

Improvement of L-Valine Production by Atmospheric and Room Temperature Plasma Mutagenesis and High-Throughput Screening in *Corynebacterium glutamicum*

Guoqiang Han,^{||} Ning Xu,^{||} Xieping Sun, Jinzhao Chen, Chun Chen, and Qing Wang*



Cite This: <https://dx.doi.org/10.1021/acsomega.9b02747>



Read Online

ACCESS |



Metrics & More

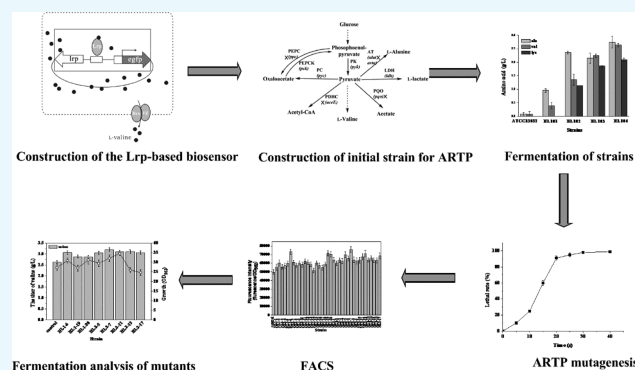


Article Recommendations



Supporting Information

ABSTRACT: As one of the branched-chain amino acids, L-valine is an essential nutrient for most mammalian species. In this study, the L-valine producer *Corynebacterium glutamicum* $\Delta ppc\Delta aceE\Delta alaT\Delta p-qo$ was first constructed. Additionally, an improved biosensor based on the Lrp-type transcriptional regulator and temperature-sensitive replication was built. Then, the *C. glutamicum* strain was mutagenized by atmospheric and room temperature plasma. A sequential three-step procedure was carried out to screen L-valine-producing strains, including the fluorescence-activated cell sorting (FACS), 96-well plate screening, and flask fermentation. The final mutant HL2-7 obtained by screening produced 3.20 g/L of L-valine, which was 21.47% higher than the titer produced by the starting strain. This study demonstrates that the L-valine-producing mutants can be successfully isolated based on the Lrp sensor system in combination with FACS screening after random mutagenesis.



INTRODUCTION

L-Valine is one of the nine essential amino acids that must be supplied in the diet.¹ It is largely produced through microbial fermentation, and the production capacity reached 2 thousand tons per year, with a market value of 45 million dollars.² Microbial production of L-valine has been established using various industrial microorganisms, including *Brevibacterium flavum*,³ *Escherichia coli*,⁴ *Corynebacterium glutamicum*,⁵ and *Bacillus subtilis*.⁶ As a well-known workhorse for the production of amino acids such as L-glutamate,⁷ L-lysine,⁸ L-isoleucine⁹ and L-threonine,¹⁰ *C. glutamicum* is also viewed as an attractive microorganism for the production of L-valine.

Industrial strains for the production of L-valine were usually developed by traditional random mutagenesis or metabolic engineering. The traditional strategies, which utilize toxic analogues, have been used to produce the most efficient amino acid producing strains of *C. glutamicum*. Because of the inefficient genetic manipulation of *C. glutamicum* and heavy workload required to identify high-yield strains from very large mutant libraries,^{11–13} metabolic engineering has emerged as a more efficient solution for the production of specific amino acids and their precursors.¹⁴ Furthermore, the genome sequences of several *C. glutamicum* strains have been reported,^{15,16} and a high L-valine production phenotype was also obtained through rational genetic engineering manipulation.^{17–22} In *C. glutamicum*, the L-valine biosynthesis pathway competes with many by-product pathways, such as L-alanine

and lactic acid. Consequently, the strategies to improve the yield of L-valine have largely focused on the enhancement of downstream synthesis pathways in combination with the inhibition of competing pathways.¹⁴

Rational design and subsequent metabolic engineering represents an important tool for developing industrial production strains.²³ However, because of the high complexity of carbon and energy fluxes in living cells, rational approaches to strain development are often restricted by the current knowledge of bacterial physiology.²⁴ However, the limits of rational strain engineering can be overcome by nonrational high-throughput approaches based on random mutagenesis and sequential efficient screening methods.²⁵ the biosensors that detect intra- and extracellular metabolites or environmental signals are ubiquitous in nature.²⁶ The use of biosensors has proven to be a highly valuable tool to specifically transform the intracellular product concentration into a screenable optical output, such as fluorescence.^{11,13,27} Transcriptional factor (TF)-based biosensors for the high-throughput screening of engineered bacteria^{11,28,29} and fast

Received: August 25, 2019

Accepted: February 13, 2020

selection of desirable mutations³⁰ are widely used because of their ability to detect a wide variety of metabolites.²⁹ In recent years, a large number of TF-based biosensors have been applied for the screening of high-value products such as amino acids,^{12,23,28,31,32} organic acids,^{33,34} and other microbial chemicals.^{12,35–41}

In this study, adaptive evolution driven by an L-valine biosensor in combination with fluorescence-activated cell sorting (FACS) approach following rational genetic manipulation and atmospheric and room temperature plasma (ARTP) mutagenesis was successfully applied to improve the L-valine production in *C. glutamicum*. This approach demonstrates the power of combining rational design with biosensor-driven laboratory evolution approaches to improve the production of specific amino acids.

MATERIALS AND METHODS

Strains and Culture Conditions. The strains and plasmids used in this study are listed in Table 1. *E. coli*

Table 1. Bacterial Strains and Plasmids Used in This Study

strain or plasmid	description	source
Strains		
DH5 α	cloning strain of <i>E. coli</i>	Novagen
ATCC13032	wild-type <i>C. glutamicum</i>	ATCC
HL101	ATCC13032 Δ ppc	18
HL102	ATCC13032 Δ ppc Δ aceE	this work
HL103	ATCC13032 Δ ppc Δ aceE Δ alat	this work
HL104	ATCC13032 Δ ppc Δ aceE Δ alat Δ pqo	this work
ATCC13032/pHL203	ATCC13032 harboring pHL203	this work
HL104/pHL203	HL104 harboring pHL203	this work
Plasmids		
pK19mobsacB	vector for gene deletion in <i>C. glutamicum</i>	45
pK19mobsacB- Δ ppc	derived from pK19mobsacB by inserting the segment ppc (up + down)	this work
pK19mobsacB- Δ aceE	derived from pK19mobsacB by inserting the segment aceE (up + down)	this work
pK19mobsacB- Δ alat	derived from pK19mobsacB by inserting the segment alat (up + down)	this work
pK19mobsacB- Δ pqo	derived from pK19mobsacB by inserting the segment pqo (up + down)	this work
pHL200	pTRCmob harboring <i>lrp-brnE-gfp</i>	this work
pHL201	pTRCmob harboring <i>rrnB</i>	this work
pHL202	pHL201 harboring <i>lrp-brnE-gfp</i>	this work
pHL203	derived from pHL202 by inserting the temperature-sensitive replication origin from pDTW109	this work

DH5 α was used for plasmid construction. *E. coli* cells were grown in an LB medium at 37 °C and 200 rpm,⁴² and *C. glutamicum* was grown in an LBG medium (an LB medium with 5 g/L of glucose) at 30 °C and 200 rpm. Modified Epo medium (5 g/L of yeast extract, 10 g/L of tryptone, 10 g/L of NaCl, 30 g/L of glycine and 0.1% Tween 80) was used for preparing competent cells of *C. glutamicum*. An LBHIS medium (2.5 g/L of yeast extract, 5 g/L of tryptone, 5 g/L of NaCl, 18.5 g/L of Brain Heart Infusion powder and 91 g/L of sorbitol) was used for *C. glutamicum* transformation. When necessary, 30 mg/L of kanamycin was added to the medium to promote plasmid maintenance.

Construction of the Lrp-Based Biosensor. For the construction of the biosensor, the *rrnB* terminator was amplified from the pTRCmob plasmid using the primer pair *rrnB*-F/R, digested with *Eco*RI and *Eco*RV, and ligated into the

same sites of the pTRCmob backbone digested, resulting in pHL201.

The fragment containing the *C. glutamicum* *lrp* ORF²⁸ and the intergenic region of *lrp-brnF* was amplified using the primers *lrp*-F and *lrp-brnF*-R. The *egfp* ORF was amplified by PCR using the primers *gfp*-F/R. The two resulting fragments *lrp-brnF* and *gfp* were ligated by overlap-extension PCR. The PCR product was digested with *Sac*I/*Bam*HI, and ligated into the same sites of pHL201, resulting in the plasmid pHL202.

The temperature-sensitive *C. glutamicum* replicon *rep* was amplified from pDTW109⁴³ using the primer pair *rep*-F/R. The PCR product was then digested with *Spe*I and *Eco*RV, and inserted into pHL202, resulting in the plasmid pHL203. The temperature sensitivity of pHL203 was tested in *C. glutamicum* ATCC13032. The strains were grown in an LBG medium in the presence of kanamycin at 30 °C for 16 h, then introduced into a fresh LBG medium without kanamycin and grown at 30 or 37 °C. Samples were taken after 12 h, diluted, and spread onto LBG plates with or without kanamycin, and grown at 30 °C for 36 h. The colonies were counted on both plates and the proportion of kanamycin-resistant cells in the sample was calculated.

C. glutamicum was seeded into an LBG liquid medium and grown overnight, then transferred to a fresh LBG medium at an initial OD₅₆₂ of 1. When the OD₅₆₂ reached 5, L-valine was added to a final concentration of 0–40 mM. The fluorescence and OD₅₆₂ were determined after continuous culture at 30 °C for 2 h. Fluorescence measurements were carried out using a SpectraMax M2 multidetection microplate reader (Molecular Devices, CA, USA). The eGFP fluorescence was measured at excitation and emission wavelengths of 488 and 530 nm, respectively.⁴⁴

Construction of the Initial *C. glutamicum* Strain for ARTP. Chromosomal inactivation of the *ppc* gene encoding phosphoenolpyruvate carboxylase (PEPC), *aceE* encoding the Elp subunit of the pyruvate dehydrogenase complex (PDHC), *alat* encoding aminotransferase (AT) and *pqo* encoding pyruvate/quinine oxidoreductase (PQO) in *C. glutamicum* ATCC13032 was performed using the suicide vector pK19mobsacB.⁴⁵ The primers used in this study were designed according to the genome sequence of *C. glutamicum* and are listed in Table 2. The upstream fragment ppcU of the *ppc* gene was amplified from the genomic DNA of *C. glutamicum* ATCC13032 using the primer pair ppcU-F/R; the downstream fragment ppcD of the *ppc* gene was amplified using the primer pair ppcD-F/R. The two fragments ppcU and ppcD were purified, mixed, ligated by overlap extension PCR, and the fusion product was ligated into the vector pK19mobsacB,⁴⁵ resulting in the plasmid pK19mobsacB- Δ ppc.

The plasmid pK19mobsacB- Δ ppc was introduced into *C. glutamicum* ATCC13032 by electroporation and transformants were selected on LBHIS agar supplemented with 30 mg/L of kanamycin.⁴⁵ The kanamycin-resistant transformants were further grown for 12 h in an LBG medium, and subsequently selected again on the LBHIS plates supplemented with 10% sucrose. The sucrose-resistant and kanamycin-sensitive colonies were further grown in an LB medium. The deletion of *ppc* was further confirmed by PCR using the primers *ppc*-V and *ppc*-R. The cells of the mutant strain HL101 grew on the plate without antibiotics. Similarly, the *aceE* gene in the genome of HL101 was deleted, resulting in the strain HL102. Next, the *alat* gene in the genome of HL102 was deleted, resulting in the strain HL103. Finally, the *pqo* gene in the genome of HL103

Table 2. Primers Used for PCR in This Study^a

name	5'–3' sequence	restriction site
<i>ppcU-F</i>	GTAGGATCCATAAGTCTGGATGAGGTTGAG	<i>Bam</i> HI
<i>ppcU-R</i>	GCAGAAGCTGTGGCCGATGCATTAGAGCAGTGGATTGG	
<i>ppcD-F</i>	CCAATCCACTGCTCTAATGCATCGGCCACAGCTTCTGC	
<i>ppcD-R</i>	GTAGGATCCGCTCTGATCGTGGAGCTTGCAG	<i>Bam</i> HI
<i>ppc-V</i>	AAGTGCACACCGAGGAACAGTG	
<i>aceEU-F</i>	GTAGGATCCAGATACGACCATTCTCAGC	<i>Bam</i> HI
<i>aceEU-R</i>	GTCCAGGTTGTTCAAGTGCCTTAGAGTCATCCGAGGGC	
<i>aceED-F</i>	CCTCGGATGACTCTAACGCACTGAACAACCTGGACAAC	
<i>aceED-R</i>	TACTCTAGATTTCCTTGAAGGTACGAACAGTC	<i>Xba</i> I
<i>aceE-V</i>	AAGAACCTTGCCAATTACCGAG	
<i>alatU-F</i>	ACGTCTAGAGGACATGTAATAGGAGAAGG	<i>Xba</i> I
<i>alatU-R</i>	GAGAGCTACCTGAATCAGCACGTCCTTCATCTTCTCC	
<i>alatD-F</i>	GATGAAGGACGTGCTGATTGAGGTAGCTCTCGGTGG	
<i>alatD-R</i>	ACGTCTAGAAATCCTTCTCTAGCATGCTGGC	<i>Xba</i> I
<i>alat-V</i>	GACGATGAACAGTGGCTTCTG	
<i>pqoU-F</i>	GTAGGATCCATGCTCCCGAGGTGATCTGCTG	<i>Bam</i> HI
<i>pqoU-R</i>	GTGCGAAGGTGTTGGCCATTAATGCGCTGTCGATCCAC	
<i>pqoD-F</i>	TGGGATCGACAGCGCATTAATGGCCAACACCTTCGCACC	
<i>pqoD-R</i>	GTAGGATCCCTTCTTTGAGGCGCTTAGAGG	<i>Bam</i> HI
<i>pqo-V</i>	CTCCGACAAAGCAGAGATTCTG	
<i>rrnB-F</i>	CGATGGTAGTGTGGGGTCTC	
<i>rrnB-R</i>	AGGAATTCAGAAAAATAAACAAAAAGAG	<i>Eco</i> RI
<i>Lrp-brnF-F</i>	ATAGAGCTCATCACACCTGGGGGCGAGCTG	<i>Sac</i> I
<i>Lrp-brnF-R</i>	TCTTCTCCTTTACTCATATATCTCCTTCTTAAAGTTCAG	
<i>gfp-F</i>	AGAAGGAGATATATGAGTAAAGGAGAAGAACTTTTCAC	
<i>gfp-R</i>	CGCGGATCCCTATTTGTATAGTTCATCCATGCCA	<i>Bam</i> HI

^aRestriction sites are underlined.

was deleted, resulting in the strain HL104. The replacement at the chromosomal loci was verified by PCR using the primer pairs *aceE-V/aceED-R*, *alat-V/alatD-R*, or *pqo-V/pqoD-R*, respectively.

Mutagenesis by ARTP and Determination of Optimal Treatment Time. An ARTP-IIIS instrument (Wuxi TMAX-TREE Biotechnology Co., Ltd, Wuxi, China) was used to carry out the ARTP mutagenesis.⁴⁶ Pure helium was used as the plasma working gas to irradiate the samples on the stainless-steel plate. The operating parameters were as follows: (1) the radio frequency power input was 100 W; (2) the distance between the sample plate and the plasma torch nozzle exit was 2 mm; (3) the helium gas flow rate was 8 SLM (standard liter per minute); and (4) the temperature of the plasma jet was at 25–30 °C. The mutagenic effect was only dependent on the treatment time.

The *C. glutamicum* strain HL104/pHL203 was grown to an OD₅₆₂ of 10 in the LBHIS medium (mid-log phase). To determine the optimal treatment time, the cells were washed twice with 50 mL of phosphate-buffered saline (PBS), after which 10 μL of the cell suspension (1 × 10⁸ cells per milliliter) was spread on the sterilized steel plate, and exposed to the ARTP nozzle exit for 0, 5, 10, 15, 20, 25, 30, and 40 s. After each treatment, the samples were washed down with 1 mL of PBS into fresh sterile tubes, properly diluted, and cultured on a solid medium with 30 mg/L of kanamycin at 30 °C for 2 days. An untreated culture was used as a control. The appropriate treatment time was selected based on the lethality rate, which was calculated as follows

$$\text{Lethality rate (\%)} = \frac{\text{control colonies} - \text{survival colonies}}{\text{control colonies}} \times 100\%$$

Fluorescence Microscopy and Spectroscopy. After ARTP treatment, HL104/pHL203 was cultivated on LBHIS plates at 30 °C for 12 h, transferred into shake flasks containing 25 mL of LBHIS with 30 mg/L of kanamycin, and grown for 2 days. The resulting cells were harvested by centrifugation at 8000g for 10 min at 4 °C, followed by washing in PBS three times.⁴⁴ The washed cells were resuspended in PBS and vortexed for 5 min to obtain a uniform suspension. The cell suspension was diluted to an optical density OD₅₆₂ of 0.1 to avoid internal interference⁴⁴ and collected to measure their GFP fluorescence by flow cytometry. The cells were screened using a MoFlo XDP Flow Cytometry Sorter (Beckman Coulter Inc., Fullerton, CA, USA). The fluorescence signal of the eGFP reporter was measured at an excitation wavelength of 488 nm, and the emission was detected using a 530 nm band-pass filter. The “purify model”⁴⁴ was used to improve the ratio of positive cells in the library. All isolated cells were cultivated on LBHIS agar plates with 30 mg/L of kanamycin at 30 °C for 48 h for further analysis. The cells were transferred into 96-deep-well plates with 500 μL of a fresh fermentation medium per well, and cultivated at 30 °C for 48 h, at a shaking frequency of 1200 rpm and a shaking diameter of 3 mm in a Microtron shaker (Infors, Switzerland).^{24,28} The biomass and the eGFP fluorescence of the cultures was measured using a SpectraMax M2 multidetection microplate reader.⁴⁴

Fermentations. The seed medium contained 25 g/L of glucose, 30 g/L of corn steep liquor (CS001, Angel Co., Ltd,

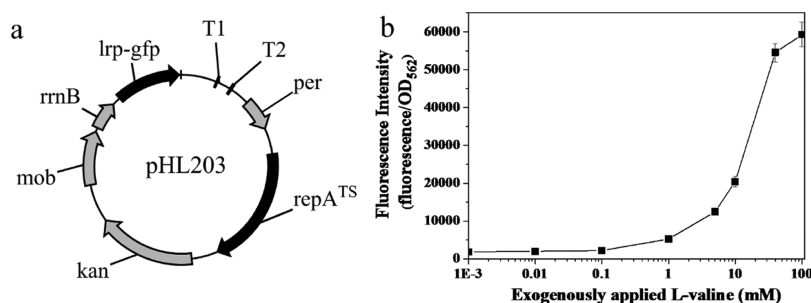


Figure 1. Construction and characterization of the Lrp-based biosensor. (a) Lrp sensor constructs with the relevant plasmid features. Lrp-PbrnF, *C. glutamicum* *lrp* open reading frame and the first 30 nucleotides of *brnF*; *gfp*, green fluorescent protein coding gene; repA, origin of *C. glutamicum* plasmid pTRCmob; *kan*, kanamycin resistance gene; repA^{TS}, temperature-sensitive *C. glutamicum* origin; *rrnB*, the terminator from plasmid pTRCmob. (b) Relationship between the concentrations of L-valine and fluorescence intensity. The data represent the means $\pm$ SEM ($n = 3$).

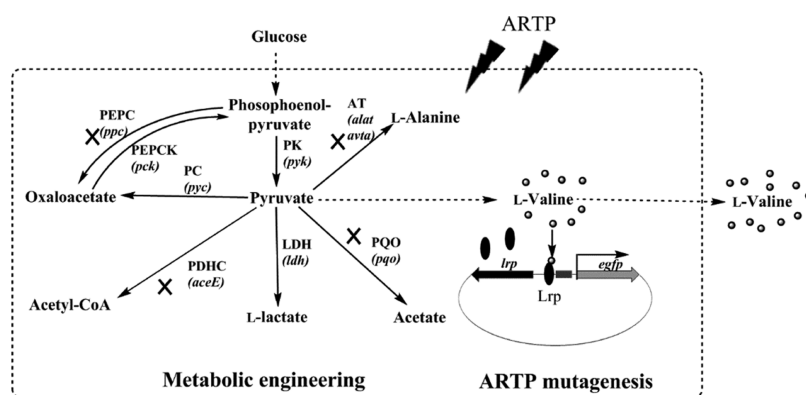


Figure 2. Biosynthetic pathways of L-valine and main by-products in *Corynebacterium glutamicum*. “X” indicates that genes were knocked out. PEPC, phosphoenolpyruvate carboxylase; PDHC, pyruvate dehydrogenase complex; AT, aminotransferase; PQQ, pyruvate/quinone oxidoreductase; PEPCCK, phosphoenolpyruvate carboxykinase; LDH, L-lactate dehydrogenase; PC, pyruvate carboxylase; and ARTP, atmospheric and room temperature plasma.

China), 5 g/L of urea, 1 g/L of $\text{KH}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$, and 0.5 g/L of MgSO_4 , and the pH was adjusted to 7.0 using 1 M KOH. The fermentation medium contained 120 g/L of glucose, 20 g/L of corn steep liquor, 20 g/L of $(\text{NH}_4)_2\text{SO}_4$, 1 g/L of KH_2PO_4 , 0.5 g/L of MgSO_4 , 0.01 g/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.01 g/L of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$. The pH was adjusted to 7.0 and 20 g/L of CaCO_3 was added to balance the pH of the medium. When necessary, 30 mg/L of kanamycin was added into the medium.

For flask cultivation, seed cultures grown at 30 °C for 12 h were used to inoculate 25 mL of fermentation medium in 250 mL flasks to an initial OD_{562} of 1.8. The cultures were incubated at 30 °C and 200 rpm in rotary shakers for 72 h. Samples were harvested for the determination of residual glucose, biomass, and amino acids as described below.

Analysis of Residual Glucose, Biomass and Amino Acids. The concentration of residual glucose was determined using the SBA-40C biosensor analyzer (Shandong Academy of Sciences, China).⁴⁷ The cell density was determined by measuring the optical density at 562 nm (OD_{562}) using a UV-1800 spectrophotometer (Beijing Ruili Analytical Instrument Co., Ltd, China). The levels of amino acids were analyzed by HPLC on a 1200 series instrument (Agilent, USA) according to the method reported by Kőrös et al.⁴⁸

RESULTS AND DISCUSSION

Construction of the Lrp-Based Biosensor. To design a biosensor that responds to elevated intracellular concentrations of L-valine, a module based on the transcriptional regulator Lrp of *C. glutamicum* was constructed. The *egfp* coding sequence

was cloned behind the plasmid region containing the open-reading frame of *lrp* and the intergenic region of *lrp-brnF*. Thus, the expression of *egfp* was placed under the control of the P_{brnF} promoter, which intrinsically responds to the intracellular concentration of L-valine. When the biosensor plasmid pHL200 was introduced into *C. glutamicum* ATCC13032, a discernible fluorescence signal above the autofluorescence of wild-type *C. glutamicum* was detected (Figure S1). To prevent this leaky expression, the *rrnB* terminator was cloned in front of Lrp to block the impact of other parts of the plasmid. Furthermore, the *rep* replicon was replaced by a temperature-sensitive replicon, resulting in the plasmid pHL203 (Figure 1a), which was again introduced into *C. glutamicum* ATCC13032. This construct effectively reduced the background fluorescence and improved the performance of the biosensor (Figure S1). The normalized fluorescence intensity (fluorescence/ OD_{562}) was very low when no L-valine was added and did not increase significantly when added L-valine concentration was below 0.1 mM. Fluorescence intensity increased significantly with the increase of the added L-valine concentration when added L-valine concentration was higher than 1 mM (Figure 1b). The results were similar to that reported in a previous study.⁴⁹ This may be attributed to the fact that the biosensor detects the intracellular rather than the extracellular L-valine concentration. Although this method is not particularly precise, it confirmed that the biosensor is functional. The results indicated that the biosensor could work to detect different levels of L-valine by generating corresponding levels of fluorescence intensity. Temperature-sensitive

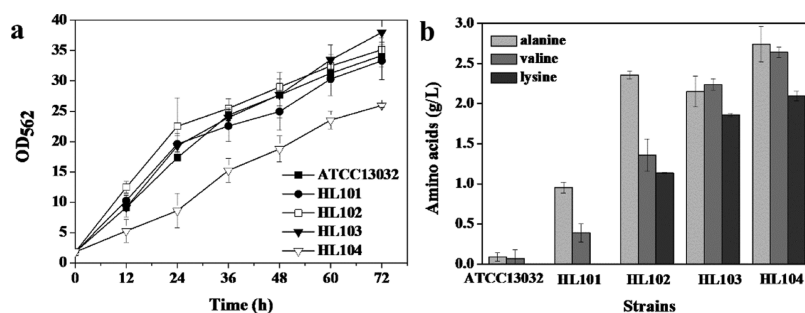


Figure 3. Growth conditions (a) and L-valine production (b) of *C. glutamicum* strains ATCC13032 (wild type), HL101, HL102, HL103, and HL104. The fermentation was carried out in 250 mL shake-flasks.

plasmids can be maintained at a given permissive temperatures,⁵⁰ which is favorable for further studies. The biosensor developed by Mustafi et al. could not be easily removed from *C. glutamicum*, which complicates the subsequent transformation for metabolic engineering.²⁸ By contrast, the plasmid pHL203 developed in this study can be easily removed by increasing the temperature. The temperature-sensitivity of pHL203 was confirmed in ATCC13032/pHL203 (data not shown) and was consistent with the results reported by Hu et al.⁴³

Construction of *C. glutamicum* Knockout Mutants.

The optimization of the intracellular pyruvate pool can increase the L-valine production in *C. glutamicum*.¹⁹ Pyruvate can be transformed into acetyl-CoA, oxaloacetate, L-alanine, acetate, and L-valine (Figure 2). PEPC encoding by *ppc* converts phosphoenolpyruvate to oxaloacetate, decreasing flow to L-valine. Deleting of gene *ppc* increased L-valine production (Figure 3b). The *elp* subunit of PDHC encoding by *aceE* converts pyruvate to acetyl-CoA. Therefore, gene *aceE* was deleted in HL102. As shown in Figure 3b, L-valine production increased and L-alanine became the major by-product of strain HL102. L-alanine was formed by catalyzing pyruvate with two enzymes encoding by *alat* and *avt*. However, L-alanine was still the major by-product of the strains HL103 and HL104, even though the *alat* gene was disrupted (Figure 3b). More L-valine was accumulated though the growth rate of HL104 was decreased because of the deletion of *pqo*. After 72 h of cultivation, HL104 produced 2.64 g/L of L-valine. Overall, the results demonstrated that the inactivation of the PEPC, PDHC, AT, and PQQ improved the L-valine production. The inactivation of PEPC (encoded by *ppc*), which converts phosphoenolpyruvate to oxaloacetate, was reported to improve the L-valine production (36). A *C. glutamicum* strain with a mutant variant of *aceE* produced more L-valine than the control.^{17,43} The elimination of PQQ, which converts pyruvate into acetate, was also reported to increase the L-valine production by increasing pyruvate levels.⁵¹ These above four candidate genes had been deleted individually, but not simultaneously for strain development in some previous studies.

Consequently, the genes *ppc*, *aceE*, *alat*, and *pqo* were deleted from *C. glutamicum* ATCC13032 before mutagenesis by ARTP. This engineering strategy, which was applied before the mutagenesis, provided a more favorable starting strain to reduce the labor-intensity of the whole project.

High-Throughput Screening of the ARTP-Mutants Using the Biosensor. To allow the high-throughput screening of strains with increased L-valine production, the plasmid pHL203 was introduced into the L-valine producer

strain HL104. Random mutations were induced by ARTP-irradiation, and high L-valine-producing mutants were screened. HL104 cells containing the biosensor plasmid were treated with helium-based ARTP for 5, 10, 15, 20, 25, 30, and 40 s, respectively. The lethality rate was over 90% when the exposure time exceeded 20 s (Figure 4). Therefore, the optimal exposure time was set at 20 s, with lethality ranging from 90 to 95%.

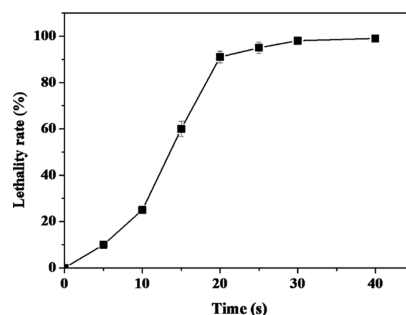


Figure 4. Lethality rate of *C. glutamicum* irradiated by ARTP. The line shows the lethality rate of cells treated by ARTP with a radio-frequency (rf) power input of 100 W for 5, 10, 15, 20, 25, 30, and 40 s. The data represent the means $\pm$ SEM ($n = 3$).

An efficient producer strain is one of the key factors that can make or break the industrialization of biotechnological processes. A rational design for the metabolic engineering of industrial strains has been proved to be a valuable approach, but the design and engineering of bacterial strains remains challenging because of the complexity of the bacterial physiology. Consequently, random mutagenesis and screening strategies are still widely used for strain development.²⁷ The generation of genetic diversity is a key first step in high-throughput screening. The ARTP mutation breeding system is an effective method to generate genetic diversity and improve product yields.^{37,46} ARTP can produce a variety of high-energy, evenly distributed particles acting on the strands of DNA in the microbial cells under atmospheric pressure, causing mutations through an incomplete gene repair process.⁴⁶ ARTP can generate hundreds of genomic modifications for FACS screening using a suitable biosensor. Moreover, beneficial mutation can be obtained by screening mutant libraries of strains that have first been modified using metabolic engineering.²⁴ The affected sites can be identified by genome sequencing and bioinformatics analysis.

After mutagenesis, *C. glutamicum* cells were incubated in LBHIS medium at 30 °C for 12 h and were then subjected to shake-flask fermentation for 2 days to promote the L-valine

production, and were then analyzed and sorted by FACS. To ensure that the mutant library is sufficiently large, about 10^8 cells were screened by FACS. The top 0.04% of the screened cells according to the fluorescence intensity were sorted and cultivated on LBHIS agar plates. The selected mutants were then seeded into individual wells of 96-deep-well plates containing a fermentation medium with 1% acetate. Acetate was added to support the growth of L-valine producing mutants with decreased pyruvate dehydrogenase activity. In this strategy, biomass is produced from acetate and the L-valine production from glucose is initiated during the stationary phase after the acetate is completely consumed.⁵²

After 2 screening rounds, 900 isolates were preliminarily selected from the genetic library, and only 53 of the selected isolates were further confirmed that they exhibited obviously increased fluorescence intensities than the starting strain in 96-well plate fermentations. Eight improved mutants with the highest relative fluorescence intensity (Figure 5a) and three negative mutants showing no obvious fluorescence change (Figure 5a) were then chosen to inoculate in a 250 mL Erlenmeyer flask, and allowed to grow for 3 days at 30 °C and 200 rpm for the production of L-valine. In order to explore whether the changes of fluorescence intensity were caused by the sequence variations of the biosensor, the *rrnB-lrp-brnE-gfp*

functional region of the plasmid in eight improved mutants was confirmed by DNA sequencing, and no mutations were found. The results demonstrated that the increase of fluorescence was caused by the change of L-valine content and not by the mutation of the biosensor.

Analysis of L-Valine Production by the Screened Mutants. Biosensor systems and high-throughput FACS screening rely on the detection of intracellular signals intensity rather than extracellular L-valine concentration. Thus, quantitative analysis of L-valine titer was also needed to determine the metabolite production in the supernatant of isolated mutants.

The biomass and amino acid production of the top eight mutants and control strain are shown in Figure 5. The top eight mutants produced more L-valine than the control strain after 72 h of fermentation. The best mutant HL2-7 produced is an L-valine titer of 3.20 g/L, which was a 21.47% improvement over the control strain (Figure 5b). After 5 rounds of cultivation, the mutant HL2-7 still produced 3.15 g/L of L-valine, suggesting that the observed production phenotype was genetically stable. As expected, the negative isolates showing no observed changes in the fluorescence properties exhibited L-valine production comparable to the starting HL104 strain. In addition, the mutants with only a moderate increase in fluorescence intensity also demonstrated varying degrees of increase in the L-valine production (Figure S2). Overall, these results suggest that the combination of ARTP with biosensor-based FACS screening can be used to improve the L-valine production in *C. glutamicum*.

It should be noted that, although the selected isolates improved the production of L-valine, the titers of the by-products alanine and lysine were still high in most mutants (Figure 5c). One possibility was that the ARTP random mutagenesis may result in specific pathway optimization by redesign of unknown regions, and allowed to more carbon fluxes for the L-valine production without disturbing the byproduct synthesis pathways. According to the previous studies, the factors affecting the production of L-valine include the expression level of L-valine biosynthesis genes, feedback inhibition of key enzymes, and the supply of NADPH.^{5,14,24,51} The sequencing of the eight mutants have been completed, and the mutation sites are still in the process of analysis and verification. Structural Variation, Single Nucleotide Polymorphism, and Small InDel results were shown in Table S1. A lot of Structural Variation at the genomic level were found in each mutant, indicating the strain was damaged greatly after the ARTP mutation. The number of SNP and Small InDel in each mutant were fewer. Some sites have been reported before, such as the mutation of *ilvBN*, which reduces the feedback inhibition regulation. Some sites have not been reported, which need further experimental verification. The sources of variability in internal L-valine accumulation among the mutants derived from HL104 will be analyzed and identified in the future studies.

In the present work, we successfully established a biosensor-driven laboratory evolution approach for the improvement of the growth and metabolite production of industrially relevant strains by directly selecting individual cells based on the fluorescence output of the biosensor using FACS.

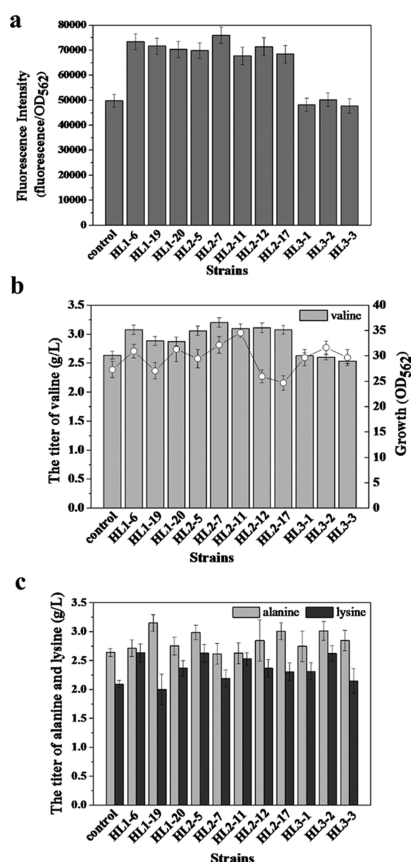


Figure 5. Normalized fluorescence intensity (fluorescence/OD₅₆₂) of mutants obtained by the ARTP treatment and fermentation analysis of *C. glutamicum* mutants. (a) Fluorescence intensity of mutants. The control is strain HL104/pHL203 with a fluorescence intensity of 49738, grown in a 96-deep-well plate at 30 °C for 72 h. (b) L-Valine production and cell growth of the strains. Strain HL104 harboring pHL203 was used as the control. (c) The titers of the by-products alanine and lysine. The data represent the means $\pm$ SEM ($n = 3$).

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.9b02747>.

fluorescence intensity of pHL203 and pHL200; normalized fluorescence intensity and fermentation analysis of *C. glutamicum* mutants; and SV test, SNP test, and small InDel test results (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Qing Wang – Life Science and Technology Institute and School of Advanced Agriculture and Bioengineering, Yangtze Normal University, Chongqing 408100, P. R. China; Phone: +86-23-72792193; Email: 767783845@qq.com

Authors

Guoqiang Han – Life Science and Technology Institute and School of Advanced Agriculture and Bioengineering, Yangtze Normal University, Chongqing 408100, P. R. China;
● orcid.org/0000-0003-1347-948X

Ning Xu – Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, P. R. China;
● orcid.org/0000-0002-4123-7827

Xieping Sun – Life Science and Technology Institute, Yangtze Normal University, Chongqing 408100, P. R. China

Jinzha Chen – Life Science and Technology Institute, Yangtze Normal University, Chongqing 408100, P. R. China

Chun Chen – Life Science and Technology Institute, Yangtze Normal University, Chongqing 408100, P. R. China

Complete contact information is available at:

<https://pubs.acs.org/doi/10.1021/acsomega.9b02747>

Author Contributions

[†]G.H. and N.X. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (31601460), the Project for the Introduction of Talents of Yangtze Normal University (2017KYQD67), the Technology Innovation and Application Development Project of Chongqing Science and Technology Bureau (cstc2019jscx-msxmX0351), and the Science and Technology Project of Chongqing Education Commission (KJQN201801429).

■ REFERENCES

- (1) Yamamoto, K.; Tsuchisaka, A.; Yukawa, H. Branched-Chain Amino Acids. *Adv. Biochem. Eng./Biotechnol.* **2017**, *159*, 103–128.
- (2) Sanchez, S.; Rodríguez-Sanoja, R.; Ramos, A.; Demain, A. L. Our microbes not only produce antibiotics, they also overproduce amino acids. *J. Antibiot.* **2018**, *71*, 26.
- (3) Xu, D.; Tan, Y.; Huan, X.; Hu, X.; Wang, X. Construction of a novel shuttle vector for use in *Brevibacterium flavum*, an industrial amino acid producer. *J. Microbiol. Methods* **2010**, *80*, 86–92.
- (4) Park, J. H.; Lee, S. Y. Fermentative production of branched chain amino acids: a focus on metabolic engineering. *Appl. Microbiol. Biotechnol.* **2010**, *85*, 491–506.
- (5) Bartek, T.; Blombach, B.; Lang, S.; Eikmanns, B. J.; Wiechert, W.; Oldiges, M.; Nöh, K.; Noack, S. Comparative ¹³C metabolic flux analysis of pyruvate dehydrogenase complex-deficient, L-valine-

producing *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* **2011**, *77*, 6644–6652.

(6) Westbrook, A. W.; Ren, X.; Moo-Young, M.; Chou, C. P. Metabolic engineering of *Bacillus subtilis* for L-valine overproduction. *Biotechnol. Bioeng.* **2018**, *115*, 2778–2792.

(7) Wen, J.; Bao, J. Engineering *Corynebacterium glutamicum* triggers glutamic acid accumulation in biotin-rich corn stover hydrolysate. *Biotechnol. Biofuels* **2019**, *12*, 86.

(8) Hoffmann, S. L.; Jungmann, L.; Schiefelbein, S.; Peyriga, L.; Cahoreau, E.; Portais, J.-C.; Becker, J.; Wittmann, C. Lysine production from the sugar alcohol mannitol: Design of the cell factory *Corynebacterium glutamicum* SEA-3 through integrated analysis and engineering of metabolic pathway fluxes. *Metab. Eng.* **2018**, *47*, 475–487.

(9) Zhao, J.; Hu, X.; Li, Y.; Wang, X. Overexpression of ribosome elongation factor G and recycling factor increases L-isoleucine production in *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 4795–4805.

(10) Petit, C.; Kim, Y.; Lee, S.-K.; Brown, J.; Larsen, E.; Ronning, D. R.; Suh, J.-W.; Kang, C.-M. Reduction of Feedback Inhibition in Homoserine Kinase (ThrB) of *Corynebacterium glutamicum* Enhances L-Threonine Biosynthesis. *ACS Omega* **2018**, *3*, 1178–1186.

(11) Eggeling, L.; Bott, M.; Marienhagen, J. Novel screening methods—biosensors. *Curr. Opin. Biotechnol.* **2015**, *35*, 30–36.

(12) Binder, S.; Schendzielorz, G.; Stäbler, N.; Krumbach, K.; Hoffmann, K.; Bott, M.; Eggeling, L. A high-throughput approach to identify genomic variants of bacterial metabolite producers at the single-cell level. *Genome Biol.* **2012**, *13*, R40.

(13) Dietrich, J. A.; McKee, A. E.; Keasling, J. D. High-throughput metabolic engineering: advances in small-molecule screening and selection. *Annu. Rev. Biochem.* **2010**, *79*, 563–590.

(14) Wang, X.; Zhang, H.; Quinn, P. J. Production of L-valine from metabolically engineered *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 4319–4330.

(15) Kalinowski, J.; Bathe, B.; Bartels, D.; Bischoff, N.; Bott, M.; Burkovski, A.; Dusch, N.; Eggeling, L.; Eikmanns, B. J.; Gaigalat, L.; Goesmann, A.; Hartmann, M.; Huthmacher, K.; Krämer, R.; Linke, B.; McHardy, A. C.; Meyer, F.; Möckel, B.; Pfefferle, W.; Pühler, A.; Rey, D. A.; Rückert, C.; Rupp, O.; Salm, H.; Wendisch, V. F.; Wiegräbe, I.; Tauch, A. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J. Biotechnol.* **2003**, *104*, 5–25.

(16) Ma, Y.; Chen, Q.; Cui, Y.; Du, L.; Shi, T.; Xu, Q.; Ma, Q.; Xie, X.; Chen, N. Comparative genomic and genetic functional analysis of industrial L-leucine- and L-valine-producing *Corynebacterium glutamicum* strains. *J. Microbiol. Biotechnol.* **2018**, *28*, 1916–1927.

(17) Chen, C.; Li, Y.; Hu, J.; Dong, X.; Wang, X. Metabolic engineering of *Corynebacterium glutamicum* ATCC13869 for L-valine production. *Metab. Eng.* **2015**, *29*, 66–75.

(18) Schwentner, A.; Feith, A.; Münch, E.; Busche, T.; Rückert, C.; Kalinowski, J.; Takors, R.; Blombach, B. Metabolic engineering to guide evolution - Creating a novel mode for L-valine production with *Corynebacterium glutamicum*. *Metab. Eng.* **2018**, *47*, 31–41.

(19) Oldiges, M.; Eikmanns, B. J.; Blombach, B. Application of metabolic engineering for the biotechnological production of L-valine. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 5859–5870.

(20) Hasegawa, S.; Suda, M.; Uematsu, K.; Natsuma, Y.; Hiraga, K.; Jojima, T.; Inui, M.; Yukawa, H. Engineering of *Corynebacterium glutamicum* for high-yield L-valine production under oxygen deprivation conditions. *Appl. Environ. Microbiol.* **2013**, *79*, 1250–1257.

(21) Holátko, J.; Eliášková, V.; Prouza, M.; Sobotka, M.; Nešvera, J.; Pátek, M. Metabolic engineering of the L-valine biosynthesis pathway in *Corynebacterium glutamicum* using promoter activity modulation. *J. Biotechnol.* **2009**, *139*, 203–210.

(22) Park, J. H.; Lee, K. H.; Kim, T. Y.; Lee, S. Y. Metabolic engineering of *Escherichia coli* for the production of L-valine based on

transcriptome analysis and in silico gene knockout simulation. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 7797–7802.

- (23) Liu, Y.; Li, Q.; Zheng, P.; Zhang, Z.; Liu, Y.; Sun, C.; Cao, G.; Zhou, W.; Wang, X.; Zhang, D.; Zhang, T.; Sun, J.; Ma, Y. Developing a high-throughput screening method for threonine overproduction based on an artificial promoter. *Microb. Cell Fact.* **2015**, *14*, 121.
- (24) Mahr, R.; Gätgens, C.; Gätgens, J.; Polen, T.; Kalinowski, J.; Frunzke, J. Biosensor-driven adaptive laboratory evolution of L-valine production in *Corynebacterium glutamicum*. *Metab. Eng.* **2015**, *32*, 184–194.
- (25) Liu, D.; Evans, T.; Zhang, F. Applications and advances of metabolite biosensors for metabolic engineering. *Metab. Eng.* **2015**, *31*, 35–43.
- (26) Williams, T. C.; Pretorius, I. S.; Paulsen, I. T. Synthetic Evolution of Metabolic Productivity Using Biosensors. *Trends Biotechnol.* **2016**, *34*, 371–381.
- (27) Schallmeyer, M.; Frunzke, J.; Eggeling, L.; Marienhagen, J. Looking for the pick of the bunch: high-throughput screening of producing microorganisms with biosensors. *Curr. Opin. Biotechnol.* **2014**, *26*, 148–154.
- (28) Mustafi, N.; Grünberger, A.; Kohlheyer, D.; Bott, M.; Frunzke, J. The development and application of a single-cell biosensor for the detection of L-methionine and branched-chain amino acids. *Metab. Eng.* **2012**, *14*, 449–457.
- (29) Liu, C.; Zhang, B.; Liu, Y.-M.; Yang, K.-Q.; Liu, S.-J. New Intracellular Shikimic Acid Biosensor for Monitoring Shikimate Synthesis in *Corynebacterium glutamicum*. *ACS Synth. Biol.* **2018**, *7*, 591–601.
- (30) Liu, D.; Xiao, Y.; Evans, B. S.; Zhang, F. Negative feedback regulation of fatty acid production based on a malonyl-CoA sensor-actuator. *ACS Synth. Biol.* **2015**, *4*, 132–140.
- (31) Zhou, L.-B.; Zeng, A.-P. Exploring lysine riboswitch for metabolic flux control and improvement of L-lysine synthesis in *Corynebacterium glutamicum*. *ACS Synth. Biol.* **2015**, *4*, 729–734.
- (32) Steffen, V.; Otten, J.; Engelmann, S.; Radek, A.; Limberg, M.; Koening, B. W.; Noack, S.; Wiechert, W.; Pohl, M. A Toolbox of Genetically Encoded FRET-Based Biosensors for Rapid L-Lysine Analysis. *Sensors* **2016**, *16*, 1604.
- (33) Chen, D.-W.; Zhang, Y.; Jiang, C.-Y.; Liu, S.-J. Benzoate metabolism intermediate benzoyl coenzyme A affects gentisate pathway regulation in *Comamonas testosteroni*. *Appl. Environ. Microbiol.* **2014**, *80*, 4051–4062.
- (34) Tang, S.-Y.; Cirino, P. C. Design and application of a mevalonate-responsive regulatory protein. *Angew. Chem.* **2011**, *50*, 1084–1086.
- (35) Tang, S.-Y.; Qian, S.; Akinterinwa, O.; Frei, C. S.; Gredell, J. A.; Cirino, P. C. Screening for enhanced triacetic acid lactone production by recombinant *Escherichia coli* expressing a designed triacetic acid lactone reporter. *J. Am. Chem. Soc.* **2013**, *135*, 10099–10103.
- (36) Siedler, S.; Schendzielorz, G.; Binder, S.; Eggeling, L.; Bringer, S.; Bott, M. SoxR as a single-cell biosensor for NADPH-consuming enzymes in *Escherichia coli*. *ACS Synth. Biol.* **2014**, *3*, 41–47.
- (37) Cai, Y.; Xia, M.; Dong, H.; Qian, Y.; Zhang, T.; Zhu, B.; Wu, J.; Zhang, D. Engineering a vitamin B12 high-throughput screening system by riboswitch sensor in *Sinorhizobium meliloti*. *BMC Biotechnol.* **2018**, *18*, 27.
- (38) Zhang, F.; others, J. M.; Keasling, J. D. Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. *Nat. Biotechnol.* **2012**, *30*, 354–359.
- (39) Watstein, D. M.; McNerney, M. P.; Styczynski, M. P. Precise metabolic engineering of carotenoid biosynthesis in *Escherichia coli* towards a low-cost biosensor. *Metab. Eng.* **2015**, *31*, 171–180.
- (40) Mahr, R.; Frunzke, J. Transcription factor-based biosensors in biotechnology: current state and future prospects. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 79–90.
- (41) Xiong, D.; Lu, S.; Wu, J.; Liang, C.; Wang, W.; Wang, W.; Jin, J.-M.; Tang, S.-Y. Improving key enzyme activity in phenylpropanoid pathway with a designed biosensor. *Metab. Eng.* **2017**, *40*, 115–123.
- (42) Sambrook, J.; Russell, D. W. *Molecular Cloning: A Laboratory Manual*, 3rd ed.; Cold Spring Harbor: New York, 2001.
- (43) Hu, J.; Tan, Y.; Li, Y.; Hu, X.; Xu, D.; Wang, X. Construction and application of an efficient multiple-gene-deletion system in *Corynebacterium glutamicum*. *Plasmid* **2013**, *70*, 303–313.
- (44) Wei, L.; Xu, N.; Wang, Y.; Zhou, W.; Han, G.; Ma, Y.; Liu, J. Promoter library-based module combination (PLMC) technology for optimization of threonine biosynthesis in *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 4117–4130.
- (45) Schäfer, A.; Tauch, A.; Jäger, W.; Kalinowski, J.; Thierbach, G.; Pühler, A. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **1994**, *145*, 69–73.
- (46) Zhang, C.; Qin, J.; Dai, Y.; Mu, W.; Zhang, T. Atmospheric and room temperature plasma (ARTP) mutagenesis enables xylitol overproduction with yeast *Candida tropicalis*. *J. Biotechnol.* **2019**, *296*, 7–13.
- (47) Han, G.; Hu, X.; Qin, T.; Li, Y.; Wang, X. Metabolic engineering of *Corynebacterium glutamicum* ATCC13032 to produce S-adenosyl-L-methionine. *Enzyme Microb. Technol.* **2016**, *83*, 14–21.
- (48) Kőrös, A.; Varga, Z.; Molnár-Perl, I. Simultaneous analysis of amino acids and amines as their o-phthalaldehyde-ethanethiol-9-fluorenylmethyl chloroformate derivatives in cheese by high-performance liquid chromatography. *J. Chromatogr. A* **2008**, *1203*, 146–152.
- (49) Chen, X. F.; Xia, X. X.; Lee, S. Y.; Qian, Z. G. Engineering tunable biosensors for monitoring putrescine in *Escherichia coli*. *Biotechnol. Bioeng.* **2018**, *115*, 1014–1027.
- (50) Okibe, N.; Suzuki, N.; Inui, M.; Yukawa, H. Efficient markerless gene replacement in *Corynebacterium glutamicum* using a new temperature-sensitive plasmid. *J. Microbiol. Methods* **2011**, *85*, 155–163.
- (51) Blombach, B.; Schreiner, M. E.; Bartek, T.; Oldiges, M.; Eikmanns, B. J. *Corynebacterium glutamicum* tailored for high-yield L-valine production. *Appl. Microbiol. Biotechnol.* **2008**, *79*, 471–479.
- (52) Blombach, B.; Schreiner, M. E.; Moch, M.; Oldiges, M.; Eikmanns, B. J. Effect of pyruvate dehydrogenase complex deficiency on L-lysine production with *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* **2007**, *76*, 615–623.