

# Enhanced glutathione production in *Saccharomyces cerevisiae* via transporter activation and catabolic pathway engineering

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## ABSTRACT

Industrial interest in glutathione, a tripeptide with key roles in cellular redox homeostasis, has increased, particularly in the food and pharmaceutical sectors. This study aimed to engineer *Saccharomyces cerevisiae* to enhance glutathione production through a dual strategy: overexpression of glutathione exporters (GXA1 and GEX2) and targeted knockout of genes involved in glutathione degradation. Overexpression of either GXA1 or GEX2 resulted in simultaneous increases in both intracellular and extracellular glutathione levels. To further enhance the intracellular glutathione content of the GXA1-overexpressing strain (G1/2-GXA1), seven candidate genes associated with glutathione catabolism were individually knocked out and the subsequent effects evaluated. Among the observed outcomes, the knockout of DUG2, which encodes a  $\gamma$ -glutamyl transpeptidase-like protein involved in glutathione degradation, and URE2, which encodes a glutathione S-transferase-like protein that also functions as a negative regulator of nitrogen catabolite repression, significantly improved glutathione accumulation under batch fermentation. Specifically, the glutathione contents in the G1/2-GXA1  $\Delta$ DUG2 and G1/2-GXA1  $\Delta$ URE2 strains increased by 27 % and 46 %, respectively, compared to the parental G1/2-GXA1 strain. Under high-cell-density fed-batch fermentation with glucose-limited feeding, the G1/2-GXA1  $\Delta$ DUG2 strain achieved a maximum dry cell weight of 97 g/L and a glutathione titer of 2.8 g/L. These values were 11 % and 14 % higher, respectively, than those of the G1/2-GXA1  $\Delta$ URE2 strain. Notably, the 2.8 g/L glutathione titer achieved by the G1/2-GXA1  $\Delta$ DUG2 strain represents the highest concentration reported to date in *S. cerevisiae* without the supplementation of pure precursor amino acids.

## 1. Introduction

Glutathione ( $\gamma$ -L-glutamyl-L-cysteinyl-glycine) is a tripeptide that plays a central role in cellular antioxidant defense by scavenging reactive oxygen species (ROS) and mitigating oxidative stress [1]. Due to its antioxidative and physiological functions, glutathione is widely used in the pharmaceutical, food, and cosmetic industries, with global demand continuing to rise [2]. *Saccharomyces cerevisiae*, a microorganism with GRAS (generally recognized as safe) status, has been extensively utilized as an industrial platform for glutathione production [2]. Indeed, glutathione in *S. cerevisiae* is synthesized via a two-step enzymatic pathway (Fig. 1): The first step is catalyzed by  $\gamma$ -glutamylcysteine synthetase (GSH1), which condenses glutamate and cysteine to form  $\gamma$ -glutamylcysteine. Subsequently, glutathione synthetase (GSH2) adds glycine to produce reduced glutathione (GSH) [3].

Following biosynthesis, excess intracellular glutathione can be exported across the plasma membrane via either the proton-coupled antiporter (GEX2) or the ATP-binding cassette transporter (GXA1) [4]. Notably, the GSH1-catalyzed reaction represents the rate-limiting step in the biosynthetic pathway and is subject to feedback inhibition by intracellular GSH [5]. Therefore, overexpression of GEX2 and GXA1 has been proposed as a strategy to decrease cytosolic glutathione levels, thereby relieving feedback inhibition and promoting continuous synthesis. This approach is expected to enhance the overall glutathione biosynthetic flux and improve total production yields in engineered strains.

In addition to controlling biosynthesis and export, *S. cerevisiae* tightly regulates intracellular glutathione homeostasis through degradation and utilization mechanisms. Under conditions of glutathione excess, catabolic pathways are activated to maintain redox balance and recycle

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amino acid components [6]. The degradation of glutathione (DUG) pathway (Fig. 1), which comprises a Cys–Gly dipeptidase (DUG1), a  $\gamma$ -glutamyl transpeptidase-like protein (DUG2), and an aminotransferase-like protein (DUG3), represents the primary route. These enzymes sequentially hydrolyze GSH into glutamate, cysteine, and glycine, serving both detoxification and nutrient salvage functions [7].

Glutathione is also consumed through conjugation with electrophilic compounds, catalyzed by glutathione S-transferases (GSTs). In *S. cerevisiae*, GTT1 plays a crucial role in the detoxification of lipid peroxides and xenobiotics through GSH conjugation [8]. Additionally, URE2, while primarily involved in nitrogen catabolite repression (NCR), exhibits GST-like activity and contributes to protection against oxidative and heavy metal stress [9]. These conjugation pathways constitute a significant sink for intracellular glutathione and, thus, influence its overall balance.

Therefore, this study aimed to enhance glutathione production in *S. cerevisiae* by employing two complementary strategies: (1) overexpression of glutathione exporters (GEX2 and GXA1), and (2) suppression of intracellular glutathione consumption by inhibiting the DUG pathway or GST activity. These approaches are anticipated to shift the intracellular glutathione balance toward accumulation, ultimately improving production efficiency.

## 2. Materials and methods

### 2.1. Strains and plasmids

The strains and plasmids used in this study are summarized in Table 1. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA) and *S. cerevisiae* D452–2 (*MAT $\alpha$* , *leu2*, *his3*, *ura1*, *can1*) [10] were used for plasmid construction and glutathione production, respectively.

### 2.2. Media and culture conditions

*E. coli* TOP10 was cultivated in Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) containing 50  $\mu$ g/mL ampicillin. *S. cerevisiae* was precultured at 30 °C and 250 rpm in YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) for

48 h. The precultured cells were harvested and inoculated into the main cultures at an initial optical density at 600 nm (OD<sub>600</sub>) of 1.0. Main fermentation experiments were conducted in a baffled flask containing 100 mL YPD50 medium (10 g/L yeast extract, 20 g/L peptone, and 50 g/L glucose) at 30 °C and 250 rpm for 72 h.

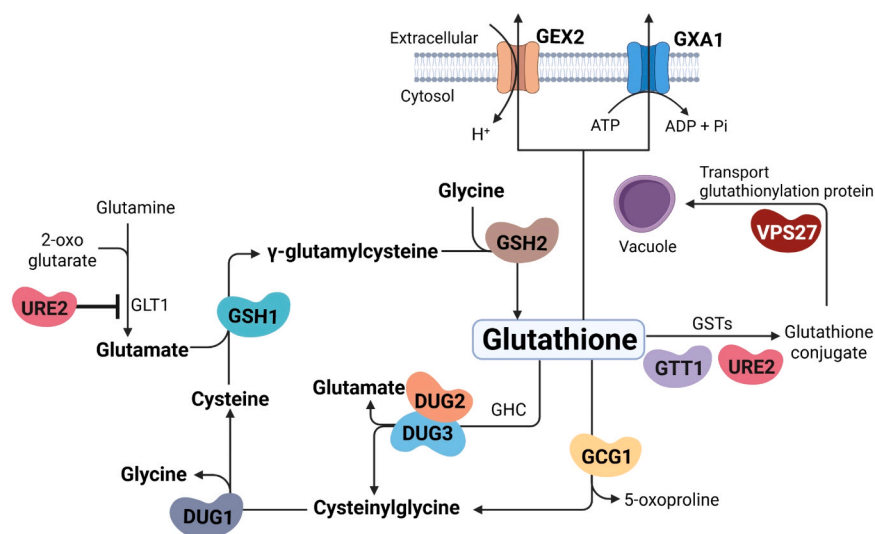
Fed-batch fermentation was conducted in a 2.5 L bioreactor (Kobitech Co., Incheon, Republic of Korea) containing 1 L of YPD medium with an initial OD<sub>600</sub> of 1.0. After the initially added glucose was depleted, a feeding solution containing 600 g/L glucose, 200 g/L yeast extract, and 100 g/L peptone was continuously administered at a rate matching the glucose consumption of the engineered strains to maintain ethanol concentrations below 10 g/L. The medium pH, temperature, agitation speed, and aeration were maintained at 5.5, 30 °C, 1000–1200 rpm, and 2.0–3.5 vvm, respectively.

### 2.3. Genetic manipulation

All gene cloning experiments were performed using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA, USA), as specified by the manufacturer. The primer sets used to amplify *GXA1*, *GEX2*, yeast integrative plasmids (YIPs), guide RNA (gRNA) plasmids, and repair DNA fragments are listed in Table S1. The resulting PCR products were combined using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs) to construct YIPs for *GXA1* and *GEX2* overexpression and to construct gRNA plasmids for the CRISPR/Cas9-based genome editing system. The sequence information for the genes whose expression was modulated in this study is provided in Table S2.

To introduce YIPs containing *GXA1* and *GEX2* into the *LEU2* locus, *S. cerevisiae* was transformed using a yeast EZ-transformation kit (BIO101, Vista, CA, USA), following the manufacturer's instructions. The overall architectures of the *GXA1* and *GEX2* overexpression cassettes are summarized in Table 1.

The CRISPR/Cas9-based genome editing system was employed to construct engineered *S. cerevisiae* strains with a targeted knockout, as previously described [11]. Briefly, repair DNA fragments were amplified using the primers listed in Table S1. The resulting repair DNA fragments and gRNA plasmids were co-transformed into *S. cerevisiae* strains harboring pCas9\_AUR to disrupt target genes.



**Fig. 1. Metabolic engineering strategies to efficiently produce glutathione in *Saccharomyces cerevisiae*.** To enhance glutathione production in *S. cerevisiae*, the glutathione exporters (GEX2 and GXA1) were overexpressed, and genes encoding proteins associated with glutathione catabolism, including *DUG1*, *DUG2*, *DUG3*, *GCG1*, *GTT1*, *URE2*, and *VPS27*, were knocked out. GSH1,  $\gamma$ -glutamylcystine synthetase; GSH2, glutathione synthase; GEX2, proton-coupled antiporter; GXA1, ABC transporter; DUG1, Cys–Gly dipeptidase; DUG2,  $\gamma$ -glutamyl transpeptidase-like protein; DUG3, aminotransferase-like protein; GCG1,  $\gamma$ -glutamylcyclotransferase-like protein; GTT1, glutathione S-transferase; URE2, glutathione S-transferase-like protein; VPS27, subunit of ESCRT-0 complex; GLT1, glutamate synthase; GHC, glutathione hydrolase complex; GSTs, glutathione S-transferases.

**Table 1**  
*Saccharomyces cerevisiae* strains and plasmids used in this study.

| Name                      | Description                                                                                           | Reference  |
|---------------------------|-------------------------------------------------------------------------------------------------------|------------|
| <b>Strains</b>            |                                                                                                       |            |
| D452–2                    | <i>MATa</i> , <i>leu2</i> , <i>his3</i> , <i>ura3</i> , and <i>can1</i>                               | [10]       |
| G1/2                      | D452–2 <i>HIS3::tHXT7p-GSH1-CYC1t</i> , <i>URA3::tHXT7p-GSH2-CYC1t</i>                                | [14]       |
| G1/2-GXA1                 | G1/2 <i>LEU2::tHXT7p-GXA1-CYC1t</i>                                                                   | this study |
| G1/2-GEX2                 | G1/2 <i>LEU2::tHXT7p-GEX2-CYC1t</i>                                                                   | this study |
| G1/2-GXA1 $\Delta$ DUG1   | G1/2-GXA1 with <i>DUG1</i> disruption                                                                 | this study |
| G1/2-GXA1 $\Delta$ DUG2   | G1/2-GXA1 with <i>DUG2</i> disruption                                                                 | this study |
| G1/2-GXA1 $\Delta$ DUG3   | G1/2-GXA1 with <i>DUG3</i> disruption                                                                 | this study |
| G1/2-GXA1 $\Delta$ GCG1   | G1/2-GXA1 with <i>GCG1</i> disruption                                                                 | this study |
| G1/2-GXA1 $\Delta$ GTT1   | G1/2-GXA1 with <i>GTT1</i> disruption                                                                 | this study |
| G1/2-GXA1 $\Delta$ URE2   | G1/2-GXA1 with <i>URE2</i> disruption                                                                 | this study |
| G1/2-GXA1 $\Delta$ VPS27  | G1/2-GXA1 with <i>VPS27</i> disruption                                                                | this study |
| <b>Plasmids</b>           |                                                                                                       |            |
| pRS405HXT                 | <i>LEU2</i> , <i>tHXT7</i> promoter, <i>CYC1</i> terminator, an integrative plasmid, Amp <sup>R</sup> | [38]       |
| pKCK18                    | pRS405HXT7p harboring <i>GXA1</i>                                                                     | this study |
| pKCK20                    | pRS405HXT7p harboring <i>GEX2</i>                                                                     | this study |
| pCas9_AUR                 | Aur <sup>R</sup> , p414- <i>TEF1p-Cas9-CYC1t</i> , modified Cas9 expression plasmid, Amp <sup>R</sup> | [11]       |
| pgRNA-TRP1-HYB            | Hyg <sup>R</sup> , 2 $\mu$ origin, <i>SNR52p-gTRP1-SUP4t</i> , Amp <sup>R</sup>                       | [39]       |
| pgRNA- $\Delta$ DUG1-HYB  | Hyg <sup>R</sup> , 2 $\mu$ origin, <i>SNR52p-gDUG1-SUP4t</i> , Amp <sup>R</sup>                       | this study |
| pgRNA- $\Delta$ DUG2-HYB  | Hyg <sup>R</sup> , 2 $\mu$ origin, <i>SNR52p-gDUG2-SUP4t</i> , Amp <sup>R</sup>                       | this study |
| pgRNA- $\Delta$ DUG3-HYB  | Hyg <sup>R</sup> , 2 $\mu$ origin, <i>SNR52p-gDUG3-SUP4t</i> , Amp <sup>R</sup>                       | this study |
| pgRNA- $\Delta$ GCG1-HYB  | Hyg <sup>R</sup> , 2 $\mu$ origin, <i>SNR52p-gGCG1-SUP4t</i> , Amp <sup>R</sup>                       | this study |
| pgRNA- $\Delta$ GTT1-HYB  | Hyg <sup>R</sup> , 2 $\mu$ origin, <i>SNR52p-gGTT1-SUP4t</i> , Amp <sup>R</sup>                       | this study |
| pgRNA- $\Delta$ URE2-HYB  | Hyg <sup>R</sup> , 2 $\mu$ origin, <i>SNR52p-gURE2-SUP4t</i> , Amp <sup>R</sup>                       | this study |
| pgRNA- $\Delta$ VPS27-HYB | Hyg <sup>R</sup> , 2 $\mu$ origin, <i>SNR52p-gVPS27-SUP4t</i> , Amp <sup>R</sup>                      | this study |

2.4. Analytical methods

Cell growth was monitored by measuring the OD<sub>600</sub> using a spectrophotometer (OPTIZEN POP, Mecasys, Republic of Korea). Dry cell weight (DCW) was estimated by multiplying the OD<sub>600</sub> value by a conversion factor of 0.3 g/L/OD<sub>600</sub>. The glucose, glycerol, acetate, and ethanol concentrations were determined using a high-performance liquid chromatography (HPLC) system (Ultimate 3000, Thermo Fisher Scientific, USA) equipped with a Rezex ROA-Organic Acid H<sup>+</sup> column (Phenomenex, USA). Elution was performed using 5 mM H<sub>2</sub>SO<sub>4</sub> at 60 °C and a flow rate of 0.6 mL/min, with detection performed by a refractive index detector.

Intracellular glutathione was extracted from *S. cerevisiae* to determine its concentration using the method previously described by Hara et al. [12]. The levels of reduced (GSH) and oxidized (GSSG) glutathione were quantified using an HPLC system (Ultimate 3000, Thermo Fisher Scientific) equipped with a YMC-Pack ODS-A column (4.6 × 150 mm, 5  $\mu$ m, 120 Å; YMC Co., Kyoto, Japan). Chromatographic separation was conducted at 30 °C under isothermal conditions, using a mobile phase composed of 5 % (v/v) acetonitrile, 0.05 % (v/v) trifluoroacetic acid, and 0.1 M sodium perchlorate, delivered at a flow rate of 0.5 mL/min. Ultraviolet detection was performed at a wavelength of 220 nm. Glutathione content was normalized to DCW and expressed as a percentage.

To quantify the extracellular glutathione, culture supernatants

obtained by centrifugation were directly subjected to HPLC analysis using the same YMC-Pack ODS-A column. The separation was performed at 30 °C, consistent with the condition used for intracellular glutathione analysis. The mobile phase consisted of 45 mM potassium dihydrogen phosphate, 8.3 mM sodium 1-heptanesulfonate, and 21 % (v/v) methanol in ultrapure water, with the pH adjusted to 2.8 using phosphoric acid. The elution was performed at a flow rate of 0.3 mL/min, and detection was conducted at 210 nm, following the method described by Kobayashi et al. [13].

2.5. Statistical analysis

All statistical analyses were performed using SPSS Statistics version 28.0 (IBM Corp., Armonk, NY, USA). Results are expressed as mean values with corresponding standard deviations. Statistical differences between the two groups were assessed using Student’s *t*-test. Statistical significance was considered at a *p*-value < 0.05.

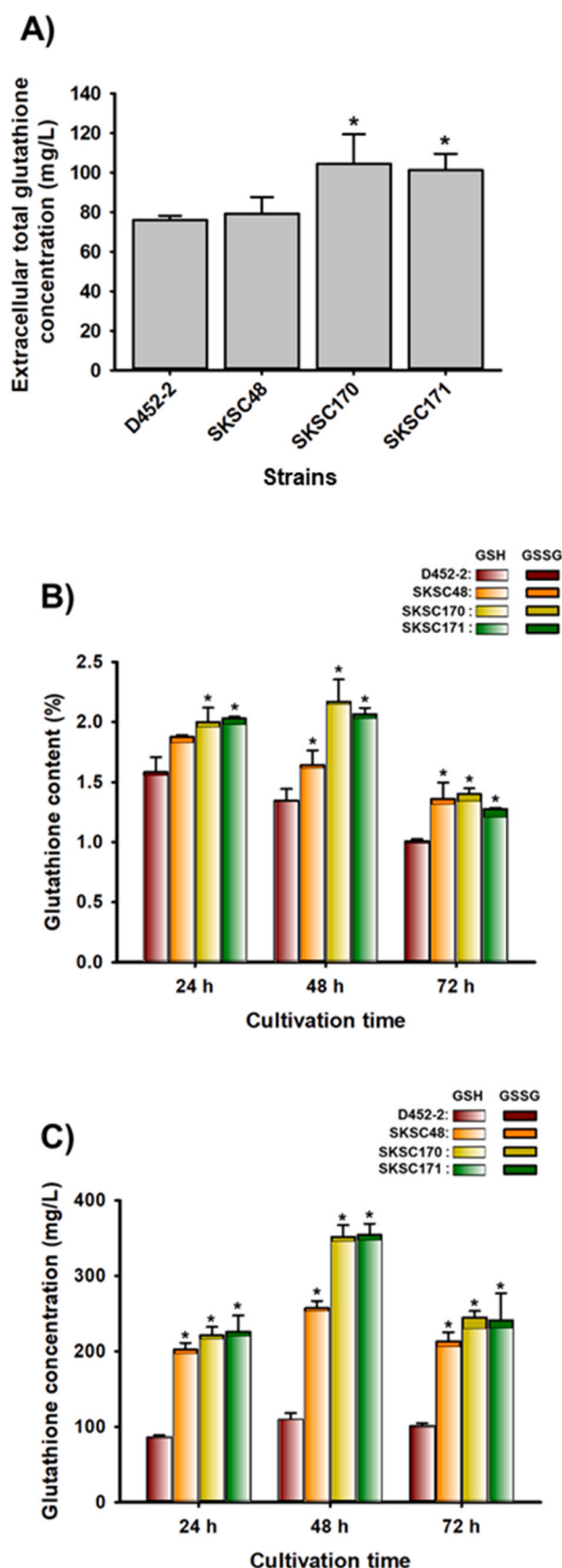
3. Results and discussion

3.1. Effects of glutathione exporter overexpression on intracellular and extracellular glutathione accumulation in *S. cerevisiae*

The *S. cerevisiae* strain G1/2 had been previously engineered by optimizing the expression of two key enzymes involved in glutathione biosynthesis, GSH1 and GSH2, resulting in a 2.7-fold increase in intracellular glutathione levels compared to the wild-type strain [14]. However, regulating only the biosynthetic enzymes proved insufficient to further enhance glutathione production. To overcome this limitation, we investigated an alternative strategy based on the overexpression of glutathione exporters.

Previous studies have demonstrated that transporter overexpression can enhance both extracellular accumulation and overall production of target compounds. In *Corynebacterium glutamicum* and *E. coli*, transporter engineering has successfully improved the secretion of amino acids and organic acids by mitigating intracellular stress and feedback inhibition [15–17]. Notably, the overexpression of *ccmABC* in engineered *E. coli*, which encodes a heme exporter, significantly increased extracellular heme levels and facilitated recovery [18]. Similarly, transporter engineering in *S. cerevisiae* has been applied to improve the secretion and titers of various metabolites, including polyamines and terpenoids [19,20]. Based on these findings, we investigated whether the overexpression of glutathione exporters in *S. cerevisiae* could likewise promote both the secretion and overall yield of glutathione.

To investigate the effect of glutathione exporter overexpression on glutathione production, we performed batch fermentation using the wild-type *S. cerevisiae* D452–2 strain, the previously constructed G1/2 strain with enhanced *GSH1* and *GSH2* expression, and G1/2 derivatives overexpressing either *GXA1* (G1/2-GXA1) or *GEX2* (G1/2-GEX2). Intracellular and extracellular glutathione levels were measured and compared across the strains (Fig. 2). In line with expectation, the concentration of glutathione secreted into the medium after 72 h of cultivation was increased in both G1/2-GXA1 and G1/2-GEX2 strains, by 32 % and 28 %, respectively, compared to the parental G1/2 strain (Fig. 2A). Notably, in addition to extracellular levels, intracellular glutathione content and concentration were also elevated in both exporter-overexpressing strains. Both strains exhibited the highest intracellular glutathione content and concentration at 48 h of fermentation (Fig. 2B and C). Specifically, the G1/2-GXA1 strain showed 32 % and 37 % higher glutathione content and concentration, respectively, compared to the background G1/2 strain. Similarly, the G1/2-GEX2 strain exhibited 26 % and 38 % increases in glutathione content and concentration, respectively, relative to the G1/2 strain. These results align with a previous study by Kiriya et al. [21], which reported that *GXA1* overexpression increased glutathione levels both within and outside the cell. Moreover, this study demonstrated that *GEX2*



(caption on next column)

**Fig. 2. Comparison of glutathione production between the control *Saccharomyces cerevisiae* G1/2 strain and engineered strains over-expressing glutathione exporters.** (A) Extracellular glutathione concentration at 72 h of fermentation, presented as the sum of the reduced and oxidized forms. (B and C) Time-course analysis of intracellular glutathione content (B) and concentration (C). G1/2, *S. cerevisiae* D452-2 overexpressing *GSH1* and *GSH2*; G1/2-GXA1, G1/2 overexpressing *GXA1*; G1/2-GEX2, G1/2 overexpressing *GEX2*; GSH, reduced glutathione; GSSG, oxidized glutathione. Results are presented as the mean of at least two independent experiments, with error bars indicating standard deviation. Asterisk (\*) indicates a significant difference compared to the control group at  $p < 0.05$ , as determined by Student's *t*-test.

overexpression contributes to elevated intracellular and extracellular glutathione levels.

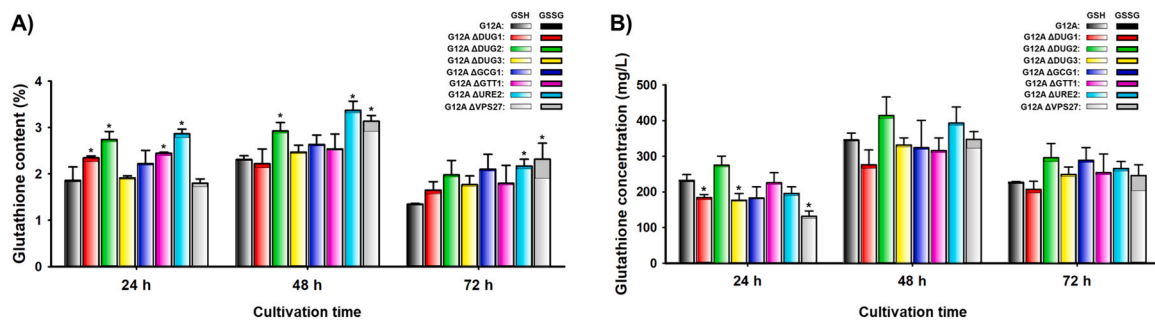
Nonetheless, while the precise mechanisms underlying this phenomenon require further investigation, two potential explanations are proposed: (1) The overexpressed glutathione exporters may localize to the plasma membrane and the vacuolar membrane [22,23], and although intracellular glutathione is typically trafficked to the vacuole for degradation, such localization may have enabled its reverse transport back into the cytosol, thereby further contributing to intracellular accumulation. (2) Exported glutathione may relieve feedback inhibition of its biosynthesis, allowing sustained production, and a portion of the extracellular glutathione may later be reimported into the cytosol via glutathione importers such as OPT1 [24], further contributing to intracellular accumulation and increasing total glutathione levels across both compartments.

### 3.2. Effects of gene knockouts on intracellular glutathione accumulation in *S. cerevisiae*

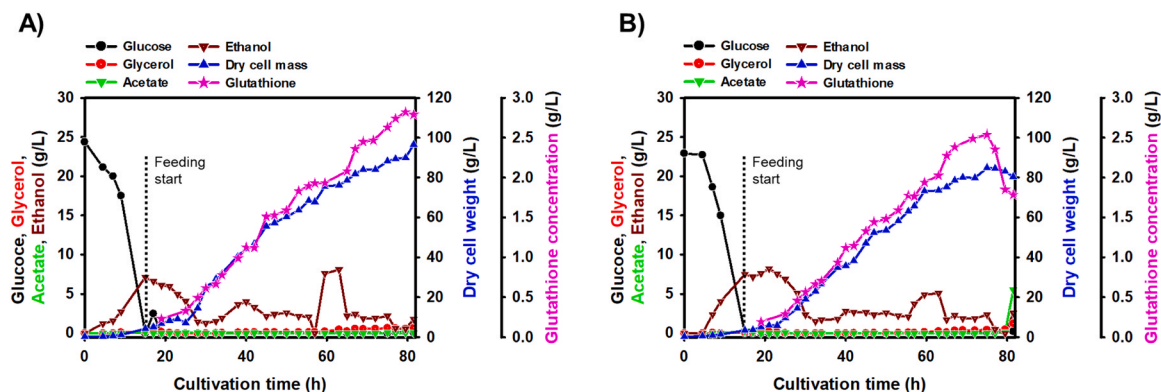
*S. cerevisiae* strains that overproduce glutathione are primarily utilized in the food industry in the form of yeast extract [25]. Therefore, this study aimed to enhance the intracellular glutathione content and concentration of the G1/2-GXA1 strain. Thus, the following genes encoding proteins known to interfere with intracellular glutathione accumulation were selected for knockout: (1) *DUG1*, *DUG2*, and *DUG3*, which formulate the DUG pathway responsible for glutathione catabolism [7]; (2) *GTT1* and *URE2*, which encode proteins exhibiting GST activity [9,10]; (3) *GCG1*, which encodes a ChaC2-like cytosolic  $\gamma$ -glutamylcyclotransferase involved in basal glutathione degradation [26]; (4) *VPS27*, which encodes a component of the endosomal sorting complex required for transport-0 (ESCRT-0) complex involved in the recognition and endosomal sorting of ubiquitinated membrane proteins into multivesicular bodies in *S. cerevisiae* [27].

To evaluate the effect of each gene disruption, all seven target genes were individually disrupted in the G1/2-GXA1 genome using the CRISPR/Cas9 genome editing system. The resulting strains were subjected to batch fermentation, and the intracellular glutathione content and production titers were comparatively analyzed. Considering the temporal profile of glutathione content and production titer, intracellular glutathione accumulation was enhanced in both the G1/2-GXA1  $\Delta DUG2$  and G1/2-GXA1  $\Delta URE2$  strains (Fig. 3). These two strains exhibited the highest glutathione content and titer at 48 h of fermentation. Specifically, the G1/2-GXA1  $\Delta DUG2$  strain showed a 26 % increase in intracellular glutathione content and a 20 % increase in production titer compared to the parental G1/2-GXA1 strain. Similarly, the G1/2-GXA1  $\Delta URE2$  strain exhibited 46 % and 14 % improvements in glutathione content and titer, respectively.

*DUG2* is a key component in the DUG pathway, forming a heterodimer with *DUG3* to catalyze the hydrolysis of glutathione. Both the present study and a previous study [6] have demonstrated that *DUG2* knockout enhances glutathione biosynthesis efficiency in *S. cerevisiae*. Although *DUG1* lacks intrinsic enzymatic activity for glutathione degradation, it plays a complementary role within the pathway [6].



**Fig. 3.** Comparison of glutathione contents (A) and concentrations (B) between the control *Saccharomyces cerevisiae* G1/2-GXA1 strain and its derivatives with gene knockouts. GSH, reduced glutathione; GSSG, oxidized glutathione. Results are presented as the mean of at least two independent experiments, with error bars indicating standard deviation. Asterisk (\*) indicates a significant difference compared to the control group at  $p < 0.05$ , as determined by Student's *t*-test.



**Fig. 4.** Glucose-limited fed-batch fermentation of the G1/2-GXA1  $\Delta DUG2$  (A) and G1/2-GXA1  $\Delta URE2$  (B) strains. Upon depletion of the initial glucose, the feeding solution (600 g/L glucose, 200 g/L yeast extract, and 100 g/L peptone) was introduced into the bioreactor as indicated by the black dotted lines. The medium pH, temperature, agitation speed, and aeration were maintained throughout the cultivation at 5.5, 30 °C, 1000–1200 rpm, and 2.0–3.5 vvm, respectively. All values were measured in duplicate; the average values with standard deviations of less than 5 % are provided.

Therefore, while a single knockout of *DUG1* had only a minimal effect, its combined knockout with *DUG2* or *DUG3* may lead to a more complete disruption of the DUG cascade [8,28]. Future studies should investigate the impact of simultaneously knocking out *DUG2* and either *DUG1* or *DUG3* on intracellular glutathione production.

Among the genes encoding proteins with GST activity in *S. cerevisiae*, only the deletion of *URE2*—and not *GTT1*—resulted in increased intracellular glutathione levels (Fig. 3). This effect may be attributed to the dual role of *URE2*, which, in addition to its GST activity, functions as a repressor of NCR, potentially altering the expression of genes involved in glutathione biosynthesis. For instance, *URE2* knockout may promote an indirect increase in *GLT1* expression, which encodes glutamate synthase. *URE2* functions as a negative regulator of NCR, and its deletion may activate the general amino acid control pathway or derepress genes under NCR-sensitive regulation [29]. Thus, as *GLT1* is known to be transcriptionally regulated in response to nitrogen availability, derepression of NCR-responsive transcription factors in the absence of *URE2* could enhance *GLT1* expression. The resulting increase in glutamate availability—a key precursor for glutathione biosynthesis—may contribute to elevated intracellular glutathione levels observed in the *URE2*-deficient strain.

Notably, the knockout of *GCG1*, which encodes a ChaC2-like cytosolic  $\gamma$ -glutamylcyclotransferase involved in basal glutathione degradation, did not significantly increase the intracellular glutathione levels under the tested conditions. This result suggests that the *GCG1* contribution to the overall glutathione turnover in *S. cerevisiae* may be limited or context-dependent. One possible explanation is that the activity of *GCG1* is relatively low under standard growth conditions, consistent with a previous report describing ChaC2-like proteins as having lower catalytic efficiency compared to the ChaC1-type [26]. Therefore, the

physiological role of *GCG1* may be restricted to maintaining basal glutathione turnover rather than mediating rapid degradation. Conversely, the knockout of *VPS27*, a core subunit of the ESCRT-0 complex involved in endosome-to-vacuole trafficking and multivesicular body formation [27], increased intracellular glutathione levels, consistent with a previous report [30]. However, the loss of *VPS27* function disrupts proteostasis and cell cycle progression by causing the accumulation of misfolded and S-glutathionylated proteins [31], ultimately impairing cell growth. Indeed, the maximum DCW of the G1/2-GXA1  $\Delta VPS27$  strain was reduced by 36 % compared to the parental G1/2-GXA1 strain. Subsequently, the final glutathione production titers of the two strains were nearly identical, despite the elevated intracellular content observed in the *VPS27*-deficient strain.

### 3.3. Fed-batch cultivation and final strain selection for maximized glutathione production in *S. cerevisiae*

To select the final production strain among the two top-performing strains that exhibited the highest intracellular glutathione production efficiency during batch fermentation, the growth and glutathione production of G1/2-GXA1  $\Delta DUG2$  and G1/2-GXA1  $\Delta URE2$  were compared under high-cell-density cultivation conditions. Thus, glucose-limited fed-batch fermentation was conducted to achieve high cell density. In this mode of cultivation, an excess glucose supply is known to induce ethanol production in *S. cerevisiae*, a Crabtree-positive yeast, thereby reducing metabolic efficiency and inhibiting cell growth [32]. To prevent this, the feeding solution was supplied at a rate matching the glucose consumption rate of the engineered strains, thereby maintaining ethanol concentrations below 10 g/L and promoting efficient cell growth and glutathione production. After depleting the initial glutathione

(~20 g/L), the feeding solution consisting of 600 g/L glucose, 200 g/L yeast extract, and 100 g/L peptone was introduced at a rate of 4.8–17.2 mL/h for the G1/2-GXA1  $\Delta$ DUG2 strain and 2.4–9.1 mL/h for the G1/2-GXA1  $\Delta$ URE2 strain (Fig. S1). Under these conditions, both strains exhibited comparable intracellular glutathione content. However, the maximum DCW was 14 % higher for the G1/2-GXA1  $\Delta$ DUG2 strain compared to the G1/2-GXA1  $\Delta$ URE2 strain. Hence, the G1/2-GXA1  $\Delta$ DUG2 strain produced 2.8 g/L of glutathione after 80 h of fermentation, representing an 11 % improvement over the G1/2-GXA1  $\Delta$ URE2 strain. The reduced growth of the G1/2-GXA1  $\Delta$ URE2 strain under high-cell-density cultivation conditions may be attributed to the pleiotropic roles of URE2 in maintaining cellular homeostasis [33]. Meanwhile, in addition to its GST activity, URE2 is a key negative regulator of NCR, which enables cells to adapt to fluctuating nitrogen sources [29]. Thus, the absence of URE2 may lead to dysregulated nitrogen metabolism and the inefficient utilization of available nitrogen, especially under nutrient-limited conditions, such as those found in fed-batch fermentation. Moreover, URE2 has been implicated in the cellular response to oxidative stress [34]. Meanwhile, URE2 knockout has been shown to increase sensitivity to ROS [35], which can accumulate under high-density growth due to elevated respiratory activity and limited oxygen availability. The combination of impaired nitrogen regulation and sensitivity to oxidative stress likely contributes to the diminished growth capacity observed in the URE2-deficient strain during prolonged fermentation. In support of this hypothesis, the G1/2-GXA1  $\Delta$ DUG2 strain consumed 496 g of glucose under high-cell-density fermentation conditions, whereas the G1/2-GXA1  $\Delta$ URE2 strain consumed only 272 g (Fig. S1), indicating a markedly reduced metabolic activity consistent with the proposed mechanism.

The glutathione production titer of 2.8 g/L achieved in this study represents the highest level reported to date using *S. cerevisiae* as a host, without the supplementation of pure precursor amino acids beyond those inherently present in complex medium components. Although Rollini et al. [36] reported a higher glutathione titer of 3.2 g/L using *S. cerevisiae*, their result was obtained after supplementing the medium with cysteine and glycine, direct precursors of glutathione, which significantly limits the economic feasibility of the process. Comparatively, the highest glutathione concentration reported in previous studies using *S. cerevisiae* under conditions without precursor supplementation only reached 2.4 g/L [37].

#### 4. Conclusion

This study demonstrates a successful metabolic engineering approach for enhancing glutathione production in *S. cerevisiae* through coordinated manipulation of both biosynthetic regulation and degradation pathways. The overexpression of GSH exporters in combination with gene knockouts targeting GSH catabolism significantly increased intracellular GSH accumulation. Among various engineered strains, the G1/2-GXA1  $\Delta$ DUG2 strain exhibited the highest production efficiency, achieving 2.8 g/L of glutathione under glucose-limited fed-batch conditions. These findings highlight the efficacy and economic viability of our strategy, offering promising insights for the scalable and cost-effective biosynthesis of glutathione using GRAS microorganisms.

#### CRedit authorship contribution statement

**Sun-Ki Kim:** Writing – review & editing, Formal analysis, Conceptualization. **Jong Myoung Park:** Validation, Supervision. **Chan-Hong Ahn:** Validation, Project administration. **Yong Jun Sim:** Visualization, Methodology. **Sang-Hun Do:** Writing – original draft, Investigation.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2026.01.001.

#### Data availability

Data will be made available on request.

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