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Metabolic Engineering and Genome-Wide Adaptive Evolution for Efficient Reduction of Glycerol in Industrial *Saccharomyces cerevisiae*

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ABSTRACT: The production of glycerol as a major by-product during yeast-based bioethanol fermentation arises directly from the need to re-oxidize excess NADH, which reduces conversion efficiency. In this study, an optimized Cas9-based genome editing method was performed to develop a mixotrophic CO_2-fixing industrial *Saccharomyces cerevisiae* by heterologous expression of ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO form *Pseudomonas* sp.) and phosphoribulokinase (PRK form *Spinach*). Additionally, the gene encoding alcohol dehydrogenase (ADH2) responsible for converting ethanol to acetaldehyde was deleted, while the great wall-family protein kinase *Rim15* gene was overexpressed to facilitate the reduction in glycerol content. The resulting CO_2-fixing yeast M-2 led to a 21.5% reduction of the by-product glycerol in corn mash fermentation cultures at 39 ℃. Moreover, we established a novel gene mutators mediated genome-wide mutations system that accumulates distinct mutations in the industrial *S. cerevisiae* strains under the stress conditions to improve the robustness in the *S. cerevisiae* strains efficiently.

Keywords: *Saccharomyces cerevisiae*; Gene editing; Glycerol yield; Genome-wide mutation; Adaptive evolution; Robustness

1. Introduction

Ethanol, predominantly manufactured by the yeast *Saccharomyces cerevisiae*, has extensive application in various industries, including chemicals, beverages, bioethanol, pharmaceuticals, and cosmetics. It has been estimated that up to 4% of the sugar feedstock is converted into glycerol in the fermentation process of ethanol production by *S. cerevisiae* [1]. Although glycerol plays a physiological role in osmoregulation and redox balance, excessive production of glycerol can reduce the efficiency of sugar utilization, ultimately influencing the rate of ethanol production [2,3]. Bro et al. [4] overexpressed non-phosphorylating $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase(GAPN) from *Streptococcus* mutants to replace the NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase in *S. cerevisiae*, which the glycerol yield decreased by 40% and the ethanol yield increased by 3%.

In anaerobic cultures of *S. cerevisiae* that produce ethanol, the excess NADH produced during biosynthetic processes, including NAD^+-dependent oxidative decarboxylations involved in the synthesis of acetyl-CoA and 2-oxoglutarate precursors, is re-oxidized by reducing part of the sugar substrate to glycerol [5]. Currently, utilizing CO_2 as an electron acceptor for the reoxidation of NADH is a highly attractive metabolic engineering strategy, especially when CO_2 reduction can be synchronized with the formation of the desired product [6]. Among the six naturally existing CO_2 fixation pathways, the Calvin-Benson-Bassham(CBB) cycle is the most widely exploited for developing CO_2-fixing *S. cerevisiae* strains [7,8]. Previous studies have demonstrated that certain strains of *S. cerevisiae* are capable of utilizing CO_2 following the heterologous expression of the CBB cycle-associated enzymes, specifically ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and phosphoribokinase (PRK) [9,10]. Additionally, the incorporation

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of numerous replicates of a bacterial RubisCO expression cassette within *S. cerevisiae*, along with the GroEL/GroES chaperonin system from *E. coli* and the anaerobic inducible DAN1 promoter regulating PRK expression, results in an engineered *S. cerevisiae* strain that exhibits a 31% reduction in glycerol production [11]. When the alcohol dehydrogenase II (ADH2) gene was deleted in genetically modified *S. cerevisiae*, the resulting ethanol content increased by 18.58% with the decrease of glycerol, acetic acid, and lactic acid contents by 22.32%, 8.87%, and 16.82%, respectively [12]. Meanwhile, glycerol is primarily exported across the plasma membrane via the protein channel Fps1, which is regulated by extracellular osmolarity [13]. Fps1 is a member of the major intrinsic protein (MIP) family of channel proteins and contains six transmembrane domains [12]. The Δ*FPS1* mutant of *S. cerevisiae* exhibits intracellular accumulation of glycerol, and the accumulation of glycerol further triggers other regulatory systems to reduce glycerol biosynthesis, leading to an increase in ethanol production [14].

Due to the complexity of metabolic and regulatory networks in microbial systems, obtaining phenotypes with good robustness through rational design is challenging. Currently, genome-scale evolution strategies such as Synthetic Chromosome Rearrangement and Modification by LoxP-mediated Evolution (SCRaMbLE) [15] in synthetic *S. cerevisiae*, Oligonucleotide-mediated Genome Engineering (YOGE and eMAGE) [16,17], and CRISPR-based multi-loci editing (CHAnGE, MAGESTIC, Target-AID, and yEvolvR) [18–20] have been extensively studied for improving the robustness of the strain. Genetic engineering via the CRISPR system is relatively straightforward in *S. cerevisiae* due to its excellent homologous recombination capability. However, industrial yeast strains are genetically more complex and often polyploid, making them more difficult to genetically manipulate than haploid laboratory strains [21]. To efficiently and rapidly manipulate the genome of industrial *S. cerevisiae*, Stovicek et al. [22] constructed the multi-copy 2μ plasmids to expressed *S. pyogenes* Cas9 protein and the corresponding gRNA, along with 90 bp double-stranded DNA as a repair template, and successfully achieved gene knockout with an efficiency of 65% to 78%. Lian et al. [23] successfully constructed higher-copy gRNA plasmids by optimizing the length of the resistance marker promoter and achieved a 100% efficiency in one-step knockout of four genes in diploid and triploid yeast.

In this study, we first optimized the CRISPR editing method in industrial *S. cerevisiae*. Subsequently, we developed a mixotrophic CO_2-fixing industrial *S. cerevisiae* by heterologous expression of ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) and phosphoribulokinase (PRK) and modified endogenous metabolic pathway with additional metabolic engineering strategies, including the knockout of *ADH2*, downregulation of *FPS1* and overexpression of the transcription regulatory gene *Rim15*. The fermentation characteristics of the engineered *S. cerevisiae* strain were investigated to analyze the effect of gene editing on the production of ethanol and by-products. Finally, we developed a novel genome-wide mutation system that accumulates distinct mutations in the industrial *S. cerevisiae* to improve robustness under stress conditions efficiently.

2. Materials and Methods

2.1. Strains, Medium and Culture Condition

E. coli DH5α was employed for plasmid construction and cultured in LB medium with 100 mg/L ampicillin. The industrial *S. cerevisiae* M (From Angel Yeast) was used as the parent strain routinely cultured in YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose). Recombinant *S. cerevisiae* strains were screened in YPD solid medium containing 50 mg/L nourseothricin, and 200 mg/L hygromycin B or 200 mg/L G418, and corn mash medium was used for the verification of yeast fermentation performance.

2.2. Plasmid Construction

The truncated fragments of the TEF promoter were cloned into the pSCM-N20 plasmids using Gibson assembly methods. The codon-optimized genes of ribulose-1,5-bisphosphate carboxylase-oxygenase (*RuBisCO*) from *Pseudomonas* sp. and phosphoribulokinase (*PRK*) from spinach were synthesized by Jinkairui Biological Engineering Co., Ltd. (Wuhan, China). The codon-optimized *Escherichia coli* chaperones *GroEL* and *GroES* were synthesized by Jinkairui Biological Engineering Co., Ltd. (Wuhan, China). The key genes that regulate glycerol or ethanol yield, including *FPS1* and the great wall-family protein kinases gene *Rim15* were all amplified from the chromosome of the *S. cerevisiae* and subsequently cloned into the donor helper plasmids using digestion/ligation and/or Gibson assembly methods. Benchling CRISPR tool (https://benchling.com) was used to design gRNAs, which were cloned into *Bsa I* digested pSCM-gRNA. All the DNA polymerase, T4 DNA ligases, and restriction enzymes were purchased from New England Biolabs. Primers were synthesized by Tsingke Biotech Co., Ltd. (Wuhan, China).

All the plasmids used in this study were listed in Supplementary Table S1. All the primers used for plasmid construction and genome integration are listed in Supplementary Table S2. The coding sequences of heterologous genes are listed in Supplementary Table S3.

2.3. Strain Construction

The integration of related genes mentioned above into the chromosome of *S. cerevisiae* was performed through the CRISPR/Cas9 method [24]. The gene expression cassettes, together with the homology arms and the corresponding gRNA plasmids, were co-transformed into the *S. cerevisiae* strain harboring the SpCas9 plasmid. The *ADH2* deletion was accomplished by substituting donor DNA containing 500 bp upstream and 500 bp downstream homologous arms of the target sequence, and the *FPS1* promoter was replaced with the weak-intensity promoter MITp. The transformation of *S. cerevisiae* was performed using the LiAC method [25]. In this study, all the constructed strains were listed in Table 1.

Table 1. *S. cerevisiae* strains constructed in this study.

Strain	Genotype	Source
M-1	*S. cerevisiae* M(wild type)	From Angel yeast
M-2	M-1-Site 21:: *PGKp-PRK-ADH1t-CIT2p-Cbbm-CYC1t*::Site 10::*GAPp-GroES-CYC1t-TEF1p-GroEL-ADH1t*	This study
M-3	M-2-Site 5::*CIT2p-Cbbm-CYC1t*	This study
M-4	M-1Δ*ADH2*	This study
M-5	M-1-Δ*FPSp*::*MIT1p*	This study
M-6	M-1-Site 19::*TEF1p-Rim15-ADH1t*	This study

2.4. Small-Scale Corn Fermentation

An engineered single colony strain was inoculated into YPD test tubes containing 5 mL of YPD medium and cultured overnight at 30 °C with a rotation speed of 200 r/min. Then all the cells in the test tube were transferred to a 500 mL shaker flask containing 200 mL YPD at the same culture conditions. After that, the cell culture is centrifuged at 5000 r/min for 5 min, and the supernatant is discarded. The cell pellet is resuspended by adding the sterile water equal to the mass.

The fermentation method was adopted from Khatibi et al. with some modifications [26]. Briefly, a corn mash consisting of 100 g of ground corn and 170 mL of water underwent a pretreatment process, in which 150 μL of amylase was added and stirred at 40 rpm for 90 min at a temperature of 101 °C. This pretreatment was intended to enhance the digestibility and subsequent utilization of the corn mash. Then 440 μL of glucoamylase and 640 μL of cell suspension were added to corn mash, and the net weight was fixed at 285 g by water. Fermentation flasks were incubated at 30 °C with 170 rpm for 72 h. At time points 0 h, 24 h, 48 h, and 72 h, the sample was weighed for analysis of weightlessness, and the flasks were immediately returned to the incubator. The alcohol content is determined at the end of fermentation at 72 h.

2.5. Analytical Methods

Once the fermentation process is finished, gently stir the fermentation mixture and then carefully pour 100 mL of it into a 1000 mL distillation flask. Simultaneously, 100 mL of tap water, along with two drops of defoamer, will be added to initiate the distillation process. The distilled liquid is then collected into a 100 mL volumetric flask using a cold water bath, ensuring that the temperature of the distillate remains below 25 ℃. A high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) with a 300-mm Aminex HPX-87H column (0.6 mL/min, 5 mM H_2SO_4 at 65 °C; Bio-Rad, CA, USA) and a refractive index detector (Agilent, CA, USA) was used to detect fermentation metabolites.

2.6. Adaptive Evolution of Engineered Strains under the Multiple Stresses

The adaptive evolution based on pH-dCas9-*Mre11* plasmid system was introduced into the engineered *S. cerevisiae* by cultivating the cells at a temperature gradually increasing from 33 °C to 38 °C during 30 series of subculture, along with the addition of 1% lactic acid (LA) and 3% ethanol. The M-2 strain was grown for inoculum preparation until the late exponential phase was reached. This culture was used to inoculate flasks with YPD to an initial OD_{600}nm of 0.2. When the culture reached the midlog phase, an aliquot was transferred to another flask with fresh medium. Each batch

was started with a low initial biomass concentration (OD_{600}nm ≈ 0.2) to select cells with better adaptability in 1% lactic acid and 3% ethanol, along with different temperatures.

3. Results

3.1. Efficient Gene Editing in Industrial S. cerevisiae Using the CRISPR/Cas9 System via an Optimized gRNA Plasmid

To improve the gene editing efficiency of industrial *S. cerevisiae* M, we used the CRISPR/Cas9 system via an optimized gRNA plasmid (Figure 1A,B). In this study, the copy number of optimized CRISPR-gRNA plasmid is regulated by a truncated TEF promoter. The *ade2* gene was chosen to test the gene editing efficiency in industrial *S. cerevisiae* M by the optimized CRISPR system, of which the disruption of *ade2* in *S. cerevisiae* resulted in a distinct pink colony phenotype caused by the accumulation of purine precursors in the vacuoles of yeast cells (Figure 1D). With the CRISPR-optimized gRNA plasmid system, the editing efficiency of the *ade2* knockout in industrial *S. cerevisiae* increased to 60%, compared to only 20% in the control group (Figure 1C). The results showed that the optimized CRISPR/Cas9 system could significantly improve the gene editing efficiency of industrial *S. cerevisiae* M.

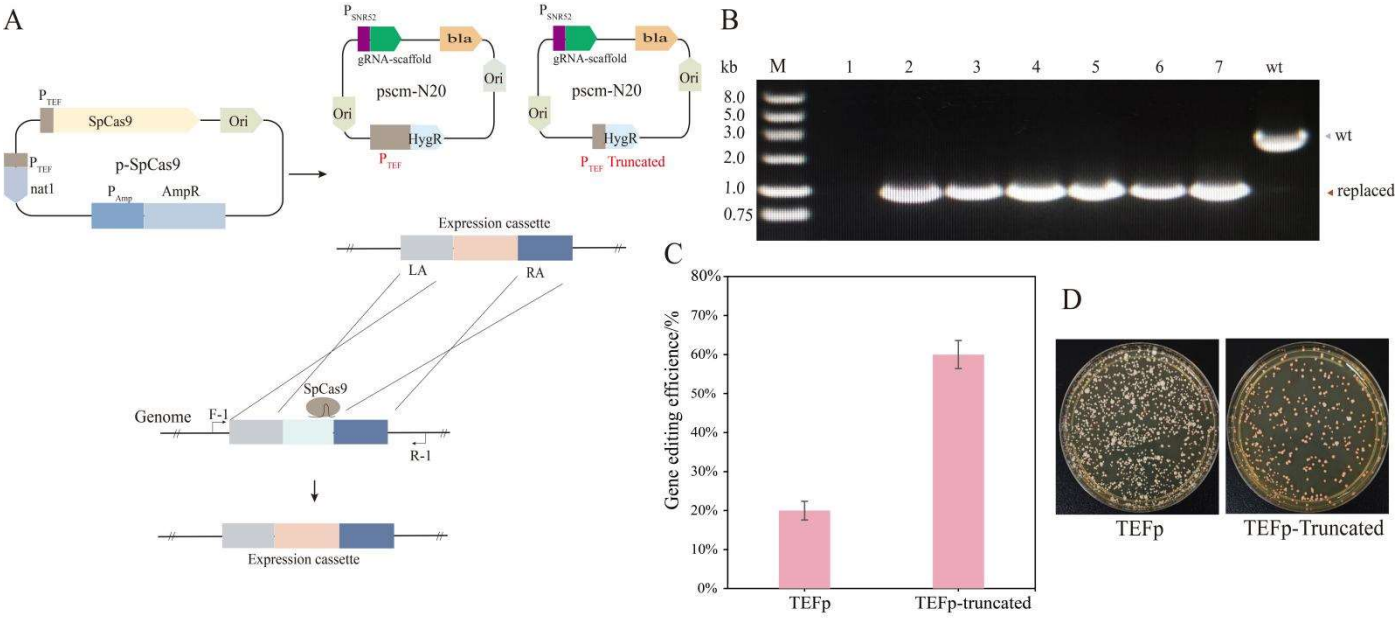

Figure 1. Evaluation of the CRISPR/Cas9 editing efficiency with optimized gRNA plasmid. (**A**) Editing schematic of CRISPR-Cas system with different types of gRNA plasmids in industrial *S. cerevisiae*. (**B**) Disruption of the *ade2* gene mediated by the CRISPR/Cas9 system with a truncated TEF promoter of gRNA plasmid in industrial *S. cerevisiae* M. (**C**) Comparison of the editing efficiency with the truncated TEF promoter and conventional TEF promoter of gRNA plasmid. (**D**) Growth of strains with disruption of the *ade2* gene.

3.2. Multi-Strategies for the Construction of Engineered Yeast Strains for Reducing Glycerol Production

To reduce the production of glycerol, a CO_2-fixation pathway was constructed in industrial *S. cerevisiae* M by heterologous expression of codon-optimized *RuBisCO* and *PRK* in combination with the codon-optimized chaperone *GroEL*/*GroES* from *E. coli* to obtain engineered strain M-2 (Figure 2A). Furthermore, the M-3 strain was obtained by increasing the two copies of *RuBisCO* gene to improve the efficiency of CO_2 fixation further. In the *S. cerevisiae*, the enzyme known as alcohol dehydrogenaseII (ADH2) plays a crucial role in oxidizing ethanol to acetaldehyde. Therefore, decreasing the activity of ADH2 results in a lower consumption of ethanol during the engineering of metabolic pathways. Consequently, we successfully developed the engineered strain M-4 by deactivating the *ADH2* gene (Figure 2B). Meanwhile, the *S. cerevisiae* Δ*FPS1* mutant strain M-5 was constructed to achieve intracellular glycerol accumulation and trigger the reduction of glycerol biosynthesis in other regulatory systems, which then led to an increase in ethanol production (Figure 2B). Previous studies have shown the *Rim15* gene as a crucial regulator in controlling the transcription levels of genes related to stress tolerance in yeast cells. To improve the yeast strains' resistance to external environmental stress, we have successfully developed a new strain named M-6 through the overexpression of the *Rim15* gene (Figure 2C).

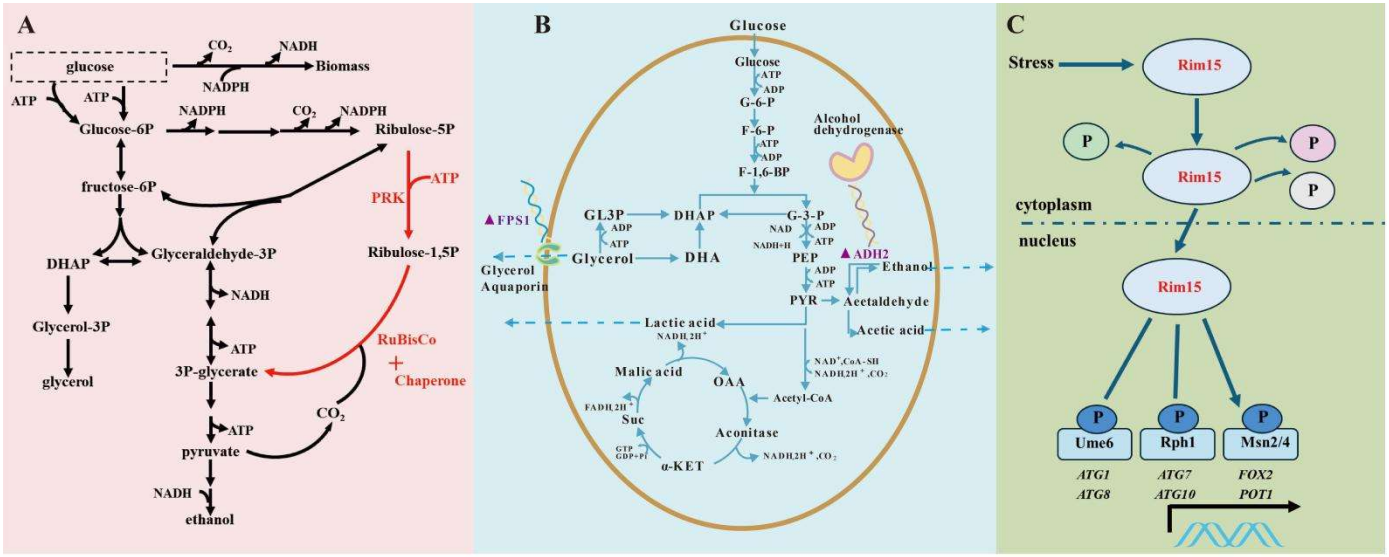

Figure 2. Metabolic engineering of industrial *S. cerevisiae* for reducing the glycerol content. Genes overexpressed and knocked-out in the present study were shown in red and purple, respectively. (**A**) Modification of the carbon metabolism pathway of *S. cerevisiae* is needed to reduce the ethanol yield in this study. (**B**) Schematic representation of central carbon metabolism and the introduced Calvin-cycle enzymes in *S. cerevisiae*. Phosphoribulokinase (PRK from *Spinacia oleracea*) and ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO form *Thiobacillus denitrificans*) in combination with the *E. coli* chaperones GroEL/GroES assist the folding of RuBisCO protein. (**C**) A schematic diagram showing the key genes related to Rim15-mediated regulation of stress response. In response to external stress, Rim15 is retained in the nucleus where it phosphorylates the transcriptional repressors (Ume6, Rph1) and activators (Msn2/4), promoting the upregulation of stress response genes.

3.3. Small-Scale Corn Fermentation for Lower Production of Glycerol

The yeast *S. cerevisiae* M used in this study is a commercial, industrial diploid yeast strain with relatively strong thermal tolerance, and the fermentation temperature can be maintained at up to 40 °C in practical production. Since the higher temperature is one of the key process parameters affecting fermentation efficiency, ethanol yield, and the formation of by-products (such as glycerol content). Therefore, we tested the fermentation parameters of the engineered yeast strains at 36 °C and 39 °C. The glycerol and ethanol yield in engineered strains mentioned above at the end of the fermentation were investigated compared to the wild-type strain. The results indicated that the glycerol content of the engineered strains decreased to some extent under fermentation conditions at 36 °C. Compared to the original strain, the glycerol content of M-2 significantly decreased by 18.6% (Figure 3A). Similarly, after raising the fermentation temperature to 39 °C, the glycerol yield of strains M-2 to M-6 underwent a reduction in varying magnitudes compared to the original strain. Among them, the glycerol content of M-2 significantly decreased by 21.5% in comparison to that of the original strain (Figure 3B).

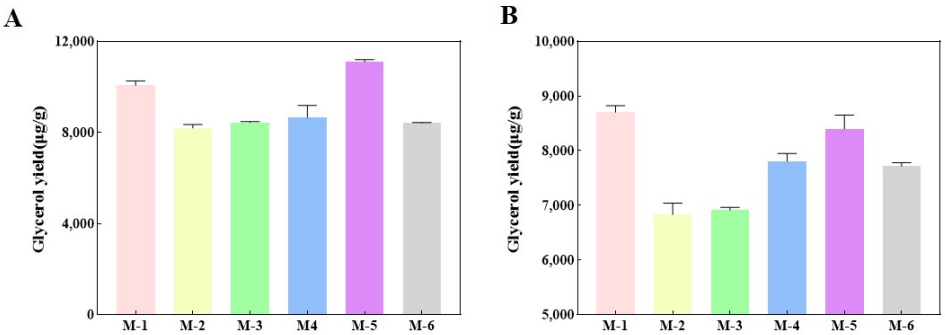

Figure 3. The glycerol yield in engineered strains at the endpoint of the fermentation. (**A**) Glycerol yield in engineered strains fermented at 36 °C; (**B**) Glycerol yield in engineered strains fermented at 39 °C.

However, the ethanol production of the engineered strain did not increase significantly compared with the original strain, and even the ethanol content of M-2, M-3, and M-6 decreased slightly at 36 °C and 39 °C (Figure S1B,D). Further analysis revealed that the cumulative weight loss of the engineered strains, except for M-4 was reduced in comparison to that of the original strain, indicating a decline in the growth performance of the strains (Figure S1A,C).

3.4. Construction and Characterization of Random Diversification in the S. cerevisiae Genome for Adaptive Evolution of Engineered Strains

To further enhance the robustness of the engineered *S. cerevisiae* strains, genome maintenance genes mediated genome-wide mutations system (*GWMS*) serves as an effective method to diversify genes in their native context. In this study, the target gene *upp* locus, which encodes a uracil phosphoribosyltransferase whose inactivation ablates the ability of cells to uptake 5-fluorouracil (5-Flu), was tested. Only cells with *upp* disabled survive when grown on media containing 1.0 mg/mL 5-Flu, where the 5-Flu is not converted by a toxic 5-fluoro-2′-deoxyuridine-5′-monophosphate(5-F-dUMP). Thus, the *upp* gene serves as a negative selection to measure *mre11-GWMS* mutagenesis at this locus. Utilizing a gRNA targeting *mre11* or *msh2*, liquid cultures were plated on SCD plates supplemented with 5-fluorouracil after 96 h of growth. 5-fluorouracil resistant (5-FluR) CFUs were quantified. The *mre11* targeting mutation efficiency showed a 2.28-fold that of *msh2* targeting mutation efficiency (Figure 4A,B). Next, we used *mre11*-GWMS for random diversification in the *S. cerevisiae* genome by adaptive evolution, which cultivating the cells at a temperature gradually increasing from 33 °C to 38 °C, along with the addition of 1% lactic acid (LA) and 3% ethanol (Figure 4C). After 30 series of subcultures, the cell growth of evolved strain 1A4e showed a significant increase compared with those of the unevolved strain (Figure 4D,E). However, there was no significant increase in ethanol production compared with the non-evolved strain (Figure S2).

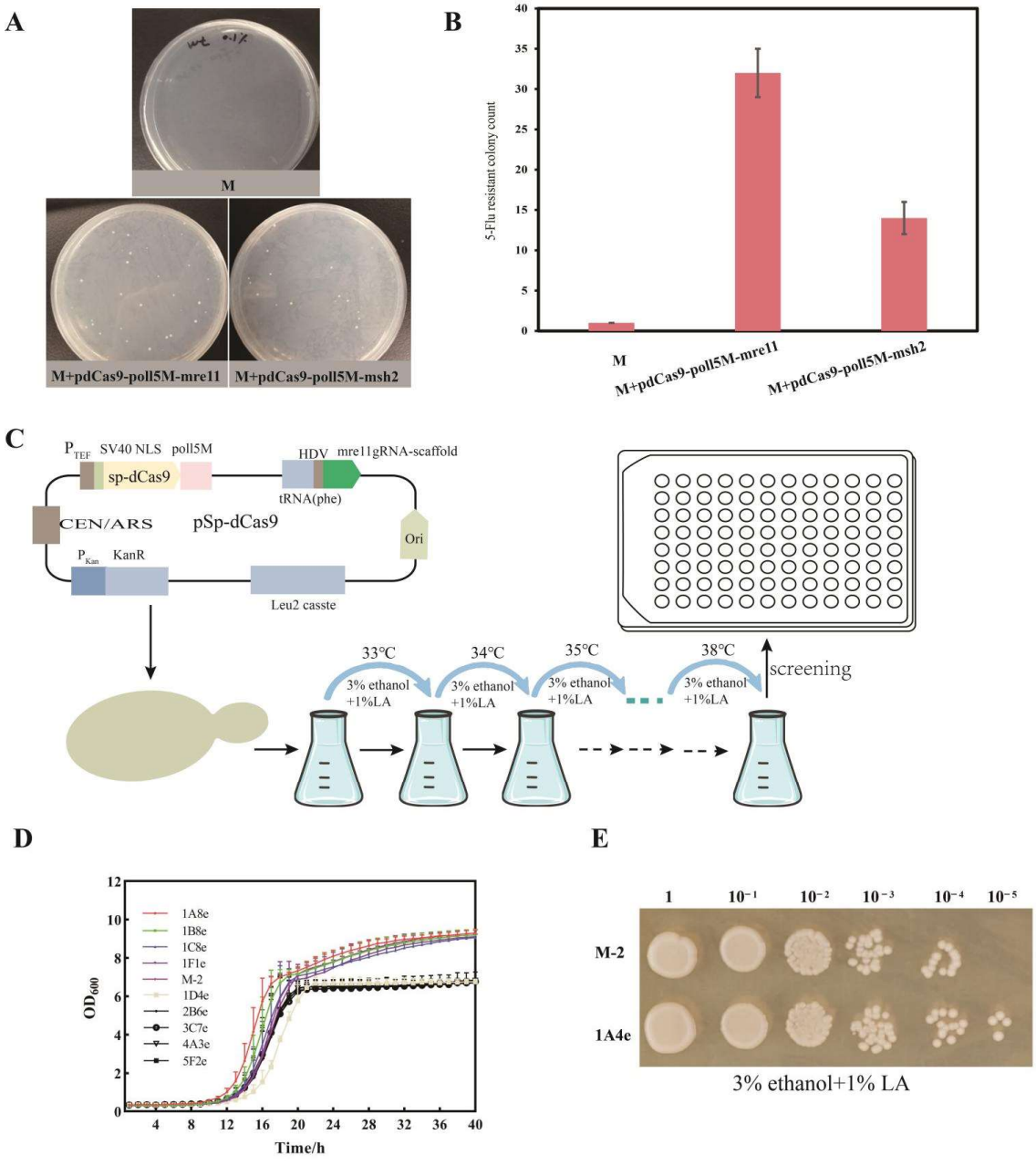

Figure 4. Enhancing the robustness of the engineered strain by genome maintenance gene *mre11* mediated genome-wide mutations system adaptive evolution. (**A**) Gene mutators induced different numbers of colonies in the *S. cerevisiae* targeted the *upp* gene on

the SCD medium containing 5-fluorouracil; (**B**) Gene mutators induced different relative mutation rates in the *S. cerevisiae* targeted the *upp* gene on the SCD medium containing 5-fluorouracil; (**C**) Schematic illustration of adaptive evolution and strain screening process; (**D**) The growth curve of different strains after evolution. (**E**) Ethanol and lactate tolerance after strain evolution.

In summary, these results indicate that *Mre11*-GWMS mediated adaptive evolution is an effective way to screen strains with good growth performance. However, further research is needed to improve the ethanol production of the strains.

4. Discussion

During the fermentation process for ethanol production, glycerol is typically formed as a by-product to maintain osmotic pressure and prevent water loss under high osmolarity conditions [27]. However, excessive accumulation will inevitably have a detrimental effect on ethanol production [27]. To date, many researchers have utilized CRISPR-Cas9 technology to construct engineered strains of *S. cerevisiae* for the regulation of glycerol production [28,29]. Functional expression of the PRK and Rubisco in *S. cerevisiae* led to a 90% reduction of the by-product glycerol and a 10% increase in ethanol production in sugar-limited chemostat cultures on a mixture of glucose and galactose [30]. The deletion of *FPS1* increased ethanol production by 10% and reduced glycerol yield by 18.8% [14], and *ADH2* deletion in *S. cerevisiae* also resulted in the improvement of ethanol yield [28]. In this study, the glycerol production of engineered strains M-2, M-3, M-4 and M-6 decreased to different degrees, in which the glycerol production of M-2 decreased by 21.5% (Figure 3), but the ethanol production did not exhibit a significant increase. Further analysis of the strains' cumulative weight loss revealed a decrease in all cases, likely due to reduced growth performance, which hindered ethanol production.

Adaptive evolution has been proven to be an effective method for selecting yeast populations resistant to various stress conditions and expanding their tolerance range [30,31]. With the development of new strategies and tools for adaptive evolution, the efficiency of modifying metabolic pathways and enhancing cellular tolerance has been significantly accelerated [32]. To further enhance the growth performance of the strain, the adaptive evolution based on the *mre11-GWMS* was performed. The cell growth of evolved strains showed better growth performance compared with those of the unevolved strain (Figure 4D), indicating that the adaptive evolution based on the *mre11-GWMS* is an effective method to improve the tolerance and growth ability of strains. However, with the progression of adaptive evolution technologies, the process of constructing and screening cell factories frequently encounters limitations imposed by the assessment of targeted phenotypes. The greatest challenge consists of developing high-throughput screening methods to identify the optimal mutants from millions of potential candidates. Further improvements and optimizations of existing technologies are necessary to convert difficult-to-detect phenotypes into rapidly measurable ones, integrating adaptive evolution with high-throughput screening to drive the further development of this technology.

In this study, we optimized the gene editing efficiency of industrial *S. cerevisiae* and reduced the glycerol content of engineered strains to varying degrees through various metabolic engineering strategies. The resulting CO_2-fixing yeast M-2 led to a 21.5% reduction of the by-product glycerol in corn mash fermentation cultures at 39 ℃, and further adaptive evolution based on the *mre11-GWMS* can effectively improve the strain's tolerance and growth performance.

Supplementary Materials

The following supporting information can be found at: https://www.sciepublish.com/article/pii/462, Figure S1. Cumulative weightlessness and ethanol yield in engineered strains. (A) Difference in cumulative weightlessness at 36 ℃. (B) Difference in ethanol yield at 36 ℃. (C) Difference in cumulative weightlessness at 39 ℃. (D) Difference in ethanol yield at 39 ℃; Figure S2. The ethanol yield of the evolved strains and the parental strain. (A) Ethanol yield of the evolved strains and the parental strain fermented at 35 °C. (B) Ethanol yield of the evolved strains and the parental strain fermented at 38 °C; Figure S3. Residual sugar and acetic acid in fermentation of engineered strains. (A) Residual sugar and acetic acid in fermentation of engineered strains at 36 °C. (B) Residual sugar and acetic acid in fermentation of engineered strains at 39 ℃; Table S1. A list of plasmids constructed in this study; Table S2. A list of primers used in this study; Table S3. A list of heterologous genes coding sequences used in this study.

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Author Contributions

N.X. and X.G. designed the study, analyzed the data, and wrote the paper. N.X., Y.Y., H.C., Y.Z., B.L., N.P. and Y.W. conducted the experiments. All the authors approved the manuscript.

Ethics Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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Declaration of Competing Interest

The authors declare no competing financial interest.

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