Journal of Industrial Microbiology & Biotechnology (2019) 46:221–230
https://doi.org/10.1007/s10295-018-2113-6

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GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL ORGANISMS - ORIGINAL PAPER

High-yield production of L-serine from glycerol by engineered *Escherichia coli*

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Received: 25 April 2018 / Accepted: 20 November 2018 / Published online: 2 January 2019

Abstract

L-Serine is widely used in pharmaceutical, food and cosmetic industries, and the direct fermentation to produce L-serine from cheap carbon sources such as glycerol is greatly desired. The production of L-serine by engineered *Escherichia coli* from glycerol has not been achieved so far. In this study, *E. coli* was engineered to efficiently produce L-serine from glycerol. To this end, three L-serine deaminase genes were deleted in turn, and all of the deletions caused the maximal accumulation of L-serine at 0.06 g/L. Furthermore, removal of feedback inhibition by L-serine resulted in a titer of 1.1 g/L. Additionally, adaptive laboratory evolution was employed to improve glycerol utilization in combination with the overexpression of the cysteine/acetyl serine transporter gene *eamA*, leading to 2.36 g/L L-serine (23.6% of the theoretical yield). In 5-L bioreactor, L-serine titer could reach up to 7.53 g/L from glycerol, demonstrating the potential of the established strain and bioprocess.

Keywords Adaptive laboratory evolution · *Escherichia coli* · Genetic engineering · Glycerol · L-Serine

Introduction

L-Serine is widely used in the pharmaceutical, food and cosmetic industries. Compared with other amino acids, accumulation of L-serine is more difficult in wild-type strains because it is an intermediate metabolite [2]. Following metabolic engineering of *Corynebacterium glutamicum* (*C. glutamicum*) ATCC13032, 36.5 g/L L-serine was produced [15]. In our laboratory, the *C. glutamicum* SYPS-062-33aΔSSAAI strain was constructed by random mutagenesis and metabolic engineering, which produced 42.62 g/L of L-serine with 21.3% mass yield [20, 26], and this was the highest titer produced by organism.

Compared with *C. glutamicum*, *E. coli* has a higher growth rate, and has also been widely used in the synthesis of amino acids [13, 16, 23]. However, L-serine is not accumulated by wild-type *E. coli*. By deleting the three L-serine deaminase genes, *sdaA*, *sdaB*, and *tdcG*, recombinant *E. coli* NW-7 could produce 3.8 mg/L L-serine from glucose [6]. Recombinant *E. coli* DH5α YF-7 was constructed by overexpressing L-serine synthetic genes, blocking the L-serine degradation pathway, and regulating the glyoxylate cycle, and the resulting strain could produce 8.45 g/L L-serine from glucose in fed-batch fermentation [5]. Recently, the recombinant *E. coli* MG1655 strain was engineered in which L-serine degradation was prevented, and *eamA* (a cysteine/acetyl serine transporter) were overexpressed, resulting in L-serine titer of 11.7 g/L from glucose with threonine addition in fed-batch fermentation [11]. Further, they obtained an evolved strain using adaptive laboratory evolution, and the strain accumulated 37 g/L L-serine from glucose with a 24% mass yield [12], is the highest L-serine value produced by *E. coli.* However, the strain was glycine auxotroph, therefore, the addition of glycine was required to maintain the fermentation.

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All aforementioned studies were based on the production of L-serine using glucose as a carbon source. The direct fermentative production of L-serine from glycerol is greatly desired (Fig. 1), because glycerol is a renewable resource generated as a by-product from the biodiesel production process [21]. Moreover, glycerol assimilation is initiated by its ATP-dependent phosphorylation via glycerol kinase [4, 14], with no phosphoenolpyruvate (PEP) consumed in this process. It is worthy to note that higher PEP concentration is favourable for L-serine production, since PEP is a precursor of L-serine. In contrast, PEP was consumed when glucose is the carbon source, and glucose is phosphorylated by a glucose-specific phosphotransferase system (PTS) to produce glucose-6-phosphate with PEP as a phosphoryl donor. Using glycerol as a carbon source is a potential strategy for enhancing L-serine production. Inactivation of the PTS to save PEP in *C. glutamicum* SYPS-062 improved L-serine titer has been done in our laboratory (unpublished data). Glycerol-based production of L-glutamate, L-lysine and succinate has been achieved [7, 14]. Nevertheless, successful production of L-serine by engineered *E. coli* from glycerol has not been achieved so far.

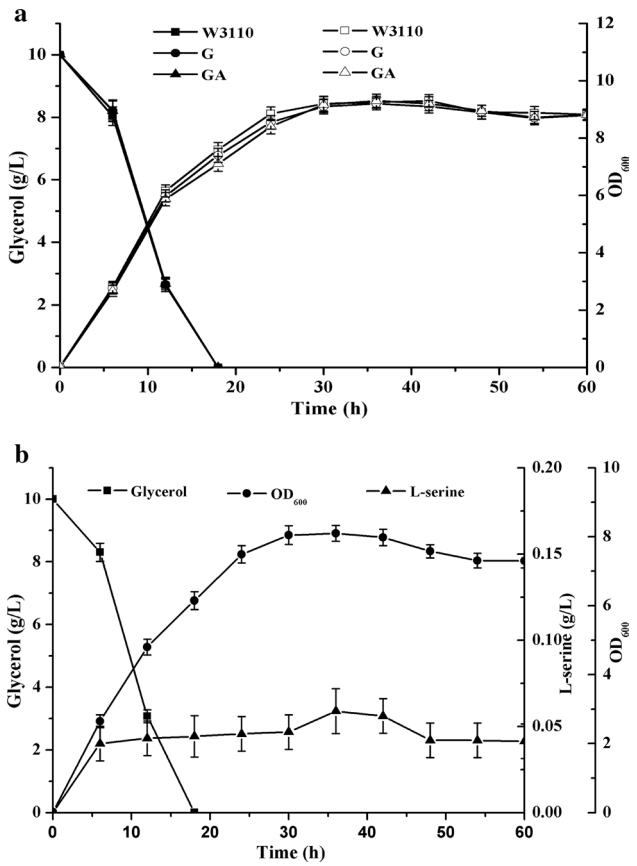

Fig. 1 L-serine biosynthesis pathways from glycerol in *E. coli*. Genes and enzymes: glycerol facilitator (*glpF*); glycerol kinase (*glpK*); glycerol 3-phosphate dehydrogenase (*glpD*); 3-phosphoglycerate dehydrogenase (*serA*); phosphoserine aminotransferase (*serC*); phosphoserine phosphatase (*serB*); cysteine/acetyl serine transporter (*eamA*); serine hydroxymethyltransferase (*glyA*); L-serine deaminases (*sdaA*, *sdaB*, and *tdcG*). Intermediates: dihydroxyacetone phosphate (DHAP); phosphoenolpyruvate (PEP); tricarboxylic acid cycle (TCA). Highlighted arrows indicate pathways in which genes were upregulated by overexpression *eamA*. Red crosses on solid lines (⊗) indicate genes that were deleted. Crosses on dashed lines (⊗) indicate the removal of feedback inhibition (color figure online)

In this study, *E. coli* was engineered to efficiently produce L-serine from glycerol. To this end, three L-serine deaminase genes (*tdcG*, *sdaA* and *sdaB*) were deleted in turn. Furthermore, remove of feedback inhibition by L-serine through the mutation (H344A, N346A and N364A) of 3-phosphoglycerate dehydrogenase. Two strategies were attempted to improve glycerol utilization, overexpression of glycerol metabolism pathway genes (*glpD*, *glpK)* or adaptive laboratory evolution was employed respectively, to improve L-serine titer further, overexpression of the cysteine/acetyl serine transporter gene *eamA* was performed. The effects of these modifications on L-serine production and cell growth were investigated and discussed in detail.

Materials and methods

Strains and plasmids

Strains and plasmids used in the work are listed in Table 1. Primers for gene cloning and deleting are listed in Table 2. *E. coli* JM109 was used for plasmid construction. The vector pDXW-10 [19] was used to provide the tac-M promoter and the corresponding rrnB terminator for *eamA* expressing, and *eamA* was amplified from chromosomal DNA of *E. coli* W3110 using primers pDXW-10-*eamA*-F and pDXW-10-*eamA*-R. The *eamA* PCR product was digested with *Not* I and *BgI* II (TaKaRa, Dalian, China) and ligated into pDXW-10 using T4 DNA ligase (TaKaRa, Dalian, China). The resulted pDXW-10-*eamA* was used as a template for amplifying the tac-M-*eamA*-rrnB fragment. The compatible pACYCDuet-1 vector was used for overexpression of *eamA*.

Engineering of strains

All target genes deletion was performed using the λRed system [3]. Plasmid pKD46 was used as the Red recombinase expression vector, pKD3 and pKD4 were used as templates for PCR amplification of disruption cassettes containing the chloramphenicol or kanamycin resistance gene, PCP20 was used to cure the kanamycin or chloramphenicol marker via the flippase mediated-recombination of FRT sequence flaking the antibiotic resistance gene. For *sdaA* and *sdaB* deletions, *sdaA*-F and *sdaA*-R, *sdaB*-F and *sdaB*-R and the pKD4 template were used to obtain linearized PCR products consisting of 50 nt of sequence homologous to the target

Table 1 Strains and plasmids used in this study

Strains and plasmids	Genotype	Source
Strains		
E. coli JM109	*recA1, endA1, gyrA96, thi-1, hsd R17(rk− mk+)supE44*	Invitrogen
E. coli W3110	Wide type, λ-F-mcrA mcrB IN (rrnD-rrnE)1	Invitrogen
G	W3110Δ*tdcG*	This study
GA	GΔ*sdaA*	This study
GAA	GAΔ*sdaB*	This study
4W	GAA $serA^{dr}$	This study
4WA	A mutant derived from 4W	This study
4WR	4W/*ompX, opgE, rhtA, rybA and mntS*	This study
4WE	4W/pACYCDuet-*eamA*	This study
4WAE	4WA/pACYCDuet-*eamA*	This study
Plasmids		
pKD4	*bla*, FRT-kan-FRT	[3]
PKD3	*bla*, FRT-cmr-FRT	[3]
pCP20	*Bla* and cmr, FLP recombinase helper plasmid	[3]
pKD46	*bla*, helper plasmid for recombination	[3]
pDXW-10	*E. coli–C. glutamicum* shuttle vector, amp, kan, L-MCS, *tac-M* promoter	[19]
pDXW-10-*eamA*	pDXW-10 carrying *eamA* gene	This study
pACYCDuet-1	Double T7 promoters, P15A ori, cmr	Invitrogen
pACYCDuet-*eamA*	pACYCDuet-1 carrying tac-M-*eamA*	This study

locus flanked by the Flp recognition target (FRT). These were electroporated into strains containing the pKD46 plasmid. Transformants were selected on kanamycin plates and verified by PCR using the corresponding primers Δ*sdaA*-F and Δ*sdaA*-R, or Δ*sdaB*-F and Δ*sdaB*-R.

Feedback inhibition of PGDH was prevented by mutating three residues H344, N346 and N364 into alanine [1] using site-directed mutagenesis to generate $PGDH^{dr}$. The method used for site-specific mutagenesis was the same as described above for genes deletion, but with primers Del-*serA*-up-P1, Del-*serA*-up-P2, Del-*serA*-up-P3, Del-*serA*-up-P4, *serA*-FRT-P1, *serA*-FRT-P2, Del-*serA*-do-P1, and Del-*serA*-do-P2.

Media

Luria–Bertani (LB) medium used for plasmid construction contained (per L) 5.0 g yeast extract, 10.0 g tryptone, and 10.0 g NaCl. When appropriate, chloramphenicol (34 μg/mL) or kanamycin (50 μg/mL) was added. For L-serine fermentation, mineral AM1 medium [9] supplemented with 1 g/L yeast extract and 10 g/L glycerol was used.

Cultivation conditions

E. coli was cultured in 10 mL Luria–Bertani (LB) media with the corresponding antibiotics at 37°C and 220 rpm for 12 h, and 2 mL was inoculated into 50 mL of fermentation medium in 250 mL shake flasks and cultured for 4 h. Expression was induced by adding isopropyl β-d-1-thiogalactopyranoside (IPTG) at the final concentration of 0.5 mM, and culturing continued for a further 30 h at 30°C and 220 rpm. For L-serine production, samples were withdrawn from cultures and used to measure the concentration of amino acids and glycerol, and the biomass.

The batch and fed-batch cultivation were accomplished in a 5-L bioreactor (BIOTECH-50JSA, China) containing 3 L of the fermentation medium with an initial glycerol concentration of 15 g/L at 37 °C. When glycerol was consumed, all the feed was started with feeding medium that contained 300 g/L glycerol. The pH was controlled at 7.0 by automatically feeding concentrated $NH_3 \cdot H_2O$. Antifoam was added to control foaming.

Adaptive laboratory evolution to improve glycerol utilization

Adaptive laboratory evolution of *E. coli* strain 4W was performed by culturing cells in LB medium, transferring a 10% (v) inoculum into a fresh AM1 medium containing 10 g/L glycerol, and growing for 10 generations. Then, the cells were inoculated into fresh AM1 medium containing 20 g/L glycerol and grown for genetic screening. Successive rounds of adaptive evolution were carried out with the glycerol concentration increased stepwise (10, 20, 40, 60,

Table 2 Primers used in this study

Primers	Sequence
pDXW-10-*eamA*-F	GCGGCCGCAGAAGGAGATATAGGATGTCGCGAAAAGATGGGGTGT (*Not* I)
pDXW-10-*eamA*-R	GAAGATCTTTAACTTCCCACCTTTACCGCT (*BgI* II)
pACYCDuet-*eamA*-F	GAATTCCATAGTGTCGGAAGCTGTGGTATGG (*Nde* I)
pACYCDuet-*eamA*-R	CCCTCGAGAGATAAAACGAAAGGCCCAGTCT (*Xho* I)
sdaA-F	TGTTATTAGTTCGTTACTGGAAGTCCAGTCACCTTGTCAGGAGTATTATCGTGTAGGCTGGAGCTGCTTC
sdaA-R	AAAGCGGGTATAAATTCGCCCATCCGTTGCAGATGGGCGAGTAAGAAGTAATGGGAATTAGCCATGGTCC
sdaB-F	CGCGCCGCTTTCGGGCGGCGCTTCCTCCGTTTTAACGCGATGTATTTCCTGTGTAGGCTGGAGCTGCTTC
sdaB-R	GGATGAGAAATCGGGAAGAGGCCTCGCAAAACGAGGCCTTTGGAGAGCGAATGGGAATTAGCCATGGTCC
Del-*tdcG*-up-P1	AACGTAATGAATCGGGAAAT
Del-*tdcG*-up-P2	GAAGCAGCTCCAGCCTACACATCCACACCCTCGGATTG
tdcG-FRT-P1	CCGAGGGTGTGGATGTGTAGGCTGGAGCTGC
tdcG-FRT-P2	GATGAAAGCTGACAGCAATGATGGGAATTAGCCATGGTCC
Del-*tdcG*-do-P1	GGACCATGGCTAATTCCCATCATTGCTGTCAGCTTTCATC
Del-*tdcG*-do-P2	CAGGTAGGAAGCACTATCGT
Del-*serA*-up-P1	CTGAATATGA GCGATGTGGT
Del-*serA*-up-P2	ATTTTGTTCAGCGCAGTTAGCACGCCCGGACGAGCTTCAGCGATGTGCAT
Del-*serA*-up-P3	CTAACTGCGCTGAACAAAATCTTCGCCGAGCAGGGCGTCGCTATCGCCGC
Del-*serA*-up-P4	GAAGCAGCTCCAGCCTACACTTAGTACAGCAGACGGGCGC
serA-FRT-P1	GCGCCCGTCTGCTGTACTAAGTGTAGGCTGGAGCTGCTTC
serA-FRT-P2	GCGCCCGTCTGCTGTACTAAGTGTAGGCTGGAGCTGCTTC
Del-*serA*-do-P1	GGACCATGGCTAATTCCCATTTCCCCTTCTCTGAAAATCA
Del-*serA*-do-P2	GCAAAGCGAACTGGTGATGA
Del-ROORM-up-P1	ATCCGATACCAACAAGAATT
Del-ROORM-up-P2	GAAGCAGCTCCAGCCTACACTTAAAGGGAGAATGACTAAA
ROORM-FRT-P1	TTTAGTCATTCTCCCTTTAAGTGTAGGCTGGAGCTGCTTC
ROORM-FRT-P2	GCATTTTACGTAATGAACCAATGGGAATTAGCCATGGTCC
Del-ROORM-do-P1	GGACCATGGCTAATTCCCATTGGTTCATTACGTAAAATGC
Del-ROORM-do-P2	GCGCCAGTAGCTGTATGGGT
Δ*sdaA*-F	TTGGTGTGAAACCCTGACTATAC
Δ*sdaA*-R	GTGCGCAAATCGTACAAACAATC
Δ*sdaB*-F	CGATGATCCTGTTCCTGATGCCGAT
Δ*sdaB*-R	TGGCAGGTTTCGACACAGGGCGACC
Δ*tdcG*-F	GATCTGAATGATTTTGCCACCAT
Δ*tdcG*-R	CTACGTTTTTGCAGGCGAGTTATTAG
Δ*serA*-F	TTCAGCGTAGTATTGGGAAGAGGAAGTT
Δ*serA*-R	TACCCGCGCTTTCTTTAAAGTGATTACT
ΔROORM-F	AAGTCAGAAATCAGCTCAACGTAATCAT
ΔROORM-R	AGCGCACAGCCGGTTAAATCGACAT
pKD46-F	TTACCAATGCTTAATCAGTGAG
pKD46-R	ATGAGTATTCAACATTTCCGTG

Underlined sequences indicate the additional restriction sites

and 80 g/L). Among the evolved strains, the most efficient strain was selected for further investigation and named 4WA.

Analytical methods

Cell growth was measured as the optical density at 600 nm (AOE UV-1200S, China) and correlated with the dry cell weight (DCW) using the formula DCW (g/L) = $0.36 \times OD_{600}$. The concentration of glycerol was determined using an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a Cosmosil Sugar-D column (4.6 × 250 mm) and a refractive index detector (RID). The mobile phase consisted of acetonitrile (80:20, v/v), and the flow rate was adjusted to 1 mL/min. For amino acid analysis,

phenylisothiocyanate (PITC) was used as a pre-column derivatization agent. The amount of L-serine and other amino acids was determined by HPLC as previously described [25].

Results

Construction of a L-serine producing stain from glycerol

L-Serine deaminase, encoded by *sdaA*, *sdaB* and *tdcG* in *E. coli*, converts L-serine into pyruvate, and thus reduces the accumulation of L-serine. In this study, we first knocked out *tdcG* in *E. coli* W3110 to generate the G strain, but there was no L-serine detected in the fermentation broth. Deletion of *sdaA* in the G strain resulted in the double knock out GA strain, but again, L-serine was barely detected in the culture. However, when *sdaB* was deleted as well, the triple deletion (Δ*sdaA*, Δ*sdaB*, Δ*tdcG*) strain GAA accumulated 0.06 g/L L-serine with the biomass (OD_{600}) reached 8.1, and all glycerol was consumed after 18 h (Fig. 2).

Furthermore, removal of feedback inhibition by L-serine through mutating three residues (H344A, N346 A and N364A) in strain GAA, resulted in strain 4W. As shown in Fig. 3, strain 4W accumulated 1.1 g/L L-serine at 54 h, which is 18-fold higher than GAA (0.06 g/L L-serine), and the maximal biomass (OD_{600}) reached 4.8. Specific production in the 4W strain was 0.66 g/L/DCW, which is 33-fold higher than the parent strain GAA (0.02 g/L/DCW). However, the rate of glycerol utilization by 4W was decreased significantly, and

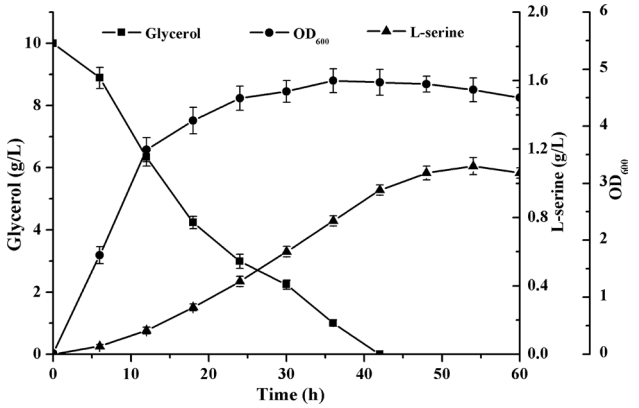

Fig. 2 Comparison of glycerol consumption, cell growth, and L-serine production of strains W3110, G, GA and GAA. **a** Profiles of residual glycerol and cell density with strains W3110, G, and GA. Squares represent W3110, circles represent G, triangles represent GA, open symbols represent OD_{600}, closed symbols represent residual glycerol. **b** Profiles of glycerol consumption, cell growth and L-serine production in GAA. Squares represent residual glycerol, circles represent cell growth, triangles represent L-serine. Values denote the average of three independent experiments, and error bars indicate standard deviation

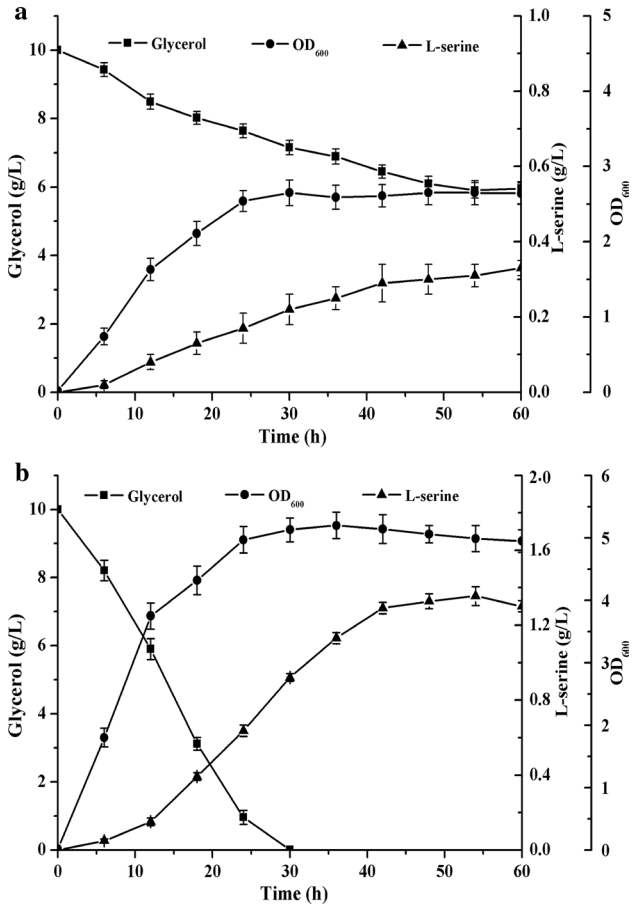

Fig. 3 Time course of L-serine production in the 4W strain during batch fermentation. Squares represent residual glycerol, circles represent cell growth, triangles represent L-serine. Values denote the average of three independent experiments, and error bars indicate standard deviation

at 18 h, the glycerol concentration was 4.24 g/L, whereas GAA consumed all glycerol by this time. How to improve glycerol utilization was the key to be solved.

Improvement of glycerol utilization

To improve glycerol utilization, two strategies including metabolic engineering and adaptive laboratory evolution were performed separately. Two genes encoding enzymes mediating glycerol dissimilation (*glpD* and *glpK*) were overexpressed in strain 4W, resulting in strain 4W-*glpD–glpK* (Fig. 4a). To our surprise, overexpression of *glpD* and *glpK* decreased the cell growth significantly compared with the parental strain 4W, and the maximal biomass (OD_{600}) was only 2.65 at 30 h, while 5.95 g/L glycerol was consumed at 60 h, presumably because the weak cell growth affected glycerol utilization.

Adaptive laboratory evolution was, therefore, performed to improve glycerol utilization, and the overall schematic

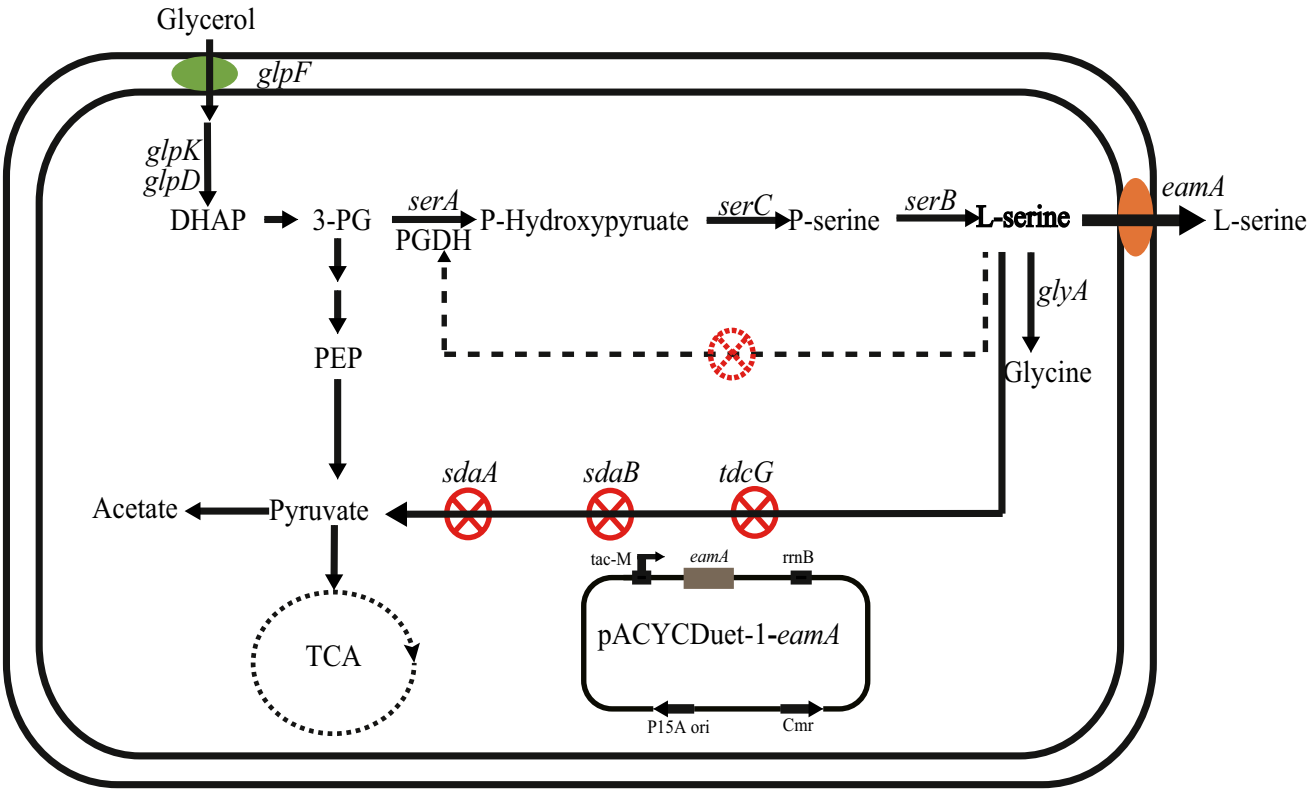

Fig. 4 Comparison of glycerol consumption, cell growth and L-serine production in strains 4W-*glpD–glpK* and 4WA. **a** Profiles of 4W-*glpD–glp*K. **b** Profiles 4WA. Squares represent residual glycerol, circles represent cell growth, triangles represent L-serine. Values denote the average of three independent experiments, and error bars indicate standard deviation

of the process is shown in Fig. 1. Five populations evolved in this step and the glycerol concentration was increased gradually (10, 20, 40, 60, and 80 g/L) of evolution (Fig. 4b). The cells were continuously transferred into fresh media during the exponential growth phase. At the end of the experiment, the ALE resultant strain, named 4WA exhibited faster growth on glycerol, as shown in Fig. 4b, and reached a maximal biomass (OD_{600}) of 5.2 at 36 h, 1.35 g/L L-serine, with all glycerol consumed at 30 h, while the parental 4W strain consumed 7.76 g/L of glycerol at this time. At the same time, the serine tolerance of those strains were evaluated (Fig. 5).

Overexpression of *eamA* in 4WA enhances L-serine titer

Overexpression of *eamA* in the evolved strain 4WA generated strain 4WAE, the cell growth, glycerol consumption and L-serine production in 4WAE were evaluated with glycerol as the sole carbon source (Fig. 6). L-Serine accumulated to 2.36 g/L at 36 h, with a maximal biomass (OD_{600}) of 5.57, a specific production of 1.18 g/L/DCW, and a yield of 23.6%, which was significantly higher than the parent strain 4WA (1.35 g/L L-serine and 0.75 g/L/DCW). Furthermore, all glycerol was consumed at 24 h by 4WAE, and the parent strain finished glycerol at 30 h. This result showed that overexpression of *eamA* improved L-serine titer.

Production of L-serine by 5-L bioreactor

The fermentation profiles of 4WAE were examined in 5-L bioreactor. As shown in Fig. 7a, the maximal biomass (OD_{600}) of 4WAE reached 9.4 at 24 h, which was 54.1% higher than that obtained under shake-flask conditions. The engineered strain exhibited higher glycerol consumption rate and the highest L-serine titer of 3.23 g/L was achieved. Meanwhile, the L-serine titer was improved by 36.9%, when compared with that obtained under shake-flask conditions.

As shown in Fig. 7b, the engineered strain 4WAE exhibited maximal biomass (OD_{600}) of 15.48 at 42 h, which was 90.1% higher than that of batch fermentation, and the highest L-serine titer of 7.53 g/L was reached with a yield of 21.5% at 42 h after overall feeding of 4 times of glycerol, which was 133% higher than that of batch fermentation.

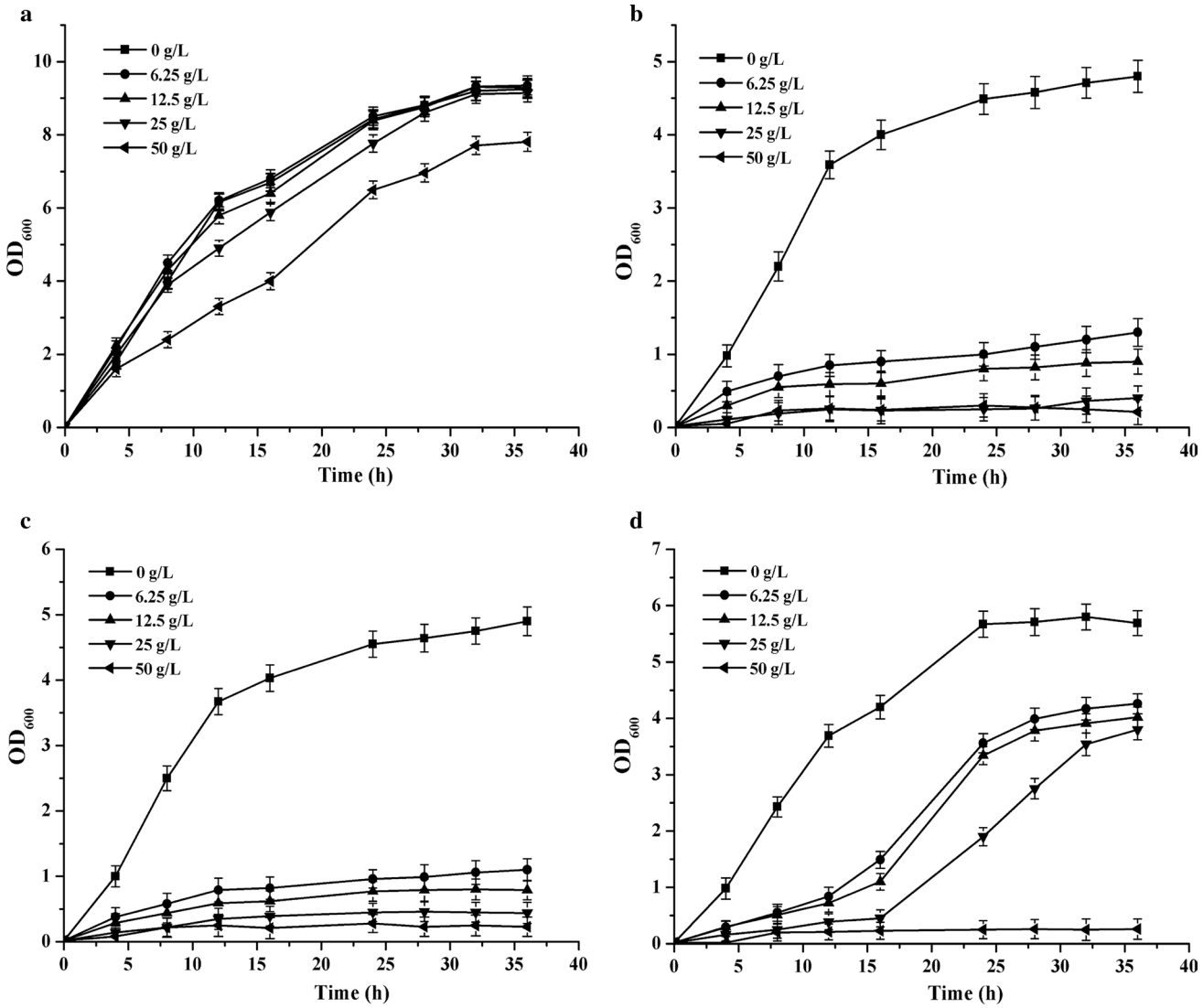

Fig. 5 Comparison of the growth profiles of strains W3110, 4W, 4WR and 4WE in AM1 containing different concentrations of L-serine. **a** Cell density profile of W3110. **b** Cell density profile of 4W. **c** Cell density profile of 4WR. **d** Cell density profile of 4WE. Values denote the average of three independent experiments, and error bars indicate standard deviation

Discussions

Direct fermentative production of L-serine is attracting growing attention, and glycerol is considered as a potentially ideal carbon source to convert to value-added bio-products. In this study, a series of genetic manipulations in *E. coli* were performed to improve L-serine production from glycerol (Table 3). Overexpression of *eamA*, deletion of *sdaA*, *sdaB* and *tdcG*, and alleviation of feedback inhibition by *serA,* combined with adaptive evolution, together enhanced L-serine production to 2.36 g/L, with a yield of 23.6%. The results clearly demonstrate the feasibility of our approach for engineering *E. coli* for L-serine production from glycerol.

In this study, three L-serine deaminase genes *tdcG, sdaA*, *sdaB* was knocked out in turn, we initially knocked out *tdcG* in *E. coli* W3110 to generate the G strain, but L-serine accumulation remained undetectable. Deletion of *sdaA* in the G strain resulted in the double knock out of GA strain, but again, L-serine was not detected (Fig. 2a). Further, when *sdaB* was also deleted, the triple deletion (Δ*sdaA*, Δ*sdaB*, Δ*tdcG*) strain GAA accumulated 0.06 g/L L-serine. Combined with alleviation of the feedback inhibition by *serA*, resulting in strain 4W, 1.1 g/L L-serine was achieved, However, the glycerol utilization rate in 4W was lower than the parental strain *E. coli* W3110.

The *glpK–glpD* pathway mediates glycerol dissimilation under aerobic conditions, overexpressing *glpK* and *glpD* can increase glycerol flux in *E. coli* [10, 18, 22]. In this study, to improve glycerol utilization, *glpD* and *glpK* were overexpressed in strain 4W first, resulting in strain

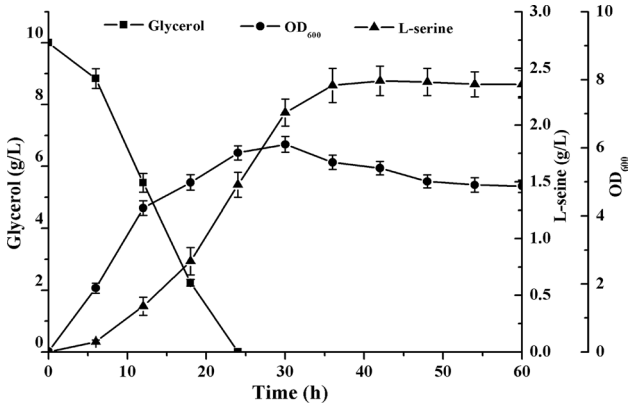

Fig. 6 Time course of L-serine production in the 4WAE strain. Squares represent glycerol, circles represent cell growth, triangles represent L-serine. Values denote the average of three independent experiments, and error bars indicate standard deviation

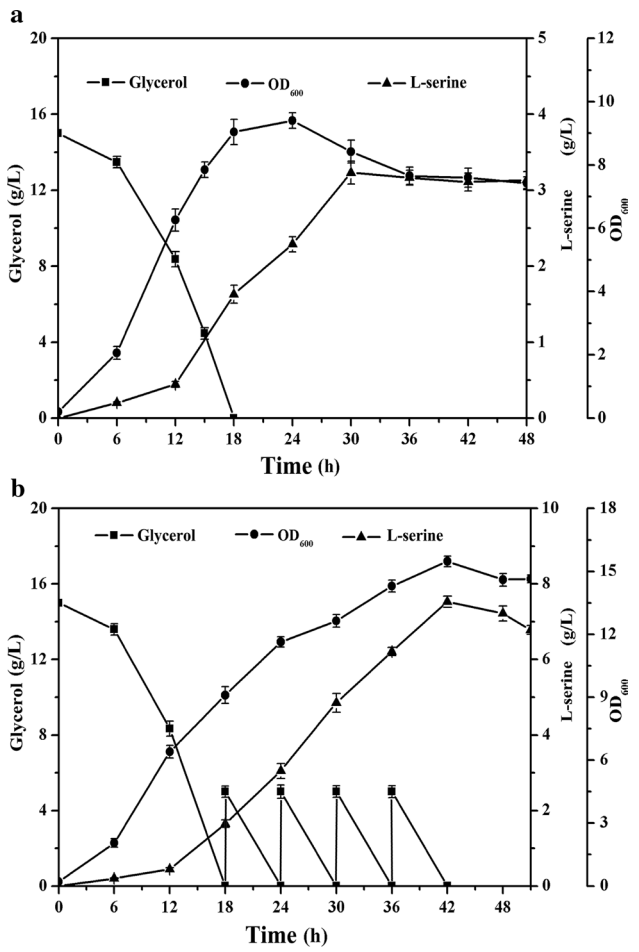

Fig. 7 Time course of L-serine production in the 4WAE strain during batch and fed-batch fermentation. **a** Time course of L-serine production in the 4WAE strain during batch fermentation. **b** Time course of L-serine production in the 4WAE strain during fed-batch fermentation. Squares represent glycerol, circles represent cell growth, triangles represent L-serine. Values denote the average of three independent experiments, and error bars indicate standard deviation

Table 3 Cell growth, L-serine titer and yield of engineered strains

Strains	Time (h)	OD_{600}	L-serine (g/L)	Y (g/g)
W3110	36	9.01	0	0
GAA	36	8.10	0.06	0.01
4W	54	4.64	1.10	0.11
4WR	48	4.66	1.04	0.10
4WA	54	4.99	1.35	0.14
4WE	36	5.69	1.96	0.20
4WAE	36	5.57	2.36	0.24

4W-*glpD–glpK* (Fig. 4a). To our surprise, overexpression of *glpD* and *glpK* decreased the cell growth significantly compared with the parental 4W strain, and the maximal biomass (OD_{600}) was only 2.65 at 30 h, while glycerol consumption was 5.95 g/L at 60 h, presumably because the weak cell growth affected glycerol utilization. The negative effect of *glpD* and *glpK* overexpression on the cell growth was observed in other study, where *E. coli* BL21 (DE3) DK DCW achieved a biomass of 4.2 g/L, compared with the parent strain 5.3 g/L [22]. It is possible that the plasmids used for overexpression burdened the host and lowered the growth rate.

Moreover, adaptive laboratory evolution was used to improve glycerol utilization, and the resulting strain 4WA was improved further for L-serine production. To illustrate the mutations causing the increased glycerol utilization, the whole genome of 4WA was sequenced on the Illumina HiSeqX10 platform with over 1.59 million reads. A comparative genomics analysis of 4WA and the parental *E. coli* W3110 strain was performed. The results revealed single-nucleotide variants, which included one synonymous mutation, nine non-synonymous mutations, and other mutations located in the intergenic region. Further studies will be needed to explore the reason for phenotypic differences.

Compared with sugar, glycerol dissimilation is initiated by its ATP-dependent phosphorylation via glycerol kinase [4, 14], whereas glucose is initially phosphorylated by the glucose-specific PTS to glucose 6-phosphate with phosphoenolpyruvate (PEP) as phosphoryl donor. A high glucose uptake rate promotes a high pyruvate and low PEP concentration in the cell, which is unfavourable for L-serine production in *E. coli*, because PEP is the substrate for anaplerotic C3 carboxylation via phosphoenolpyruvate carboxylase (PEPCx) or phosphoenolpyruvate carboxykinase (PEPCk) [24]. Conveniently, tremendous growth in the biofuels industry has created a surplus of glycerol, resulting in a dramatic decrease in crude glycerol prices [8]. Thus, using glycerol as a carbon source in the fermentation production of value-added chemicals such as 1,3-propanediol, ethanol, and amino acids is a feasible approach to enhance the economic efficiency of biofuel production processes [17]. Successful

production of L-serine by engineered *E. coli* from glycerol has not been achieved so far.

Recently, Mundhada [12] obtained a strain accumulated 37 g/L L-serine from glucose with 24% mass yield, which was the highest L-serine production by *E. coli* so far. Although the L-serine titer achieved by 4WAE in the present study was not significantly higher than the previous one, the resulting yield (23.6%, mass yield) was similar to highest yield (24%) reported by the previous study, in which glucose was used as a substrate.

The present study demonstrates the feasibility of engineering the L-serine biosynthetic pathway to make use of the abundant, renewable and affordable glycerol substrate. At present, microbial fermentative production of glycerol remains more expensive than chemical production due to a lower product yield, but if the cost of raw materials drops even further, the fermentation directly using glycerol as a carbon source could become competitive [8]. Moreover, the crude glycerol is much cheaper than glucose right now, therefore, it is a prime candidate substrate for future fermentation production processes.

Acknowledgements This work was financially supported by the National Natural Science Foundation of China (Nos. 31300027, 31600044), the Natural Science Foundation of Jiangsu Province (No. BK20150151).

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