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Enhancement of glutathione production in *Saccharomyces cerevisiae* through inverse metabolic engineering

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ABSTRACT

Glutathione is an important tripeptide with a variety of health-promoting effects. Currently, glutathione is produced industrially through a fermentation process using *Saccharomyces cerevisiae* with high glutathione content. However, the glutathione production yield and titer are relatively low compared to using bacteria as a host strain. The underlying reason for this limitation is that previous studies have mainly focused on gene targets directly related to glutathione production. To overcome this limitation, we aimed to identify novel gene targets capable of enhancing glutathione production in *S. cerevisiae*. To this end, the #ACR3–12 mutant, exhibiting 1.8-fold higher glutathione content than the wild-type D452–2 strain, was isolated after two rounds of acrolein resistance-mediated screening. Next, the genes responsible for the increased glutathione production were identified by analyzing mutations that occurred in the #ACR3–12 mutant. Notably, the *SSD1* and *YBL100W-B* genes, which encode a translational repressor of cell wall protein synthesis and a *Ty2* retrotransposon, respectively, played a crucial role in enhancing glutathione production efficiency. In particular, the D452–2 strain overexpressing the *YBL100W-B* gene exhibited 1.6- and 2.1-fold higher maximum dry cell weight and glutathione concentration than the wild-type D452–2 strain.

1. Introduction

Glutathione (γ-L-glutamyl-L-cysteinylglycine) is a biologically active sulfur-containing tripeptide with important physiological functions in all living organisms [1]. At the cellular physiological level, the main functions of glutathione include antioxidant activity to protect cells from reactive oxygen species (ROS)-mediated damage, detoxification of heavy metals and xenobiotics, and immune-boosting effects in mammalian cells [2–4]. In particular, sustained ROS imbalance caused by dysregulation of glutathione homeostasis is linked to a wide range of pathologies, including cancer, diabetes mellitus, and neurodegenerative disorders [5]. As such, glutathione can be used as an important biomarker to detect the onset of disease and as a potential drug or prodrug for specific treatments [6]. Owing to its broad applications in food, medicine, and cosmetic industries, glutathione has experienced an increased demand in recent years, with more than 200 tons of pure crystalline glutathione produced globally per annum [7].

Currently, glutathione is mainly produced through *Saccharomyces cerevisiae* fermentation because chemical synthesis of glutathione results in the production of glutathione in the form of a racemic mixture, and enzymatic synthesis requires an expensive ATP supply [8,9]. Previous studies have employed fermentation process optimization, metabolic engineering, and classical screening strategies to enhance glutathione content in *S. cerevisiae* [8,10,11]. However, its titers and yields remained inferior to those of bacterial systems. For instance, the highest glutathione titer (3.2 g/L) produced by *S. cerevisiae* supplemented with surfactant (lauroyl sarcosine) and precursors (cysteine and glycine) [12] was significantly lower than the titer (15.2 g/L) produced by engineered *Escherichia coli* [13]. Nonetheless, *S. cerevisiae* remains the preferred host for industrial production due to its GRAS (Generally Recognized As Safe) status, compatibility with food-grade processes, and well-established large-scale fermentation infrastructure [14].

A rational metabolic engineering approach is limited in its ability to enhance the production yields of target metabolites because it relies on

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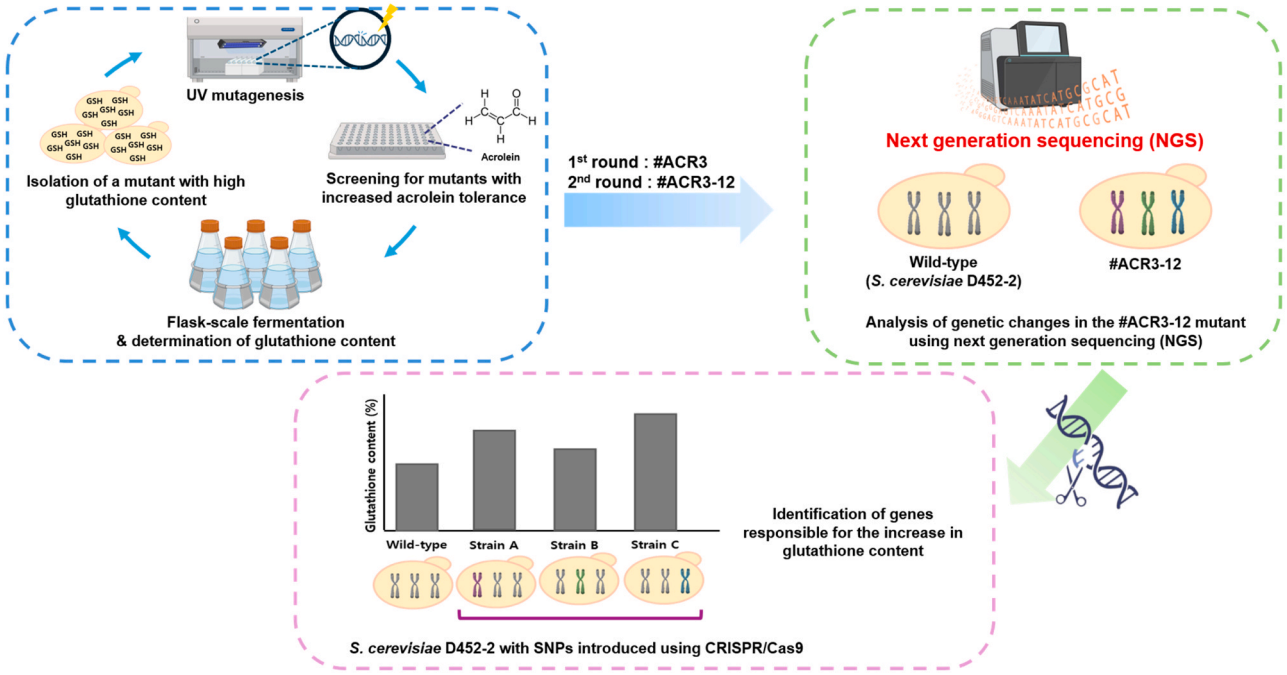

Fig. 1. Overall process of the inverse metabolic engineering employed in this study. The mutants generated by UV irradiation were cultured in parallel in the medium containing high concentrations of acrolein using 96-well plates. Only variants that can grow under these conditions were isolated and analyzed for glutathione content. The entire process was performed once to isolate the #ACR3 mutant and twice consecutively to isolate the #ACR3–12 mutant. The mutations found in the #ACR3–12 mutant were individually introduced into the parental D452–2 strain, using the CRISPR/Cas9 genome editing system to investigate their effects on glutathione production.

already-known metabolic pathways involved in the production of the target metabolites [15]. To overcome this limitation, this study applied an inverse metabolic engineering approach that generates a genome-wide perturbation library and then identifies clones with the desired phenotype [16]. Typically, the most challenging step in inverse metabolic engineering experiments is the efficient isolation of variants that exhibit the desired phenotype. Previous studies reported that glutathione played an important role in cellular defense against the toxicity of acrolein, a type of α,β-unsaturated aldehyde that binds to sulfhydryl groups of proteins [17–19]. Using the property of increased acrolein resistance as a function of intracellular glutathione concentration, Patzschke et al. [17] performed adaptive laboratory evolution (ALE) to isolate mutants with improved glutathione accumulation, in which a mutant with a 3.3-fold increase in glutathione production over its parental strain was selected after 250 generations of continuous evolution under gradually increasing acrolein concentrations. However, that same study could not identify genetic factors associated with high glutathione production because genomic or transcriptomic analyses of the selected strains were not performed.

Of two approaches (selection and screening) for isolating mutants with a desired phenotype, selection is almost always preferred whenever possible, as screening is labor-intensive and inefficient [20]. Accordingly, an ALE strategy consisting of culturing *S. cerevisiae* in increasing concentrations of acrolein was employed to enrich mutants with increased glutathione content. However, the ALE strategy, based on cultivating cells in an acrolein-containing medium, was only successful in some strains because other factors can increase resistance to acrolein, including increased expression of an NADPH-dependent oxidoreductase (OYE2), as well as a high glutathione content [17]. Therefore, to specifically enrich for mutants with enhanced glutathione production, we employed an acrolein resistance-mediated screening approach.

In this study, based on the inverse metabolic engineering approach, we first generated an *S. cerevisiae* mutant library using UV radiation and then analyzed the resistance of mutants against acrolein to isolate the variants with enhanced glutathione content (Fig. 1). After isolating the #ACR3–12 mutant with high glutathione content through two iterations of the overall screening procedure, its genome was analyzed using next-generation sequencing to reveal the genetic changes that occurred in the #ACR3–12 mutant. These analyses laid the foundation for identifying novel genetic determinants associated with enhanced glutathione production.

2. Materials and methods

2.1. Strains and plasmids

The strains and plasmids used in this study are summarized in Table 1. *E. coli* TOP10 (Invitrogen, Carlsbad, CA, USA) was used for plasmid construction. *S. cerevisiae* D452–2 (*MATα*, *leu2*, *his3*, *ura3*, *can1*) [21] was employed as a host for glutathione production.

2.2. Acrolein resistance-mediated screening

A mutant library was constructed by applying UV exposure, according to a previous study [22]. To assess the survival rates of *S. cerevisiae* strains after UV exposure, cells were first cultured in 5 mL of YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) at 30 °C and 250 rpm for 48 h. The cultured cells were then transferred into PCR tubes containing 200 μL of YPD medium, adjusted to an optical density at 600 nm (OD_{600}) of 1.0. The tubes were placed 20 cm below a UV lamp (G30T8, Sankyo Denki, Hiratsuka, Japan) and irradiated for varying durations. After exposure, the cultures were spread onto YPD agar plates and incubated at 30 °C. Colony counts were performed to determine the survival rates. Then, 1140 colonies for the first-round screening and 1128 for the second-round screening were randomly picked from solid YPD plates. The colonies were inoculated into 200 μL YPD medium containing various concentrations of acrolein and cultivated at 30 °C and 900 rpm for 48 h. The variants that could grow in these conditions were selected and cultured at the flask level, and their glutathione concentrations were measured.

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

Name	Description	Reference
Strains		
D452–2	*MATα*, *leu2*, *his3*, *ura3*, and *can1*	[21]
#ACR3	The variant with improved glutathione content isolated from the first-round screening	this study
#ACR3–12	The variants with improved glutathione content isolated from the second-round screening	this study
SSD1_SNP1*	D452–2 with the *SSD1*^{A811G} mutation	this study
SSD1_SNP2*	D452–2 with the *SSD1*$^{AA3428–3429GT}$ mutation	this study
RNR1*	D452–2 with the *RNR1*^{C91T} mutation	this study
YBL100W-B*	D452–2 with the *YBL100W-B*^{T1591C} mutation	this study
TGL4*	D452–2 with the *TGL4*^{G774C} mutation	this study
AMS1*	D452–2 with the *AMS1*^{A241T} mutation	this study
YBL100W-B*/ SSD1_SNP1*	D452–2 with the *YBL100W-B*^{T1591C} and *SSD1*^{A811G} mutations	this study
ΔSSD1	D452–2 Δ*SSD1*	this study
SSD1_over	D452–2 *LEU2::tHXT7p-SSD1-CYC1t*	this study
YBL100W-B_over	D452–2 *URA3::tHXT7p-YBL100W-B-CYC1t*	this study
SKSC48	D452–2 *HIS3::tHXT7p-GSH1-CYC1t*, *URA3*:: *tHXT7p-GSH2-CYC1t*	[11]
SKSC49	D452–2 *HIS3::tHXT7p-GSH1-CYC1t*, *URA3*:: *GPDp-GSH2-CYC1t*	[11]
Plasmids		
pRS405tHXTp	*LEU2*, *tHXT7* promoter, *CYC1* terminator, an integrative plasmid, Amp^R	[30]
pRS406tHXTp	*URA3*, *tHXT7* promoter, *CYC1* terminator, an integrative plasmid, Amp^R	[30]
pSNK04	pRS405tHXT7p harboring *SSD1*	this study
pSNK05	pRS406tHXT7p harboring *YBL100W-B*	this study
pCas9_AUR	Aur^R, p414-*TEF1p-Cas9-CYC1t*, modified Cas9 expression plasmid, Amp^R	[23]
pgRNA-*TRP1*-HYB	Hyg^R, 2 μ origin, *SNR52p-gTRP1-SUP4t*, Amp^R	[31]
pgRNA-*SSD1*_SNP1*-HYB	Hyg^R, 2 μ origin, *SNR52p-gSSD1*_SNP1-*SUP4t*, Amp^R	this study
pgRNA-*SSD1*_SNP2*-HYB	Hyg^R, 2 μ origin, *SNR52p-gSSD1*_SNP2-*SUP4t*, Amp^R	this study
pgRNA-Δ*SSD1*-HYB	Hyg^R, 2 μ origin, *SNR52p-gSSD1-SUP4t*, Amp^R	this study
pgRNA-*RNR1*-HYB	Hyg^R, 2 μ origin, *SNR52p-gRNR1-SUP4t*, Amp^R	this study
pgRNA-*YBL100W-B*-HYB	Hyg^R, 2 μ origin, *SNR52p-gYBL100W-B -SUP4t*, Amp^R	this study
pgRNA-*TGL4*-HYB	Hyg^R, 2 μ origin, *SNR52p-gTGL4-SUP4t*, Amp^R	this study
pgRNA-*AMS1*-HYB	Hyg^R, 2 μ origin, *SNR52p-gAMS1-SUP4t*, Amp^R	this study

2.3. Media and culture conditions

E. coli was cultivated in Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) containing 50 μg/mL ampicillin. *S. cerevisiae* strains were pre-cultured at 30 °C and 250 rpm for 48 h in YPD medium. Cells in the stationary phase of the pre-culture were harvested and inoculated into the main cultures at an initial OD_{600} of 1.0. Main fermentation experiments were conducted at 30 °C and 250 rpm for 96 h in a baffled flask containing 100 mL YP50D medium (10 g/L yeast extract, 20 g/L peptone, 50 g/L glucose).

2.4. Genetic manipulation

All gene cloning experiments were carried out using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA, USA), as specified by the manufacturer. The primer sets used to amplify guide RNA (gRNA) plasmids, repair DNA fragments, and yeast integrative plasmids (YIps) are listed in Table S1. The resulting PCR products were combined using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs) to construct YIps for *SSD1* and *YBL100W-B* overexpression and, additionally, to construct gRNA plasmids for the CRISPR/Cas9-based genome editing system.

To construct engineered *S. cerevisiae* strains with single nucleotide polymorphisms (SNPs) and *SSD1* knockout strain, the CRISPR/Cas9-based genome editing system was used, as previously described [23]. Briefly, repair DNA fragments were amplified with primers listed in Table S1. The resulting repair DNA fragments and gRNA plasmids were co-transformed into *S. cerevisiae* strains harboring pCas9_AUR to construct recombinant strains with SNPs and disrupt *SSD1*.

2.5. Genome sequencing

The genomes of *S. cerevisiae* strains were sequenced and analyzed according to a previous study [22]. Briefly, the genomic DNA (gDNA) of the D452–2 and its mutant strain (#ACR3–12) were prepared using the gDNA preparation kit (Zymo Research Co., Irvine, CA, USA), as specified by the manufacturer. The resulting gDNAs were sent to Macrogen (Seoul, Republic of Korea) for library construction and whole-genome resequencing. The libraries were sequenced by a HiSeq 4000 system (Illumina, San Diego, CA, USA). The genetic variations in the #ACR3–12 strain were identified using CLC GenomicsWorkbench (CLC Bio, Aarhus, Denmark).

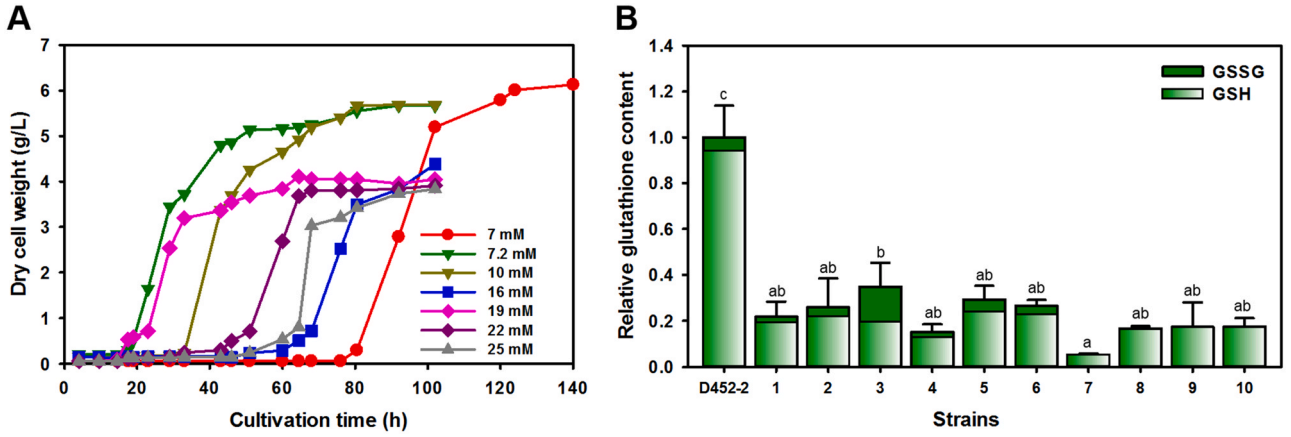

Fig. 2. Effects of acrolein resistance-mediated adaptive laboratory evolution on glutathione content of *Saccharomyces cerevisiae*. (**A**) The growth curves of the *S. cerevisiae* D452–2 strain grown serially with gradually increasing concentrations of acrolein from 7 to 25 mM. (**B**) Comparison of glutathione contents in the control *S. cerevisiae* D452–2 and its mutants isolated by the adaptive laboratory evolution. The relative fold change in glutathione content was calculated by comparing the sample strains to the *S. cerevisiae* D452–2 strain. GSH, reduced glutathione; GSSG, oxidized glutathione. Results represent the mean of three experiments, with error bars indicating standard deviation. Different letters indicate significantly different means (Tukey's HSD tests, $p < 0.05$).

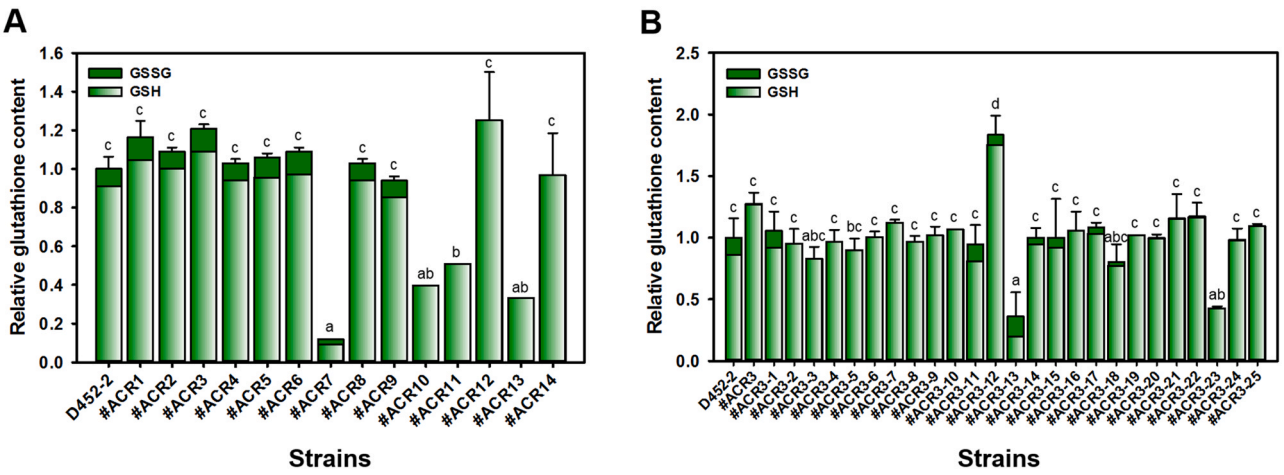

Fig. 3. Comparison of glutathione contents in the control *Saccharomyces cerevisiae* D452–2 and its mutants isolated after first (A) and second (B) rounds of acrolein resistance-mediated screening. The relative fold change in glutathione content was calculated by comparing the sample strains to the *S. cerevisiae* D452–2 strain. GSH, reduced glutathione; GSSG, oxidized glutathione. Results represent the mean of two experiments, with error bars indicating standard deviation. Different letters indicate significantly different means (Tukey's HSD tests, $p < 0.05$).

2.6. Analytical methods

Glutathione extraction from *S. cerevisiae* cells for the determination of glutathione concentration was performed according to a previous study [24]. Concentrations of the reduced (GSH) and oxidized (GSSG) forms of glutathione were measured using an HPLC system (Thermo Fisher Ultimate 3000) equipped with a YMC-Pack ODS-A column (5 μm, 120 Å, 4.6 × 150 mm; YMC, Kyoto, Japan). Glutathione was separated at a constant temperature (30 °C) in a mobile phase consisting of 5 % (v/v) acetonitrile, 0.05 % (v/v) trifluoroacetic acid, and 0.1 M sodium perchlorate at a flow rate of 0.5 mL/min, and then passed through a UV detector at 220 nm. The glutathione content (%) was expressed as the glutathione concentration per dry cell weight.

2.7. Statistical analysis

Statistical analyses were conducted using SPSS Statistics software (v.28.0; IBM Corp., Armonk, NY, USA). Data are presented as the mean ± standard deviation. A one-way analysis of variance was performed, and statistical significance was assessed using Tukey's honestly significant difference (HSD) test at a significance level of $p < 0.05$.

3. Results and discussion

3.1. Isolation of mutants with improved glutathione production via acrolein resistance-mediated screening

Because of the difficulty in predicting whether ALEs based on acrolein resistance could be applied to *S. cerevisiae* D452–2, we decided to validate the effects of the ALE strategy on glutathione content in the D452–2 strain. To this end, the D452–2 strain was serially cultured in gradually increasing concentrations of acrolein from 7 to 25 mM. As the serial culture progressed, *S. cerevisiae* cells exhibited a relatively short lag phase and high specific growth rate even at high concentrations of acrolein (Fig. 2A). This result suggested that as ALE progressed, variants with increased resistance to acrolein were enriched in the culture. Next, we measured the glutathione contents of 10 different single colonies obtained from the last culture containing 25 mM acrolein. However, all 10 variants selected through the acrolein resistance-mediated ALE process had lower glutathione content than the parental D452–2 strain (Fig. 2B). The most likely reason is that the D452–2 strain has other important genes involved in acrolein resistance in addition to glutathione production, and variants containing mutations in these genes dominated the culture.

Because selecting for high glutathione production strains through the acrolein resistance-based ALE approach seemed ineffective, we sought to screen for high glutathione production variants using acrolein resistance. Before performing the screening, we investigated whether the higher glutathione content of the D452–2 strain would increase its resistance to acrolein. To this end, the growth of the D452–2 strain and metabolically engineered SKSC48 and SKSC49 strains [11], which exhibited 2.7- and 2.3-fold higher glutathione content than the D452–2 strain, were compared in the medium containing 7 mM acrolein. The SKSC48 and SKSC49 strains were engineered to overexpress γ-glutamylcysteine synthetase (GSH1) and glutathione synthetase (GSH2), key enzymes involved in the glutathione biosynthetic pathway. In SKSC48, GSH1, which catalyzes the rate-limiting step of the pathway, is expressed under the transcriptional control of a stronger promoter than in SKSC49, resulting in higher intracellular glutathione content compared to SKSC49 [11]. As expected, higher glutathione content decreased the lag phase and increased the specific growth rate in the medium containing 7 mM acrolein (Fig. S1). Given the positive correlation between glutathione content and acrolein resistance, it was hypothesized that although the D452–2 strain could not grow in medium with a high acrolein concentration, mutants with increased glutathione content could grow in the same medium. To validate this hypothesis, the parental D452–2 strain and a total of 1140 variants, which were randomly selected from the mutant library constructed by UV irradiation, were cultivated in parallel in 96-well plates containing 200 μL of YPD medium with 4 −6 mM acrolein. Although the D452–2 strain and most of its variants could not grow in this harsh environment, a total of 14 variants showed growth. Some of these strains showed improved glutathione content, albeit not statistically significant (Fig. 3A). The third mutant (#ACR3), which exhibited 21 % higher glutathione content than the parental D452–2 strain, was selected; then, we performed the overall process consisting of the #ACR3 mutant library construction coupled with acrolein resistance screening once more to further enhance glutathione content. As a result, 25 out of a total of 1128 variants were isolated with growth in medium supplemented with 14 mM acrolein, in which the #ACR3 strain was unable to grow. Notably, the twelfth of the 25 variants (#ACR3–12) had 44 % higher glutathione content than the parental #ACR3 strain (Fig. 3B).

Table 2
Genetic variation observed in the #ACR3-12 mutant compared to its parental D452-2 strain.

Gene	Nucleotide change	Amino acid change	Function
SSD1 (*YDR293C*)	$A^{811} \rightarrow G$ $A^{3428} \rightarrow G$ $A^{3429} \rightarrow T$	$Asn^{271} \rightarrow Asp$ $Lys^{1143} \rightarrow Ser$	Translational repressor of cell wall protein synthesis
RNR1 (*YER070W*)	$C^{91} \rightarrow T$	$Pro^{31} \rightarrow Ser$	Major isoform of large subunit of ribonucleotide-diphosphate reductase
YBL100W-B	$T^{1591} \rightarrow C$	$Ser^{531} \rightarrow Pro$	*Ty2* retrotransposon
TGL4 (*YKR089C*)	$G^{774} \rightarrow C$	$Glu^{258} \rightarrow Asp$	Triacylglycerol lipase
AMS1 (*YGL156W*)	$A^{241} \rightarrow T$	$Ile^{81} \rightarrow Phe$	Vacuolar α-mannosidase

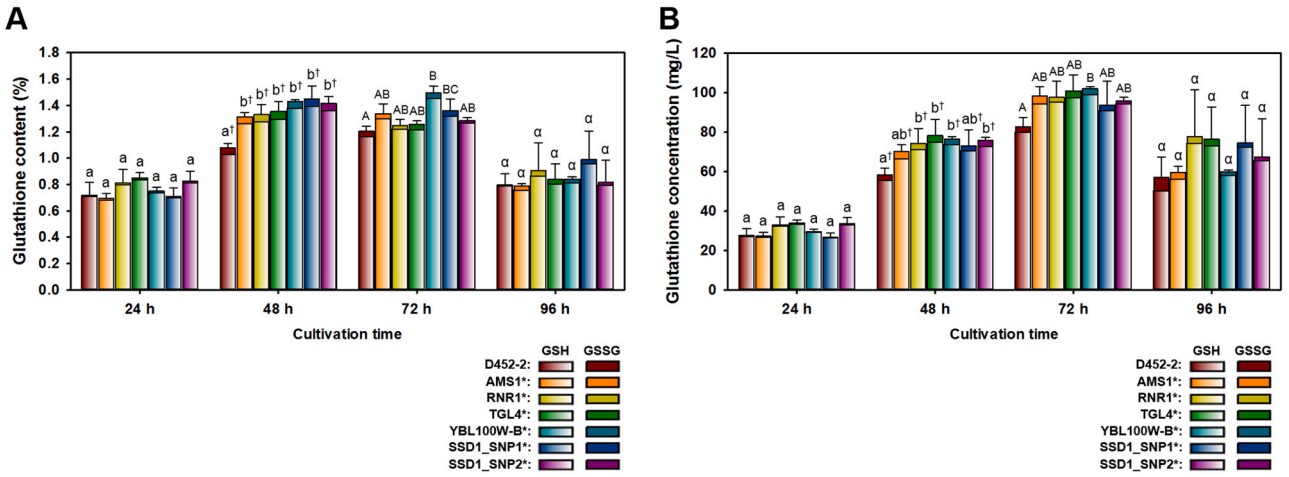

Fig. 4. Comparison of glutathione contents (A) and concentrations (B) in the control *Saccharomyces cerevisiae* D452–2 and various engineered strains. AMS1*, D452–2 with *AMS1*^{A241T} mutation; RNR1*, D452–2 with *RNR1*^{C91T} mutation; TGL4*, D452–2 with *TGL4*^{G774C} mutation; YBL100W-B*, D452–2 with *YBL100W-B*^{T1591C} mutation; SSD1_SNP1*, D452–2 with *SSD1*^{A811G} mutation; SSD1_SNP2*, D452–2 with *SSD1*$^{A3428G,\ A3429T}$ mutation; GSH, reduced glutathione; GSSG, oxidized glutathione. Results represent the mean of $n \geq 2$ experiments, with error bars indicating standard deviation. Different letters indicate significantly different means (Tukey's HSD tests, $p < 0.05$).

3.2. Identification of genes responsible for the increase in glutathione production

In this study, we sought to identify genetic changes associated with high glutathione production rather than simply screening for high glutathione production strains. To this end, the genomes of the parental D452–2 strain and the final high glutathione-producing #ACR3–12 strain were comparatively analyzed by next-generation sequencing. The #ACR3–12 mutant had seven SNPs in the coding regions of genes (Table 2). The SNPs located in the *SSD1*, *RNR1*, *YBL100W-B*, *TGL4*, and *AMS1* genes resulted in the following missense mutations: (1) N271D and K1143S mutations in SSD1, (2) P31S mutation in RNR1, (3) S531P mutation in YBL100W-B, (4) E258D mutation in TGL4, and (5) I81F mutation in AMS1. The six missense mutations identified in the #ACR3–12 mutant were introduced individually into the parental D452–2 strain using the CRISPR/Cas9-mediated genome editing system. The results showed that the missense mutation-introduced strains exhibited an overall higher glutathione content and production concentration compared to the parental D452–2 strain (Fig. 4). Among them, the SSD1_SNP1* strain with the N271D mutation in SSD1 and the YBL100W-B* strain with the mutation in YBL100W-B had the largest increase in glutathione content. The SSD1_SNP1* and YBL100W-B* strains exhibited a 20 % and 24 % increase in maximum glutathione content, respectively, compared to the D452–2 strain. Although the N271D mutation in SSD1 was combined with the mutation in YBL100W-B, no synergistic effect was observed (Fig. S2). In conclusion, using the inverse metabolic engineering approach, we identified *SSD1* and *YBL100W-B* as novel genes involved in increased glutathione production.

SSD1 is a repressor that inhibits the expression of various cell wall proteins at the translational level [25]. Therefore, it was predicted that the SNP in the *SSD1* gene in the #ACR3–12 mutant would either decrease the expression of glutathione exporters in the cell wall or increase the expression of glutathione importers, resulting in enhanced intracellular glutathione content. Meanwhile, the *YBL100W-B* gene encodes a *Ty2* retrotransposon, and transposons are characterized by transposition and insertion into new locations in the genome [26]. *S. cerevisiae* has five families of transposable elements (*Ty1*−*Ty5*), all of which are long terminal repeat retrotransposons [27]. Among them, *Ty1* and *Ty2* are the major and active families, accounting for approximately 75 % of *Ty* insertions in the *S. cerevisiae* genome [28; 29]. Therefore, we speculated that the SNP in *YBL100W-B* improved glutathione biosynthesis by increasing the transposition efficiency of the *Ty2* retrotransposon, thereby inactivating genes that inhibit glutathione biosynthesis.

3.3. Functional validation of SNPs in SSD1 and YBL100W-B genes

Although we confirmed that the missense mutations in the *SSD1* and *YBL100W-B* genes enhanced glutathione production, additional experiments were required to determine whether these mutations increased or decreased the activity of the SSD1 and YBL100W-B proteins. Accordingly, the *SSD1* gene was overexpressed and disrupted in the D452–2 strain to increase and eliminate the activity of the SSD1 repressor protein, respectively. Meanwhile, for the *YBL100W-B* gene, knocking it out was not technically feasible due to its multiple presence in the D452–2 genome; hence, an overexpression strain was constructed. When *SSD1* was overexpressed or inactivated in the D452–2 strain, lower glutathione content and concentration were observed than in the SSD1_SNP1* strain with the N271D mutation in SSD1 (Fig. 5). This

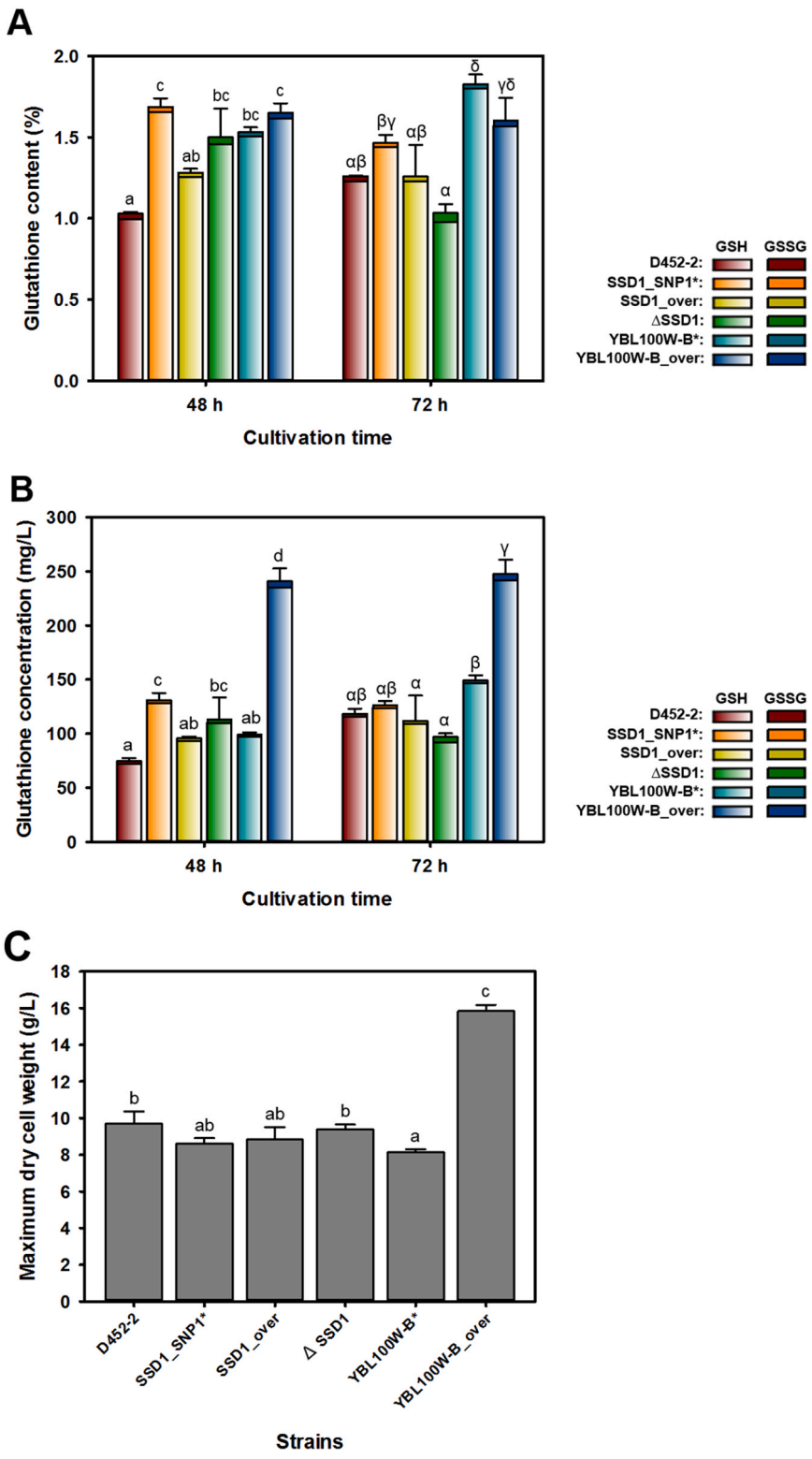

Fig. 5. Comparison of fermentation parameters by the control *Saccharomyces cerevisiae* D452–2 and various engineered strains. (**A**) Glutathione content. (**B**) Glutathione concentration. (**C**) Maximum dry cell weight. SSD1_SNP1*, D452–2 with *SSD1*^{A811G} mutation; SSD1_over, D452–2 with *SSD1* overexpression; ΔSSD1, D452–2 with *SSD1* knockout; YBL100W-B*, D452–2 with *YBL100W-B*^{T1591C} mutation; YBL100W-B_over, D452–2 with *YBL100W-B* overexpression; GSH, reduced glutathione; GSSG, oxidized glutathione. Results represent the mean of $n \geq 2$ experiments, with error bars indicating standard deviation. Different letters indicate significantly different means (Tukey's HSD tests, $p < 0.05$).

indicates that the effect of increasing glutathione production was not significant in the case of the *SSD1* gene, or its overexpression did not enhance SSD1 activity because the amount of intracellular expression is already sufficient. Meanwhile, *YBL100W-B* overexpression was associated with a significant increase in the maximum dry cell weight and glutathione production. Specifically, the *YBL100W-B*—overexpressing strain (YBL100W-B_over) exhibited 1.9 and 1.7 times higher maximum dry cell weight and glutathione concentration than the YBL100W-B* strain, respectively (Fig. 5). Although the glutathione contents of the YBL100W-B_over and YBL100W-B* strains were almost identical (Fig. 5A), the cell growth of the YBL100W-B_over strain was much higher than that of the YBL100W-B* strain (Fig. 5C). As a result, the glutathione production concentration of the YBL100W-B_over strain was greatly enhanced compared to the YBL100W-B* strain (Fig. 5B). Although the exact mechanism is not yet understood, it is speculated that the *YBL100W-B* overexpression activates *Ty2*-mediated transposition, which in turn suppresses the expression of genes associated with growth inhibition or promotes the expression of genes associated with growth promotion. Further studies will analyze where the *Ty2* retrotransposon is inserted in the D452–2 genome to identify genes involved in cell growth and glutathione production.

We previously reported that the SKSC222 strain, which synthesizes glutathione using a synthetic isozyme system composed of a novel bifunctional enzyme (GshF) from Gram-positive bacteria—possessing both GSH1 and GSH2 activities—in addition to endogenous GSH1/GSH2, produced 2.0 g/L of glutathione under fed-batch fermentation conditions [11]. Based on the findings of this study, introducing *YBL100W-B* overexpression into the SKSC222 strain is expected to further enhance glutathione production efficiency.

4. Conclusions

In this study, we demonstrated a novel strategy to unveil the genes involved in the high production efficiency of glutathione. Two iterative rounds of the UV radiation-mediated mutant library construction and acrolein resistance-mediated screening allowed the isolation of the #ACR3–12 mutant with improved glutathione production. We subsequently identified the *SSD1* and *YBL100W-B* genes as critical targets that enhanced glutathione production through the inverse metabolic engineering approach. Considering that the *YBL100W-B* overexpression significantly enhanced the growth of *S. cerevisiae* in addition to glutathione production, the strategy employed in this study can be applied to enhance the production of other high-value compounds.

CRediT authorship contribution statement

Jong Myoung Park: Supervision, Project administration. **Young-Oh Lee:** Investigation. **Chan-Hong Ahn:** Supervision, Project administration. **Soo Bin Nho:** Investigation. **Sang-Hun Do:** Writing – original draft, Methodology. **Sun-Ki Kim:** Writing – review & editing, Formal analysis, Conceptualization.

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.2025.05.001.

Data availability

Data will be made available on request.

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