Microbial Cell Factories

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Boosting succinic acid production of *Yarrowia lipolytica* at low pH through enhancing product tolerance and glucose metabolism

Yutao Zhong[1], Changyu Shang[1], Huilin Tao[1], Jin Hou[1], Zhiyong Cui[1*] and Qingsheng Qi[1*]

Abstract

Background Succinic acid (SA) is an important bio-based C4 platform chemical with versatile applications, including the production of 1,4-butanediol, tetrahydrofuran, and γ-butyrolactone. The non-conventional yeast *Yarrowia lipolytica* has garnered substantial interest as a robust cell factory for SA production at low pH. However, the high concentrations of SA, especially under acidic conditions, can impose significant stress on microbial cells, leading to reduced glucose metabolism viability and compromised production performance. Therefore, it is important to develop *Y. lipolytica* strains with enhanced SA tolerance for industrial-scale SA production.

Results An SA-tolerant *Y. lipolytica* strain E501 with improved SA production was obtained through adaptive laboratory evolution (ALE). In a 5-L bioreactor, the evolved strain E501 produced 89.62 g/L SA, representing a 7.2% increase over the starting strain Hi-SA2. Genome resequencing and transcriptome analysis identified a mutation in the 26S proteasome regulatory subunit Rpn1, as well as genes involved in transmembrane transport, which may be associated with enhanced SA tolerance. By further fine-tuning the glycolytic pathway flux, the highest SA titer of 112.54 g/L to date at low pH was achieved, with a yield of 0.67 g/g glucose and a productivity of 2.08 g/L/h.

Conclusion This study provided a robust engineered *Y. lipolytica* strain capable of efficiently producing SA at low pH, thereby reducing the cost of industrial SA fermentation.

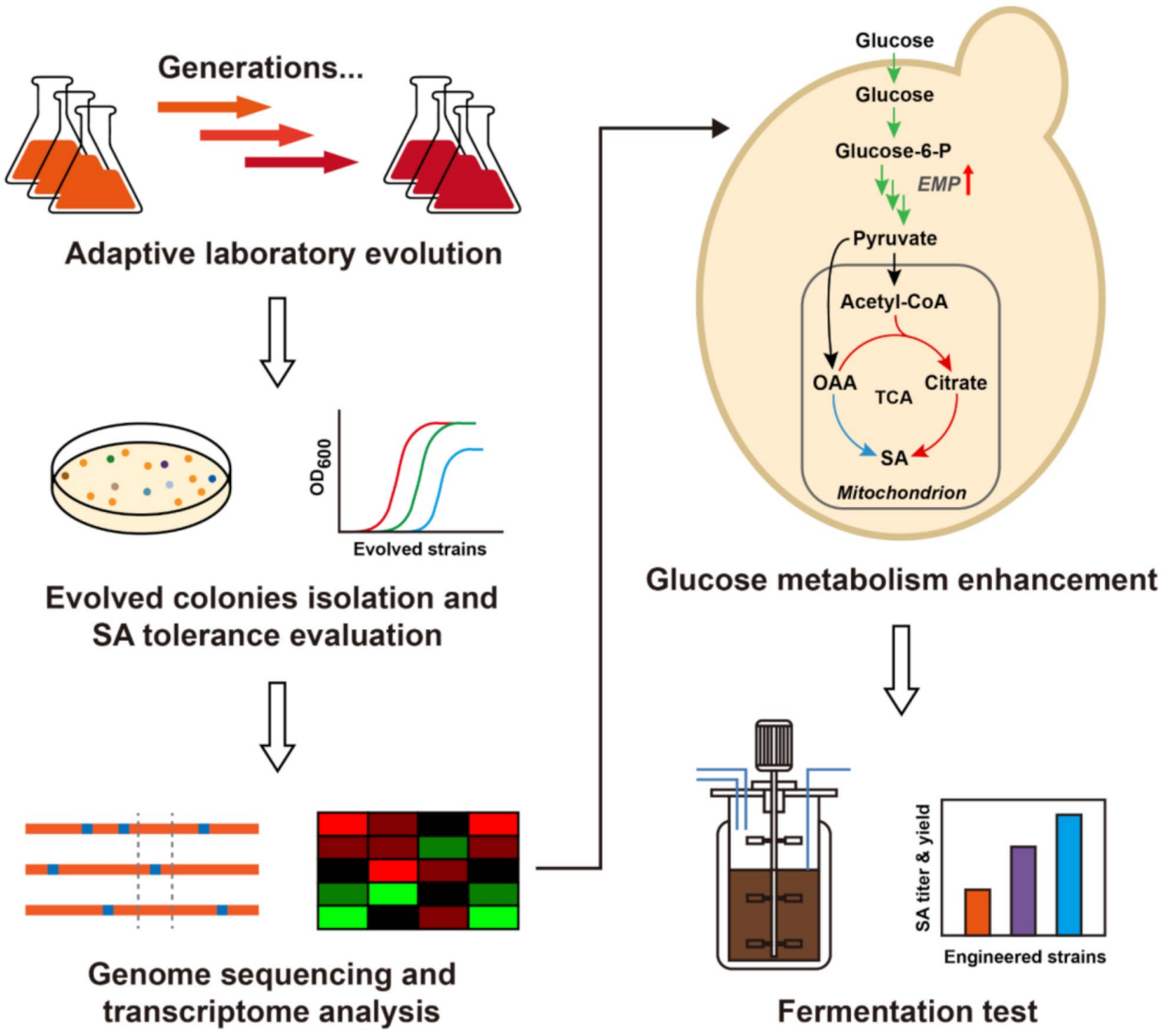
Background

Succinic acid (SA), a four-carbon dicarboxylic acid, has emerged as a versatile platform chemical with a wide range of applications in industries such as pharmaceuticals, food additives, biodegradable plastics, and green solvents [1–3]. Due to its significant potential as a value-added compound that derived from biomass, SA has been identified by the US Department of Energy as one of the top twelve building block chemicals [4–6]. According to Grand View Research, the global SA market size was valued at USD 222.9 million in 2021 and is expected to expand at a compound annual growth rate (CAGR) of 9.7% from 2022 to 2030 (https://www.grandviewresearch.com/industry-analysis/succinic-acid-market). Traditional chemical synthesis routes for SA heavily rely on nonrenewable resources and often require reactions under high temperature and pressure, leading to environmental challenges and increased production costs [7]. In contrast, microbial fermentation offers a promising alternative for sustainable SA production from renewable feedstocks,

*Correspondence:
Zhiyong Cui
cuizhiyong@sdu.edu.cn
Qingsheng Qi
qiqingsheng@sdu.edu.cn

Full list of author information is available at the end of the article

Graphical abstract



Keywords Succinic acid, *Yarrowia lipolytica*, Adaptive laboratory evolution, Multi-omics analysis, Glycolysis

aligning with the principles of a circular bioeconomy [8–10].

Fermentative production of SA under acidic conditions offers distinct advantages, particularly by reducing the need for large quantities of neutralizing agents, thereby minimizing the generation of waste salts [11–13]. Furthermore, maintaining a lower pH with increased acidity acts as a protective barrier against the proliferation of harmful bacterial species, thereby ensuring the safety of the fermentation process and the product quality [14]. The non-conventional yeast *Yarrowia lipolytica* has garnered substantial interest as a robust cell factory for producing various organic acids due to its unique metabolic capabilities, strong tolerance to low pH environments, and Generally Recognized as Safe (GRAS) status [15–17]. Our previous metabolic engineering efforts have been successfully equipped *Y. lipolytica* strains with the ability to produce SA via the reductive branch of tricarboxylic acid (TCA) cycle [18]. Unlike bacterial hosts that typically conduct fermentations at neutral pH, the engineered *Y. lipolytica* strains possess the distinctive capability to produce SA without pH adjustment, with the final pH of the fermentation broth being below 3.0 [19–22]. This low pH SA fermentation process is anticipated to reduce downstream extraction costs. However, the continuous accumulation of SA under acidic conditions can impose

significant stress on microbial cells, leading to reduced cell viability, impaired metabolic activity, and ultimately compromised production performance [23]. One of the major challenges in microbial SA production is the inhibitory effect of accumulated product and low pH conditions on cell growth and glucose metabolism. Therefore, developing effective strategies to enhance the robustness and productivity of engineered strains is essential for the sustainable and cost-effective production of SA.

Adaptive laboratory evolution (ALE) is a powerful approach for enhancing microbial robustness. It involves exposing microbial populations to selective pressures across successive generations, ultimately leading to the enrichment of evolved strains with desirable tolerance phenotypes [24–26]. This strategy has been successfully employed to improve the tolerance of microbial cell factories to organic acids, including SA [27, 28]. To improve acid tolerance and increase SA production, Zhang et al. employed adaptive evolution to develop an SA-tolerant mutant strain BC-4 of *Actinobacillus succinogenes*, which offers potential industrial advantages in cost efficiency and contamination resistance [29]. In *Y. lipolytica*, a similar strategy of metabolic evolution was implemented using an in situ fibrous bed bioreactor (*is*FBB) to obtain an SA-tolerant strain PSA3.0 [20]. This evolved strain exhibited significant advancements in SA production at low pH, achieving a high SA titer of 78.6 g/L in fed-batch fermentation. However, its productivity and yield remained insufficient for industrial-scale application.

While adaptive evolution can yield strains with enhanced SA tolerance, further rational engineering is still necessary to fine-tune metabolic pathways and boost SA biosynthesis. Given the pivotal role of glycolysis in regulating central carbon flux, engineering glucose transport and metabolism can improve the availability of precursors and energy for the biosynthesis of desired products in various microbial hosts [30–33]. By modulating glycolytic flux, Lim et al. successfully maximized the product yield and productivity while minimizing by-product formation in engineered *Escherichia coli* strains [34]. By adjusting the untranslated region (UTR) of the glucose transporter encoding gene *ptsG*, they fine-tuned glucose uptake rates and overall glycolytic activity, leading to improved cellular performance in terms of yield and productivity for the synthesis of n-butanol, butyrate, and 2,3-butanediol. Furthermore, genes related to glucose transport and key enzymes in the glycolytic pathway were overexpressed in engineered *Aspergillus niger* to increase malic acid production. The resulting strain S1149 achieved a high malic acid titer of 201.13 g/L with an increased yield of 1.64 mol/mol glucose in fed-batch fermentation [35]. These results indicate that the rational regulation of glucose metabolism is beneficial for the production of chemicals by microbial cell factories.

In this work, an ALE strategy was performed to obtain evolved strains of *Y. lipolytica* exhibiting enhanced SA tolerance. Genome resequencing and transcriptome profiling were then employed to reveal the candidate genes responsible for SA stress adaptation. Subsequently, the rate-limiting enzymes of glycolytic pathway were co-overexpressed in the SA-tolerant strain, resulting in the engineered strain E501XF. In fed-batch fermentation, this strain produced 112.54 g/L SA, demonstrating a yield of 0.67 g/g and a productivity of 2.08 g/L/h at pH 3.5. To the best of our knowledge, this represents the highest reported SA production for low pH fermentation. Our study provides a promising industrial *Y. lipolytica* strains with superior SA production performance at low pH.

Methods

Strains, reagents and medium

The *Y. lipolytica* strain Hi-SA2, constructed in our previous study, served as the background strain for all genetic manipulations and strain construction [18]. Table 1 lists the yeast strains used in this study. All restriction enzymes were supplied by Thermo Fisher Scientific (Shanghai, China). Phanta® Max Super-Fidelity DNA Polymerase (Vazyme, Nanjing, China) or 2×Taq Plus Master Mix II (Dye Plus) (Vazyme, Nanjing, China) were used for PCR amplifications. DNA gel purification and plasmid extraction kits were obtained from Omega Biotek. Chemical standards were purchased from Sigma-Aldrich unless otherwise specified.

Routine cultivation of *E. coli* was performed in Luria-Bertani (LB) medium, which contained 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. *Y. lipolytica* was cultivated in YPDG medium, consisting of 10 g/L yeast extract, 20 g/L tryptone, 20 g/L glycerol, and 20 g/L glucose. In certain cases, ampicillin (100 μg/mL) and nourseothricin (350 μg/mL) were added as required. Modified CM1 medium (0.8 g/L $Na_2HPO_4{\cdot}12H_2O$, 3.6 g/L KH_2PO_4, 1.2 g/L $MgSO_4.7H_2O$, 2.8 g/L $(NH_4)_2SO_4$, 6.0 g/L corn steep powder, 80 g/L glucose) was used for fermentation in this study. An appropriate amount of SA was added to the culture medium to evaluate SA tolerance.

Plasmid construction and genetic engineering

Plasmid construction was performed using *E. coli* DH5α. Several native genes were cloned from the genomic DNA of *Y. lipolytica* Po1f. The plasmids used in this study are listed in Table S1, and the primer sequences are provided in Table S2.

To obtain integration fragments containing the expression cassette and selection marker, the target genes were amplified from the corresponding plasmids or genomic DNA. These target genes were then assembled with expression vectors that had been digested with

Table 1 Yeast strains used in this study

Strains	Descriptions	Sources
Hi-SA2	*MatA*, *xpr2-322*, *axp-2*, *leu2-270*, *ura3-302*, *ΔSdh5::loxP*, *ΔAch1::loxP*, *YlPyc*, *TbFrd*, *EcFum*, *YlMdh1*, *Pgl1*^{G75S}, *mTbFrd*, *YlMdh2*, *SpMae1*, *mYlPyc*, *mYlFum*	[18]
E301	Evolved strain derived from Hi-SA2 strain at an SA concentration of 30 g/L	This study
E302	Evolved strain derived from Hi-SA2 strain at an SA concentration of 30 g/L	This study
E303	Evolved strain derived from Hi-SA2 strain at an SA concentration of 30 g/L	This study
E501	Evolved strain derived from Hi-SA2 strain at an SA concentration of 50 g/L	This study
E502	Evolved strain derived from Hi-SA2 strain at an SA concentration of 50 g/L	This study
E501H1	E501::*YlYht1* (YALI0C06424g)	This study
E501H3	E501::*YlYht3* (YALI0F19184g)	This study
E501H4	E501::*YlYht4* (YALI0E23287g)	This study
E501X	E501::*YlHxk1* (YALI0B22308g)	This study
E501F	E501::*YlPfk1* (YALI0D16357g)	This study
E501Y	E501::*YlPyk1* (YALI0F09185g)	This study
E501XF	E501::*YlHxk1* and *YlPfk1*	This study
E501XY	E501::*YlHxk1* and *YlPyk1*	This study
E501FY	E501::*YlPfk1* and *YlPyk1*	This study

restriction enzymes. The expression vectors used in this process included JMP-nat-GPD-TEF [18].

For gene overexpression in *Y. lipolytica*, a homology-independent genome integration approach was employed [36]. The linearized DNA fragments or plasmids were transformed to *Y. lipolytica* using the Frozen-EZ yeast transformation II kit (Zymo Research, catalog number: T2001). After transformation, positive transformants were identified by screening them on suitable solid plates and confirming their presence through colony PCR. From the pool of transformants, ten strains were selected for further evaluation based on their potential phenotypes.

Adaptive laboratory evolution

To initiate the experiment, a single colony of the Hi-SA2 strain was activated overnight and then inoculated into CM1 medium with six replicates. The CM1 medium contained SA concentrations ranging from 20 g/L to 50 g/L. The evolution process took place in shaking flasks at 30℃ and 220 rpm. The initial SA concentration was gradually increased in steps, starting from 20 g/L and progressing to 35 g/L and then 50 g/L. Once the cells reached the logarithmic growth phase, indicated by an OD_{600} of approximately 8.0, they were transferred to fresh CM1 medium with an initial OD_{600} of 0.5.

Each round of cultivation lasted approximately 48–96 h. During the evolution process, samples of the culture were collected every 24 h to measure OD_{600} and SA production. Once cell growth and SA accumulation reached a stable state, individual clones were isolated and their SA tolerance and production performance were evaluated.

Genome resequencing analysis

The control strain Hi-SA2 and the evolved strain E501 were selected for genome resequencing. Genomic DNA was extracted from the strains and fragmented into 200–300 base pair fragments using a Biorupter ultrasonic fragmentation machine. After end-repair, an "A" base was added to the 3' ends of the DNA fragments. DNA linkers containing an Index sequence were then added to both ends of the DNA fragments using TA ligation. The resulting libraries were quantified using a Qubit 2.0 Fluorometer from Thermo Scientific. Whole genome resequencing was performed using the Illumina HiSeq/Nova platform with a read length of 2×150 bp at Azenta Life Sciences (Suzhou, China). The sequencing data underwent several processing steps, including obtaining the raw data, filtering to remove connectors, decontamination, and alignment with the reference genome. The results were further analyzed to identify and remove repetitive sequences resulting from PCR amplification in each library. Single nucleotide variants (SNVs) and insertions/deletions (InDels) relative to the reference genome were then calculated. By comparing the evolved strain with the control strain, missense mutations in the coding regions were identified.

Transcriptome analysis

Total RNA was extracted from the yeast samples using TRIzol reagent (Invitrogen, CA, USA) following the manufacturer's protocol. The purity and concentration of the extracted RNA were assessed using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), which measures absorbance at different wavelengths to determine the RNA integrity and quantify RNA concentration. The quality and integrity of the RNA samples were further evaluated using an Agilent 2100 Bioanalyzer (Agilent

Technologies, Santa Clara, CA, USA), which provides electrophoretic separation and analysis of RNA fragments. To construct the RNA-seq libraries, the VAHTS Universal V6 RNA-seq Library Prep Kit was employed, following the manufacturer's instructions. This kit utilizes a streamlined and efficient protocol for library preparation, including steps such as RNA fragmentation, cDNA synthesis, adapter ligation, and library amplification. The resulting libraries were then subjected to transcriptome sequencing. The transcriptome sequencing and subsequent data analysis were performed by OE Biotech Co., Ltd. (Shanghai, China).

The libraries were sequenced on an Illumina NovaSeq 6000 platform, generating 150 bp paired-end reads. Approximately 49 M raw reads were generated for each sample. To ensure the reliability and accuracy of subsequent analyses, the raw reads in fastq format were initially processed using fastp, a software tool that performs quality control and removes low-quality reads. This process resulted in approximately 47 million clean reads per sample, ensuring high-quality data for further analysis. The HISAT2 alignment software was employed to map the clean reads to the reference genome [37]. Gene expression levels were quantified using the Fragments Per Kilobase of transcript per Million fragments mapped (FPKM) method, which accounts for transcript length and the total number of mapped reads. Read counts for each gene were obtained using the HTSeq-count tool [38]. Principal Component Analysis (PCA) was performed using R (version 3.2.0) to evaluate the biological duplication of the samples.

To identify significantly differentially expressed genes (DEGs), the DESeq2 software was employed [39]. A q-value threshold of less than 0.05 and a fold change threshold of greater than 2 or less than 0.5 were set to define the DEGs. Hierarchical cluster analysis was performed using R (version 3.2.0) to visualize the expression patterns of the DEGs in different groups and samples. Additionally, a radar map of the top 30 up-regulated or down-regulated DEGs was created using the R package ggradar.

To gain insights into the functional implications of the DEGs, enrichment analysis was performed using the hypergeometric distribution. Gene Ontology (GO) and KEGG pathway enrichment analyses were conducted to identify significantly enriched terms associated with the DEGs. Various types of diagrams were generated using R (version 3.2.0) to visualize the results of the enrichment analysis.

Batch fermentation in shaking flasks

The SA-producing strains were first pre-cultured in YPDG medium for 18–24 h. Subsequently, they were transferred into 300 mL shaking flasks containing 50 mL of CM1 medium. The fermentation process for SA was conducted at 30°C and 220 rpm for 72–120 h. The initial glucose concentration in the medium was 80 g/L, and 1–2 mL of a glucose stock solution (500 g/L) was periodically added as needed during the fermentation process. To monitor the progress of the fermentation, samples were collected at regular intervals of 24 h. These samples were then analyzed to measure biomass, residual glucose levels, and organic acid concentrations.

Fed-batch fermentation in 5-L bioreactors

The *Y. lipolytica* strains, stored at -80℃, were streaked and cultivated on YPDG solid plates for 48 h. Single colonies were then selected and inoculated into 50 mL shaking flasks containing 10 mL of YPDG medium. The flasks were incubated at 30℃ and 220 rpm for 24 h. Following the initial cultivation, a 2% culture from this step was transferred into 400 mL of YPD medium in 2 L shaking flasks to serve as the seed culture. This seed culture was further incubated at 30°C and 220 rpm. Subsequently, 10% of the second seed culture was inoculated into a 5-L bioreactor (BXBIO, Shanghai, China) with a working volume of 4 L. The conditions for the fed-batch fermentation were maintained at 30℃, with a stirring speed of 400 rpm, and a gas flow rate of 1 vvm. When necessary, the pH of the fermentation broth was maintained at 3.0 or 3.5 by adding NH_4OH. The initial medium for fed-batch fermentation consisted of modified CM1 medium with a glucose concentration of 80 g/L. During the fermentation process, the glucose concentration was measured every 4–6 h. When the residual glucose concentration fell below 20 g/L, glucose stock (500 g/L) was supplemented to maintain the desired glucose level. Concurrently, the SA titer and biomass were regularly monitored to assess the progress of the fermentation.

Metabolite extraction and quantification

To analyze the metabolites in the fermentation broth, including glucose, glycerol, and organic acids, a Shimadzu HPLC system (Shimadzu Co., Kyoto, Japan) equipped with an Aminex HPX-87 H column (Bio-Rad, Inc., Hercules, CA) and a Shimadzu refractive index detector (Shimadzu Co., Kyoto, Japan) was employed. Prior to analysis, the extract solution was filtered through a 0.22 μm filter to remove any particulate matter. The HPLC analysis was conducted using a mobile phase consisting of 5 mM H_2SO_4 at a flow rate of 0.6 mL/min, with the column temperature maintained at 65℃. For the measurement of cell growth, optical density measurements were taken at a wavelength of 600 nm (OD_{600}) using a Shimadzu UV-1800 spectrometer (Shimadzu Co., Kyoto, Japan).

Results

Improving SA tolerance of *Y. lipolytica* engineered strain Hi-SA2 through adaptive laboratory evolution

The initial SA-producing strain Hi-SA2 was developed by enhancing the oxidative TCA cycle and localizing the reductive TCA cycle to the mitochondrial matrix of succinate dehydrogenase-deficient *Y. lipolytica*. While the Hi-SA2 strain produced high levels of SA at low pH, it was noted that excessive accumulation of SA could negatively impact the cell growth and metabolic processes [18]. To evaluate cell metabolism and growth performance under high SA concentrations, the resistance of *Y. lipolytica* Hi-SA2 to different SA concentrations ranging from 0 to 100 g/L was investigated through shake flask cultivations. As shown in Fig. 1a, the SA titer and glucose consumption rate of the Hi-SA2 strain gradually declined with increasing extracellular SA supplementation. In the absence of added SA, the Hi-SA2 strain achieved an SA titer of 41.8 g/L in 48 h, with a glucose consumption rate of 1.0 g/L/h. However, with an initial addition of 20 g/L SA to the medium, the SA titer and glucose consumption rate significantly decreased to 25.5 g/L and 0.6 g/L/h, respectively. Further increasing the initial SA concentration to 40 g/L in the medium caused severe inhibition of cell growth and glucose consumption (Fig. 1b and Fig. S1). The trajectory of SA production closely paralleled that of cell growth, with an observable decrease in SA titer once the extracellular SA exceeded 40 g/L (Fig. S1). These results indicate that high extracellular SA levels can induce cytotoxicity, representing a significant challenge for achieving efficient SA production at low pH.

To enhance the SA tolerance of the Hi-SA2 strain, an ALE cultivation was conducted by progressively increasing the initial SA addition from 20 to 50 g/L. Following

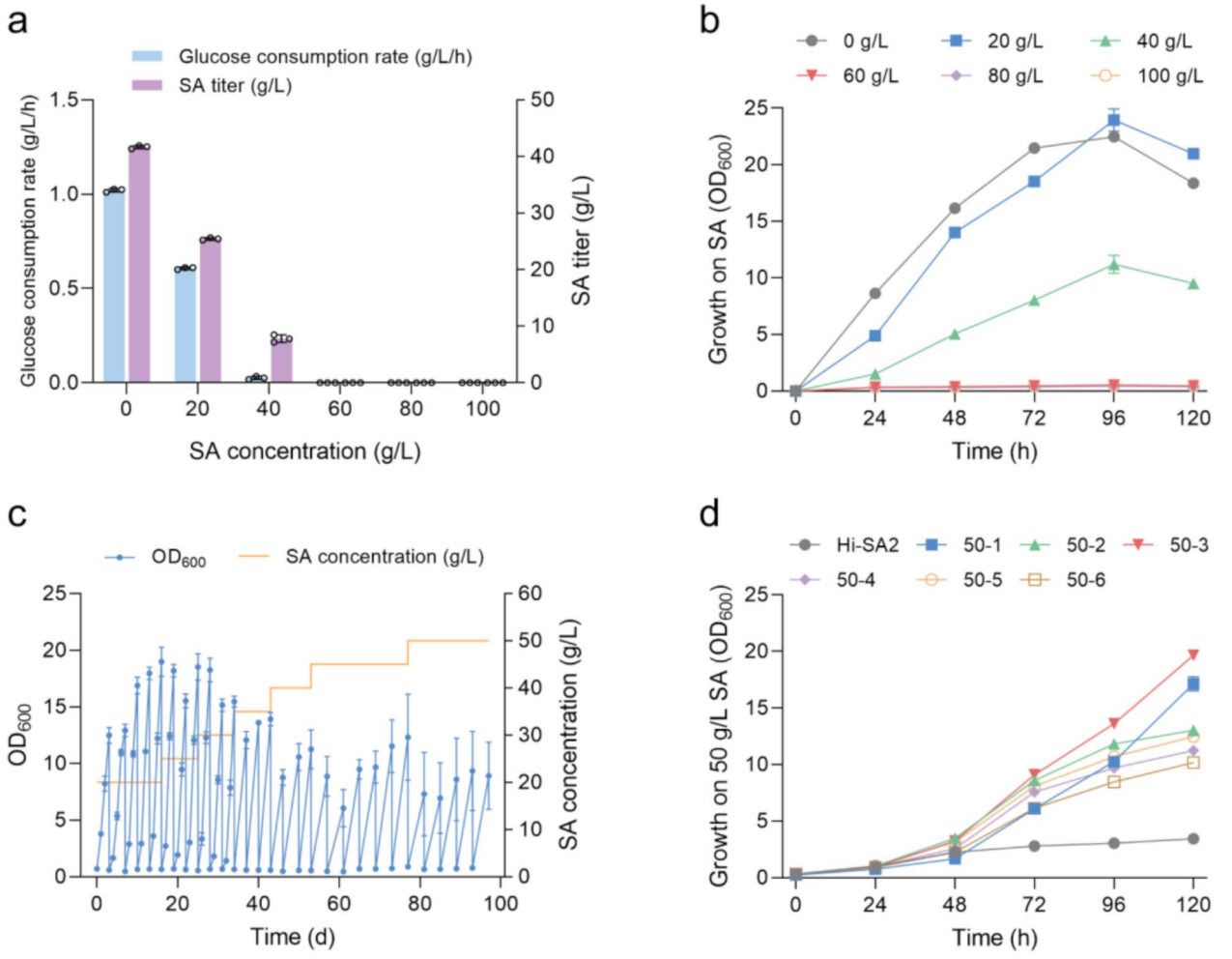


Fig. 1 Enhancing SA tolerance of the engineered *Y. lipolytica* Hi-SA2 by adaptive evolution. (**a**) SA production and glucose consumption rate of the Hi-SA2 strain with different initial SA concentrations. (**b**) Growth curves (OD_{600}) of the Hi-SA2 strain with different initial SA concentrations. (**c**) Time-course of growth (OD_{600}) and SA concentrations during the ALE process. (**d**) Growth curves (OD_{600}) of the evolved endpoint populations under an initial SA concentration of 50 g/L. Error bars indicate standard deviations of three biological replicates

approximately 27 serial subcultures, equivalent to around 80 generations, all six groups gradually acclimated to the presence of SA and restored cell growth. Noticeable improvements in cell growth on day 25 and day 77 indicated the potential for obtaining mutants with enhanced SA tolerance (Fig. 1c). Subsequent evaluation of the evolved endpoint populations in CM1 medium supplemented with 50 g/L SA demonstrated significant growth recovery across all groups. Group 50-3 exhibited superior growth, reaching a final OD_{600} of 19.7, followed closely by group 50-1 with a final OD_{600} of 17.1 (Fig. 1d). These results indicate that the ALE strategy effectively enhanced SA tolerance in the engineered strains.

Genome resequencing and transcriptome profiling revealed the potential genes associated with SA tolerance in the evolved strains

Subsequently, individual colonies were isolated from different stages of the evolved populations and cultivated in CM1 medium supplemented with 50 g/L SA. From 240 SA-tolerant candidate strains, five strains, namely E301, E302, E303, E501, and E502 were selected for their superior SA tolerance compared to the engineered strain Hi-SA2 (Fig. 2a). Among them, the evolved strain E501 exhibited the highest SA production, generating 36.5 g/L within 96 h, and demonstrated continuous growth in SA-containing medium (Fig. 2b). Furthermore, in the absence of external SA supplementation, the E501 strain produced 102.1 g/L SA, attaining a maximum OD_{600} of 27.5 within 120 h (Fig. S2). Although the SA titer did not show a significant improvement compared to Hi-SA2, the SA productivity increased by 3.5%. These results suggest that the evolved strain E501 acquired not only enhanced SA production performance but also improved overall cellular fitness in response to high SA concentrations.

To identify candidate genes associated with enhanced SA tolerance, genome resequencing was conducted on the parent strain Hi-SA2 and the evolved strain E501. Among the 9 SNVs and InDels in gene coding regions, a nonsynonymous mutation in the gene *Rpn1* (YALI0B02860g), encoding the 26S proteasome regulatory subunit, was exclusively identified in strain E501. PCR amplification and Sanger sequencing further confirmed the presence of the mutation in Rpn1 (Fig. S3). Rpn1 is involved in proteasome assembly and protein degradation, containing potential polyUb/UBL recognition motifs known as PC repeats, responsible for proteasomal signal interactions [40]. In *Saccharomyces cerevisiae*, these PC repeats are located in two regions of Rpn1: residues 366 to 625 (PC repeat I) and residues 736 to 909 (PC repeat II) [41]. In the evolved strain E501, a mutation at amino acid position 740 of Rpn1 altered leucine to phenylalanine, a conserved residue within the PC repeat II region across different yeast species (Fig. S3). Furthermore, protein structural analysis using I-TASSER revealed that the mutation site (labeled in blue) was located at a recognition site for polyUb and UBL signals (Fig. 3a). This mutation may enhance the degradation of SA stress-induced misfolded proteins via the ubiquitin-mediated proteasome pathway [42–44].

The transcriptome analysis was then conducted under SA supplementation to elucidate DEGs ($q<0.05$ and $|\log2$ fold change$|>1$) between the evolved strain E501 and the unevolved strain Hi-SA2. A total of 363 candidate genes were identified, comprising 75 upregulated genes and 288 downregulated genes (Fig. S3, Table S3). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways

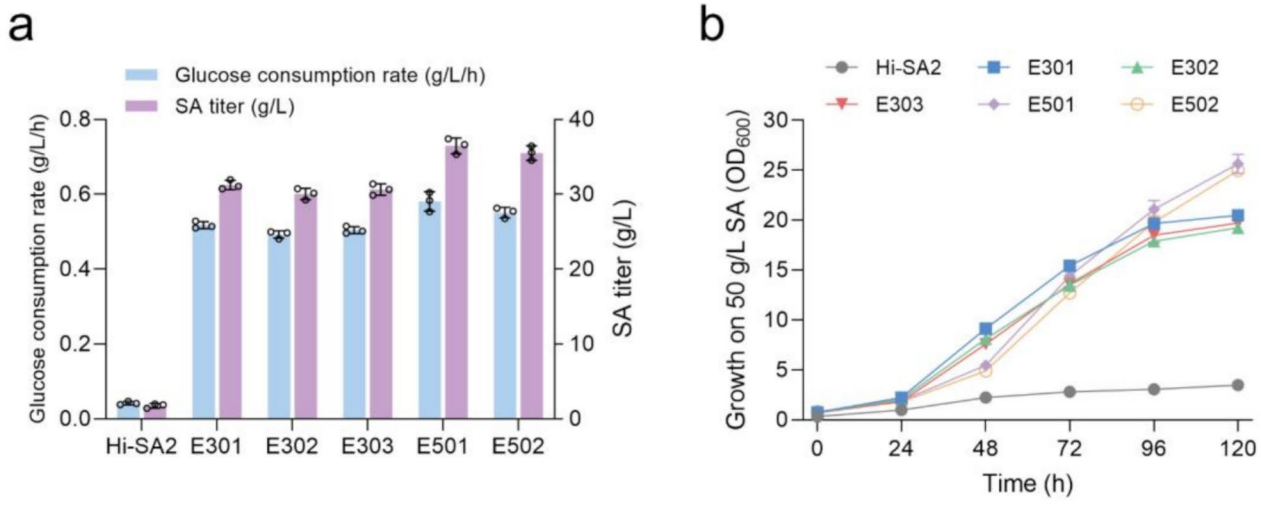


Fig. 2 Metabolic characterization of evolved strains with increased SA tolerance. (**a**) SA production and glucose consumption rate of the original and evolved strains under an initial SA concentration of 50 g/L. (**b**) Growth curves (OD_{600}) of the original and evolved strains under an initial SA concentration of 50 g/L. Error bars indicate standard deviations of three biological replicates

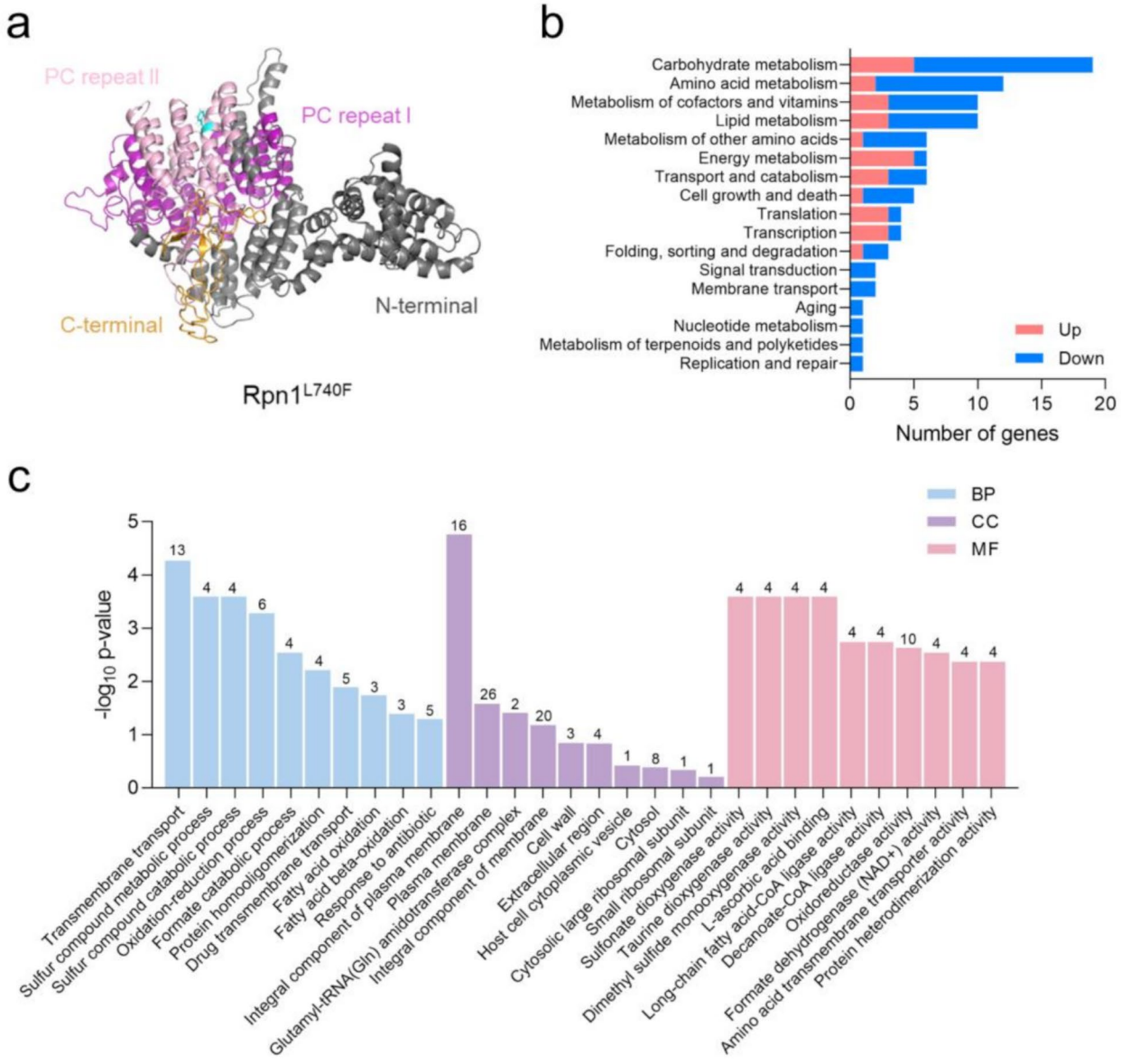


Fig. 3 Genome resequencing and transcriptome analysis of evolved strains with increased SA tolerance. (**a**) Protein structural analysis of the Rpn1^{L740F} mutant, with the amino acid residue at position 740 highlighted in light blue. (**b**) KEGG pathway enrichment analysis of DEGs. (**c**) GO enrichment analysis of DEGs

contained 93 genes associated with specific biological processes, including 30 upregulated and 63 downregulated genes (Table S3). Notably, transcriptional changes of genes were primarily concentrated in carbohydrate metabolism, energy metabolism, lipid metabolism, and the metabolism of cofactors and vitamins (Fig. 3b). The most significant number of DEGs were associated with glycolysis and gluconeogenesis, potentially facilitating the utilization of glucose for SA biosynthesis in the evolved strain E501. Furthermore, Gene Ontology (GO) annotation and classification analysis were performed to elucidate the potential functions of the DEGs under SA stress. As shown in Fig. 3c, the DEGs were categorized into three main groups based on their functions: biological processes (BP), cellular components (CC), and molecular functions (MF). Terms such as transmembrane transport, plasma membrane, and ATP binding were prominently represented in each of the three GO categories. Among these GO categories, a substantial number of DEGs were associated with transport, including the downregulation of the SA import protein Jen1 (YALI0C15488g), potentially reducing the influx of extracellular SA into the cell and mitigating acid stress-induced cell damage [45, 46]. Moreover, other DEGs related to transport could also serve as potential carboxylic acid transporters. We speculate that the enhanced SA tolerance and improved SA

production performance of the evolved strains stem from variations in the expression levels of genes implicated in transport and cell metabolism [47].

Overexpressing enzymes involved in glucose metabolism enhanced central carbon flux for efficient SA production

Several studies have reported improvements in glucose uptake in yeast strains through rational metabolic modification. For instance, a global metabolic engineering strategy was implemented by integrating multiple copies of 13 glycolysis-related genes in *S. cerevisiae*. The resulting strain YPH499/dPdA3-34 exhibited a 1.3-fold increase in glucose consumption rate compared to the control [31]. Real-time PCR analysis showed that the transcription levels of key glycolytic genes, such as *Hxk2*, *Pfk1*, *Pfk2*, and *Pyk2*, were upregulated in the engineered strain. Another study demonstrated that overexpression of different hexose transporters (HXTs) with varying glucose affinities could boost glucose uptake and ethanol production in *S. cerevisiae*, with the high-affinity HXT7 transporter being most effective [30]. To further optimize glucose metabolism, endogenous hexose transporters (YlYht1, YlYht3, and YlYht4) and key enzymes from glycolytic pathway (YlHxk1, YlPfk1, and YlPyk1) were overexpressed in the evolved strain E501 (Fig. 4a). Although individual overexpression of these enzymes did not significantly improve cell growth and SA production, the combinatorial overexpression of YlHxk1 and YlPfk1 resulted in an SA titer of 77.99 g/L, representing a 5% increase compared to the initial strain E501 (Fig. 4b). Furthermore, the engineered strain co-overexpressing YlHxk1 and YlPyk1 also demonstrated favorable performance, producing 76.57 g/L SA with a yield of 0.91 g/g glucose. These results indicate that enhancing the glycolytic pathway can improve glucose utilization and promote metabolic flux into SA biosynthesis.

Fed-batch fermentations of SA-producing strains

After obtaining both the evolved strain E501 and the engineered strain E501XF with improved SA productivity, fed-batch fermentations were conducted in 5-L bioreactors using CM1 medium without pH adjustment. As shown in Fig. 5, the pH of the Hi-SA2, E501, and E501XF strains were dropped to 2.5–3.0 at the end of fermentation. The original strain Hi-SA2 exhibited limited capacity for SA synthesis at low pH, producing only 83.59 g/L of SA after 48 h of fermentation, with a yield of 0.65 g/g glucose and a productivity of 1.74 g/L/h (Fig. 5a). In

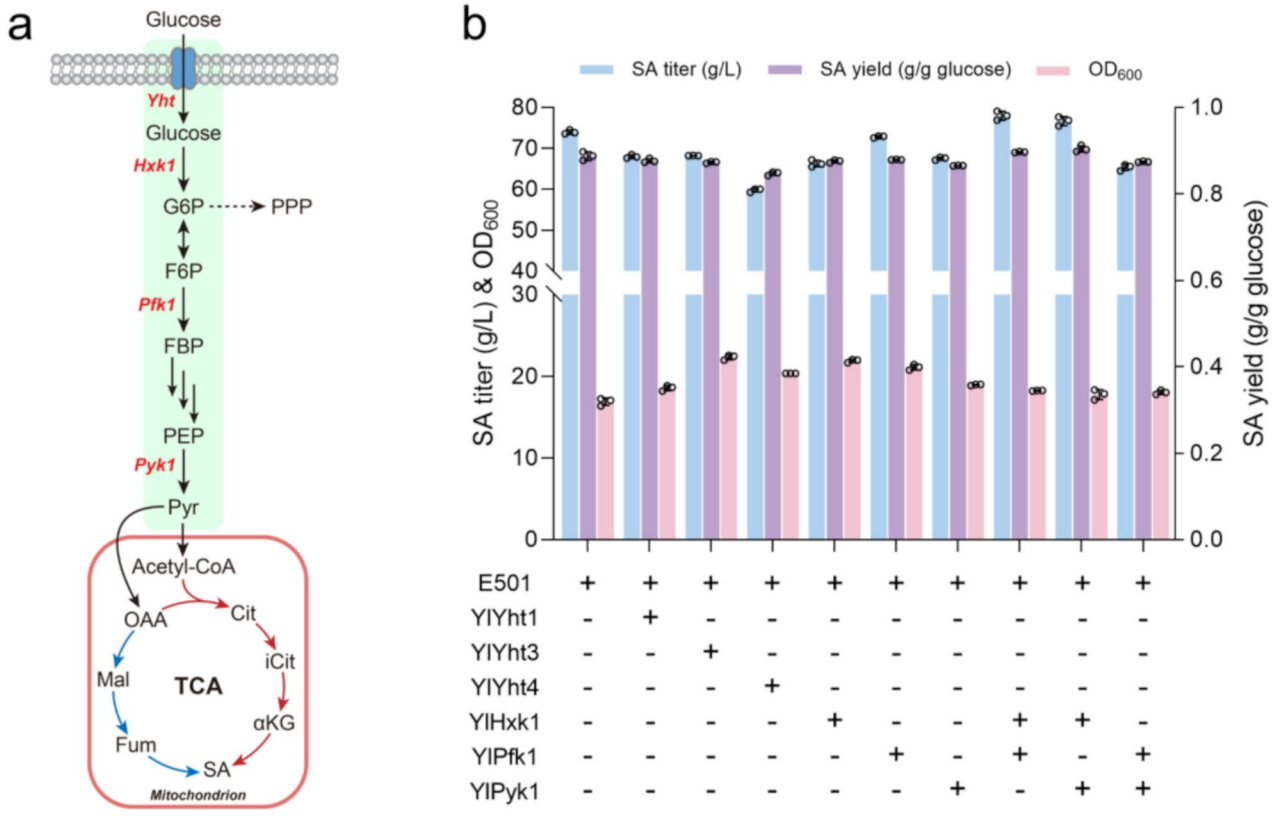


Fig. 4 Enhancing SA production of the SA-tolerant strain E501 through overexpressing genes related to glucose transport and metabolism. (**a**) A schematic diagram of engineering glucose metabolism to enhance SA production in the evolved strain E501. (**b**) Combinatorial overexpression of genes involved in glucose metabolism in the evolved strain E501. Error bars indicate standard deviations of three biological replicates

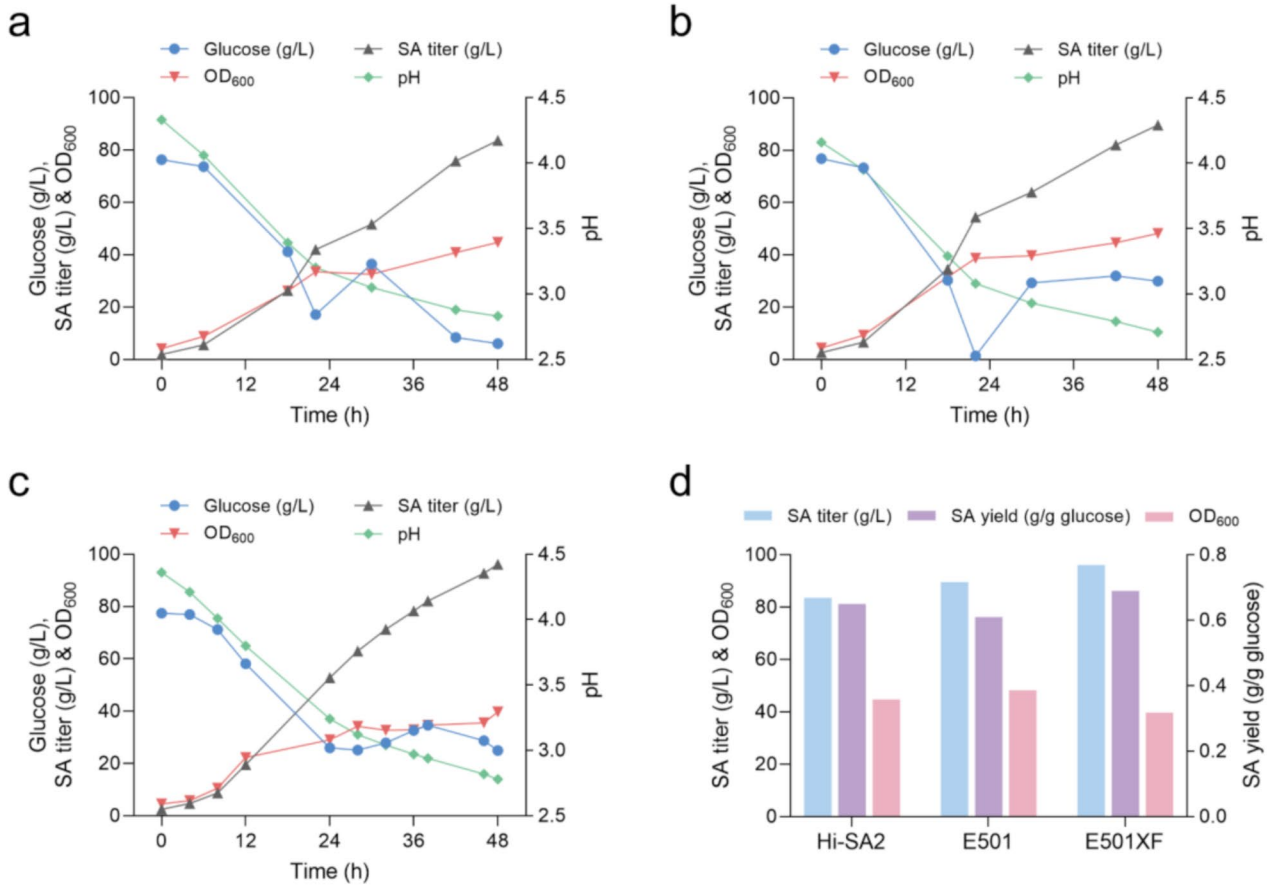


Fig. 5 Fed-batch fermentation profiles of different engineered strains without pH adjustment. (**a**) Fed-batch fermentation profile of the initial strain Hi-SA2 in a 5-L bioreactor. (**b**) Fed-batch fermentation profile of the evolved strain E501 in a 5-L bioreactor. (**c**) Fed-batch fermentation profile of the engineered strain E501XF in a 5-L bioreactor. (**d**) Comparison of SA titer, SA yield and cell growth within 48 h of fermentation

contrast, the evolved strain E501 demonstrated better cell growth in the acidic environment compared to Hi-SA2. The maximum OD_{600} of the strain E501 reached 48.24, producing 89.62 g/L SA with a yield of 0.61 g/g glucose and a productivity of 1.87 g/L/h (Fig. 5b). These results indicate that enhancing product tolerance is critical for cell growth and SA production at low pH. Further genetic modification by co-overexpressing YlHxk1 and YlPfk1 in the evolved strain E501 resulted in an improved SA titer of 96.16 g/L, with a yield of 0.69 g/g glucose and a productivity of 2.0 g/L/h (Fig. 5c). This optimization promoted the metabolic conversion of glucose into SA, leading to increased SA productivity (Fig. 5d). Due to the pKa of SA being 4.21, SA predominantly exists in the form of free acids, thereby obviating the need for acidification treatment in downstream processes [23]. When the pH was maintained at 3.0 and 3.5 (pH < pKa) during fermentation, a further enhancement in the titer and productivity of SA was observed (Fig. 6). The highest SA titer of 112.54 g/L was achieved by strain E501XF at pH 3.5, with a yield of 0.67 g/g glucose and a productivity of 2.08 g/L/h (Fig. 6b).

Discussion

The production of SA through microbial fermentation has garnered substantial interest as a sustainable alternative to traditional chemical synthesis routes, which often rely on non-renewable resources and operate under harsh reaction conditions [7, 8]. Among the diverse microbial hosts explored for SA production, the non-conventional yeast *Y. lipolytica* has emerged as a particularly promising candidate, owing to its unique metabolic capabilities, robust tolerance to acidic environments, and GRAS status [15, 16]. Engineered *Y. lipolytica* strains have been shown to efficiently synthesize SA without pH adjustment, with the final pH of the fermentation broth reaching as low as 3.0. This reduces the need for neutralizing agents, minimizes the generation of waste salts, and provides a natural defense against bacterial contamination, thereby promoting bioprocess sustainability [11–14]. Eukaryotic cells can maintain a near-neutral intracellular pH (~6.8) under extreme acidic conditions (pH 3.0), but at a significant metabolic cost [48]. Sekova et al. reported that the reference strain *Y. lipolytica* W29 exhibited maximal linear growth at pH 5.5, with no growth observed

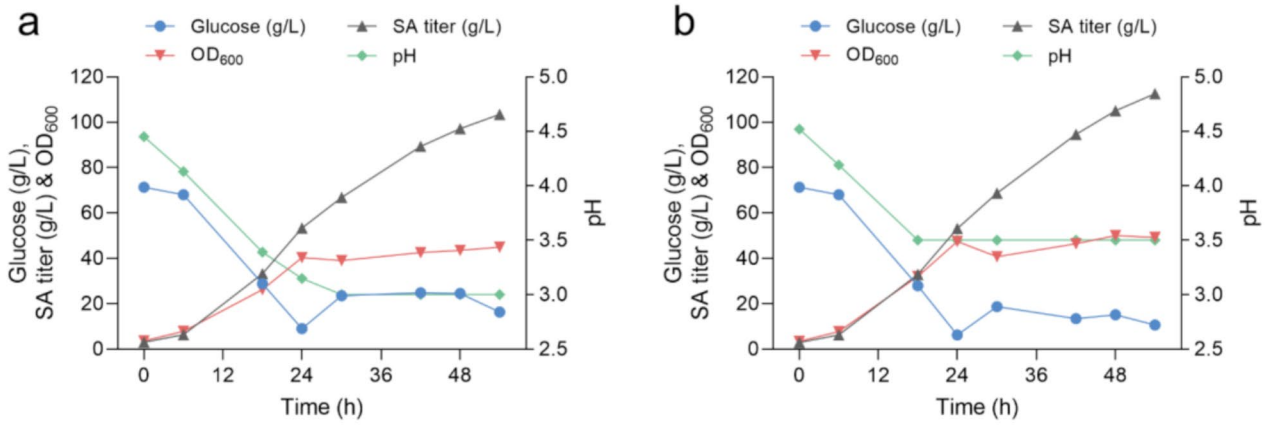


Fig. 6 Fed-batch fermentation profiles of the engineered strain E501XF at low pH in a 5-L bioreactor. (**a**) The pH was maintained at 3.0. (**b**) The pH was maintained at 3.5

below pH 3.0 or above pH 10.5 [49]. Despite its broad pH tolerance, the metabolic capacity of *Y. lipolytica* varies substantially with ambient acidity [50].

In this study, the engineered *Y. lipolytica* strain Hi-SA2, constructed through previous metabolic engineering efforts, exhibited notable limitations in its ability to withstand high extracellular SA concentrations, particularly under acidic conditions (Fig. 1a and b). This finding underscores a common challenge faced by microbial cell factories for organic acids production: the inhibitory effects of the accumulated product and the associated reduction in pH can significantly impair cell growth, glucose metabolism, and overall biosynthetic performance. Several strategies can be employed to improve the microbial tolerance and robustness, including random mutagenesis, ALE, and systems metabolic engineering [27, 51, 52]. To enhance the SA tolerance of *Y. lipolytica* strains, an ALE process was conducted by gradually exposing the parental strain to progressively increasing SA concentrations. Ultimately, evolved variants with improved acid resistance were enriched and selected (Fig. 1c and d). The resulting strain E501 displayed superior growth and SA production capabilities in the presence of high SA levels. These results again suggest that ALE is an effective strategy to develop more robust phenotype in *Y. lipolytica*.

Genome resequencing and transcriptome analysis of the evolved strain E501 provided valuable insights into the potential mechanisms underlying the enhanced SA tolerance. A key finding was the identification of a missense mutation in the gene encoding the 26S proteasome regulatory subunit Rpn1. Structural analysis revealed that the amino acid substitution (L740F) occurred within the PC repeat II domain of Rpn1, which is responsible for recognizing polyubiquitin and ubiquitin-like signals (Fig. 3a) [40, 41]. This mutation may have improved the degradation of SA stress-induced misfolded proteins via the ubiquitin-mediated proteasome pathway, thereby enhancing cellular fitness under acidic conditions [42–44]. Transcriptome analysis further highlighted significant transcriptional changes in genes associated with various metabolic pathways, including carbohydrate metabolism, energy metabolism, lipid metabolism, and the metabolism of cofactors and vitamins (Fig. 3b). Notably, a substantial number of DEGs were related to transmembrane transport, including the downregulation of the SA importer Jen1 (Fig. 3c). This downregulation may have contributed to reduced SA influx and alleviated acid stress-induced cellular damage [45, 46]. Additionally, the upregulation of several potential dicarboxylic acid transporters could enhance the efflux of SA, further improving tolerance.

Robust glucose metabolism is crucial for the biosynthesis of bulk chemicals, including organic acids. Wang et al. demonstrated that enhancing glycolysis could significantly improve glucose-based lactic acid production in engineered *Corynebacterium glutamicum* strains under oxygen-deprived conditions [53]. Similarly, Xu et al. boosted the glycolytic flux in *A. niger* to enhance malic acid biosynthesis [35]. Thus, the glycolytic flux of the evolved *Y. lipolytica* E501 strain was fine-tuned in this work, to increase the availability of precursors and energy for SA biosynthesis (Fig. 4). By overexpressing the genes encoding hexokinase (YlHxk1) and phosphofructokinase (YlPfk1), the engineered strain E501XF achieved a remarkable SA titer of 112.54 g/L, with a productivity of 2.08 g/L/h under low pH conditions (Fig. 6). This high level of SA production demonstrates the efficacy of our strategies in overcoming the challenges associated with high product titers and acidic fermentation environments.

Conclusion

In this study, an SA-tolerant *Y. lipolytica* strain E501 with significant SA production performance was obtained through ALE experiments. In fed-batch fermentation, the evolved strain E501 achieved an SA titer of 89.62 g/L, with a yield of 0.61 g/g glucose and a productivity of 1.87 g/L/h. Despite the final pH of the fermentation broth dropping to 2.71, the OD_{600} steadily increased to 48.24, demonstrating improved SA tolerance. Genome resequencing and transcriptome analysis revealed that the 26S proteasome regulatory subunit Rpn1, along with several potential dicarboxylic acid transporters, may be responsible for enhanced SA tolerance. Further optimization by overexpressing YlHxk1 and YlPfk1 to enhance glucose uptake resulted in a highest SA titer of 112.54 g/L, with a productivity of 2.08 g/L/h. Overall, our strategies successfully improved the SA tolerance and production of engineered *Y. lipolytica* strains, ensuring the efficiency of large-scale SA fermentation processes at low pH.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-024-02565-0.

Supplementary Material 1

Supplementary Material 2

Acknowledgements
Not applicable.

Author contributions
Yutao Zhong, Zhiyong Cui, and Qingsheng Qi conceived the study. Yutao Zhong designed and performed most of the experiments. Jin Hou, Zhiyong Cui, and Qingsheng Qi supervised the project. Changyu Shang and Huilin Tao assisted with experimental performance. Yutao Zhong, Zhiyong Cui, and Qingsheng Qi wrote and revised the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]State Key Laboratory of Microbial Technology, Shandong University, Qingdao 266237, P. R. China

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