

Metabolic engineering of *Escherichia coli* to enhance L-serine biosynthesis

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Abstract

L-serine, an important non-essential amino acid, is widely used in the food, pharmaceutical, feed, and cosmetic industries. However, previous studies on fermentative L-serine production by *Escherichia coli* have mainly focused on improving titer, whereas insufficient attention has been paid to the conversion yield, limiting its industrial economic feasibility. In this study, systematic metabolic engineering was performed to improve L-serine yield. Using H5 as the parental strain, the L-serine re-uptake-related genes *sstT* and *tdcC* were deleted based on the CRISPR/Cas9 system to generate strain H8, in which the L-serine titer increased to 5.58 g/L and the conversion yield reached 0.37 g/g. Subsequently, the L-serine biosynthetic module, L-serine transport module, and cofactor supply module were individually enhanced in H8, resulting in recombinant strains H8-*abc*, H8-*be*, and H8-*pntab*, respectively. The resulting strains H8-*abc*, H8-*be*, and H8-*pntab* produced 6.40, 5.89, and 5.59 g/L L-serine, with yields of 0.43, 0.39, and 0.37 g/g, respectively. Further combination of effective strategies in the H8 chassis generated strain H8-*abc-be*; however, its L-serine production decreased. Finally, H8-*abc* was selected for fermentation medium optimization. After optimization, the L-serine titer reached 7.20 g/L in shake-flask fermentation, with a yield of 0.48 g/g, representing a 71.84% increase compared with the starting strain H5. These results provide a useful reference for the development of efficient *E. coli* strains for industrial L-serine production.

1. Introduction

L-serine has been widely used in food, medicine, feed, cosmetics, and other fields ^[1, 2, 3]. With the continuous expansion of downstream application scenarios, the market demand for L-serine is increasing rapidly, making it necessary to develop efficient, low-cost and sustainable production technologies ^[4]. From the perspectives of cost-effectiveness, production efficiency, environmental impact, and technical feasibility, chemical synthesis, protein extraction, and enzymatic catalysis are all restricted to varying degrees in L-serine production, and are still difficult to meet the requirements of large-scale industrial production ^[5]. In contrast, microbial fermentation can directly synthesize L-serine from inexpensive renewable carbon sources under mild conditions, while producing only the L-enantiomer, avoiding the separation and purification problems associated with racemic mixtures ^[6]. In addition, fermentation processes generally exhibit lower production costs than other routes and possess advantages such as environmental friendliness and high sustainability. Therefore, microbial fermentation has become the mainstream approach for industrial L-serine production ^[7].

At present, *E. coli* and *Corynebacterium glutamicum* are two important chassis microorganisms for constructing amino acid cell factories ^[8]. *E. coli* is characterized by rapid growth, a clear genetic background, abundant genetic tools, and high flexibility in metabolic engineering, making it suitable for precise multi-site genome editing and complex metabolic network reconstruction. Therefore, it plays an important role in mechanistic studies and strain development for high-level L-serine production. *C. glutamicum*, on the other hand, exhibits strong adaptability to industrial fermentation, high biosafety, and

extensive experience in amino acid production, showing broad prospects for industrial applications ^[9]. However, compared with *E. coli*, *C. glutamicum* grows more slowly, requires a longer fermentation period, and has relatively higher cultivation demands. By comparison, *E. coli* is more amenable to targeted metabolic engineering and facilitates the rapid accumulation of target products during high-cell-density fermentation. Therefore, *E. coli* was selected in this study as the host for L-serine fermentation production. The biosynthetic pathway of L-serine in *E. coli* has been systematically investigated. When glucose is used as the carbon source, L-serine biosynthesis mainly involves two modules: glycolysis and the L-serine biosynthetic module. In *E. coli*, L-serine is synthesized through the classical phosphorylated pathway, in which three consecutive enzymatic reactions are catalyzed by 3-phosphoglycerate dehydrogenase (PGDH, encoded by *serA*), phosphoserine aminotransferase (PSAT, encoded by *serC*), and phosphoserine phosphatase (PSP, encoded by *serB*) ^[10, 11]. In this pathway, 3-phosphoglycerate serves as the initial substrate. It is first oxidized by PGDH to form 3-phosphohydroxypyruvate. This step is generally considered the rate-limiting reaction and represents a key regulatory node in L-serine biosynthesis ^[12]. Subsequently, PSAT catalyzes the conversion of 3-phosphohydroxypyruvate to 3-phosphoserine, using glutamate as the amino donor. Finally, PSP dephosphorylates 3-phosphoserine to generate L-serine (Fig. 1).

In recent years, studies aimed at improving L-serine production have mainly focused on constructing high-producing strains. In *E. coli*, common strategies include strengthening the L-serine biosynthetic pathway, blocking degradation pathways and feedback inhibition, improving strain tolerance to L-serine through adaptive laboratory evolution, developing high-throughput screening methods and optimizing fermentation processes to obtain strains with better overall performance. Rennig et al. ^[13] deleted *sdaA*, *sdaB*, *tdcG*, and *glyA*, and subsequently enhanced the expression of three key genes in the L-serine biosynthetic pathway, namely *serA*, *serC*, and *serB*. After TARSyn-based screening and optimization, a high-producing L-serine strain was obtained. In fed-batch fermentation, the L-serine titer reached 50 g/L, with a yield of 0.36 g/g. Since excessive intracellular accumulation of L-serine can exert toxic effects on bacterial cells, Mundhada et al. ^[14] improved L-serine tolerance through adaptive laboratory evolution (ALE), and further increased L-serine accumulation by overexpressing the transporter gene *eamA*. In addition, Chen et al. ^[15] optimized the induction time, induction temperature, and dissolved oxygen level during fed-batch fermentation, and applied transcriptome sequencing to the fermentation process to reveal key metabolic pathways affecting L-serine production. Finally, the L-serine titer was increased to 45.66 g/L, with a yield of 0.39 g/g. Wang et al. ^[16] relieved L-serine-mediated feedback inhibition in *E. coli* W3110, strengthened the biosynthetic pathway, introduced a *glyA* mutant and blocked other degradation pathways. They also engineered the L-serine uptake-related genes *sdaC*, *cycA*, *sstT* and *tdcC*, and the optimized strain produced 35 g/L L-serine in a 5 L fermenter.

Although previous studies have enabled *E. coli* to achieve relatively high L-serine titers during fermentation, whereas comparatively less attention has been paid to the conversion yield from an industrial perspective, low conversion yield is a common challenge ^[17]. This study employed the L-serine-producing strain H5 as the starting strain and systematically explored several strategies to improve both

L-serine titer and conversion yield. First, the product uptake-related genes *sstT* and *tdcC* were deleted to reduce the re-uptake and reutilization of extracellular L-serine, resulting in strain H8. Subsequently, using H8 as the chassis strain, several metabolic engineering strategies were evaluated. The *serA-serC-serB* biosynthetic module from *C. glutamicum* was introduced to enhance the metabolic flux from 3-phosphoglycerate toward L-serine biosynthesis, generating strain H8-*abc*. In parallel, the transporters *EamB* and *SerE* genes were co-expressed to promote L-serine export and alleviate metabolic stress caused by intracellular product accumulation, resulting in strain H8-*be*. In addition, to enhance cofactor supply, *pntAB* was overexpressed in H8, resulting in strain H8-*pntab*. Effective strategies were further combined to construct strain H8-*abc-be*, and their effects on L-serine production performance were systematically compared. Finally, the best-performing strain for L-serine biosynthesis, H8-*abc*, was selected for fermentation medium optimization. After optimization, H8-*abc* produced 7.20 g/L L-serine in shake-flask fermentation, with a yield of 0.48 g/g, demonstrating promising potential for industrial application.

2. Materials and Methods

2.1. Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table S1. *E. coli* H5, an L-serine-producing strain previously maintained in our laboratory, was used as the parental strain. In this strain, the major degradation pathways converting L-serine to glycine and pyruvate had been blocked, and the feedback inhibition of PGDH by L-serine had been relieved through enzyme modification^[18]. *E. coli* JM109 was used for plasmid construction. The pET28a plasmid carrying the E-10 promoter was used for gene overexpression. The recombinant plasmid pDXW-10-*serA-serC-serB*, derived from *C. glutamicum* and used to enhance the L-serine biosynthetic pathway, was provided by our laboratory. Gene deletions were performed using a CRISPR/Cas9-based genome editing system.

2.2. Strain and Plasmid Construction

The full-length coding sequences of target genes were obtained according to the genome annotation information of *E. coli* K-12, and specific primers were designed accordingly. Primers were designed using SnapGene software (version 6.0, GSL Biotech) with the following criteria: melting temperature 58–60°C, GC content 40%-60%, and product size matching the predicted gene lengths. A homologous arm sequences complementary to the linearized plasmid were introduced at the 5' end of the primers, laying the foundation for subsequent homologous recombination ligation.

PCR amplification was performed in a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following conditions: initial denaturation at 95°C for 10 min; 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s/kb; and a final extension at 72°C for 10 min, then stored at 4°C.

Recombinant plasmids were constructed by homologous recombination. The total volume of the reaction system was 10 µL, containing 40 ng/1000 bp of target gene, 20 ng/1000 bp of linearized

plasmid, and an equal volume of homologous recombinase C116. The system was placed in a PCR amplifier and incubated at 50°C for 10 min; immediately after the reaction, it was stored at 4°C to prevent degradation of the recombinant product.

The recombinant plasmids were introduced into competent *E. coli* JM109 cells using the standard heat-shock transformation method. The transformed cells were recovered in antibiotic-free LB medium, then spread on LB solid plates supplemented with 50 µg/mL kanamycin and incubated at 37°C in a constant temperature incubator (Shanghai Boxun, China). Transformants were selected and verified by colony PCR and sequencing to ensure correct insertion of the target genes without DNA mutations. The verified recombinant plasmids were then transformed into competent *E. coli* cells. Transformants were selected from agar plates and verified by colony PCR. Colonies showing correct bands by agarose gel electrophoresis were regarded as successfully constructed recombinant strains. Sequencing and primer synthesis services were provided by Talen-Biological Technology (Wu Xi, China). PrimeSTAR Max DNA Polymerase, C116 homologous recombinase, plasmid extraction kits, and gel extraction kits were purchased from Vazyme Biotech (Nanjing, China).

2.3. Medium

Luria–Bertani (LB) medium was used for routine cultivation, DNA manipulation, and seed culture. The LB medium contained the following components (g/L): peptone 10, yeast extract 5, and NaCl 10. The fermentation medium used for shake-flask cultivation was a modified AMI medium based on a previously reported medium^[19]. The AMI medium contained the following components (g/L): glucose 15, diammonium hydrogen phosphate 8.6, ammonium dihydrogen phosphate 3.9, KCl 0.15, 3-(N-morpholino) propanesulfonic acid (MOPS) 20.9, and yeast extract 1. The components were dissolved in deionized water, and the pH was adjusted to 7.0 using Na₂CO₃ solution. Trace element solution and glycine solution were added at the time of inoculation.

The AMI trace element solution contained the following components (g/L): FeCl₃·6H₂O 2.4, CoCl₂·6H₂O 0.3, ZnCl₂ 0.3, Na₂MoO₄·2H₂O 0.3, H₃BO₃ 0.07, MnCl₂·4H₂O 0.5, and CuCl₂·2H₂O 0.15. These components were dissolved in 120 mM HCl solution and sterilized by filtration through a 0.22 µm sterile aqueous membrane. The trace element solution was added to the fermentation medium at a ratio of 0.1% (v/v, 30 µL). A 2 M glycine stock solution (150 g/L) was prepared and added to the fermentation medium at a ratio of 0.1% (v/v, 30 µL).

2.4. L-serine fermentation in *E. coli*

The cultivation methods used in this study were as follows. For shake-flask cultivation, frozen glycerol stocks were streaked onto LB agar plates containing the corresponding antibiotics and incubated at 37°C for 12 h. A single colony was inoculated into 10 mL LB medium supplemented with glycine and cultured at 37°C and 220 rpm for 12 h. Subsequently, 50 µL of the culture was transferred into 50 mL LB medium at an inoculation ratio of 0.1% (v/v) and cultivated at 37°C and 220 rpm for another 12 h in a rotary shaker. The seed culture was then inoculated into fermentation medium (30 mL in a 250 mL shake

flask) to an initial OD₆₀₀ of approximately 0.20. Sterile trace element solution and glycine solution were added simultaneously. Fermentation was performed at 37°C and 220 rpm for 60 h.

For strains harboring inducible plasmids, IPTG was added when the OD₆₀₀ of the culture reached approximately 1.0, and the fermentation temperature was then decreased to 30°C for induction.

2.5. Single-factor optimization of the fermentation medium

To further improve the L-serine production performance of the engineered strain, the composition of the fermentation medium was optimized using a one-factor-at-a-time strategy. In each experiment, only one factor was varied while all other medium components and fermentation conditions were kept constant. L-serine titer, cell growth, and glucose-to-L-serine conversion yield were used as the main evaluation indicators. The effects of glycine concentration, carbon source type, glucose concentration, Vitamin B1 concentration, Vitamin H concentration, and betaine concentration on the fermentation performance of engineered strain were investigated sequentially. Each round of optimization was performed based on the optimal condition obtained from the previous round, with only the tested factor being changed and all other components remaining unchanged. All shake-flask fermentation experiments were conducted under identical culture conditions, and each group was performed in triplicate.

2.6. Analytical methods

Cell growth was monitored by measuring the optical density at 600 nm (OD₆₀₀) using a UV-1800 spectrophotometer (Shimadzu, Japan). Fermentation broth samples were centrifuged at 12,000×g for 5 min using an Eppendorf 5424R refrigerated centrifuge (Eppendorf, Germany), and the supernatants were collected and diluted appropriately. Glucose concentration in the fermentation broth was determined using an M-100 biosensor analyzer (Shenzhen, China), with glucose standard solution used as the reference. The L-serine titer during fermentation was determined according to a previously published method ^[20]. All measurements were performed in triplicate.

3. Results and Discussion

3.1. Effect of L-serine re-uptake gene knockout on strain growth and L-serine production in *E. coli*

To reduce product loss caused by the re-uptake and subsequent metabolism of extracellular L-serine, the L-serine re-uptake-related genes *sstT*, *tdcC*, and *cycA* were sequentially deleted using a CRISPR/Cas9-based genome editing system in strain H5. First, deletion of *sstT* generated strain H7, then deletion of *tdcC* in H7 yielded the double-knockout strain H8. Further deletion of *cycA* in H8 resulted in, the triple-knockout strain H9 (Fig. 2A). Fermentation results showed that H7 produced 5.38 g/L L-serine, representing a 28.40% increase compared with the parental strain H5 (4.19 g/L). Then the double-knockout strain H8, in which the L-serine titer reached 5.58 g/L, corresponding to a 3.72% increase

relative to H7, with the yield of 0.37 g/g, which was 3.72% higher than that of H7 (0.36 g/g). However, L-serine titer of H9, decreased to 4.20 g/L (Fig. 2B).

These results indicate that moderate attenuation of L-serine re-uptake is beneficial for extracellular product accumulation, whereas excessive disruption of amino acid transport systems may adversely affect cellular physiology and metabolic balance. Previous studies have shown that membrane transporter genes such as *sstT*, *tdcC*, and *cycA* participate in the transmembrane transport of L-serine and related amino acids. During fermentation, extracellularly secreted L-serine may be re-imported into the cell and subsequently enter catabolic pathways or other metabolic branches, thereby reducing its net accumulation in the fermentation broth. Therefore, blocking re-uptake pathways is an effective strategy for improving extracellular product accumulation. Wang et al. [21] individually and combinatorially deleted L-serine uptake genes in *E. coli* W3110 and found that the triple-gene deletion strain lacking *sstT*, *tdcC*, and *sdaC* showed the highest L-serine of 34.8 g/L in a 5-L fermenter. Then, deletion of the fourth gene, *cycA*, in the *sstT*, *tdcC*, and *sdaC* deletion background resulted in decreased L-serine production and biomass. These transport systems also contribute to amino acid uptake and the maintenance of intracellular metabolic homeostasis; thus, deletion of a greater number of transporter genes is not necessarily more favorable for product synthesis. Considering the balance between L-serine accumulation and cellular physiological stability, the double-knockout strain H8 exhibited the best overall fermentation performance and was therefore selected as the chassis strain for subsequent metabolic engineering.

3.2. Effect of enhancing the L-serine biosynthetic pathway on L-serine production in *E. coli*

In this study, a recombinant plasmid carrying the *serA*, *serC*, and *serB* genes from a high L-serine-producing *C. glutamicum* was introduced into strain H8, to generate the recombinant strain H8-*abc*. The L-serine titer of H8-*abc* increased to 6.40 g/L, representing a 14.70% improvement over H8(5.58 g/L L-serine with a yield of 0.37 g/g), while the conversion yield reached 0.43 g/g, corresponding to a 14.70% increase. In addition, the OD₆₀₀ of H8-*abc* showed only a slight difference from that of the parental strain H8, indicating that moderate enhancement of the L-serine biosynthetic pathway can improve product formation without significantly impairing cell growth (Fig. 3A).

L-serine biosynthesis mainly proceeds through the phosphorylated pathway, in which the three key enzymatic steps are crucial for L-serine production in *E. coli* and *C. glutamicum*. Therefore, strengthening the *serA-serC-serB* module is generally considered a primary strategy for improving L-serine biosynthetic capacity. Although these three genes catalyze the same reactions in the two species, differences still exist in their enzymatic properties and regulatory mechanisms. Among them, 3-phosphoglycerate dehydrogenase, encoded by *serA*, is widely recognized as the key rate-limiting enzyme in the L-serine biosynthetic pathway.

In previously reported studies on L-serine production in *E. coli*, enhancement of the endogenous *E. coli* *serA-serC-serB* module has commonly been adopted to improve L-serine biosynthesis. However, PGDH in *E. coli* is subject to feedback inhibition by L-serine. To relieve this end-product inhibition, feedback-resistant variants of *E. coli serA* have often been constructed through site-directed mutagenesis. Overexpression of feedback-resistant *serA* mutants, combined with further strengthening of *serC* and *serB* expression, can significantly promote L-serine accumulation. Nevertheless, excessive expression of *serA* may also lead to metabolic imbalance, thereby impairing cell growth and L-serine production. Therefore, although previous studies have demonstrated the importance of reinforcing the L-serine biosynthetic module for increasing metabolic flux toward L-serine, most of these strategies have been limited to the modification of endogenous homologous genes in *E. coli*. Their improvement is still constrained by the intrinsic catalytic properties and feedback regulation of native *E. coli* enzymes [22].

In contrast, PGDH derived from *C. glutamicum* exhibits distinct regulatory characteristics. Its C-terminal domain is closely associated with L-serine-mediated feedback inhibition, but is not essential for catalytic activity [23]. Therefore, C-terminal truncation can be used to relieve feedback inhibition. Previous studies have shown that the C-terminal 197 amino acids of *C. glutamicum* PGDH are mainly involved in L-serine feedback inhibition [24], and deletion of this region has only a limited effect on catalytic activity while markedly reducing the sensitivity of PGDH to L-serine. In addition, PGDH activity can also be optimized through promoter engineering or genomic expression regulation, Gao et al. [25] characterized the native promoters of *serA*, *serC*, and *serB* in *C. glutamicum* and found that *serA* exhibited the highest promoter strength. Replacement of the native promoters of these three genes further showed that overexpression of *serA* caused a significant change in L-serine titer, indicating the pivotal role of *serA* as a major control point in the L-serine biosynthetic pathway.

In the present study, instead of directly strengthening the endogenous *serA-serC-serB* module of *E. coli*, we introduced a heterologous L-serine biosynthetic module derived from *C. glutamicum*. This strategy provides a new enzyme source for L-serine biosynthesis and helps overcome the limitations imposed by the catalytic properties and feedback regulation of native *E. coli* enzymes. The experimental results showed that introduction of the *C. glutamicum*-derived *serA-serC-serB* module effectively improved both L-serine titer and the conversion yield. These results suggest that the heterologous biosynthetic module can more efficiently redirect carbon flux toward L-serine synthesis and effectively break through the limitation of the native biosynthetic flux in *E. coli*.

3.3. Effect of enhancing the L-serine transport pathway on L-serine production in *E. coli*

To investigate the effects of enhancing L-serine transporters on the cell growth and L-serine production, the transporter genes *eamA*, *eamB* from *E. coli*, and *serE* from *C. glutamicum* were selected to overexpress in strain H8. Recombinant strains with individual enhanced expression, H8-*serE*, H8-*eama*, and H8-*eamb*, as well as strains with random combinatorial enhanced expression, H8-*be* (*eamb* and *serE*), H8-*ae* (*eama* and *serE*), H8-*ba* (*eamb* and *eama*), and H8-*abe* (*eama*, *eamb*, and *serE*), were

constructed. (Fig. 4A). The results showed that the OD₆₀₀ values of the single-transporter overexpression strains were not significantly different from that of the parental strain H8 (5.22), indicating that overexpression of a single transporter had little effect on cell growth. In contrast, strains carrying combinatorial transporter modules exhibited a more pronounced growth advantage. In particular, the OD₆₀₀ values of H8-ae and H8-be increased by 4.89% and 4.69%, respectively, compared with H8 (Fig. 4B). In terms of L-serine production, the strain H8-be, showed the best performance. The L-serine titer of H8-be reached 5.89 g/L, representing a 5.56% increase over that of H8, while the yield reached 0.39 g/g, corresponding to a 5.56% improvement (Fig. 4C).

In addition to the biosynthetic pathway, the transport pathway is another key factor affecting L-serine production. Transmembrane transport of amino acids plays an important role in maintaining intracellular homeostasis and promoting extracellular product accumulation, therefore transporter engineering has become an important strategy for improving amino acid production [26]. Previous studies have shown that excessive intracellular accumulation of L-serine in engineered strains exerts toxic effects on cells, which not only inhibits cell growth but also restricts further product synthesis. Thus, enhancing export capacity is an effective approach to relieve the metabolic stress caused by intracellular L-serine accumulation. In *E. coli*, no transporter specifically dedicated to L-serine export has been clearly identified. Due to the structural similarity between L-serine and cysteine, the cysteine transporters EamA and EamB have been reported to also mediate L-serine transport. Mundhada et al. [14] found that overexpression of *eamA*, which encodes a cysteine exporter in *E. coli*, significantly increased cellular tolerance to L-serine and improved its accumulation, suggesting that reinforcement of the export system can alleviate intracellular product inhibition and promote L-serine secretion. More recently, Gao and Huang et al. [27, 28] through comparative transcriptomic analysis and functional characterization of differentially expressed genes, demonstrated that besides the previously reported ThrE and SerE, NCgl0254 and NCgl0255 are also involved in L-serine transport in *C. glutamicum*, and further identified SerE as the major L-serine exporter in this organism.

Compared with previous studies, the results likewise demonstrate the importance of transport engineering for improving L-serine production. Most earlier studies mainly focused on functional characterization of a single exporter or on strengthening native export systems in a homologous host. In contrast, this study simultaneously introduced and enhanced exporters from different origins in an *E. coli* chassis, including the endogenous *E. coli* exporters EamA and EamB as well as the *C. glutamicum*-derived exporter SerE. Therefore, our strategy was not simply to strengthen one individual export route, but rather to construct a composite transport system composed of both endogenous and heterologous exporters in order to increase the extracellular export of L-serine. In comparison, strains individually overexpressing *eamA*, *eamB*, or *serE* all showed lower L-serine production than the combined-expression strain, indicating that the transport capacity of a single exporter may be insufficient to fully support the efflux of intracellularly accumulated L-serine. The combined strain H8-be showed a 7.46% increase in L-serine production, further suggesting that coordinated expression of multiple exporters can synergistically enhance export capacity and promote product secretion more effectively than expression

of any single exporter alone. This result showed that the close relationship between transporter engineering and cellular physiological state. Once the export capacity is improved, intracellular L-serine accumulation is reduced, feedback inhibition is relieved, and central metabolic pathways such as glycolysis and the tricarboxylic acid cycle can operate more efficiently, thereby supporting simultaneous improvement in both cell growth and L-serine production. Nevertheless, optimization of the transport system is not simply a matter of increasing export or completely blocking uptake. Membrane transport processes are closely associated with cell growth, amino acid homeostasis, and overall metabolic balance, and excessive weakening of uptake capacity or overexpression of membrane proteins may adversely affect cellular physiology.

3.4. Effect of cofactor supply enhancement on L-serine production in *E. coli*

To improve intracellular cofactor availability, four key genes—(*nadE*, which participates in the de novo biosynthesis of NAD⁺, *nadK*, which catalyzes the phosphorylation of NAD(H) to NADP(H), *Pos5p* a mitochondrial NADH kinase-derived gene that can provide NADPH, and *pntAB*, which encodes the membrane-bound pyridine nucleotide transhydrogenase and enables proton-coupled conversion between NADH and NADPH) involved in cofactor regulation were selected for overexpression. And the single overexpression strains H8-*nade*, H8-*nadk*, H8-*pos5p*, and H8-*pntab* were constructed (Fig. 5A). Under different cofactor engineering strategies, enhancement of cofactor supply led to a certain increase in biomass in all recombinant strains. Among them, strain H8-*pntab*, with enhanced expression of *pntAB*, showed the highest L-serine production. The L-serine titer of this strain reached 5.59 g/L, and the conversion yield was 0.37 g/g. Meanwhile, the two genes showing the most obvious improvement in L-serine production, *pos5P* and *pntAB*, were combined for further enhancement to generate strain H8-*pp*, the L-serine titer of H8-*pp* decreased to 4.64 g/L (Fig. 5B).

L-serine biosynthesis is closely related to the intracellular redox state. In the biosynthetic pathway, the oxidation of 3-phosphoglycerate catalyzed by *serA* requires NAD⁺ as a cofactor, while cellular biosynthetic processes require a large amount of NADPH for reductive reactions. When cofactor supply is insufficient, the cells may exhibit slow growth, reduced glycolytic flux, and limited L-serine production capacity [29]. Previous studies have shown that regulating the biosynthesis, regeneration, and interconversion of nicotinamide cofactors can effectively improve the product synthesis capacity of microbial cell factories [30]. Therefore, in this study, from the perspective of cofactor regeneration, NAD(H)/NADP(H)-related metabolism in *E. coli* was enhanced to improve cell growth and L-serine biosynthetic capacity.

Among the cofactor engineering strategies tested, *pntAB* overexpression maintained L-serine production at a level comparable to that of H8, whereas the other strategies showed limited or negative effects. This result suggests that the key constraint on L-serine biosynthesis may not simply be an insufficient total intracellular supply of NAD(H) or NADP(H), but rather an inadequate coordination between these two reducing cofactor pools, that is, the lack of an effective dynamic balance between NADH and NADPH.

As a transmembrane transhydrogenase, PntAB catalyzes the reversible conversion between NADH and NADPH and can regenerate NADPH from NADH when the cellular demand for NADPH increases, thereby optimizing the distribution of reducing power. Therefore, compared with strategies that merely increase the level of a single cofactor, the balancing effect mediated by PntAB is better able to meet the dynamically changing cofactor requirements during L-serine biosynthesis, and thus more effectively produces L-serine. It is worth noting that L-serine biosynthesis involves the balance of cofactors such as NADH/NAD⁺, and the effect of cofactor engineering usually depends on the overall metabolic state and redox demand of the chassis strain ^[31]. If intracellular cofactor supply is not the main bottleneck at the current stage, individual enhancement of transhydrogenase systems such as *pntAB* may not significantly improve target product synthesis and may even disturb the original intracellular redox homeostasis.

3.5. Construction of an L-serine-producing recombinant *E. coli* strain and fermentation optimization

To further improve L-serine production, strain H8 was used as the chassis, and the effective strategies identified in the previous experiments were combined. The biosynthetic pathway and transport pathway were simultaneously enhanced in H8, resulting in the recombinant strain H8-*abc-be*. The L-serine titer of H8-*abc-be* was 4.41 g/L, and the yield reached 0.29 g/g, showing no obvious improvement compared with H8. To further improve the L-serine titer and yield of the recombinant strain, the best-performing strain H8-*abc* was selected for single-factor optimization of the fermentation medium. The effects of glycine concentration, carbon source type, glucose concentration, vitamin B1 concentration, vitamin H concentration, and betaine concentration on fermentation performance were investigated individually. The results showed that the optimal fermentation conditions were as follows: 0.2 g/L glycine, glucose as the carbon source, 15 g/L glucose, 1 mg/L vitamin B1, 2.5 mg/L vitamin H, and 0.8 g/L betaine. Under these conditions, the L-serine titer of strain H8-*abc* reached 7.2 g/L, and the yield was 0.48 g/g (Fig. 6). Under these conditions, both the cell growth and L-serine production were maintained at relatively favorable levels, and the medium components showed a good synergistic effect, which could better meet the demands of cell growth and L-serine biosynthesis.

L-serine biosynthesis is not only determined by the diversion of the glycolytic intermediate 3-phosphoglycerate, but is also closely associated with cell growth, energy metabolism, cofactor balance, and one-carbon unit supply ^[32]. Although optimization of the biosynthetic module and the export module may each exert a positive effect when implemented individually, their combined effects are not necessarily additive. On the one hand, accelerated export may alter intracellular L-serine homeostasis and thereby affect the regulatory state of enzymes involved in related metabolic pathways. On the other hand, simultaneous strengthening of multiple modules may disturb the original metabolic balance, creating new trade-offs among carbon flux distribution, serine homeostasis, and cell growth, which in turn limits further improvement in production. Therefore, the major limiting step should be identified according to the metabolic characteristics of different chassis strains, rather than simply stacking all potentially beneficial modules.

In addition to metabolic engineering, which can improve the intrinsic production capacity of the strain, fermentation conditions also play an important role in fully releasing strain performance^[33]. Rational optimization of medium composition can also effectively release the L-serine production potential of recombinant strains, allowing the intrinsic effects of metabolic engineering to be fully manifested during actual fermentation and ultimately achieving efficient conversion of glucose into L-serine. Therefore, in future studies, beneficial pathways could be integrated into the host genome. At the same time, the recombinant strain H8-*abc* could be evaluated in fermenter, combined with fed-batch fermentation strategies, dissolved oxygen regulation, and process parameter optimization, to further explore its potential for improved production.

4. Conclusion

In this study, the L-serine-producing strain H5, was used as the starting strain. The L-serine re-uptake-related genes were deleted based on the CRISPR/Cas9 system to generate the best chassis strain H8 firstly. Then, systematic engineering was carried out at three levels, including strengthening the biosynthetic pathway, optimizing the transport system, and regulating cofactor supply, and the optimal host strain H8-*abc* was obtained. After further optimization of the fermentation medium, the strain H8-*abc* could produce 7.2 g/L L-serine with yield of 0.48 g/g, representing a 71.84% increase compared with the starting strain H5. These results provide a new strategy for constructing efficient industrial L-serine-producing strains.

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Declarations

Author Contribution

Conceptualization: K. X., Y. H., X. Z., M. X., G. X., X. Z., H. L., J. S. and Z. X. Methodology: K. X., Y. H., H. L., Z. X. and X. Z. Formal analysis and investigation: K. X., Y. H., H. L., Z. X. and X. Z. Writing—original draft preparation: K. X., Y. H. and X. Z. Writing, review, and editing: K. X., Y. H., X. Z., G. X., M. X., X. Z., H. L., J. S. and Z. X. Funding acquisition: X. Z. and Z. X. Resources: X. Z. and Z. X. Supervision: Z. X.

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Figures

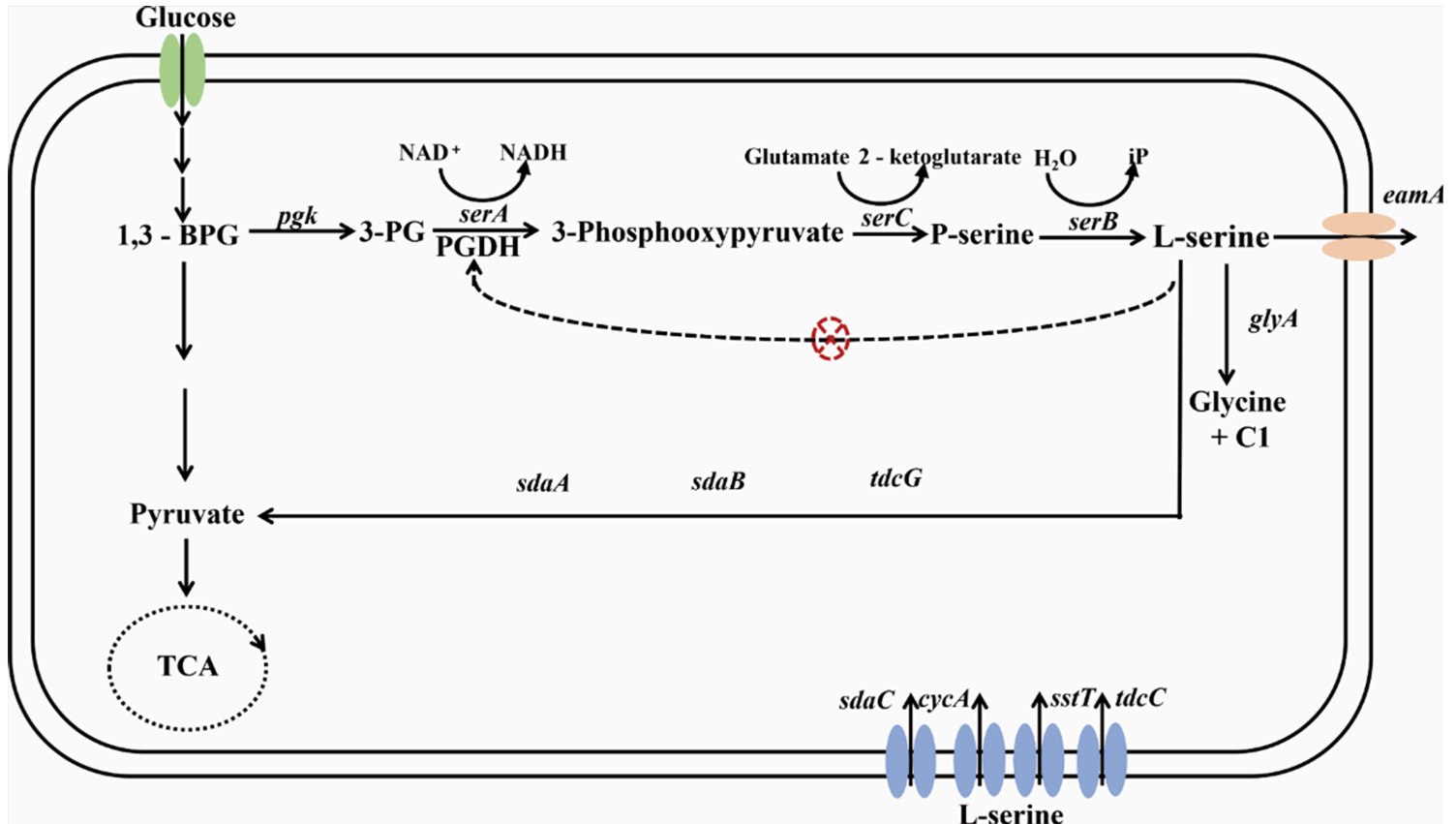


Figure 1

Biosynthetic and metabolic pathway of L-serine. Each gene encodes the following: *pgk*: phosphoglycerate kinase; *serA*: 3-phosphoglycerate dehydrogenase; *serC*: phosphoserine aminotransferase; *serB*: phosphoserine phosphatase; *glyA*: serine hydroxymethyltransferase; *sdaA*, L-serine deaminase A; *sdaB*: L-serine deaminase B; *tdcG*: threonine/serine deaminase; *eamA*: L-serine exporter; *sdaC*: serine transporter; *cycA*: glycine/serine/alanine transporter; *sstT*: serine/threonine transporter; *tdcC*: threonine/serine transporter. Abbreviations: 1,3-BPG: 1,3-bisphosphoglycerate; 3-PG: 3-phosphoglycerate; PGDH: 3-phosphoglycerate dehydrogenase; P-serine: O-phospho-L-serine; iP : inorganic phosphate; C1: one-carbon unit; TCA: tricarboxylic acid cycle; NAD^+ : oxidized nicotinamide adenine dinucleotide; NADH : reduced nicotinamide adenine dinucleotide.

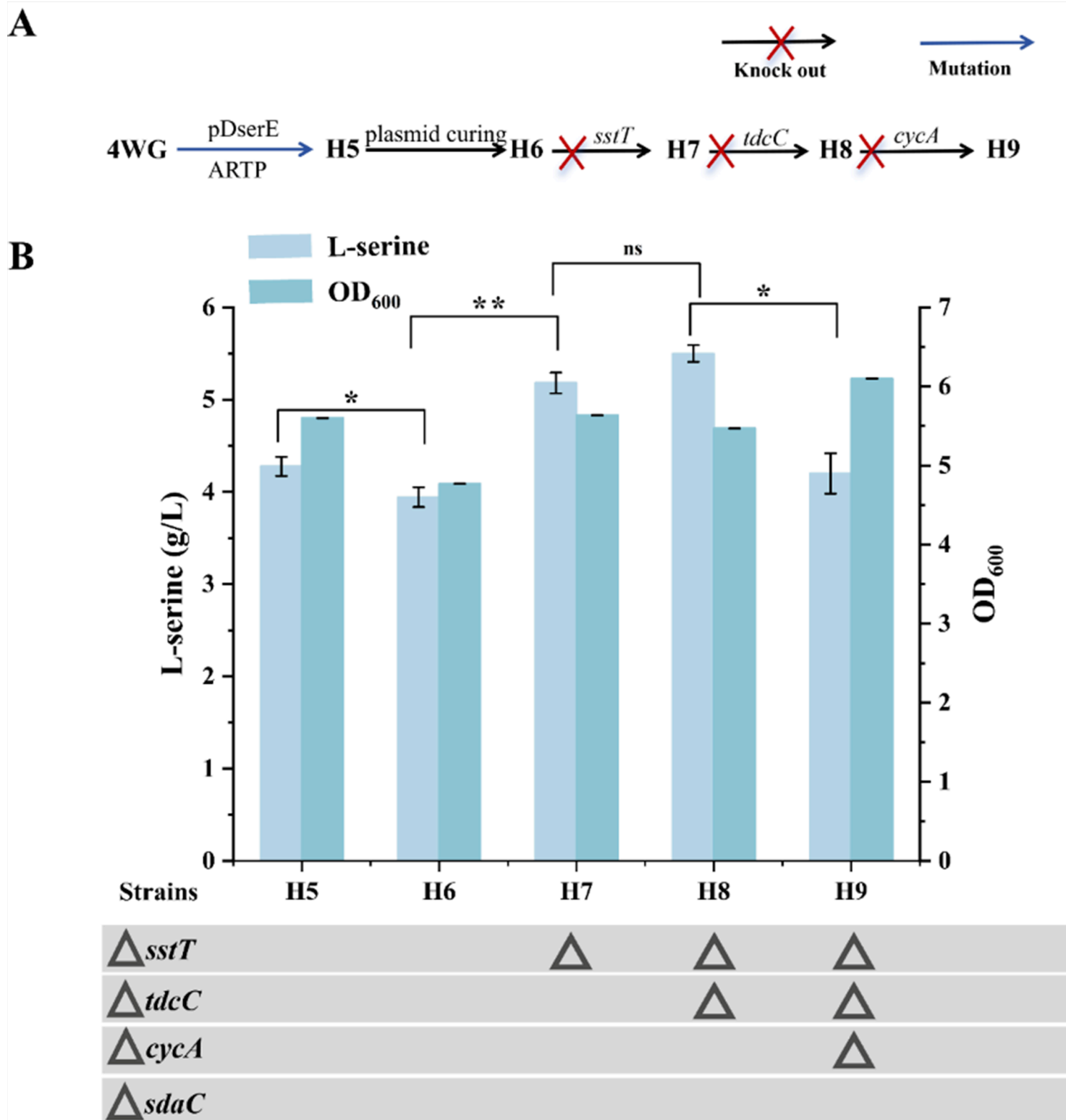


Figure 2

Effects of knockout of genes associated with product re-uptake. (A) Construction of serine-uptake gene-knockout strains in *E. coli*. (B) Results of shake flask fermentation of the *E. coli* engineered strains. Data are presented as mean $\pm$ SD ($n = 3$). Statistical significance was evaluated using Student's t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant)

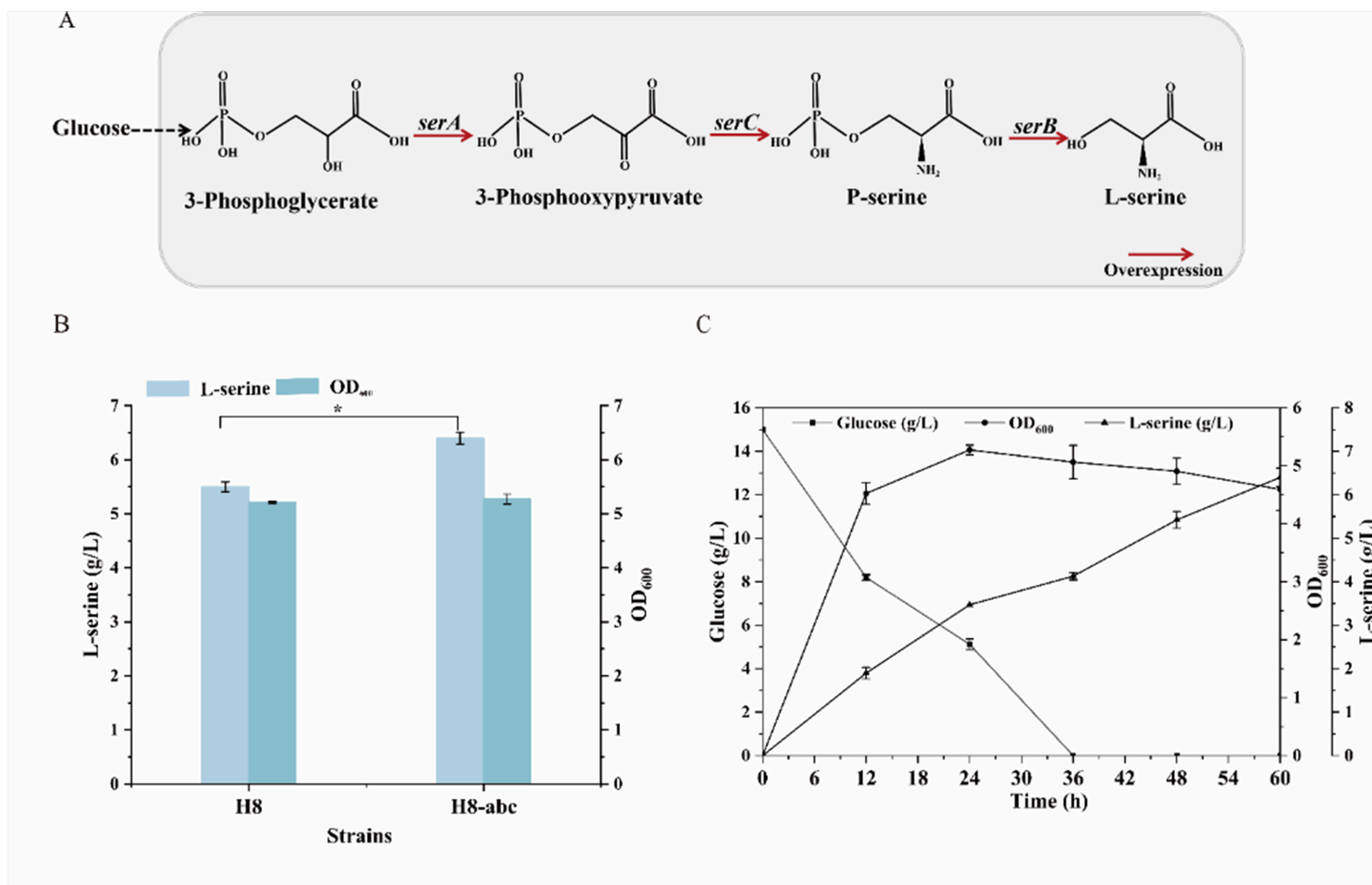


Figure 3

Effects of enhancing the biosynthetic pathway on L-serine production. (A) Schematic diagram for enhancing the serine biosynthetic pathway. Red arrows represent gene overexpression. (B) Shake-flask fermentation results of *E. coli* engineered strains. (C) Analysis of the fermentation process of *E. coli* H8-abc. Data are presented as mean $\pm$ SD ($n = 3$). Statistical significance was evaluated using Student's *t* test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant)

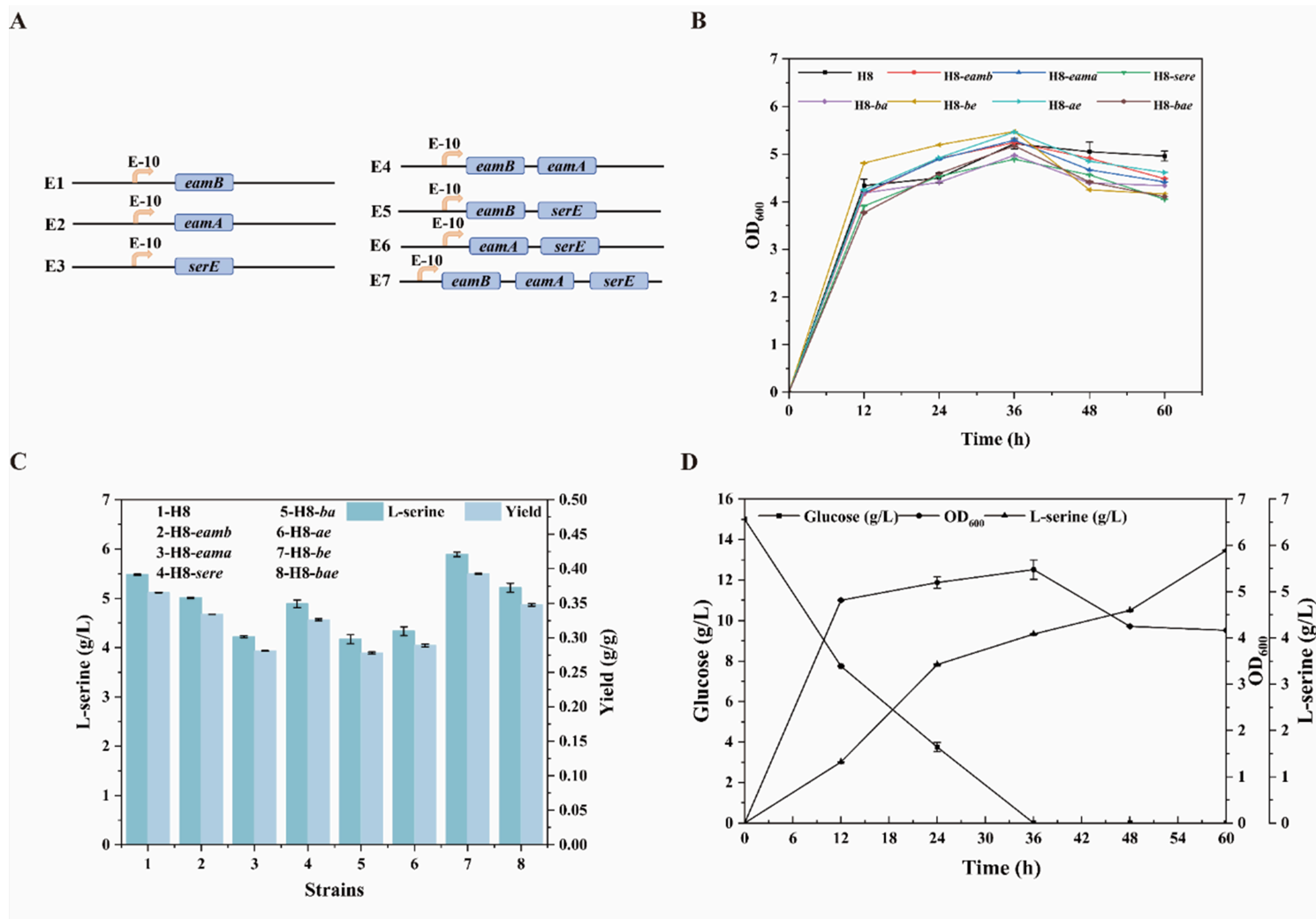


Figure 4

Effects of enhancing the L-serine transport pathway on L-serine production. (A) Construction of recombinant plasmids for overexpression of serine transporters. (B) The cell growth OD_{600} of the *E. coli* engineered strains. (C) The L-serine titer and conversion yield of the *E. coli* engineered strains. (D) Analysis of the fermentation process of *E. coli* H8-be. Data are presented as mean $\pm$ SD ($n = 3$).

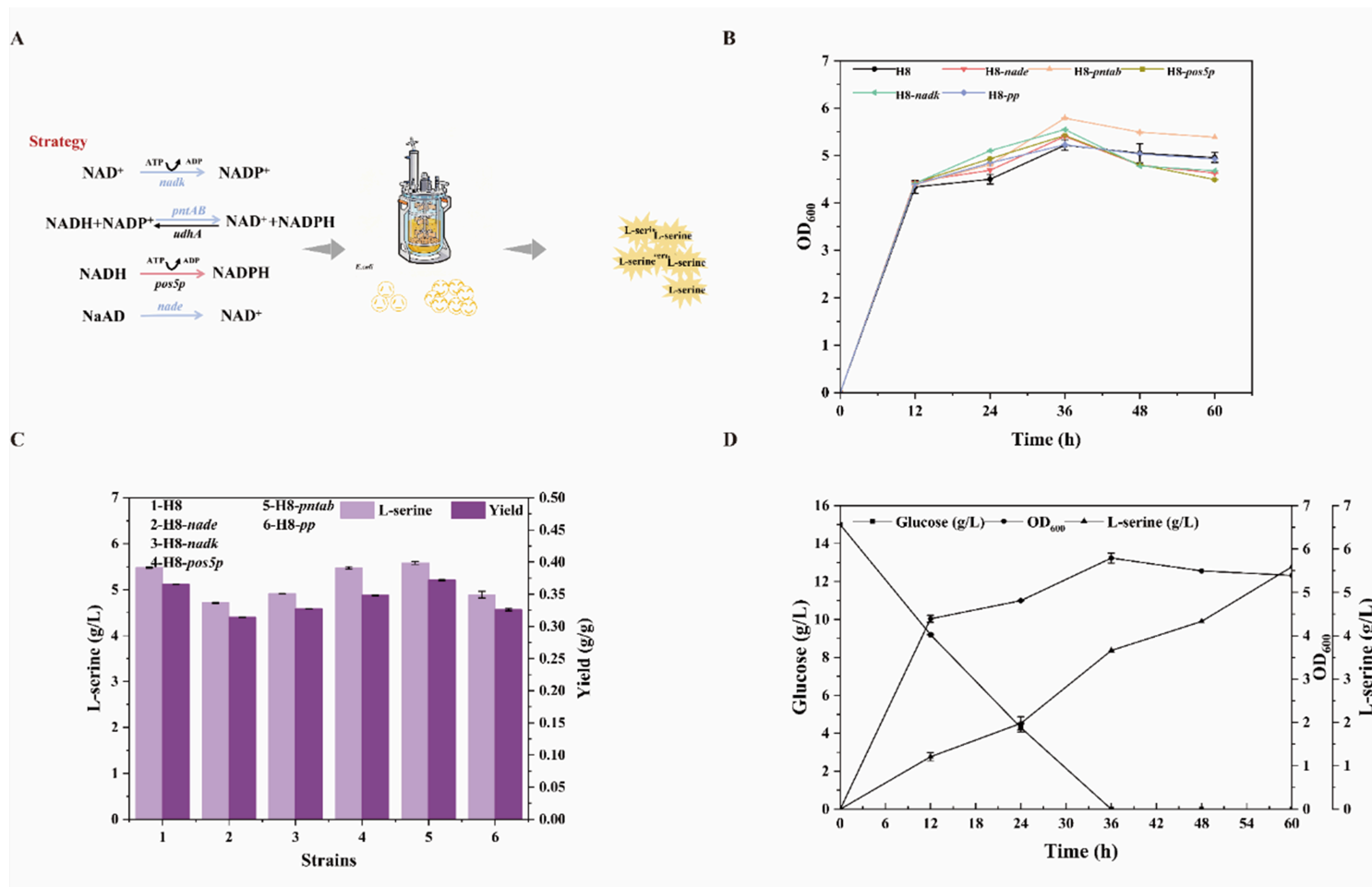


Figure 5

Effects of cofactor supply enhancement on L-serine production. (A) Strategies for enhancing intracellular cofactor supply of *E. coli*. (B) The cell growth OD₆₀₀ of the *E. coli* engineered strains. (C) The L-serine titer and conversion yield of the *E. coli* engineered strains. (D) Analysis of the fermentation process of *E. coli* H8-pntab. Data are presented as mean $\pm$ SD ($n = 3$).

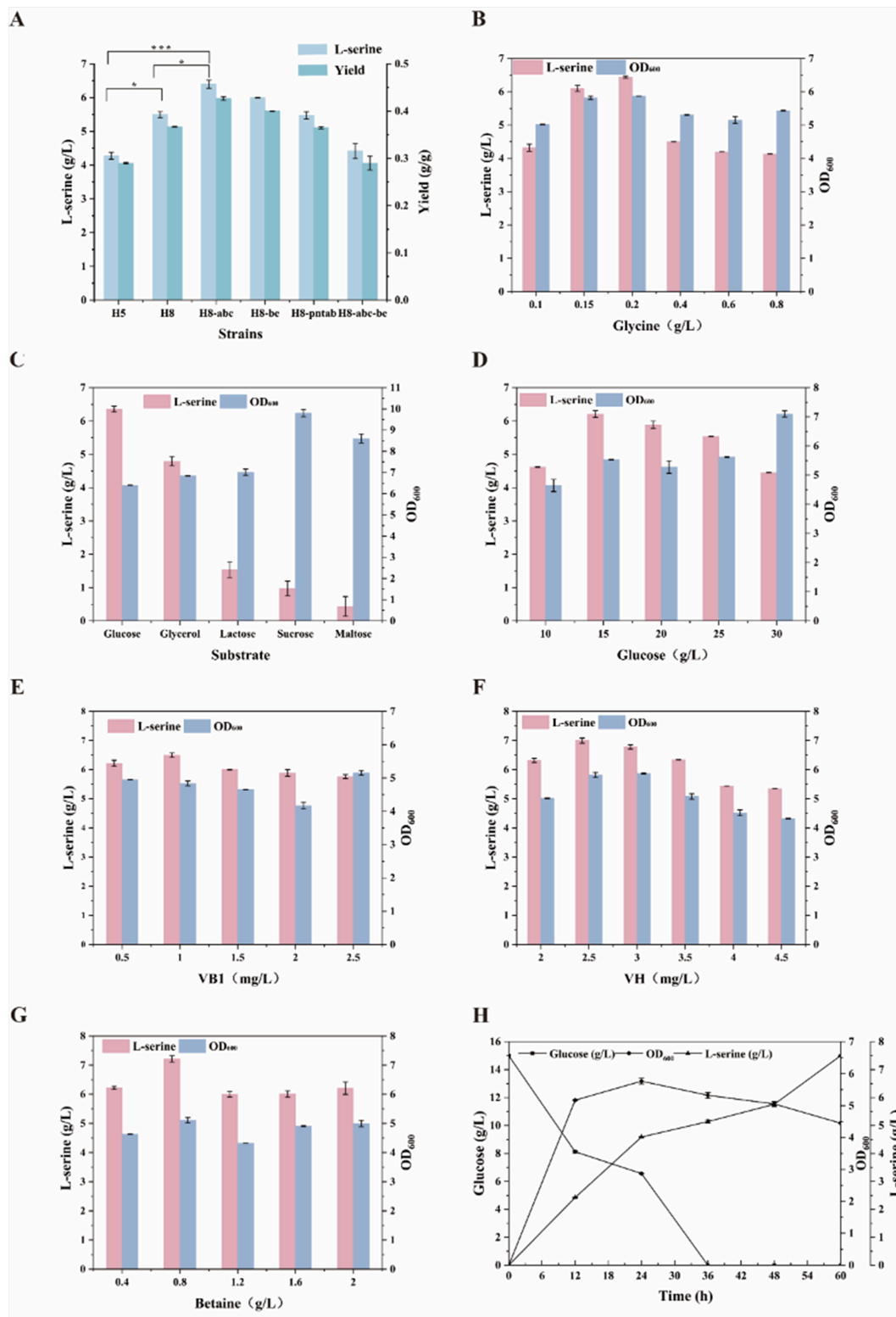


Figure 6

Construction of L-serine-producing *E. coli* strains and Optimization of fermentation media. (A) Results of shake flask fermentation of the *E. coli* engineered strains. (B) Fermentation results of *E. coli* H8-abc under different glycine concentrations. (C) Fermentation results of *E. coli* H8-abc under different different types of carbon sources. (D) Fermentation results of *E. coli* H8-abc under different glucose concentrations. (E) Fermentation results of *E. coli* H8-abc under different VB1 concentrations. (F) Fermentation results of *E.*

*coli*H8-*abc* under different VH concentrations. (G) Fermentation results of *E. coli* H8-*abc* under different betaine concentrations. (H) Analysis of the fermentation process of *E. coli* H8-*abc*. Data are presented as mean $\pm$ SD ($n = 3$). Statistical significance was evaluated using Student's t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant)

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