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Combinatorial metabolic engineering and process optimization enables highly efficient production of L-lactic acid by acid-tolerant *Saccharomyces cerevisiae*

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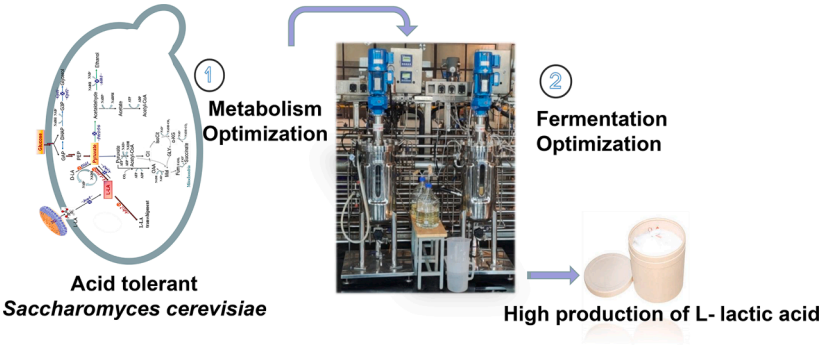
HIGHLIGHTS

- Engineered an acid-tolerant *S. cerevisiae* for efficient L-LA synthesis.
- Increased L-LA production by metabolic regulation and fermentation optimization.
- Engineered *S. cerevisiae* produced 192.3 g/L L-LA in a 15-L bioreactor at pH 4.5.

GRAPHICAL ABSTRACT



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ABSTRACT

L-lactic acid (L-LA) is widely used in the food, pharmaceutical, and cosmetic industries. In recent years, the production of L-LA using microbial fermentation has been favored. Herein, a *Saccharomyces cerevisiae* TAM strain tolerant to pH 2.4, was used as the starting strain. Exogenous L-lactate dehydrogenase expressing *S. cerevisiae* TAM strain with downregulated glycerol and ethanol synthesis pathways produced an L-LA titer of 29.8 g/L, and it increased to 50.5 g/L after carboxylic acid transport pathway modulation at the shake-flask level. Subsequently, increased energy supply and redox balancing increased the L-LA titer to 72.7 g/L in shake-flask fermentation without a neutralizer, with the yield of 0.66 g/g. Finally, optimization of the fermentation conditions, such as the seed quantity, oxygen level, and pH in a 15-L bioreactor, increased the L-LA titer to 192.3 g/L at pH 4.5, with a yield of 0.78 g/g. Overall, this study proposes an efficient L-LA bioproduction method.

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1. Introduction

L-Lactic acid (L-LA, $CH_3CHCOOH$) is a natural organic acid that is widely used in the food, pharmaceutical, cosmetics, tobacco, and chemical industries (Stoica et al., 2020; Bai et al., 2020; Ranakoti et al., 2022). The main L-LA production methods are chemical synthesis, enzymatic conversion, and microbial fermentation (Liu et al., 2022; Costa et al., 2021). Chemical synthesis has several limitations, including substrate toxicity, hydrocyanic acid production, and high production cost (Kulkarni et al., 2021). Although enzymatic production can yield L-LA with high optical specificity, the requirements of this process are stringent and tedious; therefore, it is not widely used in industries (Nuzzo et al., 2019). Conversely, microbial fermentation has become the mainstream method for L-LA production because it can utilize a wide range of raw materials, has low production cost, provides yield with high optical purity, and ensures product safety (Bichot et al., 2018; Mazzoli, 2020).

Currently, *Saccharomyces cerevisiae* has been widely used in the biosynthesis of various organic acids, such as L-malic acid, L-LA, and muconic acid, because of its acid tolerance and clear genetic background (Li et al., 2022; Kang et al., 2022; Wang et al., 2020). The introduction of L-lactate dehydrogenase (L-LDH) into *S. cerevisiae* allows L-LA biosynthesis (Zou et al., 2013). On this basis, several metabolic regulation tactics have been applied to the construction of *S. cerevisiae* cell factories dedicated to L-LA production, including the enhanced expression of the key enzyme L-LDH, the weakening of byproduct synthesis pathways, and the acceleration of extracellular transport (Liang et al., 2016). For example, using an integrated expression strategy to replace *PDC1* with *LDH* from *Lactobacillus helveticus*, a mutant strain carrying *LDH* and a deletion of *PDC1* was constructed, which yielded an L-LA titer of up to 52.2 g/L (Wu and Zhang, 2012). However, the initial fermentation condition requires a neutralizer to maintain the pH between 5 and 6, which easily causes resource waste and environmental pollution.

To solve the abovementioned problems, in recent years, several studies have focused on the selection and isolation of acid-tolerant *S. cerevisiae* strains (Cheng et al., 2017; Haitani et al., 2012). For example, through adaptive laboratory evolution (ALE), Jang et al. obtained an acid-tolerant (pH 4.2) strain (*S. cerevisiae* BK01), which produced an increase of 17% in the L-LA titer from 102 to 119 g/L (Jang et al., 2021). Mitsui et al. (2020) improved lactic acid tolerance of yeast by genome evolution based on the CRISPR system. The resultant engineered yeast produced about 33.9 g/L of LA from 100 g/L glucose in a non-neutralized condition and 52.2 g/L of LA from 100 g/L glucose with 20 g/L $CaCO_3$ in a semi-neutralized condition. In our previous study, an *S. cerevisiae* mutant (TAM) tolerant to low pH (pH 2.4) was isolated using ALE (Sun et al., 2023). Herein taking TAM as the starting strain, exogenous lactate dehydrogenase (Bc*LDH* and Lac*LDH*) was employed to achieve the expression of L-LA. After that, by weakening the branching pathways of pyruvate, ethanol, and glycerol synthesis, regulating the carboxylic acid transport pathway, balancing the homeostasis of cofactors, and increasing the supply capacity of ATP, the biosynthesis efficiency of L-LA was significantly improved. Combined with process optimization in a 15-L bioreactor, the engineered strain TAM-L17 produced the highest titer of L-LA in *S. cerevisiae* reported so far. In general, this study provides a major step toward the production of L-LA using acid-tolerant yeast cell factories.

2. Materials and methods

2.1. Strains and reagents

The chassis cell used in this study was *S. cerevisiae* TAM (acid-stable *S.* cerevisiae screened by ALE) (Sun et al., 2023). Table 1 summarizes the *S. cerevisiae* strains used and constructed via recombination in this study. Engineered yeasts with loss of the selective flag were selected using solid synthetic dropout (SSD) medium containing 1 mg/mL 5-fluoroorotic acid (Nakazawa et al., 2016). Restriction enzymes, T4 DNA ligase, Ex-Taq DNA polymerase, Pyrobest™ DNA Polymerase, the plasmid miniprep kit, the DNA fragment purification kit, and the SteadyPure DNA gel recovery kit were purchased from Bao Biological Engineering (Dalian) Co., Ltd. Genomic DNA and plasmid DNA extraction kits were purchased from G-biosciences (St Louis, Mo, USA). Primer synthesis, gene synthesis, and Sanger sequencing were performed by Suzhou Geminiz Biotechnology Co., Ltd. Standard L-LA was purchased from Beijing Solaibao Technology Co., Ltd. Glucose, glycerin, ethanol, and sucrose were purchased from Shanghai Sinopharm Group. Soybean peptone was purchased from Wokai Biologics Co., Ltd. Tryptone and yeast powders were purchased from OXOID British Biologics. Other conventional reagents were imported or were domestic reagents with analytical pure grade.

2.2. Construction of recombinant plasmids

The construction of the gene expression box was based on the initial plasmid. Promoter and terminator domains (TEF1 and BLA; TDH3 and CYC1, respectively) were expanded from the *S. cerevisiae* CEN.PK2-1C genome. A new plasmid of LDH was constructed on PY13TEF1 plasmid, expressed in the large intestine, and verified by sequencing (see supplementary materials). LB medium was required for the culture of *Escherichia coli* and was used for the transformation of plasmids after the addition of glucose (20 g/L), yeast extract (5 g/L), peptone (10 g/L), and NaCl (10 g/L). Solid medium was prepared by adding 20 g/L of agar powder.

2.3. Molecular manipulation and expression of key genes

Primers were designed based on the whole genome of *S. cerevisiae* S288C (see supplementary materials). Heterologous genes encoding L-LDH were cloned from *Lactococcus lactis* and *Bacillus coagulans* and those encoding phosphofructokinase (PFK) were cloned from *E. coli.* The genes encoding the plasm.

a membrane carboxylic acid transporter protein (ADY2) and lactate and pyruvate permease (JEN1) were overexpressed by inserting the strong promoter *TEF1 in* front of the gene locus. Efficient conversion via the lithium acetate (Li Ac) method was used for gene knockdown and expression (Yarimizu et al., 2017). Subsequently, PCR was performed in a reaction volume of 50 μL using the following amplification conditions: predenaturation for 5 min at 94 °C, followed by 34 cycles of denaturation at 94 °C for 30 s, 60 s of annealing at 55 °C, and 60 s of decompression at 72 °C and a final 7-min heating at 72 °C. The gene fragments obtained by PCR were transformed into set positions in *S. cerevisiae*, the transformed strains were plated on HIS^-/LEU^- plates, and the cultured

Table 1
Yeast strains used in this study.

Strains	Host strain	Relevant genotype	Reference
TAM			(Sun et al., 2023)
TAM-L1	TAM	*416d*::Lac*LDH*	This study
TAM-L2	TAM	*416d*::Bc*LDH*	This study
TAM-L3	TAM-L2	Δ*Cyb2*::Lac*LDH*	This study
TAM-L4	TAM-L3	Δ*PDC1*::Lac*LDH*	This study
TAM-L5	TAM-L3	Δ*PDC6*::Lac*LDH*	This study
TAM-L6	TAM-L3	Δ*PDC1*::Lac*LDH*Δ*PDC6*::Lac*LDH*	This study
TAM-L7	TAM-L6	Δ*Adh1*::Lac*LDH*	This study
TAM-L8	TAM-L7	Δ*Gpd1*::Lac*LDH*	This study
TAM-L9	TAM-L7	Δ*Gpd2*::Lac*LDH*	This study
TAM-L10	TAM-L7	Δ*Gpd1*::Lac*LDH*Δ*Gpd2*::Lac*LDH*	This study
TAM-*L*11	TAM-*L*10	Δ*Jen1*::Lac*LDH*	This study
TAM-L12	TAM-*L*10	::*Jen1*::Lac*LDH*	This study
TAM-L13	TAM-*L*11	::*ADY2*	This study
TAM-L14	TAM-L13	Δ*NDE1*	This study
TAM-L15	TAM-L13	Δ*NDE2*	This study
TAM-L16	TAM-L13	Δ*NDE1* Δ*NDE2*	This study
TAM-L17	TAM-L16	::*pfkA*	This study

colonies were validated using the designed primers. Tag elimination was performed by adding the receptor to the Cre plasmid, and the final strain obtained after tag removal was grown only on YPD plates and not on HIS^-/LEU^- plates.

2.4. Shake-flask and fed-batch fermentation

First, seed cultures with 2-mL YPD broth were performed in 5-mL culture tubes, which were incubated for 28 h at 30 °C with shaking at 220 rpm. Subsequently, 30 mL of YPD medium (glucose, 20 g/L; trypsin, 20 g/L; and yeast powder, 10 g/L) was added to a 250-mL shaker flask for L-LA production tests.

Amplified fermentation was performed in a 15-L bioreactor. In the first step, seed cultures were prepared. Colonies were selected on plates, inoculated in tubes containing 3-mL YPD medium, and incubated for 24 h at 30 °C. In the second step, a subculture was performed. About 1.5–2 mL of primary seed culture was aspirated and added to 150 mL of fresh YPD medium. In the third step, inoculation was performed in a 15-L bioreactor. After culturing the secondary inoculum for 18–36 h, 10% of it was inoculated into 15 L of optimized medium (glucose, 20 g/L; tryptone, 20 g/L; yeast powder, 10 g/L; soy peptone, 25 g/L; K_2HPO_4, 1 g/L; KH_2PO_4, 1 g/L; L-arginine, 374.4 mg/L; L-tryptophan, 179.3 mg/L; Tween-80, 0.5 mL/L; and vitamin B1, 500 mg/L) for enhanced fermentation and regulation. Fermentation was performed at 30 °C and at 300–800 rpm. The dissolved oxygen (DO) level was adjusted according to the requirements of the experiment. Glucose at a concentration of 600 g/L was fed-batch added to the bioreactor as a carbon source supplement. The concentration of glucose was maintained at 15–20 g/L. In the presence of neutralizers, $CaCO_3$ was added as a buffer to maintain the pH at 5.8 or 4.5. The fermentation status was monitored according to the OD_{600} value of cell growth and the residual glucose concentration of the fermentation broth.

2.5. Analytical methods

Measurement of growth: the absorbance was measured at 600 nm using a Type 721 spectrophotometer.

Residual sugar determination method: a biosensor M−100 (Sieman) was used to determine the concentration of residual sugar in the fermented broth.

Organic acid determination method: high-performance liquid chromatography was performed using an organic acid column, a mobile phase of 0.05 mol/L H_2SO_4, a flow rate of 0.6 mL/min, an injection volume of 20 μL, a column temperature of 55 °C, and an ultraviolet detector with a detection wavelength of 210 nm.

2.6. Statistical analysis

Data were statistically analyzed using the GraphPad Prism 8.0.0 and SPSS 26.0 software, and all values were tested in three independent replicates. *P*-values of *< 0.05, **0.01, and ***<0.001 were considered statistically significant.

3. Results and discussion

3.1. Heterologous expression of L-LDH for L-LA synthesis

To realize the biosynthesis of L-LA in the acid-tolerant *S. cerevisiae* TAM strain, the L-LDH gene from *L. lactis* (Lac*LDH*) and *B. coagulans* (Bc*LDH*) was introduced into the yeast cells to obtain the TAM-L1 and TAM-L2 strains, respectively (Fig. 1A). As shown in Fig. 1B, the TAM-L1 strain produced 6.20 g/L of L-LA, which represented a higher titer than that produced by TAM-L2 (5.78 g/L of L-LA). Therefore, the TAM-L1 strain was selected as the chassis cell to further enhance the ability of L-LA production capacity. In addition, considering that the demand of yeast for biosynthetic oxygen is typically low (Mooiman et al., 2021), an anaerobic shake flask was used for subsequent fermentation.

*Cyb*2 encodes L-lactate cytochrome-c oxidoreductase, which catalyzes the conversion of lactate to pyruvate (Engqvist et al., 2015). In the TAM-L1 strain, *Cyb*2 was replaced by Lac*LDH* to prevent the conversion of L-LA into pyruvate, thus promoting the accumulation of L-LA. The engineered strain was named TAM-L3 (TAM-L2Δ*Cyb*2::Lac*LDH*), and it could produce 6.80 g/L L-LA in a triangle shaker; the produced by this strain in anaerobic shake flask culture increased to 7.76 g/L (Fig. 1C).

3.2. Modification of the pyruvate shunt metabolic pathway

The introduced exogenous L-LA synthesis pathway competes with the ethanol pathway, as they share the intracellular pyruvate and NADH (Fig. 1A). Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) catalyze the conversion of pyruvate to acetaldehyde and acetaldehyde to ethanol, respectively (Ke et al., 2015). PDC consists of three highly homologous subtypes encoded by *Pdc*1, *Pdc*5, and *Pdc*6. However, because respiratory function is naturally inhibited by glucose, the growth of *Pdc*1–*Pdc*5 double-deficient strains stagnates under anaerobic conditions (Ishida et al., 2006). Therefore, in this study, the *Pdc*1, *Pdc*6, and *Adh*1 loci were replaced with Lac*LDH* using the Cre-loxP system, which weakened the ethanol synthesis pathway while ensuring the growth activity of the strain and increasing the number of copies of L-LDH (Fig. 2A). The TAM-L4 (TAM-L3Δ*Pdc*1::Lac*LDH*) and TAM-L5 (TAM-L3Δ*Pdc*6::Lac*LDH*) strains were obtained by replacing *Pdc*1 and *Pdc*6, respectively, with Lac*LDH*, which yielded L-LA titers of 7.8 and 8.4 g/L, respectively (Fig. 2B). The TAM-L6 strain (TAM-L3Δ*Pdc*1::Lac*LDH*Δ*Pdc*6::Lac*LDH*) was obtained by replacing *Pdc*1 and *Pdc*6 with Lac*LDH* simultaneously. The L-LA titer produced by this strain increased to 12.3 g/L in shake-flask fermentation at 72 h (Fig. 2C). Moreover, knockout of *Adh*1 (ADH-encoding gene) to prevent the synthesis of the ethanol byproduct from acetaldehyde led to the generation of the TAM-L7 strain (TAM-L6Δ*Adh*1), which increased the L-LA titer to 22.56 g/L after 72 h of shake-flask fermentation (Fig. 2C). After the metabolic regulation described above, the synthesis of ethanol was successfully suppressed, with a reduction from 10.58 to 4.28 g/L (Fig. 2D).

3.3. Disabling glycerol triphosphate dehydrogenase (GPD) to block the glycerol synthesis pathway

In classical anaerobic fermentation, *S. cerevisiae* consumes excess NADH in the cell through the glycerin synthesis pathway to maintain the balance of intracellular cofactors; however, the synthesis and accumulation of glycerin, which is a byproduct of this pathway, reduces the yield of L-LA (Jiang et al., 2021). To weaken the competing pathway and prevent glycerol production, *Gpd*1 and *Gpd*2, which are genes encoding glycerol triphosphate dehydrogenase, were knocked out. The L-LA titers of the resulting *Gpd1*-knockout strain TAM-L8 and *Gpd2*-knockout strain TAM-L9 were 25.4 and 25.5 g/L, respectively. Subsequently, *Gpd*1 and *Gpd*2 were knocked out simultaneously to produce the strain TAM-*L*10 (TAM-L7Δ*Gpd*1Δ*Gpd*2). Without an appreciable effect on strain growth (Fig. 3A–B), the L-LA titer produced by the recombinant strain TAM-*L*10 increased to 29.8 g/L, and the yield productivity was 0.82 g/L/h in shake-flask fermentation, which was almost 30-fold that of the initial acid-tolerant TAM strain (Fig. 3C).

3.4. Transporter engineering for L-LA synthesis

sc*Jen1* (systematic name, YKL217w) was first found to encode lactate and pyruvate permeases in *S. cerevisiae*, which participate in the intracellular transportation of lactic acid (Casal et al., 1999). However, a contradictory report suggested that *JEN1* and *ADY2* both encode monocarboxylate transporters and may be used to control lactic acid export in *S. cerevisiae* cells. *Ady*2 encodes a plasma membrane carboxylic acid transporter protein that participates in the extracellular transport of lactic acid (Pacheco et al., 2012). In this study, both overexpression and

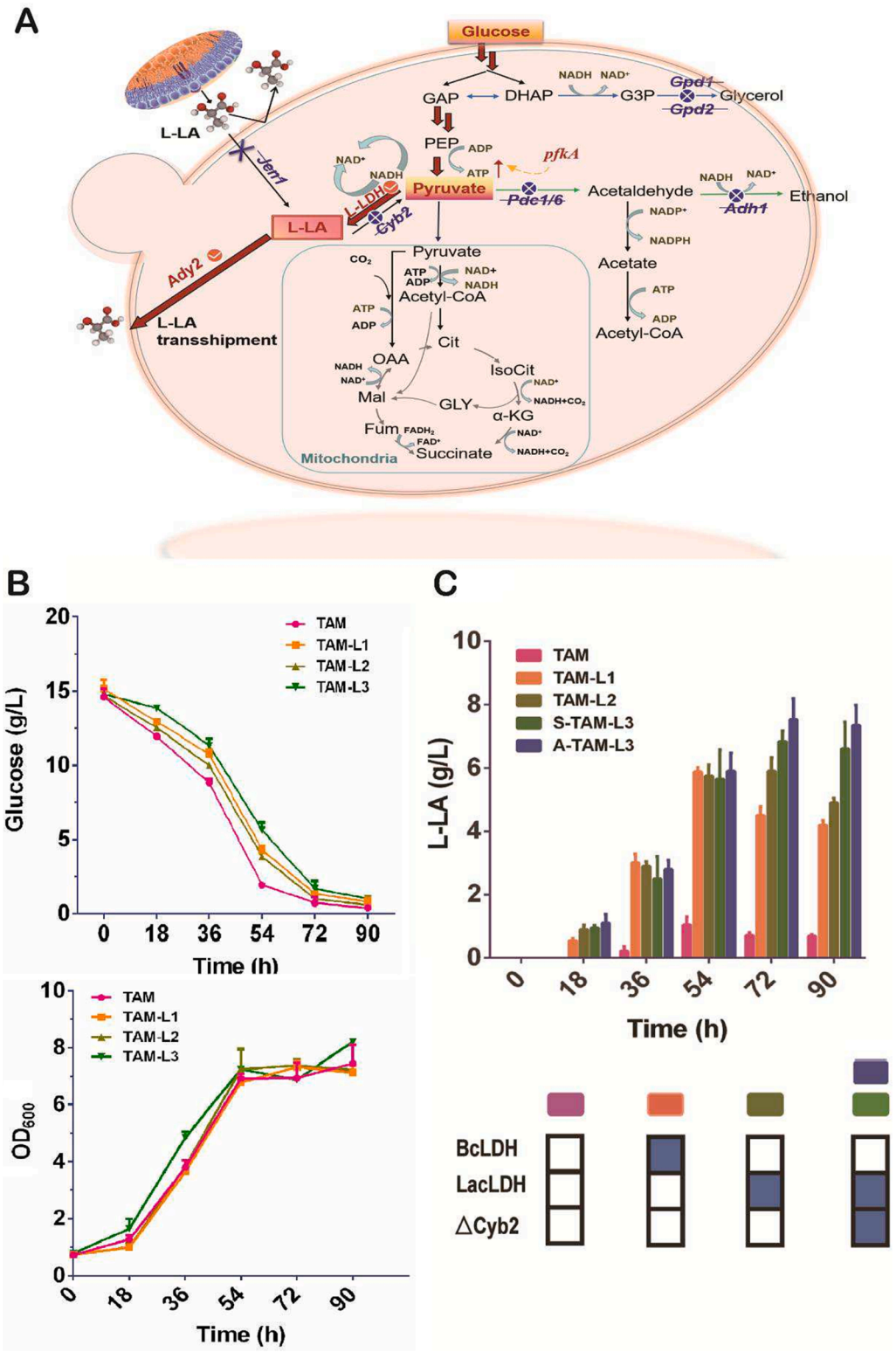

Fig. 1. L-LA bioproduction in *S. cerevisiae* strains that heterologously synthesized the lactate dehydrogenase genes Bc*LDH* and Lac*LDH*. (A) Illustration of the metabolism and related cytoplasmic synthesis pathways in *S. cerevisiae* using glucose as a carbon source. Red arrows represent the major metabolic pathways for L-LA production from glucose. Blue and green arrows and × marks represent branching paths that needed to be weakened or blocked. GAP: phosphoglyceraldehyde; DHAP: dihydroxyacetone phosphate; G3P: glyceraldehyde-3-phosphate; L-LDH: L-lactate dehydrogenase; Cyb2: cytochrome b2 (L-lactate cytochrome-c oxidoreductase); Dld1: L-lactate dehydrogenase; Pdc1/6: indolepyruvate decarboxylase 1/6; Adh1: alcohol dehydrogenase; Gpd1: glycerol-3-phosphate dehydrogenase 1, Gpd2: glycerol-3-phosphate dehydrogenase 2. (B) Plot of glucose consumption and OD_{600} trends according to growth time in strains TAM (original acid-resistant strain), TAM-L1 (TAM::Bc*LDH*), TAM-L2 (TAM::Lac*LDH)*, and TAM-L3 (TAM-L2Δ*Cyb2*). (C) Histogram trend of the titer of L-LA produced by fermentation of the strains TAM-L1, TAM-L2, and TAM-L3 in a shake flask. Among them, TAM-L3 was subjected to conventional triangular shake-flask fermentation (S) and anaerobic shake-flask fermentation (A).

knockout experiments of *Jen1* were conducted, for comparison; the strain carrying *Jen1* knockout was termed TAM-*L*11 (TAM-*L*10 Δ*Jen*1) and had a higher L-LA titer, at 34.9 g/L, than the strain overexpressing *Jen*1, which was termed TAM-L12 (TAM-*L*10::*Jen*1; 23.7 g/L at 90 h; Fig. 4A–B). These results revealed that in the absence of glucose, L-LA was able to re-enter the cell as a carbon source to replenish intracellular transport. Subsequently, *Ady*2 was overexpressed in TAM-*L*11 to further promote the efflux of L-LA. The L-LA titer produced by the TAM-L13 strain (TAM-L11Δ*Jen*1::*Ady*2) generated in this manner was further increased to 50.5 g/L after 72 h of shake-flask fermentation (Fig. 4C–D).

The transfer rate of L-LA was determined as the ratio of the L-LA titer measured in the supernatant of the fermented culture (U) to the fragmentized liquid of the fermented culture (C) using high-performance liquid chromatography. The transfer rate of L-LA increased from 69.3% to 97.6%, and almost all of the L-LA could be transferred out. The increased rate of L-LA transfer suggests that both *Jen*1 and *Ady*2 play

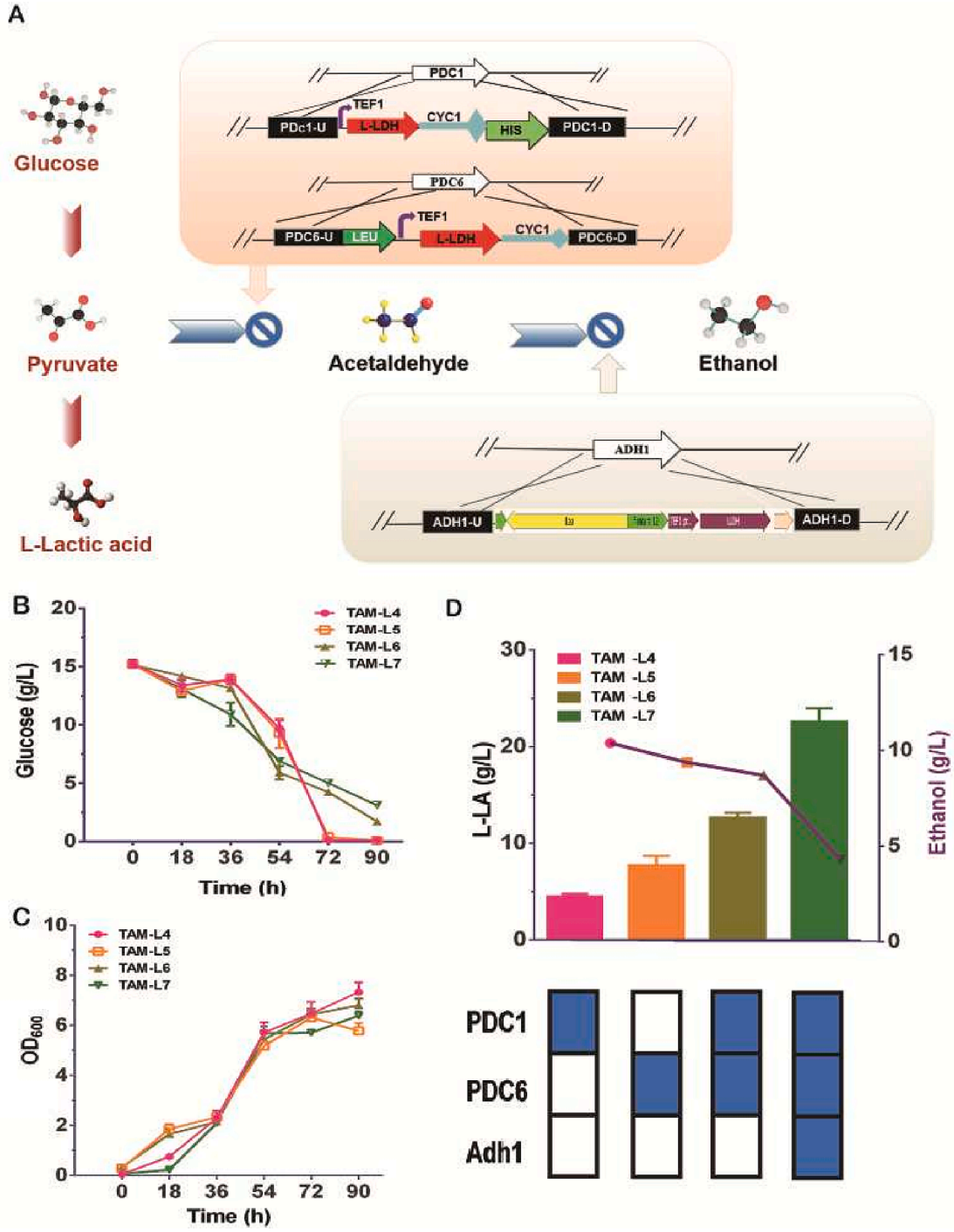

Fig. 2. Modification of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) to enhance metabolic control and promote high-efficiency synthesis of L-LA. (A) Metabolic scheme used to prevent the production of acetaldehyde by TAM-L4 (TAM-L3Δ*Pdc*1), TAM-L5 (TAM-L3Δ*Pdc*6), and TAM-L6 (TAM-L3Δ*Pdc*1 Δ*Pdc*6) by knocking out *Pdc*1 and *Pdc*6. The formation of ethanol by TAM-L7 (TAM-L6Δ*Adh*1) was prevented by knocking out *Adh1*. (B) Plot of glucose consumption trends according to growth time in the TAM-L4–TAM-L7 strains. (C) Plot of strain OD_{600} trends according to growth time in the TAM-L4–TAM-L7 strains. (D) Histogram trend of the titer of L-LA produced by the fermentation of the TAM-L4–TAM-L7 strains for 72 h. The purple line represents the decreasing trend in ethanol levels.

particularly critical roles in the intracellular and extracellular transport of L-LA.

3.5. Energy and redox cofactor compensation in L-LA bioproduction

In *S. cerevisiae*, redox cofactors play a key role in coupling catabolism with anabolism and energy production. Based on available reports, *NDE*1 and *NDE*2 affect product profiles and intracellular metabolite levels (Maeda et al., 2020). In oxygen-limited culture conditions, deletion of the NADH dehydrogenase gene reduced NADH consumption and promoted the increase in cytosolic NADH levels; moreover, it increased L-LA production in a manner dominated by cellular respiration. Therefore, the two NADH dehydrogenase genes (*NDE*1 and *NDE*2), which are involved in the regulation of the redox equilibrium, were deleted, to redistribute the cytosolic NADH, yielding strains TAM-L14 (Δ*NDE1*), TAM-L15 (Δ*NDE2*), and TAM-L16 (Δ*NDE*1 Δ*NDE2*); these strains

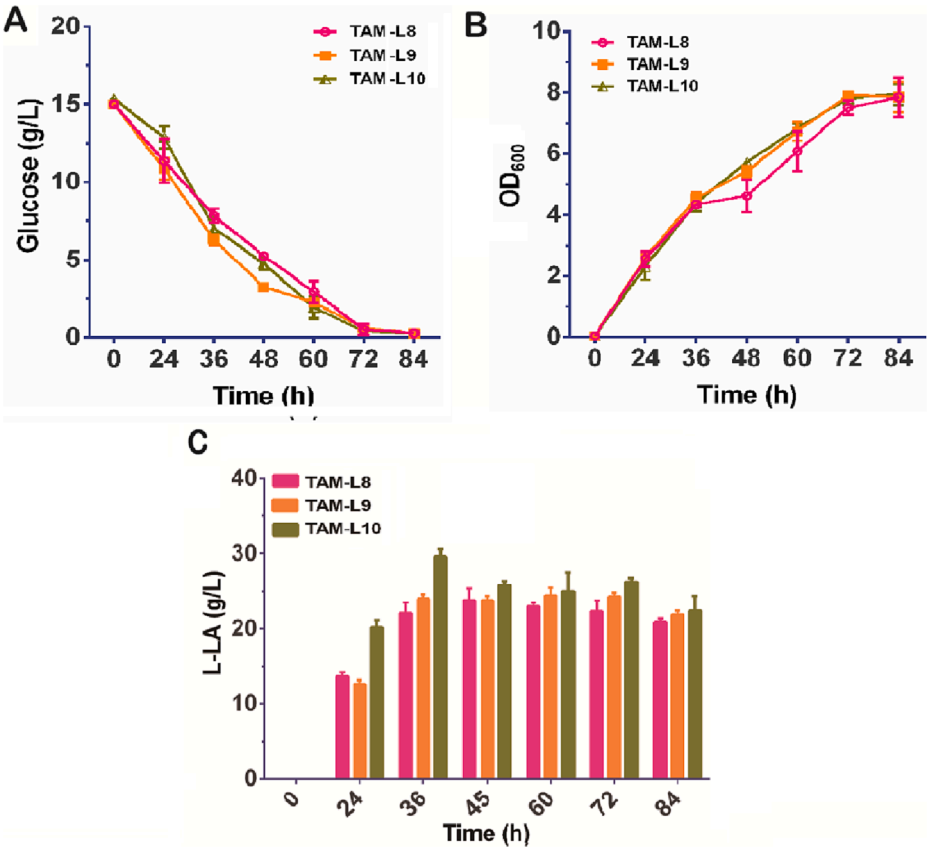

Fig. 3. Modification of glycerol triphosphate dehydrogenase (GPD) for weakening the competing pathway of pyruvate to L-LA. (A) Plot of glucose consumption trends according to growth time in strains TAM-8 (TAM-7Δ*Gpd*1), TAM-9 (TAM-7Δ*Gpd*2), and TAM-10 (TAM-7Δ*Gpd*1Δ*Gpd*2). (B) Plot of strain OD_{600} trends according to growth time in strains TAM-L8–TAM-*L*10. (C) Histogram trend of the titer of L-LA produced by the fermentation of strains TAM-L8–TAM-*L*10.

produced titers of 52.4, 53.1, and 63.3 g/L, respectively (Fig. 5A, 5B). In addition, phosphofructokinase (PFK), which is encoded by *pfkA*, catalyzes the conversion of fructose-6-phosphate to fructose-1,6-diphosphate, is one of the rate-limiting steps in the Embden-Meyerhof-Parnas (EMP) pathway; this pathway determines the efficient utilization of glucose by microorganisms (Tsuge et al., 2015). Therefore, fructose 6-phosphokinase-encoding *pfkA* from *E. coli* was heterologously expressed in the TAM-L16 strain to generate the TAM-L17 (TSL-13Δ*NDE*1Δ*NDE*2::*pfk*A) strain (Fig. 5C). The L-LA titer produced by the TAM-L17 strainLincreased to 72.7 g/L, consuming 90 g/L of glucose after 84 h, with the conversion rate reaching 0.66 g/g (Fig. 5D). Hence, from the perspective of energy regulation, the heterologous expression of *pfkA* promoted the efficient synthesis of L-LA.

3.6. L-LA overproduction by process optimization in a 15-L bioreactor

To improve its suitability for industrial production, the TAM-L17 strain was grown and fermented in a 15-L bioreactor, and four aspects of its growth conditions were optimized: seed culture growth, DO control, feed control, and pH control.

3.6.1. Effect of seed culture growth on the fermentation-based production of L-LA

To obtain a high starting fermentation level, the seed-growth times of 18, 24, and 30 h were selected for inoculation (I-18H, I-24H, I-30H). The results revealed that the transfer and growth of the seed solution in a 15-L bioreactor yielded the best growth status of the strain seed solution over a 24-h seed-growth period (Fig. 6A). Moreover, the 15-L bioreactor, the biomass of the seed liquid grown for 18 h was insufficient, and the adaptation period was relatively long, which easily allowed bacteria to prevail. However, strains subjected to a seed-growth time of 30 h had a lengthy delay period and a moderate growth rate. The seed solution fermented for 24 h in shaking flask (I-24H) was extended to 15 L bioreactor. After 24 h of natural growth of the strain in the 15 L bioreactor, L-LA began to accumulate when the OD_{600} value exceeded 10 (Fig. 6A). After 36 h, L-LA reached a stable level, with OD_{600} values fluctuating around 17. As the strain entered the stable phase, the accumulation rate of L-LA accelerated, increasing at the rate of 2 g/h, until an L-LA titer of 72.3 g/L at 72 h was achieved.

3.6.2. Effect of dissolved oxygen levels on the production of L-LA

Whole fermentation in anaerobic conditions yielded a higher L-LA titer than whole fermentation in aerobic conditions; however, in the absence of oxygen, the strain grew extremely slowly, which markedly affected L-LA production. Although the strain grew quickly and the OD_{600} reached a value of about 30 under full oxygenation, the fermentation results were not ideal, and we found that the carbon flow was diverted to another substance (see supplementary materials), resulting in a low titer of L-LA (only 35.8 g/L). Conversely, the strain exhibited a stronger L-LA-producing ability under slightly anaerobic conditions compared with both the entirely ventilated and fully anaerobic conditions (Fig. 6B). Fig. 6B shows that *S. cerevisiae* grew faster in the presence of oxygen and that the OD_{600} was higher in this condition than in anaerobic and microanaerobic conditions at 12 h. Therefore, we concluded that as much oxygen as possible should be provided during the early growth period, with reduction of the oxygen supply in the later stage, to allow *S. cerevisiae* to produce L-LA. The manner in which growth and production are balanced at the oxygenation level is critical to determine the timing of this oxygen-supply switch. After several prefermentation tests, it was found that in the early stage of

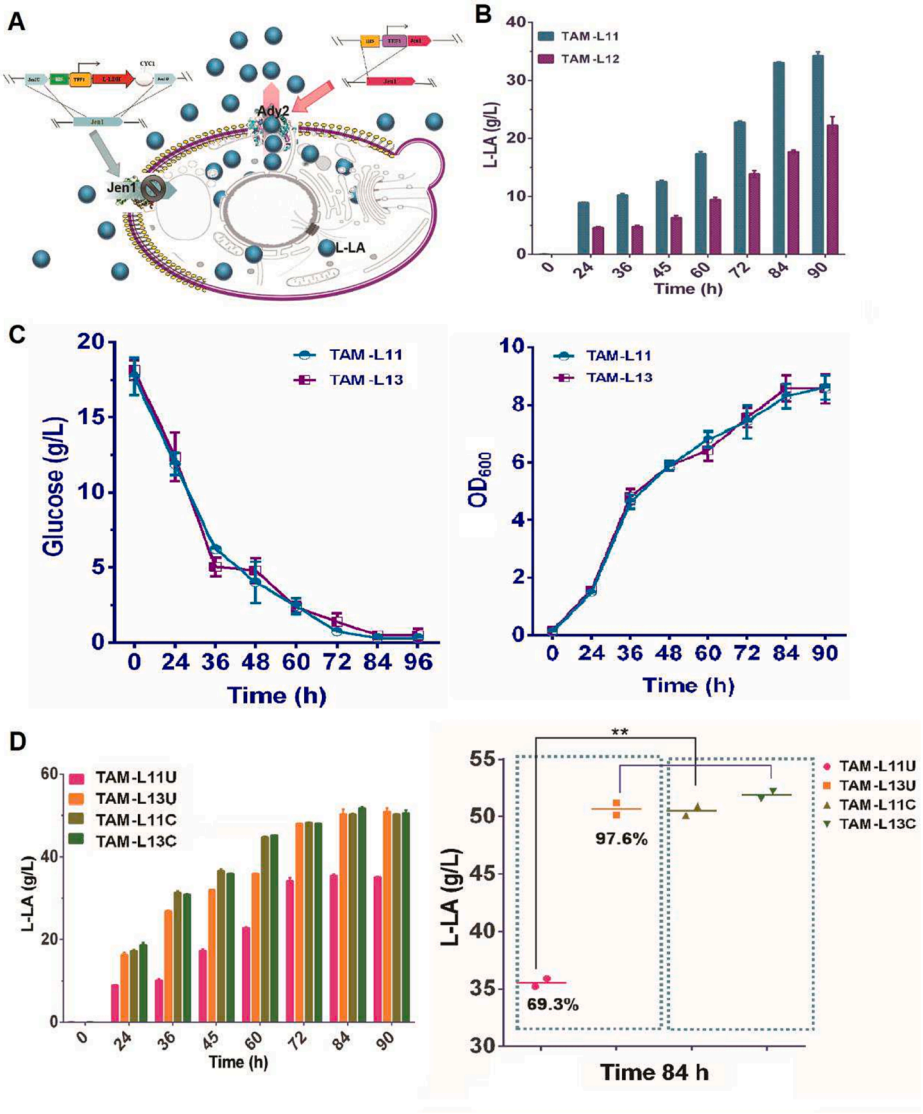

Fig. 4. Regulation of intracellular and extracellular transport promoted L-LA synthesis in *S. cerevisiae*. (A) Schematic diagram of the regulation of L-LA bioproduction by extracellular transport. (B) Results of L-LA bioproduction in the presence of knockout of the gene encoding hydroxytransferase Jen1 in TAM-11 and overexpression of the same gene in TAM-12. (C) OD_{600} values and glucose levels of TAM-13, which overexpressed *Ady*2 upon *Jen*1 knockout. (D) L-LA content in the fermentation broth supernatant (U) and fragmentized liquid of the fermented culture (C) of strains TAM-11 and TAM-13.

fermentation, oxygen was first passed to allow the strain to grow, with the OD_{600} value reaching a range of 20–22. When the glucose was almost exhausted about at 24 h and the oxygen supply was turned off to continue fermentation (O-A, pre-oxygenation and post-anaerobic), the L-LA yield was considerably improved (up to 86.2 g/L; Fig. 6B).

3.6.3. Effect of fed-batch culture changes on the fermentation-based production of L-LA

During fermentation in the 15-L bioreactor, a single glucose concentrate was previously added to the feed. To further increase the vitality of the strain during fermentation, an 8-fold concentrated optimized medium was supplemented with anaerobic growth factors (L-arginine, L-tryptophan, Tween-80, and vitamin B1) (L1). In fed-batch culture, we found that the production strength of L-LA was the best when the glucose level was maintained in the range of 15–20 g/L (L2). As shown in Fig. 6C-D, the total biomass of the strain increased after the feed was changed, with the maximum OD_{600} value reaching 22 and the L-LA titer reaching 111.0 g/L. These results suggest that the addition of anaerobic growth factors to the 15-L bioreactor of *S. cerevisiae* for L-LA production may prolong the cell density of the strain and increase the productivity of L-LA.

3.6.4. Effect of different pH control methods on the fermentation-based production of L-LA

During the process of fermentation, we found that when L-LA accumulation decreased the pH of the fermentation broth to 3.2 (at 66–72 h), the cell cell density began to decrease, the OD_{600} value mostly ceased to increase (or even decreased), and L-LA did not accumulate as rapidly as that recorded before this time point. Therefore, we adjusted the fermentation conditions and recovered the fermentation pH to 5.8 by adding 35.0 g/L $CaCO_3$ as a neutralizer. Moreover, the strain exhibited some restoration of the L-LA production capacity. The titer of L-LA reached 129.0 g/L after 96 h of fermentation (Fig. 6E). These results indicated that a pH adjustment enabled strain TAM-L17 to yield a better L-LA production capacity, with the titer of L-LA increasing by 16.2% after a single addition of 35 g/L $CaCO_3$ as a neutralizer compared with that observed without the addition of the neutralizer. According to the experimental evidence described above, the pH of the fermentation broth was regulated throughout the fermentation process. L-LA bioproduction was performed by adding $CaCO_3$ to control the pH, which was maintained at around 4.5 throughout the process. After 114 h of fermentation, the maximum titer of L-LA was 192.3 g/L, the yield was 0.78 g/g, and the productivity was 1.6 g/L/h, with a total $CaCO_3$ addition of 65.0 g/L (Fig. 6E). That is, strain TAM-L17 achieved satisfactory L-LA production by adding neutralizers to maintain a pH of

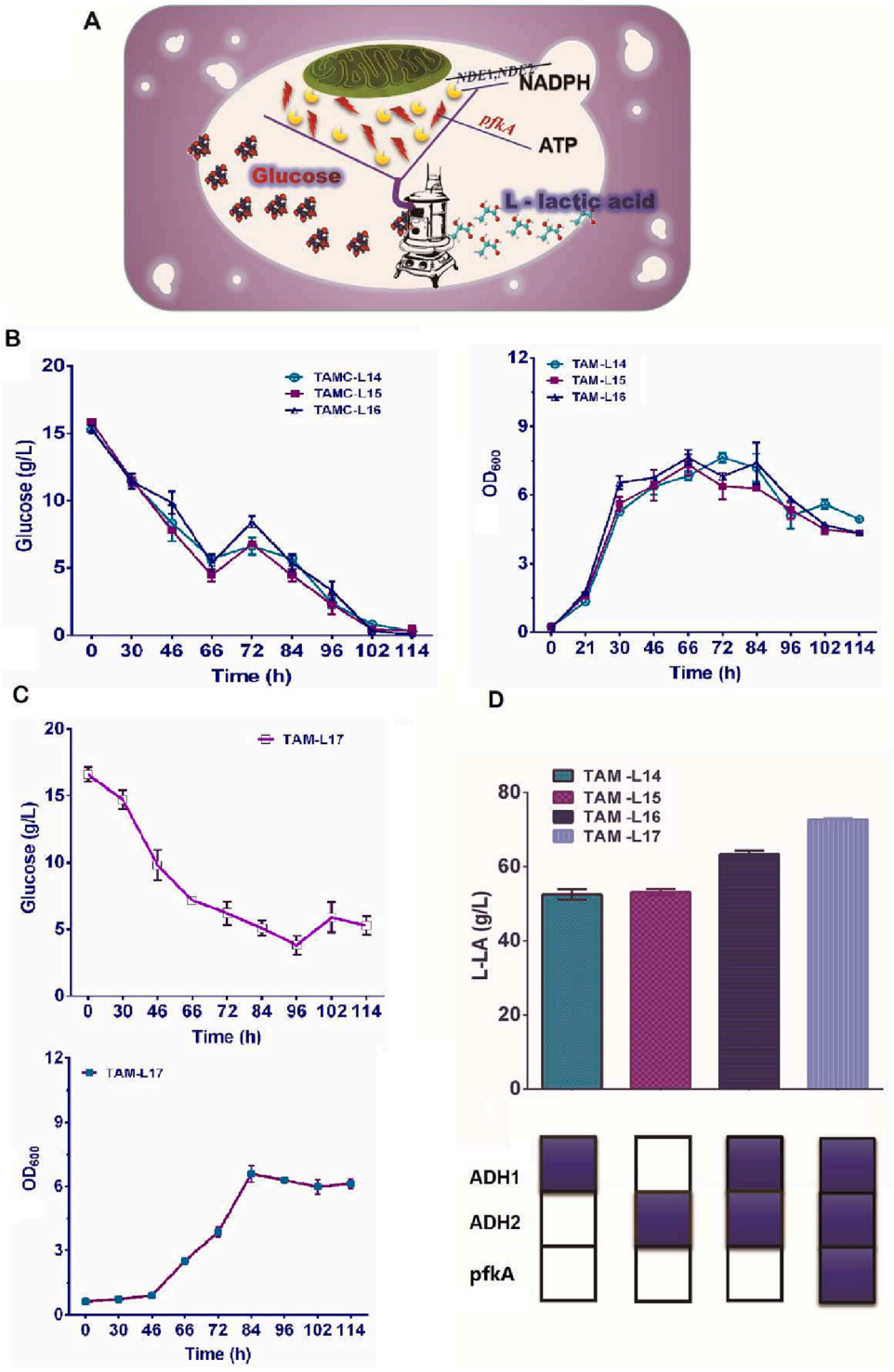

Fig. 5. Regulation of energy and redox cofactor compensation to promote L-LA bioproduction. (A) Schematic diagram of L-LA bioproduction regulated by energy and REDOX factors. (B) OD_{600} values and glucose consumption of strains TAM-L14 (TAM-L13Δ*NDE*1), TAM-L15 (TAM-L13Δ*NDE*2), and TAM-L16 (TAM-L13Δ*NDE*1Δ*NDE*2). (C) OD_{600} values and glucose consumption of strain TAM-L17 (TAM-L16::*pfk*A). (D) L-LA bioproduction results of strains TAM-L14–TAM-L17.

around 4.5 instead of restoring a neutral culture environment. In addition, the stability of the strains was analyzed and the results showed that after 100 generations, strain TAM-L17 still had stable heritability (see supplementary materials). In brief, the pH of the fermentation environment is a key factor impacting the fermentation of strains, and acid-tolerant strains are essential for the efficient production of L-LA. In the late-stage studies, L-LA can be used as a screening pressure for ALE in the hope of obtaining an engineered strain that efficiently produce L-LA without adding any neutralizer. In addition, reducing production costs through the use of inexpensive carbon and nitrogen sources will further promote the green bioproduction of L-LA.

4. Conclusion

In this study, the acid-tolerant *S. cerevisiae* TAM was used as the chassis for efficient synthesis of L-LA. The metabolic pathway of L-LA

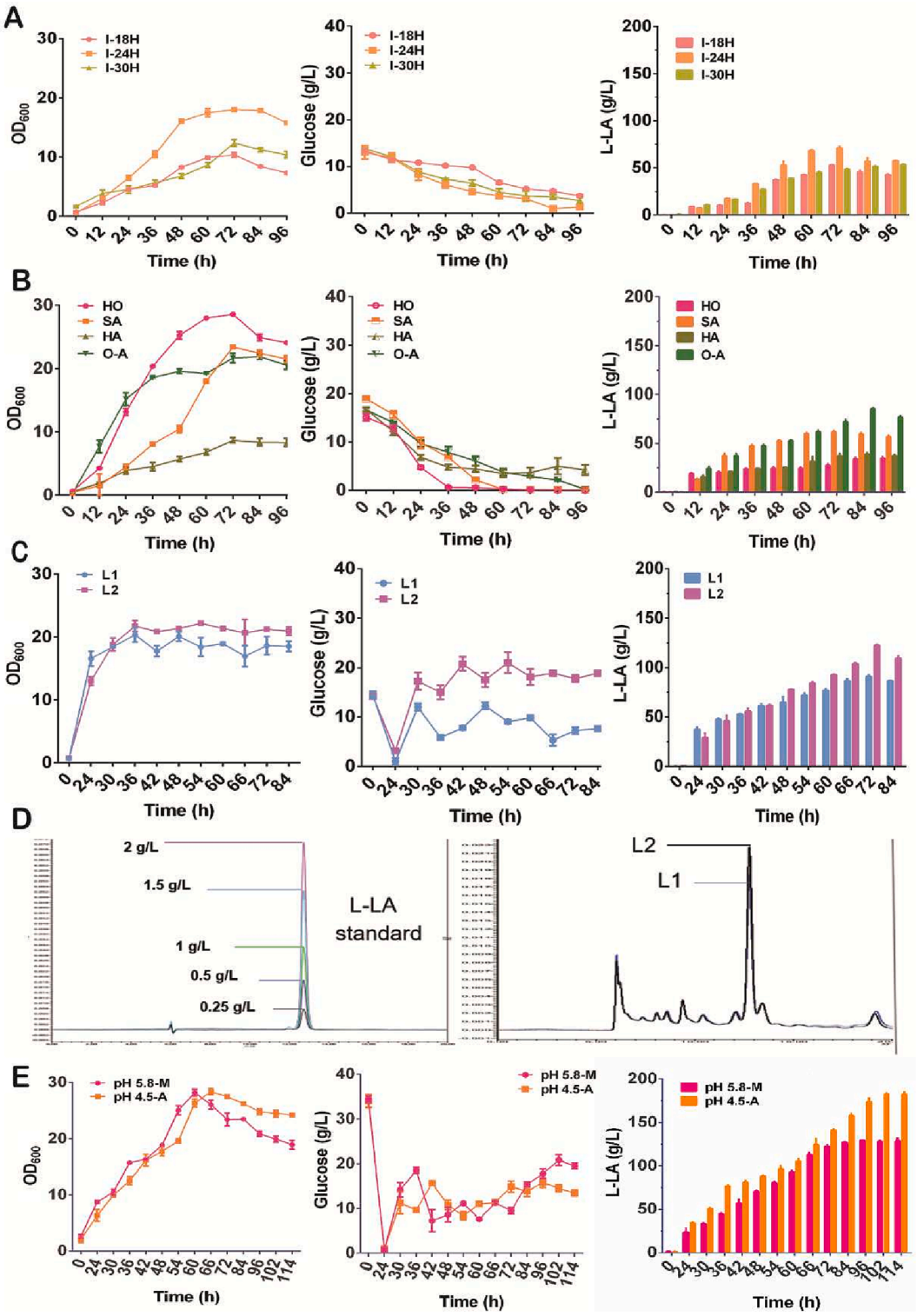

Fig. 6. Optimization of the fermentation conditions of the 15-L bioreactor. (A) Regulation of the seed liquor fermentation time. (B) Control of the oxygen volume and ventilation time. HO: whole-process oxygen; SA: slightly anaerobic; HA: whole-process anaerobic; O-A: preoxygenation and postanaerobic. (C) Regulation of the feed fermentation. L1: addition of anaerobic factors; L2: maintenance of the glucose concentration control. (D) HPLC diagrams of the fermentation results. (E) pH-regulated fermentation to maximize L-LA yield. pH 5.8-M: the pH of L-LA was restored to 5.8 by adding a neutralizer once during the fermentation process; pH 4.5-A: neutralizers were added throughout the entire fermentation of L-LA to maintain the pH at 4.5.

synthesis in *S. cerevisiae* was optimized by genetic engineering, and fermentation conditions were adjusted. The obtained acid-tolerant *S. cerevisiae* TAM-L17 successfully achieved a high level of L-LA production, with a titer of 192.3 g/L (yield 0.78 g/g) in a 15-L bioreactor at pH 4.5. In summary, this study demonstrated a regulatory strategy based on a combination of metabolic engineering and fermentation regulation in acid-tolerant strains and is beneficial to explore the production of L-LA by *S. cerevisiae*.

CRediT authorship contribution statement

Tiantian Liu: Analysis of results, data compilation, writing - original draft. **Li Sun:** Screening and experimental help of original acid resistant strains S. cerevisiae strains. **Cheng Zhang:** Data collation. **Yanfeng Liu:** Writing revision. **Jianghua Li and Guocheng Du:** Supervision & administration. **Xueqin Lv:** Framework adjustment, edit, and revision. **Long Liu:** Experimental supervision & design.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.129023.

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