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Metabolic engineering of *Corynebacterium glutamicum* for improved L-arginine synthesis by enhancing NADPH supply

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Abstract

Corynebacterium glutamicum SNK 118 was metabolically engineered with improved L-arginine titer. Considering the crucial role of NADPH level in L-arginine production, *pntAB* (membrane-bound transhydrogenase) and *ppnK* (NAD^+ kinase) were co-expressed to increase the intracellular NADPH pool. Expression of *pntAB* exhibited significant effects on NADPH supply and L-arginine synthesis. Furthermore, *argR* and *farR*, encoding arginine repressor ArgR and transcriptional regulator FarR, respectively, were removed from the genome of *C. glutamicum*. The competitive branch pathway gene *ldh* was also deleted. Eventually, an engineered *C. glutamicum* JML07 was obtained for L-arginine production. Fed-batch fermentation in 5-L bioreactor employing strain JML07 allowed production of 67.01 g L^{-1} L-arginine with productivity of 0.89 g L^{-1} h^{-1} and yield of 0.35 g g^{-1} glucose. This study provides a productive L-arginine fermentation strain and an effective cofactor manipulating strategy for promoting the biosynthesis of NADPH-dependent metabolites.

Keywords *Corynebacterium glutamicum* · NADPH · $NADP^+$ transhydrogenase · NAD^+ kinase · L-Arginine

Introduction

L-Arginine (Arg) is a semi-essential amino acid and has numerous applications in health supplements and pharmaceutical industries [18, 29]. In human, L-arginine is a conditionally essential amino acid for protein synthesis, and its metabolite also gives rise to nitric oxide, a key component of endothelium-derived relaxing factor. Thus, L-arginine can also be utilized in many clinical areas such as relax and dilate blood vessels [19]. *Corynebacterium glutamicum*, a non-pathogenic and safe Gram-positive soil bacterium [24, 30], exhibits numerous ideal intrinsic attributes as a microbial factory [11] upon which one after another efficient production processes for amino acids such as L-glutamate, L-glutamine, L-lysine, L-valine, and L-arginine have been developed [20, 28].

Initially, the industrial L-arginine production began with strains obtained from random mutagenesis [23, 28], without understanding their mechanism of L-arginine biosynthesis. In recent years, metabolic engineering has contributed significantly to the performance of *C. glutamicum* in its industrial applications, using strategies involving regulation of the carbon flux of L-arginine synthesis pathway such as replacing native promoters of *argCJBDF* and *argGH* operons with stronger promoters (such as EF-Tu, sod) [18], removal of feedback inhibition and regulatory repressors [9], overexpression of the biosynthetic operons and elimination of undesired by-products [28]. Besides, cofactor availability has been demonstrated to be critical in microbial production of various natural products [3].

In *C. glutamicum*, L-arginine biosynthesis involves nine reaction steps, starting from the central precursor α-ketoglutarate. The first four enzymes, glutamate dehydrogenase (GDH), *N*-acetylglutamate synthase (ArgJ), *N*-acetylglutamate kinase (ArgB), and *N*-acetyl-gamma-glutamyl-phosphate reductase (ArgC) sequentially catalyze the transformation of α-ketoglutarate into acetylglutamate semialdehyde. The remaining five enzymes in the L-arginine biosynthesis pathway are acetylornithine aminotransferase

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(ArgD), bifunctional ornithine acetyltransferase (ArgJ), ornithine carbamoyltransferase (ArgF), argininosuccinate synthase (ArgG), and argininosuccinate synthase (ArgH). In this nine-step pathway, two enzymes GDH and ArgC are NADPH dependent [23] and all enzymes are negatively regulated by ArgR and FarR (Fig. 1). In standard aerobic conditions, glucose catabolism produce less NADPH than that required for arginine biosynthesis. It is therefore speculated that NADPH may be a limiting factor during L-arginine synthesis. In *C. glutamicum*, NADPH is mainly generated through pentose phosphate pathway (PPP), consisting of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase [22]. The NADPH availability in vivo could generally be enhanced by redirecting the metabolic fluxes from glycolysis toward the PPP through down regulating the phosphoglucose isomerase (PGI) [19], and overexpression of NAD^+ kinase or $NAD(P)^+$ transhydrogenases [15, 36]. For example, PGI has been applied to improve the L-arginine accumulation in *C. glutamicum* and *C. crenatum* [19]. However, the effects of NAD^+ kinase and $NADP^+$ transhydrogenase on NADPH supply and L-arginine synthesis have not been investigated in *C. glutamicum*. The NAD^+ kinase encoded by *ppnK* could produce $NADP^+$ from NAD^+ using ATP and poly(P) as phosphoryl donors [16, 36], while the membrane-bound transhydrogenase encoded by the *pntAB* can convert $NADP^+$ to NADPH by oxidation of NADH [10].

Corynebacterium glutamicum SNK 118 is an L-glutamate and L-arginine-producing industrial strain. In this study, the NADPH supply of *C. glutamicum* SNK 118 was improved by overexpression of *pntAB* from *Escherichia coli* and *ppnK* from *C. glutamicum* ATCC 13032 (Fig. 1). The influence of PntAB and PpnK on the synthesis of L-arginine was also evaluated. In contrast to the oxidative PPP, this strategy is not accompanied by a loss of substrate carbon. Meanwhile, the strict feedback repression of L-arginine biosynthesis was eliminated by deleting the regulatory repressors. In addition, L-lactate dehydrogenase encoded by endogenous *ldh* was knocked out to reserve more precursors and energy for L-arginine synthesis. Finally, the metabolically engineered *C. glutamicum* JML07 was constructed and evaluated in L-arginine production.

Materials and methods

Bacterial strains, media, and cultivation conditions

Escherichia coli JM109 was used as the host for gene cloning. Genome of *E. coli* was used as template for amplification of *pntAB*. For cultivation of *E. coli*, Luria–Bertani (LB) medium was used at 37 °C and 120 rpm. *C. glutamicum* SNK 118 is an arginine-producing industrial strain obtained by random mutagenesis. Genomic DNA of *C. glutamicum* ATCC 13032 was used as template for amplification of *ppnK*. *C. glutamicum* was grown in BHI medium (10 g L^{-1} of peptone, 5 g L^{-1} of sodium chloride, 18.5 g L^{-1} of brain heart infusion, and 10 g L^{-1} of beef extract, pH 7.2) at 30 °C and 120 rpm. Where appropriate, media were supplemented with antibiotics, histidine (0.3 g L^{-1}), and/or 1.8% agar. The final concentrations of antibiotics for *E. coli* were

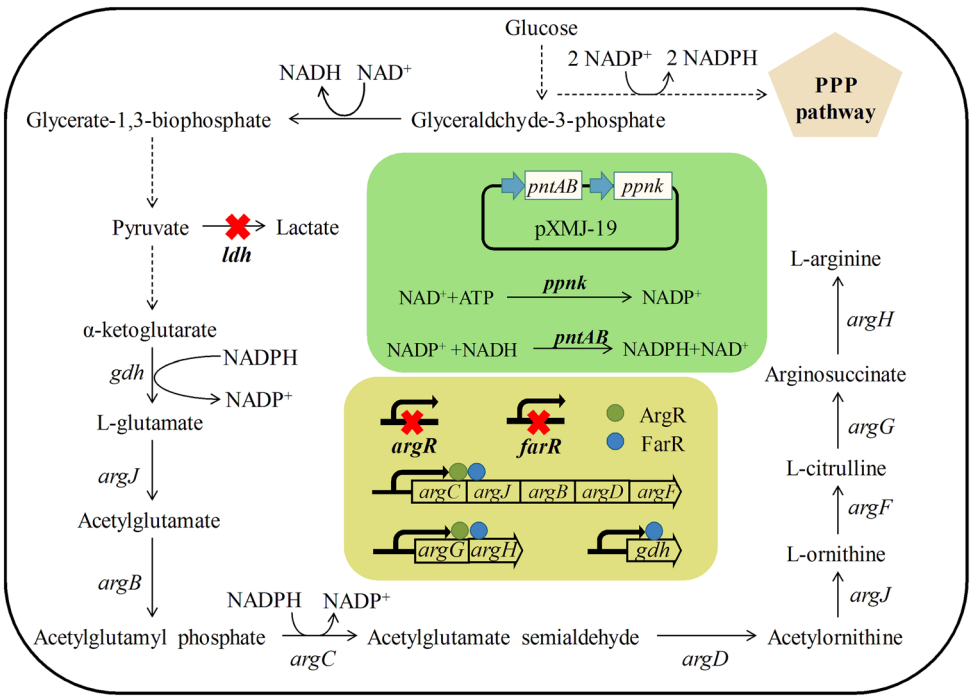

Fig. 1 Strategies adopted in metabolic engineering of *C. glutamicum* SNK 118 for improved L-arginine production

100 μg mL^{-1} of ampicillin, 50 μg mL^{-1} of kanamycin, and 34 μg mL^{-1} of chloramphenicol. With regard to *C. glutamicum,* the final antibiotic concentrations were 30 μg mL^{-1} of kanamycin, and 10 μg mL^{-1} of chloramphenicol.

Construction of plasmids and strains

Strains and plasmids used in this study are summarized in Table 1. The *E. coli*/*C. glutamicum* shuttle vector pXMJ-19 [5, 6] was used for gene expression under tac promoter in *C. glutamicum* SNK 118. For the expression of *pntAB* in *C. glutamicum* SNK 118, the *pntAB* (Gene IDs: 946628 and 946144) was PCR amplified from the genomic DNA of *E. coli* using the primers *pntAB*-F (5′-CCCAAGCTTAAAGGAGGACA-CGCATGCGAATTGGCATACCAAGAGAAC-3′) contained a *Hin*dIII site (underlined), and *pntAB*-R (5′-CGCGGATCCTTACAGAGCTTTCAGGATTGCATCC-3′) contained a *Bam*HI site (underlined), then ligated into vector pXMJ-19 to generate the plasmid pXMJ-19-*pntAB*. Gene *ppnK* (Gene ID: 1019388) was PCR amplified from the genomic DNA of *C. glutamicum* ATCC13032 using the primers *ppnK*-F (5′-CAGGTCGACTCTAGAGGATCCAGAAGGAGATATAGGATGACTGCAC-CCACGAACGC) contained a *Bam*HI site (underlined) and *ppnK*-R (5′-AGCCAAGCTGAATTCGAGCTCTTACCCCGCTGACCTGGG) contained a *Sac*I site (underlined), then ligated into vector pXMJ-19 to generate the plasmid pXMJ-19-*ppnK*. Subsequently, the *ppnK* with tac promoter sequence (tac-*ppnK*) was amplified from the plasmid pXMJ-19-*ppnK* using primers of tac-F (5′-TTGACAATTAATCATCGGCTCGTAT-3′) and *ppnK*-R. The resultant tac-*ppnK* was further adopted in PCR as template using primers of *ppnK*-tac-F (5′-CTGAAAGCTCTGTAAGGATCC-TTGACAATTAATCATCGGCTCG-3′) contained a *Bam*HI site (underlined) and *ppnK*-tac-R (5′-AGCCAAGCTGAATTCGAGCTCTTACCCCGCTGACCT-GGG-3′) contained a *Sac*I site (underlined), and then ligated into linearized pXMJ-19-*pntAB* double digested with *Bam*HI and *Sac*I to generate the plasmid pXMJ-19-*pntAB*-tac-*ppnK*. Finally, the plasmids pXMJ-19-*ppnK*, pXMJ-19-*pntAB* and pXMJ-19-*pntAB*-tac-*ppnK* were transformed into *C. glutamicum* SNK 118, respectively, to generate recombinant strains JML01 (harboring *pntAB*), JML02 (harboring *ppnK*), JML03 (harboring both *pntAB* and *ppnK*).

Genomic modifications were introduced using the Cre/loxP-mediated recombination system [4]. All DNA manipulations were performed by standard procedures, including purification and analysis of plasmid DNA, transformation of *E. coli* and *C. glutamicum* strains via electroporation, and extraction of *C. glutamicum* genomic DNA [33, 34].

Table 1 Construction of plasmids and strains used in this study

Strains and plasmids	Description	References
Strains		
E. coli JM109	*E. coli* cloning strain	Takara
C. glutamicum ATCC 13032	*C. glutamicum* model strain	ATCC
C. glutamicum SNK 118	Arg-producing *C. glutamicum* mutant strain	This lab
JML00	*C. glutamicum* SNK 118 harboring pXMJ-19	This study
JML01	*C. glutamicum* SNK 118 harboring pXMJ-19-*pntAB*	This study
JML02	*C. glutamicum* SNK 118 harboring pXMJ-19-*ppnK*	This study
JML03	*C. glutamicum* SNK 118 harboring pXMJ-19-*pntAB*-tac-*ppnK*	This study
JML04	*C. glutamicum* SNK 118 Δ*argR*	This study
JML05	*C. glutamicum* SNK 118 Δ*argR* Δ*farR*	This study
JML06	*C. glutamicum* SNK 118 Δ*argR* Δ*farR* Δ*ldh*	This study
JML07	JML06 harboring pXMJ-19-*pntAB*-tac-*ppnK*	This study
Plasmids		
pXMJ-19	*Corynebacterium* inducible expression vector, Cm^r	This study
pXMJ-19-*pntAB*	pXMJ-19 carrying *E. coli pntAB* gene	This study
pXMJ-19-*ppnK*	pXMJ-19 carrying *C. glutamicum* ATCC 13032 *ppnK* gene	This study
pXMJ-19-*pntAB*-tac-*ppnK*	pXMJ-19 carrying *E. coli pntAB* gene and *C. glutamicum* ATCC 13032 *ppnK* gene	This study
pBlueScript	Cloning vector, ColE1, lacZ, Amp	[4]
pDTW202	pBlueScript harboring a kan^R gene flanking with loxPLE/LoxPRE sites	[4]
pDTW109	pDTW109 harboring a cre gene	[4]
pDTW203	pBlueScript harboring *argR* gene flanking regions and loxPLE/PRE-kan	This study
pDTW204	pBlueScript harboring *farR* gene flanking regions and loxPLE/PRE-kan	This study
pDTW205	pBlueScript harboring *ldh* gene flanking regions and loxPLE/PRE-kan	This study

Genotypes of recombinant strains were verified by PCR and DNA sequencing.

Enzyme activity assay

For activity assay of transhydrogenase, cells were harvested at the exponential phase of batch fermentation by centrifugation (5000×*g* for 10 min at 4 °C), and washed twice with 30 mL 50 mM Tris–HCl buffer (pH 7.0). The cells were treated following the protocol of a EnzyChrom NADP/NADPH assay kit from BioAssay Systems (Hayward, CA). Briefly, around 20 mg cells were suspended in a 1.5-mL tube with 100 µL NADP or NADPH extraction buffer, then treated at 60 °C for 5 min followed by addition of 20 µL assay buffer and 100 µL of the opposite extraction buffer to neutralize the extracts. The supernatant of the extracts was used for NADP/NADPH assays. The protein concentration was determined by Bradford method. Appropriate amount of cell debris (containing 5–10 µg proteins) was added into a 1-mL assay mixture (pre-warmed at 30 °C) containing 50 mM potassium phosphate (pH 7.0), 100 mM NaCl, 0.1 mM NADH, 0.1 mM $NADP^+$ and 10 mM β-mercaptoethanol [12]. The activity of transhydrogenase was defined as the amount of enzyme that required for the formation of 1 µmol NADPH per minute at 30 °C.

Flask cultivation and fed-batch fermentation

For flask fermentation, the glycerol stock of each strain was streaked on BHI medium (with 0.5% glucose) agar slant in a test tube at 30 °C for 36 h, and all the colony were transferred into 25 mL BHI broth in a 250-mL flask. Furthermore, 5 mL culture was inoculated into a 500-mL flask containing 70 mL seed medium and grown at 30 °C and 120 rpm for 16 h. The seed medium comprised of 5 g L^{-1} of $(NH_4)_2SO_4$, 30 g L^{-1} of glucose, 0.8 g L^{-1} of $MgSO_4{\cdot}7H_2O$, 1.5 g L^{-1} of $KH_2PO_4{\cdot}3H_2O$, 0.5 g L^{-1} of K_2HPO_4, 2.5 g L^{-1} of corn steep liquor, 1.5 g L^{-1} of urea, 0.3 g L^{-1} of histidine, and 50 µg L^{-1} of biotin. After 18 h of cultivation, 2 mL seed culture was transferred into 20-mL flask fermentation medium (FFM) in a 250-mL flask. The FFM medium consisted of 40 g L^{-1} of $(NH_4)_2SO_4$, 100 g L^{-1} of glucose, 20 g L^{-1} of corn steep liquor, 1.0 g L^{-1} of $MgSO_4{\cdot}7H_2O$, 1.5 g L^{-1} of $KH_2PO_4{\cdot}3H_2O$, 0.5 g L^{-1} of K_2HPO_4, 1.5 g L^{-1} of urea, 50 g L^{-1} $CaCO_3$, 100 µg L^{-1} of biotin, and 200 µg L^{-1} thiamine. After 72 h of fermentation at 30 °C and 120 rpm, the cells were harvested for further analysis.

Fed-batch fermentation was carried out in 5-L bioreactors with 2.5 L working volume. The medium consisted of 40 g L^{-1} of $(NH_4)_2SO_4$, 100 g L^{-1} of glucose, 0.8 g L^{-1} of $MgSO_4{\cdot}7H_2O$, 1 g L^{-1} of $KH_2PO_4{\cdot}3H_2O$, 1 g L^{-1} of K_2HPO_4, 20 g L^{-1} of corn steep liquor, 0.8 of g L^{-1} urea, 15.2 g·L^{-1} of sodium glutamate, 0.075 g L^{-1} of histidine, 50 µg L^{-1} of biotin, and 20 µg L^{-1} thiamine. The temperature and the aeration were maintained at 30 °C and 1 vvm, respectively. Dissolved oxygen level was controlled at 30% by adjusting the agitation speed, and the pH was maintained at 6.7 by addition of 7.5 N ammonia solution. When the residual glucose concentration was below 10 g L^{-1}, concentrated glucose (833 g L^{-1}) was added. All 5-L fermentation was performed in at least three independent experiments.

Analytical procedures

Cell concentration was measured at 600 nm (OD_{600}) using a Mapada UV-1800 spectrophotometer (Shanghai, China). Residual glucose concentrations were measured by dinitrosalicylic acid method [21]. The concentration of L-arginine was measured using HPLC [23]. The intracellular $NADP^+$ and NADPH levels of *C. glutamicum* cells were measured using a EnzyChrom $NADP^+$/NADPH assay kit from BioAssay Systems (Hayward, CA, USA) according to the manufacturer's instructions with the samples withdrawn at exponential phase during the 5-L batch fermentation by centrifugation (5000×*g* for 10 min at 4 °C) [31, 35]. Each assay was measured in three parallel and the data were calculated and presented as mean ± standard deviation.

Results and discussion

Effect of NADPH precursors on L-arginine synthesis of *C. glutamicum* SNK 118

Corynebacterium glutamicum SNK 118 was used as the starting strain due to its high arginine production capacity. In 5-L fed-batch fermentation, *C. glutamicum* SNK 118 could produce 40.47 g L^{-1} L-arginine with a yield of 0.24 g g^{-1} glucose after 72 h of cultivation. To understand the genetic background of the *C. glutamicum* SNK 118, its genome was sequenced by Illumina HiSeq 4000 sequencing platform from the Beijing Genomics Institute. The genome sequence of *C. glutamicum* SNK 118 was deposited in NCBI under accession No: PRJNA428026. Compared with type strain *C. glutamicum* ATCC13032, SNK 118 possesses different genetic background. Based on the sequencing results, six genes of L-arginine biosynthesis pathway were found to contain nonsynonymous SNPs in the *C. glutamicum* SNK 118, including *argC*, *argJ*, *argD*, *argF*, *argR* (arginine repressor) and *argH*, resulting in changes in amino acids (see Supplementary Data 1). While the other genes contain synonymous SNPs, including *argB*, *argG*, and *farR* (transcriptional regulator). It is worth mentioning that *ppnK* gene is missing in the genome of *C. glutamicum* SNK 118, according to the genome sequence.

Biosynthesis of L-arginine from glucose typically utilizes cofactor NADPH, and the formation of 1 mol of L-arginine requires 3 mol of NADPH [23]. As a result, availability of NADPH level is supposed to be crucial especially for the synthesis of related amino acids such as L-arginine, L-citrulline, and L-ornithine, which require large quantities of NADPH [14]. To evaluate the effects of intracellular NADPH on arginine biosynthesis, shake flask experiments were performed. After 72 h of fermentation, *C. glutamicum* SNK 118 allowed production of 11.04 g L^{-1} L-arginine when fourfold of KH_2PO_4 and K_2HPO_4 were supplemented in the fermentation medium, which is 89% higher than that of control medium. In addition, when nicotinamide, NAD(P)H precursor, was added to the fermentation medium, the L-arginine titer increased by 32% compared with control (Fig. 2). These results demonstrate that increasing NADPH precursors such as nicotinamide and phosphate could improve L-arginine synthesis. Therefore, in this study, NAD^+ kinase coding gene (*ppnK*) from *C. glutamicum* ATCC 13032 as well as transhydrogenase coding genes (*pntAB*) from *E. coli* were cloned and expressed in *C. glutamicum* SNK 118, respectively. Transhydrogenase (PntAB) could convert $NADP^+$ to NADPH by oxidation of NADH to NAD^+ [10, 26], while NAD^+ kinase (PpnK) could transform NAD^+ into $NADP^+$ using ATP and poly(P) as phosphoryl donors [16, 17, 38]. Furthermore, their influence on NADPH supply and L-arginine production were also evaluated by co-expression of *ppnK* and *pntAB*.

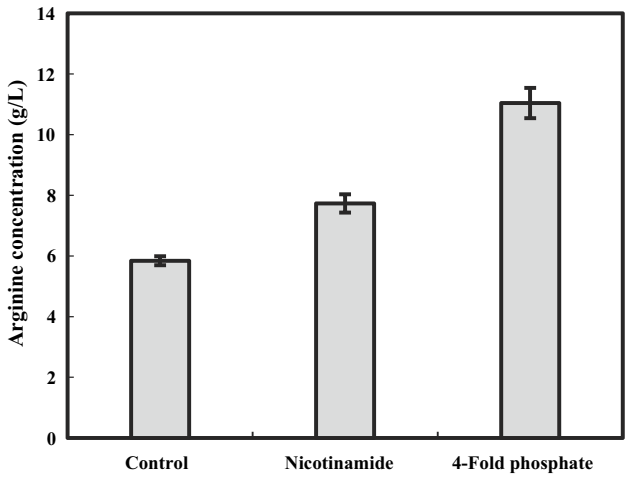

Fig. 2 Effect of NADPH precursor on L-arginine production by *C. glutamicum* SNK 118. Control: FFM medium; nicotinamide: FFM medium with 250 μg L^{-1} nicotinamide; fourfold phosphate: FFM medium with fourfold phosphates (6.0 g L^{-1} of $KH_2PO_4{\cdot}3H_2O$, 2.0 g L^{-1} of K_2HPO_4)

Heterologous expression of *pntAB* improves NADPH supply and L-arginine biosynthesis

To improve NADPH regeneration and L-arginine biosynthesis, transhydrogenase from *E. coli* was introduced into *C. glutamicum* SNK 118 to provide an additional source of NADPH. PntAB is a transhydrogenase that catalyzes the direct conversion of $NADP^+$ to NADPH. It represents an alternate route for generating NADPH from PPP, without consuming carbon source [12, 25].

The specific activity of recombinant PntAB in *C. glutamicum* JML01 (harboring pXMJ-19-*pntAB*) after IPTG induction was determined to be 0.32 ± 0.05 U mg^{-1} membrane protein. While *C. glutamicum* with empty pXMJ-19 (JML00) under the same induction exhibited none transhydrogenase activity. This result indicates that the PntAB coding genes from *E. coli* could be functionally expressed in *C. glutamicum* SNK 118. Furthermore, the intracellular $NADP^+$ and NADPH concentrations of strains JML00 and JML01 were measured after 48 h of cultivation when arginine was rapidly accumulated (Table 2). The intracellular $NADP^+$ concentration in JML01 was 51 μmol g^{-1} lower in comparison with JML00, whereas the intracellular NADPH level in JML01 was increased by 66 μmol g^{-1} DCW (36%). The ratio of NADPH to $NADP^+$ was enhanced by 10% in JML01 strain than that of JML00. All above result demonstrate that expression of *pntAB* in *C. glutamicum* SNK 118 via inducible vector pXMJ-19 effectively improved NADPH supply.

Considering the enhanced NADPH level and NADPH/$NADP^+$ ratio, effect of PntAB on cell growth and arginine synthesis of strain JML01 was evaluated in fed-batch

Table 2 Intracellular $NADP^+$ and NADPH concentrations of various recombinant *C. glutamicum* strains

Strains	$NADP^+$ (μmol g^{-1})	NADPH (μmol g^{-1})	$NADP^+$ and NADPH (μmol g^{-1})	NADPH/$NADP^+$
JML00	900 ± 45	182 ± 17	1082 ± 62	0.20
JML01	849 ± 15	248 ± 14	1097 ± 29	0.29
JML02	950 ± 19	201 ± 32	1151 ± 51	0.21
JML03	900 ± 28	280 ± 41	1180 ± 69	0.31
JML07	805 ± 48	300 ± 28	1105 ± 76	0.37

Cells were collected after 48 h of fermentation at 30 °C, and the intracellular $NADP^+$ and NADPH concentrations of cells were measured as described in the "Materials and Methods". SDs were calculated based on three biologically independent experiments

fermentation. As illustrated in Fig. 3a, b, the OD_{600} of strain JML01 could reach 97 at 80 h fermentation, similar to 98.5 of strain JML00, indicating this recombinant PntAB has little effect on the growth of *C. glutamicum*. The L-arginine production of JML01 was 56.6 g L^{-1} at 72 h which is 40% greater than that produced by JML00. The overall L-arginine yield was 0.30 g g^{-1} glucose with a productivity of 0.78 g L^{-1} h^{-1} (Table 3). L-Arginine began to be synthesized at the middle exponential stage, when the glucose consumption rate was high. Compared with the L-arginine yield of 0.19 g g^{-1} glucose of JM00, more glucose was metabolically flowed toward L-arginine synthesis. Herein, *pntAB* (encoding transhydrogenase) from *E. coli* was heterologously expressed in *C. glutamicum* JM00, and the L-arginine titer was significantly enhanced for 40%. Transhydrogenase could provide an alternative source of NADPH without loss of carbon flux, and has been applied in *C. glutamicum* to enhance the production of acetic acid [36], succinic acid [37], L-lysine [8], L-valine [1], and isobutanol [2]. To the best of our knowledge, there has been no report on the recombinant expression of *pntAB* in *C. glutamicum* for L-arginine production.

Table 3 Parameters of L-arginine production by various *C. glutamicum* strains

Strains	Arginine titer (g L^{-1})	Yield of arginine (g g^{-1})	Productivity (g L^{-1} h^{-1})
JML00	41.50	0.19	0.58
JML01	56.60	0.30	0.78
JML02	42.94	0.27	0.60
JML03	61.00	0.34	0.76
JML04	48.23	0.30	0.67
JML05	51.96	0.30	0.72
JML06	52.70	0.31	0.73
JML07	67.01	0.35	0.89

Overexpression of *ppnK* improves NADPH/$NADP^+$ supply and L-arginine biosynthesis

NAD^+ kinase (PpnK) could catalyze the phosphorylation of NAD^+ to form $NADP^+$ [36]. Overexpression of PpnK can regulate the concentration of NAD^+/$NADP^+$, which might influence the product synthesis of cofactor dependent pathways [13, 27]. To explore the role of PpnK in $NADP^+$/NADPH supply and arginine biosynthesis, *C. glutamicum* SNK 118 (JML02) harboring *ppnK* was constructed. $NADP^+$ concentration in JML02 strain was increased by 5% in comparison with the control strain JML00 (Table 2). Overexpression of PpnK can convert NAD^+ into $NADP^+$, leading to increase of $NADP^+$. Meanwhile, the concentration of NADPH was also slightly increased. It was speculated that high $NADP^+$ concentration could result in high concentration of NADPH through PPP. Due to the more

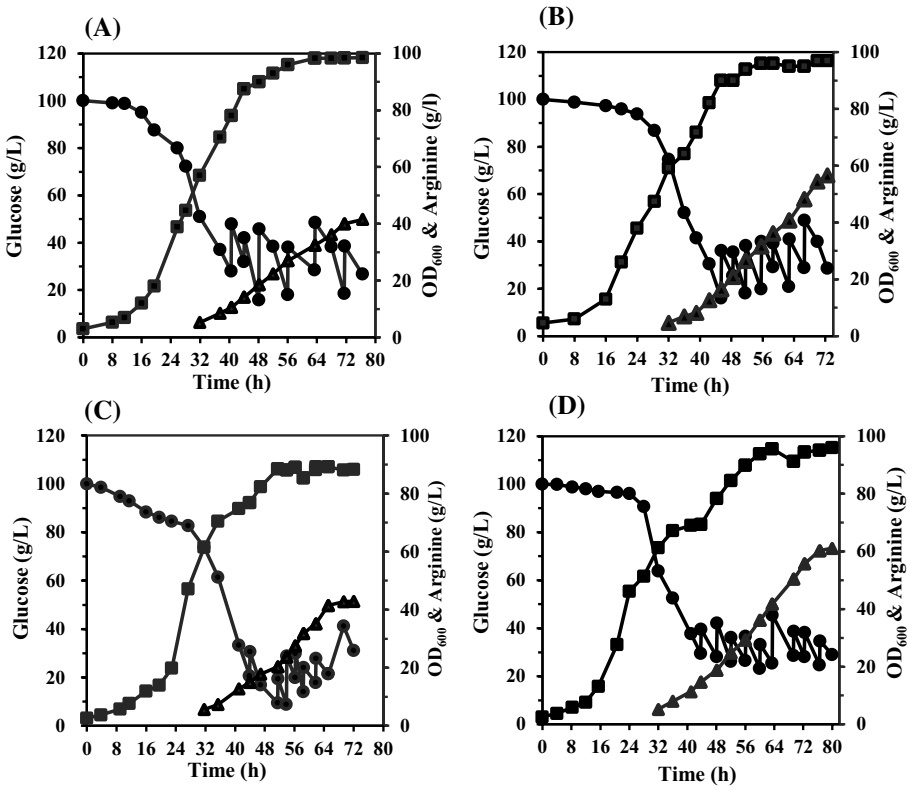

Fig. 3 Time course of L-arginine production by *C. glutamicum* strains JML00 (**a**), JML01 (**b**), JML02(**c**), JML03 (**d**) in 5-L bioreactors. Squares, cell growth (OD_{600}); circle, glucose concentration; triangle, arginine concentration

sufficient NADPH supply, more L-arginine was synthesized as expected. After 72 h of fermentation, the *ppnK* overexpression strain JML02 allowed production of 42.94 g L^{-1} of L-arginine (Fig. 3c), which was improved marginally compared with the control strain JML00 as well as higher yield of 0.27 g g^{-1} than 0.19 g g^{-1} of JML00 strain (Table 3). However, both cell growth and glucose consumption rate were decreased. These results demonstrate that increased flux towards the PPP is effective in improving the intracellular NADPH pool, which leads to appreciable increase in L-arginine. Above all, the introduction of NAD^+ kinase could positively contribute to L-arginine biosynthesis.

Co-expression of *pntAB* and *ppnK* on NADPH supply and L-arginine biosynthesis

Considering the positive roles of transhydrogenase (PntAB) and NAD^+ kinase (PpnK) in enhancing the NADPH supply and L-arginine production, the synergistic effect of PntAB and PpnK in improving NADPH/$NADP^+$ levels was evaluated. Hence, *C. glutamicum* SNK 118 strain co-expressing *pntAB* and *ppnK* (JML03) was developed. Compared with the control strain JML00, the glucose consumption and cell growth of JML03 was not influenced by PntAB and PpnK. The coexpression of *pntAB* could compensate the adverse effect of PpnK on the cell growth. The intracellular $NADP^+$ and NADPH concentrations were also measured (Table 2). JML03 showed a remarkable increase in NADPH level, which was enhanced by 13% and 39% than that of JML01 and JML02, respectively. Moreover, the intracellular NADPH level and NADPH/$NADP^+$ ratio in JML03 were increased by 54% and 11% compared with the control strain JML00. In addition, the total amount of NADPH and $NADP^+$ also increased slightly.

The effect of increased NADPH pool on L-arginine synthesis was also investigated. After fed-batch fermentation for 80 h, strain JML03 allowed production of 61.13 g L^{-1} L-arginine with a yield of 0.34 g g^{-1} glucose (Fig. 3d), representing the highest titer among three recombinant strains. Remarkably, the L-arginine titer was enhanced for 47% compared with that of JML00, along with 78.9% and 31.0% increase in yield and productivity (Table 3). Above results are consistent with the positive role of enhanced NADPH pool in the biosynthesis of other natural products such as isobutanol and isoleucine [12, 28]. It also indicates that both NAD^+ kinase and transhydrogenase could improve NADPH supply, and co-expression of *pntAB* and *ppnK* could further enhance L-arginine biosynthesis. Enhancing NADPH pool by NAD^+ kinase and transhydrogenase is therefore an effective strategy in L-arginine production. This is mainly because more carbon flux could bypass the pentose phosphate pathway where carbon is lost as carbon dioxide. Therefore, co-expression of *pntAB* and *ppnK* could significantly improve NADPH supply and L-arginine biosynthesis.

Deletion of transcriptional repressors to increase L-arginine production

Both *argR* and *farR* have negative regulatory effects on L-arginine biosynthesis. ArgR, a global transcriptional regulator, binds to the operator of *argCJBDF* and *argGH* operons (Fig. 1) that maintains the L-arginine biosynthetic pathway within its threshold concentration [23]. Furthermore, the binding of ArgR on the upstream sequence of the *argB* gene also plays an important role in L-arginine biosynthesis by *C. glutamicum* [32]. Deletion of *argR* is therefore an effective strategy for enhancing the expression level of *argCJBDF* and *argGH* operons. Thus, the *argR* of *C. glutamicum* SNK 118 was deleted to generate strain JML04. After fed-batch fermentation for 72 h, L-arginine titer of 48.23 g L^{-1} was achieved by JML04 with a yield of 0.30 g g^{-1} (Fig. 4a), which was 19% higher than that of *C. glutamicum* SNK 118. No negative effect of *argR* deletion was observed on cell growth, glucose consumption, and L-arginine production.

FarR regulates the transcription of *argC, argB,* and *argGH* operons by repressing the expression of *argB*, *argC*, *argG*, and *argH* (Fig. 1) in the presence of high intracellular L-arginine concentration [7]. In addition, FarR, as a repressor, binds to upstream of the *gdh* gene which responsible for the conversion of α-ketoglutarate to L-glutamate. Thus, to alleviate its negative regulation on L-arginine synthesis, *farR* was knocked out in JML04 to construct *C. glutamicum* Δ*argR* Δ*farR* (JML05). In fed-batch fermentation, 51.96 g L^{-1} of L-arginine was reached by JML05 (Fig. 4b), which is slightly higher than that obtained by JML04 (Fig. 4a).

In *C. glutamicum*, NADH-dependent lactate dehydrogenase (LDH) was disadvantageous for L-arginine synthesis since it could competitively convert the precursor pyruvate into L-lactic acid under anaerobic or aerobic conditions [30]. Hence, deletion of *ldh* was performed in strain JML05 to generate *C. glutamicum* Δ*argR* Δ*farR* Δ*ldh* (JML06). It is presumed that *ldh* knockout might affect the carbon fluxes and redox balance as well as L-arginine production. As shown in Fig. 4c, L-arginine titer of strain JML06 was slightly increased from 51.96 g L^{-1} (JML05) to 52.70 g L^{-1} in fed-batch fermentation. At the same time, similar L-arginine yield of 0.31 g g^{-1} glucose was achieved. Thus, the deletion of *ldh* had no significant effect on L-arginine production.

Fed-batch fermentation of engineered *C. glutamicum* strain JML07

Above results revealed that co-expression of *pntAB* and *ppnK* could efficiently improve NADPH supply

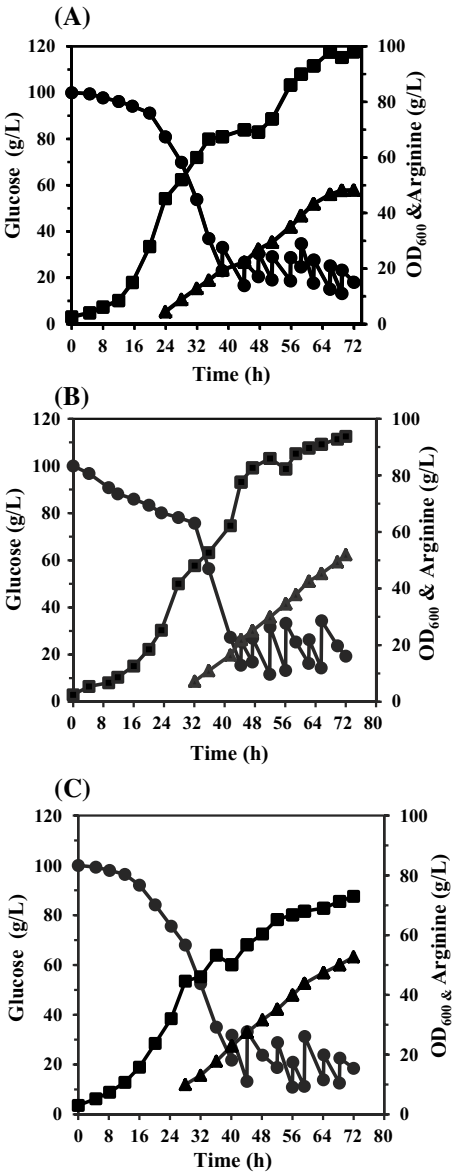

Fig. 4 Time course of L-arginine production by *C. glutamicum* strains JML04 (**a**), JML05 (**b**), and JML06 (**c**) in 5-L bioreactors. Squares, cell growth (OD_{600}); circle, glucose concentration; triangle, arginine concentration

and L-arginine biosynthesis. Additionally, deletion of transcriptional repressors (ArgR and FarR) of arginine metabolism and blocking of competitive pathway for precursors and energy (*ldh*) could further improve L-arginine production. Consequently, recombinant strain JML07 (*C. glutamicum* SNK 118 Δ*argR* Δ*farR* Δ*ldh*/pXMJ-19-*ppnK-tac-pntAB*) was constructed. After 75 h of fermentation, 67.01 g L^{-1} of L-arginine with a volumetric productivity of 0.89 g L^{-1} h^{-1} and yield of 0.35 g g^{-1} glucose was achieved by JML07 (Fig. 5, Table 3). In comparison, *C. glutamicum* SNK 118 could only produce 40.47 g L^{-1}

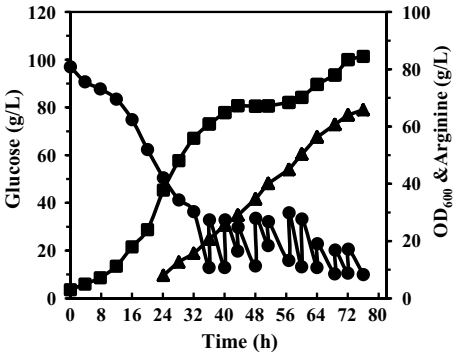

Fig. 5 Time course of L-arginine production by *C. glutamicum* strain JML07 in 5-L bioreactors. Squares, cell growth (OD_{600}); circle, glucose concentration; triangle, arginine concentration

arginine with a yield of 0.24 g g^{-1} and productivity of 0.56 g L^{-1} h^{-1} after 72 h. The results demonstrate that strain JML07 developed by strengthening NADPH supply allowed efficient L-arginine production.

By metabolic engineering of *C. glutamicum* ATCC 21831, the highest L-arginine titer of 92.5 g L^{-1} with a yield of 0.40 g g^{-1} and productivity of 1.28 g L^{-1} h^{-1} have been reported. Strategies including inactivating regulatory repressors, enhancing NADPH level, and blocking L-glutamate exporter have been adopted [23]. In our future study, L-arginine biosynthesis could be further enhanced by strategies such as replacing native promoter of *argCJBDF* and *argGH* operons and glucose uptake genes with strong promoters (such as EF-Tu, sod), enhancing L-arginine biosynthetic pathway flux.

Conclusions

Here, an efficient arginine producer, *C. glutamicum* strain JML07, was constructed. The cofactor imbalance problem in L-arginine production was circumvented through co-expression of membrane-bound transhydrogenase and NAD kinase to increase intracellular NADPH level. The expression of *pntAB* alone could markedly increase L-arginine titer from 41.5 to 56.6 g L^{-1}, suggesting its critical effect on NADPH supply and L-arginine production. In addition, genes encoding repressors and lactate dehydrogenase were deleted to further increase L-arginine production. In fed-batch fermentation, 67.01 g L^{-1} of L-arginine with a volumetric productivity of 0.89 g L^{-1} h^{-1} and yield of 0.35 g g^{-1} glucose were achieved by strain JML07 in a 5-L bioreactor. Consequently, the strain improvement strategies in this study would be useful for engineering strains with enhanced production of L-arginine as well as other industrially important chemicals.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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