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Research Article

Investigating formate tolerance mechanisms in *Saccharomyces cerevisiae* and its application

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ABSTRACT

Current global energy and environmental crisis have spurred efforts towards developing sustainable biotechnological solutions, such as utilizing CO_2 and its derivatives as raw materials. Formate is an attractive one-carbon source due to its high solubility and low reduction potential. However, the regulatory mechanism of formate metabolism in yeast remains largely unexplored. This study employed adaptive laboratory evolution (ALE) to improve formate tolerance in *Saccharomyces cerevisiae* and characterized the underlying molecular mechanisms. The evolved strain was applied to produce free fatty acids (FFAs) under high concentration of formate with glucose addition. The results showed that the evolved strain achieved a FFAs titer of 250 mg/L. Overall, this study sheds light on the regulatory mechanism of formate tolerance and provides a platform for future studies under high concentrations of formate.

1. Introduction

The issue of energy shortage and greenhouse gas emissions has spurred widespread attention. To address this problem, scientists have proposed the concept of the third-generation biorefinery, aiming to use CO_2 as the carbon source for the production of chemicals and biofuels [1,2]. Compared with other one-carbon sources such as formate and methanol, CO_2 has low solubility and low fixation efficiency through microbial transformations [3,4]. Formate is a one-carbon substrate demonstrated in various applications, due to its ability to be produced through the conversion of CO_2 using photocatalysis [5,6] and electrocatalysis [7].

Among various CO_2 and formate conversion pathways, the Calvin-Benson-Bassham cycle and the reductive glycine (rGly) pathway have demonstrated certain advantages [1]. Through the expression and optimization of these pathways, strains that used CO_2 or formate as sole carbon source have been demonstrated in both prokaryotic organism *Escherichia coli* [8–10] and eukaryotic organism *Pichia pastoris* [11,12]. *Saccharomyces cerevisiae* had been reported that possesses an endogenous formate utilization pathway [13,14]. Yet, due to the frequent formate supplementation, strains currently grew poorly [13], restricting them for potential industrial applications.

Compared to natural evolution, which is characterized by high randomness and long cycles, adaptive laboratory evolution (ALE) is a powerful tool in microbiology research that promotes the evolution of microbes through the application of artificial interference and the regulation of growth correlated mechanisms [15]. ALE can enhance the benign phenotypes [16], improve robustness [17], and optimize toward favorable phenotypes [18,19]. Application of ALE technology in microbial adaptation of one-carbon chemicals was summarized in Table 1.

Here, we applied the ALE approach to enhance the tolerance of yeast to high concentrations of formate by incrementally elevating the formate concentrations in the culture medium. This led to the isolation of a robust strain capable of withstanding up to 50 g/L formate. Subsequently, we employed omics analyses to interrogate underlying mechanisms associated with the formate tolerance of yeast (Fig. 1). In order to validate the suitability of the evolved strains, we specifically chose to focus on the production of FFAs as a target. FFAs are crucial as precursors for a wide range of applications including biofuels, aviation fuels, and precursor chemicals for the production of renewable diesel [20]. By successfully demonstrating the production of FFAs using the evolved strains, we can establish the applicability and potential of these strains for the sustainable production of valuable bio-based products. Notably, the evolved strain was capable of

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Table 1
Application of ALE technology in microbial transformation of one-carbon chemicals.

C1 sources	Expected phenotype	Organism/strain	ALE strategy	Outcome	Ref.
Methanol	Increased growth and utilization of methanol using methanol and xylose as substrates	*E. coli*	• 4 g/L methanol and 4 g/L xylose in CGXII micro medium for 27 generations	• Growth rate: 0.03/h • Cell concentration: 1.17 ± 0.03 gCDW/L	[21]
	Improvement of methanol assimilation	*E. coli*	• Methanol and gluconate for evolution	• Growth rate: 0.081 ± 0.002/h	[22]
	Growing on methanol as the sole carbon source	*E. coli*	• 400 mM MeOH and 20 mM xylose for MeOH auxotrophy • Gradually decreasing HDA and increasing MeOH for growth	• Doubling time: 8.5 h • Methanol tolerance: 1.2 M • Maximum OD600: ~ 2	[23]
	Growing on methanol as the sole carbon source	*S. cerevisiae*	• Serial transfers in shake flasks in five stages • Terminated upon reaching a stable growth rate in the MM with 1 % methanol and 0.2 % xylose	• Revealed that the evolved glyoxylate-based serine pathway plays an important role in methanol-dependent growth • Increased concentration of squalene and ergosterol in evolved strain.	[24]
	Growing on methanol as the sole carbon source	*S. cerevisiae*	• YNB medium containing 1 g/L yeast extract or 1 g/L yeast extract with 2 % methanol (48 h) pulsing with YNB medium with 1 % glucose (24 h)	• No metabolic engineering modification required, only achieved through ALE	[25]
	Improvement of methanol tolerance and utilization	*C. glutamicum*	• CGXII minimal medium supplemented with 15 g/L methanol and 4 g/L xylose	• Growth rate: 0.052 h^{-1} • Methanol consumption: 203.75 mM • Production: 230 mg/L L-glutamate	[26]
	Improvement of methanol utilization	*Y. lipolytica*	• Continuous culturing in medium containing 2 % methanol by varying CSM	• Methanol assimilation: 1.1 g/L (Every 72 h)	[27]
Formate	Improvement of formate utilization	*Thermococcus onnurineus*	• Consecutive transfers for 156 times	• Cell density, H_2 production and formate consumption rates were increased by 1.71, 1.93 and 1.91 times, respectively	[28]
	Improvement of formate utilization	*E. coli*	• Gradually decreasing yeast extract and increasing formate over 150 generations	• Growth rate: 0.035/h • Formate utilization rate: 0.05 g/l/h	[29]
	Growing on formate as the sole carbon source	*E. coli*	• Adding formate once every 3–6 days to increase formate by 30 mM and repeating for 13 cycles	• Formate yield: 2.3 g CDW/mol • Doubling time: < 8 h	[9]
	Growing on formate as the sole carbon source	*E. coli*	• 20 mM glycerol and 70 mM formic acid for 3 weeks • 20 mM pyruvate and 70 mM formic acid for 5 weeks • Gradually increasing formic acid and decreasing glycine to negligible for 90 days • Increasing the growth rate under formic acid for 60 days	• Growing on formate at ambient CO_2 concentration	[30]
	Improving formate tolerance	*C. beijerinckii*	• 10 g/L glucose with 0–10 g/L sodium formate for evolution	• Formate consumption: 4 g/L within 48 h.	[31]
	Improving the growth rate using formate as the sole carbon source	*Cupriavidus necator H16*	• 50 mM sodium formate for 400 generations	• Maximum growth rate was increased by 1.15–2.18 times • Maximum OD_{600} was increased by 10–34 %	[32]
CO_2	Growing semi-autotrophic with CO_2 as carbon source and pyruvate as energy source	*E. coli*	• Xylose restricted constantiser for about 2 months (about 150 generations)	• Growing on basal medium with pyruvate and high CO_2 concentration • Doubling time: 6 h	[33]
	Growing autotrophically with CO_2 as the sole carbon source	*E. coli*	• Cultured in a xylose-restricted constantiser with 10 % CO2 with a concentrated jet of air • No xylose added around day 400 of evolution	• Heterotrophic to fully autotrophic strain on laboratory time scales	[8]
	Developing of potential CO_2 fixation pathways	*E. coli*	• Xylose and E4P at an elevated CO_2 concentration of 20 % for 2 weeks	• Opening the GED pathway • Doubling time: 5.3 h	[34]
	Growing autotrophically with CO2 as the sole carbon source	*P. pastoris*	• Excess CO2 for 27–29 generations in continuous batch culture	• Growth rate: 0.018/h	[11]
CO	Improving CO tolerance	*Eubacterium limosum*	• Syngas (44 % CO, 22 % CO_2, 2 % H_2, and 32 % N_2) for evolution	• Growth rate: 0.089/h • CO consumed rate: 0.058 ± 0.003 mmol/h • Maximum OD_{600}: 0.639 ± 0.016	[35]
Formate	Improving formate tolerance	*S. cerevisiae*	• 20 g/L glucose with 0–50 g/L sodium formate for evolution	• Growth at 20 g/L glucose with 50 g/L formate in MM medium • Maximum OD_{600}: 6.88 • FFAs production: 250 mg/L	This study

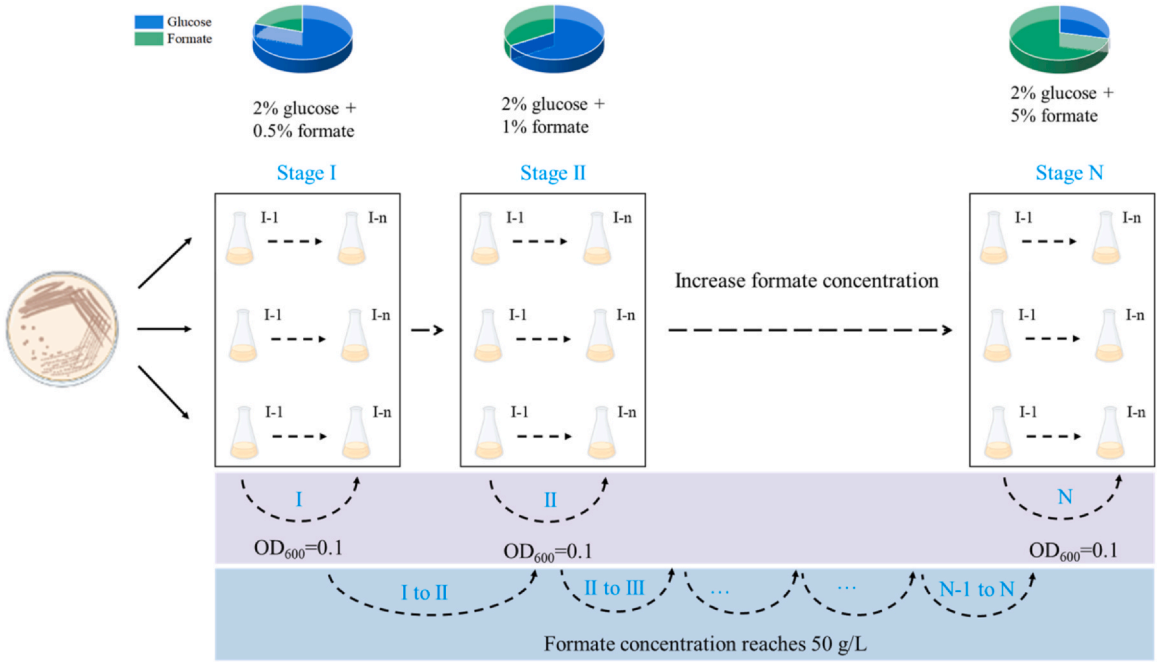

Fig. 1. Schematic diagram of high formate tolerance and omics analysis of *Saccharomyces cerevisiae* by the ALE strategy.

generating FFAs under high formate concentrations, yielding 250 mg/L of FFAs in shaking flasks with 20 g/L glucose. The basic and applied results gained here lay a foundation for future industrial applications under high concentrations of formate.

2. Materials and methods

2.1. Strains, media and growth conditions

Plasmids, strains, and primers used in this study are listed in Tables S1–S3. *S. cerevisiae* CEN.PK 113-5D *LEU2*Δ (MATa MAL2-8c SUC2 ura3-52 LEU2Δ) strain (ORI) was employed for the investigation. Liquid cultures utilized Yeast Extract Peptonen (YPD) medium at a temperature of 30 °C with continuous stirring at 200 rpm. Solid cultures used YPD agar plates supplemented with 50 g/L formate.

YPD medium supplemented with 20 g/L formate was used for the cultivation of the seed culture. The shaking flask fermentation conditions were as followed: MM medium (5 g/L $(NH_4)_2SO_4$, 14.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, with trace metal and vitamin solutions [20], pH = 5.6) containing 20 g/L glucose and 50 g/L formate, 0.2 g/L uracil and 0.4 g/L leucine. The fermentation was carried out at 30 °C and 200 rpm, with an initial OD_{600} of 0.1.

2.2. Laboratory directed evolution

During the adaptive laboratory evolution process, formate was added to an MM medium containing 20 g/L glucose, 0.2 g/L uracil, and 0.4 g/L leucine. The concentration of formate was gradually increased in a stepwise manner with a gradient of 5 g/L until reaching a final concentration of 50 g/L. The strain was continuously adapted at each gradient of formate, and prior to moving to the next gradient, the strain was primarily cultured for a minimum of 40–60 generations to ascertain its growth stability at that concentration.

2.3. Analytical methods

2.3.1. Measurement of biomass and extracellular substances

Biomass was measured by the optical density at 600 nm (OD_{600}) using an EU-2600 Visible Spectrophotometer (Shanghai Onlab instrument, China).

Glucose and formate were quantified using HPLC (Shimadzu LC-20AT, Japan) equipped with refractive index (RID) and UV detectors at 210 nm. The analysis was performed using a 5 mM H_2SO_4 eluent and an Aminex HPX-87 H column (Bio-Rad) maintained at 65 °C with a flow rate of 0.6 mL/min. The injection volume was 10 μL. HPLC standards were prepared by diluting a mixture of 20 g/L glucose and 10 g/L formate into six gradients using the eluent.

FFAs samples were taken at 120 h and measured using gas chromatography–mass spectrometry (QP2020, Shimadzu, Japan) after derivatization, as stated in our recent paper [13]. In short, the procedure was initiated by transferring 200 μL of cell culture into a glass vial. Immediately afterwards, 10 μL of 40 % tetrabutylammonium hydroxide and 200 μL of methylation reagent were added. The mixture was shaken for 30 min, followed by centrifugation at 5000 g for 3 min. Next, 100 μL of the upper liquid which contained the extracted methyl esters, was transferred into a GC vial. The sample was then evaporated for 6 h and resuspended in 100 μL of hexane. GC-MS analysis (QP2020, Shimadzu, Japan) was performed with a DB-5MS column (30 m × 0.250 mm × 0.25 μm, Agilent). The temperature program was as follows: an initial temperature of 40 °C was maintained for 2 min, followed by a linear ramp at a rate of 5 °C per minute to reach 130 °C. Subsequently, the temperature was increased at a rate of 10 °C per minute until it reached 280 °C, which was then maintained for 3 min. The inlet, mass transfer line, and ion source temperatures were set at 280 °C, 300 °C, and 230 °C, respectively. The carrier gas flow rate was 3.0 mL/min, and the injection volume was 1 μL. Data collection was performed using the full inspection mode (50–650 *m/z*) and analyzed with GCMS solution 4.4 software.

2.3.2. Omics analysis

For the omics analysis, the origin strain ORI was grown in MM medium containing 20 g/L glucose, 0.2 g/L uracil and 0.4 g/L leucine; the final strain ALEF-5 % was grown in MM medium containing 20 g/L glucose, 50 g/L formate, 0.2 g/L uracil and 0.4 g/L leucine at 30 °C, 200 rpm. for 72 h. Samples were collected at 72 h. RNAseq was completed by Novogene Biotech Co., Ltd. Transcriptome data was analyzed using the NovoMagic platform provided by Novogene Biotech Co., Ltd. The transcription factors were determined by aggregating the differentially expressed genes and processing them

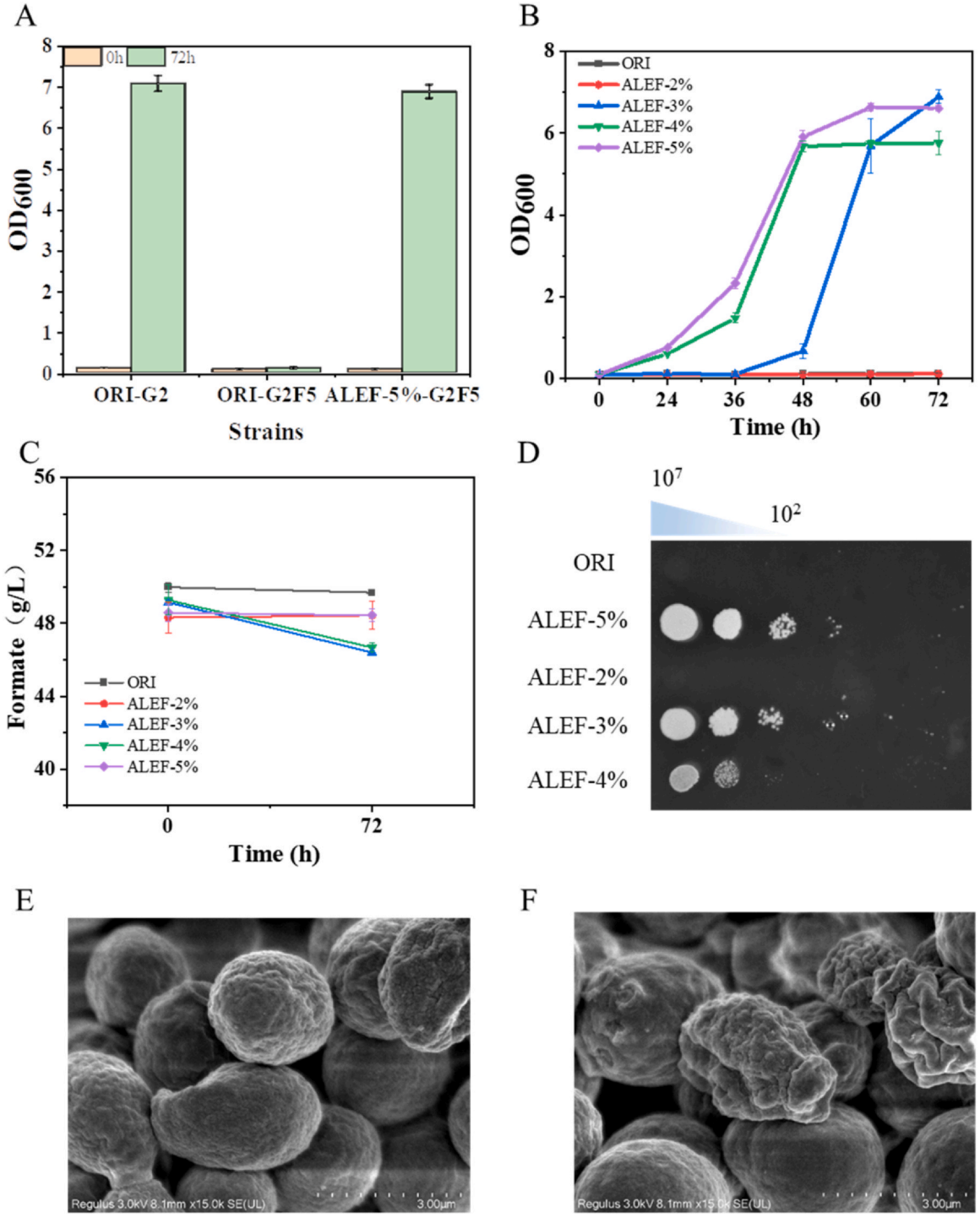

Fig. 2. Strain growth and formate consumption. (A) Comparison of growth between ORI and ALEF-5 % in different media, G2: 20 g/L glucose, G2F5: 20 g/L glucose and 50 g/L formate; (B) Growth comparison of different evolutionary strains in MM medium containing 20 g/L glucose and 50 g/L formate, ALEF-2 %: The strain ALEF-2 % represented a strain that was capable of growth in MM medium containing 20 g/L formate and 20 g/L glucose. The numerical value represents the concentration of formate. It was worth noting that the tested evolved strains may also possess the ability to grow under conditions of 50 g/L formate; (C) Formate consumption of different evolutionary strains in MM medium containing 20 g/L glucose and 50 g/L formate. (D) Plate assay of ORI and ALEF-2–5 % strains in YPD containing 50 g/L formate at 72 h. (E) SEM of ALEF-5 % strain in MM medium containing 20 g/L glucose at 72 h. (F) SEM of ALEF-5 % strain in MM medium containing 20 g/L glucose and 50 g/L formate at 72 h. Strains were cultivated in shake flasks for 72 h at 200 rpm, 30 °C. All data represent the mean ± s.d. of at least triplicate experiments.

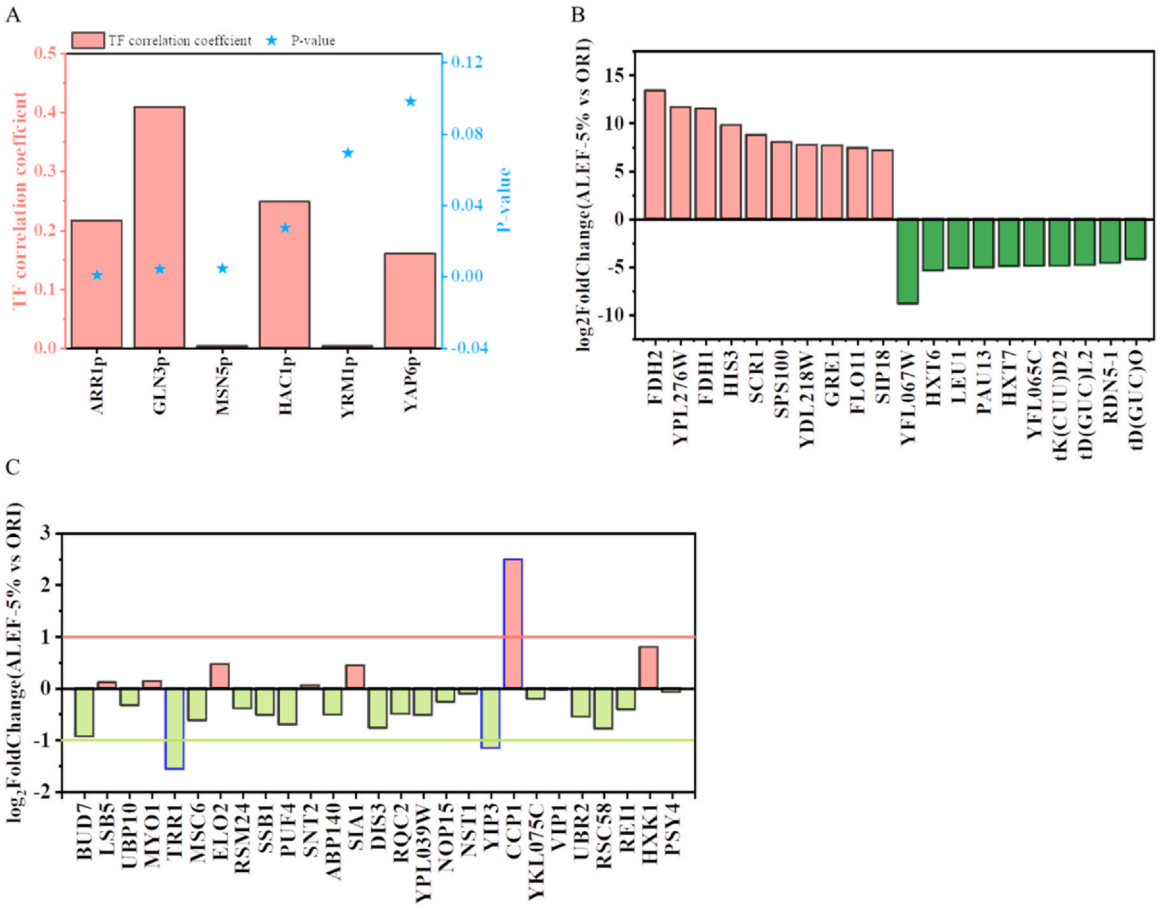

Fig. 3. Transcriptomic analysis between strains ALEF-5 % and ORI. (A) Screening of transcription factors of differentially expressed genes. (B) Summary of TOP10 genes up and down at transcriptional level (C) Comparison of transcriptional levels of mutant genes.

through the website: http://www.yeastract.com/formfindregulators.php. Metabolomics samples were analyzed using LC-Q TOF (1290 Infinity II UHPLC-6546 Q TOF LCMS system, Singapore, Agilent Technology, INC.) equipped with an InfinityLab Poroshell 120 HILIC-Z column (2.1 × 150 mm, 2.7 μm, PEEK-lined.). The binary pump parameters and MS parameters were as stated in our recent paper [13].

3. Results and discussion

3.1. Adaptive laboratory evolution enables growth of Saccharomyces cerevisiae under high formate concentrations

To achieve a high tolerance of formate in yeast, adaptive laboratory evolution techniques were employed, and the ORI strain was selected as the starting strain. By continually increasing the concentration of formate in MM medium with 20 g/L glucose, the growth of yeast ALEF-5 % in high concentrations of up to 50 g/L formate was achieved (Fig. 2A). Furthermore, evolved strains were selected from various stages, such as ALEF-2 %, ALEF-3 %, ALEF-4 %, and ALEF-5 %, which were capable of growth in the presence of 20, 30, 40, and 50 g/L formate, respectively. These strains were compared in MM medium containing 20 g/L glucose and 50 g/L formate. No significant growth changes were observed within 72 h of fermentation for the ORI strain and ALEF-2 %. A longer lag phase was observed for ALEF-3 %, and it only exhibited significant growth after 48 h. Both ALEF-4 % and ALEF-5 % showed more pronounced growth tendencies, with ALEF-5 % exhibiting faster growth and the final OD_{600} approaching 7 (Fig. 2B). Regarding formate consumption, no significant formate consumption within 72 h was observed for the starting strain, strains ALEF-2 % and ALEF-5 %, while some formate consumption was observed for ALEF-3 % and ALEF-4 % strains (Fig. 2C). Taking into consideration the prolonged lag phase, formate consumption, and subsequent growth, we have chosen the ALEF-5 % strain for further experimental investigations. Simultaneously, the results were also confirmed by the plate assay (Fig. 2D). Moreover, the scanning electron microscope (SEM) observations revealed that under standard culture conditions, the ALEF-5 % strain displayed a comparatively smooth cellular surface (Fig. 2E). Nevertheless, when cultivated with high formate concentration, the strain demonstrated notable morphological alterations and a wrinkled cell surface (Fig. 2F).

3.2. Genomic and transcriptomic analysis

To investigate the mechanisms that enable yeast tolerate to high concentrations of formate, genome sequencing was conducted on the starting strain ORI and the evolved strain ALEF-5 %. The ORI strain was sequenced through the third-generation sequencing and served as the reference template, while the evolved ALEF-5 % strain was sequenced through the second-generation sequencing. Nine individual clones from three shake flasks were selected and analyzed, and 35 key mutations were identified (Fig. S1). Further screening of these 35 mutated genes based on their frequency of occurrence among the nine strains resulted in the selection of 32 genes with a frequency of occurrence greater than or equal to 1 (Table S4). In particular, *MMM1p*, an outer mitochondrial membrane protein, associated with mitochondrial DNA (mtDNA) nucleoids and is essential for maintaining mtDNA and the structure of mitochondria stability [36,37]. This stability is crucial for providing the cellular basis for energy production and cell passage under high concentrations of formate. Direct mutation or reverse mutation

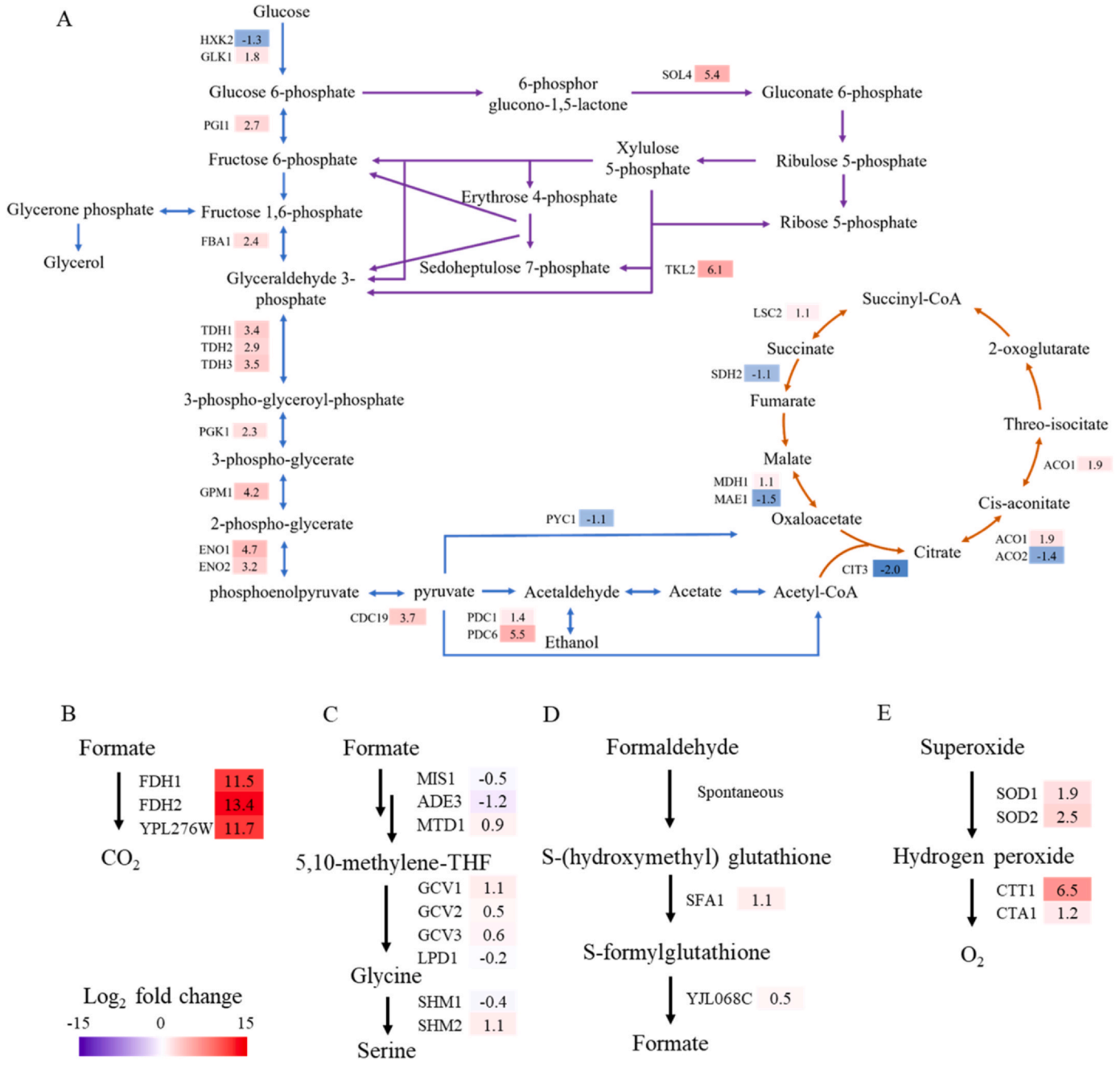

Fig. 4. Transcriptome analysis of metabolic pathway between strains ALEF-5 % and ORI. Regulated genes related to central carbon metabolism(A), one-carbon metabolism(B, C, D) and superoxide radical degradation pathway(E). The color key represented a transcriptional comparison between ALEF-5 % and ORI. The ORI strain was grown for 72 h in MM medium supplemented with 20 g/L glucose, while the ALEF-5 % strain was grown for 72 h in MM medium containing 20 g/L glucose and 50 g/L formate. All data represents the mean of at least triplicate experiments.

experiments will be performed in future research to assess the impact of these genes on formate tolerance. This additional investigation aims to provide a more comprehensive understanding of the regulatory mechanisms involved and strengthen the conclusions of our study.

To further understand the regulatory mechanism of the evolved strain at the transcriptional level, mRNA sequencing was conducted on the starting strain ORI (cultivated without formate to ensure normal growth of the strain) as the control group and the evolved strain ALEF-5 % (cultivated with 50 g/L formate) after 72 h of fermentation. As illustrated in Fig. S2A, Principal Component Analysis (PCA) of the samples showed clear clustering of the samples. It was found that the evolved strain up-regulated 1006 genes and down-regulated 452 genes compared to the ORI strain, with 4850 genes showing no significant difference in transcription (Fig. S2B). Upon analysis of the differentially expressed genes, it was discovered that significantly up-regulated processes involved in carbohydrate metabolic and oxidation-reduction processes of biological process aspect, membrane part of cellular component aspect and oxidoreductase activity of molecular function aspect, while significantly down-regulated processes involved in carboxylic acid and organic acid metabolic processes of biological process aspect, membrane part of cellular component aspect, oxidoreductase activity and co-factor binding of molecular function aspect (Fig. S3). These results indicated that the evolved strain mainly achieved tolerance to high concentrations of formate by regulating genes related to cell membranes (possibly related to osmotic pressure) and oxidation-reduction processes. Additionally, a query and sorting of transcription factors (http://www.yeastract.com/) were performed on the differentially expressed genes, and 6 transcription factors

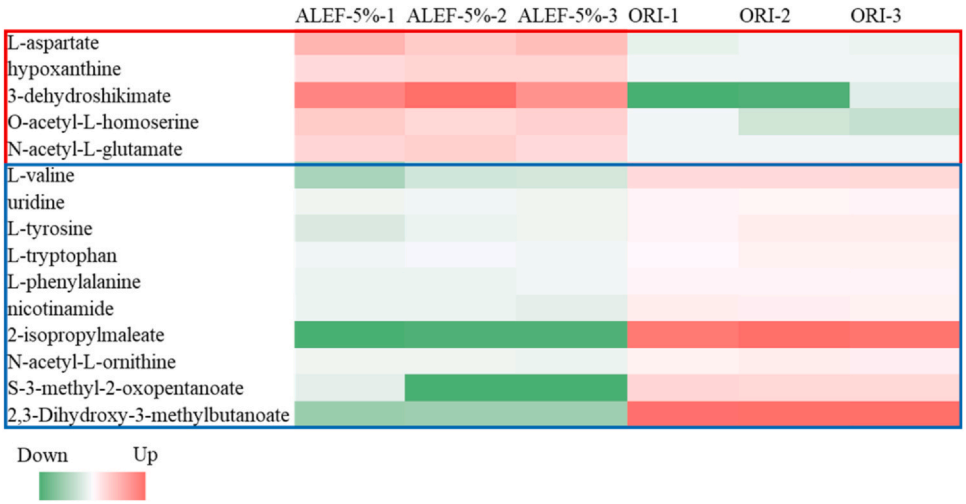

Fig. 5. Non-targeted metabolomic analysis between strains ALEF-5 % and ORI. Differentially expressed metabolites between ALEF-5 % and ORI strains at 72 h. Red indicates overexpression, and green indicates less-expression. All data represent the mean of at least triplicate experiments.

with a P-value less than 0.1 were identified (Fig. 3A).

Furthermore, analysis was performed on the top 10 upregulated genes and top 10 downregulated genes, where differential expression was most evident. As shown in Fig. 3B, it was found that the top three significantly upregulated genes were *FDH1*, *YPL276W* [38], and *FDH2*, all of which encode formate dehydrogenases, with their upregulation levels approximately 3000-fold higher than those of the ORI strain, indicating their crucial roles in formate tolerance.

Intriguingly, the upregulation of *FDH* did not correspond with formate utilization (Fig. 2C), which may be attributed to the cells primarily employing it as a means to counteract external stress rather than serving as a cofactor in this high formate milieu. This observation also underscores the importance of optimizing formate supplementation levels in standard fermentation processes. Also, the genome sequences including promoters and terminators of these three genes were not mutated, indicating that the transcription regulation of these three genes were caused by secondary effects, such as transcription factors, etc. Other upregulated genes mainly included those involved in regulating cell membrane and cell wall composition (such as *SPS100* [39], *FLO11* [40]), as well as those associated with regulating osmotic pressure (such as GRE1 [41], SIP18 [42]). Among the most significantly downregulated genes, *YFL067W* with unknown function exhibited significantly reduced transcription. Other downregulated genes mainly included those involved in high-affinity glucose transporters (*HXT6* and *HXT7*) [43,44]. *YFL067W* (downregulated under low calcium conditions) [45], *LEU1* (involved in leucine biosynthesis) [46], *PAU13* (associated with cell division) [47], and other genes of unknown functions, including *tD(GUC)L2*, *tD(GUC)O*, and *RDN5–1*, were demonstrated considerable downregulation in expression.

Combining the genomic and transcriptomic data (Fig. 3C), three genes (*TRR1*, *YIP3*, and *CCP1*) were both mutated and regulated. Among them, the transcription levels of *TRR1* and *YIP3* genes were significantly downregulated, while *CCP1* was significantly upregulated. *TRR1* is a gene encoding cytoplasmic thioredoxin reductase, acting as a disulfide reductase system that protects cells from oxidative and reductive stress. The downregulation of *TRR1* may primarily regulate the balance between the disulfide reduction system and the formate dehydrogenase reduction system in the cell [48,49]. *YIP3* is a gene encoding Ypt-interacting protein, which is reported to participate in COPII vesicle transport between the endoplasmic reticulum and the Golgi, interacting with the Rab-GTPase family, Yip1p members, and Rtn1p [50,51]. However, the specific function of the downregulation of this gene in formate tolerance is unknown. *CCP1* is a gene encoding cytochrome c peroxidase that degrades reactive oxygen species in mitochondria, acting as a peroxide sensor in mitochondria [52,53]. The significant upregulation *CCP1* may be used to eliminate reactive oxygen species generated by high concentrations of formate, as well as to regulate the redox balance within the cell.

One of the key objects of our attention during metabolic engineering is the changes in central carbon metabolism. Therefore, an in-depth analysis of the transcription levels of genes related to central carbon metabolism was carried out. As shown in Fig. 4A, significant upregulations were observed in genes related to the glycolysis pathway in the evolved strain. In the pentose phosphate pathway, significant upregulation was also observed in *SOL4* and *TKL2*. The transcription levels of genes related to the tricarboxylic acid cycle exhibited mixed regulation. We consider the significant downregulation of *CIT3* to be more critical, as it would lead to less metabolic flow of acetyl-CoA towards the TCA cycle, and we speculated that acetyl-CoA could be converted into lipids such as fatty acids and participate in the formation of cell membranes.

Moreover, the transcription levels of genes related to one-carbon metabolism were analyzed. It was found that the transcription level of formate dehydrogenase in the evolved strain was significantly increased, which can convert formate to CO_2 and generate reducing power (Fig. 4B). Notably, it was found that key genes (*GCV1*, *GCV2*, and *GCV3*) in the rGly pathway were significantly upregulated (Fig. 4C), which increased the efficiency of formate assimilation. The transcription levels of genes related to the formaldehyde detoxification pathway were also upregulated (Fig. 4D), which increased the strain's tolerance to formate. High concentrations of formate may lead to the production of reactive oxygen species. Therefore, we also analyzed genes related to the superoxide scavenging pathway. Indeed, it was found that the transcription levels of these genes were significantly upregulated (Fig. 4E).

3.3. Metabolomic difference analysis

After analysis at the genomic and transcriptomic level, analysis was also conducted on the metabolomic level. Non-targeted metabolomic analysis was performed on the original strain ORI and the evolved strain ALEF-5 % to explore differences in their metabolic synthesis using LCMS-QTOF. A total of 15 metabolites with significant differences in production levels were identified through the METLIN database. Five metabolites were overexpressed and 10 metabolites were less-expressed (Fig. 5). In the overexpressed products, L-aspartate is a crucial substance for growth and defense [54]. Hypoxanthine prevents oxidative damage by inhibiting the activation of nucleoside diphosphate (ADP-ribose) polymerase [55]; O-acetyl-L-homoserine is a precursor to the biosynthesis of L-methionine and S-

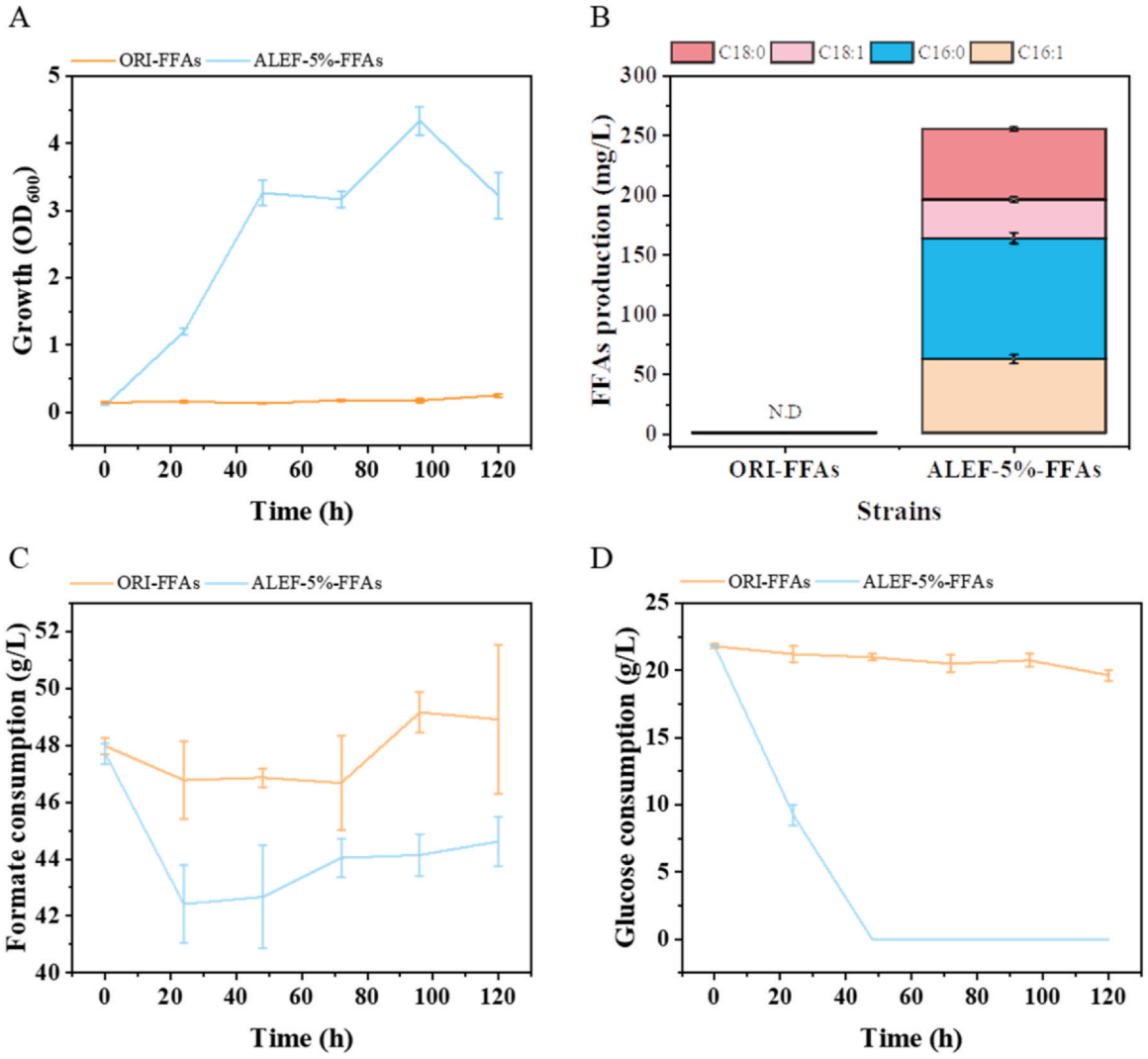

Fig. 6. The evolved strain ALEF-5 %-FFAs exhibited resecured cell growth and FFAs production via glucose under high formate concentration. (A) Growth; (B) FFAs production; (C) Formate consumption and (D) Glucose consumption. The strains were cultivated in MM medium containing 20 g/L glucose and 50 g/L formate for 120 h at 200 rpm, 30 °C. All data represent the mean ± s.d. of at least triplicate experiments.

adenosyl-L-methionine, contributing to the formaldehyde detoxification [56]; 3-dehydroshikimate is an effective antioxidant [57]; N-acetyl-L-glutamate serves as a substrate for de novo arginine synthesis, which plays a crucial role in preserving the integrity of cell walls and plasma membranes [58,59]. These overexpressed metabolites were generally associated with the antioxidant activity, suggesting that the strain achieved high tolerance to formate by regulating metabolites productions. In the less-expressed metabolites, we observed a decrease in the synthesis of most amino acids, which is consistent with the conclusions obtained from transcriptome data (Fig. S4).

3.4. Free fatty acids production via glucose under high formate concentration

In order to verify the applicability of the evolutionary strain ALEF-5 %, production of FFAs in ALEF-5 %-FFAs was validated under the condition of MM medium containing 20 g/L glucose and 50 g/L formate, with an initial OD_{600} of 0.1. We also deleted *FAA1*, *FAA4* and *POX1* genes in the ORI strain, generating strain ORI-FFAs, as the control strain for fermentation validation.

The control strain- ORI-FFAs could not grow nor produce FFAs under the fermentation conditions, while the strains ALEF-5 %-FFAs was able to consume glucose, grow to final OD_{600} of around 4 (Fig. 6A), and produce FFAs (Fig. 6B). This was not as good as the growth of the ALEF-5 % strain (OD_{600} reached around 7 at 72 h, as shown in Fig. 2A), which may be due to the knockout of the fatty acid degradation pathway affecting the supply of cell membrane components to the strain. In addition, it was observed that formate was slightly consumed by the ALEF-5 %-FFAs strain within 20 h (approximately 4 g/L, as shown in Fig. 6C). Interestingly, there was a slight increase in formate concentration in the following time period, which could be due to cellular regulation of osmotic pressure, leading to the extracellular release of a portion of the internally produced formate. Compared to the ALEF-5 %-FFAs strain, the ORI-FFAs strain exhibited negligible glucose consumption, which was consistent with the observed growth results (Fig. 6D).

4. Conclusions

This study focused on enhancing the tolerance of *S. cerevisiae* to high formate concentrations (50 g/L) using the ALE technique. Genomic, transcriptome, and metabolomics approaches were employed to investigate the mechanisms underlying this improved tolerance. Additionally, through metabolic engineering, we successfully enabled the production of free fatty acids (FFAs) in the presence of high formate concentrations. These findings not only contribute to our understanding of the molecular mechanisms involved in formate tolerance but also

have implications for the future development of formate-based bioprocesses.

Moving forward, there are several avenues for future research in this field. Firstly, further optimization of the engineered *S. cerevisiae* strains could be pursued to enhance formate conversion efficiency and increase the yield of target products such as FFAs, farnesene, and olefins. Additionally, exploring the potential synergistic effects of combining microbial one-carbon fixation with other bioprocesses, such as cellulose treatment with formic acid, holds promise for more sustainable and efficient biotechnological applications.

Authors contributions

Kai Wang: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Yining Liu, Zhuoheng Wu, Yilu Wu, Haoran Bi:** Formal analysis, Investigation. **Biqiang Chen, Meng Wang and Yanhui Liu:** Methodology, Funding acquisition. **Jens Nielsen:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision. **Zihe Liu:** Conceptualization, Methodology, Project administration, Supervision, Funding acquisition. **Tianwei Tan:** Project administration, Supervision, Funding acquisition.

Declaration of Competing Interest

Tianwei Tan is honorary editor-in-chief for Green Carbon and was not involved in the editorial review or the decision to publish this article. Jens Nielsen is an advisory board member for Green Carbon and was not involved in the editorial review or the decision to publish this article. 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.greenca.2023.08.003.

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