Reconstruction of the Diaminopimelic Acid Pathway to Promote L-lysine Production in Corynebacterium Glutamicum

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Research

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Abstract

Background: The dehydrogenase pathway and the succinylase pathway are involved in the synthesis of L-lysine in *Corynebacterium glutamicum*. Despite the low contribution rate to L-lysine production, the dehydrogenase pathway is favorable for its simple steps and great potentials to increase the production of L-lysine.

Results: The aim of this work is to enhance the carbon flux in dehydrogenase pathway to promote L-lysine production. Firstly, the effect of ammonium (NH_4^+) concentration on L-lysine biosynthesis was investigated, and the results indicated that the biosynthesis of L-lysine can be promoted in high NH_4^+ environment. In order to reduce the requirement of NH_4^+, the nitrogen source regulatory protein AmtR was knocked out, resulting in an 8.5% increase in L-lysine production (i.e., 52.3±4.31 g/L). Subsequently, the dehydrogenase pathway was upregulated by blocking or weakening tetrahydrodipicolinate succinylase (DapD)-coding gene *dapD* and overexpressing the *ddh* gene to further enhance L-lysine biosynthesis. The final strain XQ-5-W4 could produce 189±8.7 g/L L-lysine with the maximum specific rate ($q_{Lys,max.}$) of 0.35±0.05 g/(g·h) in a 5-L jar fermenter.

Conclusions: The L-lysine titer and $q_{Lys,max}$ achieved in this study is about 25.2% and 59.1% higher than that of the original strain without enhancement of dehydrogenase pathway, respectively. The results indicated that the dehydrogenase pathway could serve as a breakthrough point to reconstruct the diaminopimelic acid (DAP) pathway and promote L-lysine production.

Introduction

L-lysine is an essential amino acid widely used in food, animal feed, medicine, cosmetics, and other industries[1]. Methods to produce L-lysine include albuminolysis, chemical method, enzymic method, and microbial fermentation. Microbial fermentation uses renewable feedstock and produces low amounts of pollutants[2]. Therefore, L-lysine is mainly produced by microbial fermentation in the industries where *C. glutamicum* and *Escherichia coli* are the most commonly used strains[1].

The L-lysine biosynthesis includes two pathways, i.e., the diaminopimelic acid (DAP) pathway and the α-aminoadipic acid (AAA) pathway. In the AAA pathway, L-lysine is synthesized from α-ketoglutarate and acetylcoenzyme A (acetyl-CoA), in which α-aminoadipic acid serves as an intermediate metabolite. The AAA pathway is commonly found in yeast, fungi, and some species in the domain of Archaea[3, 4]. In the DAP pathway, however, L-lysine is synthesized from aspartate and pyruvate, in which *meso*-diaminopimelic acid (*meso*-DAP) serves as an intermediate metabolite(Fig. 1) [5]. Commonly found in archaea, algae, fungi, plants, and bacteria[6],the DAP pathway starts with the biosynthesis of L-Δ^1-tetrahydrodipicolinate (THDPA) from L-aspartate, which is then converted into *meso*-DAP, and finally, L-lysine is produced with diaminopimelate deacetylase (DAPDC, EC:4.1.1.20) as catalyst[7]. The conversion into *meso*-DAP is the essential step that distinguishes the four DAP pathway variations, i.e., the succinylase pathway, acetylase pathway, dehydrogenase pathway, and aminotransferase pathway[8]. The four variants share the common steps of converting L-aspartate to THDPA catalyzed by aspartokinase (AK, EC:2.7.2.4), aspartic semialdehyde dehydrogenase (AsaDH, EC:1.2.1.11), dihydrodipicolinate synthetase (DHDPS, EC:4.3.3.7), and dihydrodipicolinate reductase (DHDPR, EC:1.17.1.8) in turn. And then different enzymes are involved in the four different variant pathways to produce *meso*-DAP (Fig. 1). The succinylase pathway is the most common in most bacteria including *Escherichia coli*[9].The acetylase pathway is only found in some *Bacillus*, and the dehydrogenase pathway only exists in some Gram-positive bacteria (i.e., *Corynebacterium* and *Bacillus*)[10] and plants (i.e., *Glycine* and *Zea*)[11]. The aminotransferase pathway is found in

Cyanobacteria, *Chlamydia*, *Methanothermobacter thermautotrophicus*, and *Arabidopsis thaliana*[12–14]. Among the four variants, THDPA is directly converted into *meso*-DAP in the dehydrogenase pathway. Therefore, the dehydrogenase pathway is more favorable in situations where energy is limited[15]. However, L-lysine accumulation in the dehydrogenase pathway requires high NH_4^+ concentrations[16] due to the low affinity of diaminopimelate dehydrogenase (DapDH, E.C. 1.4.1.16) to the substrate. Thus, the application of the dehydrogenase pathway is limited[15].

C. glutamicum is a Gram-positive bacterium isolated from soil in 1957[17, 18]. *C. glutamicum* is often used to produce amino acids in the industries, such as L-glutamic acid, L-lysine and L-arginine[19]. Interestingly, two variants of the DAP pathway are found in *C. glutamicum*, i.e., the succinylase pathway and the dehydrogenase pathway. According to Fig. 1, the *meso*-DAP is biosynthesized from THDPA in one step via the dehydrogenase pathway[20] and in four steps via the succinylase pathway, both of which involve NH_4^+. In addition, the L-lysine biosynthesis efficiency of both pathways depends on the concentration of NH_4^+ in the substrate[16]. L-lysine was mainly produced through the dehydrogenase pathway at first but then entirely through the succinylase pathway[16] as the concentration of NH_4^+ decreases along with the fermentation, resulting in the decrease in the dehydrogenase activity[16, 21]. Sonntag et al. reported that 33% of L-lysine was synthesized via the dehydrogenase pathway while 66% via the succinylase pathway in *C. glutamicum*[22]. Although the succinylase pathway is important for increasing the titer of L-lysine, the dehydrogenase pathway has great potential in improving the production intensity of L-lysine as only one step is required to biosynthesize *meso*-DAP. Hence the question: does upregulating the dehydrogenase pathway promote L-lysine production in *C. glutamicum*?

In this study, the *C. glutamicum* XQ-5 strain was developed, which took the dehydrogenase pathway for *meso*-DAP biosynthesis to promote L-lysine production. The DAP pathway of *C. glutamicum* XQ-5 was reconstructed to upregulate the dehydrogenase pathway in L-lysine biosynthesis during the fermentation process. The strategies of this study include: (1) investigating the effect of different NH_4^+ concentrations on L-lysine production; (2) alleviating the nitrogen limitation to improve the efficiency of L-lysine production; (3) rationally regulating the two pathways to promote L-lysine production. As a result, a recombinant strain *C. glutamicum* XQ-5-W4 (i.e., *C. glutamicum* XQ-5-*dapD*^{W}Δ*amtR*/pEC-*ddh*) derived from *C. glutamicum* XQ-5 was obtained, which produced 189 ± 8.7 g/L L-lysine with the maximum specific rate ($q_{Lys,max}$.) of 0.35 ± 0.05 g/(g·h) in a 5-L jar fermenter, which were 25.2% and 59.1% higher than that of the original strain *C. glutamicum* XQ-5, respectively.

Methods

Strains, growth medium and culture conditions

L-lysine producing strain *C. glutamicum* XQ-5 derived from the wild-type strain *C. glutamicum* ATCC 13032 after multiple rounds of random mutagenesis. Strains and plasmids used in this study are listed in Table 4. Oligonucleotides used in this study are listed in Supplementary material (Table S2). The L-lysine high-producing strain *C. glutamicum* XQ-5 was derived from the wild-type strain *C. glutamicum* 13032[41]. Molecular reagents (isopropyl B-D-1-thiogalactopyranoside (IPTG), 2 x Phanta Max Master Mix, 2 x Tag Max Master Mix (Dye Plus), and kanamycin) were purchased from Vazyme Biotech Co., Ltd (Nanjing, China). Restriction endonucleases and the DNA Ligase were purchased from Thermo Fisher Scientific Shanghai Instruments Co. Ltd. (shanghai, China). Other chemical reagents, including yeast extract, tryptone, and NaCl, were purchased from China National Pharmaceutical Group Corporation (Shanghai, China). The cell concentration was measured with a spectrophotometer (721N,

shanghai, China). The glucose and L-lysine concentrations were measured with an SBA-40E immobilized enzyme biosensor (Shandong, China).

Table 4
The main strains and plasmids used in this study

strains and plasmids	characters	reference
C. glutamicum **strains**		
XQ-5	*C. glutamicum* AEC^r 2-TA^r MF^r Met^s, L-lysine-producing bacteria derived from strain *C. glutamicum* ATCC13032	[41]
XQ-5-1	strain XQ-5 harboring plasmid pEC-XK99E-*ddh*	this study
XQ-5-2	derivative of strain XQ-5 with deletion of *amtR*	this study
XQ-5-3	strain XQ-5-2 harboring plasmid pEC-XK99E-*ddh*	this study
XQ-5-4	derivative of strain XQ-5 with deletion of *dapD*	this study
XQ-5-5	derivative of strain XQ-5 with deletion of *ddh*	this study
XQ-5-6	derivative of strain XQ-5-2 with deletion of *dapD*	this study
XQ-5-7	strain XQ-5-4 harboring plasmid pEC-XK99E-*ddh*	this study
XQ-5-8	strain XQ-5-6 harboring plasmid pEC-XK99E-*ddh*	this study
XQ-5-W1	derivative of strain XQ-5-3 with weaking of *dapD*(T1-Terminator)	this study
XQ-5-W2	derivative of strain XQ-5-3 with weaking of *dapD*(T2-Terminator)	this study
XQ-5-W3	derivative of strain XQ-5-3 with weaking of *dapD*(T3-Terminator)	this study
XQ-5-W4	derivative of strain XQ-5-3 with weaking of *dapD*(T4-Terminator)	this study
XQ-5-W5	derivative of strain XQ-5-3 with weaking of *dapD*(T5-Terminator)	this study
XQ-5-W6	derivative of strain XQ-5-3 with weaking of *dapD*(T6-Terminator)	this study
Plasmids		
pEC-XK99E	Kan^r, Expression vector with *pMB1* replicon	stratagene
pK18mobSacB	Kan^r, Integration vector	stratagene
pEC-XK99E-*ddh*	pEC-XK99E carrying gene *ddh* from *C.glutamicum*	this study
pK18*mobsacB*-Δ*amtR*	pK18*mobsacB* carrying *amtR*-L and *amtR*-R fragments	this study
pK18*mobsacB*-Δ*dapD*	pK18*mobsacB* carrying *dapD*-L and *dapD*-R fragments	this study
pK18*mobsacB*-Δ*ddh*	pK18*mobsacB* carrying *ddh*-L and *ddh*-R fragments	this study
pK18*mobsacB*-T1	a derivative of pK18*mobsacB*, harboring the fragment of inserting T1 terminator in front of *dapD*	this study
pK1*8mobsacB*-T2	a derivative of pK18*mobsacB*, harboring the fragment of inserting T2 terminator in front of *dapD*	this study
pK18*mobsacB*-T3	a derivative of pK18*mobsacB*, harboring the fragment of inserting T3 terminator in front of *dapD*	this study
pK18*mobsacB*-T4	a derivative of pK18*mobsacB*, harboring the fragment of inserting T4 terminator in front of *dapD*	this study

strains and plasmids	characters	reference
pK18*mobsacB*-T5	a derivative of pK18*mobsacB*, harboring the fragment of inserting T5 terminator in front of *dapD*	this study
pK18*mobsacB*-T6	a derivative of pK18*mobsacB*, harboring the fragment of inserting T6 terminator in front of *dapD*	this study

Escherichia coli grew in Luria-Bertani (LB) medium at 37°C. *C. glutamicum* grew in LB-glucose (LBG) medium at 30°C[42]. EPO medium and LB-Brain Heart Infusion-Sorbitol (LBHIS) medium were used to construct the recombinant bacteria[43]. In addition, a 50 μg/mL kanamycin solution was used to build the plasmids and a 25 μg/mL kanamycin solution was used to screen the recombinant strains. A 1 mmol/L IPTG solution was used to induce gene overexpression. Samples were taken from the shake flasks or fermenters every four hours.

The single colony was inoculated in LBG liquid medium and incubated at 30°C for 12 h with rotation speed 100 r/min. Next, 5 mL of the seed culture was transferred to 50 mL of the fermentation medium in a standard 500 mL shake flask and was cultured for 72 h at 30°C with rotation speed 100 r/min. The fermentation medium contained (per liter) 100 g glucose, 8 g corn steep liquor, 40 g $(NH_4)_2SO_4$ (≈ 300 mM), 0.02 g Fe^+, 0.02 g Mn^+, 450 μg VB_1, 8 mg VB_3, 850 μg VH, 0.6 mg Zn^+, 0.53 g KCl, 1 g KH_2PO_4, 1 g K_2HPO_4, 4 g $MgSO_4{\cdot}7H_20$, 50 mg betaine, 8 mL beet molasses and 40 g $CaCO_3$. Both media were adjusted to pH 7.3 with NaOH. Fermentation conditions: initial pH 7.3 and 10% of inoculation volume.

Fed-batch fermentation was carried out in a 5-L jar fermenter (BLBio-5GJ-2-H, Bailun Bi-Technology Co. Ltd., Shanghai, China). The fermentation medium contained (per liter): 70 g glucose, 20 g corn steep liquor, 2 g KH_2SO_4, 50 g beet molasses, 40 g $(NH_4)_2SO_4$, 1.5 g $MgSO_4.7H_20$, 0.03 g $FeSO_4$, 0.02 g $MnSO_4$, 0.03 g glycine betaine, 600 ug biotin, 300 ug thiamine-HCl, and 2 ml antifoam. Ammonia was used to control pH 7.0 and provide nitrogen source for bacteria. The relative dissolved oxygen was controlled at 20–30% by stirring speed and ventilation. The temperature is maintained at 30 °C by jacket cooling. OD_{600}, residual sugar concentration and L-Lysine concentration were determined every 4 h during fermentation. The prepared feed solution[44] was used to control the glucose concentration at about 5 g/L by adjusting the feeding rate. L-lysine concentration was determined as lysine·HCl in duplicates.

Analytical methods

The cell concentration after 25-fold dilution was measured at OD_{600} using a spectrophotometer. The correlation coefficient between the dry cell weight (DCW) and OD_{600} was 0.32 (1 OD_{600} = 0.32g DCW). After sample dilution of 100 times, glucose and L-lysine concentrations were measured with an SBA-40E immobilized enzyme biosensor. The concentration of the by-products was measured with high performance liquid chromatography (HPLC)[44]. Cell morphology was observed via field emission scanning electron microscopy (FESEM). Cells of *C. glutamicum* in the mid-log phase were collected by centrifugation and rinsed three times in physiological saline (pH 7.0). Bacterial cells were spread onto a small silicon platelet and air dried under room temperature, followed by in-situ fixation with a 2.5% glutaraldehyde solution in a 0.15 M sodium phosphate buffer (pH7.4) for 10 min. The samples were coated with gold and transferred to FESEM (SU8220, Hitachi, Japan) for observation at an accelerating voltage of 3 kV.

Construction of C. glutamicum **recombinant strains**

Restriction endonucleases and the DNA Ligase were used to construct the plasmids. In this study, the plasmid pEC-XK99E was used for gene overexpression in *C. glutamicum*. The suicide plasmid pK18*mobsacB* was used for gene knockout in *C. glutamicum.* Firstly, the constructed plasmid was electroporated into *C. glutamicum*, and then the positive transformants were screened with a 25 μg/mL kanamycin solution in LBH medium. The final positive transformants were obtained by eliminating the plasmids according to the sucrose lethal principle. The deletions in the chromosome were verified by PCR analysis.

Real-time PCR

In order to analyze RNA, cells in exponential phase were collected during shake flask fermentation for mRNA isolation. RNA was extracted with an RNAiso Plus reagent (Takara, Dalian, China). The cDNA was synthesized with RevertAid™ First Strand cDNA synthesis kit (Fermentas, Shanghai, China). The Ct values of the 16S rDNA gene and those of the *ddh* and *dapD* genes were obtained by RT-qPCR using a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Hercules, CA) with SYBR Premix Ex Taq™ II (Takara, Dalian, China). The primer sequences used for RT-qPCR and RT-PCR are shown in Table S3. Each sample was analyzed in triplicate.

Preparation of crude extracts and enzyme assays

Crude enzyme solution was prepared to measure the activities of DapDH and DAPD. The preparation method was based on a previous report[45]. Enzyme activity was analyzed in triplicate.

DapDH activity was measured at 30 ℃. The forward reaction mixture contained 200 mM glycine-KOH (pH 10.5), 100 mM $(NH_4)_2SO_4$, 0.3 mM NADPH, 5 mM THDPA and crude enzymes extract. The reverse reaction mixture contained 200 mM glycine/NaOH (pH 10.5), 2 mM $NADP^+$, 4 mM *meso*-DAP and crude enzymes extract. One unit is defined as the amount of enzyme which catalyzes the formation or decrease of 1 μmol NADPH (340nm) per minute[46].

DapD activity was measured by the formation of free coenzyme A (CoA) at 412 nm. The reaction mixture contained 0.1 M Tris-HCl (pH 8.0), 0.5 mM DTNB, 0.2 mM succinyl-CoA, 5 mM 2-aminopimelate and crude enzymes extract. One unit is defined as the amount of enzyme which catalyzes the formation of 1 μmol CoA per minute[32].

Results And Discussions

The effects of different ammonium (NH_4^+) concentrations on L-lysine production

Nitrogen is one of the essential nutrients for living cells. The nitrogen utilization is very important for the growth of bacteria. Ammonium (NH_4^+) is the standard component of growth medium and the preferred nitrogen source for many bacteria. Both variants of DAP pathway in *C. glutamicum* require the participation of NH_4^+. Therefore, the effect of NH_4^+ on L-lysine production was investigated. To do this, eight NH_4^+ solutions of different concentrations (i.e., 50, 100, 200, 250, 300, 350, 400, 500 mM) were added to the culture mediums and the cell growth, L-lysine production as well as q_{Lys} were monitored. The results showed that the growth of strain XQ-5 was significantly inhibited at high NH_4^+ concentration, but the effect of low NH_4^+ concentration is relatively insignificant (Fig. 2a). Xu et al. observed similar results, in which high NH_4^+ concentration was toxic to the bacteria[23]. Interestingly, too high or too low NH_4^+ concentration is not conducive to L-lysine accumulation (Fig. 2b). The L-lysine yield was relatively

high at NH_4^+ concentrations of 250 mM, 300 mM, and 350 mM, which were 42.3 ± 2.31 g/L, 48.2 ± 3.54 g/L, and 43.9 ± 3.78 g/L, respectively. Interestingly, the maximum specific rate ($q_{Lys,max}$.) was found with a 350 mM NH_4^+ solution (i.e., 0.22 ± 0.03 g/(g·h)), which was 10% higher than that with a 300 mM NH_4^+ solution (i.e., 0.20 ± 0.01 g/(g·h)) (Fig. 2c). These results indicated that an appropriate increase in NH_4^+ supply is necessary for the effective biosynthesis of L-lysine. Previous research reported that the dehydrogenase pathway is only active at high NH_4^+ concentration[22]. Therefore, increasing the concentration of NH_4^+ may upregulate the dehydrogenase pathway and promote L-lysine biosynthesis. The transcription level of the DapDH-coding gene *ddh* was measured. As might be expected, the transcription level of *ddh* gene increased with the increase of NH_4^+ concentration within a certain range (Fig. 2d). It's safe to conclude that the increase of $q_{Lys,max}$ at high NH_4^+ concentration was related to the increase in *ddh* gene transcription level, and upregulating the dehydrogenase pathway is conducive to promoting L-lysine biosynthesis.

Effect of the upregulated dehydrogenase pathway on L-lysine biosynthesis

As mentioned above, upregulating *ddh* gene promotes the biosynthesis of L-lysine. It is speculated that upregulating the dehydrogenase pathway may further increase L-lysine production. In order to enhance the effect of dehydrogenase pathway on L-lysine production, the *ddh* gene was overexpressed to investigate whether the q_{Lys} and L-lysine production were improved. The *ddh* gene was ligated into the plasmid pEC-XK99E, which was then introduced into strain XQ-5 to give the target strain XQ-5/pEC-*ddh* (i.e., strain XQ-5-1) (Fig. 3a). The growth rate, L-lysine production, and q_{Lys} of strain XQ-5-1 were measured at shake flask fermentation for 72 h, and the effect of the upregulated dehydrogenase pathway on L-lysine production was investigated.

According to Fig. 3b, the growth of strain XQ-5-1 was not affected, remaining almost the same as the original strain. In addition, the final L-lysine production of strain XQ-5-1 was 49.3 ± 3.21 g/L, slightly increased compared with strain XQ-5 (i.e., 48.2 ± 3.54 g/L) (Fig. 3c). The results showed that the upregulated dehydrogenase pathway had little effect on L-lysine production. Similar results were also found in previous reports, in which the overexpression of *ddh* gene on plasmid had no effect on the L-lysine production of *C. glutamicum*[24]. It is speculated that the intracellular NH_4^+ concentration limits the increase of L-lysine production in strains with overexpressed *ddh*. Due to the low affinity to NH_4^+, the dehydrogenase pathway is only adoptable at high NH_4^+ concentration[16]. However, it is heartening that XQ-5-1 produced the same amount of L-lysine as the original strain in a shorter time (Fig. 3c). With the same NH_4^+ concentration, the $q_{Lys,max}$. of XQ-5-1 was 5%-10% higher than that of the original strain (Fig. 2c, 3d). Previous research suggested that the level of extracellular NH_4^+ concentration is higher than the intracellular NH_4^+ concentration as the utilization of nitrogen is controlled by AmtR[25]. Then, is the increase in intracellular NH_4^+ concentration beneficial L-lysine production?

Effect of gene amtR deletion on NH_4^+ utilization and L-lysine synthesis

As mentioned above, L-lysine production showed no significant increase with the overexpression of *ddh*, possibly due to the low intracellular NH_4^+ concentration. To address this limitation, the regulatory proteins controlling the utilization of NH_4^+ should be inactivated. In *C. glutamicum*, the expression of genes responding to nitrogen utilization is controlled by the TetR-type regulator AmtR[25]. In contrast with most TetR-type regulators, the

dissociation of AmtR from its target promoters is triggered by complex formation with the PII-type signal transduction protein GlnK rather than by the binding of a low-molecular mass ligand[26]. The transcriptional regulator AmtR responses to changes in nitrogen levels, thus at least 35 genes involved in nitrogen utilization and metabolism needed to be regulated[27]. In order to increase the concentration of NH_4^+, the AmtR-coding gene *amtR* was deleted to give the target strain *C. glutamicum* XQ-5-Δ*amtR* (i.e., XQ-5-2) (Fig. 4a). Interestingly, the $q_{Lys,max}$ of strain XQ-5-2 at 250 mM NH_4^+ (i.e., 0.19 ± 0.02 g/(g·h)) was similar as the strain XQ-5 at 300 mM NH_4^+ (i.e., 0.20 ± 0.01 g/(g·h)). Moreover, the $q_{Lys,max}$ of strain XQ-5-2 was almost the same at 300 mM (i.e.,0.22 ± 0.03 g/(g·h)) and 350 mM NH_4^+ (i.e., 0.23 ± 0.04 g/(g·h)) (Fig. 4b). In addition, the highest L-lysine production (i.e., 52.3 ± 4.31 g/L) of strain XQ-5-2 was obtained at 300 mM NH_4^+, which was 8.5% higher than that of strain XQ-5 (i.e., 48.2 ± 3.54 g/L) (Fig. 4d). Previous results also indicated that deletion of *amtR* gene increased the yield of L-lysine[28]. These results indicated that the alleviation of nitrogen restriction increased the intracellular NH_4^+ concentration.

Subsequently, the plasmid pEC-XK99E-*ddh* was introduced into strain XQ-5-2 to give the target strain XQ-5-2/pEC-*ddh* (i.e., strain XQ-5-3). As expected, the growth of strain XQ-5-2 was inhibited at high NH_4^+ concentration (Fig. 4c). It should be noted that the L-lysine yield of strain XQ-5-3 was 53.8 ± 3.98 g/L, which is similar to that of strain XQ-5-2 (i.e., 52.3 ± 4.31 g/L) (Fig. 4d). These results indicated that the overexpression of *ddh* gene did not significantly increase the L-lysine production in strain XQ-5-2, possibly due to the fact that the dehydrogenase pathway is still not the dominant pathway for L-lysine production in strain XQ-5-3. According to Fig. 1, the dehydrogenase pathway is a reversible reaction. In addition, previous reports pointed out that DapDH is highly specific to *meso*-DAP, while *L*,*L*-DAP and *D*,*D*-DAP are competitive inhibitors[29]. Theoretically, *L*,*L*-DAP, an intermediate in the succinylase pathway, inhibits the reverse reaction of DapDH[29]. Thus, the succinylase pathway was upregulated to supply *L*,*L*-DAP during the overexpression of *ddh*. In order to confirm this conjecture, the transcription levels of *ddh* and *dapD* were measured in strain XQ-5-3. The results showed that the transcription level of *ddh* and *dapD* increased with the increase of NH_4^+ concentration within a certain range (Fig. 4e). The transcription level of *ddh* was 82% higher at high NH_4^+ concentration (i.e., 500 mM) than that at low NH_4^+ concentration (i.e., 50 mM). Similarly, the transcription level of *dapD* was 92% higher at high NH_4^+ concentration (i.e., 500 mM) than that at low NH_4^+ concentration (i.e., 50 mM). Moreover, it is reported that the expression level of *dapD* gene increases after the deletion of *amtR* gene[28]. These results indicated that the upregulated *dapD* expression level comes with the upregulated *ddh* expression level. However, DapD (tetrahydrodipicolinate succinylase, E.C. 2.3.1.117) has a high affinity with the substrate THDPA[15], making the succinylase pathway the main pathway for biosynthesizing L-lysine rather than the dehydrogenase pathway in spite of the overexpression of *ddh*.

Blocking the succinylase pathway to upregulate the dehydrogenase pathway

Based on the above results, increasing the expression of *ddh* gene in the dehydrogenase pathway showed no significant effect on L-lysine production, possibly due to the fact that the expression level of *dapD* is increased with the overexpression of *ddh*. In order to upregulate the dehydrogenase pathway, the succinylase pathway was blocked. The succinylase pathway involves enzymes such as DapC (succinyl-amino-ketopimelate-coding gene *dapC*), DapE (*N*-succinyl-diaminopimelate desuccinylase-coding gene *dapE*), and DapF (diaminopimelate epimerase-coding gene *dapF)*, respectively[30–32]. Previous research indicated that the genes *dapC* and *dapE* are dispensable for L-lysine overproduction in shake-flask cultures[33, 34], whereas the genes *dapF* and *dapD* are indispensable for the succinylase pathway[15, 33]. DapD is the first key enzyme in the succinylase pathway, thus

DapD was inactivated to block the succinylase pathway, hence the DapD-deficient strain XQ-5-Δ*dapD* (i.e., strain XQ-5-4). As a control, the DapDH-deficient strain XQ-5-Δ*ddh* (i.e., strain XQ-5-5) was also constructed. The cell morphology of the three strains (i.e., a:strain XQ-5, b:strain XQ-5-4, c:strain XQ-5-5) was observed with FESEM, and the results indicated almost no change in cell morphology compared to the original strain (Fig. 5a, b, c). It is well-known that the *meso*-DAP connects the glycan backbone on the cell wall of many bacteria to give them shape and rigid structure[8]. These results showed that the *meso*-DAP synthesized by either of these two pathways had met the needs of cell structure.

In order to investigate the L-lysine production of different strains under the different NH_4^+ concentrations, three NH_4^+ solutions of different concentrations (i.e., 250 mM, 300 mM and 350 mM) were used in the test. According to Fig. 5d, L-lysine production of XQ-5-4 decreased significantly, especially at low NH_4^+ concentration (i.e., 17.6 ± 5.11 g/L), indicating that *dapD* gene is essential for the succinylase pathway and L-lysine production. By contrast, the L-lysine production of strain XQ-5-5 (i.e., 39.3 ± 4.11 g/L) decreased slightly. These results have once again proven that the succinylase pathway is the main pathway for L-lysine production rather than the dehydrogenase pathway[22]. It should be noted that the L-lysine production of strain XQ-5-4 increased at high NH_4^+ concentration (Fig. 5d), possibly due to the fact that the one-step dehydrogenase pathway compensated the L-lysine production at high NH_4^+ concentration when the succinylase pathway was downregulated[34]. Among the recombinant strains, strain XQ-5-Δ*dapD*Δ*amtR*/pEC-*ddh* (i.e., strain XQ-5-8) accumulated the highest L-lysine production (i.e., 41.9 ± 4.57 g/L) if not counting the strains with succinylase pathway (Fig. 5d). It is worth noting that the overexpression of *ddh* gene in strain XQ-5-6 increased the L-lysine production (300 mM) from 29.3 ± 3.49 g/L (strain XQ-5-6) to 41.9 ± 4.57 g/L (strain XQ-5-8). Interestingly, although the L-lysine yield of strain XQ-5-8 (i.e., 41.9 ± 4.57 g/L) was lower than that of strain XQ-5-3 (i.e., 53.8 ± 3.98 g/L), the $q_{Lys,max}$. of strain XQ-5-8 (i.e., 0.30 ± 0.04 g/(g·h)) was 20% higher than that of strain XQ-5-3 (i.e., 0.25 ± 0.03 g/(g·h)) (Fig. 5e), possibly due to the fact that the L-lysine precursor (i.e., *meso*-DAP) was biosynthesized in one step rather than four steps[35] since the dehydrogenase pathway is the only pathway for L-lysine production in strain XQ-5-8. These results also showed that the dehydrogenase pathway has potential to increase L-lysine production. Taken together, these results indicated that blocking the succinylase pathway is beneficial to upregulating the dehydrogenase pathway, thus improving the $q_{Lys,max}$.. However, the L-lysine production in strains with blocked succinylase pathway decreased. Two reasons were speculated: (1) More NH_4^+ were required to produce L-lysine in the dehydrogenase pathway. Previous researches indicated that *C. glutamicum* strain with the dehydrogenase pathway alone cannot produce L-lysine at low NH_4^+ concentration[36] due to the low affinity of dehydrogenase pathway to NH_4^+in the substrate[16]; (2) The dehydrogenase pathway is a reversible process. *L*,*L*-DAP cannot be synthesized while blocking the succinylase pathway, thus relieving the competitive inhibition of the reverse reaction[29]. These speculated reasons have been validated by examining the concentration of the by-products (Table 1). As might be expected, strain XQ-5-8 had the highest by-product concentration at high NH_4^+ concentration (Table 1). All of the above mentioned results indicated that blocking the succinylase pathway is beneficial to improving the production intensify of L-lysine, but unhelpful for increasing the yield of L-lysine.

Table 1
By-products of *C. glutamicum* strains XQ-5-1, XQ-5-3 and XQ-5-8

	XQ-5-1 (XQ-5/pEC-ddh)			XQ-5-3 (XQ-5-ΔamtR/pEC-ddh)			XQ-5-8 (XQ-5-ΔamtRΔdapD/pEC-ddh)		
by-products	200mM	300mM	400mM	200mM	300mM	400mM	200mM	300mM	400mM
glutamate	0.26 ± 0.05	0.35 ± 0.11	0.54 ± 0.02	0.49 ± 0.06	0.97 ± 0.11	1.23 ± 0.02	1.07 ± 0.04	1.75 ± 0.15	2.01 ± 0.02
pyruvate	1.94 ± 0.04	2.08 ± 0.14	2.36 ± 0.02	2.21 ± 0.04	2.65 ± 0.03	3.32 ± 0.04	3.01 ± 0.12	3.43 ± 0.09	3.91 ± 0.12
isoleucine	1.06 ± 0.16	1.33 ± 0.11	1.35 ± 0.12	1.57 ± 0.02	2.03 ± 0.03	2.37 ± 0.02	2.31 ± 0.04	2.93 ± 0.11	3.51 ± 0.02
aspartate	2.06 ± 0.06	2.87 ± 0.05	2.92 ± 0.07	2.98 ± 0.08	3.52 ± 0.12	3.91 ± 0.12	3.88 ± 0.16	4.01 ± 0.11	4.22 ± 0.12
methionine	1.12 ± 0.11	1.33 ± 0.07	1.41 ± 0.15	1.22 ± 0.02	1.54 ± 0.11	1.87 ± 0.08	1.46 ± 0.02	1.98 ± 0.05	2.31 ± 0.06
threonine	0.98 ± 0.13	1.15 ± 0.14	1.23 ± 0.02	1.06 ± 0.16	2.01 ± 0.11	3.91 ± 0.12	2.17 ± 0.13	3.23 ± 0.11	3.91 ± 0.12

*All data represent values of three determinations of triplicate independent experiments.

Weakening the dapD gene makes the metabolic flux of the two pathways reach the best balance

As mentioned above, L-lysine production decreased significantly while blocking the succinylase pathway (Fig. 5d) and the amount of by-products increased because of the lack of *L*,*L*-DAP (Table 1). To address this problem, the key enzyme DapD in the succinylase pathway was weakened to balance the flux between the succinylase pathway and the dehydrogenase pathway.

There are many ways to weaken genes, including the ones from the level of transcription or translation[37]. At the translation level, weakening the gene is often achieved by replacing RBS to change the gene expression level[38]. In this experiment, terminators were inserted in front of the gene to weaken its expression as the terminators are essential elements controlling the normal transcription of genes[37]. Six terminators with different intensities were selected (Fig. 6a) and inserted in front of the *dapD* gene by secondary homologous recombination using pK18*mobSacB*[39]. The sequences of the six terminators are listed in Supplementary material (Table S1). Six recombinant bacterias with different weakening degrees were derived from strain XQ-5-3, i.e., XQ-5-W1, XQ-5-W2, XQ-5-W3, XQ-5-W4, XQ-5-W5, and XQ-5-W6. The L-lysine production of strain XQ-5-W1 (i.e., 48.7 ± 4.12 g/L), XQ-5-W2 (i.e., 50.2 ± 5.31 g/L), XQ-5-W5 (i.e., 48.3 ± 5.87 g/L), and XQ-5-W6 (i.e., 41.1 ± 6.44 g/L) were lower than that of the strain XQ-5-3 (i.e., 53.8 ± 3.98 g/L). Conversely, the L-lysine production of strain XQ-5-W3 (i.e., 54.2 ± 4.76 g/L) and XQ-5-W4 (i.e., 58.5 ± 5.43 g/L) were higher than that of strain XQ-5-3 (i.e., 53.8 ± 3.98 g/L), especially strain XQ-5-W4 (Fig. 6b). These results indicated that the introduction of terminators in front of the *dapD* gene could change the translation level of *dapD*, thus affecting the flux in the succinylase pathway. The similar results were also found in previous reports[40]. The highest L-lysine yield was found in strain XQ-5-W4 (i.e., 58.5 ± 5.43 g/L), which was 21.4% higher than that of the original strain XQ-5 (i.e., 48.2 ± 3.54 g/L). In addition, the $q_{Lys,max}$ of strain XQ-5-W4 (i.e., 0.31 ± 0.04 g/(g·h)) and strain XQ-5-8 (i.e., 0.30 ± 0.04 g/(g·h)) were similar, about 55 % higher than that of strain XQ-5 (i.e., 0.20 ± 0.01 g/(g·h)) (Fig. 6c). As expected, the activity of DapD decreased with the increase of terminator strength (i.e., 5.8 ± 0.13 mU/mg-1.3 ± 0.42 mU/mg) (Table 2). In addition, the forward reaction of DapDH increased

with the weakening of the succinylase pathway and the reverse reaction was also enhanced (Table 2). It is worth noting that the best balance in strain XQ-5-W4 (i.e., XQ-5-*dapD*^{W}Δ*amtR*/pEC-*ddh*) resulted in the best L-lysine yield (i.e., 58.5 ± 5.43 g/L) and $q_{Lys,max}$ (i.e., 0.31 ± 0.04 g/(g·h)). At the same time, the by-products of the six strains were measured. In comparison, strain XQ-5-W4 has fewer by-products. (Table 3).

Table 2
The activity DapDH and DapD different recombinant *C. glutamicum* strains

strains	specific activity (mU/mg of protein)		
	DapD	DapDH(F-reaction)	DapDH (R-reaction)
XQ-5-W1	5.8 ± 0.13	201 ± 13.6	130 ± 13.8
XQ-5-W2	5.3 ± 0.21	227 ± 22.1	138 ± 18.1
XQ-5-W3	4.5 ± 0.39	241 ± 26.8	145 ± 17.4
XQ-5-W4	3.3 ± 0.26	260 ± 18.4	151 ± 21.2
XQ-5-W5	2.1 ± 0.22	268 ± 19.3	177 ± 19.3
XQ-5-W6	1.3 ± 0.42	275 ± 20.2	194 ± 18.9
*All data represent values of three determinations of triplicate independent experiments.			

Table 3
By products of *C. glutamicum* strains XQ-5-W1, XQ-5-W2, XQ-5-W3, XQ-5-W4, XQ-5-W5 and XQ-5-W6

strains	by-products (g/L)					
	glutamate	pyruvate	isoleucine	aspartate	methionine	threonine
XQ-5-W1	0.4 ± 0.03	2.11 ± 0.14	1.5 ± 0.09	2.93 ± 0.18	1.54 ± 0.04	1.26 ± 0.08
XQ-5-W2	0.54 ± 0.04	2.23 ± 0.13	1.64 ± 0.07	2.85 ± 0.12	1.25 ± 0.02	1.75 ± 0.11
XQ-5-W3	0.64 ± 0.12	2.41 ± 0.10	1.32 ± 0.05	2.42 ± 0.12	1.05 ± 0.06	1.35 ± 0.02
XQ-5-W4	0.78 ± 0.04	2.32 ± 0.13	1.24 ± 0.09	2.15 ± 0.11	0.85 ± 0.12	1.65 ± 0.08
XQ-5-W5	1.32 ± 0.07	2.87 ± 0.11	2.01 ± 0.12	2.78 ± 0.05	1.05 ± 0.10	2.75 ± 0.02
XQ-5-W6	1.64 ± 0.12	3.12 ± 0.03	2.64 ± 0.14	3.65 ± 0.02	1.75 ± 0.04	3.01 ± 0.12
*All data represent values of three determinations of triplicate independent experiments.						

Fed-batch fermentation of C. glutamicum **XQ-5-W4**

The production performance of strain XQ-5-W4 was investigated in a fed-batch process. As a comparison, fed-batch fermentation of the original strain XQ-5 was also conducted. Figure 7 shows the time profiles of fed-batch fermentations in a 5-L jar fermenter. Fed-batch fermentation of XQ-5-W4 resulted in 189 ± 8.7 g/L of L-lysine with a $q_{Lys,max}$ of 0.35 ± 0.05 g/(g·h) (Fig. 7b, c). However, fed-batch fermentation of XQ-5 resulted in 151 ± 9.3 g/L of L-lysine with a $q_{Lys,max}$ of 0.22 ± 0.02 g/(g·h) (Fig. 7a, c). Consistent with the results of production intensity of L-lysine in shake flasks, the $q_{Lys,max}$ of strain XQ-5-W4 was higher than that of strain XQ-5 (0.35 ± 0.05 g/(g·h) vs. 0.22 ± 0.02 g/(g·h)) (Fig. 7c). In addition, the L-lysine yield of strain XQ-5-W4 stabilized faster, about six hours earlier than that of strain XQ-5. However, the dry weight of strain XQ-5-W4 was 38.9 ± 5.12 g/L, 13.9% lower than that of the original strain (i.e., 45.2 ± 7.64 g/L) (Fig. 7a,b). Previous research also found the similar result, in which the biomass

decreased while enhancing the yield of the target products[23]. Taken together, these results demonstrated that the final strain XQ-5-W4 shows an efficient L-lysine production under fed-batch fermentation, making it a very promising platform for L-lysine production.

Conclusion

For the first time, DAP pathway was reconstructed to optimize L-lysine production in *C. glutamicum*, which demonstrated that the dehydrogenase pathway is promising for promoting L-lysine production. In *C. glutamicum*, both the dehydrogenase pathway and the succinylase pathway are involved in the production of L-lysine, but the relative proportion of each pathway on L-lysine biosynthesis is different because of the different demand for NH_4^+ concentration[16]. The proportion of the dehydrogenase pathway on L-lysine production increased when increasing the NH_4^+ concentration (Fig. 2d). Since the L-lysine biosynthesis in the dehydrogenase pathway has less steps, the strain showed the highest $q_{Lys,max}$ at high NH_4^+ concentration (Fig. 2c). The similar results were also found in the AmtR-deficient strain (Fig. 4b), as NH_4^+ was efficiently transferred into the cell during inactivation of AmtR[25]. The L-lysine yield and $q_{Lys,max}$ in strain XQ-5-2 reached 52.3 ± 4.31 g/L and 0.22 ± 0.03 g/(g·h), which were 8.5% and 10% higher than that of the original strain XQ-5, respectively. Although the dehydrogenase pathway is promising in promoting L-lysine production, redirecting the flux into the dehydrogenase pathway while blocking the succinylase pathway is counterproductive to L-lysine production (Fig. 5d). Fortunately, this problem can be overcome by weakening the succinylase pathway (Fig. 6b). The target strain *C. glutamicum* XQ-5-W4 produced 58.5 ± 5.43 g/L L-lysine with a $q_{Lys,max}$. (i.e., 0.31 ± 0.04 g/(g·h)) in shake-flask fermentation, which were 21.4% and 55% higher than that of strain XQ-5. In addition, fed-batch fermentation of strain XQ-5-W4 resulted in 189 ± 8.7 g/L of L-lysine with a $q_{Lys,max}$ of 0.35 ± 0.05 g/(g·h). These results indicated that the reconstruction of DAP pathway to switch the flux in the variants of DAP pathway has great potential to promote L-lysine production in *C. glutamicum*.

Abbreviations

meso-DAP: *meso*-diaminopimelic acid; DapD: Tetrahydrodipicolinate *N*-succinyltransferase; DapDH: diaminopimelate dehydrogenase; DAP: diaminopimelic acid.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors give consent to publish the research in Microbial Cell Factories.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and the additional file. The authors are willing to provide any additional data and materials related to this research that may be requested for research purposes.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

J.X. and Z.R. conceived the experiments. N.L., T.Z. and W.Z. designed and performed the experiments and analyzed the data. N.L. and J.X. wrote the paper. All authors read and approved the final manuscript.

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References

1. Félix F, Letti L, Vinícius de Melo Pereira G, Bonfim P, Soccol V, Soccol C. L-lysine production improvement: a review of the state of the art and patent landscape focusing on strain development and fermentation technologies. Crit Rev Biotechnol. 2019;39(8):1031–55.
2. Leuchtenberger W, Huthmacher K, Drauz K. Biotechnological production of amino acids and derivatives: current status and prospects. Appl Microbiol Biotechnol. 2005;69(1):1–8.
3. Nishida H, Nishiyama M, Kobashi N, Kosuge T, Hoshino T, Yamane H. A prokaryotic gene cluster involved in synthesis of lysine through the amino adipate pathway: a key to the evolution of amino acid biosynthesis. Genome Res. 1999;9(12):1175–83.
4. Velasco AM, Leguina JI, Lazcano A. Molecular Evolution of the Lysine Biosynthetic Pathways. J Mol Evol. 2002;55(4):445–9.
5. Jetten M, Sinskey A. Recent advances in the physiology and genetics of amino acid-producing bacteria. Crit Rev Biotechnol. 1995;15(1):73–103.
6. Scapin G, Blanchard JS. Enzymology of Bacterial Lysine Biosynthesis. Adv Enzymol Relat Areas Mol Biol. 1998;72(1):279–324.
7. Liu Y, White RH, Whitman WB. *Methanococci* Use the Diaminopimelate Aminotransferase (DapL) Pathway for Lysine Biosynthesis. J Bacteriol. 2010;192(13):3304–10.
8. Born TL, Blanchard JS. Structure/function studies on enzymes in the diaminopimelate pathway of bacterial cell wall biosynthesis. Curr Opin Chem Biol. 1999;3(5):607–13.
9. Cirilli M, Zheng RJ, Scapin G, Blanchard JS. Structural symmetry: the three-dimensional structure of *Haemophilus* influenzae diaminopimelate epimerase. Biochemistry. 1998;37(47):16452–8.
10. White PJ. The Essential Role of Diaminopimelate Dehydrogenase in the Biosynthesis of Lysine by Bacillus sphaericus. J Gen Microbiol. 1983;129(3):739–49.
11. Wenko LK, Treick RW, Wilson KG. Isolation and characterization of a gene encoding *meso*-diaminopimelate dehydrogenase from Glycine max. Plant Mol Biol. 1985;4(4):197–204.

12. Mccoy AJ, Adams NE, Hudson AO, Gilvarg C, Maurelli AT. *L,L*-diaminopimelate aminotransferase, a trans-kingdom enzyme shared by Chlamydia and plants for synthesis of diaminopimelate/lysine. Proc Natl Acad Sci U S A. 2018;103(47):17909–14.
13. Dobson RCJ, Irma G, Fernando OHA. RL. *L,L*-Diaminopimelate Aminotransferase from *Chlamydomonas reinhardtii*: A Target for Algaecide Development. PLoS One. 2011;6(5):e20439.
14. Watanabe N, Cherney MM, Belkum MJV, Marcus SL, Flegel MD, Clay MD, et al. Crystal structure of *L,L*-diaminopimelate aminotransferase from Arabidopsis thaliana: a recently discovered enzyme in the biosynthesis of L-lysine by plants and Chlamydia. J Mol Biol. 2007;371(3):685–702.
15. Wehrmann A, Phillipp B, Sahm H, Eggeling L. Different modes of diaminopimelate synthesis and their role in cell wall integrity: a study with *Corynebacterium glutamicum*. J Bacteriol. 1998;180(12):3159–65.
16. Sahm H, Eggeling L, de Graaf A. Pathway analysis and metabolic engineering in *Corynebacterium glutamicum*. Biol Chem. 2000;381:899–910.
17. UDAKA S. Screening method for microorganisms accumulating metabolites and its use in the isolation of *Micrococcus glutamicus*. J Bacteriol. 1960;79:754–5.
18. Burillo S, Luque I, Fuentes I, Contreras A. Interactions between the nitrogen signal transduction protein PII and *N*-acetyl glutamate kinase in organisms that perform oxygenic photosynthesis. J Bacteriol. 2004;186(11):3346–54.
19. Bott M. Offering surprises: TCA cycle regulation in *Corynebacterium glutamicum*. Trends Microbiol. 2007;15(9):417–25.
20. Misono H, Togawa H, Yamamoto T, Soda K. Occurrence of *meso*-alpha, epsilon-diaminopimelate dehydrogenase in *Bacillus sphaericus*. Biochem Biophys Res Commun. 1976;72(1):89–93.
21. Rehm N, Burkovski A. Engineering of nitrogen metabolism and its regulation in *Corynebacterium glutamicum*: influence on amino acid pools and production. Appl Microbiol Biotechnol. 2011;89(2):239–48.
22. Sonntag K, Eggeling L, Graaf AA, Sahm H. Flux partitioning in the split pathway of lysine synthesis in *Corynebacterium glutamicum*. Quantification by 13C- and 1H-NMR spectroscopy. Eur J Biochem. 2010;213(3):1325–31.
23. Xu M, Li J, Shu Q, Tang M, Rao Z. Enhancement of l-arginine production by increasing ammonium uptake in an AmtR-deficient *Corynebacterium crenatum* mutant. J Ind Microbiol Biotechnol. 2019;46(8):1155–66.
24. Yeh P, Sicard A, Sinskey A. General organization of the genes specifically involved in the diaminopimelate-lysine biosynthetic pathway of *Corynebacterium glutamicum*. MolGenGenet. 1988;212(1):105–11.
25. Carles P, Vicente R. Structure of AmtR, the global nitrogen regulator of *Corynebacterium glutamicum*, in free and DNA-bound forms. FEBS J. 2016;283:1039–59.
26. Rehm N, Georgi T, Hiery E, Degner U, Schmiedl A, Burkovski A, et al. L-Glutamine as a nitrogen source for *Corynebacterium glutamicum*: derepression of the AmtR regulon and implications for nitrogen sensing. Microbiology. 2010;156(10):3180–93.
27. Marc J, Reinhard K, Andreas B. Nitrogen regulation in *Corynebacterium glutamicum*: isolation of genes involved and biochemical characterization of corresponding proteins. FEMS Microbiol Lett. 1999;173(2):303–10.
28. Buchinger S, StröSser J, Rehm N, Hänßler E, Hans S, Bathe B, et al. A combination of metabolome and transcriptome analyses reveals new targets of the *Corynebacterium glutamicum* nitrogen regulator AmtR. J Biotechnol. 2009;140(1–2):68–74.

29. Scapin G, Cirilli M, Reddy SG, Gao Y, Vederas JC, Blanchard JS. Substrate and Inhibitor Binding Sites in *Corynebacterium glutamicum* Diaminopimelate Dehydrogenase. Biochemistry. 1998;37:3278–85.
30. Debodyuti D, Sabyashachi M. The structural and energetic aspects of substrate binding and the mechanism of action of the DapE-encoded *N*-succinyl-*L,L*-diaminopimelic acid desuccinylase (DapE) investigated using a hybrid QM/MM method. Phys Chem Chem Phys. 2014;16:26348–58.
31. Sagong HY, Kim KJ. Structural basis for redox sensitivity in *Corynebacterium glutamicum* diaminopimelate epimerase: an enzyme involved in L-lysine biosynthesis. Rep. 2017;7:42318.
32. Sagong H, Kim K. Crystal Structure and Biochemical Characterization of Tetrahydrodipicolinate *N*-Succinyltransferase from *Corynebacterium glutamicum*. J Agric Food Chem. 2015;63(49):10641–6.
33. Hartmann M, Tauch A, Eggeling L, Bathe B, Möckel B, Pühler A, et al. Identification and characterization of the last two unknown genes, *dapC* and *dapF*, in the succinylase branch of the L-lysine biosynthesis of *Corynebacterium glutamicum*. J Biotechnol. 2003;104:199–211.
34. Shaw-Reid C, McCormick M, Sinskey A, Stephanopoulos G. Flux through the tetrahydrodipicolinate succinylase pathway is dispensable for L-lysine production in *Corynebacterium glutamicum*. Appl Microbiol Biotechnol. 1999;51(3):325–33.
35. Eggeling L, Bott M. A giant market and a powerful metabolism: L-lysine provided by *Corynebacterium glutamicum*. Appl Microbiol Biotechnol. 2015;99(8):3387–94.
36. Misono H, Soda K. Purification and Properties of *meso*-α, ε-Diaminopimelate D-Dehydrogenase from *Bacillus sphaericus*. Agric Biol Chem. 1980;44(1):227–9.
37. He Z, Duan Y, Zhai W, Zhang X, Shi J, Zhang X, et al. Evaluating Terminator Strength Based on Differentiating Effects on Transcription and Translation. ChemBioChem. 2020;21:2067–72.
38. Man Z, Xu M, Rao Z, Guo J, Yang T, Zhang X, et al. Systems pathway engineering of *Corynebacterium crenatum* for improved L-arginine production. Sci Rep. 2016;6:28629.
39. Chen YJ, Liu P, Nielsen AAK, Brophy JAN, Clancy K, Peterson T, et al. Characterization of 582 natural and synthetic terminators and quantification of their design constraints. Nat Methods. 2013;10(7):659–64.
40. Zhang B, Yu M, Zhou Y, Ye BC. Improvement of L-ornithine production by attenuation of *argF* in engineered *Corynebacterium glutamicum* S9114. Amb Express. 2018;8(1):26.
41. Wang L, Yu H, Xu J, Ruan H, Zhang W. Deciphering the crucial roles of AraC-type transcriptional regulator Cgl2680 on NADPH metabolism and L-lysine production in *Corynebacterium glutamicum*. World J Microbiol Biotechnol. 2020;36(6):82.
42. Xu J, Han M, Ren X, Zhang W. Modification of aspartokinase III and dihydrodipicolinate synthetase increases the production of L-lysine in *Escherichia coli*. Biochem Eng J. 2016;114:79–86.
43. Rest MEVD, Lange C, Molenaar D. A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Appl Microbiol Biotechnol. 1999;52(4):541–5.
44. Xu J, Wu Z, Gao S, Zhang W. Rational modification of tricarboxylic acid cycle for improving L-lysine production in *Corynebacterium glutamicum*. Microb Cell Fact. 2018;17(1):105.
45. Wang YY, Zhang F, Xu JZ, Zhang WG, Chen XL, Liu LM. Improvement of L-Leucine Production in *Corynebacterium glutamicum* by Altering the Redox Flux. Int J Mol Sci. 2019;20(8).
46. Cremer J, Treptow C, Eggeling L, Sahm H. Regulation of Enzymes of Lysine Biosynthesis in *Corynebacterium glutamicum*. J Gen Microbiol. 1988;134(12):3221–9.

Figures

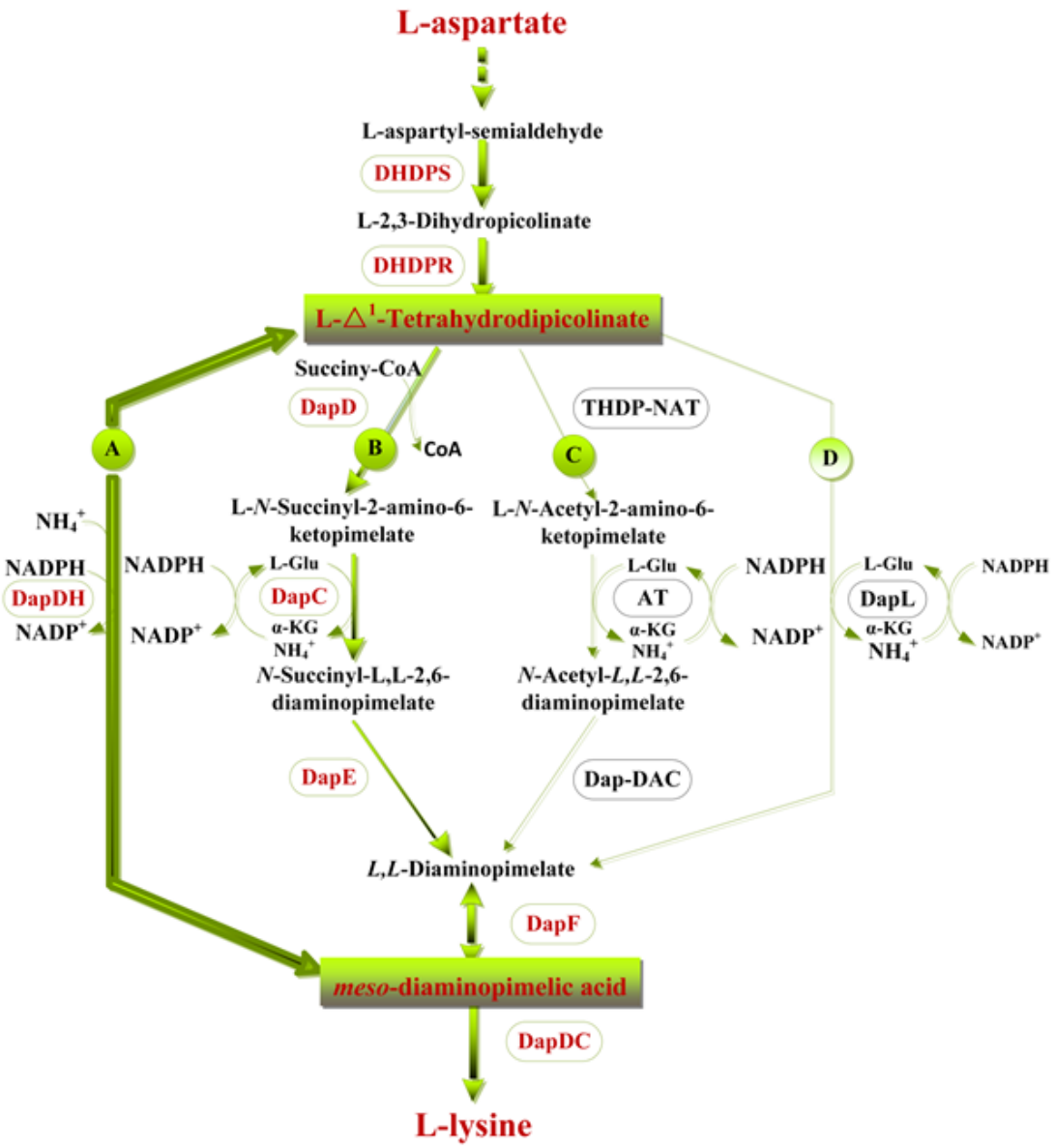

Figure 1

The DAP pathway of L-lysine synthesis. (A) dehydrogenase pathway (B) succinylase pathway, (C) acetylase pathway and. (D) aminotransferase pathway. Abbreviations: DHDPS Dihydrodipicolinate synthetase, DHDPR Dihydrodipicolinate reductase, DapDH Diaminopimelate dehydrogenase, DapD Tetrahydrodipicolinate N-succinyltransferase, DapC Succinyl-amino-ketopimelate transaminase, DapE N-succinyl-diaminopimelate desuccinylase, DapF Diaminopimelate epimerase, THDP-NAT Tetrahydrodipicolinate acetylase, AT N-acetylaminoketopimelate aminotransferase, NAD-DAC N-acetyl-diaminopimelate deacetylase, DapL Tetrahydrodipicolinate aminotransferase.

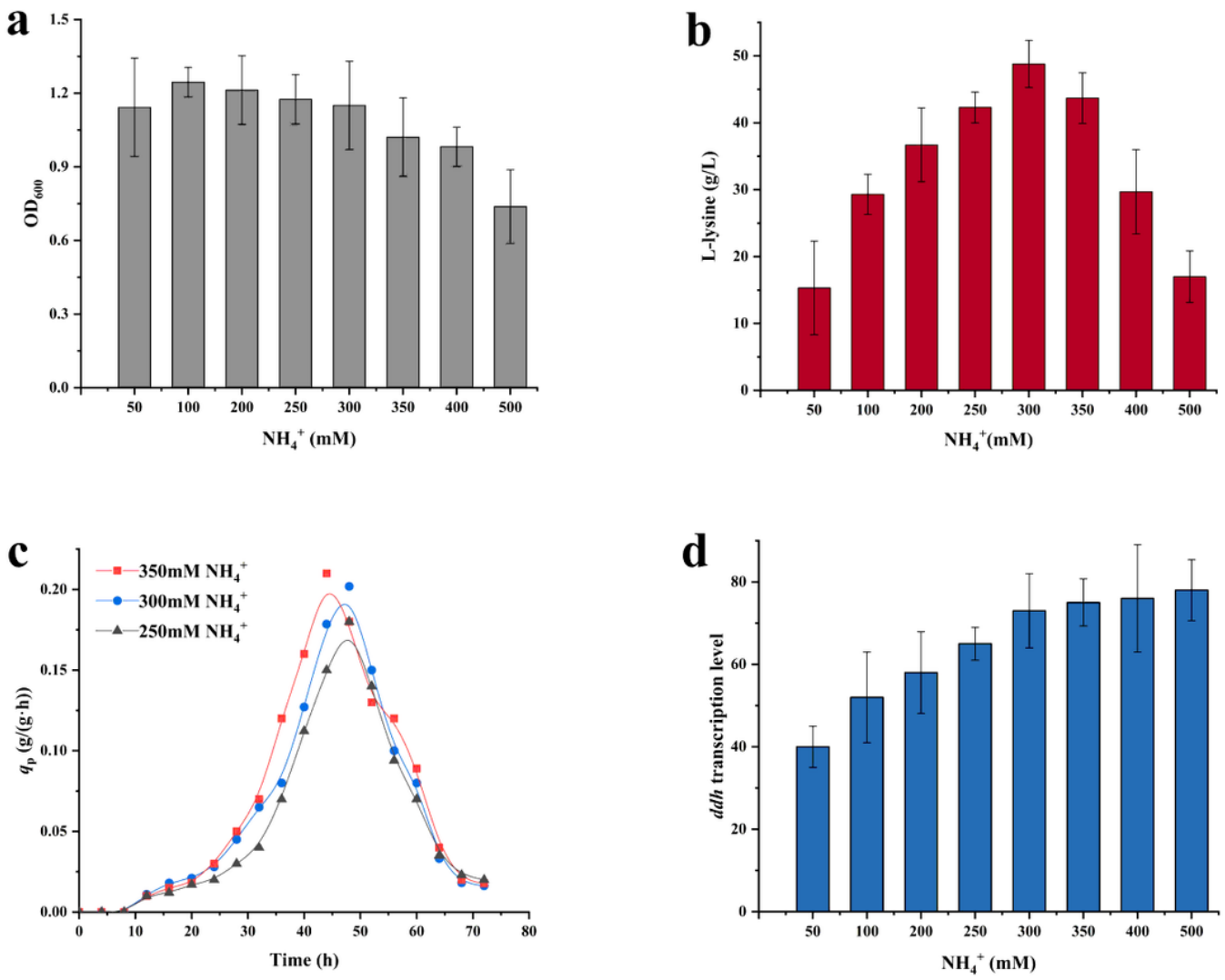

Figure 2

The effects of different ammonium (NH4+) concentrations on L-lysine synthesis. (a) Cell growth (OD600) of strain XQ-5 at different NH4+ concentrations. The cell concentration after 25 fold dilution was measured at OD600. (b) L-lysine production of strain XQ-5 at different NH4+ concentrations. (c) The qLys of strain XQ-5 at different NH4+ concentrations. (d) Transcription level of gene ddh at different NH4+ concentrations. All data represent values of three determinations of triplicate independent experiments.

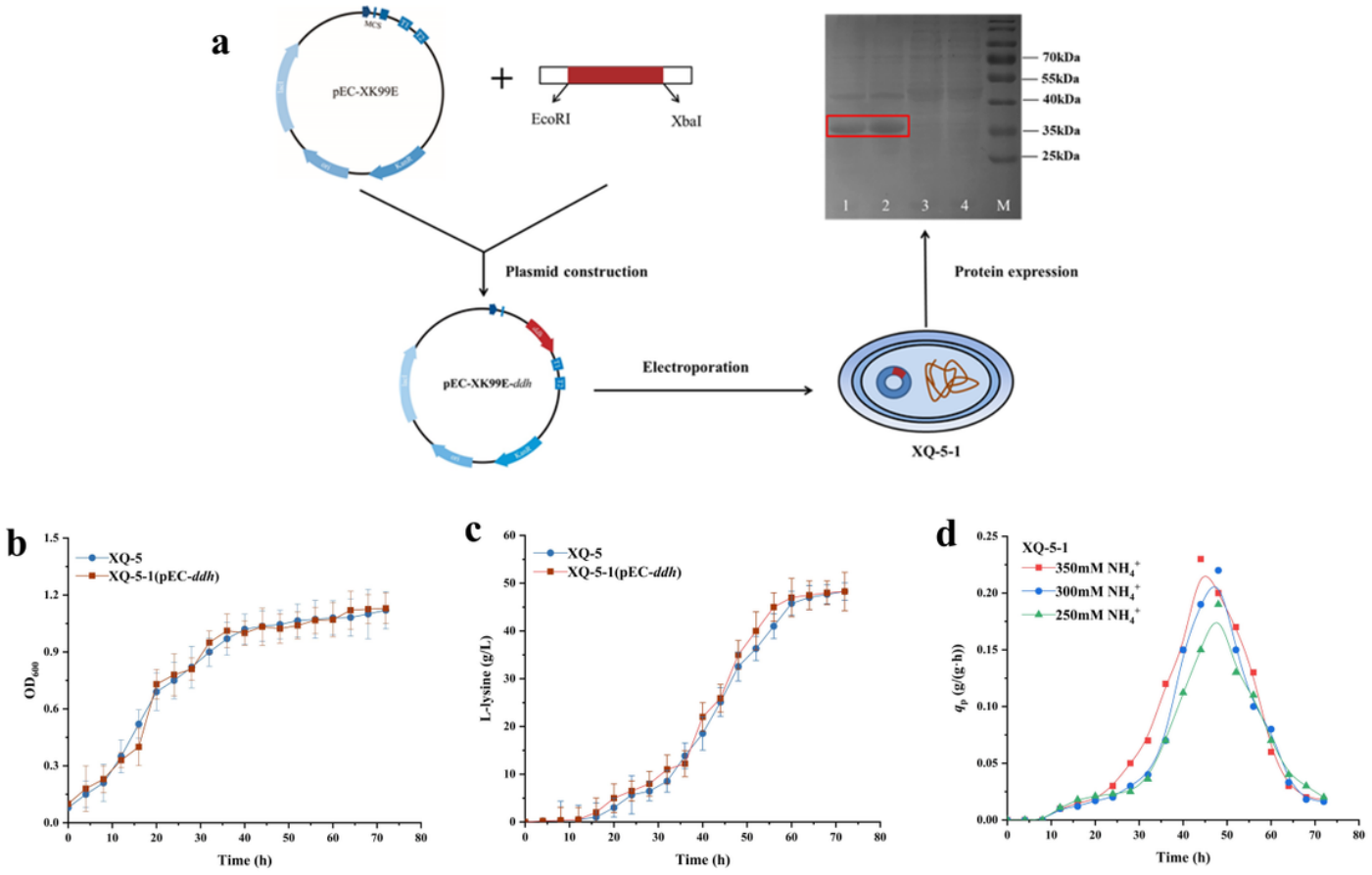

Figure 3

Effect of dehydrogenase pathway on L-lysine synthesis. (a) The construction process of strain XQ-5-1 and SDS-PAGE analysis of DapDH (i.e., DapDH: 35 kDa). Lane M, protein ruler; lane 1,2: crude enzyme extract (i.e., strain XQ-5-1); lane 3,4: crude enzyme extract (i.e., strain XQ-5). (b) Cell growth (OD600) of strain XQ-5 and XQ-5-1 in shake flask culture. The cell concentration after 25 fold dilution was measured at OD600. (c) L-lysine production of strain XQ-5 and XQ-5-1 in shake flask culture. (d) The qLys of strain XQ-5-1 in shake flask culture. The data represent mean values and standard deviations obtained from three independent cultivations.

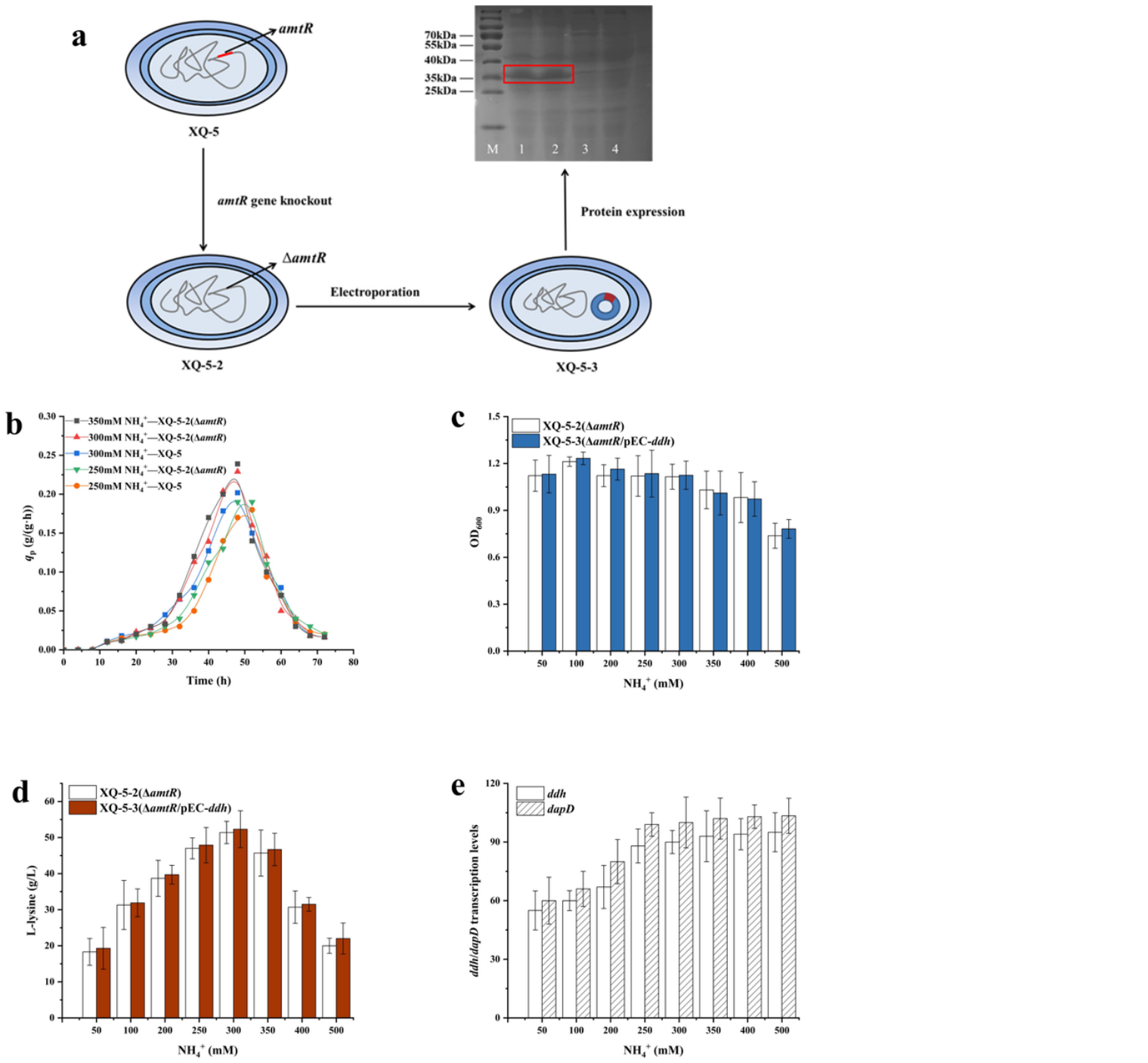

Figure 4

Effect of alleviating nitrogen source restriction on L-lysine synthesis. (a) The construction process of strain XQ-5-3 and SDS-PAGE analysis of DapDH (i.e., 35 kDa). Lane M, protein ruler; lane 1,2: crude enzyme extract (i.e., strain XQ-5-3); lane 3,4: crude enzyme extract (i.e., strain XQ-5). (b) The qLys of strain XQ-5 and strain XQ-5-1 under different NH4+ concentrations. (c) Cell growth (OD600) of strain XQ-5-1 and XQ-5-2 in shake flask culture. The cell concentration after 25 fold dilution was measured at OD600. (d) L-lysine production of strain XQ-5-1 and XQ-5-2 in shake flask culture. (e) Transcription levels of ddh and dapD in strain XQ-5-3. All data represent values of three determinations of triplicate independent experiments.

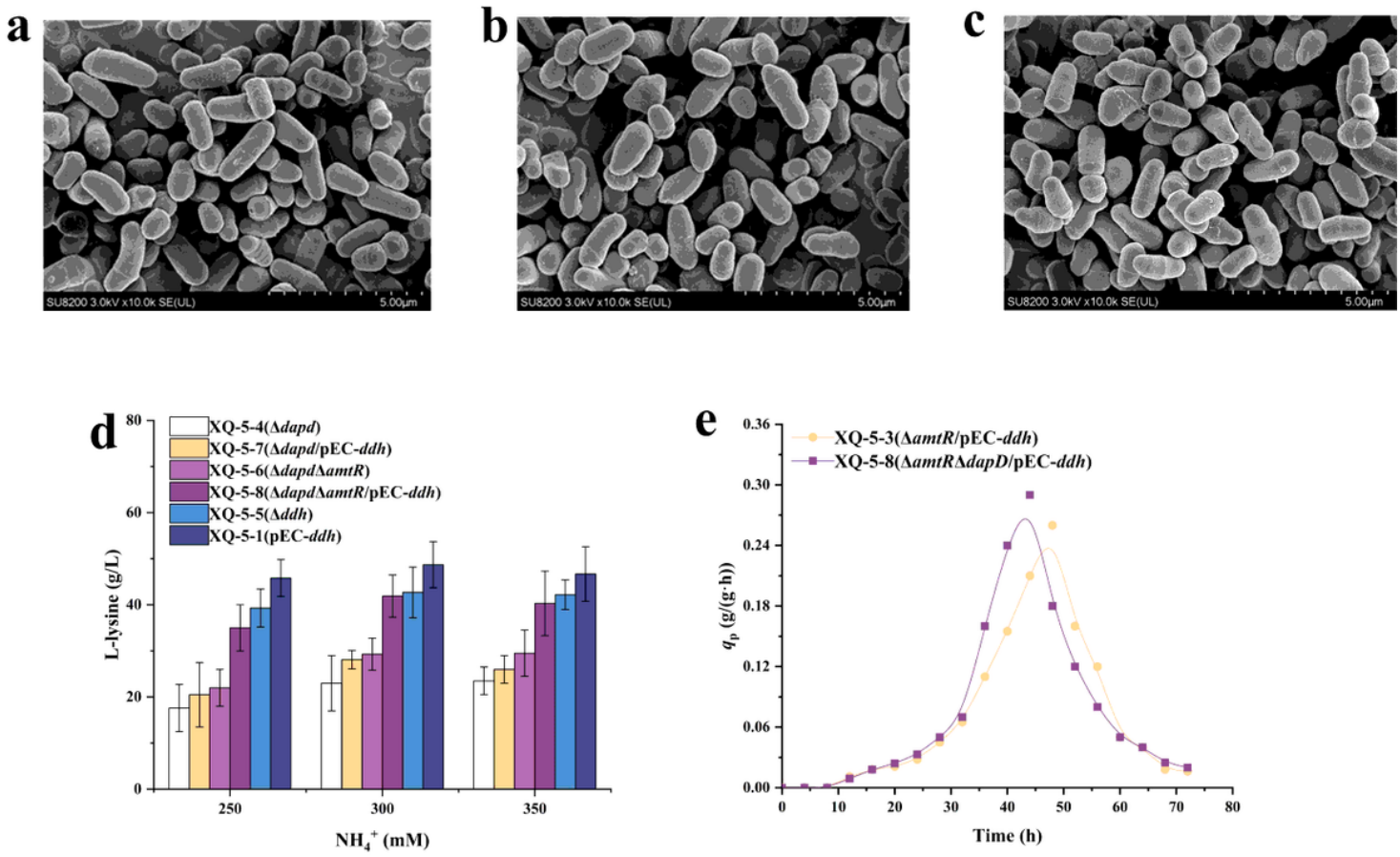

Figure 5

Blocking succinylase pathway to redirection of flux in dehydrogenase pathway. (a) FESEM of strain XQ-5. (b) FESEM of strain XQ-5-4. (c) FESEM of strain XQ-5-5. (d) L-lysine production of strain XQ-5-1, XQ-5-4, XQ-5-5, XQ-5-6, XQ-5-7, XQ-5-8 in shake flask culture. (e) The qLys of strain XQ-5-3 and strain XQ-5-8 at 300 mM NH4+ concentration. All data represent values of three determinations of triplicate independent experiments.

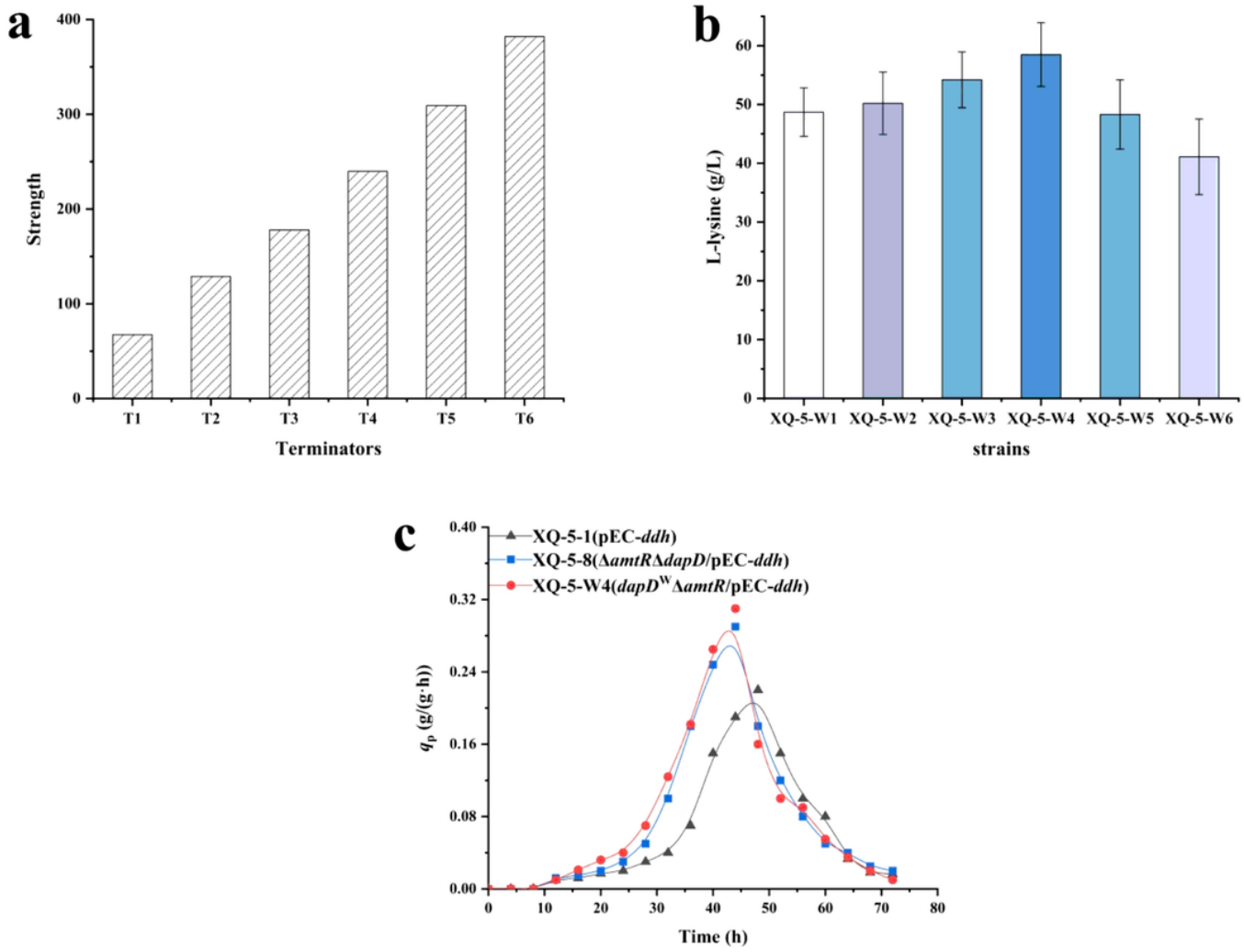

Figure 6

Figure 6

Balancing the flux in the two variants of DAP pathway to promote L-lysine production. (a) Different strengths of six terminators. (b) L-lysine production of strain XQ-5-W1, XQ-5-W2, XQ-5-W3, XQ-5-W4, XQ-5-W5 and XQ-5-W6 in shake flask culture. (c) qLys of strain XQ-5-1, XQ-5-8 and XQ-5-W4 at 300 mM NH4+ concentration. All data represent values of three determinations of triplicate independent experiments.

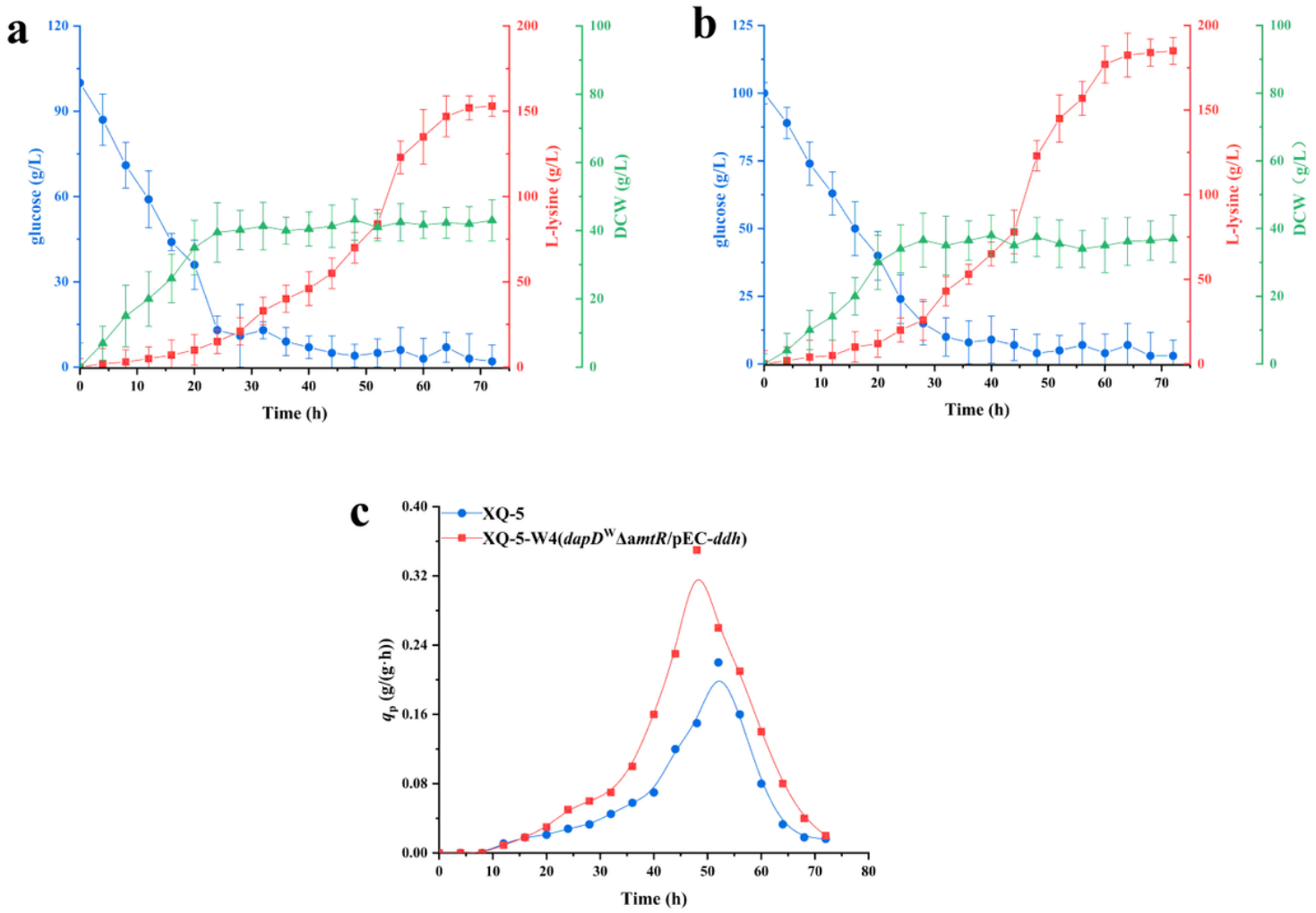

Figure 7

Time course of L-lysine fed-batch fermentations of strains XQ-5 (a) and XQ-5-W4 (b) in 5-L fermentors. The L-lysine production (squares, red), DCW (triangle, green) and glucose (circle, blue) of strains cultivated in 5-L fermentors. (c) qLys of strain XQ-5 and XQ-5-W4 at 300 mM NH4+ concentration in fed-batch fermentations. All data represent values of three determinations of triplicate independent experiments.

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