De novo biosynthesis of α-aminoadipate via multi-strategy metabolic engineering in *Escherichia coli*

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Abstract
As a non-protein amino acid, α-aminoadipate is used in the fields of medicine, chemical engineering, food science, and others. For example, α-aminoadipate is an important precursor for the production of β-lactam antibiotics. Currently, the synthesis of α-aminoadipate depends on chemical catalysis that has the disadvantages of high cost, low yield, and serious pollution. In this study, we construct a biosynthesis pathway of α-aminoadipate in *Escherichia coli* using lysine as a precursor. In addition, we regulate the cell metabolism to improve the titer of α-aminoadipate via multi-strategy metabolic engineering. First, a novel synthetic pathway was constructed to realize de novo synthesis of α-aminoadipate with titers of 82 mg/L. Second, the key enzymes involved in enhancing precursor synthesis were overexpressed and the CO₂ fixation process was introduced, and these led to 80% and 34% increases in the α-aminoadipate concentration, reaching 147 and 110 mg/L, respectively. Third, cofactor regulation was used to maintain the coupling balance of material and energy, with the intracellular α-aminoadipate concentration reaching 140 mg/L. Fourth, the weakening of the synthesis of acetic acid was used to strengthen the synthesis of α-aminoadipate, and this resulted in the enhancement of the α-aminoadipate concentration by 2.2 times, reaching 263 mg/L. Finally, combination optimization was used to promote the production of α-aminoadipate. The titers of α-aminoadipate reached 368 mg/L (strain EcN11#) and 415 mg/L (strain EcN11##), which was 3.5 and 4 times higher than that of the parent strain. With these efforts, 1.54 g/L of α-aminoadipate was produced under fed-batch conditions by strain EcN11#. This study is the first to present the effective biosynthesis of α-aminoadipate in *E. coli* using multi-strategy metabolic engineering.

**KEYWORDS**

*Escherichia coli*, metabolic engineering, multi-strategy regulation, α-aminoadipate
1 | INTRODUCTION

Note, α-aminoadipate is a non-protein amino acid that was first discovered in corn seeds and the urine of humans or guinea pigs. And, α-aminoadipate is the precursor for the synthesis of lysine in fungi. However, in plants and mammals, α-aminoadipate is produced by lysine degradation (K. Zhang et al., 2010). Also α-aminoadipate has several applications. For example, it is used for the treatment of eye diseases because it can specifically act on retinal Mullerian cells. It is also used in applications. For example, it is used for the treatment of eye diseases (Brown & Kretzschma, 1998). Currently, α-aminoadipate is still synthesized using chemical methods that produce low yields, cause serious environmental pollution, and are not cost-effective. Therefore, we hope to realize the green synthesis of α-aminoadipate by constructing microbial cell factories.

The design and construction of new cascade reactions in microorganisms for biosynthesis have been proven to be feasible in many studies, and this process can supply cofactors for product synthesis (M. Wang, Chen, et al., 2017) and serve as microbial cell factories to produce high value-added chemicals (Ko et al., 2020). As a C5 amino acid, α-aminoadipate has a structure similar to that of lysine. We hope to use lysine as a precursor to finding a suitable enzyme to convert lysine to α-aminoadipate. Therefore, we will summarize the biosynthesis and degradation pathways of lysine. In microorganisms, the synthesis of lysine has two completely different pathways: one is the dianaminopimelic acid (DAP) pathway, and the other is the α-aminoadipate pathway. The DAP pathway exists in bacteria, fungi, and plants, and it uses aspartate as a precursor to synthesize lysine through a 10 step enzymatic reaction, including aspartate transaminase (encoded by aspC), aspartate kinase (encoded by lysC), aspartate semialdehyde dehydrogenase (encoded by asd), 4-hydroxy-tetrahydropicolinate synthase (encoded by dapaA), 4-hydroxy-tetrahydropicolinate synthase (encoded by dapaB), 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase (encoded by dapD), N-succinyl-diaminopimelate aminotransferase (encoded by dapaC), succinyl-diaminopimelate desuccinylase (encoded by dapaE), diaminopimelate epimerase (encoded by dapaF), and diaminopimelate decarboxylase (encoded by lysA) (H. Xu et al., 2006). The DAP biosynthetic pathway has been extensively studied in Escherichia coli and Corynebacterium glutamicum and has been engineered for the industrial production of lysine. Unlike the DAP pathway in E. coli, there is a multifunctional enzyme in C. glutamicum (meso-diaminopimelate dehydrogenase, encoded by the gene ddh) that can replace DapD, DapC, DapE, and DapF to directly reduce L-piperidine-2,6-dicarboxylic acid to L-diaminopimelate (Ishino et al., 1984; and Misono et al., 1986). The α-aminoadipate pathway exists in eucalyptus and higher fungi that use ketoglutarate as the precursor, and it is gradually catalyzed by homoisocitrate synthase, cis-aconitase, and isocitrate dehydrogenase to produce α-aminoadipate. Next, α-aminoadipate is catalyzed by α-aminoadipate reductase, saccharopine dehydrogenase, and saccharopine reductase to generate lysine (Burk et al., 2007; Hermann, 2003; Zabriskie & Jackson, 2000). However, lysine is degraded by the reverse α-aminoadipate pathway in plants and animals (Arruda et al., 1982, 2000). It has been reported that lysine is catalytically converted to saccharopine by lysine-ketoglutarate reductase (LKR) and then catalytically converted to α-aminoadipate semialdehyde by saccharopine dehydrogenase (SDH). Finally, α-aminoadipate semialdehyde is catalytically converted to α-aminoadipate by α-aminoadipate semialdehyde dehydrogenase (Arruda et al., 2000; de Mello Serrano et al., 2012). These above enzymes probably originated from Proteus vulgaris and are also found in animals and plants. It has been determined that lysine is directly converted to α-aminoadipate semialdehyde by lysine dehydrogenase with NAD(P)⁺ as a cofactor in prokaryotes. It is then oxidized to α-aminoadipate (Misono & Nagasaki, 1982).

In summary, α-aminoadipate is both the precursor for lysine synthesis and an intermediate for lysine degradation. Therefore, in this study, we combine the DAP pathway with the reverse α-aminoadipate pathway to design a biosynthesis pathway for α-aminoadipate (Figure 1). In this study, we select E. coli as the host and glucose as the carbon source to produce α-aminoadipate by fermentation through the above pathway. And, multi-strategy metabolic engineering was used to regulate microbial cell factories to realize the efficient and green synthesis of α-aminoadipate.

2 | MATERIALS AND METHODS

2.1 | Flux balance analysis (FBA) and OptForce on iML1515

Both FBA (Orth et al., 2010) and OptForce algorithm (Ranganathan et al., 2010) were performed using the Gurobi and COBRApy toolkit via Python to determine the flux spans of each reaction. The glucose uptake rate was set at 10 mmol/gDCW/h. The upper limit for biomass in the overproducing strain was set to 5% of the theoretical maximum for biomass flux.

2.2 | Media, strains, plasmids, materials, and growth conditions

The E. coli Trans 10 and its derivatives were cultured in the lysogeny broth (LB) medium with 200 rpm at 37°C, which contained 5 g yeast extract, 5 g NaCl, and 10 g tryptone per liter. The recombinant E. coli strains were cultured in the M9 medium with 200 rpm at 37°C for feeding experiments or de novo production for culturing cells and at 30°C for inducing gene expression. The M9 medium contained 10 g glucose, 3 g yeast extract, 1 g NH4Cl, 6.78 g Na2HPO4, 3 g KH2PO4, and 0.5 g NaCl per liter. For the fed-batch cultivation, culture conditions referred to the strategy described in Li et al. (2019). Ampicillin, kanamycin, chloramphenicol, or spectinomycin was added to screen the recombinant strains with final concentrations of 100, 50, 30, or 50 mg/L, respectively.
The *E. coli* JM109(DE3) was used as the host strain to produce \( \alpha \)-aminoadipate. The kits for genomic DNA isolation, plasmid extraction, and DNA recovery were purchased from Omega Bio-Tek, and all of the enzymes were purchased from New England Biolabs. All of the strains and plasmids used are listed in Table A1. The knockout strains were constructed using RED recombination.

### 2.3 Plasmid construction

All of the primers used for gene cloning are listed in Table A2. The l-lysine 6-dehydrogenase encoding gene *lysDH* from *Bacillus thermothyrolyovorans* 1A1 (GenBank: CEE01557.1) and the aminoadipate semialdehyde dehydrogenase coding gene *Psefu_1272* (GenBank: AEF21248.1) from *Pseudomonas fulva* 12-X were codon-optimized for *E. coli*, which were synthesized and respectively ligated into the plasmids pUC57-*lysDH* and pUC57-*pse* by BGI. Both *lysDH* and *Psefu_1272* were amplified by polymerase chain reaction (PCR) using pUC57-*lysDH* and pUC57-*pse* as the templates, respectively. The PCR products were subcloned into plasmid pETDuet-1 to generate the plasmid pET-T7-*lysDH*-pse by the Gibson assembly reaction (Gibson et al., 2009). Then, the plasmids pET-tac-*lysDH*-pse, pET-trc-*lysDH*-pse, pET-lacUV5-*lysDH*-pse, and pET-LacO1-*lysDH*-pse were obtained by replacing the T7 promoter with the different promoters.

### 2.4 Measurement of the biomass, substrate consumption rate, and metabolic products

The cell density was determined by measuring the turbidity of the culture medium at 600 nm using a spectrophotometer (Thermo Fisher Scientific).

The concentrations of glucose and organic acids were determined by the UltiMate 3000 HPLC (Thermo Fisher Scientific) using the following apparatus and operating conditions: Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories) with RID and UV detectors; column temperature of 65°C; and 0.6 ml min\(^{-1}\) of 5 mM sulfuric acid as the mobile phase. The concentration of \( \alpha \)-aminoadipate was analyzed by LC-MS using the AB SCIEX QTRAP 5500 system (AB SCIEX) equipped with a HILIC-Z column (2.1 × 100 mm) (Agilent Technologies). The mobile phase was: A: water containing 0.1% (v/v)
formic acid, B: acetonitrile containing 0.1% (v/v) formic acid, and 20 mM ammonium acetate with a flow velocity of 0.3 mL min\(^{-1}\) at 40°C. Also, α-amino adipate was further identified by gas chromatography-mass spectrometry (GC-MS) using the Agilent Technologies 7890B-5977A system (Agilent Technologies) in which the appropriate amount of fermentation supernatant was dried by vacuum centrifugation or freeze-drying and extracted using a silylation reagent at a ratio of N, O-bis(trimethylsilyl) trifluoroacetamide to trimethylchlorosilane of 99:1.

3 RESULTS AND DISCUSSION

3.1 Designing an optimal E. coli α-amino adipate producer via FBA and OptForce

We added two reactions catalyzed by lysine dehydrogenase and aminoadipate semialdehyde dehydrogenase and the α-amino adipate exchange reaction to iML1515. Both FBA and OptForce were used to calculate and predict the metabolic strategies of α-amino adipate overproduction. As shown in Figure 2a, nine reactions in the lysine biosynthesis pathway were found to require amplification. The pentose phosphate pathway (PPP) was required to be upregulated because the PPP could provide NADPH for lysine synthesis. In addition, we introduced the exogenous reaction catalyzed by pyruvate carboxylase (encoded by pyc) into the model and calculated the theoretical yield of α-amino adipate. When the above reaction was added to the model, the yield was 90% (mol/mol), which was 9.8% higher than that of the wild type (the yield was 82% [mol/mol]). This is because pyruvate carboxylase enhanced the carbon flux of α-amino adipate biosynthesis by the CO\(_2\) fixation. The reaction of glucose 6-phosphate to fructose 6-phosphate catalyzed by glucose-6-phosphate isomerase (encoded by pgi) in glycolysis is the competitive pathway of PPP, which is required to be downregulated. The α-amino adipate flux was calculated at different levels of the PPP (in terms of G6PDH2r) and the glycolysis pathway (in terms of PGI).

![Diagram](image)

**FIGURE 2** α-Aminoadipate overproducing strategies in recombinant Escherichia coli. (a) Metabolic interventions prediction via OptForce based on iML1515. ackA, encoding acetate kinase; poxB, encoding pyruvate oxidase; pgi, encoding glucose-6-phosphate isomerase; ppc, encoding phosphoenolpyruvate carboxylase; pta, encoding phosphate acetyltransferase; pyc, encoding pyruvate carboxylase. (b) The influences of glycolysis and the PPP flux on the α-amino adipate flux. G6PDH2r, the reaction catalyzed by glucose-6-phosphate 1-dehydrogenase (encoded by zwf); PGI, the reaction catalyzed by glucose-6-phosphate isomerase (encoded by pgi). (c) The effect of the biomass flux and the acetate flux on α-amino adipate flux; gdcw, gram of dry cell weight.
In Figure 2b, the higher α-aminoadipate flux was obtained when the PG1 flux was decreased and the PPP was upregulated. In addition, Figure 2c shows the negative effect of the biomass and the acetate pathway on α-aminoadipate flux.

3.2 Constructing the bioproduction pathway of α-aminoadipate in E. coli

Lysine dehydrogenase with NAD(P)⁺ as a cofactor in prokaryotes can directly convert lysine into aminoacidimide semialdehyde (Misono & Nagasaki, 1982). Subsequently, aminoacidimide semialdehyde is converted to α-aminoacipate by aminoacidimide semialdehyde dehydrogenase. Searching through the UniProt database (https://www.uniprot.org/), we selected lysine dehydrogenase encoded by the lysDH gene from Bacillus thermoamylovorans 1A1 and aminoacidimide semialdehyde dehydrogenase encoded by the Psefu_1272 gene from Pseudomonas fulva 12-X to construct the biosynthetic pathway of α-aminoacipate in E. coli (Figure 1).

The lysDH from Bacillus thermoamylovorans 1A1 and Psefu_1272 from Pseudomonas fulva 12-X were codon-optimized for E. coli and ligated into the pETDuet-1 plasmid. Their expressions were induced using isopropyl β-D-1-thiogalactopyranoside (IPTG). The E. coli JM109(DE3) cells harboring the pET-T7-lysDH-pse plasmid were designated as strain EcETTT7N, and this strain produced 236.5 mg/L of α-aminoacipate in M9 at 72 h by feeding experiments (feeding lysine at a final concentration of 5 g L⁻¹ to the M9 medium) (Figure 3a). The product was verified by GC-MS, and the result showed that the product from the fermentation broth was α-aminoacipate (Figure B1).

To determine the optimal expression level of heterologous enzymes catalyzing the reactions for α-aminoacipate synthesis in E. coli, the lysDH, and Psefu_1272 genes were ligated into plasmids with different promoters that included the tac, trc, lac UV5, and lacO1 promoters. Thus, α-aminoacipate titers produced by feeding experiments with the recombinant strains of EcETTn, EcETTrcN, EcETlacUV5N, and EcETlacO1N (Table A1) were compared. The results showed that the trc promoter (pET-trc-lysDH-pse) was more suitable for α-aminoacipate production than the other promoters. The strain EcETTrcN effectively converted lysine to α-aminoacipate with titers of 315.5 mg/L (Figure 3c). To explore the de novo biosynthesis of α-aminoacipate, the strain EcETTrcN was cultivated in an M9 medium with 10 g L⁻¹ glucose, which produced 82 mg/L of α-aminoacipate at 72 h (Figure 3d).

3.3 Increasing the supply of precursors and introducing the CO₂ fixation process

According to the results from FBA and OptForce, the lysine synthesis pathway should be upregulated. In E. coli, lysine is synthesized through the DAP pathway that involves two key enzymes, namely, aspartate kinase (encoded by lysC) and 4-hydroxy-tetrahydrodipicolinate synthase (encoded by dapA) (Contador et al., 2009; J. Wang et al., 2015; J. Xu et al., 2014). Some studies have shown that aspartate kinase is inhibited by the feedback of lysine. We overexpressed the above two genes to strengthen the two key enzymes. In addition, the meso-diaminopimelate dehydrogenase from Corynebacterium glutamicum can replace the functions of four enzymes (2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase, N-succinyldiaminopimelate aminotransferase, succinyl-diaminopimelate desuccinylase, diaminopimelate epimerase) in E. coli to directly convert L-piperidine-2,6-dicarboxylic acid into D-, L-diaminopimelate. Therefore, we hoped to replace the original four-step enzyme by introducing meso-diaminopimelate dehydrogenase (encoded by dhd) from Corynebacterium glutamicum ATCC 13032 to shorten the metabolic pathway.

Five candidate genes derived from E. coli or Corynebacterium glutamicum were constructed in the plasmid pACYC-tac-T7-T7ter or pACYCDuet-1 and electrotansformed into the strain EcETTrcN to obtain the strain EcN01*EcN03* (Table A1). The results of fermentation in the shake flasks showed that the titer of α-aminoacipate produced by the strain EcN02* was higher, reaching 147 mg/L (Figure 4a). These results demonstrated that overexpression of lysC and dapA promoted the synthesis of α-aminoacipate. The mutant lysCT311I with anti-feedback inhibition and dapA from Corynebacterium glutamicum ATCC 13032 were better than the lysC and dapA from E. coli, and the titer of α-aminoacipate was 80% higher than that of the strain EcETTT7N. Next, we hoped to introduce meso-diaminopimelate dehydrogenase (encoded by dhd) from Corynebacterium glutamicum ATCC 13032 to shorten the synthesis pathway of the precursor lysine. However, the strain EcN03* produced the same amount of α-aminoacipate compared with the strain EcETTrcN (Figure B2), speculating that the insufficient supply of cofactors and the prior balance may have been disrupted.

In addition, it has been confirmed that oxaloacetate is another important precursor for the synthesis of lysine or lysine derivatives. Increasing the replenishment of oxaloacetate is beneficial for improving lysine and lysine derivative production. Pyruvate carboxylase catalyzes the transformation of pyruvate to oxaloacetate, and the overexpression of its coding gene, pyc, can enhance the supply of oxaloacetate. The mutant pycP458S is a beneficial mutation that can improve the activity of pyruvate carboxylase (Xiong et al., 2021). And FBA calculation results also showed that the theoretical yield of α-aminoacipate increased by nearly 10% after the introduction of pyruvate carboxylase. In addition, the catalytic reaction by pyruvate carboxylase is a CO₂ fixation process, which could achieve the intracellular CO₂ reutilization to enhance the carbon flux of α-aminoacipate biosynthesis. Thus, pyc from Corynebacterium glutamicum ATCC 13032 and its mutant pycP458S were constructed in the plasmid pRSF-tac-T7-T7ter to obtain plasmids pRSF-tac-pyc and pRSF-tac-pycP458S and to finally obtain the recombinant strains EcN04* EcN05*. The results of the shake flask fermentation showed that expression of the pyc gene mutant (pycP458S) encoding pyruvate carboxylase from Corynebacterium glutamicum ATCC 13032 could promote the synthesis of α-aminoacipate. The titer of α-aminoacipate reached 110 mg/L (Figure 4c), which was 34% higher than that of the strain EcETTrcN. Furthermore, endogenous
phosphoenolpyruvate carboxylase encoded by ppc catalyzed the conversion of phosphoenolpyruvate (PEP) to oxaloacetate. However, the glucose transport system of *E. coli* was mainly the phosphoenolpyruvate–carbohydrate phosphotransferase system (PTS), which consumed PEP to assist glucose transport and produce pyruvate. Therefore, to increase the availability of oxaloacetate, expressing pyc instead of ppc was probably better. Some experiments were carried out to compare the effects of overexpression of ppc and expression of pyc on the synthesis of α-amino adipate. The results of the shake flask fermentation showed that overexpression of ppc also could promote the synthesis of α-amino adipate. Among them, the titers of α-amino adipate of the strain EcN05*# were equivalent to that of the strain EcN05*. The biomass and glucose utilization were also similar. However, coexpression of ppc and pyc was detrimental to the
synthesis of α-aminoadipate (strain EcN05##). The coexpression of ppc and pyc resulted in the imbalance of upstream and downstream metabolic flux. Although it promoted the utilization of glucose, excessive oxaloacetate did not effectively enter the synthesis pathway of α-aminoadipate, but promoted the TCA cycle, increasing the biomass of strain EcN05## (Figure B3).

3.4 | Regulating intracellular cofactors

Cofactors (e.g., NADH, NADPH, and ATP) are important metabolic factors in microbial cells. As the most important redox carriers in cell metabolism, NADH/NAD⁺ and NADPH/NADP⁺ not only act as electron acceptors for catalyzing substrate catabolism but also provide reducing power for energy-dependent redox reactions. ATP/ADP from substrate-level phosphorylation and oxidative phosphorylation can enter the metabolic network of microorganisms in a variety of ways, such as substrates, products, activators, or inhibitors, to control the physiological function of cells and participate in the formation of the cytoskeleton system. Therefore, the control of the intracellular cofactor balance is a basic requirement to maintain the normal metabolism of cells and achieve a balance of material and energy couple. Currently, the primary regulation strategies of cofactors can be divided into four categories: regulation of endogenous cofactor systems, regulation of heterologous cofactor regeneration systems, modification of cofactor preferences, and creation of synthetic-cofactor systems (M. Wang, Chen, et al., 2017; Y. Zhang et al., 2021).

In the synthesis pathway of α-aminoadipate constructed in this study, the synthesis of the precursor lysine required a significant amount of NADPH, and the conversion of lysine to aminoadipic acid required NAD⁺ as the cofactor. Therefore, it was necessary to regulate the level of intracellular cofactors to achieve cofactor balance to promote the synthesis of α-aminoadipate. In this study, the cofactor level was regulated by three strategies: (1) introducing the Entner–Doudoroffed pathway (ED pathway, Figure 5a) (M. Wang, Zhou, et al., 2017); (2) introducing the transhydrogenase system (Figure 5b) (Y. Chen et al., 2015); and (3) enhancing the pentose phosphate pathway (by deleting the pgi gene that encodes glucose-6-phosphate isomerase, Figure 5c) (Marx et al., 2003). The recombinant
strains EcN06, EcN07, EcN07*, and EcN07** were obtained. The fermentation results showed that the introduction of the transhydrogenase system and the strengthening of the pentose phosphate pathway promoted the synthesis of α-aminoadipate. Then, the strains EcN07, EcN07*, and EcN07** produced 133, 140, and 136 mg/L of α-aminoadipate, respectively, Figure 5d).

3.5 | Weakening the synthesis of acetic acid and realizing combination optimization

Studies have shown that a large portion of the carbon source flows to the acetic acid synthesis pathway when *E. coli* uses glucose as the carbon source to express heterologous proteins (Bernal et al., 2016). The synthesis of acetic acid is a metabolic overflow phenomenon (Enjalbert et al., 2017). Under aerobic conditions, there are two primary ways to synthesize acetic acid. One is from pyruvate catalyzed by pyruvate oxidase (encoded by poxB). The other is from acetyl-CoA catalyzed by phosphate acetyltransferase (encoded by pta) and acetate kinase (encoded by ackA) (Schütze et al., 2020). The fermentation results of the above 10 strains showed that acetic acid was the primary by-product in the production of α-aminoadipate by *E. coli*, resulting in many ineffective carbon fluxes (Appendix B, Figure B4). In addition, studies have shown that the knockout of acetic acid synthesis pathways can promote the synthesis of some compounds such as citramalate (Parimi et al., 2017). The FBA results also showed that the flux of α-aminoadipate was negatively correlated with that of acetic acid. We first investigated the production of acetic acid, glucose consumption, and biomass changes of *E. coli* JM109(DE3) and its mutants in the M9 medium. The results showed that the knockout of poxB significantly inhibited acetic acid production, which is the key gene in acetic acid synthesis. However, the knockout of poxB affected glucose utilization, resulting in pyruvate accumulation (Figure B5). Subsequently, the poxB knockout strains were combined with the above regulatory strategies to promote the synthesis of α-aminoadipate and obtain the strains EcN08–EcN11. The strain EcN11 obtained the highest titer of α-aminoadipate by fermentation in the shake flasks, up to 263 mg/L (Figure 6a), and this was 2.2 times higher than that of the strain EcPETtrcN. Hence, the knockout of the poxB gene and enhancement of pyruvate carboxylase effectively promoted glucose utilization and pyruvate conversion, reducing acetic acid flux (Figure B4). To reduce the number of plasmids and facilitate subsequent optimization, the pycP458S gene was integrated into the plasmid PET-trc-lysDH-pse or pACYC-tac-lysC311-dapA_cg, and the strains EcN11* and EcN11** were obtained. The fermentation results showed that the strain...
EcN11* produced a higher titer of α-aminoadipate, up to 313.5 mg/L (Figure 6c). However, the titer of α-aminoadipate produced by the EcN11** strain was lower than that produced by the strain EcN11, and glucose utilization and pyruvate conversion were blocked. At 72 h of fermentation, the glucose residue was 0.68 g/L, and pyruvate accumulated to 0.94 g/L. This may have been due to the low-level expression of pyruvate carboxylase (Table A3). Finally, the titer of α-aminoadipate was further improved by combination optimization. The strains EcN11#, EcN11##, and Ec11*# are presented in (Table A3). The results demonstrated that the synthesis of

FIGURE 6  Inhibition of acetic acid synthesis and the combination optimization for improving α-aminoadipate synthesis. (a), (c), and (e) The titers of α-aminoadipate in the different strains. (b), (d), and (f) The biomass in the different strains.
α-aminoacidipate can be strengthened by combination optimization. The strains EcN11#, EcN11##, and Ec11*# produced 368, 415, and 330 mg/L of α-aminoacidipate, respectively (Figure 6e). Among them, the titer of α-aminoacidipate produced by the strains EcN11#, EcN11##, and EcN11*# strains were 17%, 32%, and 5% higher than that produced by the E. coli N11* strain, respectively. The carbon metabolisms of the EcN11## and EcN11*# strains were weakened due to the knockout of the pgI gene, and this negatively affected the growth and glucose absorption (Figures 6f and B6). To prove that the main flux of oxaloacetate in strain EcN11# came from the carbon fixation pathway catalyzed by pyruvate carboxylase, the main consumption pathway of pyruvate was blocked. In E. coli, pyruvate was mainly consumed in two pathways: (1) the conversion of pyruvate to acetic acid catalyzed by the pyruvate oxidase (encoded by poxB); (2) the conversion of pyruvate to acetyl-CoA catalyzed by the pyruvate dehydrogenase complex (its rate-limiting component was coded by aceE). Therefore, the flux of oxaloacetate was proved by the experiment of aceE knockout. We compared the growth and main metabolites of different strains. The results showed that the simultaneous knockout of poxB and aceE without the introduction of pyruvate carboxylase resulted in serious obstruction of glucose utilization, growth restriction, and serious accumulation of pyruvate, and the titer of α-aminoacidipate decreased seriously (Figure B7). This showed that pyruvate was mainly converted to acetyl-CoA by the pyruvate dehydrogenase complex, and then entered the TCA cycle to produce oxaloacetate. To further explore the metabolic flux, strain EcN11-2 was obtained. The results showed the simultaneous knockout of poxB and aceE with the introduction of pyruvate carboxylase could reduce pyruvate accumulation and restore the titer of α-aminoacidipate. Although glucose utilization and strain growth were a little improved, they were still not as good as strain EcN11#. Thus, this proved that the most of metabolic flux of oxaloacetate in strain EcN11# was from pyruvate and only a small part of that was from the TCA cycle (Figure B7).

In addition, we also explored the effect of the removal of feedback inhibition at the anaplerotic pathway on the synthesis of α-aminoacidipate through experiments. In E. coli, the conversion of oxaloacetate to PEP catalyzed by phosphoenolpyruvate carboxykinase (encoded by pck) could inhibit the replenishment of oxaloacetate, and excess oxaloacetate was introduced into gluconeogenesis through this reaction. We knocked out pck to the effect of the removal of feedback inhibition at the anaplerotic pathway on the synthesis of α-aminoacidipate. The results showed that the knockout of pck could promote the synthesis of α-aminoacidipate, but it was not as good as strengthening the replenishment pathway of oxaloacetate (The strain EcN11_pck, Figure B3). And knockout of pck and enhancement of oxaloacetate replenishment pathway resulted in the limited synthesis of α-aminoacidipate (The strain Ec11#_pck, Figure B3). This may be due to the imbalance of upstream and downstream metabolic flux and excessive OAA did not effectively enter the synthesis pathway of α-aminoacidipate but entered the TCA cycle (Figure B8).

3.6 | Fed-batch production of α-aminoacidipate

To verify the scale-up potential of α-aminoacidipate production, fed-batch experiments were carried out in 2L bioreactors using strains EcN11# and EcN11##. Initially, strain EcN11### was used for fed-batch experiments. However, the results showed that strain EcN11## was not suitable for the scale-up production of α-aminoacidipate (Figure B9), which is because its glycolytic pathway is blocked, resulting in the obstruction of glucose utilization and growth restriction. So it is difficult to realize high-density fermentation. After that, we tested the ability of strain EcN11# to produce α-aminoacidipate. We investigated the effect of dissolved oxygen (DO) on α-aminoacidipate production. The results showed that high DO is not only beneficial for cell growth but also α-aminoacidipate accumulation. When DO was set at 10%, the cell density (OD600) of strain EcN11# only reached 20 and the final titer of α-aminoacidipate was only 525 mg/L at 72 h (Figure 7a). Furthermore, at 20% set DO, the cell density (OD600) reached 50 and 1.54 g/L of α-aminoacidipate was produced at 72 h (Figure 7b).

4 | CONCLUSION

In this study, E. coli was successfully engineered to α-aminoacidipate from glucose. Two key enzymes, lysine dehydrogenase and aminoacidipate semialdehyde dehydrogenase, from Bacillus
thermoamylovorans 1A1 and Pseudomonas fulva 12-X were examined for the construction of an α-aminoadipate biosynthesis pathway. The metabolic network was optimized by enhancing the synthesis of precursor lysine, introducing the CO₂ fixation process, regulating intracellular cofactor metabolism, and inhibiting the synthesis of by-product acetic acid. By using multi-strategy metabolic engineering, the recombinant strains EcN11# and EcN11## improved α-aminoadipate production from glucose. Shake flask fermentation using the strains EcN11# and EcN11## produced 368 and 415 mg/L of α-aminoadipate. Finally, fed-batch experiments produced 1.54 g/L of α-aminoadipate by the strain EcN11#. This study is the first to achieve the green synthesis of α-aminoadipate in microorganisms. To improve the yield of α-aminoadipate from glucose, further metabolic engineering modifications would be required. At present, some studies have shown that improving transmembrane transport could strengthen mass transfer level and metabolic efficiency, and improve the production efficiency of cell factories (X. Chen et al., 2017; Fukui et al., 2019). So far, α-aminoadipate transporter has not been reported. According to the structural similarity, a glutamate transporter may be used as α-aminoadipate transporter. It has been reported that MscCG is a mechanosensitive channel of Corynebacterium glutamicum and acts as a glutamate transporter (Hashimoto et al., 2010; Nakayama et al., 2016, 2018; Yao et al., 2009). In addition, MscCG2 is a novel glutamate transporter found in Corynebacterium glutamicum which has a low amino acid sequence identity (23%) to MscCG (Wang et al., 2018). It was also demonstrated that constitutive glutamate effusion was triggered by its mutation of MscCG2 (A151V). We speculate that glutamate transporter is the possible transporter of α-aminoadipate. Therefore, strengthening the expression of α-aminoadipate transporter to improve its efflux could have a positive impact on improving the titer of α-aminoadipate. Besides, some other metabolic engineering modifications could promote the synthesis of α-aminoadipate, including stable expression of key genes through genomic integrations, regulation of the tricarboxylic (TCA) cycle, identification of unknown and potential key metabolic nodes via omics analysis such as transcriptomics or metabolomics, etc.

AUTHOR CONTRIBUTIONS
Yang Zhang: data curation (equal), project administration (equal), writing original draft (equal), writing review & editing (equal). Meng Liu: investigation (equal), methodology (equal). Binggi Cai: software (equal). Keqin He: formal analysis (equal), visualization (equal). Meng Wang: funding acquisition (equal), resources (equal). Biqiang Chen: supervision (equal). Tianwei Tan: project administration (equal), supervision (equal).

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CONFICT OF INTEREST
None declared.

DATA AVAILABILITY STATEMENT
All data are provided in full in this paper.

ETHICS STATEMENT
None required.

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APPENDIX A
(Tables A1–A3)

| Strain and plasmid | Relevant characteristic | Reference or source |
|--------------------|-------------------------|---------------------|
| E. coli Trans 10   | F-mcrΔ(mrr-hsdRMS-mcrBC)p80 lacZΔM15ΔacX74 recA1 araΔ139Δ(ara-leu)7697 galUgalKΔK30StrR endA1 nupG | Purchased from TransGen Biotech |
| E. coli JM109(DE3)  | endA1, recA1, gyrA96, thi, hsdR17(rK-, mK+), relA1, supE44, λ-, Δ(lac-proAB), [F- traD36, proAB, lacIqZΔM15], ΔE. coli ΔEcN05* | Laboratory collection |
| 109ΔpoxB          | Knockout of poxB gene in E. coli JM109(DE3) | Laboratory collection |
| 109Δpta ackA       | Knockout of pta and ackA genes in E. coli JM109(DE3) | Laboratory collection |
| 109Δpgi            | Knockout of pgi gene in E. coli JM109(DE3) | Laboratory collection |
| 109ΔpoxBΔpgi       | Knockout of poxB and pgi genes in E. coli JM109(DE3) | Laboratory collection |
| Corynebacterium glutamicum ATCC 13032 | Wild type | Laboratory collection |
| EcpETT7N           | E. coli JM109(DE3) with pET-T7-lysDH-pse | This study |
| EcpETacN           | E. coli JM109(DE3) with pET-tac-lysDH-pse | This study |
| EcpETrcN           | E. coli JM109(DE3) with pET-trc-lysDH-pse | This study |
| EcpETlacUV5N       | E. coli JM109(DE3) with pET-lacUV5-lysDH-pse | This study |
| EcpETlacO1N        | E. coli JM109(DE3) with pET-lacO1-lysDH-pse | This study |
| EcN01*             | E. coli JM109(DE3) with pET-trc-lysDH-pse and pACYC-tac-lysC_Ec-dapA_Ec | This study |
| EcN02*             | E. coli JM109(DE3) with pET-trc-lysDH-pse and pACYC-tac-lysCT311I-dapA_cg | This study |
| EcN03*             | E. coli JM109(DE3) with pET-trc-lysDH-pse and pACYC-PJ23100-ddh_cg | This study |
| EcN04*             | E. coli JM109(DE3) with pET-trc-lysDH-pse and pRSF-tac-pyc | This study |
| EcN05*             | E. coli JM109(DE3) with pET-trc-lysDH-pse and pRSF-tac-pycP458S | This study |
| EcN05**            | E. coli JM109(DE3) with pET-trc-lysDH-pse and pRSF-tac-pyc_P458S-Ec | This study |
| EcN05#             | E. coli JM109(DE3) with pET-trc-lysDH-pse and pRSF-tac-pyc_P458S-pyc_P458S_cg | This study |
| EcN06              | E. coli JM109(DE3) with pET-trc-lysDH-pse and pCDF-T7-ED | This study |
| EcN07              | E. coli JM109(DE3) with pET-trc-lysDH-pse and pCDF-T7-ffdh1-T7-pntAB | This study |
| EcN07*             | 109Δpgi with pET-trc-lysDH-pse | This study |
| EcN07**            | 109ΔpoxB with pET-trc-lysDH-pse and pACYC-tac-lysCT311I-dapA_cg and pRSF-tac-pycP458S | This study |
| EcN08              | E. coli JM109(DE3) with pET-trc-lysDH-pse and pACYC-tac-lysCT311I-dapA_cg and pRSF-tac-pycP458S | This study |
| EcN09              | 109ΔpoxB with pET-trc-lysDH-pse and pACYC-tac-lysCT311I-dapA_cg | This study |
| EcN10              | 109ΔpoxB with pET-trc-lysDH-pse and pRSF-tac-pycP458S | This study |
| EcN11              | 109ΔpoxB with pET-trc-lysDH-pse and pACYC-tac-lysCT311I-dapA_cg and pRSF-tac-pycP458S | This study |
| EcN11*             | 109ΔpoxB with pET-trc-lysDH-pse-tac-pycP458S and pACYC-tac-lysCT311I-dapA_cg | This study |
| EcN11**            | 109ΔpoxB with pET-trc-lysDH-pse and pACYC-tac-lysCT311I-dapA_cg-tac-pycP458S | This study |

(Continues)
| Strain and plasmid | Relevant characteristic | Reference or source |
|--------------------|-------------------------|---------------------|
| EcN11#             | 109ΔpoxB with pET-trc-lysDH-pse-tac-pycP458S and pACYC-tac-lysCT311I-dapaA cg and pCDF-T7-fdh1-T7-pnAB | This study |
| EcN11##            | 109ΔpoxBΔpdi with pET-trc-lysDH-pse-tac-pycP458S and pACYC-tac-lysCT311I-dapaA cg and pCDF-T7-fdh1-T7-pnAB | This study |
| EcN11*#            | 109ΔpoxBΔaceE with pET-trc-lysDH-pse and pACYC-tac-lysCT311I-dapaA cg and pCDF-T7-fdh1-T7-pnAB | This study |
| EcN11_1            | 109ΔpoxBΔaceE with pET-trc-lysDH-pse and pACYC-tac-lysCT311I-dapaA cg and pCDF-T7-fdh1-T7-pnAB | This study |
| EcN11_2            | 109ΔpoxBΔaceE with pET-trc-lysDH-pse and pACYC-tac-lysCT311I-dapaA cg and pCDF-T7-fdh1-T7-pnAB | This study |
| EcN11_pck          | 109ΔpoxBΔpck with pET-trc-lysDH-pse and pACYC-tac-lysCT311I-dapaA cg and pCDF-T7-fdh1-T7-pnAB | This study |
| EcN11#_pck         | 109ΔpoxBΔpck with pET-trc-lysDH-pse-tac-pycP458S and pACYC-tac-lysCT311I-dapaA cg and pCDF-T7-fdh1-T7-pnAB | This study |
| **Plasmid**        |                          |                     |
| pUC57              | E. coli cloning vector, pMB1 ori, AmpR | BGI collection |
| pUC57-lysDH        | pUC57 derivative; codon-optimized lysDH gene from Bacillus thermoamylovorans, AmpR | This study |
| pUC57-pse          | pUC57 derivative; codon-optimized Psefu_1272 gene from Pseudomonas fulva 12-X, AmpR | This study |
| pETDuet-1          | E. coli expression vector, T7 promoter, ColE1 ori, AmpR | Laboratory collection |
| pET-tac-T7-T7ter   | pETDuet-1 derivative; tac and T7 promoters, ColE1 ori, AmpR | Laboratory collection |
| pACYCduet-1        | E. coli expression vector, T7 promoter, P15A ori, CmR | Laboratory collection |
| pACYC-tac-T7-T7ter | pACYCDuet-1 derivative; tac and T7 promoters, P15A ori, CmR | Laboratory collection |
| pCDFduet-1         | E. coli expression vector, T7 promoter, CloDF13 ori, SmR | Laboratory collection |
| pRSFduet-1         | E. coli expression vector, T7 promoter, RSF ori, KmR | Laboratory collection |
| pRSF-tac-T7-T7ter  | pRSFDuet-1 derivative; tac and T7 promoters, RSF ori, KmR | Laboratory collection |
| pET-T7-lysDH-pse   | pETDuet-1 derivative; T7 promoter, codon-optimized lysDH gene from Bacillus thermoamylovorans and codon-optimized Psefu_1272 gene from Pseudomonas fulva 12-X, ColE1 ori, AmpR | This study |
| pET-tac-lysDH-pse  | pET-T7-lysDH-pse derivative; tac promoters, codon-optimized lysDH gene from Bacillus thermoamylovorans and codon-optimized Psefu_1272 gene from Pseudomonas fulva 12-X, ColE1 ori, AmpR | This study |
| pET-trc-lysDH-pse  | pET-T7-lysDH-pse derivative; trc promoter, codon-optimized lysDH gene from Bacillus thermoamylovorans and codon-optimized Psefu_1272 gene from Pseudomonas fulva 12-X, ColE1 ori, AmpR | This study |
| pET-lacUV5-lysDH-pse | pET-T7-lysDH-pse derivative; lacUV5 promoter, codon-optimized lysDH gene from Bacillus thermoamylovorans and codon-optimized Psefu_1272 gene from Pseudomonas fulva 12-X, ColE1 ori, AmpR | This study |
| pET-lacO1-lysDH-pse | pET-T7-lysDH-pse derivative; lacO1 promoter, codon-optimized lysDH gene from Bacillus thermoamylovorans and codon-optimized Psefu_1272 gene from Pseudomonas fulva 12-X, ColE1 ori, AmpR | This study |
| pEASY-Blunt Simple Cloning Vector | A cloning vector of E. coli, KanaR or AmpR, pUC ori | Purchased from TransGen Biotech |
| pEASYblunt-lysC    | pEASY-Blunt simple cloning vector derivative, KanaR or AmpR, harboring aspartate kinase encoding gene lysC | This study |
| Strain and plasmid | Relevant characteristic | Reference or source |
|--------------------|-------------------------|---------------------|
| pEASYblunt-lysCT311I | pEASY-Blunt simple cloning vector derivative, KanaR or AmpR, harboring the mutant of aspartate kinase encoding gene lysCT311I | This study |
| pACYC-tac-lysC_Ec-dapA_Ec | pET-tac-T7-T7ter derivative; tac promoter, lysC and dapA genes from E. coli JM109(DE3), P15A ori, CmR | This study |
| pACYC-tac-lysCT311I-dapA_cg | pACYC-tac-T7-T7ter derivative; tac promoter, lysC311I and dapA genes from C. glutamicum ATCC 13032, P15A ori, CmR | This study |
| pACYC-PJ23100-ddh_cg | pACYCDuet-1 derivative; PJ23100 promoter, ddh gene from Corynebacterium glutamicum ATCC 13032, P15A ori, CmR | This study |
| pRSF-tac-pyc | pRSF-tac-T7-T7ter derivative; tac promoter, pyc gene from C. glutamicum ATCC 13032, RSF ori, KmR | This study |
| pRSF-tac-pycP4585 | pRSF-tac-T7-T7ter derivative; tac promoter, pyc gene mutant, RSF ori, KmR | This study |
| pRSF-tac-ppc_Ec | pRSF-tac-T7-T7ter derivative; tac promoter, ppc gene from E. coli JM109(DE3), RSF ori, KmR | This study |
| pRSF-tac-ppc_cg | pRSF-tac-T7-T7ter derivative; tac promoter, ppc gene from Corynebacterium glutamicum ATCC 13032, RSF ori, KmR | This study |
| pRSF-tac-pycP4585-ppc_cg | pRSF-tac-T7-T7ter derivative; tac promoter, pyc gene mutant, ppc gene from Corynebacterium glutamicum ATCC 13032, RSF ori, KmR | This study |
| pCDF-T7-ED | pCDFDuet-1 derivative; T7 promoter, zwf, pgI, edd and eda genes from Zymomonas mobilis subsp. Mobilis CICC 41465, CloDF13 ori, SmR | Laboratory collection |
| pCDF-T7-fdh1-T7-pntAB | pCDFDuet-1 derivative; T7 promoter, fdh1 gene from Saccharomyces cerevisiae s288c, pntAB gene from E. coli JM109(DE3), CloDF13 ori, SmR | Laboratory collection |
| pET-trc-lysDH-pse-tac-pycP458S | pET-trc-lysDH-pse derivative; trc promoter, codon-optimized lysDH gene from Bacillus thermoamyllovorans and codon-optimized Psefu_1272 gene from Pseudomonas fulva 12-X; tac promoter, pyc gene mutant, ColE1 ori, AmpR | This study |
| pACYC-tac-lysC311-dapA_cg-tac-pycP458S | pACYC-tac-lysC311-dapA_cg derivative; tac promoter, lysC311I and dapA genes from Corynebacterium glutamicum ATCC 13032; tac promoter, pyc gene mutant, P15A ori, CmR | This study |
| Primers          | Sequence (5′→3′)                                 | Descriptions                                      |
|------------------|--------------------------------------------------|---------------------------------------------------|
| pET-lysDH-Gibson-F | ACTTTAAGAGGAGATATACATGAAAATTGGCGTGCTGG          | Amplification of gene lysDH                        |
| pET-lysDH-Gibson-R | TATATCTCTTTTAGCTAGATTTTCGCTAAATCAC               |                                                   |
| pET-pse-Gibson-F  | TCTGAGCTAAAAGGATATACCTGTGGAATGCGCTGTGG          | Amplification of gene pse                          |
| pET-pse-Gibson-R  | TTTACCAGACTCCAGGTACCTTAATCAAAACAATGCCCTGCGG    |                                                   |
| lacI-Apal-F       | TTAAGGCGCCCGTAAACGCGGCGATTTTGCT                | Amplification of gene lacI                         |
| tac-XbaI-R        | CTAGCTCTAGAGGGAAATTTTACGACGTGACGCGGTACATTGTT   | Amplification of tac promoter                      |
| trc-XbaI-R        | CTAGCTCTAGAGGGAAATTTTACGACGTGACGCGGTACATTGTT   | Amplification of trc promoter                      |
| lacUV5-XbaI-R     | CTAGCTCTAGAGGGAAATTTTACGACGTGACGCGGTACATTGTT   | Amplification of lacUV5 promoter                   |
| lacO1-XbaI-R      | CTAGCTCTAGAGGGAAATTTTACGACGTGACGCGGTACATTGTT   | Amplification of lacO1 promoter                    |
| lysC-F            | GTGCCGCGTGTGCTACAG                               | Amplification of gene lysC from Corynebacterium glutamicum ATCC 13032 |
| lysC-R            | TTAGCGCTGCGGCTCTCA                               |                                                   |
| lysCT311I-F       | CATCATCTCTACCTGCCCT                              | Amplification of gene lysCT311I                    |
| lysCT311I-R       | TCGGTCGTGCGCTCTCTTA                              |                                                   |
| pACYC-lysC_Ec-HindIII-F | CCCAACGCTTTGCTGAAATTGTTGCTCACAATTTTG            | Amplification of gene lysC from E. coli JM109(DE3) |
| pACYC-lysC_Ec-PacI-R | CCTTAATTAATCTCAAAACATTACTATGCGAGTTTTTGC        |                                                   |
| pACYC-RBS-dapA_Ec-PacI-F | CCTTAATTAAGAGGAGATATACCAGGCGCGGTATTGTCG        | Amplification of gene dapA from E. coli JM109(DE3) |
| pACYC-dapA_Ec-AvrII-R | GGGGAAACGTTAGTACAGAAACCACCAGGCGATC            |                                                   |
| pACYC-lysCT311I_cg-Sall-F | AGCGCTCGACATGGCGCTCAGGCA                        | Amplification of gene lysCT311I                    |
| pACYC-lysCT311I_cg-PacI-R | CCTTAATTAATCTCGCTCGTCCCCGTCGCGCGGTCTGC         |                                                   |
| pACYC-RBS-dapA_cg-PacI-F | CCTTAATTAAGAGGAGATATACCAGGCGCGGTATTGTCG        | Amplification of gene dapA from Corynebacterium glutamicum ATCC 13032 |
### Table A2 (Continued)

| Primers                  | Sequence (5'–3')                                      | Descriptions                                                                 |
|--------------------------|-------------------------------------------------------|-------------------------------------------------------------------------------|
| pACYC-dapA_cg-AvrII-R    | GGGAAACCTAGGTATAGAATCCAGCTTTTTTCATGCTCTC             |                                                                              |
| pRSF-pyc-HindII-F        | CCCAAGCTTATGCGACTCACATCTTTCAACG                      | Amplification of gene pyc from Corynebacterium glutamicum ATCC 13032         |
| pRSF-pyc-AvrII-R         | GGGAAACCTAGGTAGAAACGACGATCAAGTCG                     |                                                                              |
| pycP458S-R               | GTGATCGGAATGACATCCTCGG                                | Amplification of gene pycP458S                                              |
| pycP458S-F               | CCGGATTCAATTGCGATCACTCGCACCTCCTTCAGGCCT             |                                                                              |
| T7ter-NsiI-tac-AvrII-F   | GGGAAACCTAGGCTAGCATACAACTCCGGGCGGCTCAAGGTTTTTATGATGATTACAAATCCTGGCTG     | Amplification of gene pyc or pycP458S                                       |

### Table A3

Glucose, acetic acid, and pyruvate concentration at 72 h in strains EcN11* and EcN11**

| Strains               | The titer of glucose (g/L) | The titer of acetic acid (g/L) | The titer of pyruvate (g/L) |
|-----------------------|-----------------------------|-------------------------------|---------------------------|
| EcN11*                | 0.68                        | N.A.                          | N.A.                      |
| EcN11**               | 0.68                        | N.A.                          | 0.94                      |

Abbreviation: N.A., not available.
APPENDIX B
(Figures B1–B9)

**FIGURE B1** Identification of α-aminoadipate by gas chromatography-mass spectrometry (GC-MS). (a) The chromatogram for the standard of α-aminoadipate, triTMS. (b) The mass spectrum for the standard of α-aminoadipate, triTMS. (c) The chromatogram for the sample of α-aminoadipate, triTMS. (d) The mass spectrum for the sample of α-aminoadipate, triTMS. TIC, total ion chromatogram.

**FIGURE B2** Overexpression of meso-diaminopimelate dehydrogenase (encoded by *ddh*) from *Corynebacterium glutamicum* ATCC 13032 (a) The titers of α-aminoadipate. (b) The biomass in the different strains.
FIGURE B3  Comparison of the effects of overexpression of ppc and expression of pyc on the synthesis of α-aminoadipate. (a) The titers of α-aminoadipate in the different strains. (b) The biomass in the different strains. (c) The titers of acetic acid. (d) The titers of glucose.

FIGURE B4  Acetic acid accumulation and glucose residuals in the different strains (a) The titers of acetic acid. (b) The titers of glucose.
**Figure B5**  Acetic acid and pyruvate accumulation, glucose consumption, and the biomass changes of *E. coli* JM109(DE3) and its mutants in the M9 medium. (a) Acetic acid accumulation. (b) Glucose consumption. (c) Cell growth. (d) Pyruvate accumulation.

**Figure B6**  Glucose and pyruvate concentrations in strains EcN11#, EcN11##, and EcN11*#. (a) The titers of glucose. (b) The titers of pyruvate.
FIGURE B7  Metabolic flux analysis by experiments. (a) The titers of α-amino adipate in the different strains. (b) The biomass in the different strains. (c) The titers of pyruvate. (d) The titers of glucose.

FIGURE B8  The effect of the removal of feedback inhibition at the anaplerotic pathway on the synthesis of α-amino adipate. (a) The titers of α-amino adipate in the different strains. (b) The biomass in the different strains.
Fed-batch production of α-amino adipate in 2 L bioreactors using strain EcN11#. DO was set at 10%.