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ORIGINAL ARTICLE

Metabolic engineering *Corynebacterium glutamicum* for the L-lysine production by increasing the flux into L-lysine biosynthetic pathway

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Abstract The experiments presented here were based on the conclusions of our previous results. In order to avoid introduction of expression plasmid and to balance the NADH/NAD ratio, the NADH biosynthetic enzyme, i.e., NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GADPH), was replaced by NADP-dependent GADPH, which was used to biosynthesize NADPH rather than NADH. The results indicated that the NADH/NAD ratio significantly decreased, and glucose consumption and L-lysine production drastically improved. Moreover, increasing the flux through L-lysine biosynthetic pathway and disruption of *ilvN* and *hom*, which involve in the branched amino acid and L-methionine biosynthesis, further improved L-lysine production by *Corynebacterium glutamicum*. Compared to the original strain *C. glutamicum* Lys5, the L-lysine production and glucose conversion efficiency (α) were enhanced to 81.0 ± 6.59 mM and 36.45 % by the resulting strain *C. glutamicum* Lys5-8 in shake flask. In addition, the by-products (i.e., L-threonine, L-methionine and L-valine) were significantly decreased as results of genetic modification in homoserine dehydrogenase (HSD) and acetohydroxyacid synthase (AHAS). In fed-batch fermentation, *C. glutamicum* Lys5-8 began to produce L-lysine at post-exponential growth phase and continuously increased over 36 h to a final titer of 896 ± 33.41 mM. The L-lysine productivity was 2.73 g l^{-1} h^{-1} and the α was 47.06 % after 48 h. However, the attenuation of MurE was not beneficial to increase the L-lysine production because of decreasing the cell growth. Based on the above-mentioned results, we get the following conclusions: cofactor NADPH, precursor, the flux through L-lysine biosynthetic pathway and DCW are beneficial to improve L-lysine production in *C. glutamicum*.

Keywords *Corynebacterium glutamicum* · NAD-dependent GADPH · NADP-dependent GADPH · L-Lysine biosynthetic pathway · By-products minimization

Introduction

L-Lysine, one of the essential amino acids required for nutrition of animals and humans, is widely used as feed additives, dietary supplements as well as component of cosmetics and pharmaceuticals. L-Lysine has been mainly produced by employing mutant of bacteria, such as *Eshcherichia coli*, *Saccharomyces cerevisiae* and *Corynebacterium glutamicum* (Wendisch 2007). As shown in Fig. 1, L-lysine is biosynthesized from oxaloacetate (OAA) in aminotransferase pathway comprising seven reactions, catalyzed by aspartate aminotransferase (*aspB* gene product), aspartokinase (*lysC* gene product), aspartate semialdehyde dehydrogenase (*asd* gene product), dihydrodipicolinate synthase (*dapA* gene product), dihydrodipicolinate reductase (*dapB* gene product), *meso-*

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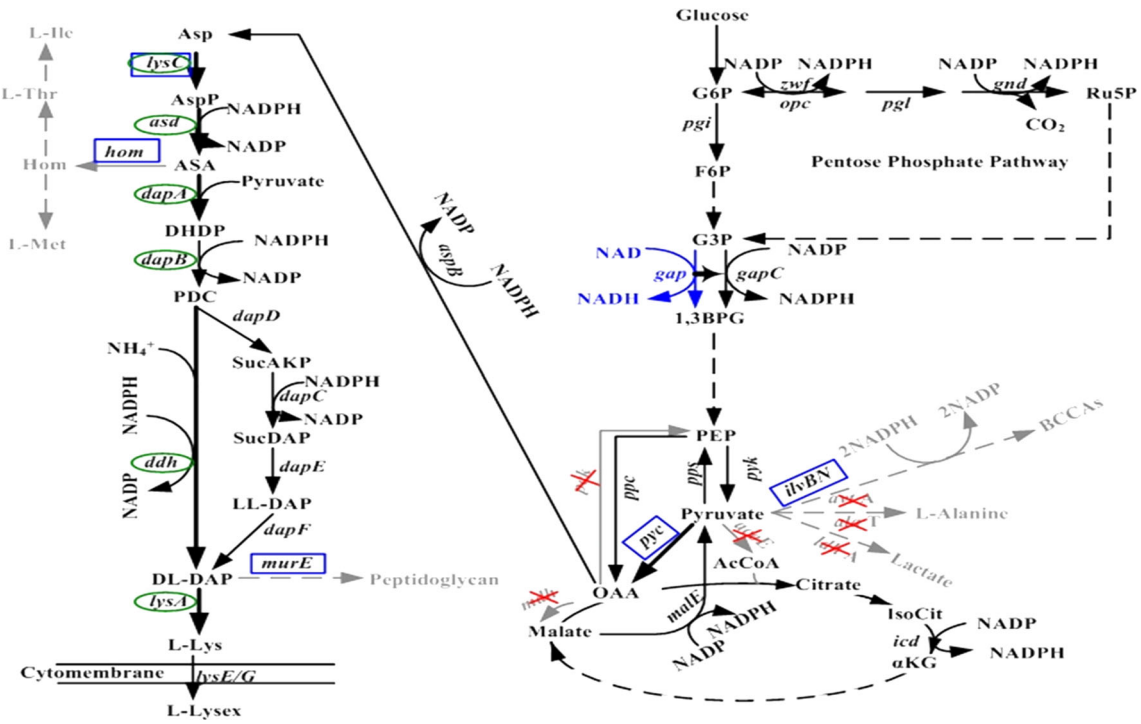

Fig. 1 Schematic representation of metabolic engineering strategy for genetically modifying *C. glutamicum* strain for L-lysine production. *G6P* glucose-6-phosphate, *F6P* fructose-6-phosphate, *G3P* glyceradehyde-3-phosphate, *1,3BPG* 1,3-diphosphoglycerate, *PEP* phosphoenolpyruvate, *IsoCit* Isocitrate, *αKG* α-oxoglutarate, *Asp* L-aspartate, *AspP* L-aspartyl-phosphate, *ASA* L-aspartyl-semialdehyde, *DHDP* L-2,3-dihydropicolinate, *PDC* L-piperidine-2,6-discarboxylate, *SucAKP* *N*-succingl-2-amino-6-ketopimetate, *SucDAP* *N*-succinyl-2,6-L,L-diaminopimetate, *L-Lys*$_{ex}$ extracellular L-lysine. *Gray arrows* represent the interrupted or attenuated pathways, *red cross* represents the gene knockout, *green ring* represent the gene over-expression, *blue line* and *box* represent the gene replacement, and the *thick arrows* represent the up-regulated pathway (color figure online)

diaminopimelate dehydrogenase (*ddh* gene product) and diaminopimelate decarboxylase (*lysA* gene product) (Wendisch 2007). Although *C. glutamicum* possesses two diaminopimelate routes used to synthesize L-lysine, i.e., aminotransferase pathway and succinylase pathway, aminotransferase pathway is a main pathway because of the high concentration of ammonium in the fermentation medium (Sahm et al. 2000). Many researchers pointed out that overexpression or deregulation the enzyme genes involved in L-lysine biosynthetic pathway are beneficial to improve L-lysine production (Pisabarro et al. 1993; Ohnishi et al. 2002), but there have been no systematic research achievements on the roles of enzymes, and most of these researches amplified the genes via plasmid-mediated. It should be noted that plasmid-mediated gene amplification against the strain breeding of L-lysine industrial fermentation (Xu et al. 2014a). We recently developed a method to genetic modification in *C. glutamicum* genome. Unlike plasmid-mediated genetic modification, the target-transformant obtained via this method does not take any genetic markers and this method cannot be restricted with the homologous fragment (Xu et al. 2014a).

Precursors of L-lysine, e.g., pyruvate and OAA, are the most important factors for L-lysine production. Increasing the replenishment and decreasing the consumption of precursors are beneficial to improve the precursor availability. Over-expression or deregulation of pyruvate carboxylase gene *pyc* is beneficial to increase the replenishment of OAA (Peters-Wendisch et al. 1998; Xu et al. 2013a). Moreover, decreasing the consumption of pyruvate and OAA is carried out by inactivating the pyruvate dehydrogenase complex (Blombach et al. 2007), phosphoenolpyruvate carboxykinase (Becker et al. 2011), aminotransferases and lactate dehydrogenase, etc. (Xu et al. 2013a). In a previous study (Xu et al. 2013a), we constructed a *C. glutamicum* Lys5 (*C. glutamicum* *lysC*^{C932T} *pyc*^{G1A,C1372T} Δ*aceE* Δ*alaT* Δ*avtA* Δ*ldhA* Δ*mdh*) which produced high L-lysine production with few by-products' accumulation.

Aside from genetically modifying the biosynthetic pathways and increasing the precursor availability, NADPH supply is also shown to be a critical factor for L-lysine production (Xu et al. 2013b). Engineering pentose phosphate pathway (PPP) is one of the major strategies to achieve more NADPH supply (Georgi et al. 2005; Becker et al. 2007, 2011). However, enhancing PPP leads to increasing the release of CO_2, thus decreasing the conversion rate of sugar to target product (Jiang et al. 2013). In

addition, Kabus et al. (2007) have demonstrated that plasmid-mediated *pntAB* gene (encoding membrane-bound transhydrogenase, PntAB) amplification increases effectively the NADPH availability by converting NADH into NADPH. However, this method will introduce a plasmid. In recent years, some reports indicated that the replacement of NAD-dependent GADPH with a NADP-dependent GADPH from *Clostridium acetobutylicum* (encoded by *gapC*; Martínez et al. 2008) or from *Streptococus mutaus* (encoded by *gapN*; Takeno et al. 2010) is beneficial to generate NADPH rather than NADH in the oxidation of glyceraldehyde-3-phosphate during glycolysis.

We recently demonstrated that inactivation of enzymes involved in by-products' biosynthesis can effectively interrupt the by-products' accumulation and increase the precursor availability. Additional plasmid-mediated *pntAB* gene amplification reduced $NADP^+$ to NADPH by the oxidation of NADH to NAD^+ and met the requirement of NADPH for L-lysine biosynthesis (Xu et al. 2014a). The transformant *C. glutamicum* $lysC^{C932T}$ $pyc^{G1A,C1372T}$ Δ*aceE* Δ*avtA* Δ*alaT* Δ*ldhA* Δ*mdh/pDXW-8-pntAB* produced up to 52.6 ± 1.9 mM L-lysine with an $Y_{P/X}$ of 6.12 mmol (g DCW)$^{-1}$. In contrast to the L-lysine producers constructed previously (Georgi et al. 2005; Becker et al. 2007, 2011), this transformant did not excrete L-alanine and lactate. Based on the previous L-lysine producer, here we highlight the importance of enzymes involved in L-lysine biosynthetic pathway by increasing the transcriptional level of these enzyme genes. Furthermore, we further reduce the by-products' accumulation by attenuation of AHAS (*ilvBN* gene product) and HSD (*hom* gene product). Finally, in order to avoid using the expression plasmid and convert NADH into NADPH, we will substitute the NADP-dependent GADPH from *C. acetobutylicum* for the native NAD-dependent GADPH.

Materials and methods

Strains, growth media and culturing conditions

Strains used in this study are listed in Table 1. The original strain *C. glutamicum* Lys5, i.e., *C. glutamicum* $lysC^{C932T}$ $pyc^{G1A,C1372T}$ Δ*aceE* Δ*alaT* Δ*avtA* Δ*ldhA* Δ*mdh*, was derived from *C. glutamicum* ATCC13032 (Xu et al. 2013a). Luria–Bertani (LB) was used as the standard medium for cultivating *E. coli* BL21. LBG (LB supplemented with 5 g l^{-1} glucose) was used to cultivate *C. glutamicum* (Hou et al. 2012). TYA (Bacto-tryptone-yeast-extract-glucose) was used to cultivate *C. acetobutylicum* ATCC 824 (Nakotte et al. 1998). The Epo medium for growing electroporation-competent cells and the LBHIS (LB, Brain Heart Infusion, Sorbitol) plates for obtaining transformants of *C. glutamicum* were prepared according to the descriptions of van der Restá et al. (1999). *E. coli* and *C. glutamicum* were, respectively, grown at 37 and 30 °C. When appropriate, *E.coli* and *C. glutamicum* were cultured with kanamycin (50 μg ml^{-1}) and 120 rpm, and a reduced concentration of kanamycin (25 μg ml^{-1}) was used to obtain recombinant strains of *C. glutamicum* (Georgi et al. 2005).

DNA manipulations and transformations

The plasmids and oligonucleotides used in this study and the functions of each oligonucleotide are listed in Table S1 and Table S2, respectively. DNA was extracted from *C.*

Table 1 Strains used in this study

Strains	Relevant characteristic(s)	Reference
E. coli BL21 (DE3)	F$^-$ ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3)	Stratagene
C. acetobutylicum ATCC 824	*C. acetobutylicum* wild-type strain	ATCC
C. glutamicum Lys5	C932T mutation in *lysC*, G1A and C1372T mutation in *pyc* gene, and *aceE*, *alaT*, *avtA*, *ldhA* and *mdh* deletion in *C. glutamicum* ATCC13032 chromosome	Xu et al. (2013a)
C. glutamicum Lys5-1	Replacement of *gapA* fragment by *C. acetobutylicum gapC* cassette in *C. glutamicum* Lys5 chromosome	This work
C. glutamicum Lys5-2	The $ilvN_{C\text{-}T}$ deletion in *C. glutamicum* Lys5-1 chromosome	This work
C. glutamicum Lys5-3	T176C mutation in *hom* of *C. glutamicum* Lys5-2 chromosome	This work
C. glutamicum Lys5-4	Replacement of *pck* fragment by $lysC^{C932T}$ cassette in *C. glutamicum* Lys5-3 chromosome	This work
C. glutamicum Lys5-5	Replacement of *mdh* fragment by *asd* cassette in *C. glutamicum* Lys5-4 chromosome	This work
C. glutamicum Lys5-6	Replacement of *alaT* fragment by *dapA* casstte and the replacement of *ldhA* fragment by *dapB* cassette in *C. glutamicum* Lys5-5 chromosome	This work
C. glutamicum Lys5-7	Replacement of *avtA* fragment by *ddh* cassette in *C. glutamicum* Lys5-6 chromosome	This work
C. glutamicum Lys5-8	Replacement of *aceE* fragment by *lysA* cassette in *C. glutamicum* Lys5-7 chromosome	This work
C. glutamicum Lys5-9	G242A mutation in *murE* of *C. glutamicum* Lys5-8 chromosome	This work

acetobutylicum ATCC 842 and *C. glutamicum* Lys5 using the Ezup-pillar Bacterial Genomic DNA Extraction Kit (Sangon, Shanghai, China). The *C. acetobutylicum* ATCC 842 DNA was used as template to amplify the *gapC* gene, and the *C. glutamicum* Lys5 DNA was used to amplify other fragments with relevant primers according to the descriptions of Table S2. The plasmid construction and transformation were performed according to the descriptions of previous reports (Sambrook and Russel 2001; Xu et al. 2014a). The build processes of plasmids were illustrated in the supplementary material. Plasmids were extracted from *E. coli* using the SanPrep Mini Plasmid Kit (Sangon, Shanghai, China) and confirmed by restriction endonuclease reaction.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total cellular RNA was extracted from cells grown for 24 h in LBG medium with the total RNA extraction kit (BioFlux, Beijing, China) as described by the manufacturer. To eliminate residual DNA, RNA preparations were treated with DNase I. The first-strand cDNA was synthesized using RevertAid^{TM} First Strand cDNA synthesis kit (Fermentas, Shanghai, China). qRT-PCR was performed using the Real Master Mix kit (TIANGEN, Beijing, China) on an iCycler iQ5 Real-Time PCR system (Bio-Rad, Richmond, USA). 100 ng of cDNA and 500 nM of each premier were added into the qRT-PCR mixture (20 μl), and the primers for qRT-PCR are listed in Table S2. The PCR procedure was prepared according to the description of previous report (Jiang et al. 2013). The target gene transcriptional levels were normalized to the 16S rRNA from the same RNA samples. Each sample was analyzed in triplicate.

Construction of *C. glutamicum* recombinant strains

The gene deletions and gene replacements were executed in *C. glutamicum* chromosome using the suicide plasmid pK18*mobsacB* (Schäfer et al. 1994). The recombinant suicide plasmids were transferred into *C. glutamicum* competent cell by electroporation, and the recombinant strains were screened on the LBHIS agar plates containing 25 μg ml^{-1} kanamycin (van der Restá et al. 1999). The second round of positive selection was carried out using sucrose resistance as selected marker (Ohnishi et al. 2002). The build process of the recombinant strain was stated in the supplementary material. The deletions in the chromosome were verified by PCR analysis using relevant primers according to the description of Table S2. The gene replacements were validated via sequencing by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

Enzyme activity assay

The crude enzyme was prepared according to the method reported by Xu et al. (2013b). After centrifugation at 4 °C for 30 min at 10,000*g*, the cell-free supernatants were immediately used to determine the enzyme activities. Protein concentrations were determined using the Bradford Protein Quantification Kit (Sangon, Shanghai, China) with bovine serum albumin as standard. The analyses of enzyme activities and protein concentrations were done in triplicate. Specific activity was given as the number of mU mg^{-1}of protein. The GADPH assay was based on the protocol of Martínez et al. (2008). Assay for AHAS (Morbach et al. 1996) and HSD (Chen et al. 2013) were performed as previously described.

Analysis of NADH/NAD and NADPH/NADP

The cells were harvested by centrifugation (4 °C, 6,000 rpm and 10 min) and washed three times with ice-cold quenching solution (60 % MeOH and 70 mM HEPES) to remove residual extracellular metabolites (Lee et al. 2010). The extraction and analysis of the relative amount of NADH/NAD and NADPH/NADP were carried out using NAD/NADH Quantification Colorimetric Kit and NADP/NADPH Quantification Colorimetric Kit following the manufacturer's instructions, respectively (BioVision, San Francisco, USA).

L-Lysine fermentation

Batch cultivations in shake flasks were carried out as described in previous reports (Xu et al. 2013a). The main culture with CgXII minimal media was inoculated with OD_{600} of 1 in 60 ml CgXIIG-media (CgXII supplemented with 40 g l^{-1} glucose, 10 g l^{-1} potassium acetate and 1 g l^{-1} L-alanine) in 500 ml Erlenmeyer flasks. The fed-batch fermentations in fermenter were carried out in a 7-l jar fermenter (KF-7l, Korea Fermenter Co., Inchon, Korea) containing 3 l media. The aeration rate, pH, dissolved oxygen, and temperature were set according the reports of Becker et al. (2011). Culture medium used for seed culture consisted of (per liter): 12.5 g soybean meal hydrolyzed, 2 g $(NH_4)_2SO_4$, 10 g CH_3COONH_4, 1 g KH_2SO_4, 0.4 g $MgSO_4$, 0.01 g $FeSO_4$, 0.01 g $MnSO_4$, 1 g L-alanine, 0.1 g aginomoto, 0.05 g nicotinamide, 2 mg thiamine-HCl, 0.5 mg biotin, 0.05 ml antifoam (BL-100, Bestn, Suzhou, China). The fermentation medium contained (per liter): 80 g glucose, 48 ml beet molasses, 60 ml corn steep liquor, 24 g $(NH_4)_2SO_4$, 24 g CH_3COONH_4, 1.5 g KH_2SO_4, 1.5 g $MgSO_4$, 0.02 g $FeSO_4$, 0.02 g $MnSO_4$, 1.5 g L-alanine, 0.05 g glycine betaine, 0.08 g nicotinamide, 4.5 mg thiamine-HCl, 8.5 mg biotin and 10 ml antifoam. Glucose

solution, contained sterile 800 g l^{-1} glucose and 400 g l^{-1} $(NH_4)_2SO_4$, was used to maintain the glucose concentration between 20 g l^{-1} and 30 g l^{-1} by adjusting the feeding rate according to glucose concentration checked every 4 h. Both media were adjusted to pH 7.0 with ammonium hydroxide.

Analytical methods

A certain amount of samples were taken from fermentation broth every 2 or 4 h. One half of samples were used to measure the biomass by a photometer at 600 nm after an appropriate dilution or by gravimetric analysis as described previously. Under these experimental conditions, the number of grams of cells (dry weight) was calculated from the OD_{600}, using a ratio of 0.34 g of cells (dry weight) l^{-1} per OD_{600} (Xu et al. 2013b). The other samples were diluted 100 times and then used to determine the glucose and L-lysine concentration by an SBA-40E immobilized enzyme biosensor (Shandong, China). In addition, the fermentation broth was also used to determine the by-products' concentration. The determination of amino acids in 1:10-diluted cultivation supernatant was carried out by high-performance liquid chromatography (HPLC, Agilent Technologies, Palo Alto, USA) with DAD detection (338 nm) after automatic precolumn derivatization with ortho-phthaldialdehyde (Xu et al. 2013a). Separation was carried out on a Thermo dC_{18} 4.6 × 250 mm column at 40 °C with a flow rate of 1 ml min^{-1}. The elution buffer of polar phase and nonpolar phase was prepared according to the description of Hou et al. (2012). Quantification was calculated the concentration using an internal standard. The organic acid concentrations in 1:10-diluted cultivation supernatant were determined by HPLC with a Kromasil C-18 4.6 × 250 mm column (AkzoNobel, Amsterdam, Netherlands) at 30 °C using isocratic elution with 20 mM KH_2PO_4 and 0.5 % acetonitrile at a flow rate of 0.8 ml min^{-1}, and subsequently detected via determination of UV absorption at 210 nm. Quantification was calculated the peak area by the external standard method.

Results

Replacement of NAD-dependent GADPH with NADP-dependent GADPH increases the glucose consumption rate and L-lysine production

In a previous study (Xu et al. 2013a), we found that heterologous expression *E. coli pntAB* gene in *C. glutamicum* Lys5 increases the glucose consumption rate, DCW and L-lysine production, indicating that the ratio of NADH/NAD in *C. glutamicum* Lys5 cell is too high to effectively assimilate glucose. NAD-dependent GADPH in *C. glutamicum* was a main enzyme for synthesizing NADH (1 mol glucose was used to produce 2 mol NADH; Takeno et al. 2010). In order to reduce the ratio of NADH/NAD and increase the NADPH availability, we replaced the native NAD-dependent GADPH with NADP-dependent GADPH from *C. acetobutylicum*, and cell growth (DCW), residual glucose and L-lysine production were monitored over the cause of the experiment. The genetically modified strain, *C. glutamicum lysC*^{C932T} *pyc*^{G1A,C1372T} Δ*aceE* Δ*alaT* Δ*avtA* Δ*ldhA* Δ*mdh* Δ*gapA::gapC*, or *C. glutamicum* Lys5-1, showed no detectable specific NAD-dependent GADPH activity but exhibited 223.5 ± 13.67 mU (mg^{-1} protein) of NADP-dependent GADPH, whereas the original strain *C. glutamicum* Lys5 showed 317.6 ± 24.56 mU (mg^{-1} protein) of NAD-dependent GADPH and no detectable specific NADP-dependent GADPH activity (Table 2). Accordingly, compared with *C. glutamicum* Lys5, the ratio of NADH/NAD was decreased, but the ratio of NADPH/NADP was increased in *C. glutamicum* Lys5-1 (Table 2). However, in contrast to NAD-dependent GADPH, the activity of NADP-dependent GAPDH was inhibited by NADPH, and not affected by NADH (Fig. 2a). In addition, *C. glutamicum* Lys5-1 showed good cell growth as compared with original strain *C. glutamicum* Lys5, 6.7 ± 0.34 g l^{-1} and 9.1 ± 0.73 g l^{-1}, respectively (Fig. 3a, b; Table 3). And the maximal glucose consumption rate of *C. glutamicum* Lys5-1 was higher than that of *C. glutamicum* Lys5

Table 2 The specific activity of enzymes and the ratio of NADH/NAD and NADPH/NADP in the original- and engineered-*C. glutamicum* strains

C. glutamicum strains	Specific activity of (mU mg^{-1} protein)				NADH/NAD ratio	NADPH/NADP ratio
	NAD-dependent GADPH	NADP-dependent GADPH	HSD	AHAS		
Lys5	317.6	ND	721.2	45.5	1.15	0.73
Lys5-1	ND	223.5	980.3	71.8	0.57	1.16
Lys5-2	ND	198.1	806.7	20.4	0.48	1.22
Lys5-3	ND	195.0	148.9	18.6	0.45	1.27

All data are mean values of three determinations of three independent experiments with errors ≤10 %

ND Not detected

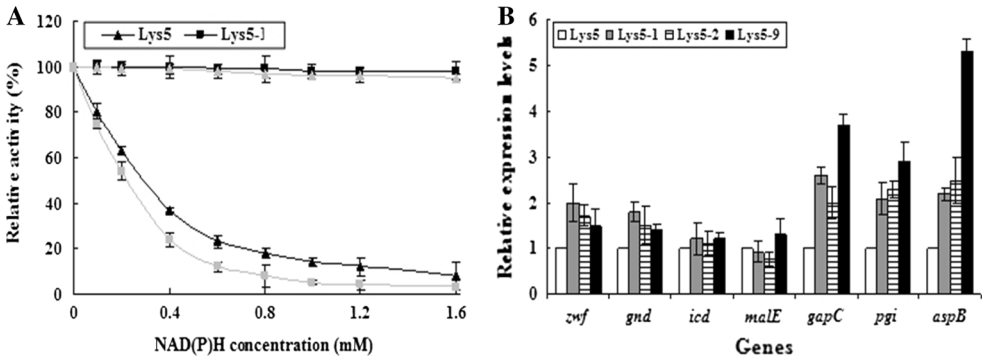

Fig. 2 **a** The relative activity of NAD-dependent GADPH using NAD^+ as cofactor in the presence of NADH (*black line*) and the relative activity of NADP-dependent GADPH using $NADP^+$ as cofactor in the presence of NADPH (*gray line*), symbols: *filled square* strain Lys5, *filled triangle* strain Lys5-1, **b** The relative expression level of genes involved in NADPH synthesis in strain Lys5 (*white bars*), Lys5-1 (*gray bars*), Lys5-2 (*lateral-cut bars*) and Lys5-9 (*black bars*). The *standard errors* are shown as *bars*

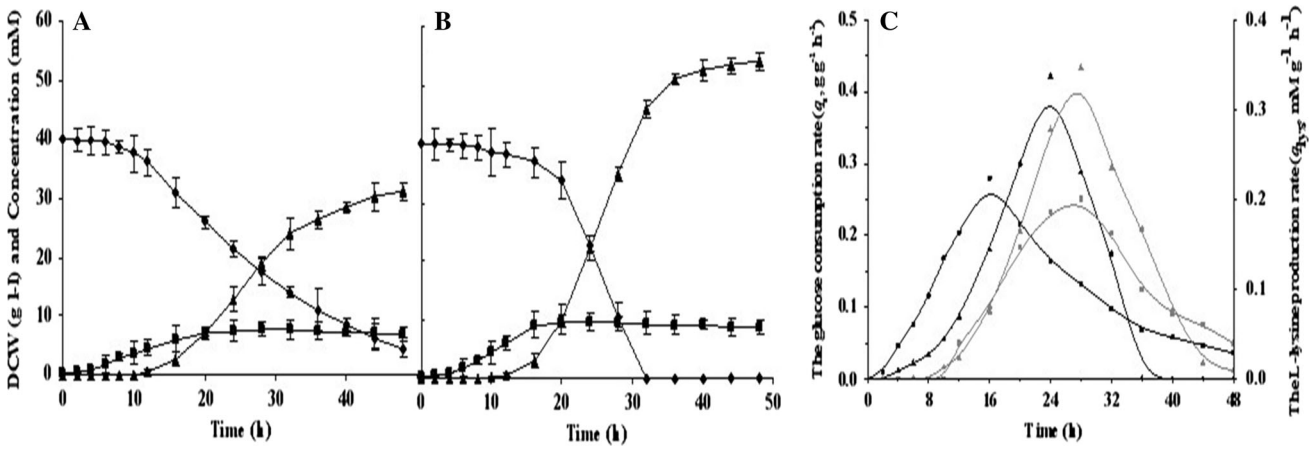

Fig. 3 Comparison of L-lysine production from glucose by strain Lys5 (**a**) and strain Lys5-1 (**b**), and the glucose consumption rate (*black lines*) and the L-lysine production rate (*gray lines*) of these two strains (**c**). Symbols: *filled square* glucose, *filled diamond* DCW, *filled triangle* L-lysine. The *standard errors* are shown as *bars*

(Fig. 3c; Table 3). The L-lysine production of *C. glutamicum* Lys5-1, of cause, was significantly increased by the replacement of GADPH. *C. glutamicum* Lys5-1 excreted 52.2 ± 5.13 mM L-lysine with resulting glucose conversion efficiency (α) of 23.96 %, whereas the *C. glutamicum* Lys5 only accumulated 31.5 ± 2.16 mM L-lysine with resulting α of 14.19 % (Table 3). The ratio of NADPH/NADP in strain Lys5 and Lys5-1 were decreased with the increase of L-lysine (Fig. S1). Interestingly, the ratio of NADH/NAD in strain Lys5 was increased after 24 h, whereas that of strain Lys5-1 was always decreased in the entire process (Fig. S1).

In order to better understand the mechanism of increased NADPH concentration, we investigated the relative expression level of the genes (*pgi*, *zwf*, *gnd*, *icd*, *malE* and *gapC*) involved in NADPH synthesis in *C. glutamicum* strains Lys5, Lys5-1, Lys5-2 and Lys5-9 (Fig. 2b). The levels of transcription of *pgi*, *zwf*, *gnd* and *gapC* in strains Lys5-1, Lys5-2 and Lys5-9 were higher than that of strain Lys5. The transcription levels of *icd* and *malE* showed little change. In addition, we also investigated the transcription levels of *aspB* involved in L-aspartate synthesis with OAA as substrate (Fig. 1). The relative expression level of *aspB* was gradually increased with the genetic modification of *C. glutamicum* Lys5 (Fig. 2b).

Attenuation of AHAS and HSD activities further decreases the by-product accumulation and improves the L-lysine production

The above-mentioned results suggested that L-valine, L-threonine, L-methionine were the major by-products and shared some of L-lysine precursor (Table 3). To decrease the L-valine, L-threonine, L-methionine accumulations and further increase the availability of precursor for L-lysine production, we attenuated the AHAS and HSD activities by deletion of C-terminal domain in *ilvN* (encoding the regulatory subunit of AHAS) and site-directed mutant of *hom*.

Table 3 L-Lysine production, by-product accumulation, final DCW, maximal specific growth rate (μ_{max}), maximal glucose consumption rate ($q_{s,\ max}$) and glucose conversion efficiency (α) of *C. glutamicum* strains

Strains	Concentration of (mM)					DCW (g l^{-1})	μ_{max} (h^{-1})	$q_{s,\ max}$ (g g^{-1} h^{-1})	α (%)[a]
	L-Lys	L-Val	L-Thr	L-Met	Pyruvate				
Lys5	31.5	8.3	1.0	0.5	44.6	6.7	0.19	0.28	14.19
Lys5-1	52.2	11.8	1.7	0.9	40.3	9.1	0.35	0.43	23.96
Lys5-2	56.8	3.2	2.6	1.5	47.5	8.3	0.31	0.42	26.04
Lys5-3	59.1	3.5	ND	ND	49.2	7.9	0.29	0.41	27.07
Lys5-4	65.7	2.6	ND	ND	33.8	8.2	0.32	0.47	29.14
Lys5-5	66.2	2.5	ND	ND	32.7	8.3	0.32	0.47	29.91
Lys5-6	71.5	1.9	ND	ND	22.4	8.0	0.29	0.43	32.21
Lys5-7	74.2	1.7	ND	ND	17.8	8.0	0.28	0.48	33.42
Lys5-8	81.0	1.1	ND	ND	8.1	7.7	0.26	0.46	36.45
Lys5-9	51.7	1.8	ND	ND	13.9	4.4	0.15	0.21	36.61

All data are mean values of three determinations of three independent experiments with errors <9 %

L-Lys L-lysine, *L-Val* L-valine, *L-Thr* L-threonine, *L-Met* L-methionine, *ND* Not detected

[a] The rate of glucose conversion into L-lysine

The resulting strains were designated as *C. glutamicum* Lys5-2 and *C. glutamicum* Lys5-3, respectively. As shown in Table 2, the activity of AHAS in strains Lys5-2 and Lys5-3 were strongly decreased and the activity of HSD in strain Lys5-3 was also strongly decreased as compared with *C. glutamicum* Lys5-1. Moreover, the concentration of L-valine in broth was sharply decreased by $\Delta ilvN_{C\text{-}T}$ as compared with *C. glutamicum* Lys5-1 (3.2 vs. 11.8 mM) and there was no detectable L-threonine and L-methionine in *C. glutamicum* Lys5-3 (Table 3). Furthermore, within 48 h, *C. glutamicum* Lys5-2 produced 56.8 ± 3.18 mM L-lysine with α of 26.04 % and *C. glutamicum* Lys5-3 produced 59.1 ± 2.87 mM L-lysine with α of 27.07 %, which were 8.81 and 13.22 % higher than that of *C. glutamicum* Lys5-1 (52.2 ± 2.13 mM L-lysine) (Table 3). For growth performance, the maximal specific growth rate (μ_{max}) was lower for strain Lys5-2 ($\mu_{max} = 0.31$) and strain Lys5-3 ($\mu_{max} = 0.29$) than for strain Lys5 ($\mu_{max} = 0.35$). This was, however, not the consequence of a reduced uptake of glucose. In fact, these strains showed relatively similar values of glucose consumption rate (Table 3).

L-Lysine production is enhanced by enhancement of L-lysine biosynthetic flux

The study of our work showed that AAT over expression has little effect on L-lysine production (Xu et al. 2014b), so five rounds of targeted genetic modifying could be used to further improve the L-lysine production (Fig. 1). Feedback-resistant aspartokinase played an important role in flux control (Becker et al. 2011). For this purpose, first, the $lysC^{C932T}$ cassette was inserted into the *C. glutamicum* Lys5-3 chromosome at *pck* gene locus resulting in *C. glutamicum* Lys5-4, thus increasing the transcriptional level of $lysC^{C932T}$ gene. Generally, the *lysC* gene and *asd* gene formed a gene cluster (Zhang et al. 1999). Therefore, the *asd* gene was also over-expressed by insertion of *asd* cassette into *C. glutamicum* Lys5-4 at *mdh* gene locus resulting in *C. glutamicum* Lys5-5. Moreover, the cassettes of *dapA* gene and *dapB* gene were, respectively, inserted into the *C. glutamicum* Lys5-5 at *alaT* and *ldhA* gene locus resulting in *C. glutamicum* Lys5-6. As shown in Table 3, the concentration of pyruvate was sharply decreased but the L-lysine production was strongly increased in *C. glutamicum* Lys5-6 as compared with *C. glutamicum* Lys5-3.

Furthermore, the next modification aimed at increasing synthetic efficiency was executed by increasing the flux in aminotransferase pathway. The *ddh* gene was over expressed by insertion of the *ddh* cassette into *C. glutamicum* Lys5-6 chromosome at *avtA* gene locus resulting in *C. glutamicum* Lys5-7 to increase the flux in aminotransferase pathway. The maximal glucose consumption rate ($q_{s,\ max}$) of strain Lys5-7 was higher than that of strain Lys5-6, 0.48 g g^{-1} h^{-1} and 0.43 g g^{-1} h^{-1}, respectively. Finally, to reduce the anabolic demand, the *lysA* gene was over expressed to channel the diaminopimelate towards L-lysine production rather than cell wall synthesis (Fig. 1). The resulting strain was designated as *C. glutamicum* Lys5-8. As shown in Table 3, *C. glutamicum* Lys5-8 exhibited poor cell growth (7.7 g l^{-1}), with μ of 0.26. In addition, *C. glutamicum* Lys5-8 produced 81.0 ± 6.59 mM L-lysine with α of 36.49 %, which was 9.16 % higher than that of *C. glutamicum* Lys5-7. Compared with the original strain *C. glutamicum* Lys5, the final strain *C. glutamicum* Lys5-8 showed higher production capacity of L-lysine (from 31.5 ± 2.16 to 81.0 ± 6.59 mM) and glucose conversion

efficiency (from 14.19 to 36.45 %) (Table 3). These results again underline the importance of precursor (i.e., pyruvate and OAA) and NADPH availability for L-lysine production. Moreover, these results also indicated that increasing the carbon flux through the L-lysine biosynthetic pathway is all good for L-lysine production.

Relevance of MurE ligase for L-lysine production with *C. glutamicum* Lys5-8

The Mur ligases play an essential role in the biosynthesis of bacterial cell-wall peptidoglycan and thus represent attractive targets for the design of novel antibacterials (Fiuza et al. 2008). MurE (encoded by *murE* gene), belongs to Mur ligase, catalyzes the ATP-dependent formation of UDP-*N*-acetylmuramic acid-tripeptide in bacterial peptidoglycan biosynthesis (Garcia et al. 2008). Binder et al. (2012) have reported that G242A mutation in *murE* from *C. glutamicum* DM1132 or DM1730 is beneficial to L-lysine production. To investigate whether down-regulation of carbon flux into peptidoglycan biosynthesis also benefits the improvement of L-lysine production by *C. glutamicum* Lys5-8, we swapped amino acid on codon 81 locus in the targeted *murE* gene, resulting in *C. glutamicum* Lys5-9. In contrast to the results of Binder et al. (2012), the L-lysine production was drastically reduced, only 51.7 ± 4.61 mM, as compared with the parental strain *C. glutamicum* Lys5-8 (81.0 ± 6.59 mM). In addition, *C. glutamicum* Lys5-9 had only consumed some glucose (25.4 ± 1.65 g in 48 h) and showed poor cell growth (4.4 g l^{-1}), the maximal specific growth rate μ of 0.15 h^{-1} (Table 3). However, the glucose conversion efficiency (α) of *C. glutamicum* Lys5-9 was higher than that of other tested strains (Table 3). It is worth pointing out that the stage of L-lysine production of *C. glutamicum* Lys5-9 was distinct from other tested strains. The L-lysine was excreted into fermentation broth at the early exponential phase by *C. glutamicum* Lys5-9 (data not shown). These results indicated the down-regulation of carbon flux into peptidoglycan biosynthesis in *C. glutamicum* Lys5-8 against the L-lysine production.

The production performance of *C. glutamicum* Lys5-8 is investigated in fed-batch fermentation in fermenter

To test the suitability of *C. glutamicum* $lysC^{C932T}$ $pyc^{G1A,C1372T}$ hom^{T176C} $\Delta ilvN_{C\text{-}T}$ *ΔaceE::lysA ΔalaT::dapA ΔavtA::ddh ΔldhA::dapB Δmdh::asd ΔgapA::gapC* $\Delta pck::lysC^{C932T}$, or *C. glutamicum* Lys5-8, the fermentation was carried out in a 7-l jar fermenter containing 3 l fermentation media. During the post-exponential phase, L-lysine began to be secreted into the broth and continuously increased over 36 h to a final titer of 896 ± 33.41 mM (converts to a 130.82 g l^{-1} L-lysine and 163.52 g l^{-1} L-lysine-HCl) (Fig. 4). The overall L-lysine productivity was 2.73 g l^{-1} h^{-1}, and the glucose conversion efficiency (α)

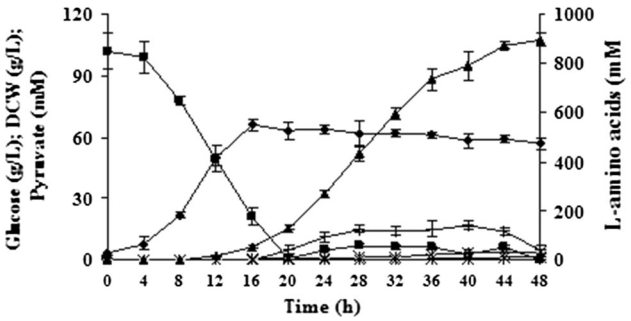

Fig. 4 DCW, glucose consumption and products (pyruvate and L-amino acids) accumulations of *C. glutamicum* Lys5-8 during fed-batch fermentation in fermentor in fermentation medium. Symbols: *filled square* glucose, *filled diamond* DCW, *plus symbol* pyruvate, *filled triangle* L-lysine, *filled circle* L-threonine, *times symbol* L-methionine, *asterick* L-valine. The *standard errors* are shown as *bars*

was 47.06 % after 48 h. After complete consumption of L-alanine (after 16 h), the biomass reached a maximum (57 ± 2.7 g l^{-1}) (Fig. 4). The residual glucose was 3 ± 2.0 g l^{-1} at the time point of 20 h. In order to maintain the glucose concentration at 20–30 g l^{-1}, the glucose solution was added into the media. After 24 hours, adding the glucose solution into the media was stopped. As shown in Fig. 4, except for L-lysine, the cell excreted about 5 ± 0.7 mM pyruvate, 8 ± 1.2 mM L-methionine, 13 ± 0.4 mM L-threonine and 33 ± 3.4 mM L-valine into the broth. However, there was not any detectable L-alanine, lactate and acetate (data not shown). Taken together, these results demonstrated that *C. glutamicum* $lysC^{C932T}$ $pyc^{G1A,C1372T}$ hom^{T176C} $\Delta ilvN_{C\text{-}T}$ *ΔaceE::lysA ΔalaT::dapA ΔavtA::ddh ΔldhA::dapB Δmdh::asd ΔgapA::gapC* $\Delta pck::lysC^{C932T}$, or *C. glutamicum* Lys5-8 is a very useful platform for optimizing L-lysine production with *C. glutamicum*.

Discussion

With respect to the availability of the cofactor NADPH, of which 4 mol is required for the production of 1 mol L-lysine, reconstructing PPP to increase the flux through PPP is a major and pervasive method to optimize the L-lysine high-yielding strain, because PPP is major pathway for NADPH formation in *C. glutamicum* (Georgi et al. 2005; Becker et al. 2007, 2011; Xu et al. 2013b). However, enhancing PPP leads to increasing the release of CO_2, thus decreasing the conversion rate of sugar to target product (Jiang et al. 2013). Although introduction of *E. coli* PntAB can be used to convert NADH into NADPH, the expression plasmid is also introduced into the *C. glutamicum* cell (Kabus et al. 2007; Xu et al. 2013a). Previous results indicated that replacement of NAD-dependent GADPH with NADP-dependent GADPH (Martínez et al. 2008;

Takeno et al. 2010) is beneficial to generate NADPH in glycolysis. As shown here, the replacement of native NAD-dependent GADPH with *C. acetobutylicum* NADP-dependent GADPH in *C. glutamicum* Lys5 not only decreases the NADH/NAD ratio but also increases the NADPH/NADP ratio, DCW and μ_{max} because of removing the inhibition of GADPH activity by NADH (Tables 2, 3). These results are consistent with the previous results (Martínez et al. 2008; Takeno et al. 2010). However, in contrast to the results reported by Takeno et al. (2010), the retardant cell growth was not observed in strain Lys5-1. The likely cause is that there is different original strain or alternative gene. In contrast to native NAD-dependent GADPH, the activity of NADP-dependent GAPDH was not be affected by NADH but strongly inhibited by NADPH (Fig. 2a). This result is consistent with the previous result (Martínez et al. 2008). This result is surprising because the replacement of GADPH in *C. glutamicum* $lysC^{C932T}$ $pyc^{G1A,C1372T}$ Δ*aceE* Δ*alaT* Δ*avtA* Δ*ldhA* Δ*mdh* or strain Lys5 resulted in about 34 % more than in *C. glutamicum* RE2/pCAK311 (52.2 vs. 39 mM; Takeno et al. 2010). This is because those gene deletions increase the precursor availability for L-lysine production. Interestingly, the replacement of GADPH in strain Lys5 increases the relative expression level of the genes (i.e., *pgi*, *zwf*, *gnd* and *gapC*) involved in NADPH synthesis except for *icd* and *malE*. This is because that high NADH concentration inhibits the cell growth. And the result indicates once again the *icd* and *malE* is not important for NADPH synthesis during L-lysine production (Georgi et al. 2005).

Although the recombinant strain Lys5-1 (i.e., *C. glutamicum* $lysC^{C932T}$ $pyc^{G1A,C1372T}$ Δ*aceE* Δ*alaT* Δ*avtA* Δ*ldhA* Δ*mdh* Δ*gapA::gapC*) excreted higher L-lysine production (up to 52.2 mM) with higher glucose conversion efficiency ($\alpha = 23.96$ %), a certain amount of L-valnine, L-threonine and L-methionine was accumulated in fermentation broth (Table 3), indicating that the enzymes AHAS and HSD play a significant role in the process of L-lysine fermentation. In addition, Dong et al. (2011) and Hasegawa et al. (2013) reported that the production of 1 mol L-valine, L-thereonine and L-methionine will consume at least 2 mol NADPH. Consequently, to minimize the carbon loss and increase the NADPH supply, we further modified the *ilvN* gene (encoding AHAS subunit) and *hom* gene (encoding HSD) in *C. glutamicum* Lys5-1 chromosome. Consistent with the previous results (Blombach et al. 2009), deletion of C-terminal of *ilvN* gene strongly decreases the AHAS activity and L-valnine production (Tables 2, 3). In addition, the ratio of NADPH/NADP was slightly increased (Table 2). Moreover, site-directed mutant of *hom* gene at gene locus 176 drastically decreases the activity of HSD and the production of L-threonine and L-methionine (Tables 2, 3). The ratio of NADPH/NADP was also increased by mutation of *hom* gene (Table 2). Interestingly, the activity of NADP-dependent GADPH was slightly decreased (Table 2). This is because that the activity of NADP-dependent GADPH was strongly inhibited by NADPH (Martínez et al. 2008). And this is why the relative expression level of *gapC* gene was decreased in strain Lys5-2 (Fig. 2b).

Increasing the flux through the L-lysine biosynthetic pathway and decreasing the flux through the counteraction of the decarboxylating reaction are the most significant for improving L-lysine production (Becker et al. 2011). To increase the availability of OAA and the conversion from OAA to L-lysine, the deletion of *pck* gene (encoding phosphoenolpyruvate carborykinase, PEPCk) and the enhancement of L-lysine biosynthetic pathway were done. As described previously, the resulting strain *C. glutamicum* $lysC^{C932T}$ $pyc^{G1A,C1372T}$ hom^{T176C} $\Delta ilvN_{C\text{-}T}$ Δ*aceE::lysA* Δ*alaT::dapA* Δ*avtA::ddh* Δ*ldhA::dapB* Δ*mdh::asd* Δ*gapA::gapC* Δ*pck::*$lysC^{C932T}$, or *C. glutamicum* Lys5-8 showed an undetectable PEPCk activity (Xu et al. 2013a) and the highest L-lysine production (up to 81.0 mM). However, the cell growth was impaired by over-expression of *lysA* gene. One possible reason is that over-expression of *lysA* gene increases the conversion between diaminopimelate and L-lysine but decreases the biosynthesis of bacterial cell-wall peptidoglycan (Fiuza et al. 2008; Becker et al. 2011).

Previously, Binder et al. (2012) have reported that mutation of *murE* gene from *C. glutamicum* DM1132 or DM1730 is beneficial to L-lysine production. These results indicate that the mutation of *murE* gene has a limited effect on cell growth and glucose consumption. However, Osman et al. (2012) pointed out that MurE (encoded by *murE*) is a crucial enzyme in the cytoplasmic step of peptidoglycan. We here show that *C. glutamicum* $lysC^{C932T}$ $pyc^{G1A,C1372T}$ hom^{T176C} $murE^{G242A}$ $\Delta ilvN_{C\text{-}T}$ Δ*aceE::lysA* Δ*alaT::dapA* Δ*avtA::ddh* Δ*ldhA::dapB* Δ*mdh::asd* Δ*gapA::gapC* Δ*pck::*$lysC^{C932T}$, or *C. glutamicum* Lys5-9 showed poor cell growth and glucose consumption, resulting in less L-lysine production (Table 3). These results indicate that *murE* mutation impairs the biosynthesis of cell-wall peptidoglycan to decrease the biomass and then decrease the glucose consumption. Interestingly, the glucose conversion efficiency (α) of *C. glutamicum* Lys5-9 was higher than that of other tested strains (Table 3). And it is worth pointing out that the phase of L-lysine production of *C. glutamicum* Lys5-9 was distinct from other tested strains. The L-lysine was excreted into fermentation broth at the early of exponential phase by *C. glutamicum* Lys5-9 (data not shown). These findings can be explained by the effect of MurE attenuation on cell growth which leads to more diaminopimelate being used to synthesize L-lysine rather than peptidoglycan during cell growth phase.

The production performance of *C. glutamicum* Lys5-8 was investigated in a fed-batch process. During fermentation in a fermentor, L-lysine production started at the post-exponential growth phase, continuously increasing over 36 h to a final titer of 896 ± 33.41 mM (Fig. 4). The overall L-lysine productivity was 2.73 g l^{-1} h^{-1}, and the glucose conversion efficiency (α) was 47.06 %. Although the L-lysine productivity of *C. glutamicum* Lys5-8 is lower, the α and total L-lysine production are higher than previous result reported by Becker et al. (2011) who enhanced the flux through the PPP to increase the NADPH supply. This is because that enhancement of PPP leads to increasing of the release of CO_2 and decreasing of the conversion rate of sugar to target product (Jiang et al. 2013). Unlike the previous result (Xu et al. 2013a), there is a negligible amount of pyruvate in fermentation broth (Table 3). Other by-products, i.e., L-valine, L-threonine and L-methionine, are seldom in fermentation broth. In addition, all genetic modification was executed in the genome such that the resulting strains are stable during cultivating in absence of selection markers conditions. This is superior to classical producers and original strain to some degree; thus *C. glutamicum* Lys5-8 is more adaptable for industrial L-lysine production.

In conclusion, we have studied on the development of a high-yielding strain for the production of L-lysine and demonstrate the importance of enzymes involved in L-lysine biosynthetic pathway for increasing the L-lysine production. Moreover, the present work points out that increasing the NADPH supply by the replacement of GADPH is superior to enhancement of PPP because of decreasing the CO_2 release. The shortened fermentation time is also important to L-lysine production (Kelle et al. 2005). Therefore, further optimizing L-lysine production with *C. glutamicum* Lys5-8 will aim at shortening the fermentation time by optimizing the formula of the fermentation medium and the culture condition.

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Conflict of interest The authors declare no commercial or financial conflict of interest.

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