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Rational engineering of multiple module pathways for the production of L-phenylalanine in *Corynebacterium glutamicum*

Chuanzhi Zhang · Junli Zhang · Zhen Kang · Guocheng Du · Jian Chen

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Abstract Microbial production of L-phenylalanine (L-Phe) from renewable sources has attracted much attention recently. In the present study, *Corynebacterium glutamicum* 13032 was rationally engineered to produce L-Phe from inexpensive glucose. First, all the L-Phe biosynthesis pathway genes were investigated and the results demonstrated that in addition to AroF and PheA, the native PpsA, TktA, AroE and AroA, and the heterologous AroL and TyrB were also the key enzymes for L-Phe biosynthesis. Through combinational expression of these key enzymes, the L-Phe production was increased to 6.33 ± 0.13 g l^{-1} which was about 1.48-fold of that of the parent strain *C. glutamicum* (pXM-*pheA*^{fbr}-*aroF*^{fbr}) (fbr, feedback-inhibition resistance). Furthermore, the production of L-Phe was improved to 9.14 ± 0.21 g l^{-1} by modifying the glucose and L-Phe transport systems and blocking the acetate and lactate biosynthesis pathways. Eventually, the titer of L-Phe was enhanced to 15.76 ± 0.23 g l^{-1} with a fed-batch fermentation strategy. To the best of our knowledge, this was the highest value reported in rationally engineered *C. glutamicum* 13032 strains. The results obtained will also contribute to rational engineering of *C. glutamicum* for production of other valuable aromatic compounds.

Keywords *Corynebacterium glutamicum* · L-phenylalanine · Shikimate · Glucose-PTS · Metabolic engineering

Introduction

L-Phe, an essential amino acid for humans and other animals, has been widely used in food and pharmaceutical industries [30]. Over the past years, microbial production of L-Phe has attracted much attention and most of the studies mainly concentrated on *Escherichia coli* [12, 34]. Applying metabolic engineering strategies, the supply of precursors phosphoenolpyruvate (PEP) [10, 13] and erythrose-4-phosphate (E4P) [18, 32] and the metabolic flux [3, 30, 35] toward L-Phe was rationally optimized. In addition, many fermentation control approaches were also introduced to accomplish the whole capacity of recombinant strains, enabling significant increase of L-Phe titer in *E. coli* [30, 49]. However, although high concentration of L-Phe has been achieved in *E. coli*, the problem of phage contamination and the concern on food safety confined its industrial scale application [39]. In contrast, *Corynebacterium glutamicum* as a Gram-positive model bacterium has been widely used in the food industry especially for the production of amino acids, such as L-glutamate, L-lysine, and L-ornithine [4, 11, 23]. Since its many advantages [4], *C. glutamicum* as a good chassis microbe has also been engineered for the production of many valuable secondary products [4, 11] and proteins [7, 8, 22].

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C. Zhang · J. Zhang · Z. Kang
Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, 1800 Lihu Road, Wuxi 214122, Jiangsu, China

C. Zhang · J. Zhang · Z. Kang · G. Du · J. Chen
Synergetic Innovation Center of Food Safety and Nutrition, 1800 Lihu Road, Wuxi 214122, Jiangsu, China

C. Zhang · J. Zhang · Z. Kang (✉) · G. Du · J. Chen
School of Biotechnology, Jiangnan University, 1800 Lihu Road, Wuxi 214122, Jiangsu, China
e-mail: zkang@jiangnan.edu.cn

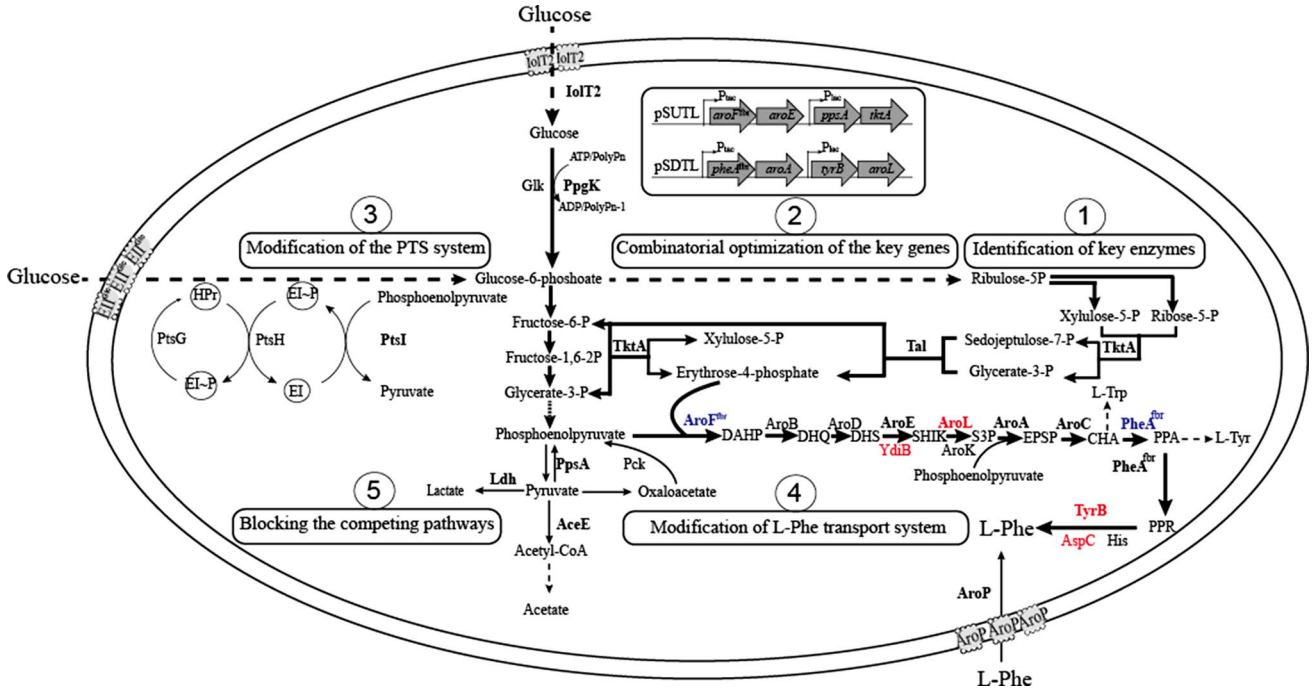

Fig. 1 Illustration of pathway engineering for L-Phe synthesis in *C. glutamicum*. Metabolites abbreviations: *DAHP* 3-deoxy-D-arabino-heptulosonate 7-phosphate, *DHQ* 3-dehydroquinate, *DHS* 3-dehydroshikimate; *SHIK* Shikimate, *S3P* Shikimate-3-phosphate, *EPSP* 5-enolpyruvoyl shikimate 3-phosphate, *CHA* Chorismate, *PPA* Prephenate, *PPR* Phenylpyruvate, *L-Phe* L-phenylalanine, *L-Tyr* L-tyrosine, *L-Trp* L-tryptophan. Enzymes abbreviations: *IolT2* major myo-inositol transporter IolT, *Ppgk* polyphosphate glucokinase, *PtsG* fused glucose-specific PTS enzyme IIBC, *PtsH* PTS system phosphohistidinoprotein-hexose phosphotransferase (HPr), *PtsI* PEP-protein phosphotransferase of PTS system (EI), *Glk* glucokinase, *PpsA* phosphoenolpyruvate synthase, *Pck* phosphoenolpyruvate carboxykinase, *AceE* pyruvate dehydrogenase subunit E1, *TktA* transketolase, *Tal* transaldolase, *AroF*^{fbr} 3-deoxy-D-arabino-heptulosonate-7-phosphate, *AroB* 3-dehydroquinate synthase, *AroD* 3-dehydroquinate dehydratase, *AroE and YdiB* shikimate 5-dehydrogenase, *AroK* shikimate kinase, *AroL* shikimate kinase II, *AroA* 5-enolpyruvylshikimate-3-phosphate synthetase, *AroC* chorismate synthase, *PheA*^{fbr} chorismate mutase-prephenate dehydratase, *His TyrB and AspC* aminotransferase, *AroP* aromatic amino acid transporter. The heterologous genes from *E. coli* W3110 were highlighted in *red*, while the genes obtained from plasmid pXM-*pheA*^{fbr}-*aroF*^{fbr} were marked in *blue* (color figure online)

In *C. glutamicum*, the L-Phe biosynthesis pathway can be divided into three parts: central carbon metabolism, shikimate, and chorismate pathways, which is similar to *E. coli* [14, 47]. Previous studies conducted in *C. glutamicum* mainly concentrated on the shikimate and chorismate pathways (Fig. 1). By overexpressing the mutants of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHPS, encoded by *aroG*) and the bifunctional enzyme chorismate mutase-prephenate dehydratase (CM-PDT, encoded by *pheA*) and deleting *tyrA* in an L-tryptophan producer, the production of L-Phe was increased to 28 g l^{-1} [16]. Similarly, the titer of L-Phe was increased by 20 % (3.97 g l^{-1}) after integrant expression of the tandem genes *aroG-pheA* into *tyrA* locus [28]. More recently, through co-overexpression of the key enzymes DAHPS (encoded by *aroH* from *E. coli*) and CM-PDT in *C. glutamicum* 13032, the production of L-Phe was also significantly improved to 4.64 g l^{-1} [47].

Although notable progress has been achieved in *C. glutamicum*, the production of L-Phe was still much lower than that in *E. coli*. In addition, the L-Phe biosynthesis pathways in *E. coli* and *C. glutamicum* seem to be different [47]. Consequently, related module pathways in *C. glutamicum* should be investigated and engineered to accomplish high-level production of L-Phe. In present study, the module pathways that involve substrate utilization, precursor supply, by-product synthesis, and target product synthesis and transportation were systematically engineered and evaluated toward L-Phe biosynthesis. Through integrating the engineered modules, the titer of L-Phe was significantly increased to 15.76 ± 0.23 g l^{-1}. To the best of our knowledge, this is the highest L-Phe production in rationally engineered *C. glutamicum* ATCC 13032.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* JM109 was chosen as the host for cloning

Table 1 Strains and plasmids used in this study

Strains and plasmids	Description	Source
Strains		
Escherichia coli JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi*-1 Δ(*lac-proAB*)/F' [*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ* ΔM15]	Lab stock
E. coli W3110	*F*-λ-*rph*-1 *INV* (*rrnD rrnE*)	Lab stock
Corynebacterium glutamicum ATCC 13032	Wild-type *C. glutamicum*	Lab stock
C. glutamicum Δ*ptsI*	Deletion of *ptsI* in *C. gltuamicum* ATCC 13032	This work
C. glutamicum Δ*ptsI*::*iolT2-ppgK*	Integration of *iolT2-ppgK* into *C. gltuamicum*	This work
C. glutamicum Δ*ptsI*::*iolT2-ppgK*Δ*aroP*	Deletion of *aroP* in *C. glutamicum* Δ*ptsI*:: *iolT2-ppgK*	This work
C. glutamicum Δ*ptsI*::*iolT2-ppgK*Δ*pheP*	Deletion of *pheP* in *C. glutamicum* Δ*ptsI*:: *iolT2-ppgK*	This work
C. glutamicum Δ*ptsI*::*iolT2-ppgK*Δ*aroP*Δ*aceE*	Deletion of *aceE* in *C. glutamicum* Δ*ptsI*:: *iolT2-ppgK*Δ*aroP*	This work
C. glutamicum Δ*ptsI*::*iolT2-ppgK*Δ*aroP*Δ*ldh*	Deletion of *ldh* in *C. glutamicum* Δ*ptsI*:: *iolT2-ppgK*Δ*aroP*	This work
C. glutamicum Δ*ptsI*::*iolT2-ppgK*Δ*aroP*Δ*aceE*Δ*ldh*	Deletion of *ldh* in *C. glutamicum* Δ*ptsI*::*iolT2-ppgK*Δ*aroP*Δ*aceE*	This work
Plasmids		
pXMJ19	Cm^r, *E. coli-C. glutamicum* shuttle expression vector	Lab stock
pEC-XK99E	Kan^r, *E. coli-C. glutamicum* shuttle expression vector	Lab stock
pK18mobSacB	Kan^r; Vector for in-frame deletion in *C. glutamicum*	Lab stock
pXM-*pheA*^{fbr}-*aroF*^{fbr}	pXMJ19 containing *pheA*^{fbr} and *aroF*^{fbr}	[46]
pEC-*tktA*	pEC-XK99E containing *tktA* (*C. glutamicum*)	This work
pEC-*tal*	pEC-XK99E containing *tal* (*C. glutamicum*)	This work
pEC-*ppsA*	pEC-XK99E containing *ppsA* (*C. glutamicum*)	This work
pEC-*pck*	pEC-XK99E containing *pck* (*C. glutamicum*)	This work
pEC-*aroB*	pEC-XK99E containing *aroB* (*C. glutamicum*)	This work
pEC-*aroD*	pEC-XK99E containing *aroD* (*C. glutamicum*)	This work
pEC-*aroE*	pEC-XK99E containing *aroE* (*C. glutamicum*)	This work
pEC-*ydiB*	pEC-XK99E containing *ydiB* (*E. coli*)	This work
pEC-*aroK*	pEC-XK99E containing *aroK* (*C. glutamicum*)	This work
pEC-*aroL*	pEC-XK99E containing *aroL* (*E. coli*)	This work
pEC-*aroA*	pEC-XK99E containing *aroA* (*C. glutamicum*)	This work
pEC-*aroC*	pEC-XK99E containing *aroC* (*C. glutamicum*)	This work
pEC-*tyrB*	pEC-XK99E containing *tyrB* (*E. coli*)	This work
pEC-*aspC*	pEC-XK99E containing *aspC* (*E. coli*)	This work
pEC-*his*	pEC-XK99E containing *his* (*C. glutamicum*)	This work
pEC-*iolT2-ppgK*	pEC-XK99E containing *iolT2* and *ppgK* (*C. glutamicum*)	This work
pEC-*yddG* (E)	pEC-XK99E containing *yddG* (*E. coli*)	This work
pSUTT	pEC-XK99E-Ptac-*aroF*^{fbr}-*aroE*-Ptac-*ppsA-tktA*	This work
pSULL	pEC-XK99E-Plac-*aroF*^{fbr}-*aroE*-Plac-*ppsA-tktA*	This work
pSUTL	pEC-XK99E-Ptac-*aroF*^{fbr}-*aroE*-Plac-*ppsA-tktA*	This work
pSULT	pEC-XK99E-Plac-*aroF*^{fbr}-*aroE*-Ptac-*ppsA-tktA*	This work
pSDTT	pXMJ19-Ptac-*pheA*^{fbr}-*aroA*-Ptac-*tyrB-aroL*	This work
pSDLL	pXMJ19-Plac-*pheA*^{fbr}-*aroA*-Plac-*tyrB-aroL*	This work
pSDTL	pXMJ19-Ptac-*pheA*^{fbr}-*aroA*-Plac-*tyrB-aroL*	This work
pSDLT	pXMJ19-Plac-*pheA*^{fbr}-*aroA*-Ptac-*tyrB-aroL*	This work
pK18mobSacB-*ptsI*	pK18mobSacB contains the up-and downstream regions of *ptsI*	This work
pK18mobSacB-*iolT2-ppgk*	pK18mobSacB contains *iolT2-ppgK* with up-and downstream regions of *ptsI*	This work
pK18mobSacB-*aroP*	pK18mobSacB contains the up-and downstream regions of *aroP*	This work
pK18mobSacB-*pheP*	pK18mobSacB contains the up-and downstream regions of *pheP*	This work
pK18mobSacB-*aceE*	pK18mobSacB contains the up-and downstream regions of *aceE*	This work
pK18mobSacB-*ldh*	pK18mobSacB contains the up-and downstream regions of *ldh*	This work

and plasmids construction. *C. glutamicum* ATCC13032 and recombinant *C. glutamicum* were used for L-Phe fermentation and analysis. Plasmids pXMJ19 and pEC-XK99E, the *E. coli* and *C. glutamicum* shuttle vectors, were used for genes expression. Plasmid pk18mobSacB [38, 43] was used for genes deletion and integration in *C. glutamicum* as well as *Brevibacteriumn flavum* by single crossover. The oligonucleotide primes used for genes amplification in this study are listed in Supplementary Table S1.

Media and culture condition

Escherichia coli was cultured in LB medium (tryptone 10.0 g l^{-1}, yeast extract 5.0 g l^{-1}, NaCl 10.0 g l^{-1}) at 37 °C. During transformation, all the *C. glutamicum* strains were grown in Luria–Bertani (LB) medium with addition of 5 g l^{-1} glucose at 30 °C. For fermentation, a 10 % (v/v) inoculum of an overnight culture (18 h) was used. Isopropyl-β-d-thiogalactopyranoside was initially added to the medium with a final concentration of 1.0 mM to induce genes expression in recombinant *C. glutamicum*. Antibiotics like ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), or chloramphenicol (34 μg ml^{-1}) was supplemented to provide selective pressure for maintaining plasmids. Flask cultivations were performed in 250-ml shake flask with 20 ml medium at 30 °C at an agitation of 200 rpm. The seed medium of *C. glutamicum* was composed of (g l^{-1}): glucose 25.0, corn steep liquor 25.0, $(NH_4)_2SO_4$ 15.0, urea 2.0, KH_2PO_4 2.0, and $MgSO_4{\cdot}7H_2O$ 1.0, pH 6.8–7.0. The fermentation medium was composed of (g l^{-1}): glucose 110.0, corn steep liquor 7.0, $(NH_4)_2SO_4$ 25.0, $MgSO_4{\cdot}7H_2O$ 1.0, KH_2PO_4 1.0, sodium citrate 2.0, glutamic acid 1.0, $CaCO_3$ 25.0, and pH 6.8–7.0.

Preparation for competent cells and transformation of *C. glutamicum*

Corynebacterium glutamicum competent cells for electroporation were prepared according to previously reported method [43]. *C. glutamicum* strains were inoculated in 25 ml of LB medium (containing 5 g l^{-1} glucose) and cultured for 16 h at 30 °C with the agitation of 200 rpm. Then 10 % of the overnight cell culture was inoculated into 50-ml medium (tryptone 10.0 g l^{-1}, yeast extract 5.0 g l^{-1}, NaCl 10.0 g l^{-1}, isonicotinic acid hydrazide 4.0 g l^{-1}, glycine 25.0 g l^{-1}, and Tween 80 0.1 %) and cultured until $OD_{600\ nm}$ reached 0.9. The chilled cells were centrifuged (4,000 rpm, 4 °C) for 10 min and washed four times with 15 ml ice-cold 10 % (v/v) glycerol. Cells were re-suspended in 0.5 ml 10 % (v/v) glycerol and 100 μl of aliquots stored at −70 °C before application.

For electro-transformation, the competent cells were thawed on ice and mixed with 1–5 μl DNA (about 1 μg) and transferred into a 4 °C pre-cooled electroporation cuvette (gap 0.1 cm). Electroporation was performed at 1.8 kV, 200 Ω, 25 μF and pulsed for twice. Subsequently 1 ml pre-warmed LB with brain heart infusion and sorbitol (LBHIS, tryptone 5.0 g l^{-1}, NaCl 5.0 g l^{-1}, yeast extract 2.5 g l^{-1}, brain heart infusion powder 18.5 g l^{-1}, and sorbitol 91.0 g l^{-1}) was immediately added into the cuvette, and the contents were mixed and then transferred into a sterile Eppendorf tube. The mixture was heated in a water bath at 46 °C for 6 min. They were allowed to regenerate and segregate for up to 2 h at 30 °C and 100 rpm. Cells were plated on LBHIS agar (LBHIS medium added 1.5 % agar) containing appropriate antibiotics and incubated at 30 °C for 2 days. As a negative control, ddH_2O was added to one aliquot of electro-competent cells.

Deletion and integration of target genes in recombinant strains

Plasmid pK18mobSacB was used for inactivation of *ptsI*, *aroP*, *pheP*, *ldh*, and *aceE* and integration of *iolT2-ppgK* in *C. glutamicum* with corresponding primers (Supplementary Table S1). Specifically, two-step homologous recombination was operated for deletion or integration of the above genes [38, 43]. First, the knockout box with kan^r as a selection marker was knocked-in the genome at the specified location. Then, the target recombinants without kan^r resistant were generated after the second exchange. All the recombinant strains constructed were further confirmed by PCR.

Analytical procedures

Growth of *C. glutamicum* was measured at $OD_{600\ nm}$ with a UV-1,700 spectrophotometer (Shimadzu, Kyoto, Japan) after appropriate dilution. Dry cell weight (DCW) was calculated with the following equation: DCW (g l^{-1}) = $OD_{600\ nm}$ × 0.31. Specifically, the conversion factor of 0.31 was experimentally determined in this study using *C. glutamicum* cultures. Residual glucose in the supernatant was detected by a glucose-glutamate analyzer SBA-40C (Biology Institute of Shandong Academy of Sciences, Jinan, China). Intracellular and extracellular amino acids, organic acids, and intermediates were analyzed by Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) according to Zhang et al. [47].

Results

Identification of key genes of the L-Phe biosynthetic pathway in *C. glutamicum*

In order to identify the key genes involved in L-Phe biosynthesis, the native genes that belong to the central carbon

Table 2 The titers of L-Phe and shikimate accumulated in *C. glutamicum* ATCC 13032 with different recombinant plasmids

Strains	L-Phe (g l^{-1})	Shikimate (g l^{-1})
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr})	4.29 ± 0.06	3.29 ± 0.12
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-XK99E)	4.21 ± 0.05	3.20 ± 0.09
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-*tktA*)	5.45 ± 0.14	4.21 ± 0.08
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-*tal*)	4.85 ± 0.12	3.33 ± 0.14
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-*ppsA*)	5.24 ± 0.15	4.44 ± 0.16
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-*pck*)	4.36 ± 0.11	2.47 ± 0.06
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-*aroB*)	4.54 ± 0.08	2.76 ± 0.11
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-*aroD*)	4.43 ± 0.21	3.03 ± 0.17
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-*aroE*)	5.31 ± 0.17	4.81 ± 0.21
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-*ydiB*)	5.21 ± 0.17	4.24 ± 0.21
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-*aroL*)	5.03 ± 0.05	2.27 ± 0.13
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-*aroK*)	4.94 ± 0.12	2.43 ± 0.08
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-*aroA*)	5.20 ± 0.08	1.65 ± 0.13
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-*aroC*)	4.69 ± 0.14	2.89 ± 0.15
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-*tyrB*)	5.06 ± 0.09	2.57 ± 0.06
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-*aspC*)	4.85 ± 0.22	2.75 ± 0.13
C. glutamicum (pXM-*pheA*^{fbr}-*aroF*^{fbr}, pEC-*his*)	4.69 ± 0.22	2.61 ± 0.08

The fermentation medium was described in "Materials and methods". Data are presented as the mean ± standard deviation (SD) ($n = 3$)

metabolic pathway (*tktA*, *tal*, *ppsA*, and *pck* encode transketolase, transaldolase, phosphoenolpyruvate synthase, and phosphoenolpyruvate carboxykinase, respectively), the shikimate pathway (*aroB*, *aroD*, *aroE*, *aroK*, *aroA*, and *aroC* encode 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, shikimate 5-dehydrogenase, shikimate kinase, 5-enolpyruvylshikimate-3-phosphate synthetase, and chorismate synthase, respectively), and the chorismate pathway (*his* encodes aminotransferase) were separately subcloned into the vector pEC-XK99E (Table 1) and overexpressed in the recombinant *C. glutamicum* (pXM-*pheA*^{fbr}-*aroF*^{fbr}) [46]. Moreover, according to the differences between *E. coli* and *C. glutamicum*, the shikimate pathway genes (*ydiB* and *aroL* encode shikimate 5-dehydrogenase and shikimate kinase II, respectively) and the chorismate pathway genes (both *tyrB* and *aspC* encode aminotransferase) from *E. coli* were also heterologously overexpressed and investigated in *C. glutamicum*. As expected, significant differences were introduced after single overexpression of the above genes. Compared with *pck* and *tal*, *ppsA*, and *tktA* showed greater positive influence on the production of L-Phe (Table 2), indicating more precursors PEP and E4P were generated after overexpression of *ppsA* and *tktA*. In addition, it could be found that although overexpression of all the above genes increased the titer of L-Phe, the endogenous genes *aroE* and *aroA* and the heterogeneous genes *aroL* and *tyrB* seem to play crucial positive roles on L-Phe synthesis. Consequently, the native genes *ppsA*, *tktA*, *aroE*, and *aroA* and the *E. coli* genes *aroL* and *tyrB* were identified as the metabolic engineering targets in addition to *aroF*^{fbr} and *pheA*^{fbr} [46]. At the same time, it was notable that overexpression of the upstream genes *tktA*, *ppsA*, and *aroE* also significantly increased the accumulation of intermediate shikimate. In contrary, overexpression of the downstream genes *aroL*, *aroA*, and *tyrB* decreased shikimate accumulation. Thus, construction of a balanced pathway by coordinated overexpression of the above key genes is crucial to efficient production of L-Phe.

Combinatorial optimization of the key genes for L-Phe production

Generally, achievement of a balanced flux toward the target compound is essential for its high production [20, 42]. In this regard, the identified key genes *ppsA*, *tktA*, *aroE*, *aroA*, *aroL*, and *tyrB* as well as *pheA*^{fbr} and *aroF*^{fbr} were overexpressed with different combinations on the plasmids pEC-XK99E and pXMJ19 which contain regulated promoters Ptac (strong) and Plac (moderate) (Table 1). The key genes were divided into two modules (the upstream shikimate module, SU; the downstream shikimate module, SD) at the intermediate shikimate for its stability and easy construction [20]. As shown in Fig. 2, different combinations of the eight key genes gave rise to distinct results. Although combination 1 generated more L-Phe compared to combination 2, higher titer of the intermediate shikimate was also concomitantly accumulated, indicating an unbalanced L-Phe biosynthetic pathway. In contrast, combinations 3 and 6 resulted in higher titer of L-Phe (6.13 ± 0.01 and 6.33 ± 0.13 g l^{-1}, respectively) and lower titer of shikimate (0.18 ± 0.12 and 0.32 ± 0.15 g l^{-1}, respectively), suggesting that moderate expression of the upstream shikimate module and strong expression of the downstream shikimate module lead to more balanced flux distribution.

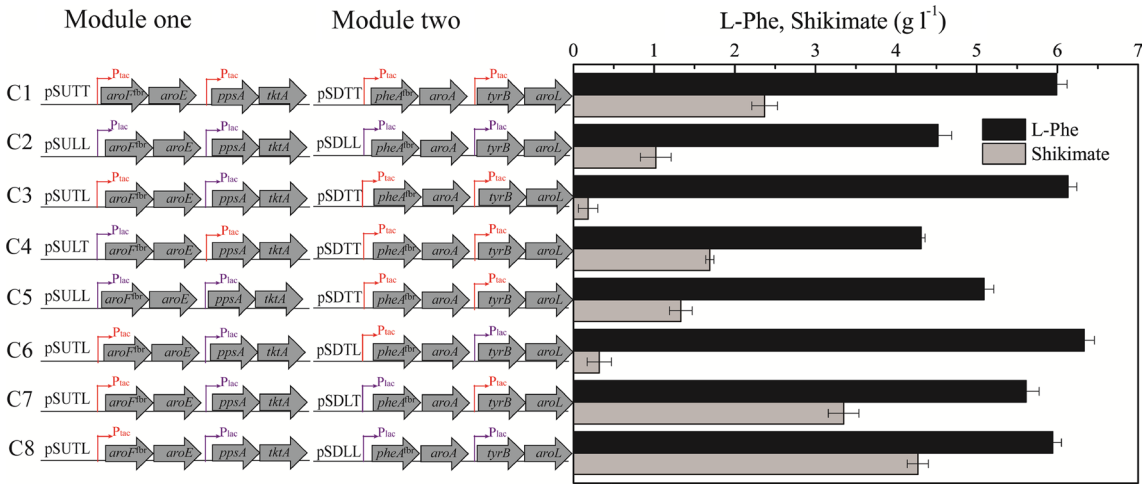

Fig. 2 Combinatorial optimization of key genes for L-Phe production in *C. glutamicum* ATCC 13032 with different recombinant plasmids. Key genes were divided into two modules, shikimate upstream module (*aroF*^{fbr}, *aroE*, *ppsA* and *tktA*) and shikimate downstream module (*pheA*^{fbr}, *aroA*, *tyrB* and *aroL*), at the intermediate shikimate according to the previous study [20] for production of L-Phe. The *open blocks* indicate the constructed plasmids, the *shaded arrows* represent the genes, and the *angled arrows* indicate the promoters (*red arrow* named T mean: Promoter Ptac, *purple arrow* named L mean: Promoter Plac). The fermentation medium was described in "Materials and methods". Data are presented as the mean ± SD ($n = 3$) (color figure online)

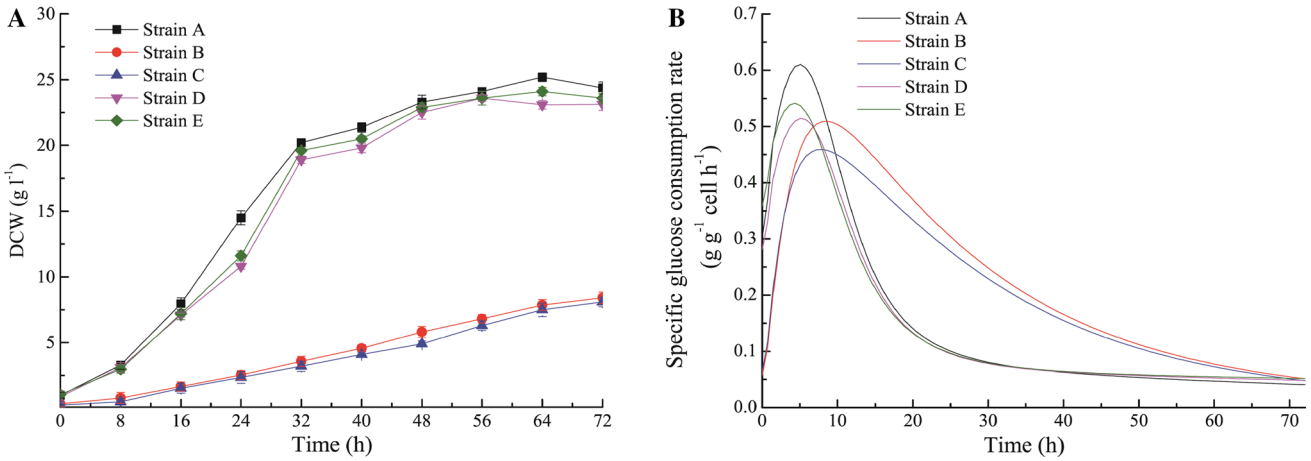

Fig. 3 Comparison of growth rate (**a**) and specific glucose consumption rate (**b**) in recombinant strains. *Strain A C. glutamicum* ATCC13032; *Strain B C. glutamicum* Δ*ptsI*; *Strain C C. glutamicum* Δ*ptsI* (pEC-XK99E); *Strain D C. glutamicum* Δ*ptsI* (pEC-*iolT2-ppgK*); *Strain E C. glutamicum* Δ*ptsI*:: *iolT2-ppgK*. The fermentation medium was described in "Materials and methods". Data are presented as the mean ± SD ($n = 3$)

Modification of the PTS system to increase the precursor PEP for L-Phe synthesis

Similar with *E. coli*, *C. glutamicum* also prefers to uptake glucose with the PTS-glucose system that depends on consumption of the high-energy compound PEP. To provide more PEP for L-Phe synthesis, we first inactivated the gene *ptsI* to block the PTS-dependent system. Unexpectedly, deletion of *ptsI* leads to slow cell growth and low biomass (Fig. 3a), indicating the critical role of PTS on cell growth. To recover glucose consumption rate, a hybrid glucose transport system was constructed by integration of the tandem genes of *iolT2-ppgK* at the *ptsI* site. As we expected, cell growth (Fig. 3a) and specific glucose consumption rate (Fig. 3b) of the recombinant strain *C. glutamicum* Δ*ptsI*:: *iolT2-ppgK* were restored to comparable levels compared with the wild-type strain *C. glutamicum* ATCC 13032 which further confirmed the excellent capacity of the *iolT2-ppgK* transport system [26]. Meanwhile, the effect of this nonnative PTS-independent glucose uptake system on the metabolites was further investigated. As shown in Table 3, compared with *C. glutamicum* (pSUTL, pSDTL),

Table 3 The titers of L-Phe and intermediary metabolism (Acetate and Lactate) accumulated by different recombinant strains

Strains[a]	L-Phe (g l^{-1})	PEP (mg l^{-1})	Acetate (g l^{-1})	Lactate (g l^{-1})
C. glutamicum ATCC13032	6.63 ± 0.13	0.63 ± 0.01	4.32 ± 0.08	2.84 ± 0.16
C. glutamicum Δ*ptsI*:: *iolT2-ppgK*	7.42 ± 0.01	0.76 ± 0.01	2.13 ± 0.16	1.76 ± 0.12
C. glutamicum Δ*ptsI*:: *iolT2-ppgK* Δ*aroP*	8.21 ± 0.10	0.78 ± 0.02	2.33 ± 0.13	1.98 ± 0.04
C. glutamicum Δ*ptsI*:: *iolT2-ppgK* Δ*aroP* Δ*aceE*	8.53 ± 0.09	0.94 ± 0.01	0.76 ± 0.05	2.47 ± 0.24
C. glutamicum Δ*ptsI*:: *iolT2-ppgK* Δ*aroP* Δ*ldh*	8.69 ± 0.03	1.12 ± 0.02	2.67 ± 0.09	0
C. glutamicum Δ*ptsI*:: *iolT2-ppgK* Δ*aroP* Δ*aceE* Δ*ldh*	9.14 ± 0.21	1.34 ± 0.03	1.23 ± 0.06	0

[a] The fermentation of recombinant strains all transformed with plasmids of pSUTL and pSDTL. The fermentation medium was described in "Materials and methods". Data are presented as the mean ± SD ($n = 3$)

the recombinant strain *C. glutamicum* Δ*ptsI*:: *iolT2-ppgK* (pSUTL, pSDTL) that harboring the combination six produced higher concentration of L-Phe (7.42 ± 0.01 g l^{-1}) and PEP (0.76 ± 0.01 mg l^{-1}). It was also notable that accumulation of the by-products acetate and lactate simultaneously decreased which suggested that modification of the glucose transport system alleviates the carbon overflow metabolism because of the declined conversion of PEP to pyruvate.

Modification of L-Phe transport system to increase the production

In order to reduce the import of extracellular L-Phe, the potential two L-Phe transport genes *aroP* and *pheP* were deleted in recombinant *C. glutamicum* Δ*ptsI*:: *iolT2-ppgK* to generate *C. glutamicum* Δ*ptsI*:: *iolT2-ppgK* Δ*aroP* and *C. glutamicum* Δ*ptsI*:: *iolT2-ppgK* Δ*pheP*, respectively (Table 1). As shown in Table 3, inactivation of *aroP* increased the accumulation of extracellular L-Phe (8.21 ± 0.10 g l^{-1}) with simultaneously decreased intracellular L-Phe (1.76 ± 0.05 mg l^{-1}) (Supplementary Table S2). In contrast, no obvious changes were detected with deletion of *pheP*. The results indicated that compared to PheP, the transporter AroP encoded by *aroP* plays a more important role in L-Phe import. In addition, the gene *yddG* from *E. coli* that encodes transporter for exporting L-Phe was also heterologously expressed in *C. glutamicum* while no effect was observed (Supplementary Table S2).

Enhancement of carbon flux to L-Phe by blocking the competing pathways

During cultivation, acetate and lactate as the major by-products were produced and accumulated (Table 3). As a result, to increase the carbon flux to L-Phe, the corresponding genes *aceE* (encodes enzyme E1p, a part of pyruvate dehydrogenase complex, PDHC) and *ldh* (encodes lactate dehydrogenase) were knocked-out in *C. glutamicum* Δ*ptsI*:: *iolT2-ppgK* Δ*aroP* (Table 1). As shown in Table 3, both deletions of *aceE* and *ldh* resulted in detectable increase of L-Phe and significant decline of acetate and lactate. Based on these results, the recombinant strain *C. glutamicum* Δ*ptsI*:: *iolT2-ppgK* Δ*aroP* Δ*aceE* Δ*ldh* with inactivation of *aceE* and *ldh* was further constructed and examined. As expected, double deletion of *aceE* and *ldh* leads to obvious increase of L-Phe (9.14 ± 0.21 g l^{-1}) and decrease of acetate (1.23 ± 0.06 g l^{-1}) and lactate (Table 3). Moreover, inactivation of *aceE* and *ldh* also enhanced the intracellular concentration of PEP which is beneficial to L-Phe synthesis.

Fed-batch fermentation of L-Phe

To evaluate the capacity of the recombinant strain *C. glutamicum* Δ*ptsI*:: *iolT2-ppgK* Δ*aroP* Δ*aceE* Δ*ldh* (pSUTL, pSDTL) regarding L-Phe, fed-batch fermentation was carried out in 3-l fermentor. As shown in Fig. 4, the production of L-Phe was significantly increased to 15.76 ± 0.23 g l^{-1} although less biomass was accumulated compared to the parent strain *C. glutamicum* (pXM-*pheA*^{fbr}-*aroF*^{fbr}) [46]. Furthermore, the titers of by-products acetate and lactate were substantially decreased. The results indicated that the recombinant strain *C. glutamicum* Δ*ptsI*:: *iolT2-ppgK* Δ*aroP* Δ*aceE* Δ*ldh* (pSUTL, pSDTL) with multiple modifications possesses a more balanced pathway to L-Phe compared with the parent strain *C. glutamicum* (pXM-*pheA*^{fbr}-*aroF*^{fbr}) [46].

Discussion

To date, intensive work was carried out in *E. coli* to identify the bottlenecks involved in the central carbon metabolic pathway and the shikimate pathway for biosynthesis of L-Phe, L-Tyr and L-Trp [12, 19, 20]. Whereas few related studies have been performed in *C. glutamicum* [15, 16, 28]. Recently, by introducing the wild-type gene *aroH* from *E. coli* into *C. glutamicum*, the production of L-Phe was significantly enhanced which further confirmed the difference

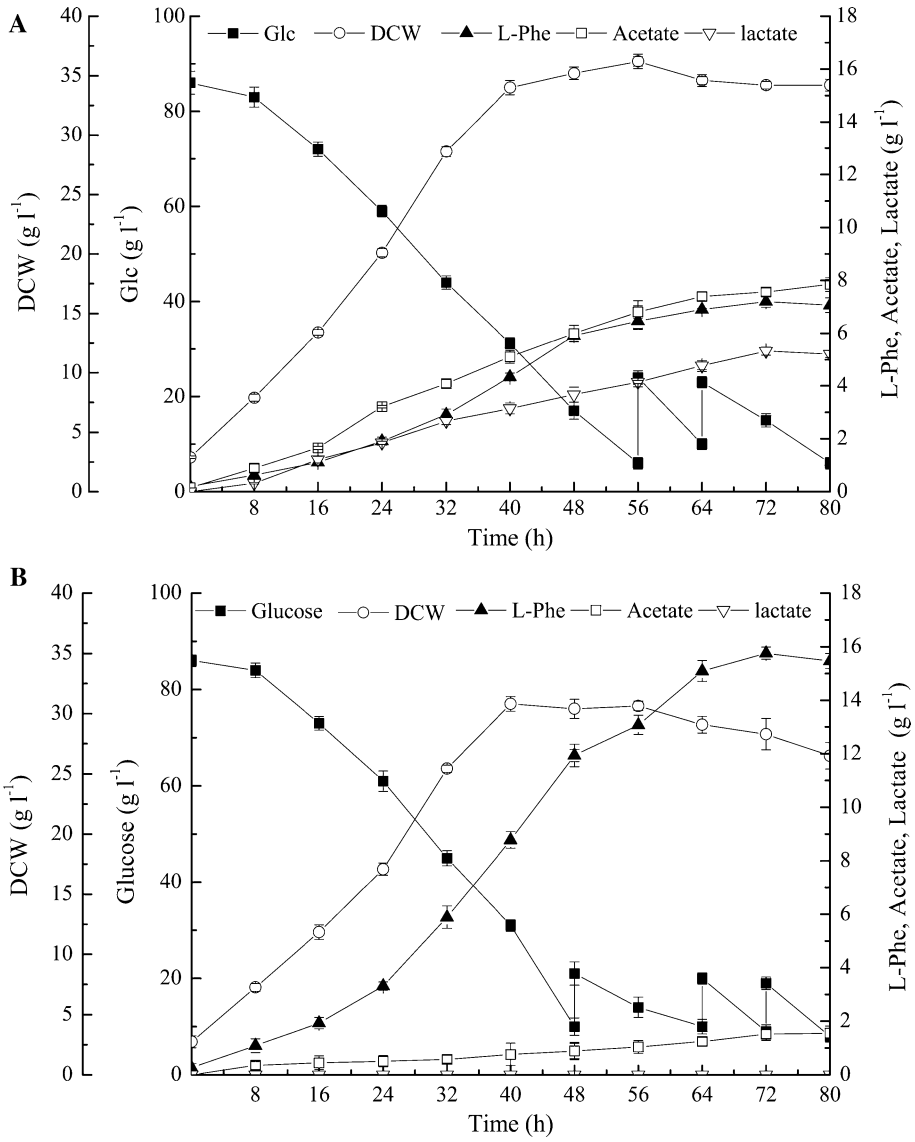

Fig. 4 Fed-batch fermentation of recombinant strains *C. glutamicum* (pXM-*pheA*^{fbr}-*aroF*^{fbr}) and *C. glutamicum* Δ*ptsI*:: *iolT2-ppgK* Δ*aroP* Δ*aceE* Δ*ldh* (pSUTL, pSDTL). **a** The fed-batch fermentation of recombinant strain *C. glutamicum* (pXM-*pheA*^{fbr}-*aroF*^{fbr}); **b** the fed-batch fermentation of recombinant strain *C. glutamicum* Δ*ptsI*:: *iolT2-ppgK* Δ*aroP* Δ*aceE* Δ*ldh* (pSUTL, pSDTL). The fermentation medium was described in "Materials and methods". Data are presented as the mean ± SD ($n = 3$)

between *E. coli* and *C. glutamicum* [47]. As a consequence, the L-Phe biosynthesis pathways in *C. glutamicum* was systematically investigated and rationally engineered for efficient production of L-Phe (Fig. 1).

To increase the pools of precursors, PEP and E4P, *ppsA*, and *tktA* have been applied and overexpressed for the production of aromatic amino acids [1, 12, 15, 18, 20]. In the present work, we also found that overexpression of *ppsA* and *tktA* lead to obvious increase of L-Phe, while no significant changes were observed when overexpressing *pck* and *tal*. The results suggested that *ppsA* and *tktA* play more important roles in synthesis of PEP and E4P (Table 2). Similarly, although two shikimate kinase isoenzymes are preserved in *E. coli*, the shikimate kinase encoded by *aroL* has been proposed as the dominant isoenzyme and overexpressed for improving chorismate biosynthesis in *E. coli* [9, 14, 24, 33]. In addition, we identified that the 5-enolpyruvoyl shikimate 3-phosphate (EPSP) synthase encoded by *aroA* is also an enzymatic bottleneck of the shikimate pathway which was consistent with previous reports in *E. coli* [24]. Interestingly, we found that overexpression of the native *aroE* gene gave rise to significant increase of L-Phe and shikimate, while no obvious changes were detected when overexpressing the heterogeneous *ydiB* gene (Table 2). In comparison, *ydiB* has been selected and overexpressed for L-tyrosine production [20, 24] due to *aroE* is feedback inhibited by the intermediate shikimate in *E. coli* [9]. Consequently, it could be concluded that the native *aroE* gene encoding shikimate dehydrogenase in *C. glutamicum* is insensitive to shikimate. Moreover, it was notable that upregulation of *tyrB* (*E. coli*) resulted in more accumulation of L-Phe, while no obvious increase was observed when overexpressing *his* (*C. glutamicum*) and *aspC* (*E. coli*). The results indicated that the *tyrB*-encoded tyrosine

aminotransferase has higher affinity to phenylpyruvate compared with His and AspC.

To achieve high production of the end-product, increase and balance of the objective biosynthesis pathways are the most critical parameters [6, 29, 45]. For this purpose, many combinatorial optimization strategies have been developed and applied more recently [2, 20, 36, 37, 44]. Here, the key genes identified above as well as *pheA*^{fbr} and *aroF*^{fbr} [46] were divided into two modules according to similar study that carried out in *E. coli* [20]. The results demonstrated that compared with the upstream enzymes (TktA, PpsA, AroF^{fbr}, and AroE), the downstream committed enzymes (AroL, AroA, PheA^{fbr}, and TyrB) should be overexpressed with higher level to balance the carbon flux to L-Ple (Fig. 2). Previously, many studies have been performed to increase the precursor PEP by blocking the glucose-PTS components (i.e., glucose-specific EIIABC component, encoded by *ptsG*) [21, 25, 40, 50] or constructing hybrid transport systems [26, 31, 41]. Similarly, a new glucose transport system was constructed in *C. glutamicum* by integrant expression of the gene cluster *iolT2-ppgK* at *ptsI* (Cgl1933) site [17, 27]. Consistent with previous results [48], introduction of this glucose transporter system leads to more intracellular PEP and extracellular L-Phe (Table 3) with normal cell growth (Fig. 3A). The results confirmed that modification of the PEP-dependent PTS with the IolT2-PpgK system is applicable approach for improving the production of aromatic amino acids.

To simultaneously decrease import and accelerate export of L-Phe, the potential transport systems were also investigated in *C. glutamicum*. Deletion results (Table 3) demonstrated that AroP plays a more important role in L-Phe import compared with PheP. Interestingly, no effect was detected when overexpressing the heterologous export transporter YddG from *E. coli*, indicating unrevealed differences between *C. glutamicum* and *E. coli* [30]. In the course of fermentation, acetate and lactate as the major by-products were always accumulated (Fig. 4a). To increase the flux toward L-Phe, both *aceE* and *ldh* were inactivated to decrease the formation of acetate and lactate. As expected, obvious increase of L-Phe accumulation was observed with double deletion of *ldh* and *aceE* (Table 3). Different from previous report [5], no defect cell growth was observed after deletion of *aceE* which should be attributed to different medium components.

Conclusion

After systematical investigation of the native three module pathways (Fig. 1) genes *tktA*, *tal*, *ppsA*, *pck*, *aroB*, *aroD*, *aroE*, *aroK*, *aroA*, *aroC*, and *his* from *C. glutamicum* as well as the heterologous genes *ydiB*, *aroL*, *tyrB*, and *aspC* from *E. coli*, we identified *ppsA*, *tktA*, *aroE* and *aroA* from *C. glutamicum*, and *aroL* and *tyrB* from *E. coli* as the engineering targets in addition to *aroF*^{fbr} and *pheA*^{fbr}. Through combinational expression of these key genes under control of promoters Ptac and Plac, the flux toward L-Phe was optimized and the production of L-Phe was increased to 6.33 ± 0.13 g l^{-1} which was about 1.48-fold of that of the parent strain *C. glutamicum* (pXM-*pheA*^{fbr}-*aroF*^{fbr}). Subsequently, the concentration of L-Phe was increased to 9.14 ± 0.21 g l^{-1} by modifying the transport systems of glucose and L-Phe and blocking the competitive pathways toward acetate and lactate. The production of L-Phe was finally improved to 15.76 ± 0.23 g l^{-1} by applying a fed-batch fermentation strategy. Further optimization of the cultivation process should enhance the L-Phe production to even higher level.

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