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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Integration of ARTP mutagenesis with biosensor-mediated high-throughput screening to improve L-serine yield in *Corynebacterium glutamicum*

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Abstract

L-Serine is widely used in the pharmaceutical, food, and cosmetics industries. Although direct fermentative production of L-serine from sugar in *Corynebacterium glutamicum* has been achieved, the L-serine yield remains relatively low. In this study, atmospheric and room temperature plasma (ARTP) mutagenesis was used to improve the L-serine yield based on engineered *C. glutamicum* ΔSSAAI strain. Subsequently, we developed a novel high-throughput screening method using a biosensor constructed based on NCgl0581, a transcriptional factor specifically responsive to L-serine, so that L-serine concentration within single cell of *C. glutamicum* can be monitored via fluorescence-activated cell sorting (FACS). Novel L-serine-producing mutants were isolated from a large library of mutagenized cells. The mutant strain A36-pDser was screened from 1.2×10^5 cells, and the magnesium ion concentration in the medium was optimized specifically for this mutant. *C. glutamicum* A36-pDser accumulated 34.78 g/L L-serine with a yield of 0.35 g/g sucrose, which were 35.9 and 66.7% higher than those of the parent *C. glutamicum* ΔSSAAI-pDser strain, respectively. The L-serine yield achieved in this mutant was the highest of all reported L-serine-producing strains of *C. glutamicum*. Moreover, the whole-genome sequencing identified 11 non-synonymous mutations of genes associated with metabolic and transport pathways, which might be responsible for the higher L-serine production and better cell growth in *C. glutamicum* A36-pDser. This study explored an effective mutagenesis strategy and reported a novel high-throughput screening method for the development of L-serine-producing strains.

Keywords L-Serine · *Corynebacterium glutamicum* · ARTP mutagenesis · High-throughput · FACS · Biosensor

Introduction

L-Serine plays major roles in the physiological metabolism of cells, in processes including metabolism, purine and pyrimidine biosynthesis, and generation of activated one-carbon (C-1) units (Beaudin et al. 2011). It is widely used in medicine and in industrial applications such as food and cosmetics (Leuchtenberger et al. 2005). The production of L-serine mainly involves enzyme or cell transformation, and glycine and methanol are used as precursors to synthesize L-serine (Izumi et al. 1993; Kubota and Yokozeki 1989). However, the low yield and the dependence on expensive substrates prevent current production methods from being easily scalable. Thus, the production of L-serine through direct fermentation has received increasing attention. L-Serine tends not to accumulate in organisms, because it is a central intermediate in several cellular reactions (Zhang et al. 2014a).

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The gram-positive soil bacterium *Corynebacterium glutamicum* has been widely used in large-scale production of L-valine and L-lysine (Blombach et al. 2008; Eggeling and Bott 2015; Koffas and Stephanopoulos 2005). However, it cannot accumulate L-serine. Peters-Wendisch et al. engineered a wild-type strain, ATCC13032, producing L-serine with a titer of 9.03 g/L, by elimination of feedback inhibition, overexpression of the synthetic pathway, knocking out or weakening the degradation pathway (Peters-Wendisch et al. 2005). In fed-batch fermentation with glucose and fructose, 36.25 g/L of L-serine can be produced by this strain (Stolz et al. 2007). In our previous study, *C. glutamicum* SYPS-062 was subjected to mutagenesis to obtain a novel strain SYPS-062-33a, and then a series of genetic modifications were performed on *C. glutamicum* SYPS-062-33a genome, resulting in *C. glutamicum* ΔSSAAI, producing 26.23 g/L L-serine in batch fermentation and 42.64 g/L of L-serine in fed-batch fermentation, with a yield of 0.21 g/g sucrose (Xu et al. 2015; Zhu et al. 2015). Although the L-serine titer of *C. glutamicum* ΔSSAAI is the highest reported in the literature, the yield and productivity are relatively low.

Microbial mutation breeding methods have been widely used in the fermentation industry. Traditional strategies to enhance the yield of wild-type strains include random mutagenesis by nitrosoguanidine (NTG) and ultraviolet treatment (UV) (Kodym and Afza 2003). In these conventional mutation methods, the issues of health and safety of the operator and the mutation efficiency are concerns. Recently, a novel mutagenesis tool of atmospheric and room temperature plasma (ARTP) has been developed. ARTP has several favorable features, such as low and controllable gas temperatures, abundant chemically reactive species, rapid mutation, security, and high operation flexibility (Zhang et al. 2014b). Many studies reported that the plasma from the ARTP system had extensive effects on organisms, through aspects including chemically active species of nitrogen or oxygen, the hydroxyl (OH) molecular band, heat, UV radiation, charged particles, and an intense electric field; all of which can destroy cell walls and membranes, as well as damage DNA and proteins, thereby creating mutations and damaging cellular genomes (Laroussi and Leipold 2004; Li et al. 2008). Therefore, ARTP might be a powerful mutagenesis tool to improve the yield of L-serine, provided an efficient method to acquire mutants with the desired phenotype. The traditional agar plate screening procedure is cumbersome and time-consuming. Hence, there is a pressing need for efficient approaches to screen high-yield mutant strains.

High-throughput screening methods with biosensors were used to screen mutant strains that overproduce molecules such as amino acids and organic acids (Binder et al. 2012; Chen et al. 2015; Cress et al. 2015; Liu et al. 2015; Schendzielorz et al. 2014; Tang et al. 2013). The biosensor devices, including riboswitches, enzymes, and transcription factors, can transform information about a specific metabolite into a graded fluorescence output. Binder et al. developed a genetic metabolite biosensor capable of detecting L-lysine in single *C. glutamicum* cells. This biosensor is based on the transcriptional regulator *lysG* of *C. glutamicum*, which activates the *lysE* promoter to drive transcription of enhanced yellow fluorescent protein (EYFP) (Binder et al. 2012). Mustafi et al. constructed a biosensor for L-methionine and other branched-chain amino acids, which is based on the transcriptional regulator *Lrp*. *Lrp* activates the expression of the *brnFE* in the presence of increasing amounts of branched-chain amino acids or L-methionine. This detection, combined with fluorescence-activated cell sorting (FACS), enables high-throughput screening of large libraries (Mustafi et al. 2012). Although the sequence of an L-serine biosensor was reported (Binder et al. 2012), it was not used to screen L-serine-overproducing strains. As novel and powerful tools for screening target strains, high-throughput FACS and ARTP are attracting increasing attention; hence, screening L-serine-overproducing strains through this method may be a feasible strategy.

In this study, ARTP mutagenesis and high-throughput screening were used to improve L-serine yield. To this end, a high-throughput screening method with a biosensor was first developed, and the correlation and specificity of the method were then verified. L-Serine-overproducing strains were selected through FACS after ARTP mutagenesis. The fermentation characteristics of the parent strain and the mutant were evaluated. Moreover, the whole genome of the mutant strain A36-pDser was sequenced, and comparative genomics analysis and reverse mutation were performed.

Material and methods

Strains and plasmids

All the strains and plasmids used in this study are listed in Table 1. *C. glutamicum* SYPS-062-33a, derived from *C. glutamicum* SYPS-062 by chemical mutagenesis, was deposited in CGMCC under accession no. 8667. The engineered *C. glutamicum* ΔSSAAI (with 591 bp of the C-terminal domain of *serA*, *sdaA*, *avtA*, and *alaT* knocked out, and *ilvN* attenuated in the genome of *C. glutamicum* SYPS-062-33a) was stored in our laboratory (Zhu et al. 2015). *Escherichia coli* JM109 was used as a host for plasmid construction.

Medium and growth conditions

The seeding medium of *C. glutamicum* contained (per liter) 20.0 g glucose, 37.0 g brain heart infusion (BHI), 10.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.3 g NaH_2PO_4, and 0.2 g K_2HPO_4. The fermentation medium contained (per liter)

Table 1 Strains and plasmids, primers used in this study

Strains/plasmids/primers	Genotype or description	Sources or reference
Strains		
E. coli		
JM109	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsd R17(rk⁻ mk⁺)supE44*	Invitrogen
C. glutamicum		
ATCC14067	A model wild-type *C. glutamicum*	Lab collection
ATCC14067-pDser	*C. glutamicum*14067with pDser	This study
ΔSSAAI	*C. glutamicum* SYPS-062-33a with deletion of the 591 bp in the C-terminus of *serA*, deletion of *sdaA*, *alaT*, *avta*, and attenuation of *ilvN*	(Zhu et al. 2015)
ΔSSAAI-pDser	*C. glutamicum* ΔSSAAI with pDser	This study
ΔSSAAI-pDser(*pabAB*426)	*C. glutamicum* ΔSSAAI-pDser with Thr-426 → Ile reverse mutation in *pabAB*	This study
Plasmids		
pDXW-11	*E. coli-C. glutamicum* shuttle vector, Km^r	(Xu et al. 2010)
pDser	Transcription factor NCgl0581 and the intergenic region of NCgl0581 NCgl0580 were ligated on plasmid pDXW-10 and a transcriptional fusion of NCgl0580 with *eyfp*	This study
pK18mob*sacB*	Integration vector, *oriV*, *oriT*, *mob*, *sacB*, Km	(Schäfer et al. 1994)
pK18mob*sacBpabAB426*	pK18mob*sacB* with the *pabAB* sequence of A36-pDser to mutate *C. glutamicum* ΔSSAAI-pDser *pabAB* sequence	This study
Primes	Sequences (5′-3′)	
L-Serine biosensor-fw	GGCCTCGAGGGATCCTTATTACTTGTACAGCT (*Xho*I)	This study
L-Serine biosensor-re	CGAGCTCACTCTACTAGACGAGCCTCC (*Sac*I)	This study
pabAB-fw	GCTCTAGACCATAATGTCGGCGAGGGGG(*Xba*I)	This study
pabAB-re	CGGAATTCAACCCCAAACAAAT(*Eco*RI)	This study

100.0 g sucrose, 30.0 g $(NH_4)_2SO_4$, 3.0 g KH_2PO_4, 0.5/1 g $MgSO_4{\cdot}7H_2O$, 20 mg $FeSO_4{\cdot}7H_2O$, 20 mg $MnSO_4{\cdot}H_2O$, 60.0 g $CaCO_3$, 30 mg protocatechuic acid, 50 μg biotin, and 450 μg thiamine.

C. glutamicum was incubated overnight in a rotary shaker (120 rpm, 30 °C) in 250-mL shake flasks until logarithmic phase (optical density at 562 nm [OD_{562}] = 25) was reached. Then, 1 mL seed liquid was inoculated in 25 mL fermentation medium in 250-mL shake flasks to make the initial OD_{562} = 1. The fermentation medium was incubated at 120 rpm at 30 °C for 120 h and then used to measure the concentrations of amino acids, biomass, and sugars. *E. coli* JM109 was grown in lysogeny broth (LB) medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) on a rotary shaker (220 rpm) and on LB agar plates at 37 °C. The medium contained 50 mg/mL kanamycin when necessary.

Construction of the pDser biosensor

Primers and restriction enzymes used for the construction of plasmids are listed in Table 1. The pDser plasmid was derived from the pDXW-11 plasmid. pDser was ligated with a fragment consisting of a NCgl0580 promoter and transcription factor NCgl0581, the intergenic region of NCgl0581 and NCgl0580, as well as eyfp, and was synthesized by Sangon Biotech, Shanghai, China (Binder et al. 2012). First, the fragment was amplified with the primer L-serine biosensor-fw/L-serine biosensor-re, after which the PCR product and pDXW-11 plasmid were digested with *Xho*I and *Sac*I and ligated to create the pDser plasmid.

EYFP expression fluorescence assay

Cells were diluted to OD_{562} of approximately 0.5 with phosphate-buffered saline (PBS, pH 7.0), placed on a microscope slide and sealed with a coverslip, and then photographed using a laser scanning confocal microscope (Leica, TCS SP8, Germany) equipped with a HC PL Apo ×63/1.40 oil CS2 oil-immersion objective, a ×4 zoom, a 488-nm excitation filter, and a 520–583-nm emission filter. Digital images were acquired and analyzed in Leica Application Suite X 2.0.

The fluorescence intensity of the strain was measured in 96-well plates. First, OD_{562} cultures were diluted in PBS to approximately 0.5 to decrease the deviation; 100 μL of strain culture was used to measure the strain density (OD_{562}) and fluorescence intensity (excitation at 488 nm, emission at 530 nm) with a microplate reader (BioTek, synergy H4, USA) (Shaner et al. 2005). The background control (PBS) was subtracted from all measured data, and the fluorescence

intensity values were divided by the OD_{562} to obtain the fluorescence intensity of the unit cell. The OD_{562} values of all the samples were approximately 0.5–1.0.

ARTP mutagenesis

We first determined the optimal treatment period to ensure that the *C. glutamicum* culture was in the logarithmic phase. Pelleted cells were washed twice with sterile saline and diluted to 10^6–10^8 cells/mL, before undergoing ARTP treatment. The ARTP mutation breeding system (Si Qing Yuan Biotechnology Co., Ltd., China) included a plasma generator, a helium gas supply and control subsystem, and a sample plate made of stainless steel, which could be moved up and down. The main parameters of mutagenesis were a radio frequency power input of 100 W, a helium gas flow rate of $Q_{He} = 10$ standard liters per minute (slpm), and 2 mm distance between the plasma torch nozzle exit and the sample plate (Cao et al. 2017; Lu et al. 2011).

For the mutation, 10 μL of the culture was placed on a stainless steel minidisc and then exposed to ARTP with irradiation for 0, 15, 30, 45, 60, 90, 120, or 150 s. The mutated cells were allowed to recover in seed medium at 30 °C and 120 rpm for 3 h, and then the cells were diluted and spread on seed medium plates. After 3 days of incubation at 30 °C, the numbers of clones, control clones (A), and survival clones (B) were counted. The lethality rates were calculated as $(A - B)/A \times 100\%$.

High-throughput screening

The cells were washed three times with PBS and diluted to OD_{562} of approximately 0.1, and then FACSCalibur (BD Biosciences, FACSCalibur, USA) was used to analyze the fluorescence intensity distribution in strains cultured for different times, with a 488-nm excitation filter and a 530-nm emission filter. BD CellQuest was used to analyze the data and construct maps. The fluorescence distributions of five different time periods for *C. glutamicum* ΔSSAAI-pDser and *C. glutamicum* 14,067-pDser were analyzed.

FACS was performed with a FACSAria II (BD Biosciences, FACSAria II, USA) with cells diluted to a density of OD_{562} below 0.1. EYFP fluorescence was detected at 488 and 530 ± 10 nm at a sample pressure of 70 psi. Sorting was performed with four-way purity as the precision mode at a threshold rate up to 10,000 events/s. The forward scattering and lateral scattering were detected under a 488-nm laser, and a gate was marked to collect cells with high fluorescence intensity. Data were analyzed using BD DIVA 6.1.3 software. Cells were sorted in a tube containing 50 mg/mL kanamycin. The collected cells were spread on seed medium plates containing kanamycin. After 72 h, clones were picked, and 24-well plates containing 3 mL seed medium per well were incubated in an Infors HT shaker (Infors, Multitron Pro, Switzerland) at 30 °C and 500 rpm. After 48 h, clones were further analyzed for fluorescence and product.

Analytical methods

The cell density was measured at OD_{562} (AOE UV-1200S, China). The concentration of sugars (sucrose, glucose, and fructose) and amino acids (including L-serine, L-alanine, and L-valine) were determined by HPLC according to a previously published method (Zhang et al. 2017). All assays were performed in triplicate.

Genome sequencing

C. glutamicum A36-pDser genome sequencing was performed by GENEWIZ, Suzhou, China, with the Illumina HiSeq ×10 platform. The whole-genome Illumina sequencing raw data of *C. glutamicum* A36-pDser was deposited in the NCBI Sequence Read Archive database (accession no. SRP126758). *C. glutamicum* SYPS-062-33a was selected as the reference strain, and it was deposited under accession no. JYEG01.

Reverse mutation of the variant gene in the genome

Reverse mutation was performed using the nonreplicable deletion vector, pK18mob*sacB*, as reported previously (Schäfer et al. 1994). First, *pabAB* was amplified from *C. glutamicum* A36-pDser chromosome using the primer pairs *pabAB*-fw/re. The *pabAB* PCR product was then digested with *Xba*I and *Eco*RI and ligated into the pK18mob*sacB* vector, which was treated similarly. The recombinant pK18mob*sacBpabAB*426 plasmid was transformed into *C. glutamicum* ΔSSAAI-pDser competent cells by electroporation. Cells with the reverse mutations were screened and verified by PCR.

Enzyme activity assay

The *C. glutamicum* cells in logarithmic growth were harvested by centrifugation, washed three times with 100 mM Tris-HCl, pH 7.0, and then disrupted by sonication for 45 min (pulse, 3 s; interval, 7 s) with an ultrasonic homogenizer. The cell debris was discarded after centrifugation (12,000 rpm, 4 °C for 10 min), and the protein concentrations in the supernatant were measured.

The serine hydroxymethyl transferase (SHMT) activity was determined according to a published method (Zuo et al. 2007) in 50 mM Hepes buffer, pH 7.5, containing 0.1 M D, L-β-phenylserine, 1 mM Na_2EDTA, 25 mM Na_2SO_4, and crude enzyme in a total volume of 1 mL. The rate of formation of the benzaldehyde product was determined at 25 °C at

279 nm. One unit of enzyme activity was defined as 1 μmol benzaldehyde formed per minute.

Results

L-Serine biosensor plasmid construction

The L-serine biosensor plasmid was constructed based on the LysR-type transcriptional regulator NCgl0581 of *C. glutamicum*. The genomic region containing the open reading frame of NCgl0581 and the promoter of NCgl0580 were cloned in front of *eyfp* so that *eyfp* expression was under the control of the NCgl0580 promoter (Fig. 1a). The biosensor plasmid was transformed into *C. glutamicum* ΔSSAAI, thus resulting in *C. glutamicum* ΔSSAAI-pDser. Afterward, *C. glutamicum* ΔSSAAI and *C. glutamicum* ΔSSAAI-pDser were photographed with a laser scanning confocal microscope under visible light and fluorescence light. As shown in Fig. 1b, *C. glutamicum* ΔSSAAI strains showed no discernable fluorescence signal. However, *C. glutamicum* ΔSSAAI-pDser showed substantial fluorescence intensity. This result demonstrated that the L-serine biosensor was successfully constructed.

Verification of the correlation and specificity of the biosensor

To verify the correlation of fluorescence intensity with L-serine titer, the fluorescence intensity was measured with a microplate reader and microscope, and L-serine titer was measured by HPLC. When the concentration of L-serine increased, the EYFP fluorescence consequently increased, indicating that the fluorescence signal from the serine biosensor correlated with the L-serine concentration (Fig. 2a, b). These results demonstrated the functionality of the biosensor.

The specificity of the L-serine biosensor was then examined. In addition to L-serine, the by-products L-alanine and L-valine were present in the *C. glutamicum* ΔSSAAI-pDser fermentation culture. Hence, we sought to determine the interference of these by-products with the L-serine biosensor. The biosensor plasmid pDser was transformed into *C. glutamicum* 14,067, which does not accumulate L-serine, but does accumulate L-alanine and L-valine. The titers of L-alanine and L-valine of *C. glutamicum* 14,067-pDser and *C. glutamicum* ΔSSAAI-pDser were all within the same range (1–10 g) (Fig. 3a). The fluorescence signal of *C. glutamicum* ΔSSAAI-pDser increased over time; in contrast, the signal of *C. glutamicum* 14,067-pDser remained constant. The minimum fluorescence value of *C. glutamicum* ΔSSAAI-pDser was also higher than the maximum fluorescence value of *C. glutamicum* 14,067-pDser (Fig. 3b). The fluorescence intensities of both strains at different times were analyzed by flow cytometry (Fig. 3c). The results showed that the peak of *C. glutamicum* ΔSSAAI-pDser migrated to the right, and the peak of *C. glutamicum* 14,067-pDser did not migrate. The fluorescence intensity of *C. glutamicum* ΔSSAAI-pDser increased, whereas the fluorescence intensity of *C. glutamicum* 14,067-pDser was unchanged. Together, these results revealed that the by-products of L-alanine and L-valine had no effect on the L-serine biosensor pDser.

Biosensor-based FACS high-throughput screening

A high-throughput biosensor system was developed to screen L-serine-overproducing mutants treated with ARTP. A flowchart of the high-throughput screening is shown in Fig. 4a, which includes mutagenesis, screening, and fermentation validation. As shown in Fig. 4b, when the exposure time was 30 s, the lethality rate reached 94.05%, a level appropriate to mutagenize the cells. Therefore, that time was chosen to mutagenize *C. glutamicum* ΔSSAAI-pDser. Subsequently, *C. glutamicum* cells were incubated in seed medium for 2 h, washed with PBS, and then analyzed and sorted with FACS.

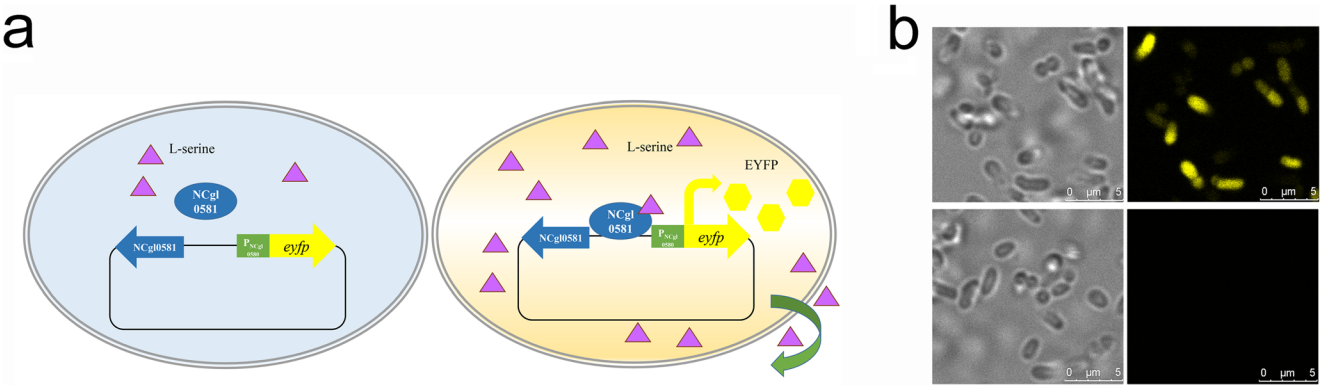


Fig. 1 Construction of the L-serine sensor pDser. **a** L-serine content is lower on the left (resting state) and higher on the right (excited state). **b** Top two images show *C. glutamicum* ΔSSAAI-pDser in visible light and fluorescent light, and the next two images show *C. glutamicum* ΔSSAAI in visible light and fluorescent light

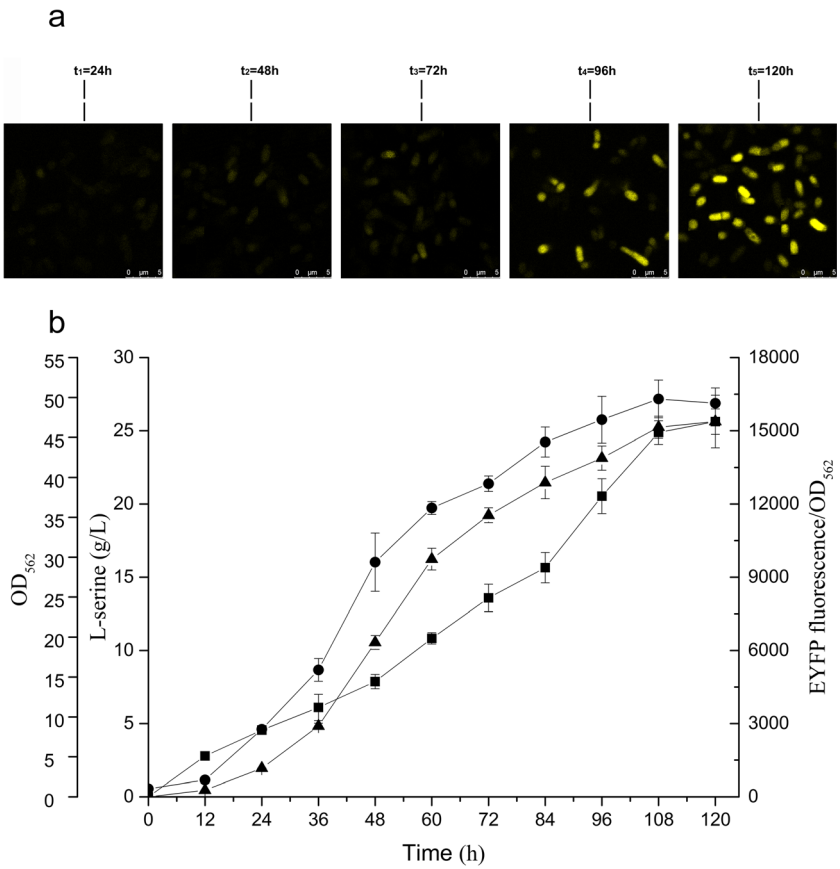


Fig. 2 Verifying the correlation of sensor pDser. **a** Fluorescence image of five time points (24 h/48 h/72 h/96 h/120 h). **b** Fluorescence intensity of unit cells (squares), the concentration of L-serine (triangles) and OD_{562} (circles) after fermentation of *C. glutamicum* ΔSSAAI-pDser

In a primary screen, 1.2×10^5 cells were collected and analyzed with FACS. The gate P1 was marked to sort cells with enhanced fluorescence emission (Fig. 4c). Nearly 510 cells were sorted in a tube with 2-mL seed medium and allowed to recover at 30 °C for 72 h. Then, the recovered strains were sorted with FACS again. The P2 gate in the second sort was the same as the P1 gate (Fig. 4c), and 170 positive cells with higher fluorescence were selected via P3 gate and then cultivated in seed medium plates at 30 °C for 72 h.

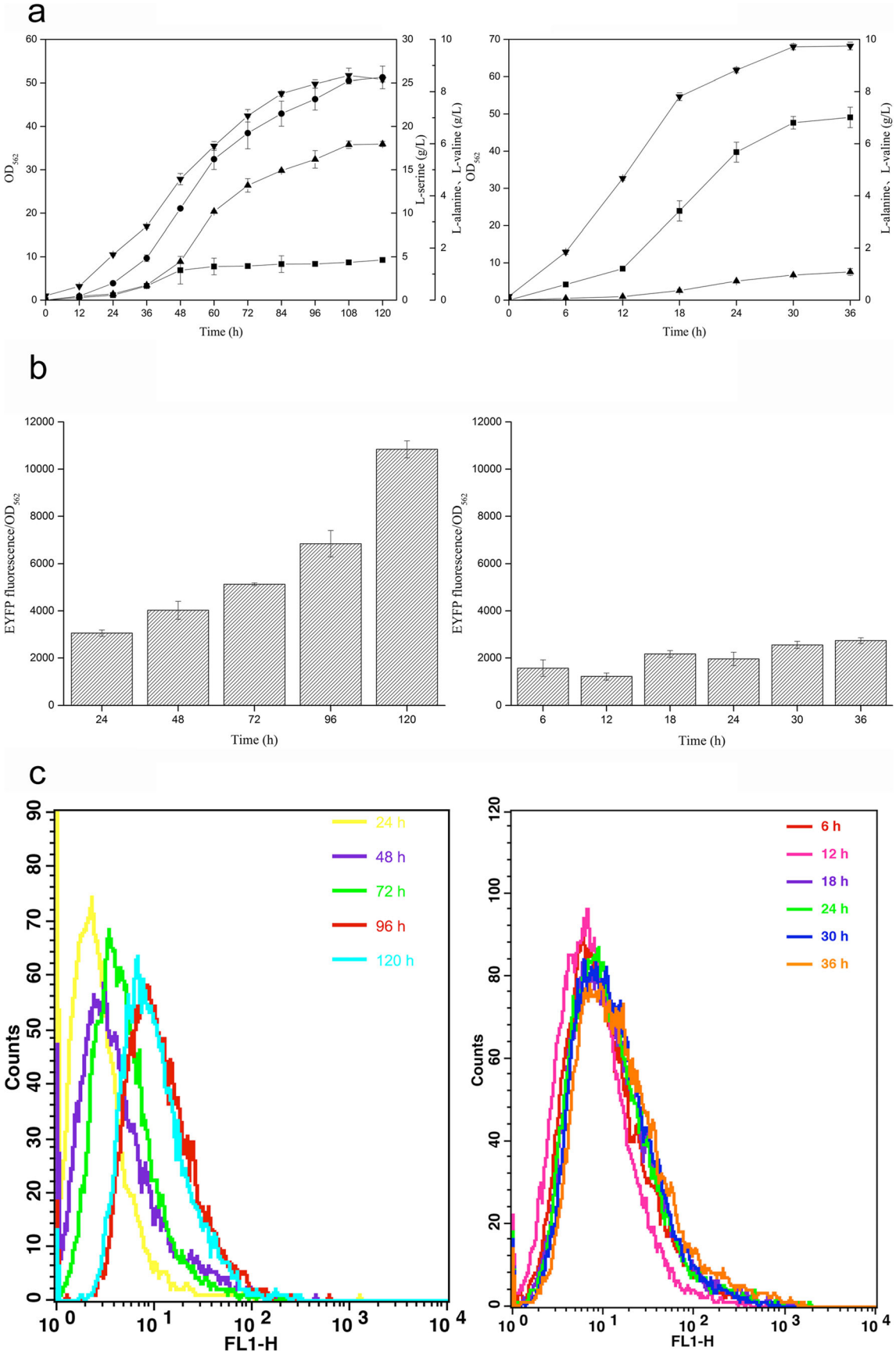
Characterization of mutant strains

After FACS screening of the mutant library, 96 mutant colonies with high fluorescence were selected and cultured in 24-well plates in an Infors HT shaker. The fluorescence of the 96 clones is shown in Fig. 5a. Among the 96 mutants, 19 mutants had much higher fluorescence than that of the parent strain. Those mutants were screened through fermentation, and amino acid concentrations were measured with HPLC (Table 2). The six strains with the highest titers were selected for fermentation, and the results are shown in Fig. 5b. Four strains had higher titers than that of *C. glutamicum* ΔSSAAI-pDser: A2-pDser (26.08 g/L), A10-pDser (28.05 g/L), A36-pDser (30.18 g/L), and A15-pDser (27.46 g/L). The mutant strain A36-pDser had the highest titer among all strains.

The fermentation characteristics of *C. glutamicum* A36-pDser

The stability of *C. glutamicum* A36-pDser in successive subcultures was evaluated through shake flask fermentation, and the L-serine titer of *C. glutamicum* A36-pDser remained stable even after ten generations (Fig. 6a), thus demonstrating that ARTP was effective and was able to improve diverse traits of bacteria in a genetically stable manner. The fermentation performance of the mutant A36-pDser and its parent strain were evaluated (Fig. 6b). As shown in Fig. 6b, *C. glutamicum* A36-pDser accumulated 30.57 g/L L-serine with a yield of 0.30 g/g sucrose, and OD_{562max} was 58.92, which were 19.5, 42.9, and 15.3% higher than those of the parent strain *C. glutamicum* ΔSSAAI-pDser. The by-product titer was essentially unchanged (data not shown). The fermentation medium was

Fig. 3 Verifying the specificity of sensor pDser. **a** The left image shows the *C. glutamicum* ΔSSAAI-pDser fermentation process, and the right image shows the *C. glutamicum* 14,067-pDser fermentation process. L-serine (circles), L-alanine (squares), L-valine (regular triangles), and OD_{562} (inverted triangles) are indicated. **b** Fluorescence intensities of unit cells at different times for *C. glutamicum* ΔSSAAI-pDser (left) and *C. glutamicum* 14,067-pDser (right). **c** Flow cytometry analysis of *C. glutamicum* ΔSSAAI-pDser (left) and *C. glutamicum* 14,067-pDser (right). The counts are the number of strains, and the FL1-H is the EYFP fluorescence intensity


a
OD562
L-serine (g/L)
L-alanine、L-valine (g/L)
Time (h)
b
EYFP fluorescence/OD562
Time (h)
c
24 h
48 h
72 h
96 h
120 h
6 h
12 h
18 h
24 h
30 h
36 h
Counts
FL1-H

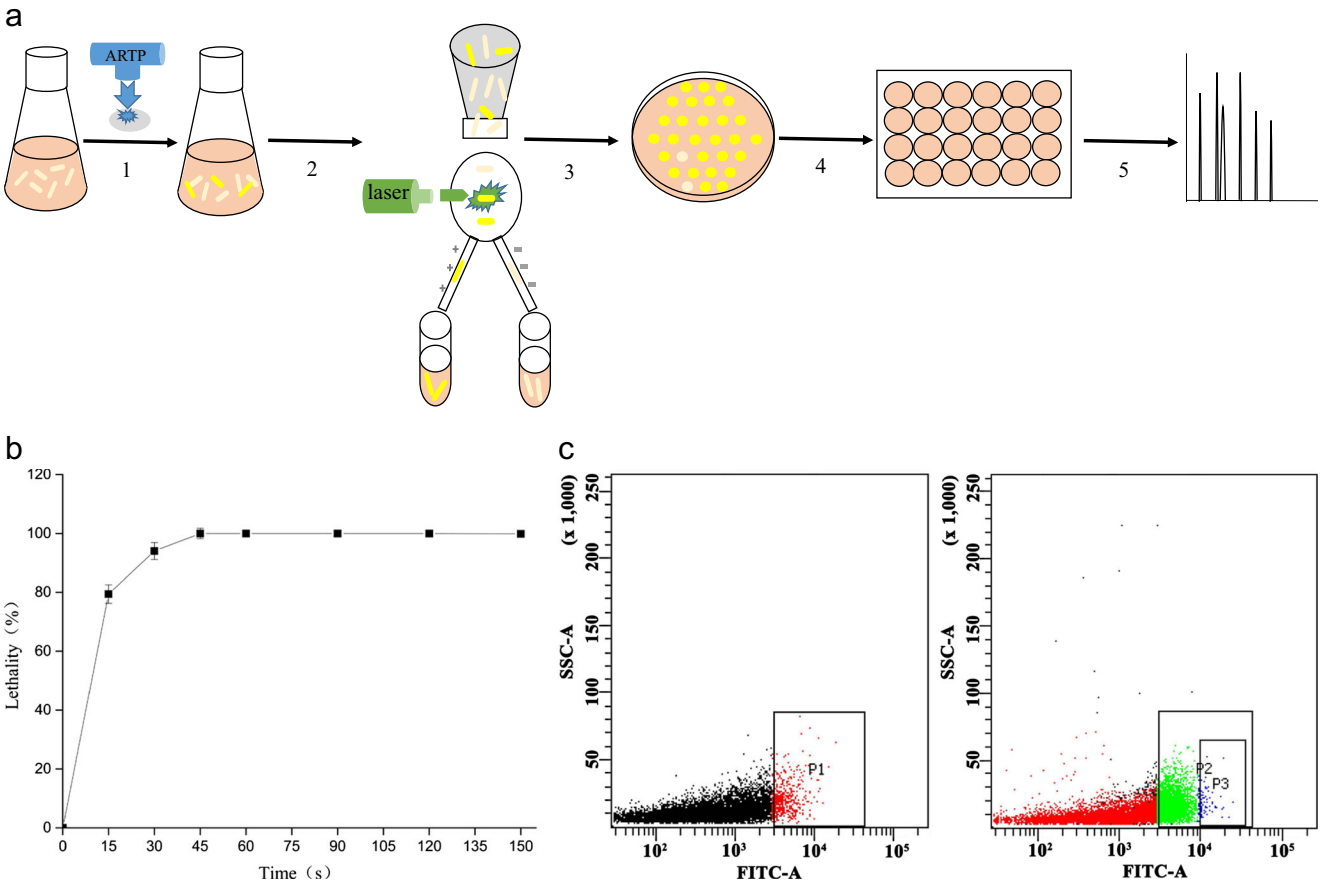


Fig. 4 High-throughput screening method based on the biosensor. **a** A flow chart of FACS screening: (1) ARTP mutagenesis, (2) sorting of nearly 510 cells with FACS in gate P1. The sorted bacteria were screened again by re-culture, and 170 cells were then acquired with higher fluorescence in gate P3. (3) Coating on seed medium plates. (4) Cells were cultured in 24-well plates. (5) The L-serine content in the supernatant was determined with HPLC. **b** The lethality of *C. glutamicum* ΔSSAAI-pDser. **c** The dots in the figure show the fluorescence distribution of the mutagenized strain. The abscissa FITC-A represents the EYFP fluorescence intensity. SSC side scatter

optimized (Supplementary Fig. S1),the result showed that the L-serine titer was further increased, when the concentration of $MgSO_4{\cdot}7H_2O$ was increased from 0.5 to 1 g/L (Fig. 6c), L-serine titer was 34.78 g/L with the yield of 0.35 g/g, and OD_{562max} was 64.46, which were 13.8, 16.7, and 9.4% higher than those of the strain *C. glutamicum* A36-pDser (0.5 g/L $MgSO_4{\cdot}7H_2O$), respectively.

The whole-genome sequence of *C. glutamicum* A36-pDser

C. glutamicum A36-pDser was re-sequenced on the Illumina HiSeq ×10 platform with over 1.59 million reads. Then, a comparative genomics analysis of *C. glutamicum* A36-pDser and *C. glutamicum* SYPS-062-33a (in which 591 bp of the C-terminal domain of *serA*, *sdaA*, *avtA,* and *alaT* was knocked out, and *ilvN* was attenuated, resulting in *C. glutamicum* ΔSSAAI) was performed. The results revealed 83 single-nucleotide variants, which included 29 synonymous mutations, 11 non-synonymous mutations, and other mutations located in the intergenic regions. The non-synonymous mutations are shown in Table 3. Four genes were associated with the folate biosynthesis, purine metabolism, and fatty acid biosynthesis metabolic pathways (SD36_05555, SD36_11550, SD36_13090, and SD36_04560), four genes were associated with the transport pathway (SD36_02790, SD36_10410, SD36_12845, and SD36_06225), and the functions of three genes were unclear.

We found that the glycine titer of *C. glutamicum* A36-pDser was lower than the parent strain *C. glutamicum* ΔSSAAI-pDser (ΔSSAAI-pDser 0.685 ± 0.010 g/L; A36-pDser 0.545 ± 0.012 g/L), and SHMT (catalyze L-serine to glycine) enzymatic activities were also measured (ΔSSAAI-pDser 0.285 ± 0.001 U/mg; A36-pDser 0.245 ± 0.001 U/mg). The gene-encoded SHMT had no difference between the two strains; however, a mutation was detected in aminodeoxychorismate synthase (*pab*AB), aminodeoxychorismate synthase is a key enzyme in the folate biosynthesis pathway, and folate is coenzyme of SHMT. Then, reverse mutation of *pab*AB (mutation of Thr-426 → Ile in *pabAB*) was performed in the genome of

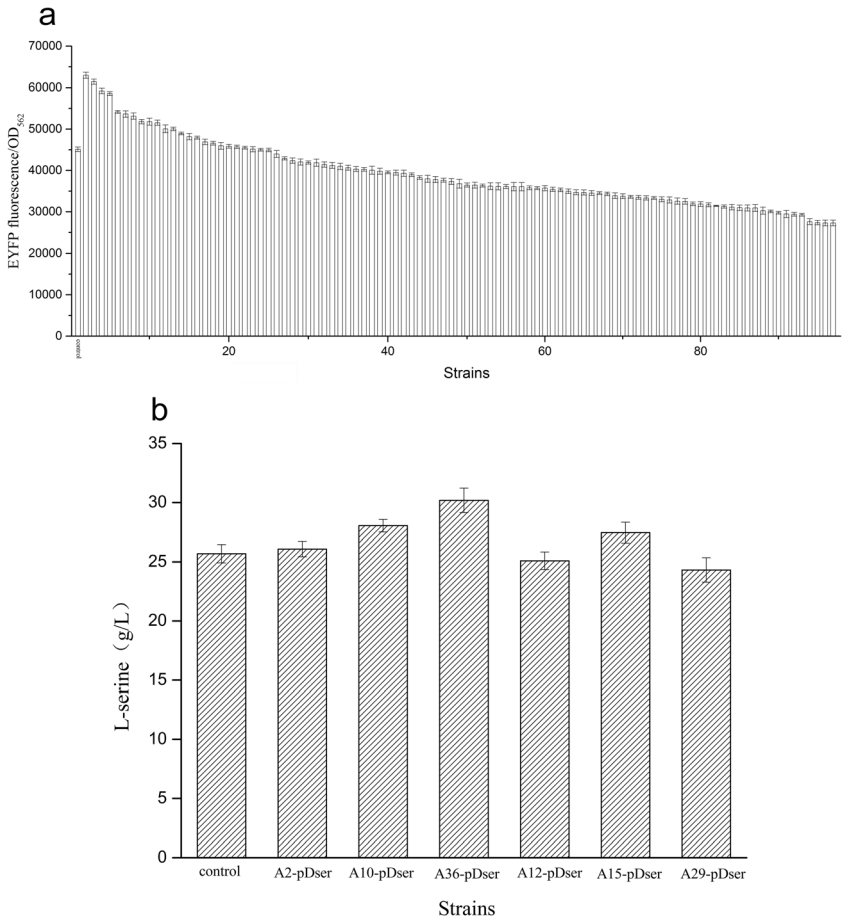


Fig. 5 Characterization of L-serine-producing mutants screened by FACS. **a** Fluorescence intensities of unit cells of the 96 cells, as detected with a microplate reader. **b** L-Serine yield of cells that were selected again from 19 cells by fermentation

Table 2 The fermentative characterization of mutants. Nineteen strains with higher fluorescence were selected from 96 strains for fermentation, and the titer of L-serine was detected by HPLC

Strain	L-serine(g/L)
ΔSSAAI-pDser	25.11 ± 0.74
A1-pDser	26.94 ± 0.43
A2-pDser	30.61 ± 0.83
A4-pDser	24.93 ± 0.39
A5-pDser	28.13 ± 0.23
A6-pDser	28.22 ± 0.42
A9-pDser	27.02 ± 0.81
A10-pDser	30.90 ± 0.78
A12-pDser	35.16 ± 0.32
A14-pDser	21.53 ± 1.13
A15-pDser	29.75 ± 0.62
A19-pDser	22.62 ± 1.02
A23-pDser	23.68 ± 0.19
A25-pDser	22.75 ± 0.39
A27-pDser	25.76 ± 0.89
A29-pDser	30.85 ± 0.62
A36-pDser	32.20 ± 0.14
A38-pDser	22.34 ± 0.81
A46-pDser	23.98 ± 0.35
A54-pDser	22.07 ± 0.98

C. glutamicum ΔSSAAI-pDser, thus resulting in the strain *C. glutamicum* ΔSSAAI-pDser (*pabAB* 426). However, we did not observe any significant variation regarding amino acid production, cell growth, and sugar consumption rate. Further studies will be needed to explore the reasons for these phenotypic differences.

Discussion

ARTP is an effective and convenient mutagenesis method, which can greatly improve the screening efficiency of mutagenic libraries, when combined with biosensor and high-throughput screening. In this way, we obtained the high-yield *C. glutamicum* A36-pDser, with a titer of 34.78 g/L and a yield of 0.35 g/g. ARTP has also been successfully applied to the microbial production of molecules such as amino acids, succinic acid, and butanol (Zhang et al. 2014b). Studies using SOS/*umu*-test indicated that ARTP had a unique mechanism of genetic damage, by decreasing the cell membrane surface potential and increasing its permeability to the active particles, and then DNA is damaged. Its effect on gene damage is greater than those of traditional mutagenesis

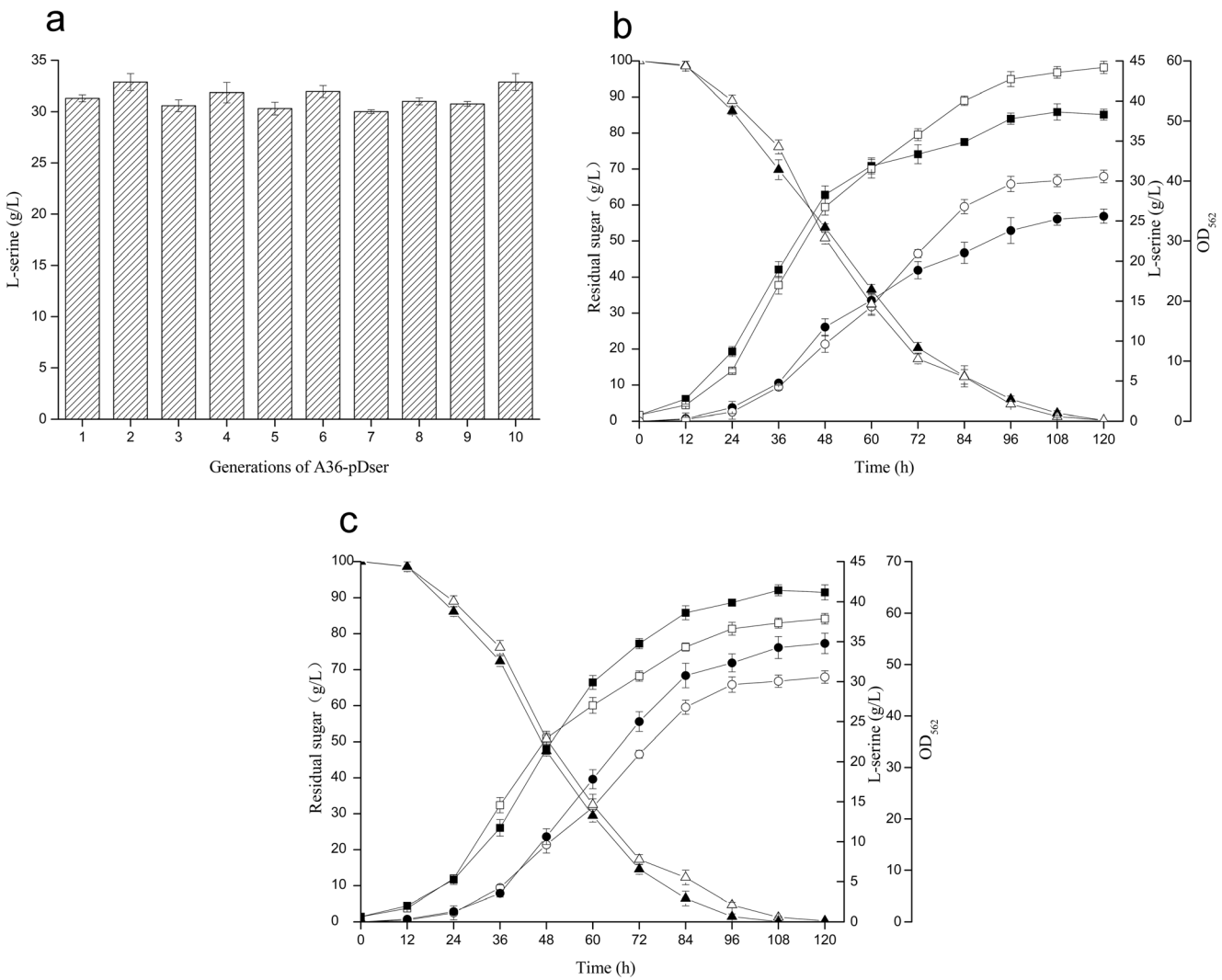


Fig. 6 Analysis of *C. glutamicum* A36-pDser. **a** Genetic stability experiments. **b** Comparison of fermentation performance between mutant strain *C. glutamicum* A36-pDser (hollow) and parental strain *C. glutamicum* ΔSSAAI-pDser (solid). The OD_{562} (squares), residual sugar (triangles), and L-serine (circles) are shown. **c** Comparison of fermentation performance of the mutant strain *C. glutamicum* A36-pDser under the conditions of 0.5 g/L $MgSO_4{\cdot}7H_2O$ (hollow) and 1 g/L $MgSO_4{\cdot}7H_2O$ (solid). The OD_{562} (squares), residual sugar (triangles), and L-serine (circles) are shown

methods such as UV and MNNG (Xue et al. 2014). Cheng et al. used ARTP to develop the mutant ARG 3–15 of *C. glutamicum* GUI089, thus increasing the titer of L-arginine by 43.79% (Cheng et al. 2016). Zheng et al. reported a mutant *Corynebacterium acetoacidophilum* D3 with 65.8 g/L of proline, 20.3% higher than that of the parent strain (54.7 g/L) (Zheng et al. 2013). In this study, the ARTP mutagenesis method was successfully applied to the L-serine-producing strain *C. glutamicum* ΔSSAAI-pDser, and the yield of L-serine increased by 66.7%. For ARTP, the power input, the gas flow rate, and the distance between the plasma torch nozzle exit and the sample plate are the key parameters affecting the bacterial mutation efficiency and were set to 100 W, 10 slpm, and 2.0 mm, respectively (Gu et al. 2017; Zhang et al. 2014b). Longer exposure times would lead to high lethality, thus affecting the efficiency of mutagenesis. Therefore, we limited the exposure time to 30 s, and the corresponding lethality rate was 94.05%.

Biosensor technology has been widely used to screen products and optimize pathway expression (Eggeling et al. 2015; Johnson et al. 2017; Xu 2017; Xu et al. 2014, 2013). Many different types of biosensors were used to detect small molecules, including RNA-based biosensors, transcription factor-based biosensors, and Förster resonance energy transfer biosensors (Jang et al. 2017; Schallmey et al. 2014; Wang et al. 2017; Yu et al. 2017). We constructed L-serine biosensor pDser, which is based on NCgl0581 (a transcription factor specifically responsive to L-serine), for high-throughput screening of high-yield L-serine strains. The key prerequisite for screening cells is the availability of biosensor with

Table 3 The non-synonymous mutations of strain A36-pDser

Gene	Definition	Amino acid change
SD36_05555	Para-aminobenzoate synthetase	T426I
SD36_11550	Folylpolyglutamate synthase	D7N
SD36_13090	Adenylosuccinate lyase	A58S
SD36_04560	Fatty acid synthase, bacteria type	D2653E
SD36_02790	Tyrosine-specific transport protein	M303I
SD36_10410	Inner membrane transporter	A151V
SD36_12845	Putative ABC transport system permease protein	G797S
SD36_06225	ATP-binding cassette, subfamily C, bacterial CydC	S454N
SD36_00970	Alkylated DNA repair protein	T179A
SD36_02075	Putative transposase	F46L
SD36_14960	UPF0176 protein	L197F

sufficient correlation and specificity. To this end, we examined the correlation of L-serine concentration and unit fluorescence intensity, and imaged the cells with a fluorescence microscope. As shown in Fig. 2, the biosensor showed a strong positive correlation with extracellular L-serine titer, and this correlation was not linear. Chen et al. constructed a high-throughput method to screen ectoine hyper-producing strains, in which a positive correlation between biosensor and extracellular ectoine titer had been observed (Chen et al. 2015). Liu et al. built an artificial fusion promoter cysJHp to gain better response to threonine. The promoter cysJHp and the reporter gene *lacZ* were linked to the pTZL1 plasmid, transformed into *E. coli* MG1655, and was used to screen high-yield strains. The fusion cysJHp promoter is also positively correlated with extracellular threonine (Liu et al. 2015). As for the specificity of the biosensor used herein, only L-serine responded to the biosensor pDser (Fig. 3), and the by-products L-alanine and L-valine had no effect on the L-serine biosensor pDser. However, previous reports showed that the biosensor pSenLys can recognize L-arginine and L-histidine in addition to L-lysine, and the Lrp-based biosensor can detect L-isoleucine, L-leucine, and L-methionine in addition to L-valine (Binder et al. 2012; Mustafi et al. 2012). Thus, these biosensors were not specific for the products L-lysine and L-valine, and this lack of specificity may decrease the efficiency of screening for the single product of interest. Nevertheless, the combination of the pDser biosensor and high-throughput FACS screened L-serine high-yield strains accurately and efficiently, and this is also confirmed by the fermentation. This efficient procedure can also be applied to other desired amino acids, and the yield could easily be increased if specific transcriptional regulators are found for other amino acids.

The L-serine biosensor pDser was constructed and successfully applied to establish a high-throughput screening protocol by FACS for screening mutants. Only 2 weeks were needed to screen high-yield strains from a 1.2×10^5 mutant library, and this procedure was approximately 10^3–10^4 times faster than traditional method. The fermentation results showed that *C. glutamicum* A36-pDser was the highest titer among all mutants.

Some mutations were detected through genome comparison between *C. glutamicum* SYPS-062-33a (in which 591 bp of the C-terminal domain of *serA*, *sdaA*, *avtA*, and *alaT* was knocked out, and *ilvN* was attenuated, resulting in *C. glutamicum* ΔSSAAI) and *C. glutamicum* A36-pDser. As a decrease in glycine titer and SHMT enzymatic activity was determined in *C. glutamicum* A36-pDser, we speculated that the folate biosynthesis pathway mutation (SD36_05555; *pabAB*) might involve an increase in L-serine production. Aminodeoxychorismate synthase (*pabAB*) is a key enzyme in folate pathway, and folate is a coenzyme of SHMT. The decreased coenzyme supply might lead to decreased SHMT activity, thus decreasing the conversion of L-serine to glycine and increasing L-serine accumulation (Stolz et al. 2007; Zhang et al. 2014a). However, there were no significant changes resulting from the reverse mutation in *pabAB* in *C. glutamicum* ΔSSAAI-pDser, possibly because the mutation may have little effect on the activity of the *pabAB*, or the mutation may not be located in the domain of the active site and the coenzyme binding site. Furthermore, SD36_10410 is the homologous sequence of the gene *rhtA*, which encodes the threonine exporter (Livshits et al. 2003). Deletion of *rhtA* results in a 6.8-fold increased growth rate in L-serine-producing *E. coli* (Mundhada et al. 2016). Thus, we speculate that the mutation of SD36_10410 might be involved in the growth of *C. glutamicum* A36-pDser. The specific mutations that improved the L-serine production in *C. glutamicum* A36-pDser remain unclear. Thus, further studies should focus on the other variant genes or variations in the intergenic regions.

This study was the first report that a novel powerful mutagenesis tool (ARTP) combined with high-throughput screening (FACS) were used to substantially improve the yield of L-serine in *C. glutamicum*. We obtained a mutant named *C. glutamicum* A36-pDser whose titer and yield were 35.9 and 66.7% higher than those of the parent strain

C. glutamicum ΔSSAAI-pDser, with the optimization of magnesium concentration. Moreover, the whole-genome sequencing identified 11 non-synonymous mutations of genes associated with metabolic and transport pathways, which might be responsible for the higher L-serine production and better cell growth in *C. glutamicum* A36-pDser.

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Compliance with ethical standards

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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