fig. 4. SDS-PAGE analysis of GOX expression in *Pichia pastoris.* (A) Protein profiles of culture supernatant from *P. pastoris* GS115-PIC9-pGAPZαA-*GOX-GCN4* (colonies 1-3), showing a 68-85 kDa band; no bands in control strains. 1 -Molecular marker, 2-3-4- Host yeast lacking glucose oxidase gene, 5- *Pichia pastoris* -PIC9- pGAPZαA -*GOX-GCN4*(2), 6- *Pichia pastoris* -PIC9- pGAPZαA -*GOX-GCN4*(1), 7- *Pichia pastoris* -PIC9- pGAPZαA -*GOX-GCN4*(3) (B) Comparison of strains confirming consistent GOX expression.1- *Pichia pastoris* -PIC9- pGAPZαA -*GOX-GCN4* (2), 2- molecular markers, 3- *Pichia pastoris* -PIC9- pGAPZαA-*GOX-GCN4* (1), 4 - *Pichia pastoris* -PIC9- pGAPZαA -*GOX-GCN4* (3), 5- *Pichia pastoris* - PIC9- pGAPZαA -*GOX-GCN4*(2)

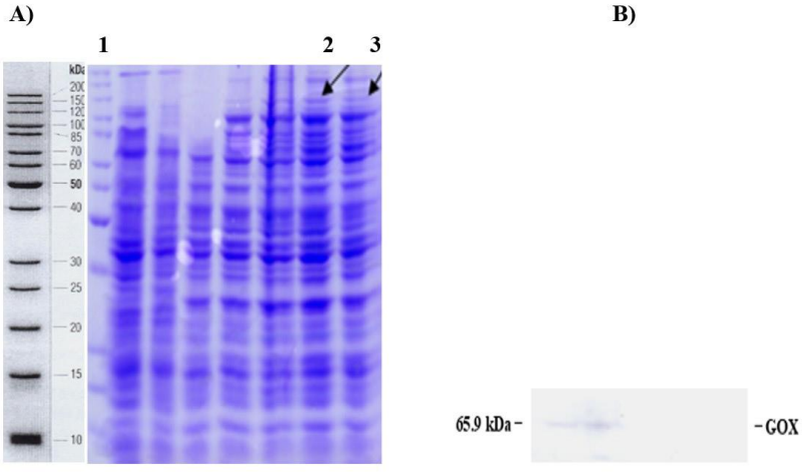

Fig. 5. Western blot analysis of GOX expression using anti-GOX antibodies. (A) Protein bands of culture supernatant from *P. pastoris* GS115-PIC9-pGAPZαA-*GOX-GCN4* (colonies 1-3) at 48 hours. 1 -Molecular marker, 2- *Pichia pastoris* -PIC9- pGAPZαA -*GOX-GCN4*(1), 3- *Pichia pastoris* -PIC9- pGAPZαA -*GOX-GCN4*(3)(B) Western blot results confirm the presence of glucose oxidase (GOX) as a distinct band in the 68-85 kDa range.

manufacturing (33). The *P. pastoris* GS115 Mut$^+$ phenotype is particularly advantageous due to its strong capacity for extracellular secretion, as demonstrated by GOX localization exclusively in the culture supernatant (34). These combined attributes position *P. pastoris* as a highly competitive host system for GOX production.

Enhanced production via a dual-promoter system. A dual-promoter strategy incorporating the constitutive *glyceraldehyde-3-phosphate dehydrogenase* promoter (p*GAP*) and the methanol-inducible *Alcohol Oxidase 1* promoter (p*AOX1*) was implemented to enhance transcriptional strength and production robustness (2-4). The p*GAP* promoter enables sustained expression on non-methanol carbon sources, eliminating methanol-induced oxidative stress and supporting stable basal production. In contrast, p*AOX1* offers strong transcriptional induction under methanol, mediated by the *AOX1/AOX2* metabolic pathway (3). The combined use of these promoters allowed modulation of metabolic burden and optimized GOX production by balancing cell fitness with high-level expression (2, 4).

***GCN4* co-expression mitigates oxidative stress.** Co-expression of the transcription factor *GCN4* significantly improved production performance by enhancing tolerance to oxidative stress associated with methanol metabolism, which generates hydrogen peroxide and formaldehyde (35, 36). *GCN4* triggers global stress-response pathways, including antioxidant defense activation, which collectively enhance cellular resilience (36). As a result, the engineered strains exhibited markedly elevated GOX titers—333 U/mL and 220.6 U/mL—corresponding to 377.4- and 249.65-fold increases relative to the control. Compared with stress regulators such as *HAC1*, which primarily activate the unfolded protein response, *GCN4* exerts broader transcriptional regulation, rendering it more effective in high-density fermentation environments (35). Consistent with the enhanced productivity, qPCR revealed increased *GCN4* copy numbers in the PIC9-containing transformants (17).

Optimization of fermentation conditions. The optimal production profile was observed at 34°C and pH 6.0, conditions aligned with previously established parameters for *P. pastoris* fermentation (28°C-30°C), pH (5.5-6.0) (29, 37-41). Aeration proved critical, given the oxygen-intensive nature of methanol metabolism and p*AOX1* induction (42-46). Insufficient oxygen availability is known to restrict *AOX1* expression due to the requirement of molecular oxygen for methanol oxidation to formaldehyde (45). Methanol induction using 5% (10% daily feeding) resulted in the highest GOX titers—16.65 µg/mL (333 U/mL)—outperforming induction with 100% methanol (0.5-3% daily feeding) by reducing methanol-associated cytotoxicity (35, 47, 48). Excess methanol (>2%) intensifies oxidative stress and disrupts protein folding, a trend consistent with previous reports that identify 0.5-1% methanol as optimal for inducible expression. The enhanced performance observed at 5% methanol in this study is likely attributable to *GCN4*-mediated stress attenuation (36).

Limitations. Several constraints remain despite a successful enhancement of GOX production. Methanol toxicity continues to pose a major bottleneck, as accumulation of oxidative byproducts significantly reduces cell viability and secretion efficiency (35, 47-50). Variable *GCN4* copy counts among transformants, as validated using qPCR, may further contribute to expression heterogeneity and restrict reproducibility (17).

From a process engineering standpoint, the substantial oxygen demand of *P. pastoris* poses challenges for large-scale bioreactors, necessitating advanced aeration strategies to maintain adequate dissolved oxygen. Moreover, potential microheterogeneity in GOX glycosylation, though not examined here, may influence enzyme stability or immunogenicity in therapeutic applications.

Future directions. Future work should explore methanol-sparing or methanol-free induction systems, including glycerol–methanol co-feeding or emerging promoters such as p*THI11*, which may enhance expression while simplifying process control (51). Improvements in bioreactor design, oxygen-transfer optimization, and intelligent feeding algorithms will be essential for scaling production to industrial levels (42-44). The dual-promoter framework presented here may also be extended to other industrial enzymes, such as lipases and amylases, thereby broadening its biotechnological relevance (47, 52). Compared with earlier GOX expression studies based on *Penicillium variabile* (53) or *A. niger* constructs (54), the integration of dual promoters and

GCN4 co-expression resulted in substantially superior yields. Addressing methanol and oxygen-related constraints will be critical for translating these findings into economically viable industrial operations.

CONCLUSION

This study establishes an effective strategy for high-level production of glucose oxidase (GOX) from *Aspergillus niger* ATCC 9029 in *Pichia pastoris* GS115 by integrating a dual-promoter expression system with targeted cellular stress mitigation. The integration of the constitutive *GAP* promoter with the methanol-inducible *AOX1* promoter facilitated continuous basal expression and strong methanol-triggered induction, while the co-expression of *GCN4* significantly improved cellular resistance to oxidative stress linked to methanol metabolism.

This coordinated design resulted in GOX activities of 333 U/mL and 220.6 U/mL in the engineered strains *P. pastoris* GS115-PIC9-pGAPZαA-*GOX-GCN4*(2) and *P. pastoris* GS115-PIC9-pGAPZαA-*GOX-GCN4*(3), representing a substantial improvement over the normal single-promoter system.

Optimal production occurred at pH 6.0, 34°C, and under high-aeration conditions, underscoring the importance of fine-tuned environmental parameters for maximizing recombinant protein output in *P. pastoris.* Collectively, these findings validate the dual-promoter/*GCN4* co-expression strategy as an effective platform for enhancing heterologous enzyme production. Future efforts should focus on process scale-up, alternative induction or co-feeding strategy, and the extension of this modular system to other industrially or clinically relevant biocatalysts.

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