

**Replacing Glycerol-3-Phosphate Dehydrogenase with NADH Oxidase: Effects on Glucose Fermentation
and Product Formation in *Saccharomyces cerevisiae***

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Abstract

The NADH/NAD⁺ balance plays a critical role in regulating cellular and metabolic pathways. In *Saccharomyces cerevisiae*, glycerol-3-phosphate dehydrogenase (ScGPD) enzymes are essential for NADH homeostasis, glycerol biosynthesis, and osmotic stress adaptation. This study investigates the replacement of ScGPD isoforms with the water-forming NADH oxidase from *Lactococcus lactis* (*L*noxE) and its effects on 10 % glucose fermentation dynamics in minimal medium under microaerobic conditions. We engineered *S. cerevisiae* strains by individually or sequentially deleting or substituting ScGPD isoforms with *L*noxE, generating strains with varying NADH oxidation levels, fermentation rates, and byproduct formation. The engineered strains exhibited three distinct fermentation profiles: faster strains (Δ GPD2 and Δ GPD1,2), five medium-speed strains (native, Δ GPD1, *L*noxE/ Δ GPD1, *L*noxE/ Δ GPD2, and *L*noxE with GPD), and three slower strains (*L*noxE/ Δ GPD1,2, *L*noxE/ Δ GPD1- Δ GPD2, and *L*noxE/ Δ GPD2- Δ GPD1). Increased NADH oxidation correlated strongly with higher acetic acid production, which inhibited cell growth and reduced fermentation speed, especially when glycerol biosynthesis was abolished. For instance, *L*noxE/ Δ GPD1 reduced glycerol production by 88% and increased ethanol yield by 6.2%, despite a 9% increase in acetic acid production. This study underscores the importance of NADH oxidation in optimizing fermentation efficiency and metabolic balance in *S. cerevisiae* strains lacking GPD during glucose fermentation.

Keywords: NADH oxidation variation, glucose fermentation, replacing glycerol-3-phosphate dehydrogenases, water-forming NADH oxidase, *Saccharomyces cerevisiae*.

Introduction

Saccharomyces cerevisiae has been widely utilized in industrial fermentation, contributing to the production of over 100 billion liters of bioethanol annually [1]. During glucose fermentation, glycerol is produced as a byproduct, playing a crucial role in maintaining redox balance and enabling osmotic stress adaptation, both of which affect fermentation efficiency [2-5]. The mitochondrial membrane of *S. cerevisiae* cells is impermeable to NADH/NAD⁺ [6], and the yeast lacks transhydrogenase activity [7]. Instead, mitochondrial external NADH dehydrogenases (ScNDE1 and ScNDE2) are responsible for oxidizing cytosolic NADH, with oxygen serving as the electron acceptor. These dehydrogenases, along with mitochondrial glycerol-3-phosphate dehydrogenase (ScGUT2), contribute to ATP production and NADH reoxidation [8]. In all eukaryotic cells, glycerol-3-phosphate dehydrogenases (GPDs) catalyze the conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P), which is subsequently dephosphorylated to glycerol [9]. Under anaerobic conditions, ScGPD2 is the primary enzyme that oxidizes cytosolic NADH, while ScGPD1 plays an essential role under aerobic and osmotic stress conditions [2, 3, 10]. This differential regulation of ScGPD isoforms allows *S. cerevisiae* to cope with various stresses and explains why less glycerol is produced under aerobic conditions than in anaerobic environments [8]. The structure of yeast as a single eukaryotic cell provides an interesting model for studying various fundamental biological phenomena [11]. Significant experiments on NADH oxidation and its relationship with the respirofermentative Crabtree effect have been conducted [12]. NADH oxidation contributes to multiple metabolic pathways and plays a key role in continuous metabolism and growth [13, 14].

Given the central role of ScGPDs in redox homeostasis and glycerol biosynthesis, numerous metabolic engineering approaches have been explored to restore redox balance and reduce glycerol biosynthesis in bioethanol industry. One common strategy is to delete the genes responsible for glycerol biosynthesis (*ScGPDs*) and/or the facilitator (*ScFPS*) and compensate for the redox imbalance through alternative pathways [5, 10, 14-22]. Another strategy involves modulating the expression of ScGPD1 to fine-tune glycerol biosynthesis [23, 24]. The *noxE* shuttle, which requires only O₂ as a substrate to oxidize NADH to NAD⁺ has attracted significant attention and represents a third approach for reducing excess NADH [12,13, 25-27]. Under batch culture conditions, comparing the expression of *Streptococcus pneumoniae noxE* (*SpnoxE*) with and without the triple deletion of ScNDE1, ScNDE2, and ScGUT2 revealed that *noxE* compensates for NADH oxidation and reduces glycerol biosynthesis by 60–80% [12]. The deletion of *ScGPDs* and the expression of *LlnoxE* have previously been used to increase the efficiency of glucose conversion to 2,3-butanediol [22, 28]. Recently, we retrofitted cytosolic NADH oxidation by replacing *ScGPD1* with *LlnoxE* to develop an efficient glycerol-converting *S.*

cerevisiae strain for biorefineries [27-30]. However, the effects of varying NADH oxidation resulting from the expression of *LlnoxE*, particularly in the absence of ScGPD, have not been explored during the fermentation of fermentable or nonfermentable carbons.

In this study, our objective was to investigate the impacts of substituting ScGPD shuttles with *LlnoxE*, which inducing different levels of NADH oxidation compared to reference strains (Table 3). The enzyme activities of *LlnoxE*, the NADH/NAD⁺ ratio, and fermentation of 10% glucose under microaerobic conditions in minimal medium were analyzed across the generated recombinant strains. Additionally, the pairwise correlation coefficients of fermentation characteristics—such as glucose consumption, ethanol production, glycerol biosynthesis, acetic acid production, and cell growth—were explored to validate the findings. This study demonstrated a positive correlation between increased NADH oxidation and acetic acid production, while a negative correlation was observed between acetic acid levels and cell growth, leading to a reduced rate of glucose fermentation. **[Please insert Fig. 1 here]**

Materials and Methods

Media compositions

YPD medium, composed of 10 g of yeast extract, 20 g of peptone, and 20 g/L of glucose, was routinely used for the cultivation and preservation of yeast strains. Agar at a concentration of 1.8% was added to all media used for plate preparation. YPD medium, supplemented with 200 mg/L geneticin (G418) and referred to as YPD_{G418} was used to select recombinant strains harboring the pCas9-gRNA-CRISPR multiplex plasmid. Selective medium for positive *ScURA3* integration, as previously described [27], consisted of 6.78 g/L yeast nitrogen base (YNB) without amino acids, 20 g/L glucose, and 20 mg/L each of leucine and histidine. For the fermentation experiments, YNB was supplemented with 100 g/L glucose (YNB_{D100}), 0.5 g/L leucine, and 0.5 g/L histidine. All the liquid media used in this study were filter sterilized. For selecting and cultivating *Escherichia coli* with ampicillin-resistance plasmids, Luria–Bertani (LB) medium was used, which contained 1% tryptone, 1% sodium chloride (NaCl), 0.5% yeast extract, and 150 µg/mL ampicillin. Additionally, 75 µg/mL kanamycin was added to LB for selecting and cultivating *E. coli* harboring the pCas9-gRNA-CRISPR multiplex plasmid [31].

Homologous repair cassette and plasmid assemblies

The primers used in this study were designed based on the sequences available in the S288C *Saccharomyces* Genome Database and are listed in Table 1. The nucleotide sequences of the DNA cassettes used for repairing double-strand breaks via homologous recombination through CRISPR were the same as previously

reported [27]. High-fidelity polymerase KOD-plus neo (Toyobo, Osaka, Japan) was used during PCR to minimize the occurrence of mutations. The combination of *TDH3p-DIT1_{t_{d22}}* containing the glyceraldehyde 3-phosphate dehydrogenase promoter (*TDH3p*) and a mutated *DIT1* terminator (*DIT1_{t_{d22}}*) as expressing system was previously shown to enhance protein production [32] and was used here as a deletion cassette. Homologous regions upstream and downstream of *ScGPD2* were added to a deleting cassette through PCR, using the template plasmid *pTDH3p-DIT1_{t_{d22}}-URA3* [27]. A synthetic codon-optimized *LlnoxE* gene from *L. lactis* subsp. *lactis* Il1403, along with its expression system (*TDH3p* and *DIT1_{t_{d22}}*), had been previously cloned into *TDH3p-noxE-DIT1_{t_{d22}}-URA3* [27], which contains the *LlnoxE* cassette flanked by the *ScGPD1* promoter and terminator sequences. PCR was used to generate the same cassette, (*TDH3p-LlnoxE-DIT1_{t_{d22}}*) with flanking regions replaced from *ScGPD1* to *ScGPD2*. The PCR-generated cassette, containing homologous regions upstream and downstream of *ScGPD2*, was digested using the *Sall* and *SacI* enzymes (Takara, Shiga, Japan). The purified DNA, obtained via the FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan), was cloned into the *Sall/SacI* sites of the pAUR101 plasmid (Takara, Japan), yielding *pTDH3p-DIT1_{t_{d22}}-AUR* and *pTDH3p-LlnoxE-DIT1_{t_{d22}}-AUR* (Table 2). *E. coli* NEB 10-beta (New England BioLabs, Tokyo, Japan) served as the recipient strain for storing and amplify the constructed plasmids following the manufacturer's instructions. After overnight cultivation in LB broth, plasmids were extracted using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany), and their construction were verified by sequencing. Once verified, the *LlnoxE* cassette with *ScGPD1* homologous regions was digested using the restriction enzymes *Sall* and *XhoI*. *Sall* and *SacI* were used to cleave the *LlnoxE* cassette containing *ScGPD2* flanking regions (Table 1). **[Please insert Table 1 here]**

To create a pCas9-gRNA-CRISPR multiplex that targets *ScGPD2*, we used our previously constructed multiplex plasmid (pCas9-gRNA-CRISPR-*ScGPD1*) as a template following, the same procedure [27]. Briefly, primers for a specific guide 20 base pairs (bp) upstream of the protospacer-adjacent motif (PAM) of *ScGPD2* were designed using the online tool <https://chopchop.cbu.uib.no/> (Table 1). The universal scaffold was generated through two rounds of PCR and inserted into a multiplex plasmid truncated at the *SmaI/PstI* sites. After retrieving the plasmid from *E. coli*, the universal scaffold sequence was verified by sequencing. **[Please insert Table 2 here]**

Genetic engineering of *S. cerevisiae* strains via the CRISPR multiplex

The CRISPR-Cas9-mediated genome editing protocol [31] was used to create a double-strand break in either *ScGPD1* or *ScGPD2*, which was then repaired through homologous recombination with the *TDH3p-DIT1_{t_{d22}}* or *TDH3p-LlnoxE-DIT1_{t_{d22}}* cassettes, resulting in the synchronous deletion of *ScGPD1* or *ScGPD2*. Initially, the *ScGPD1* or *ScGPD2* gene in the parent strain D452-2 (D) was replaced with the *TDH3p-DIT1_{t_{d22}}*

cassette, generating strains D1 and D2, respectively (Table 3). Simultaneously, substitution of the *ScGPD1* or *ScGPD2* gene was performed using the *TDH3p-LlnoxE-DIT1_{td22}* cassette, yielding strains DR1 and DR2, respectively. In the second round of genetic engineering, the *TDH3p-DIT1_{td22}* cassette was used to replace *ScGPD2* in strains D1 and DR1, generating D12 and DR1-D2, respectively. Similarly, in strain DR2, *ScGPD1* was replaced by the *TDH3p-DIT1_{td22}* and *TDH3p-LlnoxE-DIT1_{td22}* cassettes, generating DR2-D1 and DR12, respectively (Table 2). To generate the DN strain, the *TDH3p-LlnoxE-DIT1_{td22}* cassette was integrated into the *URA3* locus of the D452-2 (D) strain via homologous integration of a linearized *TDH3p-LlnoxE-DIT1_{td22}* plasmid using the *NcoI* restriction site, without deleting the *ScGPD1* or *ScGPD2* genes, as previously described [27]. This strain was selected on YNB agar medium supplemented with 20 mg/L leucine and histidine. CRISPR transformants were selected on YPD medium supplemented with geneticin [31] and verified via PCR, using one primer located on *DIT1_{td22}* in the inserted cassettes and the other primer downstream of the homologous loci in the genome. To avoid the regrowth of non-engineered cells, positive colonies were recultivated on fresh YPD_{G418} plates. The engineered strains were subsequently grown in liquid YPD without G418 to eliminate the pCas9-gRNA-CRISPR multiplex plasmids, and the final engineering was confirmed via PCR. Table 3 lists all the strains generated in this study, along with their genotypes. **[Please insert Table 3 here]**

Cultivation and batch fermentation conditions

The yeast cells were cultivated as previously described [27, 34], with some modifications. All strains listed in Table 3 were grown in 10 mL of YPD medium in 50 mL Falcon tubes (VIOLAMO, Japan) to reduce sterilization steps, minimize contamination risks, and streamline cell recovery procedures. The precultures were incubated at 30°C with orbital shaking at 200 rpm, using an approximately 45° sitting angle on a TAITEC BioShaker BR-42FL (Japan). The Falcon tube caps remained closed loosely during cultivation to allow the release of CO₂ while preventing contamination. Cell density was measured at 600 nm (OD₆₀₀) using a spectrophotometer (AS ONE, Japan) after tenfold dilutions.

For fermentation preparation, cell pellets from a 20 mL preculture (OD₆₀₀ of approximately 4) were harvested by centrifugation at 4000 rpm for 2 minutes. The cells were washed twice with 20 mL of sterilized Milli-Q water to remove any residual metabolites. The washed cells were resuspended in 20 mL of YNB_{D100} medium supplemented with amino acids, as mentioned previously. Fermentation was conducted under microaerobic conditions using 100 mL Erlenmeyer flasks, each containing 20 mL of YNB_{D100} medium. This setup restricted oxygen availability, maintaining minimal levels throughout the fermentation process. The flasks were

shaken at 150 rpm to ensure uniform mixing of the culture, minimizing oxygen diffusion beyond the controlled level.

Measurement of *L*noxE activity in recombinant strains

Cell-free extracts were prepared following to a previously described protocol [27]. A 10 mL culture (OD_{600} of approximately 5) was centrifuged at 4,000 rpm for 2 min at 4°C to harvest the cell pellets. The pellets were washed twice with 20 mL of 100 mM HEPES buffer (pH 7.4) and resuspended in 1 mL of the same buffer. The pellets were lysed in a 2 mL Eppendorf tube containing 1 mL of HEPES buffer supplemented with 1 mM $MgCl_2$ and 10 mM 2-mercaptoethanol, along with approximately 400 mg of glass beads. The lysis process consisted of six cycles of vigorous vortexing, alternating with 30-second chilling intervals on ice. Crude proteins (CP) were separated from the cell lysates and glass beads by centrifugation at 15,000 rpm at 4°C for 5 min. Any remaining cell debris was removed by an additional centrifugation step. Protein concentration in the supernatant was measured at 595 nm using the QuickStart Bradford assay (Bio-Rad, USA) with bovine albumin standards (Novagen, USA) as reference and absorbance was recorded using an Infinite M200 plate reader (Tecan, Switzerland).

The activity of *L*noxE (Table 4) was tested using a previously described method [13], with some modifications. A quartz cuvette was filled with a mixture of 79 mM HEPES buffer (pH 7.4), 0.3 mM Na-EDTA, 0.3 mM NADH, and 10 μ L of crude extract. The reduction in NADH absorbance at 340 nm was subsequently monitored using a UV-Vis spectrophotometer (UV-2700-Shimadzu, Japan).

Evaluation of the NADH/NAD⁺ ratios

NADH/NAD⁺ levels in the cells were determined photometrically using the N509 NAD⁺/NADH Assay Kit-WST (Dojindo, Japan), following the manufacturer's instructions and as previously reported [35]. The measurements were taken after 24 hours of fermentation in YNB_{D100} medium (Fig. 2).

Analysis of the fermentation metabolites

The concentrations of various metabolites, including glucose, glycerol, ethanol, acetic acid, pyruvate, succinic acid, and acetaldehyde, were measured using a previously described method [27]. Samples were analyzed with an Aminex HPX-87H column connected to an HPLC system equipped with a refractive index detector and a prominence diode array. The mobile phase used was 5 mM H_2SO_4 at a flow rate of 0.6 mL/min, with samples automatically injected into the system. To prepare the samples, 100 μ L of culture was taken from the fermentative flasks under sterile conditions, diluted with 900 μ L of Milli-Q water in Eppendorf tubes, mixed, and centrifuged at 15,000 rpm for 5 min. (Hitachi Himac CT15RE centrifuge, Japan). The resulting supernatants were filtered

using a hydrophilic 0.45-µm PTFE FILTSTAR syringe filter (Hawach Scientific, China) and transferred into fresh 2 mL HPLC glass vials using a 1 mL syringe.

Calculation of the correlation coefficient

Pearson's correlation coefficient (R) was used to assess the linear relationships between pairs of fermentation characteristics: glucose consumption versus glycerol biosynthesis, acetic acid production, ethanol production, and cell growth (OD); glycerol biosynthesis versus acetic acid production, ethanol production, and OD; and acetic acid production versus ethanol production and OD (Table 6). The coefficient values range from -1 to 1. An R value of -1 indicates a perfect negative correlation, meaning that as one variable increases, the other decreases, and vice versa. A value of 1 indicates a perfect positive correlation, representing a direct relationship between the two variables. A coefficient of zero suggests no linear relationship, or that one factor is consistently zero, as observed in some cases in this study. Lower values indicate weaker correlations, while values closer to 1 (or -1) represent stronger correlations. In this study, R values for various characteristics were calculated using the following formula for fermentation times between 24 and 42 hours. This time window was chosen because it represents the first point at which significant acetic acid production was detected, prior to the shift toward ethanol consumption.

$$R = \frac{n \sum x_i y_i - \sum x_i \sum y_i}{\sqrt{n \sum x_i^2 - (\sum x_i)^2} \sqrt{n \sum y_i^2 - (\sum y_i)^2}}$$

In this formula, n represents the number of values in each fermentation characteristics, and $\{x_1, x_2, \dots, x_n\}$ and $\{y_1, y_2, \dots, y_n\}$ denote the values of the two datasets under consideration.

Results

*L*noxE strain recombination and activity levels

To study ScGPD1 and ScGPD2 activity, we used the CRISPR-Cas9 system [31] to replace each gene with *TDH3p-DIT1_{d22}* or *TDH3p-LnoxE-DIT1_{d22}*. By employing the single-guide RNAs (sgRNAs) listed in Table 1, we successfully induced double-strand breaks in *ScGPD1* and *ScGPD2*, resulting in the generation of the recombinant strains listed in Table 3.

Investigating *L*noxE activity in the engineered strains revealed that substituting ScGPD1 with *L*noxE in the DR1 strain resulted in an activity level of 0.09 ± 0.01 (U/mg CP; Table 4). Additionally, this substitution led to a significant increase in activity in the DR2 strain, which was 22% higher than that observed in DR1 (Table 4). Interestingly, the DR1-D2 and DR2-D1 strains, where one GPD isoform was deleted and the other gene replaced,

exhibited the highest NADH oxidation activity, reaching 0.25 ± 0.03 U (Table 4). However, strains D₀, D1, D2, and D12 did not show any NADH oxidase activity. Overall, *LlnoxE* was effectively expressed at varying levels in the engineered strains (Table 4). **[Please insert Table 4 here]**

Intracellular NADH/NAD⁺ levels

To further validate the activity of *LlnoxE* in the recombinant strains (Table 2), the NADH/NAD⁺ ratio was measured after 24 h of fermentation with 10% glucose on YNB_{D100} (Fig. 2). Significant variations were observed across all the strains. Deleting *ScGPD1* in the D1 strain significantly increased the NADH content after 24 h of fermentation, leading to a 61% higher NADH/NAD⁺ ratio compared to the parent strain (D₀). In contrast, deleting *ScGPD2* in strain D2 resulted in a 25% increase in the NADH/NAD⁺ ratio, and the double deletion of *ScGPD* in the D12 strain led to a 2.2-fold increase compared to the D₀ strain (Fig. 2).

Conversely, in the DR1 strain, where *ScGPD1* was replaced with *LlnoxE*, the NADH/NAD⁺ ratio was 55% lower than that in the D₀ strain (Fig. 2). Substituting *ScGPD2* with *LlnoxE* in DR2 led to a further 14% and 61% decrease in the NADH/NAD⁺ ratio compared to the DR1 and D₀ strains, respectively. Additionally, double replacement of *ScGPD* isoforms in the DR12 strain reduced the NADH/NAD⁺ ratio by approximately 70%. Interestingly, in the DN strain, where *LlnoxE* was expressed alongside *ScGPD* isoforms, the NADH/NAD⁺ ratio remained comparable to that of the DR2 strain. This suggests that the expression of *LlnoxE* in the presence of both *ScGPD* isoforms does not lead to further significant changes in NADH oxidation, highlighting a potential balance between *LlnoxE* activity and the native *ScGPD* system. Moreover, replacing one *ScGPD* isoform and deleting the other in the DR1-D2 and DR2-D1 strains reduced the cofactor ratio by 43% and 48%, respectively (Fig. 2). The variations in the NADH/NAD⁺ ratios presented in Fig. 2 were consistent with changes in enzyme activity (Table 4) and fermentation characteristics (Table 5). The variations in *LlnoxE* activity, influencing NADH oxidation, were directly linked to byproduct formation, specifically acetic acid and glycerol. These data demonstrate how efficient NADH oxidation affects the redox balance during fermentation and influences byproduct formation under the microaerobic conditions described. **[Please insert Fig. 2 here]**

Comparative fermentation products

To investigate the extensive substitution of *ScGPD* with *LlnoxE*, which alters the NADH/NAD⁺ ratios (Fig. 2), a comparative analysis was conducted to assess its impact on fermentation characteristics under microaerobic conditions using YNB_{D100} medium. Compared with the parent D₀ strain, strain D1 produced 79% less glycerol and 17% less acetic acid (Fig. 3 and Table 5), while ethanol production and cell growth were enhanced by 4.3% and 3%, respectively (Fig. 3 and Table 5). Glucose consumption between D1 and D₀ was similar (Fig. 3a and

Table 5). Interestingly, strains D2 and D12 exhibited faster fermentation rates than strain D₀ under microaerobic conditions (Fig. 3a and e), consuming 96.2 g/L and 94.9 g/L of glucose within 30 h of fermentation, respectively. There was no significant difference in growth between the D2 and D12 strains compared to D₀ (Fig. 3). Strain D2 did not reduce glycerol secretion, whereas D12 abolished glycerol biosynthesis. Additionally, compared with D₀, D12 reduced acetic acid production by 39% and increased ethanol production by 4.3% (Table 5). **[Please insert Fig. 3 here]**

Replacing ScGPD1 with *LlnoxE* in the DR1 strain resulted in an 88% reduction in glycerol production and a 9% increase in acetic acid production compared to the reference strain D₀ (Figs. 3b and 3c; Table 5). The growth of DR1 was 8% lower than that of the D₀ strain at 30 h, further decreasing to 15% by the end of fermentation (Fig. 3e; Table 5). Despite this, ethanol production in DR1 was 6.2% higher than in the parental strain (Fig. 3d; Table 5). Substituting ScGPD2 with *LlnoxE* in the DR2 strain reduced glycerol production by 21% and increased acetic acid secretion by 9% compared to the D₀ strain. Ethanol production improved by 2%, while cell growth was reduced by 10% compared to the D₀ strain (Table 5). **[Please insert Table 5 here]**

With the double replacement of ScGPD with *LlnoxE* in the DR12 strain, 35% more acetic acid was produced, and glucose consumption decreased by 35% compared to the reference strain (Figs. 3a and 3c; Table 5). Ethanol production efficiency was similar to that of strain D₀ (Table 5). However, there was a delay in cell growth and further fluctuations after 24 hours of fermentation in strains DR12, DR1-D2, and DR2-D1 (Fig. 3e). Compared to D₀, DR1-D2 and DR2-D1 showed 25% and 27% decreases in glucose consumption, respectively, which resulted in 19% and 22% reductions in ethanol production rates, respectively (Fig. 3). Additionally, acetic acid secretion increased by 17.4% and 30% in DR1-D2 and DR2-D1, respectively, compared to D₀ (Table 5). The expression of *LlnoxE* under microaerobic conditions in the presence of native *ScGPD* shuttles in the DN strain led to slow and inconsistent initial cell growth, particularly after 24 h of fermentation (Fig. 3e). Cell growth in the DN strain was 58% of that in the parent strain (Table 5). The DN strain exhibited glucose consumption and ethanol production similar to those of the parental strain, but acetic acid production increased by 22%, while glycerol production decreased by 4% (Fig. 3, Table 5).

Correlation coefficients of fermentation parameters

The linear relationships between pairs of fermentation data sets were calculated for the following: glucose consumption versus glycerol biosynthesis, acetic acid production, ethanol production, and cell growth (OD); glycerol biosynthesis versus acetic acid production, ethanol production, and OD; and acetic acid production versus ethanol production and OD (Table 6). Due to the progressive consumption of glucose, the R values for acetate

and ethanol production showed strong negative correlations, highlighted by a reddish background in the table. The R values for glucose consumption with glycerol and OD varied between strains (Table 6). The double deletion of GPD prevented glycerol production, resulting in no R value, which is highlighted in white. A notable observation was the shift to a strong positive R value between glucose consumption and OD in strains DR12, DR1-D2, and DR2-D1. DR1 exhibited a very weak positive R value of 0.06, while the control strain D₀ showed a weak negative R value of -0.12. The R values for glycerol production in the GPD double-deletion strains were not detected, and these strains were highlighted with a white background in the table.

The R values for D₀ and DN were strongly positive for both glycerol and acetate (Gly. and Ac.) and for glycerol and ethanol (Gly. and Eth.). Compared to D₀ and DN, strains D2 and DR2 had slightly lower R values, while DR1 had even weaker R values. Conversely, D1 exhibited negative R values of -0.42 and -0.51 (Table 6). Strains D, D2, and DR2 showed positive R values between glycerol and OD of 0.33, 0.74, and 0.54, respectively, while D1 and DR1 had negative R values of -0.88 and -0.95, respectively. There were no significant changes in R values between acetate and ethanol in either the control or recombinant strains.

A notable shift was observed in the R values between acetate and OD: D₀ had an R value of 0.24, while strains DR12, DR1-D2, DR2-D1, and DN had highly negative R values of -0.98, -0.98, -0.98, and -0.83, respectively. DR1 showed a lower negative R value of -0.28. Strain D2 was similar to D₀, while D1, D12, and DR2 exhibited higher positive R values of 0.60, 0.73, and 0.87, respectively. A similar trend was observed in the correlations between ethanol concentration and OD (Table 6).

Discussion

This study highlights the effects of replacing ScGPD with *LlnoxE*, which induce varying levels of NADH oxidation, on the fermentation of 10% glucose compared to reference strains. An increase in *LlnoxE* activity (NADH oxidation) correlated with higher acetic acid concentrations, which subsequently impacted cell growth, glucose consumption, and fermentation rates—particularly when glycerol accumulation was low or absent. Notably, glycerol accumulation helped mitigate the detrimental effects of acetic acid (Fig. 3). As shown in Fig. 3, three distinct clusters emerged for the fermentation rates of the engineered strains: those with faster fermentation (D2 and D12), those with medium-speed fermentation (D₀, D1, DR1, DR2, DN), and those with slower fermentation (DR12, DR1-D2, and DR2-D1).

Replacing ScGPD1 in the D1 strain significantly decreased glycerol biosynthesis compared to D₀ and D2, indicating that ScGPD1 plays a more prominent role under microaerobic conditions (Fig. 2). This was supported by a 79% reduction in glycerol secretion compared to D₀ and D2 (Table 5). The superior contribution of ScGPD1

may be attributed to the microaerobic and hyperosmotic conditions of 10% glucose during fermentation. The expression of *ScGPD1* is known to be induced by osmotic stress and aerobic conditions, whereas *ScGPD2* expression decreases under osmotic stress and increases under anaerobic conditions. K_m for DHAP was 0.37–0.54 mM for *ScGPD1* and 0.86 for *ScGPD2*. K_m for NADH was 0.018–0.023 mM for *ScGPD1* and 0.018 mM for *ScGPD2*, indicating that the affinity for these substrates depends on the expression and fermentation conditions [2]. The removal of *ScGPD1* led to a 7% decrease in glycerol biosynthesis and a 3% increase in ethanol production under anaerobic growth conditions. Conversely, *ScGPD2* deletion caused a 79% reduction in growth rate and extended the fermentation duration of 25 g/L glucose by 13–14 h, reducing ethanol production by 4%. Similar findings have been reported under aerobic batch cultivation, where glycerol biosynthesis was reduced by 66% and ethanol production increased by 2–3% [10], which is similar to our data obtained under microaerobic conditions. This reduction in glycerol biosynthesis in D1 along with the slightly higher in acetic acid production may explain why D1 did not expedite fermentation compared with D2, where glycerol is an osmoadaptive regulator [2].

The double deletion of *ScGPD* isoforms eliminated glycerol biosynthesis, consistent with previous studies [2, 10]. Interestingly, under microaerobic conditions, the D12 strain exhibited faster fermentation, similar to D2, with a 3% increase in ethanol production and the lowest acetic acid levels among all strains (Fig. 3c). This enhanced fermentation rate may be due to compensation between *ScNDE* and *ScGUT2*, which has been reported to provide additional ATP under microaerobic conditions [8, 12, 36]. Similar results were observed under aerobic batch cultivation, where double deletion of *ScGPD* improved ethanol production by 12.7% but reduced the maximum growth rate by 29% [10]. However, under anaerobic conditions, the simultaneous deletion of *ScGPDs* led to an osmosensitive phenotype and abolished growth [24]. This cessation of growth is attributed to the absence of NADH/NAD⁺ recycling and the final electron acceptor, which can be restored by resupplementing with oxygen or introducing alternative electron acceptors, such as acetoin or acetaldehyde [2, 10]. Aeration substantially impacts cellular processes through transcriptional regulation. Genome-wide transcriptional analysis revealed that 359 genes exhibited more than a threefold change in expression between aerobic and anaerobic conditions [37]. Additionally, promoter strength varies significantly between aerobic and microaerobic conditions [38]. Oxygen levels influence redox cofactor dynamics and trigger metabolic changes during alcoholic fermentation [39]. Taken together, these findings and other factors, such as media composition and strain lineage, may explain why D12 promoted faster fermentation than D2 in this study.

A comprehensive comparison of *LlnoxE* expression in the absence of *ScGPD1*, *ScGPD2*, or both isoforms, as well as in the presence of *ScGPD* isoforms (Table 1), revealed varying enzyme activities (Table 4). These

differences in NADH oxidation levels allowed for effective comparisons (Fig. 2). Replacing ScGPD1 with *LlnoxE* in the DR1 strain decreased the NADH/NAD⁺ ratio (Fig. 2), which aligns with previous reports where *SpnoxE* or *LlnoxE* (subsp. *cremoris* MG1363) was expressed in strains where ScGPD1 was not deleted [12, 13]. Interestingly, in the absence of ScGPD1, *LlnoxE* expression improved ethanol fermentation efficiency by 6.2% compared to the D₀ and DN strains. In contrast, previous studies reported a 14–16% reduction in ethanol production when ScGPD1 was present [12, 13]. Additionally, a strain expressing *LlnoxE* (subsp. *cremoris* MG1363) without ScGPD deletion in a complex medium containing 0.09% NaCl significantly accelerated fermentation, although no data were provided on ethanol recovery per gram of consumed glucose [25]. Due to variations in strain genotypes, gene constructs, media, and aeration conditions in previous studies, this study presents more definitive conclusions. The comparison of glycerol production between DR1 and DR2 highlights the significant role of ScGPD1 over ScGPD2 and *LlnoxE*, with 22% greater activity in DR2 under the tested conditions. Glycerol production was reduced by 88.5% in DR1 compared to 21% in DR2 (Table 5). The difference in *LlnoxE* enzyme activity between DR1 and DR2 could be attributed to chromosomal positioning [40]. *LlnoxE* expression also increased acetic acid production, and a positive correlation was observed between increased cytosolic NADH oxidation and acetic acid production (Table 6), consistent with previous findings [12, 13]. Similarly, the increase in NADH oxidation due to the double replacement of ScGPD with *LlnoxE* in the DR12 strain resulted in acetic acid production exceeding 34% of that in the reference strain (Fig. 3c, Table 5).

The expression of *SpnoxE* in *S. cerevisiae* has been shown to upregulate NAD-dependent aldehyde dehydrogenase (ScALD) isoforms [12]. For example, the introduction of *LlnoxE* (subsp. *cremoris* MG1363) led to a twofold increase in the expression of NAD-dependent cytoplasmic aldehyde dehydrogenase *ALD3*, which is associated with beta-alanine biosynthesis, and a threefold upregulation of ScALD6, a key isoform responsible for converting acetaldehyde to acetate [25]. As a result, increasing NAD⁺ levels associated with *LlnoxE* expression is expected to enhance ethanol oxidation to acetaldehyde via ScADH2. Deletion of ScGPD2, ScADH2, plasma membrane channels (FPS1), and 2-hydroxyglutarate transhydrogenase, a minor D-lactate dehydrogenase, has been shown to improve ethanol production [41]. However, further studies are required to confirm this observation.

Acetic acid inhibits the growth of *S. cerevisiae* and glucose fermentation, with concentrations exceeding 20 mM inducing cell death [42-45]. The DR12 strain may have been severely affected by acetic acid, as its accumulation reached 3.1 g/L, especially in the absence of G3P and glycerol following the deletion of ScGPD isoforms (Fig. 3c, Table 5). Additionally, the expression of *SpnoxE* increases the free energy dissipated from oxidative phosphorylation through ScNDE, as *SpnoxE* is cytosolic and not associated with proton translocation

[12]. Knocking out *ScGPD* genes eliminates G3P synthesis, which serves as another energy source via *ScGUT2* (Fig. 1). G3P plays a central role in various cellular processes, including signaling, regulation, and glycerophospholipid metabolism, particularly under osmotic stress [46, 47]. This could explain the 32% reduction in cell growth (Fig. 3e, Table 5), leading to sluggish glucose consumption and fermentation (Fig. 3a). Similar severe effects were also observed in the DR1-D2 and DR2-D1 strains, where enzyme activities reached 0.25 ± 0.03 U (Fig. 3, Tables 2 and 4). Enzyme activities in the DR1-D2 and DR2-D1 strains were more than twice those in DR1 and DR2, despite all strains carrying only one copy of *LlnoxE* (Table 3). Variations in enzyme activities and NADH concentrations have been observed within the same cell during exponential growth and stationary phases [13]. The DR1-D2 and DR2-D1 strains exhibited slower growth rates than the other strains, which may explain the differences in enzyme activity between DR1-D2/DR2-D1 and DR1/DR2. Consequently, strong negative R values between acetic acid production and cell growth were observed in strains DR12, DR1-D2, and DR2-D1 (Table 6).

A 4% reduction in glycerol content was observed in the DN strain, which expresses *LlnoxE* without the deletion of *ScGPD*, compared to the control strain D₀ (Fig. 3b, Table 5). This slight reduction in glycerol biosynthesis by the DN strain indicates the dominance of *ScGPD* isoforms in the D452-2 strain over *LlnoxE* (subsp. *lactis* II1403) for NADH oxidation under the studied conditions, where the aeration level plays a critical role and influences all fermentation characteristics. The expression of *LlnoxE* (subsp. *cremoris* MG1363) in a yeast strain led to a 24% and 14.4% reduction in glycerol and ethanol, respectively, regardless of the medium or strain genotype [13]. The K_m^{NADH} of *LlnoxE* (MG1363 strain) was 4.1 μM , whereas the K_m for *ScGPD* isoforms varied from 18 to 130 μM [27, 48]. A higher level of NADH oxidation was associated with a significant reduction in cell biomass, reaching 60%, which is 45% greater than the reduction observed in this study. A reduction in the NADH/NAD⁺ ratio has also been shown to decrease the production of succinate and 2-hydroxyglutarate [13]. In a batch mineral culture medium supplemented with 40 g/L glucose, *SpnoxE* was more effective than other agents in reducing glycerol production by 80%. However, ethanol production decreased by 14%, and biomass accumulation was reduced by 9% [12].

The pairwise correlation coefficients of comparative fermentation characteristics revealed a strong inverse relationship between acetic acid production and cell growth, which consequently impacted glucose consumption and ethanol production (Table 6). These tight correlations, along with glycerol production levels, help explain the formation of three distinct clusters based on the fermentation rates of the engineered strains: strains with faster fermentation (D2 and D12), strains with medium-speed fermentation (D, D1, DR1, DR2, DN), and strains with

slower fermentation (DR12, DR1-D2, and DR2-D1). Together with the comparative data presented here, these findings indicate that replacing ScGPD1 with *LlnoxE*—an approach not previously explored—is crucial for eliminating glycerol production. However, excessively high *LlnoxE* activity results in a low NADH/NAD⁺ ratio and an undesirable increase in acetic acid secretion. NADH homeostasis is associated with more than 70 cellular cofactor cycles, which influence various metabolic pathways and cellular processes [49]. Therefore, maintaining relatively low NADH oxidase activity, under the control of a weaker promoter and combined with the deletion of key acetate biosynthesis genes such as *ALD6*, could enhance glucose fermentation efficiency.

Previously, we reported the effectiveness of replacing ScGPD1 with *LlnoxE* to enhance glycerol conversion to ethanol, as glycerol contains two extra hydrogens for reduction compared to glucose [27]. Additionally, another approach involving the deletion of ScGPD prevented glycerol biosynthesis and improved the conversion efficiency of glucose to 2,3-butanediol [26]. The recycling of NADH/NAD⁺ through the heterologous expression of oxidoreductase, xylose reductase, and xylitol dehydrogenase has also been extensively studied to improve xylose fermentation [34, 50, 51]. Therefore, the comparative data presented here could provide valuable insights for further optimizing these strategies and addressing the limitations of such approaches in future studies.

In summary, this study investigated the variation in NADH oxidation in the absence and presence of ScGPD isoforms via *LlnoxE* and its impact on the fermentation of 10% glucose under microaerobic conditions. *LlnoxE* was expressed in *S. cerevisiae* D452-2, leading to significant changes in NADH/NAD⁺ levels (Fig. 2). The recombinant strains (Table 3) displayed different fermentation characteristics and byproduct profiles. Replacing ScGPD1 with *LlnoxE* reduced glycerol production by 88% and increased ethanol and acetic acid production by 6.2% and 9%, respectively, in YNB_{D100} medium compared to the parent strain. Excess NADH oxidation, achieved by replacing both ScGPD isoforms with *LlnoxE*, resulted in increased acetic acid production and significantly inhibited cell growth, glucose consumption, and ethanol production. A strong negative correlation was observed between acetic acid production and cell growth, which, in turn, affected the positive correlation between glucose consumption and fermentation rate. This study highlights the critical role of ScGPD replacement and NADH oxidation levels in optimizing fermentation efficiency during glucose metabolism. Additionally, the findings provide valuable insights into modulating NADH oxidation when utilizing other carbon sources, such as xylose, glycerol, and acetic acid, which encounter redox imbalances during fermentation.

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Author contributions: S.M.R.K. conceived the research idea, planned the experiments, provided information regarding the procurement of strains, chemicals, and toolboxes for genetic engineering, performed the experiments, and analyzed the results. S.M.R.K. wrote, revised, and submitted the manuscript. T.W. was responsible for the financial support and provided chemicals and equipment. T.W. was also involved in formulating the research idea, planning and organization, discussion of the results, and manuscript revision and submission.

Declarations

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

The authors declare no competing interests.

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Figure legends

Fig. 1 Schematic of fermentative metabolic pathways and the main NADH/NAD⁺ cycles in *S. cerevisiae* during glucose fermentation. The black arrows represent the native routes, while the blue arrows represent the recombinant pathways.

Fig. 2 The NADH/NAD⁺ ratios in the recombinant *S. cerevisiae* strains generated in this study after 24 h of fermentation in YNB_{D100}. The data represents the average of three independent experiments $\pm$ SD.

Fig. 3 Time course fermentation of YNB_{D100} under microaerobic conditions in native and recombinant strains constructed in this study: glucose consumption (a), glycerol biosynthesis (b), acetic acid production (c), ethanol production (d), and cell density (e). The data represent the average of two independent experiments, with error bars representing half the difference between the values. Strains D12, DR12, and DR1-D2 do not produce glycerol, and as result, their data overlap with the x-axis in Fig.3b, making it not visible.

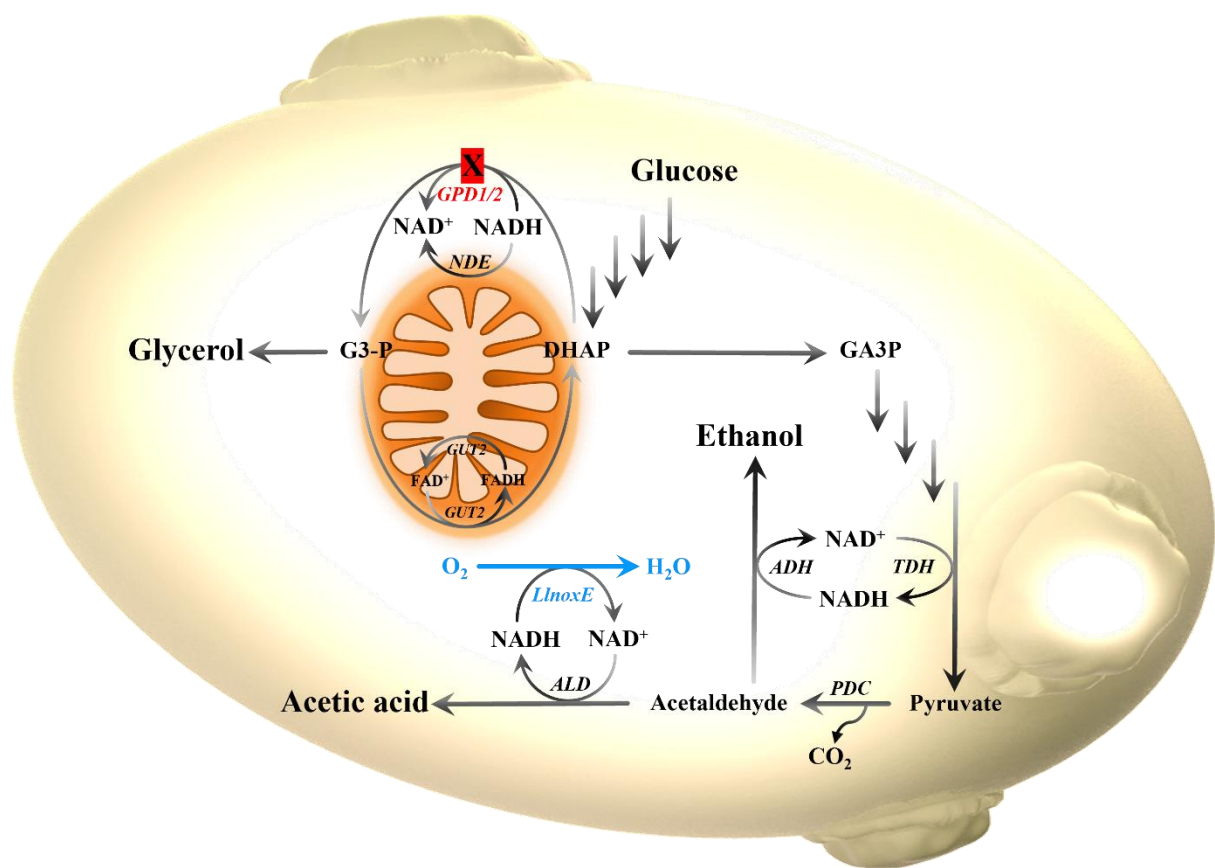


Fig. 1.

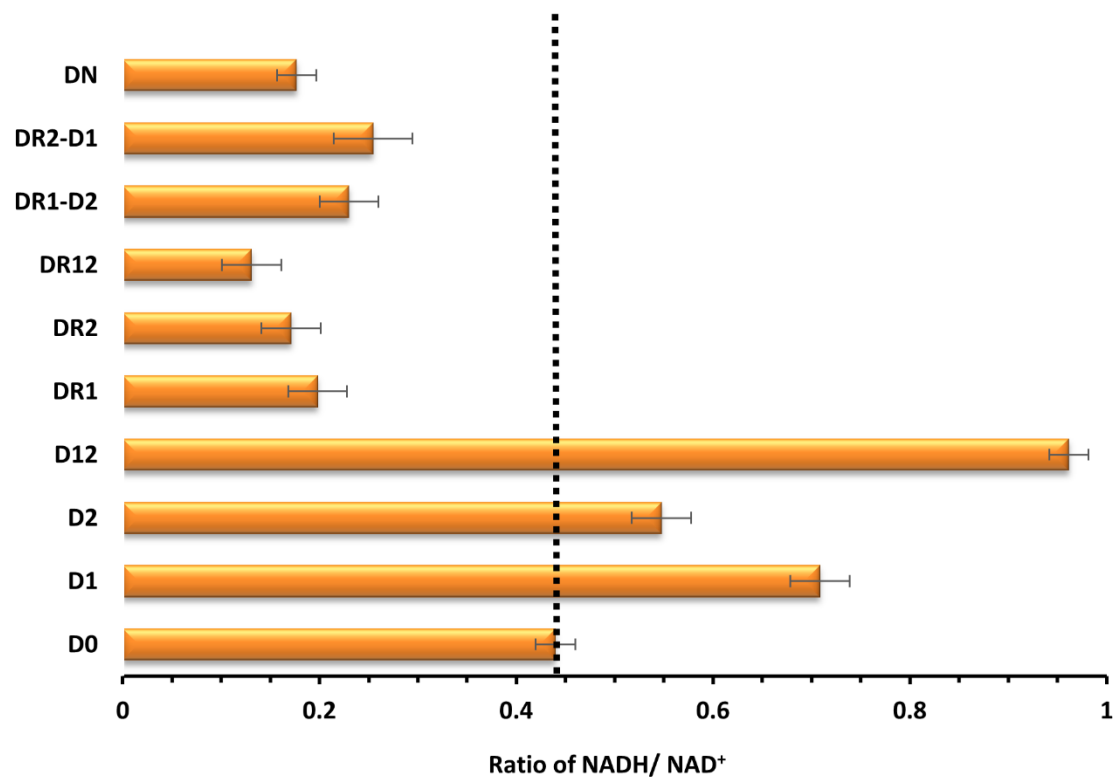


Fig. 2.

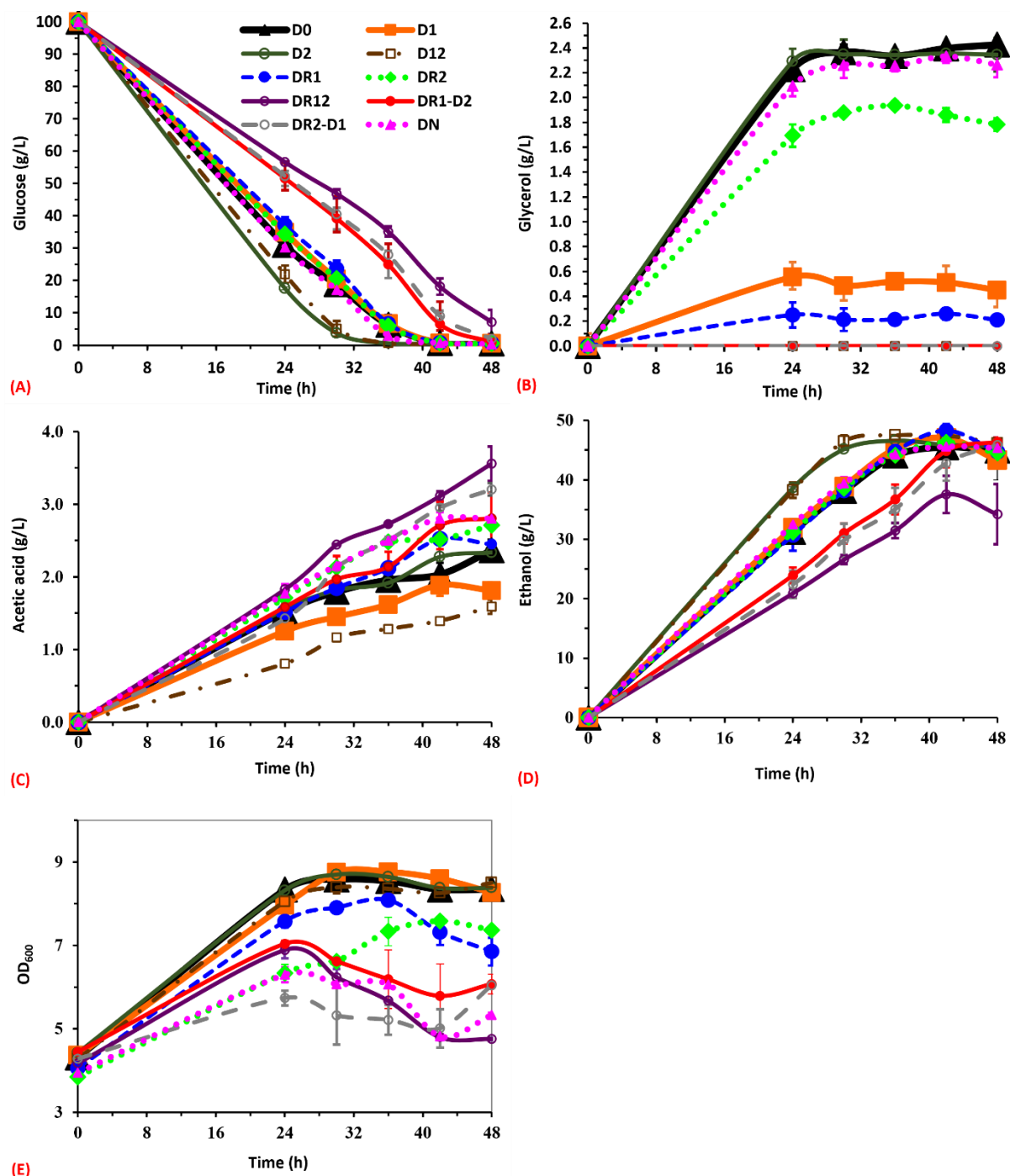


Fig. 3.

600 **Table 1.** Primers used in this study, their descriptions, and sequences.

Primers for constructing the cassettes of homologous repairing the double-strand break of <i>ScGPD1</i>	
Upstream <i>ScGPD1</i> - <i>TDH3p</i> FOR.	Aa <u>ctcgag</u> tgtatattgtacacccccccctccacaaacacaaatattgataatataaag <u>ccgaggagc</u>
	<u>tcagttcgagt</u>
downstream <i>ScGPD1</i> - <i>DIT1</i> _{td22} REV.	Tt <u>gtcgac</u> gaaaaaagtgggggaaagtatgatattgtatctttccaataaat <u>ccgagg</u> gatgaaaag
	aaaggcaaata
Primers for confirming the homologous integration during knocking out <i>ScGPD1</i>	
<i>Not1</i> - <i>BamHI</i> - <i>DIT1</i> _{td22} FOR.	Tttttcgccgcgcatcctaagtaagag
1000-downstream <i>ScGPD1</i> REV.	Ccttaacaagaacaatgtcatgacattgga
Primers for constructing the cassettes of homologous repairing the double-strand break of <i>ScGPD2</i>	
379-upstream <i>ScGPD2</i> - <i>TDH3p</i> FOR.	Aaa <u>gtcgac</u> gatggctctgccattgttatattacgttttcgpgcaggtg <u>ccgagg</u> tcagttcgagttt
	atcattatc
310-downstream <i>ScGPD2</i> - <i>DIT1</i> _{td22} REV.	Ttt <u>gagctc</u> tatatctcagtggtgggcccattgtggagaattactgcagtgaaaaag <u>ctcgag</u> gatgaaa
	agaaaggcaaa
Primers for constructing multiplex pCas9-gRNA-<i>ScGPD2</i> plasmid	
pCas9-gRNA target 267 <i>ScGPD2</i> FOR.	Gattggttctggtaactgggttttagagctagaaatag
pCas9-gRNA target 267 <i>ScGPD2</i> REV.	Agttaccagaaccaatcaaaagtccttcgccacc
pCas9 FOR.	Cggaataggaactcaagcg
pCas9 REV.	Tttttctgcagcgaggagcc
Primers for confirming the homologous integration while knocking out <i>ScGPD2</i>	
<i>Not1</i> - <i>BamHI</i> - <i>DIT1</i> _{td22} FOR.	Tttttcgccgcgcatcctaagtaagag
557-downstream <i>ScGPD2</i> REV.	Gccgctacgcagtcataatctgatcca

601

602 **Table 2.** Plasmids used in this study and their relevant genotypes.

Plasmids	Relevant genotype	Reference
pTDH3p- <i>DIT1</i> _{d22} - <i>URA3</i>	<i>URA3</i> ; <i>TDH3</i> promoter and mutated <i>DIT1</i> _{d22} terminator	[27]
pTDH3p- <i>LlnoxE</i> - <i>DIT1</i> _{d22} - <i>URA3</i>	<i>URA3</i> ; expresses <i>LlnoxE</i>	[27]
pAUR101	<i>AUR1-C</i>	Takara Bio
pTDH3p- <i>DIT1</i> _{d22} - <i>AUR</i>	<i>AUR</i> ; <i>TDH3</i> p promoter and mutated <i>DIT1</i> _{d22} terminator	This study
pTDH3p- <i>LlnoxE</i> - <i>DIT1</i> _{d22} - <i>AUR</i>	<i>AUR</i> ; expresses <i>LlnoxE</i>	This study
Multiplex pCas9/ <i>GPD1</i> -1	Multiplex expresses Cas9 and gRNA to base No. 135 of <i>ScGPD1</i>	[27]
Multiplex pCas9/ <i>GPD1</i> -2	Multiplex expresses Cas9 and gRNA to base No. 1045 of <i>ScGPD1</i>	[27]
Multiplex pCas9/ <i>GPD2</i>	Multiplex expresses Cas9 and gRNA to base No. 267 of <i>ScGPD2</i>	This study

603

604 **Table 3.** Native and recombinant *S. cerevisiae* strains used in this study and their relevant genotypes.

Strains	Progenitor strain	Relevant genotype	Reference
D (D452-2)	-	<i>MATa leu2 his3 ura3 can1</i>	[33]
D ₀	D	<i>URA3::TDH3</i> promoter and <i>DIT1</i> _{d22} terminator	[27]
D1	D ₀	$\Delta GPD1::TDH3p-DIT1$ _{t_{d22}}	This study
D2	D ₀	$\Delta GPD2::TDH3p-DIT1$ _{t_{d22}}	This study
D12	D1	$\Delta GPD2::TDH3p-DIT1$ _{t_{d22}}	This study
DR1	D ₀	$\Delta GPD1::TDH3p-LlnoxE-DIT1$ _{t_{d22}}	This study
DR2	D ₀	$\Delta GPD2::TDH3p-LlnoxE-DIT1$ _{t_{d22}}	This study
DR12	DR2	$\Delta GPD1::TDH3p-LlnoxE-DIT1$ _{t_{d22}}	This study
DR1-D2	DR1	$\Delta GPD2::TDH3p-DIT1$ _{t_{d22}}	This study
DR2-D1	DR2	$\Delta GPD1::TDH3p-DIT1$ _{t_{d22}}	This study
DN	D	<i>URA3::TDH3p-LlnoxE-DIT1</i> _{t_{d22}}	This study

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Table 4. Specific activities^a of *L*lnoxE in the strains used in this study.

Relevant strain	D ₀	D1	D2	D12	DR1	DR2	DR12	DR1-D2	DR2-D1	DN
<i>L</i> lnoxE activity ^a	ND ^b	ND ^b	ND ^b	ND ^b	0.09 ± 0.01	0.11 ± 0.01	0.23 ± 0.03	0.25 ± 0.02	0.25 ± 0.03	0.18 ± 0.01

The data represents the average of three independent experiments ± SD.

^a*L*lnoxE activity, U/mg CP. A unit was defined as the amount of CP that catalyzed the oxidation of 1 mmol of NADH to NAD⁺ at 30°C.

^bND, not detected.

Table 5. Comparison of fermentation characteristics on YNB_{D100} medium using the recombinant *S. cerevisiae* strains constructed in this study*.

Relevant strain	Consumed glucose (g/L)	Glycerol produced (g/L)	Acetic acid secreted (g/L)	Ethanol produced (g/L)	Ethanol yield g/g glucose	Optical density (OD ₆₀₀)
D₀	(99.4, 0.06)	(2.4, 0.08)	(2.3, 0.32)	(45.5, 0.20)	(0.46, 0.01)	(8.35, 0.14)
D1	(99.4, 0.02)	(0.5, 0.10)	(1.9, 0.30)	(47.6, 0.30)	(0.48, 0.01)	(8.60, 0.14)
D2	(99.4, 0.00)	(2.4, 0.14)	(2.3, 0.44)	(45.9, 0.62)	(0.46, 0.01)	(8.48, 0.28)
D12	(99.6, 0.08)	(0.0, 0.00)	(1.4, 0.02)	(47.4, 0.06)	(0.48, 0.01)	(8.26, 0.34)
DR1	(99.3, 0.08)	(0.3, 0.10)	(2.5, 0.56)	(48.3, 2.26)	(0.49, 0.01)	(7.32, 0.62)
DR2	(99.4, 0.02)	(1.9, 0.12)	(2.5, 0.12)	(46.3, 1.26)	(0.47, 0.01)	(7.58, 0.22)
DR12	(81.9, 5.02)	(0.0, 0.00)	(3.1, 0.12)	(37.6, 6.24)	(0.46, 0.01)	(4.80, 0.02)
DR1-D2	(95.5, 8.40)	(0.0, 0.00)	(2.7, 0.66)	(44.9, 5.56)	(0.47, 0.01)	(5.79, 1.54)
DR2-D1	(91.0, 8.96)	(0.0, 0.00)	(3.0, 0.08)	(42.8, 5.94)	(0.47, 0.01)	(5.01, 0.92)
DN	(99.3, 0.06)	(2.3, 0.10)	(2.8, 0.18)	(45.6, 0.40)	(0.46, 0.01)	(4.82, 0.08)

*Fermentation was conducted in YNB_{D100} medium (20 ml liquid culture:100 ml Erlenmeyer flask) at 150 rpm with orbital shaking at 30 °C. The data represent the average of two independent experiments followed by the difference between them recorded after 42 hours of fermentation.

Table 6. Pairwise *correlation coefficients (R)* during 24 h to 42 h of fermentation between the characteristics of glucose consumption, glycerol biosynthesis, acetic acid production, optical density growth, and ethanol production in the control and engineered strains generated in this study.

Var	D ₀	D ₁	D ₂	D ₁₂	DR ₁	DR ₂	DR ₁₂	DR _{1_D2}	DR _{2_D1}	DN
Glu. VS Gly.	-0.84	0.49	-0.98		-0.03	-0.80				-0.90
Glu. VS Ac.	-0.99	-0.97	-0.82	-0.98	-0.97	-0.99	-0.97	-0.99	-0.97	-0.98
Glu. VS Eth.	-0.99	-1.00	-1.00	-0.99	-1.00	-1.00	-0.99	-1.00	-0.99	-1.00
Glu. VS OD	-0.12	-0.75	-0.75	-0.85	0.06	-0.92	1.00	0.99	0.94	0.70
Gly. VS Ac.	0.89	-0.42	0.84		0.22	0.87				0.92
Gly. VS Eth.	0.85	-0.51	0.97		0.02	0.84				0.93
Gly. VS OD	0.33	-0.88	0.74		-0.95	0.54				-0.75
Ac. VS Eth.	1.00	0.95	0.76	0.96	0.98	1.00	0.99	0.99	0.99	0.97
Ac. VS OD	0.24	0.60	0.26	0.73	-0.28	0.87	-0.98	-0.98	-0.99	-0.83
Eth. VS OD	0.22	0.77	0.80	0.90	-0.07	0.91	-1.00	-1.00	-0.97	-0.70